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THE ROLE OF ARGINASE IN ENDOTHELIAL DYSFUNCTION

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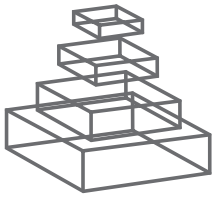

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THE ROLE OF ARGINASE IN ENDOTHELIAL DYSFUNCTION

Topic Editor:
Rudolf Lucas, Medical College of Georgia, USA

In recent years, an increasing number of manuscripts have been published addressing the deleterious role of arginase in endothelial dysfunction. ROS have been shown to play a crucial role in arginase activation, which in turn leads to eNOS dysfunction.

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Arginase in the vascular endothelium: friend or foe?

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Keywords: arginase inhibitors, eNOS, reactive oxygen species, microvascular permeability, impaired vasorelaxation, vessel wall remodeling, l-citrulline

This special issue, entitled "the role of arginase in endothelial dysfunction," assembles original contributions (1–4), as well as timely reviews (5–12) broadly related to the deleterious activities of the manganese-containing enzyme arginase in the vascular endothelium. The arginase 1 isoform is cytosolic and is mainly localized in the liver, where it performs a crucial role in eliminating nitrogen formed during amino acid and nucleotide metabolism via the urea cycle. Recent studies have demonstrated that it is also expressed in extra-hepatic tissues, including the vascular endothelium. Arginase 2 is a mitochondrial enzyme expressed in various cell types, including those in the kidneys (1) and the vasculature. In blood vessels, both isoforms likely play a role in the regulation of L-arginine homeostasis and the production of L-ornithine for subsequent polyamine and proline synthesis, which are involved in endothelial and smooth muscle cell proliferation and collagen deposition (13, 14). Polyamine and proline synthesis are crucial components of physiological and pathological vascular remodeling, a topic extensively discussed in this issue by Durante (5). Since both arginase isoforms are expressed in vascular endothelial cells, they can potentially interfere with the activity of endothelial nitric oxide synthase (eNOS), by "stealing away" the common substrate L-arginine, required to generate nitric oxide (NO) and L-citrulline. NO is a crucial mediator of endothelium-dependent vasorelaxation and restricts vascular growth and inflammation (14). Therefore, excessive activation of arginase by pathologic stimuli, including bacterial toxins [e.g., LPS and pneumolysin (6)], pro-inflammatory cytokines [e.g., TNF (15)], reactive oxygen species (ROS) (7, 11), or hyperglycemia (1, 3, 4) can potentially induce severe endothelial dysfunction. This theory is, however, complicated by observations that even during dramatically increased arginase activity, the concentrations of L-arginine in endothelial cells remain sufficiently high to conceptually support eNOS-mediated NO synthesis. As such, this has led to the suggestion that there is sub-cellular compartmentalization of L-arginine into poorly interchangeable intracellular pools, a topic discussed by Chen et al. in this issue (8). The review by Yang and Ming (7) gives an overview of the direct role of arginase in eNOS dysfunction. Indeed, a pathological increase in vascular arginase activity was shown to significantly contribute to "eNOS uncoupling," a phenomenon observed in various vascular pathologies and in aging (16), during which the enzyme generates detrimental amounts of superoxide instead of the vasoprotective NO. Decreased NO bioavailability within the vessel wall induced by competitive utilization of L-arginine by arginase and "eNOS uncoupling" can be partially circumvented by a recently discovered alternative pathway of NO generation: the reduction of nitrate and nitrite, which is the focus of the review by Madigan and Zuckerbraun (9). Since large vessel endothelial cells are functionally and morphologically distinct from microvascular endothelial cells (17), this review highlights studies on the role of arginase in both of these cell types. Using a DOCA salt-induced mouse model in wild type, $Arg1^{+/-}Arg2^{-/-}$ mice, Toque et al. present original data demonstrating an important role of arginase 1 in impaired vasorelaxation in the aorta and in hypertension (2). Bagi et al. present a concise review on the upregulation of arginase 1 expression and its effects on eNOS function in coronary arteries from diabetic patients (10). Addressing the microvascular compartments, Johnson et al. demonstrate that arginase is an essential mediator of skeletal muscle arteriolar endothelial dysfunction in diabetes, which may further compromise glucose utilization and facilitate the development of diabetes and hypertension (3). Acute pretreatment with L-arginine or with arginase inhibitors significantly improved endothelial function in skeletal arterioles. Kuo and Hein review recent data on the role of arginase in the generation of ROS and the subsequent vasomotor dysfunction of the coronary microcirculation upon angiotensin 2 receptor activation (11). Lucas et al. discuss recent findings suggesting a role for arginase 1 in pneumolysin-induced pulmonary capillary barrier dysfunction (6), which involves impairment of eNOS function (18). They demonstrate that arginase inhibitors significantly prevent pneumolysin-induced barrier dysfunction, at least in part by preventing the loss of expression of the adherens junction protein, VE cadherin (6). Comparing streptozotocin-treated diabetic wild type with diabetic $Arg1^{+/-}Arg2^{-/-}$ transgenic mice, Patel et al. demonstrate an important role for arginase in the pathogenesis of diabetic retinopathy and they extend these findings *in vitro* in high glucose-treated bovine retinal endothelial cells. Their results advance arginase as a potential therapeutic target for preserving NO bioavailability, limiting oxidative stress, and preventing early signs of diabetic retinopathy (4). Although acute treatment with L-arginine was shown to significantly improve endothelial function in several experimental studies, including those by Johnson et al. in this issue (3), a recent clinical trial in patients with acute myocardial infarction demonstrated that a 6-month chronic treatment with L-arginine in addition to standard postinfarct medications did not improve clinical outcomes. Moreover, there was an increased risk of death in older patients after infarction, which promoted the early termination of the trial (19). This study clearly indicates that the long-term treatment with L-arginine is not the optimal treatment for arginase-mediated endothelial dysfunction

and stresses the need for alternative strategies to inhibit arginase. Given the vital role that arginase plays in the detoxification of ammonia in the urea cycle, this suggests that chronic inhibition using specific arginase inhibitors should produce severe side effects. Surprisingly, this does not happen, possibly because the expression of arginase in the liver is much higher than in the vasculature (14). The review by Steppan et al. in this issue discusses the development of novel ABH-based arginase inhibitors, apart from the classically used boronic acid derivatives and moreover addresses potential disadvantages of developing isoform-specific inhibitors for macrophage function (12). As an alternative to L-arginine therapies, L-citrulline is a product of NO synthases that can be recycled to L-arginine. Romero et al. presents data demonstrating a potent protective action of L-citrulline in streptozotocin-induced diabetic nephropathy in mice, accompanied by lower albuminuria and kidney fibrosis (1). L-citrulline also restored the NO/ROS balance and barrier function in high glucose-treated monolayers of human glomerular endothelial cells. Intriguingly, L-citrulline promoted the sustained elevation of arginase 2 expression/activity in proximal tubule epithelium of kidneys from mice. This was mediated, at least in part, via a previously unappreciated immunomodulatory ability to significantly increase plasma levels of the anti-inflammatory cytokine IL-10 (20, 21). Given the weight of experimental evidence to date, there are little doubts that arginase plays a significant role in limiting L-arginine utilization by eNOS and compromising endothelial function. Currently, acute L-arginine and arginase inhibitors are effective in improving endothelial function. However, given the important contributions of arginase to vital metabolic functions, the long-term consequences of arginase inhibition remain uncertain. Improved selectivity could perhaps be obtained by elucidating and targeting the molecular mechanisms that lead to specific upregulation of arginases in the vascular compartment.

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The subcellular compartmentalization of arginine metabolizing enzymes and their role in endothelial dysfunction

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The endothelial production of nitric oxide (NO) mediates endothelium-dependent vasorelaxation and restrains vascular inflammation, smooth muscle cell proliferation, and platelet aggregation. Impaired production of NO is a hallmark of endothelial dysfunction and promotes the development of cardiovascular disease. In endothelial cells, NO is generated by endothelial nitric oxide synthase (eNOS) through the conversion of its substrate, l-arginine to l-citrulline. Reduced access to l-arginine has been proposed as a major mechanism underlying reduced eNOS activity and NO production in cardiovascular disease. The arginases (Arg1 and Arg2) metabolize l-arginine to generate l-ornithine and urea and increased expression of arginase has been proposed as a mechanism of reduced eNOS activity secondary to the depletion of l-arginine. Indeed, supplemental l-arginine and suppression of arginase activity has been shown to improve endothelium-dependent relaxation and ameliorate cardiovascular disease. However, this simple relationship is complicated by observations that l-arginine concentrations in endothelial cells remain sufficiently high to support NO synthesis. Accordingly, the subcellular compartmentalization of intracellular l-arginine into poorly interchangeable pools has been proposed to allow for the local depletion of pools or pockets of l-arginine. In agreement with this, there is considerable evidence supporting the importance of the subcellular localization of l-arginine metabolizing enzymes. In endothelial cells *in vitro* and *in vivo*, eNOS is found in discrete intracellular locations and the capacity to generate NO is heavily influenced by its localization inside the cell. Arg1 and Arg2 also reside in different subcellular environments and are thought to differentially influence endothelial function. The plasma membrane solute transporter, CAT-1 and the arginine recycling enzyme, arginosuccinate lyase, co-localize with eNOS and facilitate NO release. Herein, we highlight the importance of the subcellular location of eNOS and arginine transporting and metabolizing enzymes to NO release and cardiovascular disease.

Keywords: eNOS, l-arginine, nitric, arginase, CAT-1, ASL, ASS, l-citrulline

ENDOTHELIAL DYSFUNCTION

The past three decades have provided unprecedented gains in our understanding of vascular biology. It is now hard to conceive of a time when the vascular endothelium was thought to be a simple barrier, an inert layer of cells lining the lumen of blood vessels. However this was the prevailing view prior to 1981 and the world of vascular biology was irrevocably changed with Furchgott's discovery of an ability of the endothelium to direct changes in vasomotor function (1). In the time since, the depth and pace of research to understand the myriad functions of the endothelium has been remarkable. Not the least of these has been the discovery of endothelial nitric oxide synthase (eNOS) (2–4), an enzyme selectively expressed in the endothelial cells with the ability to generate nitric oxide (NO) and thus regulate blood vessel tone (5). Dysfunction of the vascular endothelium is considered to be the harbinger of cardiovascular disease and precedes the development of overt symptoms (6, 7). Given the importance of eNOS and endogenous NO production to endothelial function, it is not surprising that considerable effort has been focused on the mechanisms influencing eNOS activity in cardiovascular disease. The primary enzymatic function of eNOS is to catalyze the NADPH-dependent conversion of L-arginine into NO, a process shared by the two other NOS isoforms (8). Once formed, NO has an expansive array of cellular targets both locally in the endothelium to influence inflammatory signaling, metabolism, exocytosis, proliferation, motility, and survival, but also in adjacent cells such as vascular smooth muscle cells to decrease vasomotor tone, proliferation and migration, and in platelets to suppress aggregation (9). Loss of these functions promotes increased inflammation, thrombosis, high blood pressure, and vascular cell proliferation, processes that are intimately involved in the development of cardiovascular disease.

L-ARGININE

Because of the obligatory role of L-arginine in NO synthesis, considerable attention has been focused on the importance of

L-arginine availability in the vascular production of NO. Fueling this interest were early studies reporting that L-arginine could directly stimulate EDRF/NO synthesis (10–12) and that compromised endothelial function in cardiovascular disease states could be improved by supplementation with L-arginine both in animals (13–17), healthy humans (18) and those with high cholesterol (19–21), cardiac transplantation (22), peripheral artery disease (23), pulmonary hypertension (24), and angina (25). Considerable evidence pointed toward L-arginine deficiency being a major rate limiting step in the synthesis of NO. However, the affinity of eNOS for L-arginine is low (∼2–3 μM) (26) and the amount of L-arginine in endothelial cells is hundreds of times higher (∼840 μM) (27) suggesting that a substrate deficiency was an unlikely unitary cause of eNOS dysfunction and that additional mechanisms of dysfunction must exist.

SUBCELLULAR LOCALIZATION OF eNOS

The co-translational *N*-myristoylation (glycine 2) and post-translational cysteine palmitoylation of eNOS (cysteines 15 and 26) enable membrane binding and the discrete subcellular targeting (28). In the endothelial cell, eNOS can be found predominantly localized to the perinuclear Golgi (29) and microdomains of the plasma membrane, including caveolae and lipid rafts (30, 31). eNOS has also been reported in other compartments, such as the mitochondria, the nucleus and the cytoskeleton (32, 33). The importance of location to eNOS function and cellular NO release was first demonstrated by mutations that prevent both myristoylation and palmitoylation resulting in an enzyme that is catalytically competent in activity assays outside the cell, but exhibits dramatically reduced capacity to generate NO in intact cells (29). Furthermore, the relative activity of eNOS varies depending on its intracellular location with the highest activity observed from eNOS at the plasma membrane, followed by outer membranes of the cis-Golgi and very low activity in the cytosol, nucleus, and mitochondria (32, 34, 35). Given the dramatic ability of subcellular location to influence eNOS activity and NO release, it is not surprising that compartmentalization has been proposed as a major mechanism by which the local concentration of L-arginine can influence NO release (36).

L-ARGININE TRANSPORTERS

The concentration of L-arginine in human plasma is ∼100–200 μM (37) and higher concentrations, up to 840 μM (27) can be found within the endothelial cell reflecting the existence of transport processes. A number of distinct transmembrane transporters exist on the plasma membrane of endothelial cells that mediate the predominantly sodium independent import of L-arginine via y+ and y+L transporters. The major genes involved in y+ import are CAT-1 (SLC7A1) and CAT-2 (SLC7A2), whereas for y+L import, LAT1 (SLC7A7 and SLC3A2) and LAT2 (SLC7A6 and SLC3A2) are required (36). The presence of CAT-1 in plasmalemma caveolae and the ability of extracellular L-arginine to stimulate NO release in cells with abundant L-arginine levels has led to the hypothesis that L-arginine exists in poorly interchangeable subcellular compartments and reaches eNOS in sufficient concentrations via metabolite channeling (36). While there is suggestive data for the existence of these pools (38), direct evidence and a mechanism for L-arginine sequestration is lacking. A further wrinkle to this story is that the cationic amino acid transporter, CAT-1 can stimulate eNOS activity via direct binding rather than delivering abundant L-arginine to its catalytic doorstep (39).

L-ARGININE RECYCLING

Endothelial cells can maintain their L-arginine levels despite the continuous release of NO (40), suggesting the existence of mechanisms to recover substrate. Indeed, L-citrulline, the byproduct of eNOS-dependent NO generation, can be converted back to L-arginine via the sequential actions of arginosuccinate synthase (ASS) and arginosuccinate lyase (ASL) (41). The co-localization of ASS/ASL with eNOS in plasma membrane caveolae suggests that L-arginine recycling from L-citrulline is a significant source of NO (42, 43). The importance of this pathway is revealed by impaired endothelium-dependent NO generation and increased blood pressure in humans and mice with ASL deficiency (44). Not only do ASL and ASS co-localize with eNOS, but they have been shown to bind directly and regulate eNOS activity (44). A caveat to these studies is that only a fraction of eNOS is present in plasma membrane caveolae and significant amounts of eNOS can be found on endomembranes such as the Golgi. It is not yet known if eNOS at the Golgi or other organelles are regulated by arginine regulatory enzymes in the same way as the plasma membrane/caveolae bound eNOS. L-arginine can also be generated from the breakdown of proteins via both proteosomal and lysosomal pathways, which liberates L-arginine (45). The breakdown of proteins also liberates asymmetric methylated arginines, monomethylarginine (MMA), and asymmetric dimethylarginine (ADMA) which are potent substrate inhibitors of eNOS activity. The methylation of proteins is increased in cardiovascular disease, providing a source for the increased levels of MMA and ADMA via proteolysis (46). Methylated arginines are metabolized by the dimethylarginine dimethylaminohydrolases (DDAH1 and DDAH2). DDAH is found primarily in the cytosol (47) although there are reports of expression in the mitochondria (47) and nucleus (48). Accumulation of asymmetric methylated arginines results in a degree of eNOS-inhibition that is proportional to the ratio of L-arginine/methylated arginine. The inhibition of eNOS can be relieved by supplementation with L-arginine leading to increased production of NO and improvement of endothelial function (49).

ARGINASES

Arginase I and Arginase II are homologous genes encoded by different chromosomes that share the catalytic function of converting L-arginine into urea and ornithine (50). A significant difference between Arginase I and Arginase II is their distinct subcellular distribution, with Arginase I detected predominantly in the cytosol and Arginase II within the mitochondria (51, 52). As enzymes that consume the substrate for eNOS, L-arginine, the arginases have been proposed as endogenous antagonists of eNOS. Increased expression and activity of Arginase I have been implicated in numerous cardiovascular diseases including diabetic retinopathy, asthma, coronary artery dysfunction during renovascular hypertension, and sickle cell disease (53–57) and Arginase

II has been shown to be specifically increased in retinopathy of prematurity, human pulmonary arterial endothelial cells during hypertension, atherosclerosis, and in diabetic renal injury (57–60). Numerous studies have shown that increased expression of arginase correlates with impaired NO synthesis and that inhibition of arginase increases NO production (53, 61, 62). However, this seemingly simple relationship between eNOS and the arginases is complicated by enzyme kinetics and L-arginine concentrations. The affinity of eNOS for arginine is relatively high ($K_m = 3\,\mu M$), the affinity of arginase for L-arginine relatively low (2 mM) and the concentrations of intracellular L-arginine (300–800 μM) sufficient to support near maximal eNOS activity. Two explanations have been proposed to explain the inhibitory actions of arginase, one is the 1000-fold higher enzyme activity (V_{max}) and the other, the compartmentalization and regional deficiency of L-arginine (63). Vascular dysfunction achieved through the arginase-mediated depletion of L-arginine can be reversed with L-arginine supplementation (64) but this also drives increased arginase activity.

L-ARGININE SUPPLEMENTATION

The preceding evidence has emphasized the important role L-arginine plays in the maintenance of endothelial and cardiovascular function and is supported by studies showing that at least in the short-term; L-arginine supplementation can increase endothelial function and mitigate disease. However, more recent evidence suggests that chronic long term supplementation offers little benefit and may instead be harmful (65). The reasons for this are not well understood and likely to be numerous. Chronic exposure to high levels of NO can desensitize NO signaling, impair L-arginine import and increase vascular lesions and mortality (66–69). In contrast, inhibition of endogenous NO can increase sensitivity to NO donors and collectively this suggests that there is pushback when "pushing" NO signaling. Chronic supplementation with L-arginine can also influence other pathways including the greater activation of iNOS (70) which unlike eNOS, is primarily constrained by substrate availability, and the increased expression and catalytic activity of the arginases due to their higher K_m. A consequence of increased arginase activity is the production of ornithine and attendant elevation of L-proline and the polyamines which can promote cell proliferation and maladaptive vascular remodeling (71).

CONCLUSION

L-Arginine is a semi-essential amino acid with a number of important roles in the endothelium including the ability to drive NO production. The compartmentalization of arginine metabolizing and transporting enzymes has important ramifications for endothelial function and cardiovascular health. L-arginine transporters and recycling enzymes have been found in the same intracellular location as eNOS, and some have been found to directly bind eNOS. However, whether this proximity is necessary for providing eNOS with ready access to L-arginine is questionable. Catalytically inactive forms of ASL and substrate-inhibition of CAT-1 do not prevent the ability of these enzymes/transporters to stimulate NO release and this suggests they instead play a structural role in the activation of eNOS. The arginases, which compete for and metabolize L-arginine, particularly when L-arginine is in high abundance, do not reside in the same intracellular locations as eNOS (and presumably do not physically associate) and thus are unlikely to exclusively regulate L-arginine content in pools accessible to eNOS. Instead a more important role of the arginases may be to generate L-proline and polyamines that can negatively impact endothelial and vascular function. The accumulation of asymmetric methylated arginines occurs at the major sites of protein degradation, the proteasome and lysosome, and like the cytosolic DDAH, they are not thought to be in close proximity to eNOS. Frequently underappreciated is the important role eNOS subcellular location has on NO release. Targeting eNOS to the plasma membrane supports the highest levels of NO production followed by the Golgi and the cytoplasm (35). Rendering eNOS insensitive to calcium overrides the effects of intracellular location on eNOS activity and suggests that local calcium and not L-arginine, is the major determinant of efficient NO release (32, 72). While compartmentalization may not be a critical mechanisms by which L-arginine influences eNOS activity, its ability to increase NO release is well documented. However, the failure of supplemental L-arginine to improve cardiovascular health may be considered another lesson learned of why too much of a good thing can be bad. Chronic high levels of NO can result in the refractoriness of its targets to respond and is well documented in vascular smooth muscle. Mechanisms that temporarily restrict eNOS activity such as caveolin-1 or the location of eNOS on membranes of the Golgi enable efficient production of NO in the right amount at the right time for the right response.

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Arginase: the emerging therapeutic target for vascular oxidative stress and inflammation

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Oxidative stress and inflammation in the vascular wall are essential mechanisms of atherosclerosis and vascular dysfunctions associated with risk factors such as metabolic diseases, aging, hypertension, etc. Evidence has been provided that activation of the vascular endothelial cells in the presence of the risk factors promotes oxidative stress and vascular inflammatory responses, leading to acceleration of atherosclerotic vascular disease. Increasing number of studies from recent years demonstrates that uncoupling of endothelial nitric oxide synthase (eNOS), whereby the enzyme eNOS produces detrimental amount of superoxide anion O_2^- instead the vasoprotective nitric oxide ($NO^\cdot$), plays a critical role in vascular dysfunction under various pathophysiological conditions and in aging. The mechanisms of eNOS-uncoupling seem multiple and complex. Recent research provides emerging evidence supporting an essential role of increased activity of arginases including arginase-I and arginase-II in causing eNOS-uncoupling, which results in vascular oxidative stress and inflammatory responses, and ultimately leading to vascular diseases. This review article will summarize the most recent findings on the functional roles of arginases in vascular diseases and/or dysfunctions and the underlying mechanisms in relation to oxidative stress and inflammations. Moreover, regulatory mechanisms of arginases in the vasculature are reviewed and the future perspectives of targeting arginases as therapeutic options in vascular diseases are discussed.

Keywords: arginase, eNOS, superoxide, adhesion molecules, signal transduction pathway

INTRODUCTION

Atherosclerotic cardiovascular disease and vascular complications associated with risk factors such as diabetes mellitus, hypercholesterolemia, hypertension, aging, etc., remain the most important challenge for our society (Sidney et al., 2013). Mechanisms of pathogenesis of atherosclerosis are complex interplay between bloodstream cells and arterial wall components that leads to a chronic state of vascular oxidative stress and inflammation (Hansson and Hermansson, 2011). In the past decades, unambiguous evidence has been provided that heightened oxidative stress and vascular wall inflammation are the key mechanisms for initiation and progression of atherosclerosis and vascular diseases associated with the risk factors (Hansson and Hermansson, 2011). Oxidative stress not only chemically modifies native LDL to the highly atherogenic oxidized LDL which is readily taken up by infiltrated macrophages in the intima of the vascular wall, resulting in foam cell formation, but also causes vascular cell damage that triggers inflammatory responses in the vascular wall and facilitates pathogenesis of vascular diseases, leading to rupture of lipid-rich vascular lesions, the life-threatening events, such as acute myocardial infarction and stroke (Faxon et al., 2004; Hansson and Hermansson, 2011). Therefore, elucidation of mechanisms underlying oxidative stress and inflammations in the vascular wall will have important impact in understanding atherosclerosis and vascular diseases associated with cardiovascular risk factors and will eventually lead to novel and effective therapeutic modalities.

OXIDATIVE STRESS, INFLAMMATION, AND VASCULAR DISEASE

Oxidative stress is characterized with the excessive production of oxidant molecules that overwhelm the anti-oxidant defense systems, resulting in oxidative damage (Lonn et al., 2012). The oxidant molecules include radicals and non-radicals may cause damage of DNA, proteins, and lipids, leading to alterations in cellular functions or cell death (Lonn et al., 2012). Reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide ($NO^\cdot$) are important signaling molecules involved in the regulation of vascular functions, including vascular relaxations, inflammatory responses, and cell proliferation (Sundaresan et al., 1995; Yang and Ming, 2006b; Murphy et al., 2011). Under physiological conditions, the production of these molecules is spatially and temporally regulated, participating in the maintenance of homeostasis of vascular functions (Sundaresan et al., 1995; Yang and Ming, 2006b). Multiple enzymes involved in oxidative stress within the vascular wall can be stimulated or up-regulated in the presence of cardiovascular risk factors, leading to excessive production of ROS and cellular damage (Lonn et al., 2012). O_2^- is the parent ROS molecule produced by the one electron reduction of oxygen catabolized by various enzymes including NADPH oxidase, cyclooxygenase, lipoxygenases, cytochrome P450 enzymes, enzymes in the mitochondrial electron transport chain (Yang and Lüscher, 2002), and also endothelial $NO^\cdot$ Synthase (eNOS, see below). O_2^- is then dismuted by superoxide dismutase (SOD) to

H_2O_2 which is either detoxified to H_2O by peroxiredoxins, glutathione peroxidases, and catalase or metabolized to the powerful oxidant molecules such as hydroxyl radical ($OH^{\cdot}$), peroxynitrite ($ONOO^{\cdot-}$), and hypochlorous acid (HOCl) through enzymatic or non-enzymatic reactions. For detailed description on ROS generation and reaction as well as the role of oxidative stress in pathogenesis of atherosclerosis, please refer to the review article (Lonn et al., 2012).

Chronic vascular inflammation is the fundamental mechanism of vascular diseases associated with variety of risk factors, contributing to pathogenesis of atherosclerosis and plaque rupture, leading to acute coronary syndromes (Hansson and Hermansson, 2011). Macrophages, T cells and other immune cells, pro-inflammatory cytokines are found in the atherosclerotic lesions. Innate as well as adaptive immune responses are identified in atherosclerosis (Hansson and Hermansson, 2011). At the cellular and molecular levels, oxidative stress, vascular inflammation, as well as endothelial cell dysfunction which is mainly reflected by decreased vasoprotective endothelial $NO^{\cdot}$ bioavailability intertwine with each other, represent the major mechanisms leading to exaggerated atherosclerosis in the presence of risk factors (Lonn et al., 2012). Because of the complex interaction among these events, it is not easy to delineate their causal relationship in the pathogenesis of vascular diseases. Under physiological conditions, in the absence of risk factors, the endothelial cells express negligible levels of adhesion molecules such as ICAM-1 and VCAM-1 for inflammatory cells and low levels of the coagulation enzyme tissue factor (Viswambharan et al., 2004; Ming et al., 2009, 2010), whereas in the presence of the risk factors, these molecules are up-regulated in the cells, which may enhance monocyte–endothelial cell interaction and activation of coagulation cascade, participating in the initiation and progression of atherosclerotic plaque formation and thrombus formation (Camici et al., 2006). The role of inflammation and underlying mechanisms in atherogenesis and atherothrombosis are comprehensively reviewed by many articles (Faxon et al., 2004; Hansson and Hermansson, 2011; Lonn et al., 2012). In this review article, we will mainly discuss the role and mechanisms of the enzyme *arginase* in vascular endothelial dysfunction, oxidative stress, and inflammation in the pathogenesis of vascular diseases.

ENDOTHELIAL DYSFUNCTION AND eNOS-UNCOUPLING

The endothelium regulates vascular functions by multiple mechanisms (Yang and Lüscher, 2002). It is well established that the decreased bioavailability of the vasoprotective endothelial $NO^{\cdot}$ molecule best reflects dysfunctional endothelium or endothelial dysfunction under pathological conditions and in the presence of risk factors (Forstermann and Sessa, 2012). It represents one of the most important early markers and mechanisms of cardiovascular disease and also predicts the future atherosclerotic disease progression (Schachinger et al., 2000). The endothelial $NO^{\cdot}$ is produced by the eNOS from the semi-essential amino acid L-arginine in the presence of oxygen and co-factors such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH_4). Electrons from NADPH are transferred in trans from the carboxyl terminal reductase domain of one eNOS monomer, via the flavins FAD and FMN, to the heme in the amino-terminal oxygenase domain of the other monomer, where BH_4, oxygen, and L-arginine are bound (**Figure 1**). At the heme site, the electrons activate O_2, so that L-arginine is oxidized to L-citrulline and $NO^{\cdot}$. Due to the nature of the electron transfer in trans, only eNOS dimer, but not the monomer, is functional in catalyzing NO production. This process, especially the electron transfer from FMN to heme is facilitated by calmodulin binding to eNOS. An increase in intracellular Ca^{2+} concentration in the endothelial cells upon agonist stimulation enhances calmodulin binding affinity to eNOS, promoting flow of electron transfer and $NO^{\cdot}$ production (Forstermann and Sessa, 2012). In addition to intracellular Ca^{2+} concentration, eNOS also requires co-factor BH_4 for enzyme activity. Deficiency in BH_4 or inactivation of BH_4 by oxidative stress has been show to destabilize eNOS dimer and decreases $NO^{\cdot}$ production (Crabtree and Channon, 2011). Interestingly, under this condition "eNOS-uncoupling" may occur – that is, uncoupling of NADPH oxidation and $NO^{\cdot}$ synthesis, with oxygen instead of L-arginine as terminal electron acceptor, resulting in the formation of $O^{\cdot-}$ instead of $NO^{\cdot}$ from eNOS (Forstermann and Sessa, 2012) (**Figure 1**). Evidence has been shown that eNOS-uncoupling plays an important part in endothelial dysfunction in many diseases including atherosclerosis, hypertension, myocardial ischemia/reperfusion injury, diabetes mellitus, as well as aging (please refer to the review article by Kietadisorn et al. (2012). The concept to improve endothelial function under these conditions have been evolved from increasing eNOS gene expression to restoring or recoupling eNOS function, since eNOS gene expression are not decreased and even enhanced in the majority of the conditions. For example, eNOS expression in atherosclerotic arteries and arteries from diabetes mellitus as well as in arteries from aged animals is usually compensatorily increased or not changed (Cosentino et al., 1997; van der Loo et al., 2000; d'Uscio et al., 2001; Ming et al., 2004; Desrois et al., 2010; Rajapakse et al., 2011). Hence, elucidation of mechanisms of eNOS-uncoupling becomes essential for future therapeutic intervention to improve endothelial function in the clinical settings.

The mechanism of eNOS-uncoupling seems multiple and includes oxidation of the co-factor BH_4, decreased intracellular availability of the substrate L-arginine either due to increased arginase activity or accumulation of endogenous methylarginines such as asymmetric dimethyl-L-arginine (ADMA) that competes with L-arginine for eNOS binding (Forstermann and Sessa, 2012). Moreover, S-glutathionylation of eNOS has been proposed as yet another mechanism of eNOS-uncoupling (Chen et al., 2010). In this review article, we will focus on the roles of arginase in eNOS dysfunction.

ARGINASE PROMOTES eNOS-UNCOUPLING, OXIDATIVE STRESS, AND INFLAMMATION

In humans and mammals, there are two isoforms of arginases: arginase-1 (Arg-I) and arginase-II (Arg-II), which is encoded by two separate genes. The human Arg-I gene which maps to chromosome 6q23, encodes a 322 amino acid protein (Dizikes et al., 1986a,b; Sparkes et al., 1986). The human Arg-II gene which maps

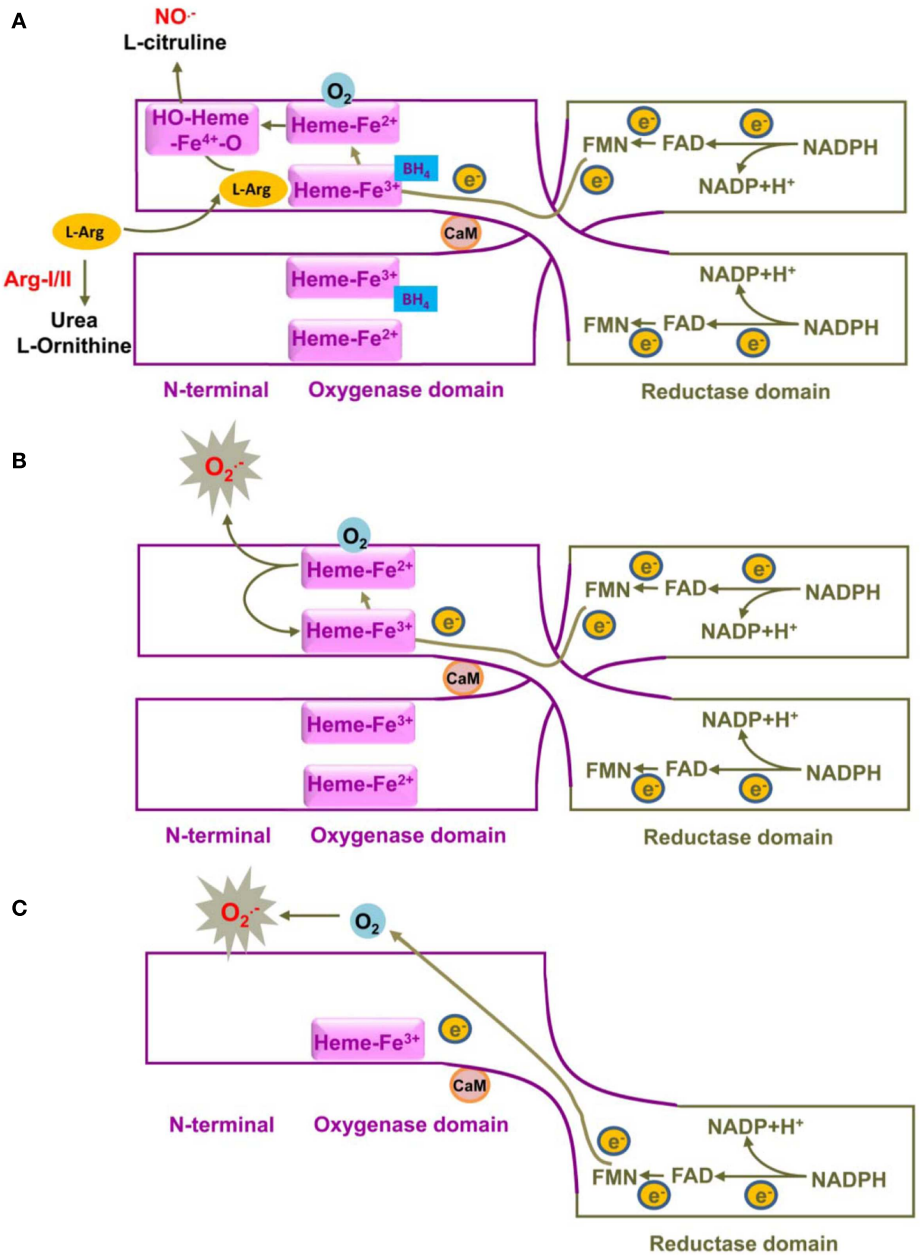

FIGURE 1 | Schematic illustration of mechanisms of eNOS in catalyzing NO· or O_2^- production. eNOS monomer consists of an N-terminal heme-containing oxygenase domain, a C-terminal flavin-containing reductase domain and a regulatory CaM-binding linker sequence. Monomer can bind to CaM, but not co-factor BH_4 or substrate L-arginine. **(A)** A functional eNOS is a homodimer and transfers electron from NADPH from the reductase domain of one monomer, via FAD and FMN, to the heme in the oxygenase domain of the other monomer, where BH_4, oxygen, and L-arginine are bound. At the heme site, the reduction of Fe^{3+} to Fe^{2+} facilitates oxygen binding to the heme group to form a transient Fe^{4+}-O_2 complex that is further reduced to form a hydroxylating heme-Fe^{4+}-oxo species, which in turn oxidizes L-arginine to NO· and L-citrulline. Due to the nature of the electron transfer in trans, only eNOS dimer, but not the monomer, is functional in catalyzing NO· production. The binding of CaM to eNOS, upon an increased intracellular Ca^{2+} concentration in response to agonist stimulation, facilitates the electron transfer from NADPH to both flavins (FAD and FMN) as well as to the heme and ultimately the NO· production. **(B,C)** Under pathological conditions that cause BH_4 deficiency or L-arginine depletion, "eNOS-uncoupling" occurs – that is, uncoupling of NADPH oxidation and NO· synthesis, with oxygen instead of L-arginine as terminal electron acceptor, resulting in the formation of O_2^- instead of NO· from eNOS. eNOS-derived O_2^- production mainly comes from uncoupled eNOS dimer **(B)**, whereas monomer has only a limited capacity to reduce molecular oxygen to O_2^- **(C)**. For simplicity and clarity, the flow of electrons in trans is only shown from one monomer to the other monomer. The diagram is not to scale and is made based on these publications (Griffith and Stuehr, 1995; Abu-Soud et al., 1997; Vasquez-Vivar et al., 1998). CaM, calmodulin; BH4, tetrahydrobiopterin; NADPH, nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; FAM, flavin mononucleotide.

to chromosome 14q24.1, encodes a 354 amino acid protein (Gotoh et al., 1996, 1997; Vockley et al., 1996) The two enzymes have similar structural properties, enzyme characteristics and share more than 50% of homology of their amino acid residues, with 100% homology in those areas critical to enzymatic function (Gotoh et al., 1996; Morris Jr. et al., 1997; Vockley et al., 1996). Arg-I is a cytosolic enzyme abundantly expressed in liver (Haraguchi et al., 1987). It hydrolyzes L-arginine to urea and L-ornithine, is the sixth and final enzyme of the hepatic urea cycle responsible for elimination of excessive nitrogen generated primarily by the metabolism of amino acids which are derived from the food intake or from endogenous protein catabolism (Crombez and Cederbaum, 2005). Arg-I knockout mice exhibited severe symptoms of hyperammonemia, and died between postnatal days 10 and 14 (Iyer et al., 2002). Arg-I deficiency due to gene mutation has been identified and characterized in humans. These patients reveal urea cycle disorder, hyperargininemia and exhibit neurologically based clinical symptoms in early childhood, including progressive neurologic impairment, development retardation, and hepatic dysfunction associated with cirrhosis and carcinoma (Crombez and Cederbaum, 2005; Tsang et al., 2012). Although this enzyme is largely confined to the liver, it is also present in many extrahepatic tissues such as stomach, pancreas, and lung (Choi et al., 2012). Arg-I gene expression is inducible by a variety of stimuli. Upregulation of Arg-I has been reported in macrophages upon stimulation by cAMP, IL-4, and TGF-β (Morris, 2000) and Arg-I expression is increased in aging vasculature of rats (White et al., 2006). Unlike Arg-I, Arg-II is a mitochondrial enzyme and most abundantly expressed in kidney and widely expressed in many extrahepatic tissues such as brain, prostate, intestine, and pancreas (Gotoh et al., 1996; Vockley et al., 1996; Choi et al., 2012) and is inducible in other organs and cells including macrophages and vascular endothelial cells (Ming et al., 2012; Yepuri et al., 2012). As compared to Arg-I, the function of Arg-II is not well characterized. Studies in the vascular endothelial cells suggest that these two isoforms share similar functions, i.e., metabolizing L-arginine to urea and L-ornithine, whereby enhanced Arg-I or/and Arg-II limits L-arginine bioavailability for $NO^{\cdot}$ production, leading to endothelial dysfunction (Xia et al., 1996; Kim et al., 2009).

This hypothesis, however, requires confirmation by further experimental evidence. Given that the concentration of L-arginine in adult human and mouse plasma (0.1 mmol/L) as well as intracellular L-arginine concentration (0.05–0.2 mmol/L) far exceed the K_m of eNOS (2–20 μmol/L) (Morris Jr., 2002), a real intracellular L-arginine depletion does not seem present. Yet acute L-arginine supplementation in patients and animals has been shown to enhance $NO^{\cdot}$ production and endothelium-dependent relaxations, despite sufficiently high concentrations of L-arginine in the extracellular space, a situation known as "arginine paradox" (Kurz and Harrison, 1997). This finding led to several hypotheses of "relative" intracellular L-arginine deficiency. One hypothesis proposes that there might be different intracellular L-arginine pools for $NO^{\cdot}$ production (Topal et al., 2006; Closs et al., 2000). While exogenous L-arginine seems channeled to eNOS to produce $NO^{\cdot}$, the putative intracellular L-arginine pool is not freely exchangeable with the extracellular L-arginine, it is however accessible to eNOS and arginase (Topal et al., 2006; Closs et al., 2000). This model could explain the "L-arginine paradox" and the observation that inhibition of arginase stimulates $NO^{\cdot}$ production and overexpression of Arg-I or -II suppresses $NO^{\cdot}$ production in the endothelial cells, which is associated with only a mild reduction in intracellular L-arginine concentration (11–25% decrease) even in the presence of high extracellular concentration of L-arginine (0.4 mmol/L) (Li et al., 2001). Yet it is highly speculative. Another explanation is a "relative" intracellular deficiency of L-arginine that could be resulted from the increased levels of ADMA, the endogenous eNOS inhibitor, which blocks intracellular L-arginine utility by eNOS to produce $NO^{\cdot}$ (Antoniades et al., 2009). It is assumable that an increase in arginase activity in the presence of ADMA in endothelial cells would further significant limit intracellular L-arginine bioavailability for eNOS to produce $NO^{\cdot}$, although the intracellular L-arginine concentration is only mildly decreased. If the hypothesis of the "relative L-arginine deficiency" is true, supplementation of L-arginine aiming to enhance endothelial $NO^{\cdot}$ production and to treat vascular disease may not work. Too much L-arginine may even cause harmful effects due to production of other undesired metabolites from L-arginine (Dioguardi, 2011). Indeed, a randomized, double-blinded, placebo-controlled study in patients with acute myocardial infarction, the VINTAGE MI study, demonstrates that 6 months oral L-arginine supplementation (3 g three times a day on top of standard postinfarction therapy) does not have any benefits on vascular stiffness and left ventricular ejection fraction, but increases mortality (Schulman et al., 2006). In line with this result, another clinical study in patients with peripheral artery disease, the NO-PAIN study, shows decreased $NO^{\cdot}$ production and shortened walking distance in patients receiving L-arginine supplementation as compared to the placebo group (Wilson et al., 2007). The impact of L-arginine supplementation, particularly chronic supplementation for treatment of cardiovascular diseases does not seem beneficial, it is rather detrimental and should not be recommended in the clinical settings.

The underlying mechanisms of the detrimental effects of chronic L-arginine supplementation in patients are not clear. Several hypotheses have been discussed. As aforementioned, too much L-arginine may lead to exaggerated production of undesired metabolites through arginase, such as L-proline and L-ornithine which is further metabolized to polyamines (Durante et al., 2001; Wei et al., 2001; Yang and Ming, 2006a). L-proline is an essential component for collagen synthesis and polyamines are important factors supporting vascular smooth muscle cell proliferation (Durante et al., 2001; Wei et al., 2001; Yang and Ming, 2006a). These effects of arginase-derived products may be involved in vascular intimal thickening and vascular stiffness associated with vascular injury and aging (Durante et al., 2001; Wei et al., 2001; Yang and Ming, 2006a; Marinova et al., 2008). The effects of the L-arginine metabolites through arginase in endothelial cells are not clear. Strong evidence shows that elevated arginase expression and/or activity in endothelial cells limit NO bioavailability through eNOS-uncoupling, leading to oxidative stress and vascular inflammatory responses (see discussion below).

The role of arginase including type-I and type-II isozyme in decreased endothelial $NO^{\cdot}$ production is well documented (please

see review articles: Yang and Ming, 2006a; Vanhoutte, 2008). This effect of arginase has been demonstrated being the consequence of eNOS-uncoupling (Ming et al., 2004; Romero et al., 2008; Kim et al., 2009; Scalera et al., 2009; Shin et al., 2012; Yepuri et al., 2012). Since endothelial $NO^{\cdot}$ is an important anti-inflammatory molecule and suppresses expression of adhesion molecules such as VCAM-1, ICAM-1 (Lee et al., 2002), a positive association between plasma arginase level or peripheral blood mononuclear cell arginase level and soluble VCAM-1 and ICAM-1 is demonstrated in patients with sickle cell anemia and overweight subjects (Morris et al., 2005; Kim et al., 2012). In cultured human endothelial cells, genetic inhibition of Arg-II prevents ICAM-1 and VCAM-1 upregulation upon persistent insulin stimulation to mimic the hyperinsulinemia condition (Giri et al., 2012) and decreases their expression in senescent endothelial cells (Yepuri et al., 2012), demonstrating that Arg-II plays a role in endothelial inflammatory responses. This conclusion is further confirmed by the fact that overexpression of Arg-II gene in the non-senescent human endothelial cells enhances VCAM-1 and ICAM-1 levels (Yepuri et al., 2012). Importantly, our study further shows that in senescent human endothelial cells and aortas of old mice, the Arg-II (but not Arg-I) gene expression and activity is augmented and genetic silencing or ablation of Arg-II in senescent human cells or in old mice recouples eNOS function, leading to inhibition of oxidative stress and decrease in adhesion molecule expression *in vitro* cell culture and *in vivo* mouse aging models, resulting in decreased monocyte-endothelial interaction (Yepuri et al., 2012). Moreover, this study shows that inhibition of Arg-II gene is able to prevent or reverse endothelial senescence phenotype markers in the aging models (Yepuri et al., 2012), demonstrating the causal role of Arg-II in cardiovascular aging. The detrimental role of Arg-II in atherosclerotic vascular disease has also been recently evidenced in mice either with systemic deficiency of Arg-II (Ming et al., 2012) or in endothelial specific Arg-II transgenic mice (Vaisman et al., 2012). Enhanced endothelial arginases thus represent an important mechanism in inducing eNOS-uncoupling and the associated oxidative stress and inflammation in vasculature contributing to the development of vascular diseases (**Figure 2**). Elucidation of the regulatory mechanisms of arginases in the vasculature would provide rationales for the development of new drugs for treatment of cardiovascular disorders.

REGULATORY MECHANISM OF ARGINASES IN VASCULAR DISEASES

Studies investigating regulatory mechanisms of arginase gene expression and enzymatic activity so far are limited to Arg-I in murine macrophages (for details please refer to the most recently published review article by Pourcet and Pineda-Torra, 2013). There is little information available regarding the upstream regulatory mechanisms involved in gene expression and enzymatic activity of arginases in vascular cells. A few stimuli have been shown to upregulate arginase gene expression and/or enzymatic activity. It has been reported that transgenic expression of IL-13

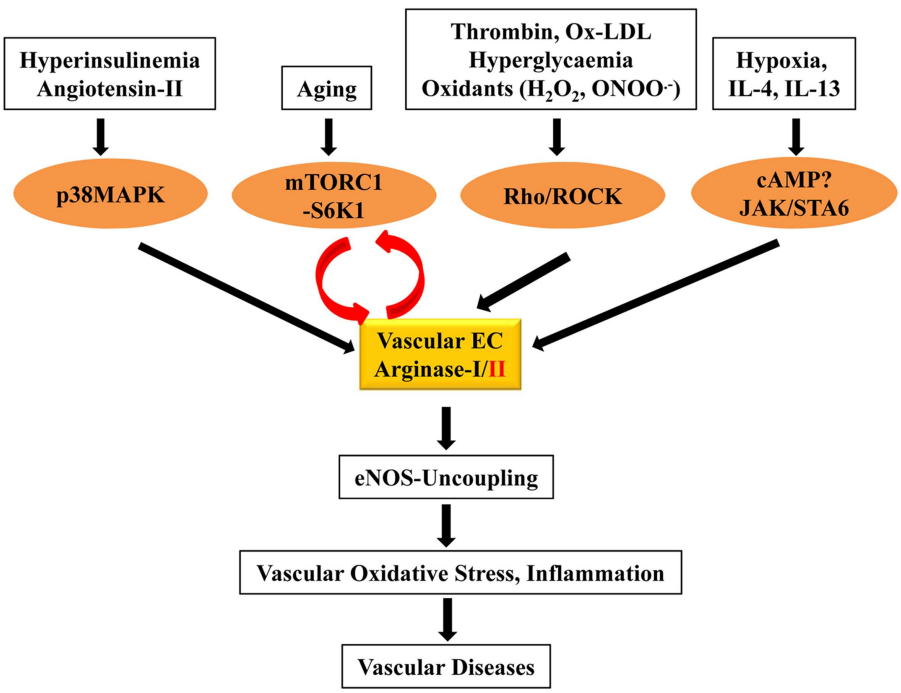

FIGURE 2 | The signaling mechanisms involved in upregulation of vascular arginase expression/activity in vascular endothelial cells (EC). Various cardiovascular risk factors such as hyperinsulinemia, aging, hyperglycemia, hypoxia, etc., upregulate Arg-I or/and Arg-II expression/activity through signaling pathways including p38MAPK, mTORC1-S6K, Rho/ROCK, and JAK/STAT6, leading to eNOS-uncoupling that ultimately causes vascular oxidative stress and inflammation contributing to the development of vascular diseases. Moreover, the mutual positive regulation between S6K1 and Arg-II gene expression accelerates oxidative stress and inflammation through eNOS-uncoupling.

in the lung of mouse, the T-helper type 2 cell effector cytokine, causes pulmonary arteriole remodeling and subsequently pulmonary hypertension, which is associated with enhanced expression of both Arg-I and Arg-II in the lung (Cho et al., 2013). Genetic deletion of Arg-II gene in this mouse partly prevents IL-13-induced pulmonary hypertensive phenotypes, suggesting the involvement of Arg-II (Cho et al., 2013). The study supports the notion of human studies showing that increased Arg-II expression in pulmonary endothelial cells is associated with pulmonary arterial hypertension (Xu et al., 2004). Whether inhibition of Arg-II reveals therapeutic effect in human pulmonary hypertension remains to be investigated and depends on the development of specific Arg-II inhibitors. In contrast to Arg-II, the role of Arg-I in this context is not known. The mechanisms of IL-13-induced Arg-II expression are not clear. In human pulmonary arterial smooth muscle cells, hypoxia is capable of inducing Arg-II expression, which is inhibited by cAMP (Chen et al., 2012). In contrast to this study, Wei et al. (2000) shows that cAMP, besides JAK/STAT6 upregulates Arg-I (not Arg-II) in rat aortic smooth muscle cells upon stimulation by IL-4 or IL-13. It is not clear whether this could be explained by the different biological properties of smooth muscle cells of different origins or species or the different stimuli used in their experimental settings.

Moreover, the GTPase RhoA and its down-stream kinase ROCK have been shown to upregulate arginase activity with or without augmentation of the corresponding gene expression of the isozymes. Oxidative stress, such as hydrogen peroxide (H_2O_2) and peroxynitrite increase Arg-I gene expression in porcine coronary arterioles (Thengchaisri et al., 2006). An increase in Arg-II but not Arg-I expression has been suggested to play a role in women with preeclampsia, which is also mediated by peroxynitrite (Sankaralingam et al., 2010). Interestingly, in both cases, Rho-ROCK is the signaling mechanism involved in Arg-I in porcine and Arg-II in human endothelial cells (Thengchaisri et al., 2006; Chandra et al., 2012). The importance of Rho-ROCK pathway in the regulation of arginase gene expression and activity is also demonstrated by other studies including ours with the stimuli such as thrombin (Ming et al., 2004), oxidized LDL (Ryoo et al., 2011) and hyperglycemia (Romero et al., 2008; Toque et al., 2013). The fact that statins inhibit arginase activity in endothelial cells involving Rho/ROCK pathway could one of the mechanisms contributing to the beneficial effects of the drugs in treatment of cardiovascular disease (Ming et al., 2004; Holowatz et al., 2011).

In addition, p38MAPK (mitogen-activated protein kinase) is also implicated in regulation of arginase expression and activity in endothelial cells. p38MAPK is a member of the superfamily of MAPKs which serves as cellular a stress sensor for a variety of cellular stresses including hyperglycemia, oxidative stress, and inflammatory cytokines (Denise et al., 2012). It has been demonstrated that activation of the p38MAPK in macrophages increases arginase activity and expression of Arg-I (Stempin et al., 2004) and Arg-II (Liscovsky et al., 2009). This seems to be true in bovine and rat aortic endothelial cells for Arg-I expression (Zhu et al., 2010) and in human endothelial cells and mouse penile tissues for Arg-II expression in response to angiotensin-II (Toque et al., 2010) and persistent exposure to insulin (Giri et al., 2012). Moreover, *in vivo* treatment of hypertensive mouse induced by angiotensin-II infusion with a p38MAPK inhibitor prevents elevation of Arg-II expression and activity and enhances endothelium-dependent relaxation (Toque et al., 2010).

Most recently, we have demonstrated a crosstalk between S6K1 (40S ribosomal protein S6 Kinase-1) and Arg-II in endothelial cells (Yepuri et al., 2012), which are importantly involved in vascular aging. Our previous study showed that S6K1 activity is persistently high in senescent human endothelial cells and in the aortas of old rodents, which plays a causal role in age-associated eNOS-uncoupling and endothelial senescence (Rajapakse et al., 2011). Interestingly, overexpression of a constitutively active S6K1 mutant upregulates Arg-II (not Arg-I) gene expression and arginase activity in non-senescent cells by stabilizing Arg-II mRNA (Yepuri et al., 2012). Conversely, silencing S6K1 in senescent cells reduces Arg-II gene expression and activity and genetic or pharmacological inhibition of S6K1 in senescent cells or in old rat aortas decreases Arg-II gene expression and activity, demonstrating a critical role of hyperactive S6K1 in up-regulating Arg-II gene expression resulting in enhanced arginase activity in endothelial aging. Furthermore, our study also shows that silencing Arg-II gene in senescent endothelial cells inhibits S6K1 activity and Arg-II gene knockout in mouse abolishes age-associated hyperactive S6K1 in the aortas, demonstrating a feedforward cycle between S6K1 and Arg-II is present in vascular endothelial aging. Interruption of this crosstalk either by inhibition of S6K1 or Arg-II can recouple eNOS function, leading to reduced oxidative stress, improved $NO^{\cdot}$ production, inhibition of endothelial adhesion molecule expression, monocyte-endothelial cell interaction, and cell senescence markers in aging. Thus, the mutual positive regulation between S6K1 and Arg-II gene expression accelerates endothelial aging through eNOS-uncoupling, leading to oxidative stress and inflammation (Yepuri et al., 2012). The results suggest that interruption of S6K1-Arg-II crosstalk may represent a promising therapeutic strategy to decelerate vascular aging and age-associated cardiovascular diseases. Future work shall investigate the exact mechanisms how S6K1 stabilizes Arg-II mRNA, and how Arg-II activates S6K1 in the endothelial cells. The signaling mechanisms that regulate vascular arginase expression/activity are also summarized in the **Figure 2**.

PERSPECTIVES OF TARGETING ARGINASE IN CARDIOVASCULAR DISEASES

Arginase-II as therapeutic target in cardiovascular diseases has shown promising beneficial effects in genetic modified mouse models. Systemic deficiency of Arg-II reduces systemic and vascular inflammations in mice fed high cholesterol diet and high fat diet, and improves endothelial function in aging, reduces atherosclerosis, and improves insulin sensitivity and glucose homeostasis (Ming et al., 2012; Yepuri et al., 2012). Conversely, endothelial specific Arg-II transgenic mice on ApoE$^{-/-}$ background show accelerated atherosclerosis (Vaisman et al., 2012). Although some studies implicate that targeting Arg-I is also of therapeutic relevance in cardiovascular diseases, the firm evidence is lacking, which is due to the fact that systemic Arg-I deficient mouse exhibited

Table 1 | Available arginase inhibitors.

Name (references)	Chemical class	Isoform-selectivity	Inhibitory mechanism
α-Difluoromethylornithine (DFMO) (Selamnia et al., 1998)	L-Ornithine analog	Non-isoform-selective $K_i = 3.9 \pm 1.0$ mM for arginase in HT-29 homogenate	Poor arginase inhibitor (commonly used as a specific ODC irreversible inhibitor)
L-Ornithine ([3]Reczkowski and Ash, 1994[1]; Colleluori and Ash, 2001; [2]Colleluori et al., 2001)		More potent in inhibiting hepatic arginase[1] $K_i = 1$ mM for Arg-I[3] $K_i > 10$ mM for hArg-II[2]	Competitive inhibition
L-Valine (Colleluori et al., 2001)	Branched-chain amino acid	Non-isoform-selective $K_i = 0.4$ mM for hArg-II	Non-competitive
L-Norvaline (Colleluori et al., 2001)	An analog of L-valine	$K_i = 0.4$ mM for hArg-II	Non-competitive
N^{ω}-Hydroxy-L-arginine (NOHA) (Boucher et al., 1994; Buga et al., 1996; [5]Custot et al., 1997; [4]Baggio et al., 1999; Cox et al., 2001)	N^{ω}-OH-based arginine analog	More potent in inhibiting hepatic arginase[4] $K_i = 10\,\mu$M for rArg-I[5] $K_i = 1.6\,\mu$M for hArg-II[1]	Competitive inhibitor. (an intermediate in NO synthesis, acts also as a substrate for the NOS)
N^{ω}-Hydroxy-nor-L-arginine (nor-NOHA) (Custot et al., 1997)	N^{ω}-OH-based arginine analog	$K_i = 0.5\,\mu$M for rArg-I[5] $K_i = 51$ nM for hArg-II[1]	Competitive inhibitor
S-(2-boronoethyl)-L-cysteine (BEC) ([6]Kim et al., 2001)	Boronic acid-based arginine analog	Non-isoform-selective. $K_i = 0.4$–$0.6\,\mu$M for rArg-I[6] $K_i = 0.31\,\mu$M for hArg-II[1]	Competitive inhibitor
2(*S*)-amino-6-boronhexanoic acid (ABH) ([7]Baggio et al., 1999; [8]Van Zandt et al., 2013)	Boronic acid-based arginine analog	More potent in inhibiting extrahepatic arginase[7]: $K_i = 190$ nM for hepatic Arg. $K_i = 18$–50 nM for extrahepatic non-isoform-selective[8]: $K_i = 1.45\,\mu$M for hArg-I $K_i = 1.92\,\mu$M for hArg-II	Competitive inhibitor
(R)-2-amino-6-borono-2-(2-(piperidin-1-yl)ethyl)hexanoic acid (compound 9) (Van Zandt et al., 2013)	Aminoethylene ABH analog (α,α-disubstituted amino acid-based)	Non-isoform-selective: $K_i = 223$ nM for hArg-I $K_i = 509$ nM for hArg-II	N/A

K_i, the inhibitor constant; ODC, ornithine decarboxylase; rArg-I, rat arginase-I; hArg-II, human arginase-II; N/A, not available. None of these inhibitors are really isoform-specific, although some of the inhibitors, such as nor-NOHA, have been reported to be more potent against one of the isoform in one study, but not in the other study. It is also to notice that the K_i is not always determined for both of the isoforms in the same study. In this case, the reference (the superscript number in italic) is given for the two K_i.

severe symptoms of hyperammonemia, and died between postnatal days 10 and 14 (Iyer et al., 2002), and endothelial specific Arg-I knockout mouse is not available, yet, and the studies are solely dependent on the pharmacological inhibitors which inhibit both isoforms of arginases (The chemical characteristics and pharmacological effects of available arginase inhibitors are summarized in the **Table 1**). Nevertheless, the therapeutic potential of targeting arginases with these inhibitors has been proved in a number of experimental models of cardiovascular disease as discussed (Yang and Ming, 2006b; Pernow and Jung, 2013). Small scale human studies with local administration of arginase inhibitors investigating vascular endothelial functions as primary end point showed promising results in improving skin blood flow in elderly human subjects (Stanhewicz et al., 2012), in hypertensives (Holowatz and Kenney, 2007), and in patients with coronary artery disease and type 2 diabetes (Shemyakin et al., 2012). These inhibitors could theoretically inhibit liver Arg-I and may lead to hyperammonemia, although this side effect has not been reported in animals treated with arginase inhibitors for studies designed to investigate the role of arginase in vascular disease (Bagnost et al., 2010). It is clear that isoform-specific arginase inhibitors should be developed.

It is however, interesting to notice that pharmacological agents that target arginase indirectly through blockade of signaling transduction pathways that regulate arginase gene expression or activity show beneficial effect on vascular functions. Early studies demonstrate that statins which inhibit arginase activity through inhibition of the small G protein or GTPase RhoA improves endothelial function (Ming et al., 2004; Holowatz et al., 2011). Similarly, pharmacological and genetic inhibition of ROCK, the down-stream kinase of RhoA, showed similar inhibitory effects on arginase activity and endothelial dysfunction in atherosclerotic, diabetic, and angiotensin-II-induced hypertensive animal models (Ming et al., 2004; Shatanawi et al., 2011; Yao et al., 2013). Moreover, rapamycin and resveratrol which are capable of inhibiting mTORC1-S6K1 signaling pathway can also inhibit

arginase activity and recouples eNOS function in aging animal models (Rajapakse et al., 2011; Yepuri et al., 2012). Furthermore, p38MAPK inhibitors have been shown to improve endothelial function also through inhibition of eNOS-uncoupling in endothelial cells or mouse aortas exposed to glucosamine (Wu et al., 2012) or inhibition of arginase in the corpora cavernosa from angiotensin-II-treated mice (Toque et al., 2010). Recently, a small clinical study has also showed that p38MAPK inhibitors improves endothelial function and reduces systemic and vascular inflammation in patients with hypercholesterolemia and coronary artery disease (Cheriyan et al., 2011; Elkhawad et al., 2012). With all these pharmacological inhibitors one can not precisely assess how much of the effects is really attributable to the inhibition of arginase activity. Depending on the functions of the signaling pathways in cardiovascular diseases, the off-target effects of these drugs could be of great therapeutic relevance in cardiovascular diseases.

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Therapeutic potential of the nitrite-generated NO pathway in vascular dysfunction

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Nitric oxide (NO) generated through L-arginine metabolism by endothelial nitric oxide synthase (eNOS) is an important regulator of the vessel wall. Dysregulation of this system has been implicated in various pathological vascular conditions, including atherosclerosis, angiogenesis, arteriogenesis, neointimal hyperplasia, and pulmonary hypertension. The pathophysiology involves a decreased bioavailability of NO within the vessel wall by competitive utilization of L-arginine by arginase and "eNOS uncoupling." Generation of NO through reduction of nitrate and nitrite represents an alternative pathway that may be utilized to increase the bioavailability of NO within the vessel wall. We review the therapeutic potential of the nitrate/nitrite/NO pathway in vascular dysfunction.

Keywords: nitrate, nitrite, nitric oxide, pulmonary hypertension, neointimal hyperplasia, peripheral vascular disease, atherosclerosis, review

INTRODUCTION

The Nobel Prize in physiology or medicine was awarded to Drs. Furchgott, Ignarro, and Murad in 1998 for their work in identifying nitric oxide (NO), previously recognized as endothelium-derived relaxing factor, as a biologic mediator of the cardiovascular system. Since that time, NO has been extensively researched and has been linked to numerous physiological and pathological processes within the cardiovascular system. Vascular dysfunction is the root cause of a variety of important disease processes, including myocardial infarction, stroke, peripheral vascular disease, pulmonary hypertension, and wound healing. This constellation of pathology imposes a significant financial burden on the healthcare system and produces significant morbidity and mortality in those affected. The underlying pathophysiology of vascular dysfunction occurs in numerous forms, and often involves a combination of dysregulated endothelial cell NO production, increased proliferation and migration of smooth muscle cells, increased formation of intimal and medial plaques, impaired collateral vessel generation, and reduced angiogenesis.

THE L-ARGININE/NITRIC OXIDE PATHWAY

Three nitric oxide synthases (NOSs), nNOS (neuronal), iNOS (inducible), and eNOS (endothelial), were identified and initially thought to be the sole producers of NO within the cardiovascular system (1). Both nNOS and eNOS are calcium-dependent and constitutively active, while iNOS is induced under inflammatory conditions and is calcium-independent. All three isoforms metabolize L-arginine, NADPH, and oxygen to L-citrulline, NADP, and NO (2) (**Figure 1**). L-arginine may alternatively be metabolized by arginase to L-ornithine and urea. When the supply of L-arginine is limited, metabolism via arginase may effectively reduce production of NO (3).

It has been suggested that the shunting of L-arginine away from the NOS/NO pathway toward the arginase/L-ornithine pathway contributes to certain vascular pathology (4–7) (**Figure 2**). Expression of arginase in the vascular wall is induced under pro-inflammatory conditions, as well as by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (8). Increased arginase activity has been associated with hypertension and coronary vascular dysfunction (9–11). Also, direct vascular injury induces a local inflammatory response. Arginase is upregulated in the vessel wall after balloon injury in the rat carotid injury model. Polyamines generated through the L-ornithine pathway form the building blocks necessary for smooth muscle cell proliferation and neointimal hyperplasia of the vessel wall (12). Peyton et al. (13) demonstrated that selective inhibitors for arginase attenuate neointimal hyperplasia in the rat carotid injury model.

Endothelial NOS is highly expressed in endothelial cells at baseline. Its metabolism of L-arginine to NO is thought to be a major contributor to plasma nitrite levels, which play an important role in baseline vasodilation (14, 15). In addition to regulating baseline vasomotor tone, eNOS is thought to help limit platelet adhesion and thrombosis (16, 17). After vessel injury iNOS is upregulated in arterial smooth muscle cells and eNOS is upregulated in the endothelium resulting in increased NO production (18). Under pathological conditions, the increased NOS activity may not translate into increased NO production. Reduced NO bioavailability through eNOS "uncoupling" is a contributing factor to reduced local NO in atherosclerosis, pulmonary hypertension, and vessel injury (7, 19). Tetrahydrobiopterin (BH_4) is an essential cofactor for the enzymatic production of NO via NOSs (20). Uncoupling occurs under conditions of reduced BH_4 availability where eNOS produces superoxide anions rather than NO (21, 22) (**Figure 3**). In addition, ROS are produced by NADPH oxidase and XOR (23, 24). ROS have been recognized as contributing to vascular dysfunction, through mechanisms including endothelial dysfunction, vascular smooth muscle cell growth, lipid peroxidation, and inflammation (25). An alternative source of

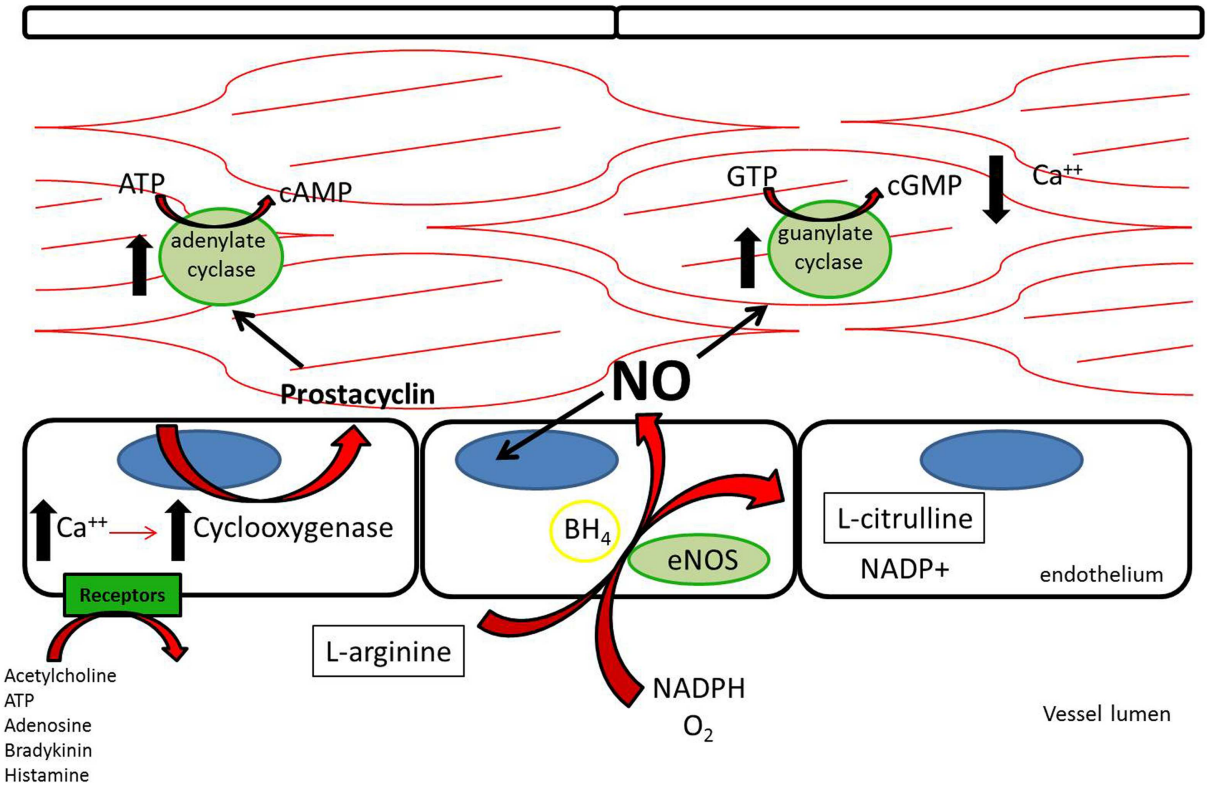

FIGURE 1 | L-Arginine is metabolized in endothelial cells via endothelial nitric oxide synthase to nitric oxide, which then acts downstream to reduce platelet adhesion, decrease leukocyte adhesion, inhibit smooth muscle proliferation and migration, and induce vasodilation. Acetylcholine, adenosine triphosphate, adenosine, bradykinin, and histamine all act on different receptors to generate downstream prostacyclin, which acts as a redundant system to induce vasodilation and platelet inhibition (112).

NO under these conditions may help restore the NO deficiency attributed to uncoupling.

NITRATE/NITRITE REDUCTION TO NITRIC OXIDE

While nitrate and nitrite were long thought of as stable end-products of NO metabolism, recent evidence supports nitrate and nitrite as potential sources of NO under appropriate conditions (12, 26–29) (**Figure 4**). As opposed to the NOS enzymes, which require oxygen as a substrate for NO generation, nitrite-generated production of NO has been shown to occur more readily under acidic and hypoxic conditions (30–32, 113). Nitrate/nitrite reduction has been shown to occur via deoxygenated hemoglobin, myoglobin, enzymatic, and non-enzymatic means (33–37). A class of molybdenum-containing enzymes, including xanthine oxidoreductase (XOR), aldehyde oxidase (AOX), and sulfite oxidase (SUOX), have been identified as enzymes that may facilitate the reduction of nitrate and nitrite to NO at the molybdenum-containing site (38). We and others have shown that XOR in particular is present within the vessel wall and tissue and contributes to NO production in intimal hyperplasia, pulmonary hypertension, and ischemia-reperfusion (12, 26, 39).

While L-arginine is a significant contributor to plasma nitrite production through the L-arginine/NOS/NO/nitrite pathway, plasma nitrite levels are also dependent on oral consumption of nitrate and nitrite (40). The Mediterranean diet, which has been associated with a lower risk of atherosclerosis and coronary artery disease, adds credence to the importance of oral nitrate/nitrite-derived NO in vascular biology (41, 42). The Mediterranean diet, known for its high content of nitrate-rich leafy green vegetables, has also been found to lower the blood pressure of healthy volunteers (40, 43). The nitrate/nitrite/NO pathway through oral ingestion is thought to rely on a symbiotic relationship with natural oral flora. Nitrate is concentrated within the salivary glands and salivary bacteria reduce nitrate to nitrite in the oral cavity (44). Once nitrite reaches the stomach, it is reduced to NO by protonation due to the stomach's low pH (45). NO then may act locally by enhancing mucosal blood flow to the stomach (45–47). Nitrite is also absorbed in the stomach where it enters the blood stream (48). Due to its relative stability, nitrite then has the ability to circulate to other areas in the body and undergo reduction to NO under acidic and hypoxic conditions (33). Acting in this way, circulating nitrite has been described as a "storage pool" for NO within the body (27).

Historically, there has been concern that oral nitrate/nitrite consumption may increase the risk of some cancers, including esophageal, stomach, and colon cancer. Some epidemiological studies have suggested that high oral intake of nitrate/nitrite correlates with increased risk of gastrointestinal malignancy, though

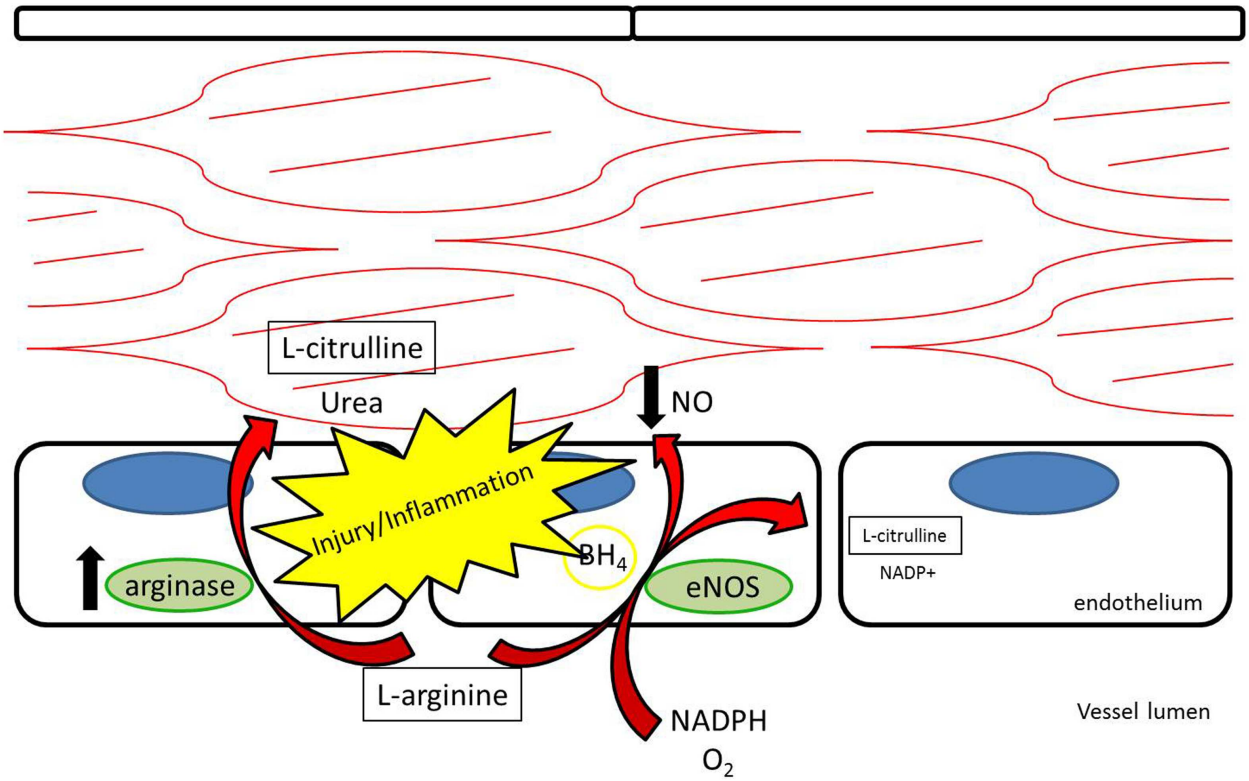

FIGURE 2 | L-arginine may be competitively metabolized by arginase to L-citrulline and urea, reducing production of nitric oxide and contributing to vascular dysfunction.

accuracy in calculating dietary exposure is difficult (49). Nitrosylation of secondary amines via nitrite occurs readily under acidic conditions, such as in the stomach, resulting in N-nitrosamines. Around 300 N-nitrosamines have been identified as carcinogenic (50). The National Toxicology Program (51) of the US Department of Health and Human Services found no evidence of carcinogenic activity in mice and rats after 2 years of exposure to oral sodium nitrite. The International Agency for Research on Cancer (52), a division of the World Health Organization, evaluated the evidence concerning dietary consumption of nitrate/nitrite and carcinogenicity in their monographs. The IARC concluded that inadequate evidence exists in humans and experimental animals for the carcinogenicity of nitrate in food and drinking water and limited evidence exists to suggest carcinogenicity of nitrite in food and drinking water (52). The IARC did, however, recognize that sufficient evidence exists in experimental animals to suggest the carcinogenicity of nitrite in combination with amines or amides and that nitrite in food is correlated with stomach cancer (52). Ongoing research will help elucidate the specific conditions in which *N*-nitrosamines may be carcinogenic in humans.

Multiple investigations have demonstrated that the nitrate/nitrite/NO pathway has vasoactive properties in the systemic and pulmonary circulations. Infusion of nitrite into the forearm brachial artery increased local blood flow and decreased blood pressure at rest and during exercise in humans (33). The infusion correlated with an increase in erythrocyte iron-nitrosylated hemoglobin, suggesting that hemoglobin may play a role in transporting NO through the bloodstream. Dietary supplementation has the potential to achieve similar results systemically. Larsen et al. (43) used a 3-day dietary supplementation of nitrate (0.1 mmol/kg body weight) in healthy volunteers and showed an increase in plasma nitrate (178 ± 51 vs. $26 \pm 11\,\mu M$) and nitrite (219 ± 105 vs. $138 \pm 38\,\mu M$). After 3 days, the volunteers also had a decrease in diastolic and mean blood pressure by 3.7 mmHg and 3.2 mmHg, respectively. In a similar study using a Japanese diet high in nitrate, Sobko et al. (53) demonstrated an increase in both salivary and plasma levels of nitrate and nitrite. These volunteers had an average 4.5 mmHg drop in diastolic blood pressure after 10 days. Dietary nitrate may also effect the pulmonary circulation. In mice exposed to hypoxia to induce pulmonary hypertension, dietary nitrate reduced vascular remodeling and right ventricular hypertrophy through pulmonary vasodilation (26). Inhaled nitrite is an alternative delivery method that has the potential to induce pulmonary vasodilation while minimizing systemic effects. Nebulized sodium nitrite reduced hypoxia-induced pulmonary hypertension in lambs by 65% with no drop in systemic blood pressure (54).

NITRIC OXIDE AND THE VESSEL RESPONSE TO INJURY

Nitric oxide has been shown to serve many vasoprotective properties that occur after vessel injury, including reduction of platelet deposition, decrease in leukocyte adhesion, inhibition of smooth

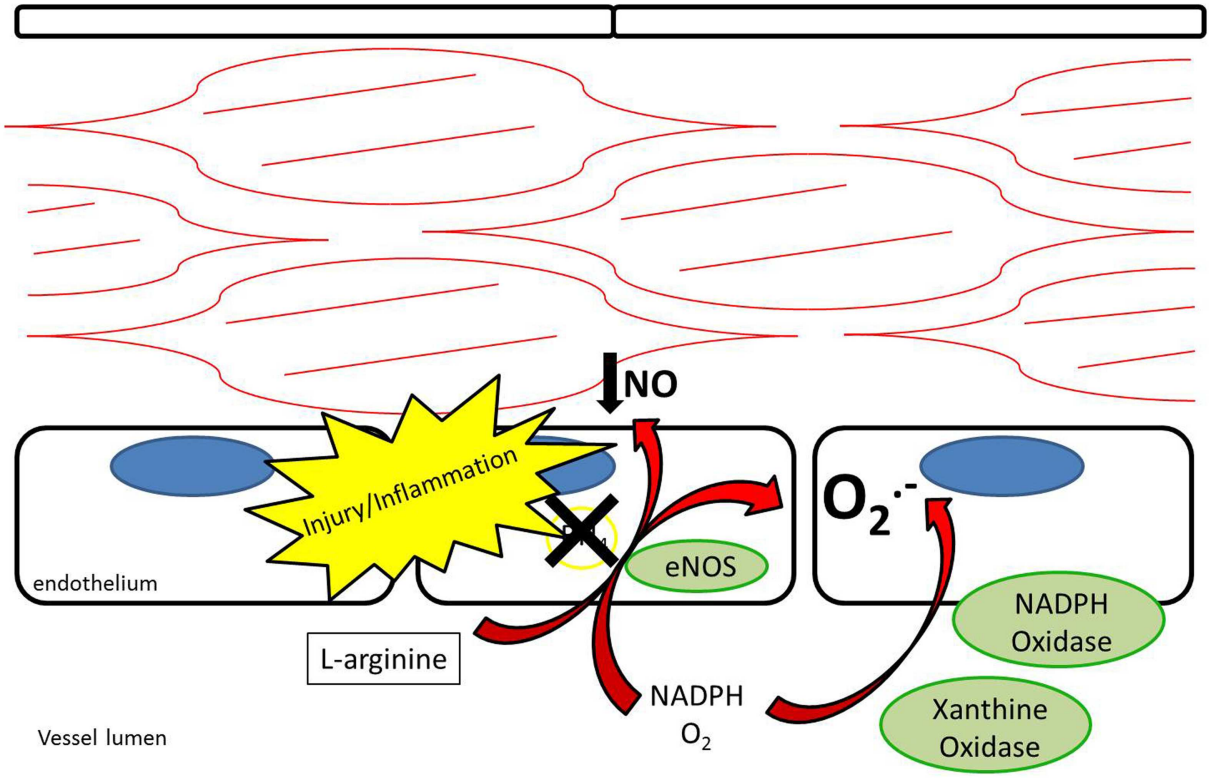

FIGURE 3 | Endothelial nitric oxide synthase uncoupling results in reduced production of nitric oxide as well as production of superoxide anions. NADPH oxidase and xanthine oxidase also contribute to production of superoxide anions.

muscle cell proliferation and migration, and induction of vasodilation (55). One of the initial responses to endothelial disruption is platelet activation and plug formation. NO and NOS expression are associated with decreased platelet adhesion at the vessel wall (56, 57). NO has been shown to be a potent inhibitor of platelet adhesion, reducing thrombosis within the vessel lumen (58, 59). NO mediates platelet adhesion though upregulation of platelet-soluble guanylate cyclase production of cyclic GMP. Nitrate and nitrite-supplemented diets increase bleeding times in mice, and there is an inverse relationship between blood nitrate/nitrite levels and platelet function (60). After platelet deposition, neutrophils and macrophages begin to infiltrate the vessel wall. NO inhibits leukocyte adhesion and the subsequent vessel inflammatory response after injury (61, 62). Once the inflammatory response sets in, smooth muscle cells infiltrate the medial layer and begin proliferating. The resulting thickened medial layer narrows the lumen and stiffens the vessel wall. NO acts to reduce the smooth muscle cell response in multiple ways. NO was first recognized as the substance responsible for calcium-dependent relaxation of the vascular smooth muscle cells (63). NO upregulates soluble guanylyl cyclase within cells and leads to increased cyclic GMP. Cyclic GMP then interacts with protein kinases to lower cytoplasmic calcium, which results in vasodilation (64). Also, it has been shown in culture that NO reversibly arrests the cell cycle of vascular smooth muscle cells (65). NO inhibits smooth muscle proliferation within the vessel wall via a p21 dependent mechanism (66–68). Overall, NO reduces smooth muscle cell migration and proliferation, which can lead to atherosclerosis and neointimal hyperplasia (69).

NITRIC OXIDE AND ATHEROSCLEROSIS

Atherosclerosis resulting in coronary artery disease and stroke are the leading causes of death in the developed world (70). Atherosclerotic plaques are formed when the endothelial layer is damaged and cholesterol accumulates within the vessel wall. Macrophages are recruited to the site of injury, form foam cells, and release cytokines leading to an inflammatory response (71). Smooth muscle cells then migrate and proliferate within the vessel wall, eventually leading to an organized plaque (72). Repeated vessel wall injury causes thrombosis and narrowing of the lumen, which leads to ischemia of the tissue bed supplied by the vessels.

While atherosclerosis is a multifactorial process, dysregulation of the arginine/NOS balance contributes to the development of atherosclerotic disease (73). For instance, iNOS inhibition in the apolipoprotein E knockout mouse model for atherosclerosis accelerates the progression of atherosclerotic disease in these mice (74). Restoring the balance of NO production at multiple points along the pathway reduces formation of atherosclerotic plaques. L-Arginine supplementation has been shown to improve vasodilation in cholesterol-fed rabbits and monkeys and reduce the progression of atherosclerosis (75–77). Also, exogenous expression of iNOS in the arteries reduces the injury response

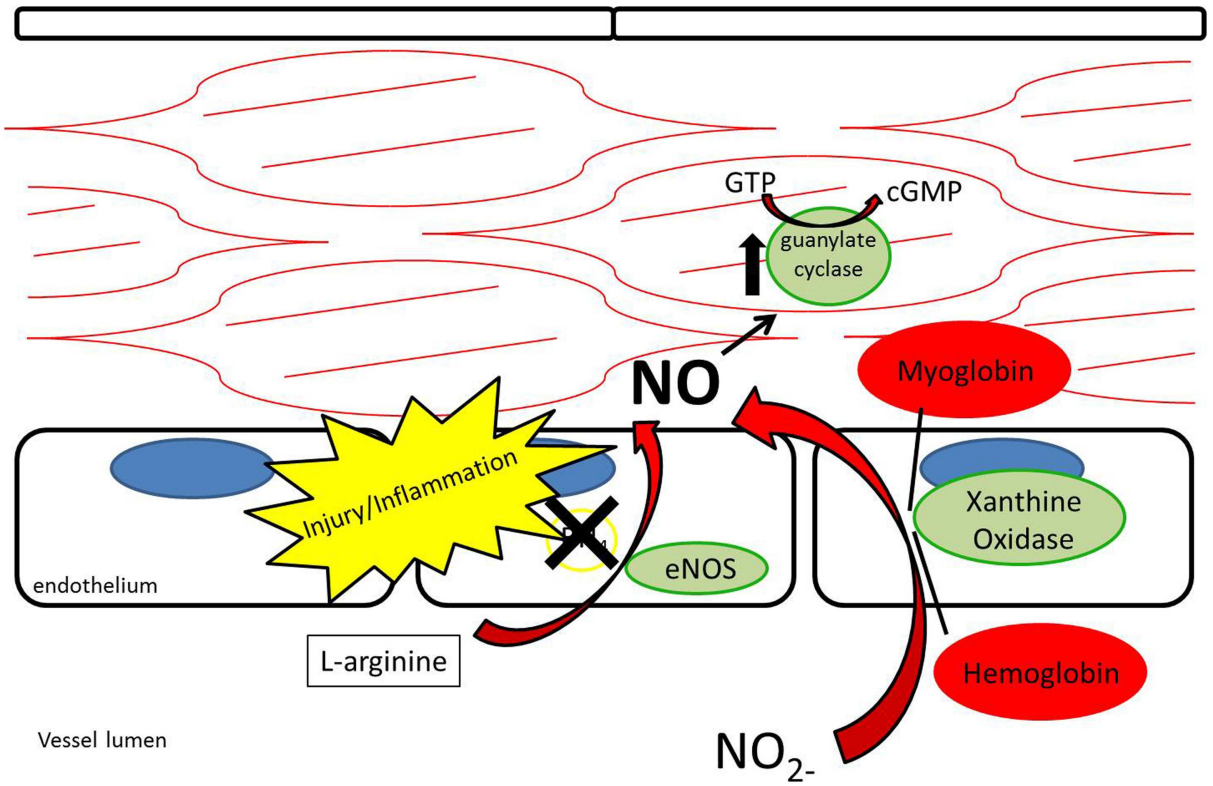

FIGURE 4 | Nitrite reduction by xanthine oxidoreductase, myoglobin, hemoglobin, and protonation results in nitric oxide production, especially under conditions of hypoxia and acidemia.

and atherosclerotic development (78). Furthermore, supplemental oral nitrite has also been shown to be beneficial in reducing vessel inflammation and endothelial dysfunction in mice treated with a high cholesterol diet (79).

NOS enzyme dysregulation results not only in reduced NO availability, but also increased superoxide anions and arginase activity, both of which are detrimental to maintaining healthy vasculature (80, 81). Oxidized low-density lipoproteins (OxLDL) caused by the interaction between LDL and superoxide anions correlate with atherosclerotic disease (82). OxLDL is taken up by macrophages, which forms foam cells on the vessel wall (73). OxLDLs also have been shown to induce apoptosis of endothelial cells and impair endothelium-dependent arterial relaxation within atherosclerotic vessels (83–85). On the contrary, NO has been shown to inhibit apoptosis in endothelial progenitor cells caused by oxidized low-density lipid proteins (86).

NITRIC OXIDE AND PERIPHERAL ARTERIAL DISEASE

Nitric oxide is an important regulator of the tissue response to peripheral arterial disease and lower extremity ischemia, specifically enhancing arteriogenesis, angiogenesis, and progenitor cell migration (4, 87, 88). Arteriogenesis is a recognized phenomenon that involves the enlargement of pre-existing collaterals as a result of increased sheer stress, often in response to stenotic or occluded primary vessels. Angiogenesis, on the other hand, is induced by vascular endothelial growth factor and occurs in response to tissue ischemia (89). As a result, new capillaries are formed (90). Endothelial NOS knockout mice show impaired arteriogenesis, angiogenesis, and pericyte recruitment after femoral artery ligation. All three processes are reversed in this model by intramuscular injection of adenovirus encoding eNOS, suggesting that NO is an important mediator of these processes during lower extremity ischemia (91).

In addition to eNOS-generated NO, the nitrite/NO pathway is functional in the peripheral vasculature. Intraperitoneal (IP) nitrite injections have been shown to improve tissue perfusion through increased collateral vessel development in the murine femoral ligation model of acute limb ischemia (92). IP delivered nitrite also improved angiogenesis and cutaneous flow in rat ischemic myocutaneous flaps, reducing tissue death via a nitrite/NO pathway (93). Nitrite therapy, delivered even in a delayed fashion, augments arteriogenesis in the mouse hindlimb ischemia model (94). Additionally, dietary nitrate supplementation increased capillary and bone-marrow derived progenitor cell density in ischemic hind-limbs, a process that was inhibited with antiseptic mouthwash (95). Antiseptic mouthwash reduces the concentration of the oral bacteria responsible for nitrate reduction to nitrite, thus disrupting the nitrate-nitrite-NO pathway (96). In a small study of healthy volunteers, antiseptic mouthwash increased systolic and diastolic blood pressure by 2–3.5 mm Hg during a 7 day course (97).

NITRIC OXIDE AND NEOINTIMAL HYPERPLASIA

Neointimal hyperplasia is an exaggerated inflammatory healing response after vascular injury. Of particular interest is neointimal hyperplasia after balloon angioplasty and vascular stent deployment, since this may limit therapeutic success. After vessel injury, platelets adhere to the vessel wall denuded of endothelium and generate a cascade of events leading to leukocyte chemotaxis, extracellular matrix modification, endothelial cell apoptosis, and vascular smooth muscle cell migration and proliferation (55). NO has been shown to limit neointimal hyperplasia through multiple levels. Similar to atherosclerosis, NO modulates neointimal hyperplasia through inhibition of platelet aggregation, decreased leukocyte chemotaxis, and reduced vascular smooth muscle cell proliferation while stimulating that of endothelial cells (57–59, 62, 65, 67, 68, 98, 99). The effects of NO may be limited by L-arginine shunting away from eNOS to arginase under pathological conditions. Arginase metabolism of L-arginine leads to the production of polyamines utilized in cell proliferation, and the expression of arginase I is increased in the proliferation of rat aortic smooth muscle cells (100). It has been demonstrated that arginase I activity is increased within the vessel wall after carotid balloon injury in rats, and that inhibition of arginase decreases neointimal hyperplasia in that model (13). Furthermore, Alef et al. (5) demonstrated that nitrite-supplemented drinking water acts to reduce intimal hyperplasia in the rat carotid injury model, and that this NO is generated through XOR.

NITRIC OXIDE AND PULMONARY ARTERIAL HYPERTENSION

Pulmonary hypertension is a vascular disease characterized by hypoxia, pulmonary vasoconstriction, increased vascular resistance, vessel remodeling, thrombosis, and right ventricular strain (7, 101). Multiple etiologies likely contribute to the development of pulmonary hypertension, but all involve increased vascular resistance as a prominent factor. NO, an important regulator of pulmonary vascular resistance, acts as a vasorelaxing agent within the pulmonary arterial system as well as a protective agent against smooth muscle cell proliferation within the vascular wall (102, 103). It has been proposed that NO may act as a "hypoxic buffer" that leads to vasodilation under hypoxic conditions, such as occurs in pulmonary hypertension (104, 105). This theory proposes that increased nitrite reduction to NO helps to counterbalance the hypoxic pulmonary vasoconstriction by generating a vasodilatory signal. Inhaled nitrite is being utilized in pulmonary hypertension as a direct means of delivering NO to the pulmonary vasculature (106). Also, dietary nitrite in mice increases pulmonary dilation, inhibits vascular remodeling, and decreases right ventricular hypertrophy. This effect was reduced in eNOS knockout mice and after allopurinol treatment (26). In a rat model of pulmonary hypertension, it has been shown that inhaled nitrite reverses the effect of hypoxia-induced pulmonary hypertension through creation of NO via XOR (103).

Investigation into the L-arginine/nitrite/NO pathway in pulmonary hypertension has led to conflicting results as far as the importance of this system. Variation in eNOS expression has been observed in human tissue studies, despite consistently elevated eNOS in animal studies (107–109). Inducible NOS has also been shown to be increased in some studies (110). The upregulation of the NOSs may be a compensatory response to upregulated arginase activity. Like other vascular disorders, arginase activity has been shown to be increased in pulmonary hypertension (111). Increased arginase may have a dual role of decreasing L-arginine metabolism to NO as well as polyamine-induced increases in smooth muscle cell proliferation within the vessel walls (7).

SUMMARY

Nitric oxide is an important regulator of vascular function. An imbalance in NO production in relation to ROSs, RNSs, and other inflammatory mediators is associated with many forms of vascular dysfunction, including atherosclerosis, peripheral arterial disease, neointimal hyperplasia, and pulmonary hypertension. The recently discovered nitrate/nitrite/NO pathway is an alternative means of delivering NO to areas of deficiency. In order to harness this pathway as a therapeutic, efficient delivery to the affected tissues must be accomplished. Because of its relatively stable nature and the recognition that nitrate, nitrite, hemoglobin, and myoglobin within the blood act as a 'storage pool' of NO, a variety of potential delivery options to areas of vascular dysfunction exist, including dietary supplementation, inhalation, and direct intravenous infusion.

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Role of arginase in vessel wall remodeling

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Arginase metabolizes the semi-essential amino acid L-arginine to L-ornithine and urea. There are two distinct isoforms of arginase, arginase I and II, which are encoded by separate genes and display differences in tissue distribution, subcellular localization, and molecular regulation. Blood vessels express both arginase I and II but their distribution appears to be cell-, vessel-, and species-specific. Both isoforms of arginase are induced by numerous pathologic stimuli and contribute to vascular cell dysfunction and vessel wall remodeling in several diseases. Clinical and experimental studies have documented increases in the expression and/or activity of arginase I or II in blood vessels following arterial injury and in pulmonary and arterial hypertension, aging, and atherosclerosis. Significantly, pharmacological inhibition or genetic ablation of arginase in animals ameliorates abnormalities in vascular cells and normalizes blood vessel architecture and function in all of these pathological states. The detrimental effect of arginase in vascular remodeling is attributable to its ability to stimulate vascular smooth muscle cell and endothelial cell proliferation, and collagen deposition by promoting the synthesis of polyamines and l-proline, respectively. In addition, arginase adversely impacts arterial remodeling by directing macrophages toward an inflammatory phenotype. Moreover, the proliferative, fibrotic, and inflammatory actions of arginase in the vasculature are further amplified by its capacity to inhibit nitric oxide (NO) synthesis by competing with NO synthase for substrate, L-arginine. Pharmacologic or molecular approaches targeting specific isoforms of arginase represent a promising strategy in treating obstructive fibroproliferative vascular disease.

Keywords: arginase, vascular remodeling, smooth muscle cell proliferation, endothelial dysfunction, nitric oxide

INTRODUCTION

Arterial remodeling is characterized by alterations in the structure and function of the vascular wall in response to specific pathophysiologic stimuli. Although vascular remodeling naturally occurs in response to aging, it also arises in response to injury and disease. The remodeling response is characterized by alterations of one or all three layers of the blood vessel wall: the adventitia, media, and intima. It is driven by numerous complex and interrelated pathological processes that influence both the cellular and non-cellular components of the vascular wall (Orford et al., 2000; Jeffrey and Wanstall, 2001; Dzau et al., 2002; van Varik et al., 2012). The proliferation of cells within the vessel wall is a major contributor to arterial remodeling. The proliferation of vascular smooth muscle cells (SMCs) and/or endothelial cells (ECs) leads to intimal thickening, hyperplasia, or hypertrophy of SMCs results in medial thickening, and fibroblast proliferation causes adventitial expansion. In addition, the recruitment of inflammatory cells from the circulation alters the cellular composition and mass within the vessel wall. These cellular modifications are often accompanied by increased deposition of extracellular matrix material, such as collagen and fibronectin, as well as the fragmentation and degradation of elastin which adversely affects the biomechanical properties of blood vessels. Furthermore, changes in cell phenotype supports vascular remodeling. The dedifferentiation of vascular SMCs from a contractile to a synthetic phenotype that occurs in response to arterial injury aids vascular remodeling by augmenting the secretion of collagen and other extracellular matrix proteins (Owens et al., 2004). Synthetic SMCs also generate matrix metalloproteinases that facilitates SMC migration from the media to the intima by detaching these cells from the basement membrane and extracellular matrix (Bendeck et al., 1996). Moreover, the phenotype of ECs plays a pivotal role in the remodeling response. In response to blood flow and shear stress, ECs release a myriad of humoral factors, including the gas nitric oxide (NO), which maintains vascular SMCs in a quiescent non-proliferative and differentiated non-secretory state (Bonetti et al., 2003; Versari et al., 2007). However, EC function decreases with age and disease resulting in diminished NO synthesis and enhanced production of cytokines and chemokines that triggers the recruitment and infiltration of immune cells into the vessel wall. Moreover, dysfunctional ECs generate various growth factors that stimulate SMCs to proliferate, dedifferentiate, and synthesize collagen. Finally, leukocytes that traffic into the vessel wall exhibit distinct phenotypes that can influence the structure of the vessel wall by enhancing or resolving vascular inflammation.

Arterial remodeling is a salient feature of aging and plays a fundamental role in the development of several vascular disorders, including atherosclerosis, restenosis after percutaneous coronary intervention, post-transplant vasculopathy, systemic and pulmonary hypertension, and aortic aneurysm and dissection (Ross, 1999; Jeffrey and Wanstall, 2001; Dzau et al., 2002; Schiffrin, 2012). The targeting of specific pathophysiological pathways that

contribute to arterial remodeling offers a potential approach for therapeutic intervention. Despite extensive investigation, the discovery and translation of potential targets of vascular remodeling to the clinic has met with limited success. However, studies in the past few years have identified arginase as a promising therapeutic target that underlies many of the pathophysiological processes that contribute to arterial remodeling (Durante et al., 2001; Wei et al., 2001; Li et al., 2002; Chicoine et al., 2004; Ryoo et al., 2008; Kim et al., 2009; Chen et al., 2012; Ming et al., 2012; Cho et al., 2013). This article will review the mechanisms by which arginase promotes aberrant vascular remodeling in arterial injury, pulmonary and systemic hypertension, aging, and atherosclerosis, focusing on the cellular actions of enzyme. In addition, it will highlight arginase as a novel therapeutic modality in the prevention and treatment of occlusive vascular proliferative disease.

L-ARGININE METABOLISM BY VASCULAR CELLS

L-Arginine is a semi-essential amino acid that is involved in numerous physiological processes. It is a necessary precursor for protein and creatinine biosynthesis and plays a role in modulating nitrogen balance. In addition, L-arginine is metabolized by vascular cells to a number of important regulatory molecules (**Figure 1**). Studies in the late 1980s, discovered that L-arginine is oxidized to NO and L-citrulline by nitric oxide synthase (NOS) (Hibbs et al., 1988; Palmer et al., 1988). L-Citrulline is subsequently recycled back to L-arginine by the successive actions of argininosuccinate synthetase and argininosuccinate lyase (Hecker et al., 1990). There are three distinct isoforms of NOS; neuronal NOS (nNOS or NOS-1), endothelial NOS (eNOS or NOS-3), and inducible NOS (iNOS or NOS-2) (Forstermann and Sessa, 2012). These isozymes display differences in tissue distribution, intracellular localization, molecular regulation, enzyme kinetics, and calcium-dependency. Aside from serving as a substrate, L-arginine plays an important structural and functional role by facilitating the intracellular assembly of the dimeric form of NOS and the proper coupling between the oxidative and reductive domains of the enzyme (Baek et al., 1993; Forstermann and Munzel, 2006). The release of NO by vascular cells plays an important homeostatic role in the circulation by inhibiting vascular tone, platelet aggregation, leukocyte adhesion and infiltration into the vessel wall, and the proliferation and migration of vascular SMCs (see Durante, 2001; Forstermann and Sessa, 2012).

Studies in the 1990s revealed that vascular cells also express the enzyme arginase that catalyzes the hydrolysis of L-arginine to L-ornithine and urea (Buga et al., 1996; Durante et al., 1997). There are two distinct isoforms of arginase, arginase I and II, which are encoded by separate genes and share approximately 60% amino acid sequence homology (Dizikes et al., 1986; Vockley et al., 1996). These isozymes exhibit differential tissue distribution, subcellular localization, and molecular regulation (Jenkinson et al., 1996). Arginase I is a cytosolic enzyme that is abundantly expressed in the liver and plays an essential role in hepatic urea cycle. In contrast, arginase II is a mitochondrial enzyme that is widely expressed outside the liver, most prominently in the kidney and prostate (Vockley et al., 1996; Morris et al., 1997). Notably, arginase I germline knockout mice die shortly after birth due to severe hyperammonemia whereas arginase II-deficient mice are viable (Shi et al., 2001; Iyer et al., 2002). The arginase product L-ornithine is further metabolized by the cytosolic enzyme ornithine decarboxylase to the polyamine putrescine which forms the successive polyamines, spermine, and spermidine (Tabor and Tabor, 1984). SMC and EC proliferation is preceded by increases in polyamine synthesis and inhibition of polyamine formation abolishes cell growth (Morrison and Seidel, 1995; Durante et al., 1996b, 1998). L-Ornithine is also catabolized by the mitochondrial enzyme ornithine aminotransferase to pyrroline-5-carboxylate, which is further metabolized to L-proline by the enzyme pyrroline 5-carboxylate reductase. L-Proline is required for the synthesis of many structural proteins, including collagen (Durante et al., 2000, 2001). Finally, L-arginine may also be metabolized by the enzyme arginine decarboxylase to agmatine, which elicits anti-proliferative effects (Regunathan et al., 1996). However, these latter findings need further corroboration.

REGULATION OF ARGINASE EXPRESSION IN VASCULAR CELLS

Recent studies have documented the presence of arginase in a multitude of blood vessels, including the aorta, carotid and pulmonary artery, retinal arteries, coronary arteries, and gracilis muscle arterioles (see Durante et al., 2007; Morris, 2009; Elms et al., 2013).

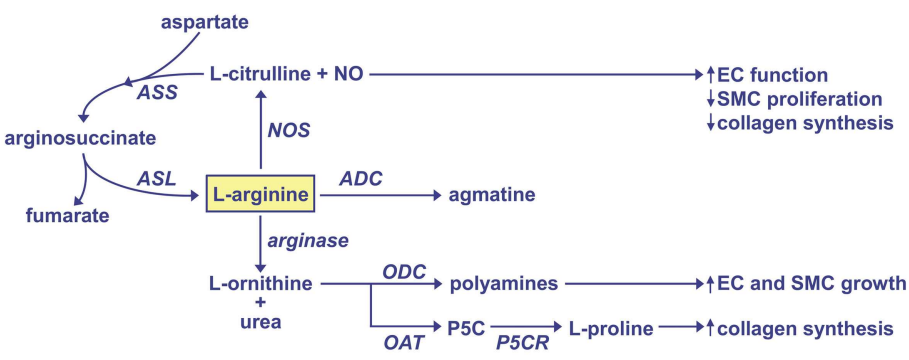

FIGURE 1 | Regulation of L-arginine metabolism by vascular cells. NO, nitric oxide; NOS, nitric oxide synthase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase; P5C, pyrroline-5-carboxylate; P5CR, pyrroline-5-carboxylate reductase.

Arginase I and II have been detected in both vascular SMCs and ECs but the abundance of each isoform is variable and likely reflects differences between animal species, vascular beds, size and function of blood vessels, and/or culture conditions. **Table 1** illustrates the regulation of arginase expression and activity in SMCs and ECs derived from various animal species in response to specific biochemical and biophysical stimuli. Rat aortic SMCs possess substantial arginase activity that is associated with the selective expression of arginase I (Durante et al., 1997, 2001; Wei et al., 2000). This contrasts with results obtained in human pulmonary artery SMCs where both isoforms are expressed (Chen et al., 2009). Several inducers of arginase have been identified in vascular SMCs. Our laboratory identified growth factors and cyclic mechanical strain as potent inducers of arginase I in rat aortic SMCs. Moreover, we showed that growth factors and hemodynamic forces stimulate the uptake of L-arginine and inhibit the expression of iNOS by vascular SMCs (Durante et al., 1996a, 1997, 2000). These coordinate actions of growth factors are synchronized to promote the proliferative capacity of SMCs by directing L-arginine metabolism from the formation of NO to L-ornithine, the first step in polyamine synthesis. Alternatively, the actions of cyclic strain are orchestrated to increase collagen synthesis by channeling L-arginine transport and metabolism to the production of L-proline. The combination of interleukin-13 (IL-13) and interleukin-4 also stimulates arginases I expression in rat aortic SMCs (Wei et al., 2000) while IL-13 induces the expression of arginase II via the IL-13 receptor α2 (IL-13Rα2) in human pulmonary artery SMCs (Cho et al., 2013). In addition, hypoxia selectively increases arginase II expression in SMCs isolated from human pulmonary arteries (Chen et al., 2009). The induction of arginase II by IL-13 or hypoxia contributes to the proliferation of pulmonary SMCs (Chen et al., 2012; Cho et al., 2013). Consistent with these *in vitro* findings, intimal hyperplasia in premenopausal human uterine arteries is paralleled by an elevation in the expression of both arginase I and II (Loyaga-Rendon et al., 2005; Marinova et al., 2008). Moreover, arginase activity positively correlates to the magnitude of intimal thickening in these blood vessels. Thus, the expression of both isoforms of arginase is regulated in a dynamic fashion in SMCs by distinct biochemical and biophysical stimuli to elicit discrete proliferative or fibrotic responses.

Endothelial dysfunction arising from impaired NO synthesis is a fundamental feature in many cardiovascular disorders. Consideration of the enzyme kinetics for NOS and arginase indicate that arginase effectively competes with NOS for L-arginine under

Table 1 | Regulation of cellular arginase expression and activity.

Cell type	Species	Isoform	Inducer or activator	Reference
SMC	Rat	I	IL-13/IL-4	Wei et al. (2000)
	Rat	I	TGF-β1	Durante et al. (2001)
	Rat	I	Cyclic strain	Durante et al. (2000)
SMC	Human	II	IL-13	Cho et al. (2013)
	Human	II	Hypoxia	Chen et al. (2012)
EC	Rat	I and II	LPS	Buga et al. (1996)
EC	Human	I and II	LPS and TNFα	Bachetti et al. (2004)
EC	Cow	I and II	LPS and TNFα	Chicoine et al. (2004)
EC	Mouse	I	TNFα	Gao et al. (2007)
EC	Human	II	Hypoxia	Toby et al. (2010)
	Human	II	Hypoxia	Krotova et al. (2010)
	Human	II	Thrombin	Ming et al. (2004)
	Human	II	Thrombin, TRAP	Yang et al. (2006)
EC	Rat	I	Thrombin	Zhu et al. (2010)
EC	Cow	I	Hydrogen peroxide	Chandra et al. (2012)
	Cow	I	Angiotensin II, peroxynitrite	Shatanawi et al. (2011)
EC	Mouse	II	Oxidized LDL	Ryoo et al. (2011)
EC	Pig	I	Oxidized LDL	Wang et al. (2011b)
EC	Rat, human	I	Hyperglycemia, high glucose	Romero et al. (2008)
EC	Mouse	I	Hyperglycemia	Yao et al. (2013)
EC	Pig	II	Shear stress	Thacher et al. (2010)
EC	Human	I	Nitric oxide	Santhanam et al. (2007)
EC	Pig	I and II	Uric acid	Zharikov et al. (2008)

SMC, smooth muscle cells; EC, endothelial cells; IL-13, interleukin-13; IL-4, interleukin-4; LPS, lipopolysaccharide; TGF-β1, transforming growth factor-β1; TRAP, thrombin receptor activating peptide; oxidized LDL, oxidized low-density lipoprotein.

physiologic conditions (Wu and Morris, 1998), providing a framework by which arginase can provoke endothelial malfunction. Indeed, arginase has been linked to endothelial dysfunction in an expanding number of vascular pathologies, including atherosclerosis, hypertension, uremia, aging, diabetes, ischemia-reperfusion, and hemorrhagic shock (see Durante et al., 2007; Johnson et al., 2010; Michell et al., 2011). Interestingly, a large number of factors that trigger endothelial dysfunction are able to stimulate endothelial arginase activity and/or expression. Several studies have reported that inflammatory mediators stimulate the expression of both arginase I and II, and this may be linked to the activation of the Src family tyrosine kinases in pulmonary ECs (Buga et al., 1996; Bachetti et al., 2004; Chicoine et al., 2004; Nelin et al., 2005; Gao et al., 2007; Chang et al., 2008). Hypoxia is also a potent inducer of arginase II in pulmonary ECs (Krotova et al., 2010; Toby et al., 2010). The induction of arginase II by hypoxia is likely mediated by hypoxia-inducible factor 2 since silencing this transcription factor negates the rise in arginase II expression. Interestingly, the serine protease thrombin stimulates endothelial arginase activity via at least two distinct mechanisms (Ming et al., 2004; Yang et al., 2006; Zhu et al., 2010). While thrombin-mediated increases in arginase II activity occurs via a Rho pathway, the induction of arginase I gene expression by thrombin is mediated through activator protein-1 activation (Ming et al., 2004; Zhu et al., 2010). The Rho pathway appears to play a central role in mediating the induction of endothelial arginase expression in response to many atherogenic stimuli, including angiotensin II, oxidized low-density lipoprotein, hyperglycemia, hydrogen peroxide, and peroxynitrite (Thengchaisri et al., 2006; Ryoo et al., 2011; Shatanawi et al., 2011; Wang et al., 2011b; Chandra et al., 2012; Yao et al., 2013). As observed in SMCs, hemodynamic forces also regulate arginase expression in ECs. Exposure of cultured ECs or isolated carotid arteries to unidirectional shear stress modestly elevates arginase II expression whereas oscillatory shear stress, a hemodynamic pattern known to favor plaque development, strongly induces the expression of arginase II, demonstrating that endothelial arginase expression is highly sensitive to disturbances in fluid flow (Thacher et al., 2010). Post-translational modes of regulating arginase have also been reported. In particular, NO release by iNOS stimulates arginase I activity in ECs by *S*-nitrosylating a specific cysteine residue of the protein (Santhanam et al., 2007). This nitrosylation event stabilizes the arginase I trimer and causes a sixfold increase in the affinity of the enzyme for L-arginine, allowing it to better compete with NOS for L-arginine. A direct interaction between the oxygenase domain of iNOS and arginase I was required for the nitrosylation of arginase I to occur (Dunn et al., 2011). Similarly, uric acid activates both arginase isoforms by enhancing their affinity for L-arginine but the underlying mechanism remains unresolved (Zharikov et al., 2008). Thus, numerous pathologic stimuli that cause endothelial dysfunction stimulate the expression and/or activity of arginase in ECs via multiple pathways.

ROLE OF ARGINASE IN VASCULAR REMODELING

ARGINASE IN ARTERIAL INJURY

Considerable evidence indicates that arginase plays an integral role in neointima formation. Intimal lesions following endothelial denudation of carotid arteries are larger in diabetic rabbits relative to normoglycemic animals and they exhibit greater arginase activity and expression (Ishizaka et al., 2007). In addition, a combined transcriptomic and proteomic study identified an increase in arginase I expression in arteriotomy-injured rat carotid arteries (Forte et al., 2008). Consistent with this investigation, we recently reported that balloon injury of rat carotid arteries results in a pronounced increase in arginase I protein expression that is coupled to a sustained increase in arginase activity (Peyton et al., 2009). Arginase I expression is detected throughout the injured blood vessel but it is especially prominent in the neointima. Although arterial injury also induces iNOS synthase expression (Yan et al., 1996; Tulis et al., 2000), the concomitant elevation in arginase I compromises NO synthesis at the site of injury (Alef et al., 2011). The underlying mechanism responsible for inducing arginase I expression is not known; however, the generation of growth factors and/or inflammatory cytokines following arterial injury may be involved.

Balloon injury of rat carotid arteries results in the development of a concentric SMC-rich neointima. However, local perivascular application of the arginase inhibitors *S*-(2-boronoethyl)-L-cysteine or hydroxy-nor-L-arginine immediately after arterial injury markedly diminishes neointima formation without affecting vessel caliber (Peyton et al., 2009). The inhibition of intimal hyperplasia is independent of any increase in apoptosis but is associated with a significant decline in medial and neointimal DNA synthesis, suggesting that arginase promotes intimal thickening by stimulating the proliferation of vascular SMCs. Indeed, transfection of cultured vascular SMCs with arginase I stimulates cell growth by increasing the production of polyamines while pharmacological inhibition of arginase suppresses polyamine synthesis and SMC replication (Wei et al., 2001). We also demonstrated that arginase promotes the entry of vascular SMCs into the cell cycle since blocking arginase activity or silencing arginase I expression arrests cells in the G_0/G_1 phase of the cell cycle (Peyton et al., 2009). The cell cycle arrest and blockade of neointimal thickening following arginase inhibition is associated with a significant increase in the expression of the cyclin-dependent kinase inhibitor p21, a known mediator of G1 arrest. The ability of arginase to suppress polyamine synthesis may contribute to the upregulation of p21 since polyamines have been demonstrated to repress p21 gene transcription (Liu et al., 2006). In addition, the discovery that arginase stimulates collagen synthesis by SMCs may further exacerbate intimal thickening by increasing collagen deposition in injured blood vessels (Durante et al., 2000, 2001). The proliferative and fibrotic actions of arginase I are further amplified by the capacity of arginase I to compete with iNOS for L-arginine and restrict the generation of NO, which is an established inhibitor of SMC proliferation and collagen synthesis (Garg and Hassid, 1989; Kolpakov et al., 1995).

ARGINASE IN PULMONARY AND ARTERIAL HYPERTENSION

Vascular remodeling is a seminal feature in pulmonary arterial hypertension (PAH) that leads to increased pulmonary vascular resistance and reduced compliance. It is characterized by the pronounced thickening of blood vessels and marked by increases in the proliferation of pulmonary artery SMCs and ECs, the extension of SMCs into smaller, non-muscular pulmonary arteries

within the respiratory sinus (neovascularization), and enhanced deposition of extracellular matrix, including collagen (Humbert et al., 2004). EC dysfunction is also observed in PAH and this can further compromise pulmonary blood flow and lead to thrombosis. Recent work suggests that arginase contributes to vascular remodeling in PAH. Both isoforms of arginase are expressed in the lungs of mice exposed to chronic hypoxia but only elevated levels of arginase II are detected in pulmonary arterial ECs of patients with PAH (Xu et al., 2004; Jin et al., 2010). The induction of arginase activity in the pulmonary circulation is paralleled by the development of EC dysfunction and decreases in NO synthesis, suggesting competition between arginase and eNOS for L-arginine (Xu et al., 2004; Sasaki et al., 2007). Increased expression of arginase II is also observed in the pulmonary vasculature of a novel genetic model of PAH in which IL-13 is specifically overexpressed in the lung (Cho et al., 2013). These transgenic mice spontaneously develop PAH with obvious vascular remodeling exemplified by lung fibrosis, prominent medial thickening of pulmonary arteries, and neovascularization of small pulmonary arteries. While the expression of both arginase isoforms is found in alveolar macrophages, only arginase II expression is noted in vascular SMCs and ECs. Interestingly, deletion of arginase II decreases medial wall thickening of pulmonary arteries and reduces the frequency of neovascularization of small pulmonary arteries in IL-13 overexpressing transgenic mice, demonstrating that arginase II contributes to pathologic vascular remodeling in these animals.

Arginase II drives arterial thickening in PAH by stimulating the proliferation of vascular SMCs. Treatment of human pulmonary artery SMCs with IL-13 induces the expression of arginase II and this is associated with a marked increase in cell growth (Cho et al., 2013). Moreover, knocking down arginase II blocks the IL-13-mediated increase in SMC proliferation. Similarly, pulmonary SMCs exposed to hypoxia exhibit increases in arginase II mRNA and protein expression (Chen et al., 2009). Arginase inhibition with *S*-(2-boronoethyl)-L-cysteine or molecular silencing of arginase II expression completely prevents hypoxia-induced SMC proliferation. Since enhanced endothelial arginase II expression is associated with prominent intimal thickening in human PAH patients (Xu et al., 2004), arginase II may also contribute to the growth of ECs in this disorder. In support of this proposal, transfection of bovine coronary ECs with arginase II increases cell proliferation in a manner that is strictly dependent on polyamine synthesis (Li et al., 2002). In addition, hypoxia-induced proliferation of human pulmonary microvascular ECs is blocked by arginase inhibition (Toby et al., 2010). Thus, the ability of arginase II to stimulate the proliferation of both SMCs and ECs may play an essential role in propelling the obstructive remodeling response observed in PAH. Furthermore, arginase-mediated increases in collagen accumulation may contribute to the development of pulmonary arterial stiffness and fibrosis in PAH (Kobs and Chesler, 2006).

Arginase also influences vascular remodeling in arterial hypertension. Arginase activity is elevated in a number of animal models of essential or secondary hypertension. We previously reported that skeletal muscle arterioles from salt-loaded, salt-sensitive hypertensive rats express higher levels of arginase I and II, and that endothelial dysfunction in this vascular bed is corrected by arginase inhibition (Johnson et al., 2005). Restoration of EC function following arginase inhibition has also been described in deoxycorticosterone acetate-salt hypertensive rats, renovascular hypertensive pigs, and spontaneously hypertensive rats (SHR) (Rodriguez et al., 2000; Zhang et al., 2004; Demougeot et al., 2005; Johnson et al., 2005). In addition, upregulation of arginase activity contributes to attenuation of cutaneous vasodilation in hypertensive patients (Holowatz and Kenney, 2007). Aside from improving endothelial function, arginase inhibitors prevent the development of hypertension when given to pre-hypertensive or young adult SHR (Bagnost et al., 2008). More recently, chronic pharmacological inhibition of arginase was also found to sustainably reduce blood pressure in fully developed hypertensive SHR (Bagnost et al., 2010). Notably, arginase inhibition prevents remodeling of the aorta in these animals. There is a significant decline in aortic medial wall thickness, aortic media to lumen ratio, and type I collagen content in SHR treated with hydroxy-nor-L-arginine. In addition, arginase inhibition dramatically increases the arterial compliance of carotid arteries in SHR. However, arginase inhibition has no effect on the remodeling of mesenteric arteries, suggesting that arginase-mediated vascular remodeling is vessel dependent in these animals. The ability of arginase inhibition to repress aortic remodeling and arterial stiffness likely occurs due to decreases in SMC proliferation and collagen synthesis, and improvements in EC function. Interestingly, arginase I is selectively induced in the vasculature of SHRs suggesting that arginase I, rather than arginase II, mediates hypertensive vascular remodeling in conduit arteries of the systemic circulation.

ARGINASE IN AGING

Aging is associated with changes in arterial wall structure and function. The most frequent modifications are luminal enlargement, vessel wall thickening due to intimal and medial expansion, elastin depletion and fragmentation, collagen and calcium deposition, glycation of proteins, and impaired vasomotor function associated with endothelial dysfunction (Virmani et al., 1991; Taddei et al., 2001; Mirea et al., 2012). These structural and functional alterations in aging contribute to increased vascular stiffness, which is an independent risk factor for cardiovascular morbidity and mortality (Sutton-Tyrrell et al., 2005; Dolan et al., 2006; Mattace-Raso et al., 2006). Accumulating evidence indicates that arginase contributes to aging-associated EC dysfunction and arterial stiffening. In aged rats, the upregulation of iNOS activity in blood vessels induces the *S*-nitrosylation and activation of arginase I (Santhanam et al., 2007). Although arginase-mediated depletion of L-arginine reduces NO synthesis, the tight physical coupling between iNOS and arginase I is likely sufficient to sustain *S*-nitrosylation and activation of arginase I (Dunn et al., 2011). Importantly, arginase inhibition restores NO synthesis and reverses endothelial dysfunction and vascular stiffness in old rats (Kim et al., 2009). Recent findings also support a role for arginase in mediating endothelial dysfunction in the circulation of aged human skin (Holowatz et al., 2006). Interestingly, increased arginase II activity contributes to endothelial dysfunction in aged mice, indicating that distinct isoforms of arginase are activated and provoke EC dysfunction in different animal species of aging (Shin et al., 2012).

ARGINASE IN ATHEROSCLEROSIS

Emerging evidence indicates that arginase also contributes to the development of atherosclerotic lesions. Endothelial arginase II activity is significantly increased in apolipoprotein E (apoE)-deficient hypercholesterolemic mice or in wild-type animals fed a high cholesterol diet in the absence of any increase in arginase II expression (Ryoo et al., 2008). Strikingly, pharmacological blockade of arginase reduces plaque burden by approximately 50% and markedly reduces average wall thickness of the ascending aorta in apoE-deficient animals. In addition, arginase inhibition improves arterial compliance in apoE-null mice to levels seen in wild-type animals. Similarly, deletion of arginase II in apoE-deficient mice fed either a high fat or high cholesterol diet results in smaller arterial lesions. In addition, the size of necrotic cores in advanced lesions is substantially reduced in arginase II-apoE double knockout mice despite comparable levels of circulating cholesterol and triglycerides (Ming et al., 2012). In contrast, apoE-deficient transgenic mice with EC-specific overexpression of arginase II exhibit increased aortic lesion development without a change in plasma lipids (Vaisman et al., 2012). Together, these animal studies demonstrate that elevations in endothelial arginase II activity or expression contributes to arterial stiffness and the development of atherosclerotic lesions that possess a vulnerable phenotype, independent of any alteration in the lipid profile.

There are several potential mechanisms by which arginase II exerts its atherogenic effect. Since impaired endothelial dysfunction and NO release plays an important role in the development and progression of atherosclerosis (Lerman and Zeiher, 2005), the ability of arginase to evoke endothelial dysfunction is highly significant. Endothelial function and NO synthesis are compromised in apoE-deficient mice but arginase inhibition or arginase II gene deletion restores NO production and endothelial function in these animals (Ryoo et al., 2008). Importantly, arginase inhibition has recently been demonstrated to acutely improve endothelial function in patients with coronary artery disease (Shemyakin et al., 2012). The beneficial effect of arginase inhibitors in these subjects is completely dependent on the increased bioavailability of NO. In-line with these findings, endothelial overexpression of arginase II induces endothelial dysfunction and hypertension in mice, further underscoring the detrimental nature of this enzyme in the cardiovascular system (Vaisman et al., 2012).

Arginase II may also promote atherogenesis by augmenting the inflammatory response of leukocytes. Monocytes and macrophages are key cellular protagonists of atherosclerosis that play a fundamental role in the initiation, progression, and rupture of atherosclerotic plaques (Libby, 2002; Mantovani et al., 2009; Shibata and Glass, 2009). Monocytes readily infiltrate vascular lesions, differentiate into macrophages, ingest lipoprotein particles, and give rise to foam cells. Macrophages are major cellular contributors to the lesion's physical bulk and contribute to the evolution of the plaque by secreting inflammatory cytokines and reactive oxygen species. They also weaken and destabilize plaque by releasing various proteases. However, monocytes and macrophages are phenotypically diverse and can express pro- and anti-atherogenic programs. Interestingly, recent work suggests that arginase influences the polarization of these cells. In particular, the expression of arginase II is coupled with the classically activated M1 macrophage phenotype, which fosters the release of inflammatory mediators and proteases, and is associated with advanced atherosclerotic lesions (Khallou-Laschet et al., 2010; Ming et al., 2012). In addition, silencing arginase II expression in human monocytes suppresses their pro-inflammatory function: both monocyte adhesion onto activated ECs and inflammatory cytokine production is blocked. The pro-inflammatory role of arginase II is also observed *in vivo*. Targeted disruption of arginase II blunts the infiltration of macrophages into various organs and the expression of inflammatory cytokines in adipose tissue of mice fed a high fat diet (Ming et al., 2012). Arginase II deficiency also limits macrophage content and cytokine expression in atherosclerotic plaques of apoE-null mice. Moreover, adoptive transfer experiments reveals that fewer donor arginase II-deficient monocytes than arginase II-competent macrophages infiltrate into the plaque of apoE-depleted mice, while apoE-arginase II double knockout mice accumulate fewer monocytes than do recipient single knockout apoE animals. Thus, arginase II may exacerbate arterial lesion formation by promoting both endothelial dysfunction and the pro-inflammatory potential of monocytes and macrophages.

Surprisingly, an atheroprotective role for arginase I has been identified in macrophages. Using subtractive suppression hybridization to screen for differentially expressed genes in macrophages obtained from two strains of rabbits with genetically determined high and low predisposition to atherosclerosis, arginase I is found to be expressed at higher levels in macrophages with low atherosclerotic response relative to those with a high response (Teupser et al., 2006). Consistent with these findings, a marked reduction in arginase I gene expression is noted in foam cell macrophages compared with non-foamy macrophages in cholesterol-fed rabbits (Thomas et al., 2007). Immunohistochemistry reveals arginase I expression in superficial and adventitial macrophages and by foam cells and SMCs underlying the fibrous cap with reduced expression deeper within the plaque of advanced rabbit lesions. In human carotid atherosclerotic plaques, arginase I is also widely distributed in the superficial cell layers but is absent from macrophages close to or within the lipid core. The expression of arginase I by macrophages is associated with the alternatively activated M2 macrophage which promotes resolution of inflammation by releasing anti-inflammatory cytokines and engulfing cellular debris (Martinez et al., 2009). Thus, arginase I may resolve inflammatory reactions within atherosclerotic lesions by regulating macrophage polarization. In support of this notion, mRNA expression profiling experiments found that macrophages from regressing murine plaques display enhanced levels of arginase I (Feig et al., 2012). Furthermore, arginase I expression modulates the inflammatory response of vascular SMCs. Overexpression of arginase I inhibits cytokine production and the activation of the pro-inflammatory transcription factor, nuclear factor-κB, by SMCs (Wang et al., 2011a). In addition, intraplaque gene delivery of arginase I reduces macrophage infiltration and inflammation in arterial lesions of rabbits while local silencing of arginase I expression aggravates these responses. Aside from attenuating inflammation, the expression of arginase I in SMCs near the fibrous cap of atherosclerotic lesions may also promote plaque stability by stimulating SMC proliferation.

MODEL FOR THE REGULATION OF VASCULAR REMODELING BY ARGINASE

Recent studies implicate arginase as a critical contributor to the structural and functional remodeling of arteries that underlies a number of vascular diseases. **Figure 2** presents a model where arginase plays a central role in vessel wall remodeling in response to arterial injury, PAH, arterial hypertension, and aging. All four of these pathologic states stimulate the activity and/or expression arginase I or II in blood vessels, while arterial injury and aging also induces the expression of iNOS. The induction of iNOS by aging or arterial injury may further augment the activation of arginase I by nitrosylating a specific cysteine residue of the enzyme. Activation and/or induction of both arginase isoforms stimulate SMC and EC proliferation, and/or collagen synthesis by increasing the production of polyamines and L-proline. These cellular actions of arginase are further amplified by the ability of arginase to limit the metabolism of L-arginine by iNOS and eNOS to NO, which is a known inhibitor of SMC proliferation and collagen synthesis. Collectively, these arginase-driven events result in neointima formation, medial wall thickening, neovascularization of small arteries, vasoconstriction, and/or arterial stiffness. Emerging work indicates that arginase also contributes to arterial remodeling in atherosclerosis; however, the role played by the two arginase isoforms appears to differ (**Figure 3**). The induction of arginase II activity in atherosclerosis stimulates plaque development and vulnerability, and vascular stiffness by triggering EC dysfunction and the inflammatory potential of macrophages. In contrast, the induction of arginase I expression promotes plaque stability by blocking the inflammatory responses of macrophages and SMCs, and by stimulating the proliferation of vascular SMCs.

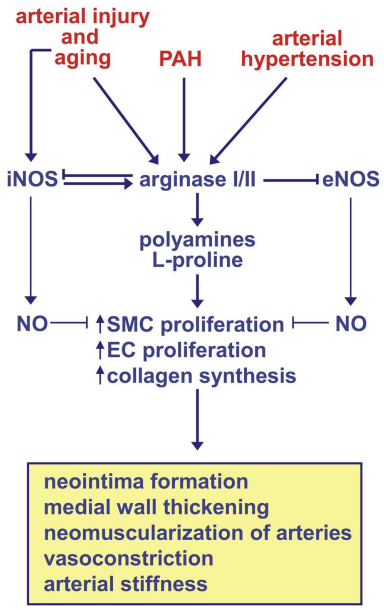

FIGURE 2 | Model for the regulation of vascular remodeling by arginase in arterial injury, pulmonary arterial hypertension (PAH), arterial hypertension, and aging. Arterial injury, PAH, arterial hypertension, and aging stimulates the activity and/or expression of arginase I or II in blood vessels, while arterial injury and aging also stimulates the expression of inducible nitric oxide (NO) synthase (iNOS). The induction of iNOS by aging or arterial injury may further augment the activation of arginase I by nitrosylating a specific cysteine residue of the enzyme. Activation or induction of both arginase isoforms stimulate vascular smooth muscle cell (SMC) and endothelial cell (EC) proliferation, and collagen synthesis by increasing the production of polyamines and L-proline. These cellular actions of arginase are further amplified by the ability of arginase to limit the metabolism of L-arginine by iNOS and endothelial NO synthase (eNOS) to NO, which is a known inhibitor of SMC proliferation and collagen synthesis. Collectively, these arginase-driven events result in neointima formation, medial wall thickening, neovascularization of small arteries, vasoconstriction, and/or arterial stiffness.

THERAPEUTIC STRATEGIES TARGETING ARGINASE IN VASCULAR REMODELING

Several approaches can be employed to target arginase in vascular remodeling. One promising approach involves the development and use of pharmacological inhibitors. While early attempts to establish a role for arginase were hampered by the lack of potent and specific inhibitors of arginase, recent development of boronic acid and N-hydroxy-guanidinium derivatives, such as 2(*S*)-amino-6-boronohexanoic acid, *S*-(2-boronoethyl)-L-cysteine and N^G-hydroxy-nor-L-arginine, has yielded highly potent competitive inhibitors that can readily be used to probe arginase function (Christianson, 2005). The effectiveness of these arginase inhibitors has been demonstrated in various *in vitro* and *in vivo* models, including humans (see Durante et al., 2007; Holowatz and Kenney, 2007; Morris, 2009; Shemyakin et al., 2012). However, further pharmacokinetic and toxicology studies are needed in order to optimize safe and effective therapeutic regimens for these inhibitors. One important limitation with currently available pharmacological inhibitors is their inability to provide isoform-selective inhibition of arginase. The development of small molecule inhibitors that discriminate between arginase I and II will be critical when targeting vascular disorders in which different arginase isoforms elicit disparate actions in blood vessels. Some concern has also been raised over possible non-specific actions of certain arginase inhibitors (Huynh et al., 2009). Given the paucity of isoform-selective arginase inhibitors, small interference RNA (siRNA) has been extensively employed to silence arginase I and II expression in cultured vascular cells. In addition, siRNA has been successfully used to knock down arginase expression in blood vessels both *ex vivo* and *in vivo* (Wang et al., 2011a; Shin et al., 2012). Although siRNA technology holds great promise, current difficulties in delivery, and potential safety and off-target effects limit the clinical efficacy of this approach (Keaney et al., 2011).

Another potential strategy in blocking arginase activity involves the use of dietary antioxidants. Cocoa flavanols lower arginase II mRNA expression and activity in cultured human ECs while oral ingestion of flavanols decreases arginase activity in rat kidney and in human erythrocytes (Schnorr et al., 2008). The catechin, epicatechin gallate, improves scar formation during incisional wound healing in rats and this is associated with a decrease in arginase I expression and activity (Kapoor et al., 2004). In addition, the intake of red wine polyphenols ameliorates endothelial dysfunction and arginase I expression in blood vessels of middle-aged rats

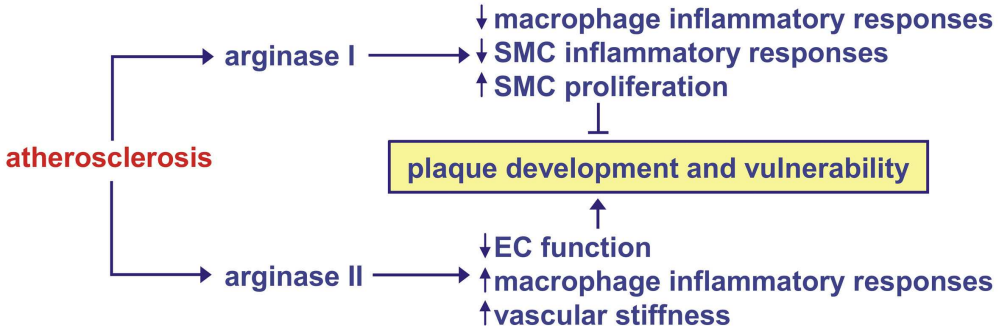

FIGURE 3 | Model for the regulation of atherosclerotic plaque progression and stability by arginase. The induction of arginase II activity in atherosclerosis stimulates plaque development and vulnerability by stimulating endothelial cell (EC) dysfunction, the inflammatory potential of macrophages, and arterial stiffness. In contrast, the induction of arginase I expression in atherosclerosis promotes plaque stability by blocking the inflammatory responses of macrophages and vascular smooth muscle cells (SMCs) and stimulating the proliferation of vascular SMCs.

(Dal-Ros et al., 2012). Furthermore, the bioflavonoid, quercetin, suppresses liver arginase activity in acute renal failure (Nikolic et al., 2003). Interestingly, Danshen, a traditional Chinese herbal medicine that is commonly used for the prevention and treatment of cardiovascular disease, may exert its beneficial vascular effects, in part, through the inhibition of arginase (Joe et al., 2012). Consequently, a variety of dietary approaches may be used in limiting arginase activation; however, detailed clinical studies are needed to establish the efficacy of any nutritional approach.

There is a growing awareness that clinically relevant drugs are able to suppress arginase activity. This is best exemplified by the statin family of drugs which lower circulating cholesterol levels by inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase. Several statins are capable of inhibiting arginase activity. Daily treatment of diabetic rats with simvastatin blunts diabetes-induced endothelial dysfunction and arginase I expression (Romero et al., 2008). In addition, lovastatin and simvastatin completely block the induction of arginase II activity by oxidized low-density lipoprotein in human ECs (Ryoo et al., 2011). Furthermore, in an acute murine model of allergic asthma, simvastatin represses arginase I protein expression and early hallmarks of airway remodeling (Zeki et al., 2010). Moreover, oral atorvastatin therapy restores cutaneous microvascular function by decreasing arginase activity in hypercholesterolemic humans (Holowatz et al., 2011), indicating that arginase inhibition contributes to the pleiotropic and anti-atherogenic actions of statins. The mechanism by which statins reduce arginase activity likely occurs through inhibition of RhoA and Rho-kinase signaling (Ryoo et al., 2011). Aside from statins, the angiotensin-converting enzyme inhibitor, lisinopril, reverses the elevation in arginase activity in erythrocytes from patients with atherosclerosis and hypertension while the phosphodiesterase type 3 inhibitor, cilostamide, inhibits hypoxia-induced arginase II expression in human pulmonary artery SMCs (Chen et al., 2012; Kosenko et al., 2012). Furthermore, oral administration of 17β-estradiol in oophorectomized rabbits fed a cholesterol enriched diet decreases atheromatous lesions and this is accompanied by reductions in both arginase I and II expression, illustrating a potential hormonal approach in targeting arginase (Hayashi et al., 2006). Thus, an increasing number of drugs used in the treatment of cardiovascular disease have been shown to inhibit arginase expression and/or activity, and some evidence suggests that arginase inhibition contributes to their therapeutic efficacy.

CONCLUSION

Recent experimental studies have implicated arginase as a key contributor to the detrimental vascular remodeling response observed in arterial injury, pulmonary and arterial hypertension, aging, and atherosclerosis. The application of potent pharmacological inhibitors of arginase has provided important novel insight into the role of arginase in regulating vascular cell function. In addition, they have proven effective in improving vascular remodeling in animal models of arterial disease and represent an attractive near-term clinical strategy. Interestingly, several natural occurring antioxidants have been demonstrated to block arginase activity and/or expression, providing a potential dietary approach in targeting the enzyme. Given the differential expression of arginase I and II between discrete vascular cells and blood vessels, and their divergent actions in certain pathological settings, the development of potent isoform-selective arginase inhibitors is highly desirable. While molecular approaches using siRNA to silence arginase expression are promising, further refinements in this technology are needed to permit the clinical targeting of distinct arginase isoforms. Future translational studies will determine the success any of these strategies in treating or preventing obstructive fibroproliferative vascular disease.

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Arginase 1 mediates increased blood pressure and contributes to vascular endothelial dysfunction in deoxycorticosterone acetate-salt hypertension

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Enhanced arginase (ARG) activity has been identified as a factor that reduces nitric oxide production and impairs endothelial function in vascular pathologies. Using a gene deletion model, we investigated involvement of arginase isoforms arginase 1 and 2 (ARG1 and ARG2) in hypertension and endothelial dysfunction in a mineralocorticoid-salt mouse model. Hypertension was induced in wild type (WT), partial ARG1$^{+/-}$ knockout (KO), and complete ARG2$^{-/-}$ KO mice by uninephrectomy and deoxycorticosterone acetate (DOCA)-salt treatment for 6-weeks. (Control uninephrectomized mice drank tap water.) After 2 weeks of DOCA-salt treatment, systolic blood pressure (SBP) was increased by ∼15 mmHg in all mouse genotypes. SBP continued to rise in DOCA-salt WT and ARG2$^{-/-}$ mice to ∼130 mmHg at 5–6 weeks, whereas in ARG1$^{+/-}$ mice SBP waned toward control levels by 6 weeks (109 ± 4 vs. 101 ± 3 mmHg, respectively). DOCA-salt treatment in WT mice increased vascular ARG activity (aorta by 1.5-fold; mesenteric artery (MA) by 2.6-fold and protein levels of ARG1 (aorta: 1.49-fold and MA: 1.73-fold) vs. WT Sham tissues. ARG2 protein increased in WT-DOCA MA (by 2.15-fold) but not in aorta compared to those of WT Sham tissues. Maximum endothelium-dependent vasorelaxation to acetylcholine was significantly reduced in DOCA-salt WT mice and largely or partially maintained in DOCA ARG1$^{+/-}$ and ARG2$^{-/-}$ mice vs. their Sham controls. DOCA-salt augmented contractile responses to phenylephrine in aorta of all mouse genotypes. Additionally, treatment of aorta or MA from WT-DOCA mice with arginase inhibitor (100 μM) improved endothelium-mediated vasorelaxation. DOCA-salt-induced coronary perivascular fibrosis (increased by 2.1-fold) in WT was prevented in ARG1$^{+/-}$ and reduced in ARG2$^{-/-}$ mice. In summary, ARG is involved in murine DOCA-salt-induced impairment of vascular function and hypertension and may represent a novel target for antihypertensive therapy.

Keywords: arginase, endothelial dysfunction, DOCA-salt, fibrosis, hypertension

INTRODUCTION

Arterial hypertension remains a major risk factor for cardiovascular disease morbidity. It affects about 26% of the adult population (1). Despite current drugs being effective in many patients, a large number of uncontrolled patients are still very evident today. Hypertension is associated with physiological and biochemical changes in the vessel wall, characterized by turbulent blood flow, fluid shear stress, vascular remodeling, and endothelial dysfunction. During the past few years, many studies have demonstrated that nitric oxide (NO) pathway is a major regulator of cardiovascular functions, and evidence has accumulated that enhanced arginase (ARG) activity is involved in the pathogenesis of several cardiovascular disorders, including hypertension (2, 3).

Arginase is a crucial manganese metalloenzyme in the hepatic urea cycle that catalyzes conversion of L-arginine to ornithine and urea. It exists in two isoforms, arginase 1 (ARG1) and arginase 2 (ARG2). Each is encoded by a separate gene and found in vascular tissues, endothelial, and smooth muscle cells, but their distribution is vessel- and species-dependent (4–6). In the endothelium ARG activity appears as a critical regulator for NO production by competing with endothelial NO synthase (eNOS) for L-arginine (7). Related studies have shown that increased ARG activity/expression is involved in many vascular pathologies including atherosclerosis (6, 8), aging (9), diabetes (10, 11), and hypertension (12–14). Blood pressure is mainly regulated by the tone of resistance vessels. Increased ARG activity and diminished NO bioavailability are observed in conduit and resistance vessels in hypertensive models (15, 16). Treatment of spontaneous hypertensive rats with arginase inhibitor (ABH) has been shown to decrease blood pressure and improve vascular function (2, 13, 14).

Both ARG1 and ARG2 are expressed constitutively in vascular tissues (7, 9). However, ARG1 has been shown to modulate vascular tone in disease conditions such as diabetes (10, 11), ischemia reperfusion (17), and hypertension (3, 13). However, the relative

contribution of the ARG isoforms in salt-sensitive hypertension remains to be determined. To address this issue, we used a genetic mouse model with either partial deletion of the ARG1 gene or complete deletion of ARG2 gene. We could not examine complete ARG1 knockout (KO) mice as they do not survive beyond 2 weeks of age due to disruption of the hepatic urea cycle and hyperammonemia (18). We hypothesized that ARG1 isoform is involved in increased blood pressure, reduced vasodilator activity, and increased reactivity to constrictor stimuli in deoxycorticosterone acetate (DOCA)-salt hypertensive mice, contributing to mineralocorticoid hypertension.

MATERIALS AND METHODS

ANIMALS

All procedures were conducted in concordance with the guiding principles in the care and use of animals, approved by the Georgia Regents University Committee on the use of Animals in Research and Education. Mice lacking one copy of ARG1$^{+/-}$ or both copies of ARG2$^{-/-}$ in a C57BL/6J background at 12 weeks of age were used in this study. The animals were housed on a 12-h light/dark cycle and fed a standard chow diet with water or saline *ad libitum*. An expanded Section "Materials and Methods" is available in the online data supplement.

DOCA-SALT HYPERTENSION

Partial ARG1$^{+/-}$, or complete ARG2$^{-/-}$ KO or control wild type (WT) mice were unilaterally nephrectomized, and DOCA (200 mg/mouse) pellets were implanted SC in the scapular region. DOCA mice received water containing 1.0% NaCl and 0.2% KCl for 6 weeks. Control mice were unilaterally nephrectomized and received silastic pellets without DOCA and tap water.

SYSTOLIC BLOOD PRESSURE MEASUREMENTS

Systolic blood pressure (SBP) was measured by tail cuff plethysmography (RTBP1001 system, Kent Scientific Corporation, Conn.) in conscious mice before and under DOCA treatment once per week thru the 6 weeks of treatment. At the end of the treatment, mice were euthanized, and aorta and mesenteric artery (MA) was isolated for further studies (see below).

VASCULAR FUNCTIONAL STUDIES

After euthanasia, thoracic aortas, and second-order branches of MA were removed and cleaned from fat tissue in ice-cold physiological saline solution. Arterial segments of aorta and MA were carefully mounted as ring preparations in myograph chambers (Danish Myo Technology A/S) filled with physiological saline solution at 37°C (pH 7.4) and continuously bubbled with 5% CO_2 and 95% O_2. Isometric force was recorded using a powerLab/8SP data system (AD Instruments, Colorado Springs, CO, USA). Tissues were adjusted to maintain a passive force of 5 mN for the aortic and 3 mN for the second-order MA rings. Vessels were equilibrated for 60 min before experiments.

After equilibration, arterial segments were contracted with KCl (80 mM) to verify viability of preparations. After washing out KCl, endothelium integrity was assessed by contracting the segments with phenylephrine (PE, 1 μM; α_1-adrenergic receptor agonist), followed by stimulation with acetylcholine (ACh; 1 μM; an endothelium-dependent vasodilator). Concentration-response curves to ACh (0.001–10 μM) were obtained in aorta or MA after precontraction with PE (1 μM). Then, following construction of control concentration-response curves to ACh in unilaterally nephrectomized or DOCA WT mice, tissues were washed several times, incubated with an ABH (100 μM, 60 min), and then a second curve was generated. Cumulative concentration-response curve to sodium nitroprusside (SNP, 0.0001–3 μM; a NO donor) were also performed in aorta and MA precontracted with PE. Additionally, concentration-response curves to PE (0.001–100 μM) were also performed in aorta or MA.

VASCULAR ARGINASE ACTIVITY ASSAY

Aorta and MA were collected and frozen in liquid nitrogen. Tissues were pulverized, homogenized in ice-cold lysis buffer (combined 1:4 w/v with 50 mM, Tris-HCl, 100 μM, EDTA, and EGTA, pH 7.5) containing protease inhibitor, PMSF, phosphatase inhibitors cocktail 2 and 3. Homogenates were sonicated and centrifuged at 14,000 × *g* for 20 min at 4°C and supernatants were collected for enzyme assay. Twenty-five microliters of supernatants in triplicate were added to 25 μL of Tris-HCl 121 (50 mM, pH 7.5) containing $MnCl_2$ (10 mM) and the mixture were activated by heating for 10 min at 55–60°C. ARG activity was assayed by measuring urea production from L-arginine as previously described (19).

Additionally, aortas from WT Sham or DOCA mice were treated with ABH (100 μM) for 60 min, then collected and frozen for ARG activity assay.

WESTERN BLOT ANALYSIS

Protein (20 μg) extracted from aortas were separated by electrophoresis on a 10% SDS-polyacrylamide pre-cast gel and transferred to polyvinylidene difluoride membrane. Non-specific binding sites were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline/Tween for 1 h at 24°C. Membranes were incubated with primary antibodies (anti-ARG1, BD Transduction Laboratories, 1:1000; anti-ARG2, Santa Cruz Biotechnology, Inc., 1:250, Cell Signaling Technology, Inc.) overnight at 4°C. After incubation with secondary antibodies, signals were visualized using an enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA), and quantified by densitometry. Results are normalized to total actin protein and expressed as arbitrary unit.

CORONARY PERIVASCULAR FIBROSIS

Hearts were embedded in paraffin blocks after fixation in 10% formalin. Paraffin-embedded sections (5 μm thick) were deparaffinized with xylene and rehydrated by immersion in a graded series of ethanol washes. Sections were stained by Picrosirius red following manufacturer's protocol (Accustain Kit, Sigma-Aldrich). Collagen deposition around the coronary vessels was detected by red staining. The area of collagen staining relative to the vessel surface area was quantified using ImageJ (NIH). Perivascular fibrosis data are expressed as the collagen-to-vessel surface area ratio.

DRUGS AND SOLUTIONS

Physiological saline solution of the following composition was used: (in mM: NaCl, 118; $NaHCO_3$, 25; glucose, 5.6; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7\ H_2O$, 1.17; and $CaCl_2 \cdot 2\ H_2O$, 2.5). ACh,

SNP, PE, phosphatase inhibitor cocktail 1 and 2 and protease inhibitor were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ABH was obtained as a gift from Dr. Daniel Berkowitz. All of the reagents were of analytic grade. Stock solutions were prepared in deionized water.

DATA ANALYSIS

Results are presented as mean ± SEM, and n represents the number of animals used in the experiments. Relaxation or contraction values were calculated relative to the maximal changes from the contraction produced by PE and KCl, respectively, taken as 100% in each tissue. Concentration-response curves were fitted using a non-linear interactive fitting program (Graph Pad Prism 4.0; GraphPad Software Inc., San Diego, CA, USA), and two pharmacological parameters were obtained: the maximal effect generated by the agonist (or E_{max}) and the negative logarithm of the concentration of agonist that produces 50% of the maximum response [−log EC_{50} (or pEC_{50})]. ARG activity data is represented as percent change respective to the control (100%). Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test was used to evaluate the results. $P < 0.05$ was considered significant.

RESULTS

BODY AND HEART WEIGHT

The body weight of WT and ARG genotype control mice and treatment groups ranged from about 25–27 g and did not differ among them (**Table 1**). The heart weight/body weight ratio was elevated in the DOCA-salt treatment groups of WT and $ARG2^{-/-}$ mice vs. their respective Sham controls (**Figure 1A**). However, this ratio was not different between DOCA and $ARG1^{+/-}$ Sham mice.

SYSTOLIC BLOOD PRESSURE

No differences in SBP measurements were observed among control WT and arginase KO mice before beginning the experiment at 12 weeks of age; SBP values ranged from 95 ± 5 to 104 ± 4 mmHg. After 2 weeks of DOCA treatment, SBP values for WT, $ARG1^{+/-}$, and $ARG2^{-/-}$ mice were higher than their Sham control (**Figure 1B**). During the third through the sixth week of DOCA-salt treatment, SBP values in WT and $ARG2^{-/-}$ mice were steadily increased, whereas SBP in $ARG1^{+/-}$ mice fell back toward control levels over this period (109 ± 4 vs. 101 ± 3 mmHg, respectively). Of particular note, SBP values for $ARG2^{-/-}$ Sham mice rose progressively and slightly less than those for $ARG2^{-/-}$ DOCA mice over the 6-week period, reaching values close to those

Table 1 | Body weight (g) did not differ among WT, $ARG1^{+/-}$, and $ARG2^{-/-}$ mice in uninephrectomized (WT Sham) or DOCA-salt treated mice.

	WT Sham	WT DOCA	$ARG1^{+/-}$ Sham	$ARG1^{+/-}$ DOCA	$ARG2^{-/-}$ Sham	$ARG2^{-/-}$ DOCA
Body weight (g)	27 ± 2	27 ± 1	26 ± 3	25 ± 2	27 ± 2	26 ± 2
Aorta E_{max} (%)	98 ± 1	101 ± 3	97 ± 1	93 ± 2	96 ± 2	95 ± 3
Aorta pEC_{50}	8.26 ± 0.07	8.37 ± 0.09	8.14 ± 0.09	8.09 ± 0.07	8.10 ± 0.07	8.17 ± 0.06
MA E_{max} (%)	100 ± 2	103 ± 2	101 ± 2	99 ± 1	101 ± 1	98 ± 2
MA pEC_{50}	8.40 ± 0.05	8.51 ± 0.06	8.33 ± 0.08	8.26 ± 0.09	8.43 ± 0.05	8.37 ± 0.09

The maximal effect (E_{max}) and potency (pEC_{50}) values obtained from concentration-response curves to a nitric oxide donor, sodium nitroprusside (SNP, 0.0001–3 μM) in aorta and second-order branches of mesenteric arteries (MA) from WT, $ARG1^{+/-}$, and $ARG2^{-/-}$ mice in uninephrectomized (WT Sham) or DOCA-salt treated mice. Data represent the means ± SEM of four experiments.

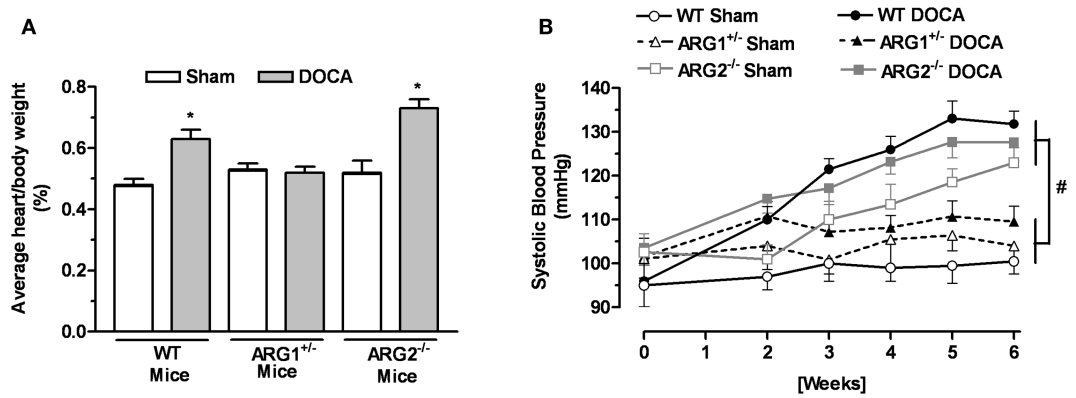

FIGURE 1 | Profile of arginase knockout (KO) mice in DOCA-salt treatment. Ratio between heart and body weight **(A)** and systolic blood pressure (SBP) **(B)** of WT uninephrectomized (WT Sham), WT uninephrectomized and DOCA-salt treated (WT DOCA), partial arginase 1 ($ARG1^{+/-}$) KO uninephrectomized ($ARG1^{+/-}$ Sham), $ARG1^{+/-}$ KO and DOCA-salt treated ($ARG1^{+/-}$ DOCA), arginase 2 ($ARG2^{-/-}$) uninephrectomized ($ARG2^{-/-}$ Sham), and $ARG2^{-/-}$ DOCA-salt treated mice. $^{*}P < 0.05$, compared with its control Sham mice. $^{\#}P < 0.05$, comparing upper and lower sets of SBP levels.

for WT-DOCA mice. A progressive rise of SBP in non-treated ARG2$^{-/-}$ mice has been reported (20).

VASCULAR ARGINASE ACTIVITY AND EXPRESSION

Arginase activities were elevated in DOCA WT aorta (1.5-fold) and MA (2.6-fold) compared to those of Sham WT mice (**Figures 2A,B**). Treatment with ABH (100 μM) reduced these elevations in vessels from WT mice. In ARG1$^{+/-}$ mice, DOCA treatment did not elevate ARG activity in aorta (**Figure 2A**) or MA (**Figure 2B**) vs. those of Sham ARG1$^{+/-}$ mice. In ARG2$^{-/-}$ DOCA mice, ARG activity was elevated in MA (by 1.55-fold), but not in aorta, compared to ARG2$^{-/-}$ Sham tissue (**Figures 2A,B**). Elevation of ARG activity induced by DOCA treatment in WT vessels was greater than observed in ARG1$^{+/-}$ aorta and ARG2$^{-/-}$ aorta and MA, but was not different from that in ARG 2$^{-/-}$ DOCA aorta.

Parallel experiments assessing protein levels of ARG1 showed significant increases in the DOCA WT aorta by 1.49-fold (**Figure 2C**) and in the MA by 1.73-fold (**Figure 2D**) compared to WT Sham. Protein levels of ARG1 were not increased in aorta or MA from DOCA ARG1$^{+/-}$ mice, but were elevated in aorta of DOCA ARG2$^{-/-}$ mice compared to their Sham tissue (**Figures 2C,D**). In DOCA WT mice, ARG2 protein was markedly increased in MA (by 2.15-fold) (**Figure 2D**). ARG 2 expression was not detected in WT aorta. Basal ARG1 expression in vessels from ARG2$^{-/-}$ mice was elevated above that of WT vessels.

Treatment of WT-DOCA aorta with ABH did not prevent elevation of ARG1 protein (not shown). These results indicate that ARG1 protein levels and activity are elevated by DOCA treatment.

VASOCONTRACTILE RESPONSES TO PHENYLEPHRINE

Concentration-response curves to PE were performed to determine ARG genotype-related regulation of vascular reactivity to contractile stimuli. Aortas from WT-DOCA-salt mice displayed a greater vasoconstriction to PE compared with WT Sham tissues [maximum efficacy (E_{max}) of 138 ± 8 and 114 ± 6%, respectively]. Contractile responses to PE were lower in the ARG1$^{+/-}$ Sham (E_{max}: 93 ± 6%) compared with those of WT Sham group, but were elevated in ARG1$^{+/-}$ DOCA mice to the level of WT Sham mice (**Figure 3A**). Aortas from ARG2$^{-/-}$ mice exhibited similar PE-induced contraction to those exhibited by aortas from WT Sham and DOCA-salt mice (**Figure 3B**).

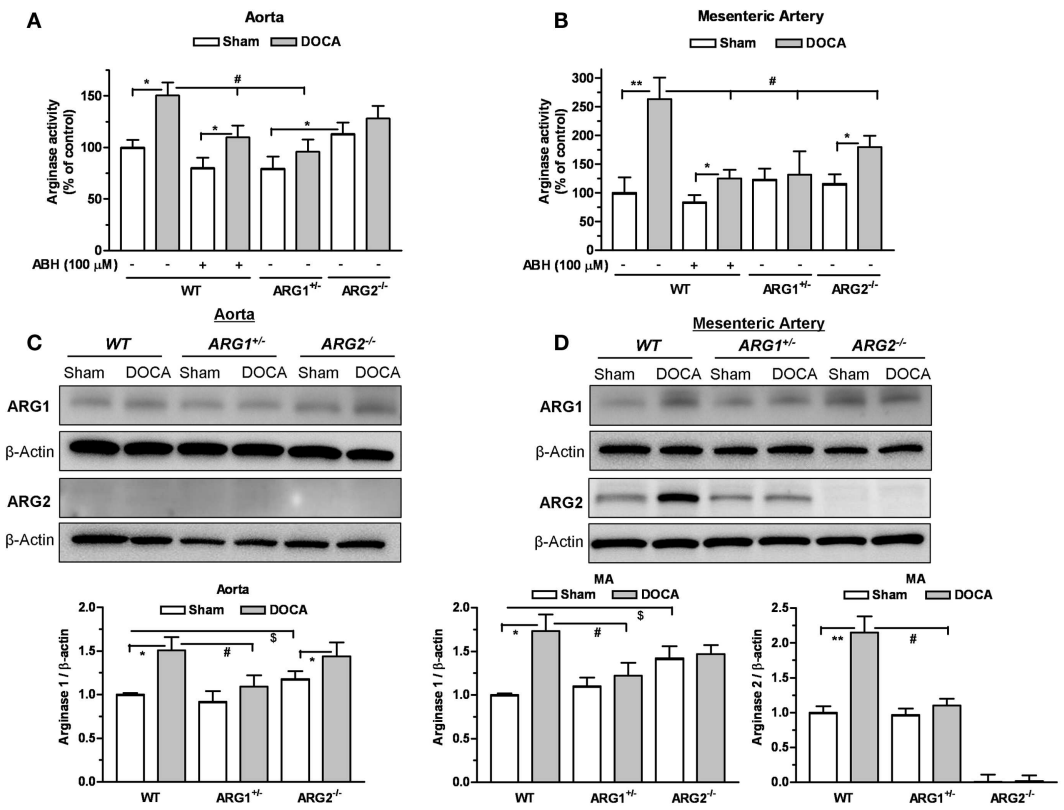

FIGURE 2 | Partial deletion of arginase 1 (ARG1$^{+/-}$) or inhibition of arginase prevents DOCA-salt-induced increase in vascular arginase activity/expression. Arginase activity measured in aorta **(A)** and mesenteric artery (MA) **(B)** in WT uninephrectomized (WT Sham), WT uninephrectomized and DOCA-salt treated (WT DOCA), partial arginase 1 (ARG1$^{+/-}$) knockout uninephrectomized (ARG1$^{+/-}$ Sham), ARG1$^{+/-}$ and DOCA-salt treated (ARG1$^{+/-}$ DOCA), arginase 2 (ARG2$^{-/-}$) uninephrectomized (ARG2$^{-/-}$ KO Sham), and ARG2$^{-/-}$ KO DOCA-salt treated mice. Pretreatment with an inhibitor of arginase (ABH, 100 μM) prevented elevation of arginase activity in aorta and MA in WT-DOCA-salt treated mice **(A,B)**. Measurement of protein expression of ARG1 and ARG2 in aorta **(C)** and MA **(D)** of animals treated with Sham or DOCA-salt treated WT, ARG1$^{+/-}$, or ARG2$^{-/-}$ mice. Arginase activity in WT Sham group was considered as 100%. Data represents mean ± SEM of five to seven experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with its respective Sham group. $^{\#}P < 0.05$, compared with WT-DOCA group. $^{\$}P < 0.05$, compared with WT Sham group.

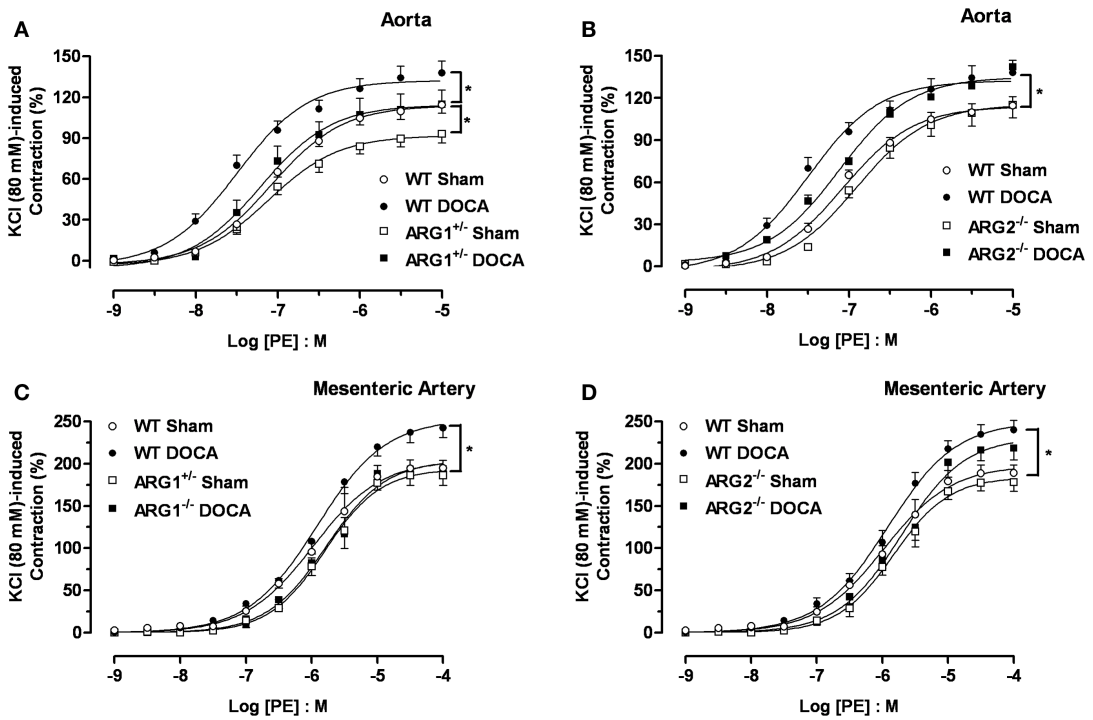

FIGURE 3 | Differences in contractile responses induced by phenylephrine (PE) in DOCA-salt treated arginase knockout (KO) mice. Concentration-response curves to PE (0.001–10 μM) in WT uninephrectomized (WT Sham), WT uninephrectomized and DOCA-salt treated (WT DOCA), partial arginase 1 (ARG1$^{+/-}$) KO uninephrectomized (ARG1$^{+/-}$ Sham), ARG1$^{+/-}$ and DOCA-salt treated (ARG1$^{+/-}$ DOCA), arginase 2 (ARG2$^{-/-}$) uninephrectomized (ARG2$^{-/-}$ Sham) and ARG2$^{-/-}$ DOCA-salt treated mice in aorta **(A,B)**, and mesenteric artery [MA; **(C,D)**]. Experimental values were calculated relative to the maximal changes from the contraction produced by KCl (80 mM), which was taken as 100%. Data represents mean ± SEM of five experiments. *$P < 0.05$, compared with WT Sham group.

As in aorta, the maximal contractile response to PE was higher in MA from WT DOCA than in WT Sham MA (E_{max}: 243 ± 12 and 195 ± 9%, respectively). However, no differences in PE-induced contraction were observed in MA between WT Sham and ARG1$^{+/-}$ Sham or DOCA mice (**Figure 3C**). Contractions induced by PE in MA were similar between WT Sham and ARG2$^{-/-}$ Sham and between WT-DOCA and ARG2$^{-/-}$ DOCA groups (**Figure 3D**).

VASORELAXATION RESPONSES TO ACETYLCHOLINE AND SODIUM NITROPRUSSIDE

Endothelial dysfunction is a well-established feature of the DOCA-salt hypertensive animal model (21, 22). To determine the effect of ARG genotype on vascular function, we compared vasorelaxation responses to ACh (0.001–10 μM) in aorta from WT, ARG1$^{+/-}$, or ARG2$^{-/-}$ mice after 6 weeks of DOCA-salt with those in Sham mice. Similar aortic vasorelaxation responses to ACh were observed between WT and ARG1$^{+/-}$ Sham mice (E_{max}: 68 ± 2%; pEC_{50}: 7.42 ± 0.09 and E_{max}: 70 ± 5%; pEC_{50}: 7.50 ± 0.07 for WT and ARG1$^{+/-}$ mice, respectively, **Figure 4A**). DOCA-salt markedly reduced maximal responses to ACh in WT aorta (E_{max}: 55 ± 4%) compared to their Sham control (E_{max}: 68 ± 2%), whereas vasorelaxation responses to ACh in ARG1$^{+/-}$ Sham and DOCA-salt vessels were not different (E_{max}: 70 ± 5 and 65 ± 2%, respectively) (**Figure 4A**). Aorta from ARG2$^{-/-}$ Sham mice showed similar E_{max} values (63 ± 4%) but displayed impaired sensitivity (pEC_{50}) to ACh compared with those of WT Sham mice (pEC_{50}: 6.94 ± 0.09 and 7.42 ± 0.09 for vessels of ARG2$^{-/-}$ and WT Sham, respectively). Aortas from ARG2$^{-/-}$ DOCA mice exhibited an impaired ACh-induced maximum vasorelaxation vs. their sham control (pEC_{50}: 6.54 ± 0.07; E_{max}: 52 ± 5%) which was not different from that of WT-DOCA aorta (**Figure 4B**).

The MA of WT-DOCA mice displayed impaired vasorelaxation to ACh (E_{max}: 78 ± 3%) compared to WT Sham (E_{max}: 95 ± 3%), but this impairment was absent in MA of ARG1$^{+/-}$ mice Sham and DOCA mice (E_{max} 93 ± 2 and 92 ± 2%, respectively) (**Figure 4C**). The MA from the ARG2$^{-/-}$ DOCA mice exhibited impairment of endothelial cell (EC)-dependent vasorelaxation similar to that of MA from WT-DOCA mice (**Figure 4D**). As in aorta, there tends to be impaired relaxation to ACh in ARG2$^{-/-}$ Sham MA.

Acute treatment with ABH (100 μM) significantly enhanced the E_{max} to ACh in aortas (**Figure 5A**) and MA (**Figure 5C**) from WT-DOCA mice (from 60 ± 5 to 79 ± 3% and from 76 ± 3 to 88 ± 2%, for aorta and MA, respectively). However, ABH did not alter the vasorelaxation to ACh in the WT Sham groups (**Figures 5B,D**).

Endothelium-independent relaxations in aorta or MA induced by the NO donor, SNP were not different between uninephrectomized (WT Sham) and DOCA-salt treatment in WT, ARG1$^{+/-}$, and ARG2$^{-/-}$ mice, respectively (**Table 1**).

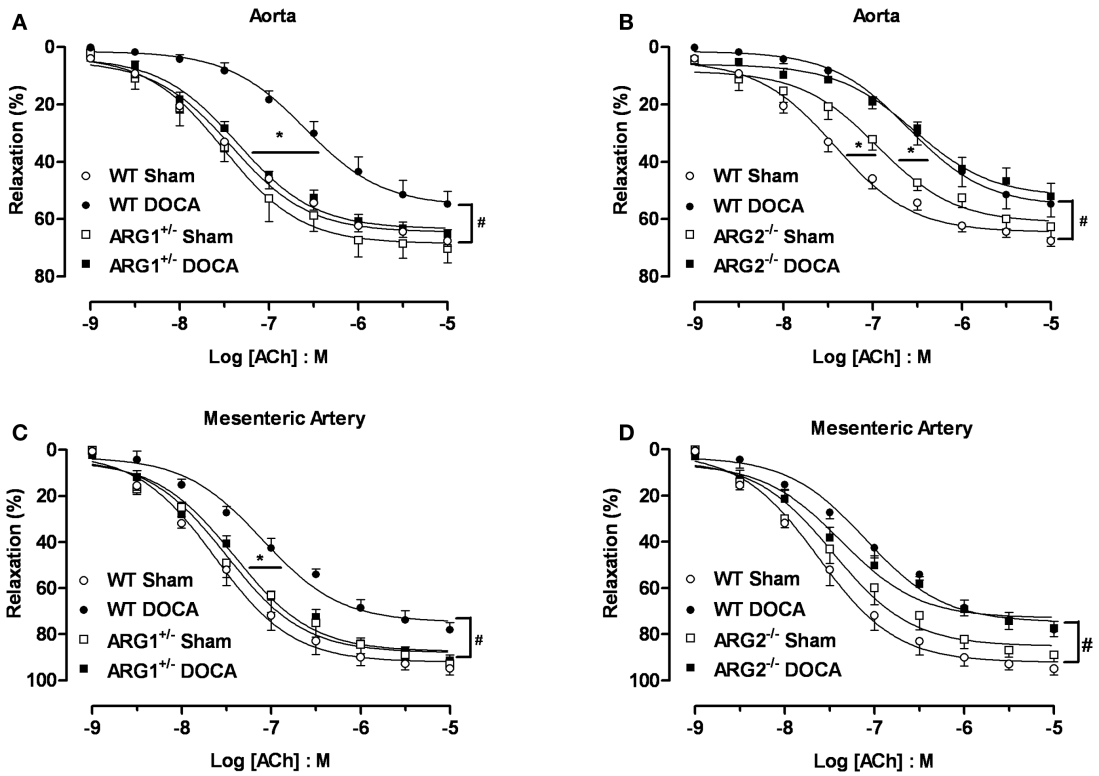

FIGURE 4 | Partial deletion of arginase 1 (ARG1$^{+/-}$) prevents DOCA-salt-induced endothelial dysfunction in aorta and mesenteric artery (MA). Concentration-response curves to acetylcholine (ACh, 0.001–10 μM) in aorta **(A,B)** and MA **(C,D)** from WT, ARG1$^{+/-}$, or ARG2$^{-/-}$ mice in uninephrectomized (WT Sham) or DOCA-salt treated mice. Data were calculated relative to the maximal changes from the contraction produced by phenylephrine (PE, 1 μM), which was taken as 100%. Data are means ± SEM of six to eight experiments. $^{*}P < 0.05$, indicates differences in pEC_{50} values of the dose-response curves. $^{\#}P < 0.05$, compared with WT Sham group.

CORONARY PERIVASCULAR FIBROSIS

Fibrosis was assessed as the amount of tissue collagen around the coronary arteries. WT-DOCA mice exhibited enhanced coronary perivascular fibrosis, as evident by the increased picrosirius red stain of collagen around the coronary vessel compared with control WT mice (**Figure 6A**). Importantly, collagen staining was not increased in ARG1$^{+/-}$ DOCA mice compared to Sham control. Additionally, the ratio of coronary perivascular fibrosis to total vessel surface area increased markedly for WT-DOCA (2.1-fold) and significantly, but to a lesser extent (1.22-fold) in the ARG2$^{-/-}$ DOCA vs. Sham mice, while no significant alteration in this ratio was observed in DOCA ARG1$^{+/-}$ or Sham mice (**Figure 6B**). Our data indicate that increase ARG activity is associated with increased collagen production.

DISCUSSION

The major findings of this study are the contributions of arginase (ARG1 and ARG2) to elevated blood pressure, impaired EC-dependent vasorelaxation, increased vasoreactivity to constrictor stimuli, and enhancement of coronary perivascular fibrosis in a model of DOCA-salt hypertension. Several important observations have been made in our study. First, we show that SBP levels in ARG1$^{+/-}$ DOCA do not rise as they do in WT-DOCA mice. The rise in SBP in WT-DOCA mice is similar to that reported by others (23, 24). In contrast, both ARG2$^{-/-}$ Sham and DOCA mice exhibited progressive elevation in SBP, with a slightly greater rise in DOCA mice. A progressive hypertension has been reported for ARG2 KO mice (20). Second, vascular ARG activity is increased in aorta and second-order resistance MA from WT-DOCA-salt mice. Reduction or lack of the ARG1 or ARG2 genes prevented the DOCA-induced increase in aortic ARG activity, but only in ARG1$^{+/-}$ aorta was activity lower than in WT aorta. Third, in WT-DOCA mice, protein levels of ARG1 are increased in aorta and both ARG1 and ARG2 are up-regulated in MA. Fourth, DOCA-induced impairment of endothelium-dependent vasorelaxation in aorta and MA is prevented in ARG1$^{+/-}$ DOCA mice, and partially so in ARG2$^{-/-}$ compared with its Sham control. These findings indicate that both ARG isoforms contribute to DOCA-induced vascular dysfunction, to varying degrees. Fifth, augmented contractile responses to the α-1-adrenergic agonist PE occur in aorta and MA from DOCA treated WT, ARG1$^{+/-}$, and ARG2$^{-/-}$ mice. Sixth, coronary perivascular fibrosis in DOCA-salt mice is prevented or reduced by deletion of ARG1$^{+/-}$ or ARG2$^{-/-}$, respectively.

Our findings indicate that enhancement of vascular ARG activity induced by ARG1 isoform has a key role in salt-induced hypertension. Salt sensitivity is associated with almost half of

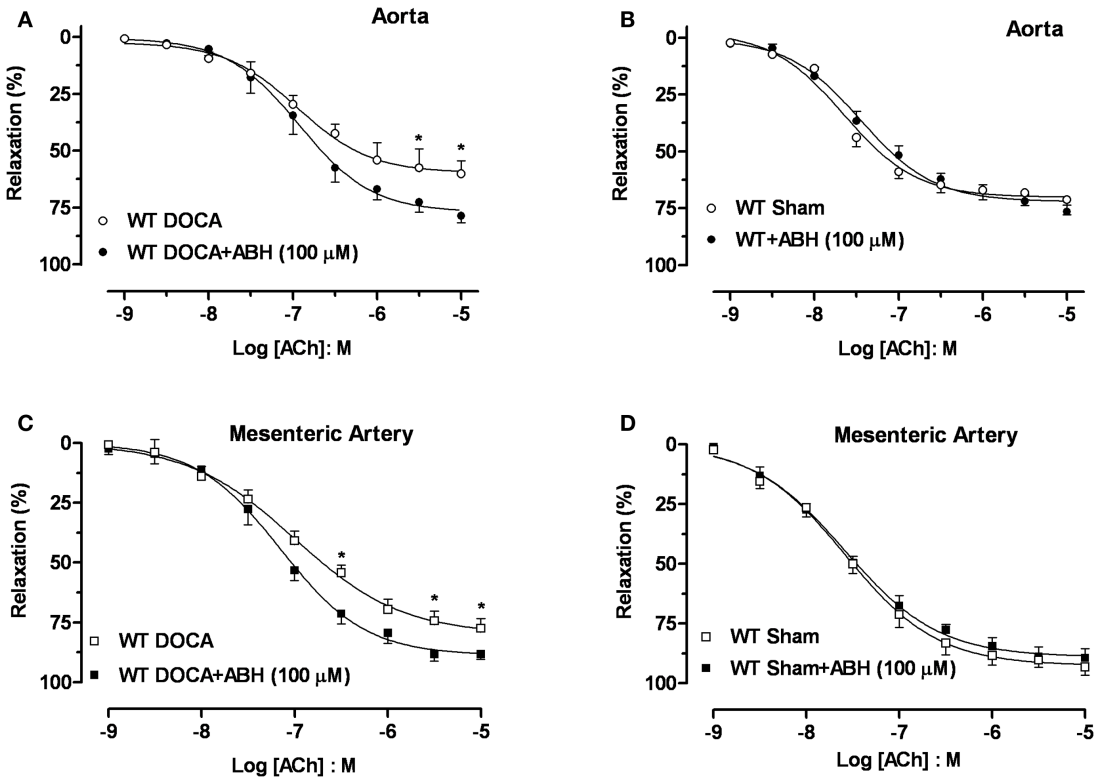

FIGURE 5 | Inhibition of arginase ameliorates DOCA-salt-induced endothelial dysfunction. Treatment with an inhibitor of arginase (ABH, 100 μM; 60 min) prevented vascular endothelial dysfunction in DOCA-salt treated mice in aorta **(A)** and mesenteric artery [MA; **(C)**]. WT Sham vessels exposed to ABH showed no change in the ACh-induced vasorelaxation in aorta **(B)** and MA **(D)**. Data were calculated relative to the maximal changes in contraction produced by phenylephrine (PE, 1 μM), which was taken as 100%. Data are means ± SEM of four experiments. $*P < 0.05$, compared with WT-DOCA + ABH group.

the cases of human hypertension (25). DOCA-salt hypertension is volume-dependent and is accompanied by low plasma renin-angiotensin system activity. The combination of DOCA-salt and unilateral nephrectomy result in hypertension and cardiac and renal hypertrophy. Increased vascular ARG has been linked in animal model for hypertension (3, 15, 16). The two isoforms of ARG have been shown to have different intracellular and tissue distributions (4, 7, 26). Depending on the disease state and tissue, ARG1, ARG2, or both may be elevated and exert prominent actions. Earlier studies have demonstrated up-regulation of both ARG isoforms in gracilis muscle arterioles and in aorta from Dahl-salt-sensitive (16) and spontaneously hypertensive rat (27). However, only ARG1 is reported to be increased in aorta from DOCA-salt rats (15), coronary arteries from pigs with aortic coarctation (13) and MA from genetic hypertensive rats (2). Our data show that absence of one copy of ARG1 gene prevents rises in SBP, indicating its involvement in DOCA hypertension. The rise in blood pressure in $ARG2^{-/-}$ mice complicates assessment of ARG2's role in DOCA hypertension. Absence of both ARG2 gene copies partially reduced but did not prevent elevation of SBP in DOCA-salt mice.

Phenylephrine-induced contractions in aorta from $ARG1^{+/-}$, but not $ARG2^{-/-}$, Sham mice were less than WT Sham mice, suggesting that activity of ARG1, but not ARG2, decreases aortic endothelial NO release in response to PE. A reduced contractile response was not observed in MA of $ARG1^{+/-}$ Sham mice, possibly due to activity of ARG2. Importantly, contractile responses in aorta of all the mouse genotypes were enhanced by DOCA treatment, indicating that the DOCA-induced enhanced contraction did not involve either ARG isoform.

Deoxycorticosterone acetate-induced vascular endothelial dysfunction was largely absent in aorta and MA of $ARG1^{+/-}$ mice. DOCA-induced dysfunction in $ARG2^{-/-}$ was less pronounced vs. its sham controls, as the $ARG2^{-/-}$ sham displayed a degree of impairment vs. WT sham. Thus, this isoform may contribute to vascular endothelial dysfunction. An important question is, how does lack of ARG2 cause this dysfunction.

Arginase can compete with NOS for the common substrate L-arginine in the vasculature. Increased vascular ARG activity/expression and decreased NO production have been observed in hypertension and diabetes (3, 10, 13, 19). The products of ARG action on L-arginine are urea and ornithine (28). Elevated ornithine levels from excessive ARG activity also could contribute to pathological vascular fibrosis and thickening by increasing the formation of polyamines and proline from ornithine. Polyamines and proline promote cell growth and

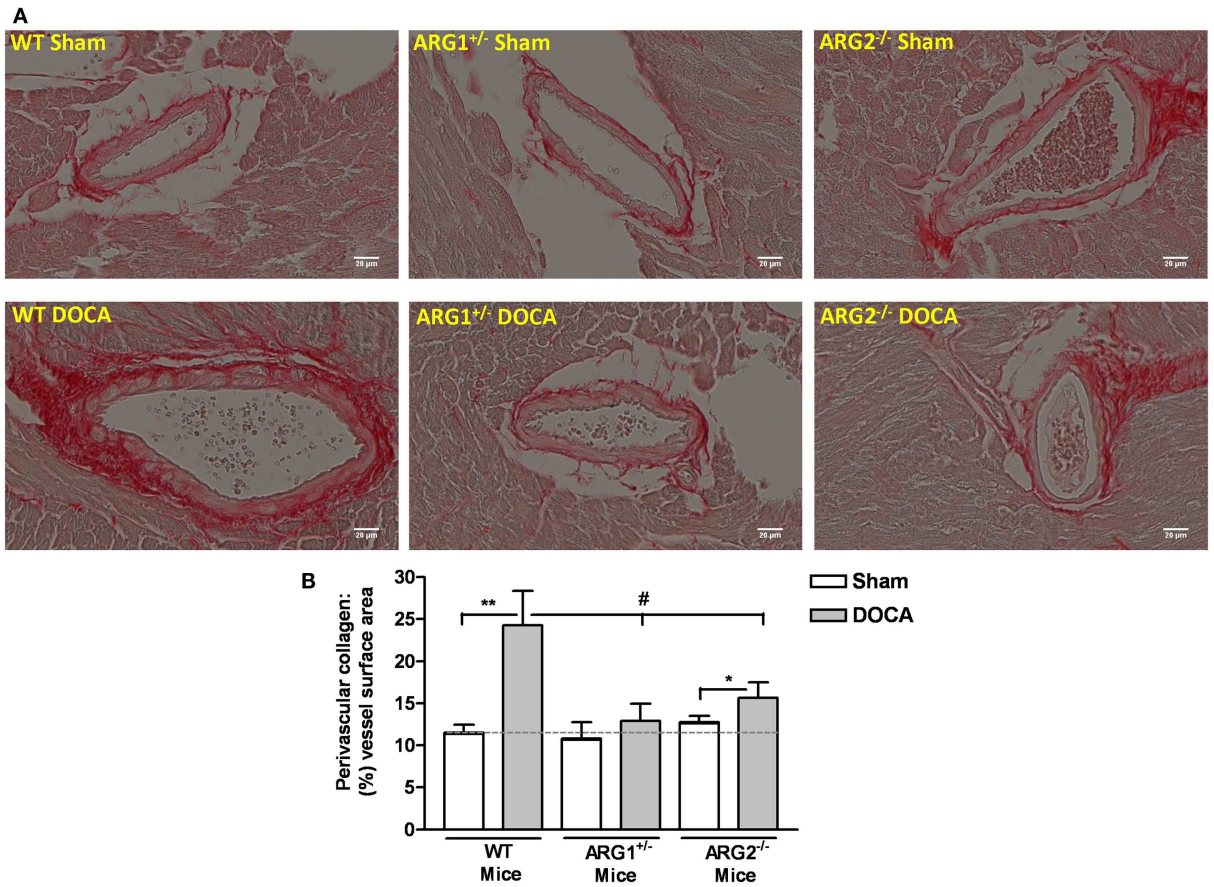

FIGURE 6 | Partial deletion of one copy of arginase 1 (ARG1) prevents coronary perivascular fibrosis in DOCA-salt treated mice. Assessment of coronary perivascular fibrosis from WT, ARG1$^{+/-}$, and ARG2$^{-/-}$ mice in uninephrectomized (WT Sham) or DOCA-salt treated mice using picrosirius red staining (red color for collagen). **(A)** Shows a representative photograph of left ventricular paraffin-embedded tissue sections. Quantitative ratio of the perivascular collagen area to the vessel wall surface area is provided in **(B)**. Data are means ± SEM of three to four experiments. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with its Sham group. $^{\#}P < 0.05$, compared with WT-DOCA group.

collagen formation, respectively (29, 30). Our findings demonstrate that DOCA-salt induces cardiac hypertrophy and increases perivascular collagen deposition in WT mice, and that these effects are absent in ARG1$^{+/-}$ mice. Lack of ARG2 also reduces DOCA-induced perivascular fibrosis. Long term treatment of SHR with an ABH has been shown to reduce blood pressure and cardiac fibrosis (2). Our data suggest that increased ARG1 mediates cardiac hypertrophy and perivascular fibrosis through increased synthesis of polyamines and proline. Elevated blood pressure/vascular resistance also contribute to cardiac hypertrophy. Earlier studies have demonstrated that hypertension, diabetes, atherosclerosis, and aging are associated with elevated vascular stiffness/decreased arterial compliance, which is recognized as an important and independent cardiovascular risk factor (31). Further studies are needed to determine whether reduction of ARG1 gene expression diminishes vascular stiffness via decreased levels of ornithine, polyamines, and proline.

The pathogenesis of DOCA-salt hypertension in conduit arteries is reported to involve increased reactive oxygen species (ROS) production (32). Superoxide and peroxynitrite contribute to increased vascular ARG activity, decreased L-arginine availability to eNOS and its uncoupling, reduced NO production, and even greater levels of these ROS (33, 34). Both peroxynitrite and hydrogen peroxide increase endothelial ARG expression/activity and inhibition of NADPH oxidase blocks this action of hydrogen peroxide (34). NOS uncoupling also would limit the production of N^{G}-hydroxyl-L-arginine (NOHA), an intermediate product in the generation of NO and a potent inhibitor of ARG. Inhibition of NADPH oxidase by apocynin has been reported to prevent elevation of ARG activity and largely prevent a lipopolysaccharide (LPS)-induced increase in ARG1 mRNA in rat alveolar macrophages (35). Additionally, exposure of cultured rat retinal cells to LPS also strongly induces expression of ARG1, which is markedly reduced by apocynin co-treatment (36). It is probable that ROS are involved in the elevation of ARG1 activity and expression observed in our study.

In our model, DOCA treatment progressively increased SBP in WT and ARG2$^{-/-}$ mice. Moreover, SBP also rose in the Sham ARG2$^{-/-}$ mice verses Sham WT mice. Huynh et al. (20)

have previously reported a progressive rise in blood pressure in ARG2$^{-/-}$ KO mice along with increased plasma levels of the norepinephrine (NE) precursor (dihydroxyphenylalanine) and its metabolite (dihydroxyphenylglycol). Their data suggest that sympathetic nervous activity is increased in the cardiovascular system and responsible for elevated blood pressure in these ARG2$^{-/-}$ mice. Further study is needed to more clearly define how ARG2 regulates blood pressure.

In summary, our results showed that ARG is involved in the pathogenesis of hypertension. Using a genetic approach, we conclude that both ARG isoforms contribute to vascular endothelial dysfunction in DOCA-salt hypertension.

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Selective up-regulation of arginase-1 in coronary arteries of diabetic patients

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Coronary artery disease (CAD) remains the leading cause of death in the Western societies. Diabetes mellitus (DM) is one of the highly prevalent diseases, which remarkably accelerates the development of CAD. Experimental evidence indicates that decreased bioavailability of coronary endothelial nitric oxide (NO) contributes to the development of CAD in DM. There are recent studies showing that a selective impairment of NO synthesis occurs in coronary arteries of DM patients, which is mainly due to the limited availability of endothelial NO synthase (eNOS) precursor, l-arginine. Importantly, these studies demonstrated that DM, independent of the presence of CAD, leads to selective up-regulation of arginase-1. Arginase-1 seems to play an important role in limiting l-arginine availability in the close proximity of eNOS in vessels of DM patients. This brief review examines recent clinical studies demonstrating the pathological role of vascular arginase-1 in human diabetes. Whether arginase-1, which is crucial in the synthesis of various fundamental polyamines in the body, will represent a potent therapeutic target for prevention of DM-associated CAD is still debated.

Keywords: diabetes mellitus, nitric oxide, arginase, endothelium, coronary artery

DIABETES LEADS TO A REDUCED AVAILABILITY OF NO IN CORONARY ARTERIES

Diabetes mellitus is associated with an increased incidence of cardiovascular diseases accounting for significant morbidity and mortality in the diabetic population. Coronary artery disease (CAD) is one of the key manifestations of diabetes-associated vascular disease, a pathology, which predisposes diabetic patients to myocardial ischemia. The underlying mechanism(s) of CAD remain incompletely understood in human diabetes, so that effective preventive therapeutic strategies cannot be adopted in diabetic patients.

The coronary flow-reserve, as defined by the ratio of coronary flow under maximal agonist-induced vasodilation to coronary flow under resting conditions, is reduced in diabetic patients, even in the absence of significant stenosis of epicardial coronary arteries (1). Nemes et al. have demonstrated that patients with type 2 diabetes exhibit a reduced coronary flow-reserve (2), a condition, which was found to be associated with increased incidence of future ischemic episode in the heart of diabetic patients (3). Patients with diabetes exhibit endothelial dysfunction, which is characterized by impaired flow- and acetylcholine (ACh)-induced relaxation of brachial artery (4) and forearm resistance vessels (5). Nitenberg et al. have demonstrated that coronary artery dilation is impaired in diabetic patients with angiographically normal coronaries (6). Kaneda et al. performed a study, in which 165 patients underwent intracoronary injection of ACh and found that diabetes was the strongest predictor for ACh-induced coronary vasospasm (7). This and other studies concluded that diabetes is associated with impaired dilator function of coronary arteries and this is manifested as a reduced vasodilator or even vasoconstrictor responses (8–10).

Previous studies have shown that animals with experimental insulin resistance and diabetes exhibit a reduced NO-mediated, agonist-induced dilation of cerebral, mesenteric, coronary, and skeletal muscle microvessels (11–16). Studies from our laboratory demonstrated that in rodent models of type 2 diabetes coronary arteries exhibit impaired ACh-induced dilation, which is primarily due to the reduced synthesis and/or bioavailability of nitric oxide (NO) (17–20). Katakam et al. have shown that prior to the impaired ACh-mediated vasodilation NO-mediated coronary dilation to insulin is reduced in obese Zucker rats (14). This seems particularly important as insulin and insulin-like growth factor I have shown to promote NO-mediated vasodilation (21).

Oxidative stress occurring in response to hyperglycemia and insulin resistance (14, 22–28) is considered to be one of the key factors leading to the reduced NO-dependent vasodilation. To support this scenario, oral administration of the antioxidant vitamin-C prevented the decreases in methacholine-induced brachial artery dilations in patients with diabetes (29). However, other studies failed to detect any beneficial effect of antioxidant therapy in the prevention of diabetes-induced vascular complications (30, 31). For instance, vitamin-E supplementation for 8 weeks did not restore the reduced ACh- and bradykinin-induced dilations of brachial arteries in diabetic patients (32). These aforementioned observations raised questions about the efficacy of antioxidant therapy in preventing diabetes-related endothelial dysfunction. To solve the apparent controversy recent studies propose a crucial role for reactive nitrogen species in the development of diabetes-related vascular complications (33). The rate

constant for the reaction between superoxide anion and NO is three to fivefold greater than the rate of superoxide anion scavenged by superoxide dismutase (34). Given that NO via interacting with superoxide anion generates various reactive nitrogen species, such as the highly reactive peroxynitrite ($ONOO^-$). $ONOO^-$ has numerous detrimental effects in the cardiovascular system and plays a crucial role in the development of diabetes-induced vascular pathology (33). $ONOO^-$ is a powerful oxidizing agent that causes rapid depletion of sulfhydryl groups, causes DNA damage, protein oxidation, and nitration of aromatic amino acid residues in proteins, specifically leading to 3-nitrotyrosin formation (33). Although the endogenous cellular mechanisms to prevent the deleterious effect of $ONOO^-$ are not clearly defined recent preclinical studies suggest that a more selective targeting of $ONOO^-$ holds considerable potential than the use of conventional antioxidants (35).

DEFICIENCY OF eNOS COFACTORS IN CORONARY ARTERY DISEASE

It is known that an adequate level of substrates and cofactors for NO synthases, such as L-arginine (36) and tetrahydrobiopterin (BH_4) is essential for NO synthesis (37, 38). Diabetes has been shown to interfere with the availability of these cofactors thereby leading to a diminished NO synthesis. To provide experimental evidence for this scenario Ihlemann et al. demonstrated that in healthy humans, oral glucose challenge-induced reduction in forearm blood flow is restored by pre-treatment with BH_4 (39). Co-infusion of BH_4 and the endothelial NO synthase (eNOS) precursor, L-arginine into the forearm of diabetic patients prevented ischemia reperfusion-induced endothelial dysfunction in the brachial artery (40). In isolated coronary arterioles of patients with atherosclerosis Tiefenbacher et al. has shown earlier that *in vitro* administration of the stable BH_4 analog, sepiapterin enhanced dilation in response to agonist (41). In a recent study oral BH_4 treatment in patients undergoing heart surgery although augmented total biopterin levels had no significant effects on dilator function of conduit vessels owing to systemic and vascular oxidation of BH_4 (42). This latter study warranted the need of further investigations aiming at effectively restoring eNOS cofactor, BH_4 levels. Underlying mechanism(s) responsible for the reduced vascular availability of BH_4 is not entirely understood in diabetes. It has been shown that $ONOO^-$ directly interacts and reduces the level of BH_4, as it has greater affinity for BH_4 than that of ascorbic acid and glutathione (43). Chen et al. demonstrated that exposure of human eNOS to $ONOO^-$ resulted in a dose-dependent loss of activity with a marked destabilization of the eNOS dimer (44). In addition, Ishii et al. has shown that insulin is a potential stimulator of BH_4, primarily via activating phosphatidylinositol 3-kinase (45). Due to the apparent lack of insulin action expression and activity of GTP cyclohydrolase-I is reduced in insulin resistance states, which ultimately leads to eNOS uncoupling and diminished NO-mediated dilation of cerebral arteries (46). Whether restoring insulin sensitivity will be associated with "re-coupling" of eNOS has yet to be elucidated.

L-ARGININE TO PREVENT ENDOTHELIAL DYSFUNCTION IN PATIENTS WITH CAD

L-arginine, the substrate for NO synthase, is the precursor for NO synthesis in the vascular endothelium. Earlier clinical studies indicated that administration of L-arginine may enhance NO bioavailability and dilate coronary arteries (47). For example, intra-coronary infusion of L-arginine in patients with CAD attenuated the vasoconstrictor response to intra-coronary ACh and increased coronary blood flow (48). Lerman et al. studied the effect of long-term administration of L-arginine (9 g/day) on patients with non-obstructive coronary disease and found a markedly improved coronary vasodilator response to ACh (49). In contrast, in 30 patients with CAD L-arginine therapy while significantly increased L-arginine plasma levels had no effect on NO bioavailability and flow-mediated dilation of brachial artery (50). Moreover, there is another clinical study, which questioned the effectiveness of L-arginine therapy in patients with CAD (50). More importantly, in the Vascular Interaction With Age in Myocardial Infarction (VINTAGE MI) study by Schulman et al. L-arginine supplementation significantly increased mortality in patients with myocardial infraction leading to early termination of the trial with the recommendation that L-arginine supplementation not to be used in patients with myocardial infarction (51). The underlying mechanism responsible for the controversial and harmful effects of L-arginine in this particular patient population remains elusive.

Limited number of studies is available to evaluate the acute and long-term effects of L-arginine treatment in patients with diabetes. In a recent large cohort, involving 2236 patients recruited within the LUdwigshafen RIsk and Cardiovascular Health (LURIC) study, patients with type 2 diabetes had a significantly lower L-arginine availability than patients without diabetes (52). A study has shown that L-arginine treatment (8.3 g/day for 21 days) improved endothelial dilator function and increased insulin sensitivity in patients with type 2 diabetes (53). The authors concluded that L-arginine exerted its beneficial effects through reducing fasting and postprandial glucose levels and normalizing adiponectin/leptin ratio (53). Thus, some evidence indicates that in diabetic patients the level of L-arginine is reduced and administering L-arginine may improve endothelial function. Whether this effect is mediated directly via enhancing the vascular availability of NO or indirectly via increasing insulin sensitivity has yet to be elucidated. Taken together, it is possible that diabetic patients may benefit from L-arginine supplementation, but several important questions still remain open including the safety and efficacy of L-arginine treatment in diabetic patients with concomitant CAD, especially in those with prior myocardial infarction.

DOES VASCULAR ARGINASE-1 SELECTIVELY UP-REGULATED IN HUMAN DIABETES?

It is known that the Km of NO synthase for L-arginine is about 2.9 μM. The intracellular concentration of L-arginine ranges from 0.1 to 1.0 mM. Given that it seemed intriguing how administration of L-arginine would increase the bioavailability of NO – the arginine-paradox. To solve this apparent controversy previous studies have shown that up-regulation of arginase, the focal

enzyme of the urea cycle, via hydrolyzing L-arginine reduces NO synthesis and, importantly, may contribute to the development of various vascular diseases (36). Detailed pathological role(s) for arginase-1 and -2 in various disease models as well as the subcellular mechanisms behind is extensively discussed in other review articles in this thematic issue. In this review we examine those few existing clinical studies that focused on altered arginase expression and its vascular consequences in man.

Vascular endothelial cells metabolize L-arginine mainly by arginase, which exists as two distinct isoforms, arginase-1 and -2. Arginase-1 is predominantly expressed in the liver and to a much lesser extent in other cell types, such as vascular endothelial cells, whereas expression of arginase-2 is more widespread (54). Circulating arginase-1 level was found significantly higher in patients with heart failure, when compared to controls; and the level of circulating arginase-1 further increased with the severity of heart failure (significant increase between NYHA I/II and NYHA III/IV groups) (55). When sublingual microcirculation was assessed by dark field intravital microscopy in heart failure patients the authors found that topical administration of the arginase inhibitor, nor-NOHA increased capillary density, a measure of tissue perfusion, in an NO-dependent manner (55). In patients with essential hypertension Holowatz and Kenney have found an attenuated NO-dependent reflex cutaneous vasodilatation, which is enhanced by arginase inhibitors, BEC, and nor-NOHA, but not with L-arginine supplementation (56). These studies provided functional evidence for the role of circulating and also tissue-expressed arginase-1, which interferes with NO-mediated tissue perfusion.

Both arginase-1 and -2 are expressed in the human heart (54). In a study by Chen et al. right atrial appendage was obtained from 13 patients undergoing heart surgery for coronary artery bypass graft (CABG) with three-vessel CAD and from 13 patients with valve replacement (non-CAD group). In the whole atrial homogenates there was a significantly reduced protein expression of both eNOS and arginase-1 in patients with CAD. Of note that in this study the CAD group had higher proportion of diabetic patients (11 out of 13), as compared to the control group (3 out of 13). Given that, the individual impact of CAD and diabetes affecting expression of arginase-1 cannot be examined in this study (57). In our recent study (10) small coronary arteries were dissected from the atrial appendage of 21 patients without and 20 patients with diabetes. Protein expression of arginase-1 in small coronary arteries was significantly higher in patients with diabetes. Arginase-1 expression was abundant in endothelial cells and was co-localized with eNOS in coronary vessels of diabetic patients, but not in non-diabetics. We demonstrated that inhibition of arginase, with L-NOHA caused restoration of endothelium-dependent, ACh-induced dilation in coronary arterioles of diabetic patients. Although we proposed an effect of diabetes causing arginase-1 up-regulation, it is of note that in the study diabetic patients exhibited a significantly greater proportion of CAD (16 out of 20 versus 9 out of 20 patients), therefore the conclusion regarding the selective up-regulation of arginase-1 in coronary arteries of diabetic patients is severely limited. In a very recent study by Shemyakin et al. the independent impact of diabetes in affecting arginase-1 expression and its functional consequence was evaluated (58). In 16 patients with CAD and 16 patients with CAD and type 2 diabetes endothelium-dependent and endothelium-independent increases in forearm blood flow were assessed during intra-arterial infusion of the arginase inhibitor, nor-NOHA. While forearm blood flow was significantly lower in both CAD and CAD plus diabetes groups, when compared to age-matched control group, nor-NOHA markedly increased blood flow with NO-dependent manner, with a significantly greater extent in patients with concomitant diabetes (58). This key observation provided functional evidence for the selectively up-regulated arginase-1 in diabetic patients, *in vivo*.

Taken together, limited number of clinical studies suggest a selective up-regulation of arginase-1, which may impair dilator function of conduit and resistance vessels in diabetic patients, independent of the presence of concomitant CAD. Further studies involving higher number of research subjects are needed to assess the independent impact of increased arginase-1 expression on coronary artery responsiveness in diabetes. Moreover, it should be noted that arginase inhibitors, nor-NOHA, and L-NOHA used in these studies do not have selectivity toward arginase isoforms (arginase-1 versus arginase-2). Thus, the functional role for arginase-2 in the development of vasomotor dysfunction cannot be entirely excluded. In this regard, a previous study has found that increased expression of arginase-2 leads to decreased NO synthesis in pulmonary endothelial cells of patients with pulmonary arterial hypertension (59). The pathological role of arginase isoforms in diabetes-related coronary microvascular dysfunction also has yet to be elucidated.

POSSIBLE MECHANISMS LEADING TO SELECTIVE UP-REGULATION OF ARGINASE-1 IN DIABETIC PATIENTS

The underlying mechanism(s) leading to selective up-regulation of arginase-1 in coronary arterioles in diabetic patients remains elusive. One obvious possibility is the known action of insulin,

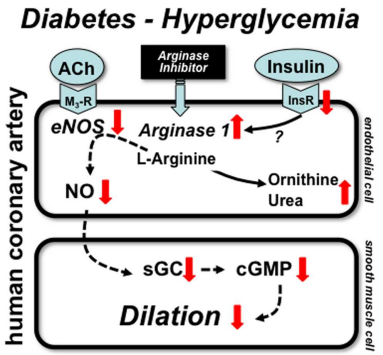

FIGURE 1 | Mechanisms of coronary artery dysfunction in diabetes. Schematic draw demonstrates proposed mechanisms by which diabetes and hyperglycemia lead to up-regulation of arginase-1 resulting in reduced NO-mediated dilation in human coronary arteries. Alterations in insulin signaling may contribute to arginase up-regulation, which reduces the level of l-arginine and thereby limit NO synthesis. ACh: acetylcholine, M_3-R: M_3 muscarinic receptor, InsR: insulin receptor, sGC: soluble guanylate cyclase, cGMP: cyclic guanosine monophosphate.

which suppresses expression and activity of enzymes of urea synthesis pathway. Since insulin signaling is impaired in type 2 diabetes (patients likely to exhibit insulin resistance) it is possible that the failure of insulin regulatory action contributes to up-regulation of arginase-1. In the clinical setting diabetic patients are on insulin sensitizing and oral anti-diabetic medication or commonly take insulin. For instance, in the study by Shemyakin et al. 31 and 56% of patients with CAD plus diabetes were on insulin and biguanides/sulfonylureas, respectively (58). In our aforementioned study all patients with diabetes had either anti-diabetic medication or were on insulin (10). Therefore, the effect of insulin resistance and the concomitant action of exogenous insulin is difficult to examine in these investigations. Also, due to the limited number of diabetic patients involved in the aforementioned studies further studies are warranted to ascertain the role of insulin and other pathological factors that could contribute to increased arginase-1 expression in diabetes. In this context, in a previous elegant study Kashyap et al. found that plasma arginase activity is increased in type 2 diabetic patients with reduced activity of eNOS in the skeletal muscle. Interestingly, these changes were detected without alterations in the plasma protein levels of arginase-1 and -2. Importantly, the increased arginase activation was correlated with the degree of hyperglycemia and was markedly reduced by 4-h insulin infusion in diabetic patients, but not in non-diabetics (60). This clinical study demonstrated the pathological role for high glucose in inducing, whereas for exogenous insulin in reducing arginase activation. The exact molecular mechanisms remained unclear. The proposed mechanisms by which diabetes and hyperglycemia lead to up-regulation of arginase-1 resulting in reduced NO-mediated dilation in human coronary arteries is depicted in **Figure 1**.

In summary, recent findings, as highlighted in this brief review underline the need for the investigations exploring the underlying mechanisms responsible for up-regulated arginase in human diabetes. Clinical studies emphasize the importance of those investigations that strive to elucidate the vascular effects of specific arginase inhibitors, including their long-term efficacy and safety in diabetic patients with CAD.

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Arginase promotes skeletal muscle arteriolar endothelial dysfunction in diabetic rats

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Endothelial dysfunction is a characteristic feature in diabetes that contributes to the development of vascular disease. Recently, arginase has been implicated in triggering endothelial dysfunction in diabetic patients and animals by competing with endothelial nitric oxide synthase for substrate L-arginine. While most studies have focused on the coronary circulation and large conduit blood vessels, the role of arginase in mediating diabetic endothelial dysfunction in other vascular beds has not been fully investigated. In the present study, we determined whether arginase contributes to endothelial dysfunction in skeletal muscle arterioles of diabetic rats. Diabetes was induced in male Sprague Dawley rats by streptozotocin injection. Four weeks after streptozotocin administration, blood glucose, glycated hemoglobin, and vascular arginase activity were significantly increased. In addition, a significant increase in arginase I and II mRNA expression was detected in gracilis muscle arterioles of diabetic rats compared to age-matched, vehicle control animals. To examine endothelial function, first-order gracilis muscle arterioles were isolated, cannulated in a pressure myograph system, exposed to graded levels of luminal flow, and internal vessel diameter measured. Increases in luminal flow (0–50 μL/min) caused progressive vasodilation in arterioles isolated from control, normoglycemic animals. However, flow-induced vasodilation was absent in arterioles obtained from streptozotocin-treated rats. Acute *in vitro* pretreatment of blood vessels with the arginase inhibitors N^{ω}-hydroxy-nor-L-arginine or *S*-(2-boronoethyl)-L-cysteine restored flow-induced responses in arterioles from diabetic rats and abolished differences between diabetic and control animals. Similarly, acute *in vitro* pretreatment with L-arginine returned flow-mediated vasodilation in vessels from diabetic animals to that of control rats. In contrast, D-arginine failed to restore flow-induced dilation in arterioles isolated from diabetic animals. Administration of sodium nitroprusside resulted in a similar degree of dilation in arterioles isolated from control or diabetic rats. In conclusion, the present study identifies arginase as an essential mediator of skeletal muscle arteriolar endothelial dysfunction in diabetes. The ability of arginase to induce endothelial dysfunction in skeletal muscle arterioles may further compromise glucose utilization and facilitate the development of hypertension in diabetes.

Keywords: arginase, arginine, diabetes, endothelial dysfunction, flow-mediated vasodilation

INTRODUCTION

Diabetes is a progressive metabolic disease that is characterized by an elevation in circulating glucose related to either insulin deficiency (type 1 diabetes) or insulin resistance (type 2 diabetes). Diabetes and its associated complications are a growing concern and represent a serious worldwide public health problem. Vascular disease is the principal cause of morbidity and mortality in patients with diabetes (Kannel and McGee, 1979; Winer and Sowers, 2004). Accelerated atherosclerosis of the large arteries results in increased risk of myocardial infarction, stroke, and limb amputation while microvascular disease is a leading cause of blindness, nephropathy, and neuropathy (Beckman et al., 2002; Porta and Bandello, 2002; Goldberg, 2003; Kikkawa et al., 2003; Duby et al., 2004). Abnormal endothelial function is a salient feature of vascular disease in diabetes that is exemplified by a decrease in nitric oxide (NO) synthesis or bioavailability. In response to shear stress or receptor stimulation, NO is produced by endothelial NO synthase (eNOS) through the oxidation of its substrate, L-arginine. The release of NO by endothelial cells plays a critical role in preserving vascular homeostasis by inhibiting vascular tone, platelet aggregation, leukocyte recruitment and infiltration into the vessel wall, and smooth muscle cell proliferation and migration (see Loscalzo and Welch, 1995; Forstermann and Sessa, 2012). Endothelial dysfunction, including blunted NO-dependent vasodilatory responses, has been documented in patients and animals with diabetes, and is believed to be an important contributor to the pathogenesis of diabetic vascular disease (Durante et al., 1988; Hattori et al., 1991; Tesfamariam and Cohen, 1992; Johnson et al., 1993; Nitenberg et al., 1993).

Although many factors have been implicated in triggering endothelial malfunction, recent studies have identified arginase as a novel mediator of endothelial dysfunction. Arginase is a metalloenzyme that hydrolyzes L-arginine to urea and L-ornithine. There are two distinct isoforms of arginase, arginase I and II, which are encoded by separate genes and share approximately 60% sequence homology (Dizikes et al., 1986; Vockley et al., 1996). Both isoforms of arginase are expressed in the vasculature but their expression is both vessel- and species-dependent (see Durante et al., 2007; Morris, 2009). Arginase elicits endothelial dysfunction by competing with eNOS for substrate L-arginine leading to a deficiency of L-arginine and diminished NO synthesis. Recent work from a number of laboratories has implicated arginase in provoking endothelial dysfunction in several pathological states, including arterial hypertension, pulmonary hypertension, atherosclerosis, aging, and hemorrhagic shock (Xu et al., 2004; Demougeot et al., 2005; Johnson et al., 2005, 2010; Ryoo et al., 2008; Kim et al., 2009). In a seminal study, Romero et al. (2008) demonstrated increased vascular arginase I expression and activity in streptozotocin-treated rats and that arginase contributes to endothelial dysfunction in coronary arteries in this animal model of type 1 diabetes. In addition, it was found that arginase stimulates endothelial dysfunction in myocardial microvessels in type 2 diabetic rats and in coronary arterioles isolated from patients with type 2 diabetes (Beleznai et al., 2011; Gronros et al., 2011). Subsequently, arginase was also shown to impair endothelial function in the aorta, retinal arteries, and corpora cavernosa of streptozotocin-induced type 1 diabetic animals (Toque et al., 2011; El-Bassossy et al., 2012; Romero et al., 2012; Elms et al., 2013). However, the involvement of arginase in mediating endothelial dysfunction in other vascular beds of diabetic animals is not known.

A majority of the studies examining the contribution of arginase to endothelial dysfunction in diabetes utilize acetylcholine to test the vasoactive function of the endothelium. However, a physiological role for acetylcholine in the local regulation of vascular resistance has not been established. Furthermore, in many vascular beds a large portion of acetylcholine-mediated vasodilation is NO-independent (Bolz et al., 1999). Thus, the use of acetylcholine may not fully reveal the nature and physiologic importance of endothelial dysfunction in diabetes. A major *in vivo* stimulus for the synthesis and release of NO by endothelial cells is luminal flow which functions to continuously modulate arterial diameter via changes in shear stress. In order to more fully evaluate the role of arginase in promoting endothelial dysfunction in type 1 diabetes, we determined the expression of arginase I and II in skeletal muscle arterioles in rats treated with streptozotocin or vehicle. In addition, we examined endothelial function in these arterioles in response to a highly relevant physiologic stimulus: luminal flow. Finally, the response of these arterioles to an endothelium-independent vasodilator was also assessed.

MATERIALS AND METHODS

MATERIALS

L-Arginine, D-arginine, glycerol, sodium dodecyl sulfate (SDS), Triton X-100, Tris, sodium acetate, streptozotocin, sodium fluoride, heparin, and sodium nitroprusside were from Sigma-Aldrich (St. Louis, MO, USA). Aprotinin and leupeptin were from Roche Applied Sciences (Indianapolis, IN, USA). N^{ω}-hydroxy-nor-L-arginine (L-OHNA) and *S*-(2-boronoethyl)-L-cysteine (BEC) were purchased from EMD Biosciences (San Diego, CA, USA). [*Guanido*-^{14}C]L-arginine (52 Ci/mmol) was from Amersham Life Sciences (Arlington Heights, IL, USA). All other chemicals were obtained from Fisher Scientific (Houston, TX, USA). Sodium nitroprusside (10 mM) stock solutions were prepared in saline and diluted in modified Krebs buffer immediately before use. L-OHNA (100 μM) and BEC (100 μM) were dissolved in Krebs buffer just before use. The composition of the modified Krebs buffer was (in mM) 118.5 NaCl, 4.7 KCl, 1.4 $CaCl_2$, 1.2 KH_2PO_4, 1.1 $MgSO_4$, 25.0 $NaHCO_3$, and 11.1 dextrose.

ANIMAL MODEL

Adult male Sprague Dawley rats between 12 and 14 weeks of age were purchased from Charles River Laboratories (Wilmington, MA, USA). Diabetes was induced by a single injection of streptozotocin (65 mg/kg, ip) dissolved in sodium citrate (50 mM). Non-diabetic, control animals were injected with an equivalent volume of vehicle. Animals were fed standard rat chow, had free access to drinking water, and were used four weeks after streptozotocin or vehicle administration. All experiments conform to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996) and were approved by the institutional care and use committee.

HEMODYNAMIC AND METABOLIC MEASUREMENTS AND TISSUE EXTRACTIONS

Four weeks after the streptozotocin or vehicle administration, rats were weighed and injected intraperitoneally with ketamine (100 mg/kg) and zylazine (7.5 mg/kg) (Butler Schein Animal Health Corporation, Dublin, OH, USA), and a carotid arterial catheter implanted for blood sample collection and blood pressure measurement. Blood samples were drawn for immediate determination of blood glucose (Accu-Chek Compact, Roche Diagnostics, Indianapolis, IN, USA), glycated hemoglobin (HbA1c, DCA 2000+ Analyzer, Bayer, Pittsburgh, PA, USA), and cholesterol (CardioChek PA Analyzer, Polymer Technology Systems, Inc., Indianapolis, IN, USA). Blood pressure was measured using a pressure transducer (TSD 104A, Biopac Systems, Santa Barbara, CA, USA) coupled to a polygraph system (Biopac Systems, Santa Barbara, CA, USA) and a personal computer. Animals were then heparinized (1000 U/kg, iv) and the thoracic aorta and gracilis anticus muscles removed and placed into ice-cold modified Krebs buffer or frozen in liquid nitrogen and stored at −70°C for later use.

ARGINASE ACTIVITY

Arginase activity was determined by monitoring the formation of [^{14}C]urea from [*guanido*-^{14}C]L-arginine, as we previously reported (Peyton et al., 2009). Blood vessels were sonicated in Tris buffer (10 mm, pH 7.4) containing Triton X-100 (0.4%), leupeptin (10 mg/mL), and aprotinin (10 mg/mL). Lysates (100 μg) were added to an equal volume of Tris buffer (10 mM, pH 7.4) containing $MnCl_2$ (10 mM) and arginase was activated by heating for 10 min at 56°C. The arginase reaction was initiated by adding Tris buffer containing L-arginine (10 mM) and [*guanido*-^{14}C]L-arginine (0.25 Ci), and samples were incubated at 37°C for

30 min. Reactions were terminated by adding ice-cold sodium acetate buffer (250 mM, pH 4.5) containing urea (100 mM). [^{14}C]Urea was separated from basic amino acids by Dowex chromatography and [^{14}C]urea formation determined by scintillation counting.

ARGINASE EXPRESSION

Arginase expression was determined by quantitative real-time PCR. Total RNA was isolated from gracilis muscle arterioles using TRIzol reagent and quantified by absorbance spectroscopy. cDNA was synthesized with 1 μg of RNA using iScript cDNA synthesis kits (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR was carried out using a SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and arginase primers using the Bio-Rad CFX96 system (Sasatomi et al., 2008). Thermal cycling was performed at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Reactions were performed in triplicate. Relative expression of arginase was analyzed using the delta–delta Ct method and results normalized with respect to 18S rRNA.

ISOLATED MICROVESSEL EXPERIMENTS

Segments of first-order gracilis muscle arterioles were isolated by microdissection and cannulated at both ends with glass micropipettes in a vessel chamber (Living Systems Instrumentation, Burlington, VT, USA). The vessel chamber was continuously perfused with Krebs buffer equilibrated with a gas mixture of 14% O_2 and 5% CO_2, balanced with N_2, in a non-recirculating system. For internal diameter measurements, the vessel chamber was mounted on a stage of an inverted microscope (TS 100-F, Nikon Instruments, Melville, NY, USA) fitted with a CCD video camera. The camera was connected to a personal computer equipped with video dimensioning software (ImagePro Express, Media Cybernetics, Bethesda, MD, USA). Images were collected at 1 frame/s and stored as digital files for documentation.

To study agonist-induced dilation, the proximal micropipette was connected to a pressure servo controller (Living Systems Instrumentation, Burlington, VT, USA) and the distal micropipette was connected to a closed stopcock to achieve 80 mmHg constant luminal pressure with no flow. Following a 60 min stabilization period, peak responses of arterioles to cumulative additions of sodium nitroprusside (10–1000 nM) were determined by adding the drug to the superfusion buffer. To study flow-induced dilation, both the proximal and distal micropipettes were connected to pressure servo controllers and to an inline micro flowmeter (Living Systems Instrumentation, Burlington, VT, USA). During a 60 min stabilization period, both proximal and distal pressures were adjusted to 80 mmHg with no luminal flow. In order to establish graded levels of luminal flow (0–50 μL), proximal and distal pressures were adjusted equally in the opposite direction maintaining midline pressure at 80 mmHg. In some experiments, arginase inhibitors or arginine was added to the superfusion buffer 20 min before arterioles were exposed to luminal flow. In order to limit the possible loss of vascular L-arginine from vessels perfused with buffer devoid of L-arginine that may potentially mask differences between the two groups of animals, the duration of experiments was restricted to a single flow-response curve per vessel.

STATISTICS

Results are expressed as mean ± SEM. Statistical analyses were performed with the use of a Student's two-tailed *t*-test and an analysis of variance with the Tukey *post hoc* test when more than two treatment regimens were compared. *p*-Values <0.05 were considered statistically significant.

RESULTS

Four weeks after administration of streptozotocin, rats develop overt diabetes as reflected by elevated fasting blood glucose and glycated hemoglobin levels compared to vehicle-treated control animals (**Table 1**). In addition, body weights of streptozotocin-diabetic rats were significantly lower than control animals. However, there was no significant difference in blood pressure, heart rate, or circulating cholesterol levels between control and diabetic rats. Vascular arginase activity was markedly increased in diabetic rats by nearly twofold (**Figure 1A**). The rise in aortic arginase activity in diabetic animals was associated with a significant increase in the expression of both arginase I and II mRNA in gracilis muscle arterioles (**Figure 1B**).

Endothelial function was examined in isolated skeletal muscle arterioles. Increases in luminal flow (0–50 μL/min) resulted in progressive vasodilation in arterioles isolated from control rats (**Figure 2**). However, flow-mediated vasodilation was absent in arterioles isolated from diabetic animals. In fact, a

Table 1 | Metabolic and hemodynamic parameters of control and diabetic rats.

	Control	Diabetic
Body weight (g)	472 ± 10	288 ± 14*
Blood pressure (mmHg)	122 ± 9	114 ± 12
Heart rate (beats/min)	328 ± 22	302 ± 15
Glucose (mg/dL)	98 ± 8	411 ± 32*
Glycated hemoglobin (%)	3.9 ± 0.1	7.9 ± 0.2*
Cholesterol (mg/dL)	78 ± 6	87 ± 11

Results are means ± SEM (n = 14–17).

**Statistically significant effect of diabetes (p < 0.01).*

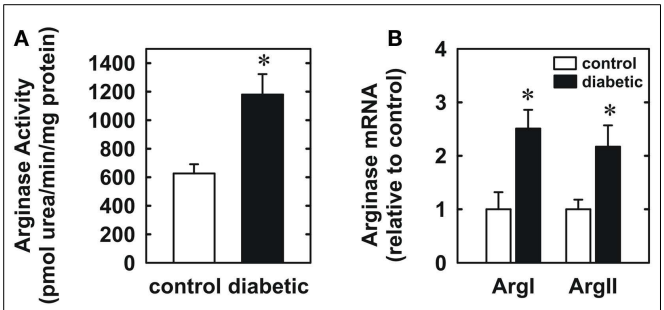

FIGURE 1 | Diabetes stimulates vascular arginase activity and expression in rats. Diabetes increases arginase activity in the aorta **(A)** and arginase I and II mRNA expression in gracilis muscle arterioles **(B)**. Results are expressed as mean ± SEM (*n* = 4–5). *Statistically significant effect of diabetes ($p < 0.05$).

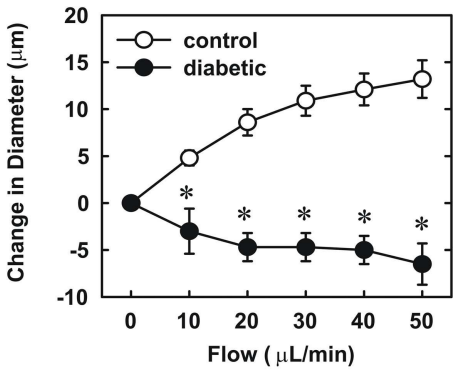

FIGURE 2 | Diabetes abolishes flow-dependent increases in internal diameter of gracilis muscle arterioles. Arterioles were isolated from control (open circles) or diabetic (closed circles) rats and exposed to graded levels of luminal flow. Results are expressed as mean ± SEM ($n = 3$–5). *Statistically significant effect of diabetes ($p < 0.01$).

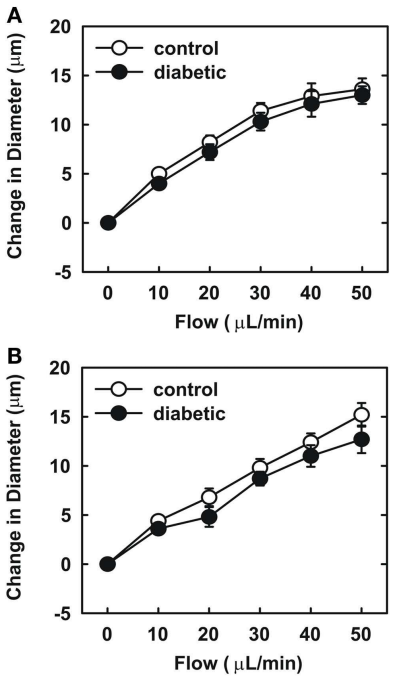

FIGURE 3 | Arginase inhibition restores flow-dependent increases in internal diameter of gracilis muscle arterioles in diabetes. Arterioles isolated from control (open circles) or diabetic (closed circles) rats were treated *in vitro* with the arginase inhibitors nor-NOHA (100 μM) **(A)** or BEC (100 μM) **(B)** and then exposed to graded levels of luminal flow. Results are expressed as mean ± SEM ($n = 5$).

slight vasoconstrictor effect was noted in diabetic vessels subjected to luminal flow. Since arginase expression was elevated in gracilis muscle arterioles of diabetic rats, we examined if arginase was responsible for impairing endothelial function in these animals. Acute *in vitro* pretreatment of blood vessels with the arginase inhibitors, L-OHNA (100 μM) or BEC (100 μM), restored flow-induced responses in arterioles from diabetic animals and abolished differences between the two groups of animals (**Figures 3A,B**). Similarly, acute *in vitro* pretreatment of vessels with the arginase and NO synthase substrate, L-arginine (1 mM), reinstated flow-induced dilation in arterioles obtained from diabetic rats and abrogated the difference between control and diabetic animals (**Figure 4A**). In contrast, D-arginine (1 mM), which is not a substrate for either enzyme, failed to restore flow-mediated responses in arterioles from diabetic rats (**Figure 4B**). Finally, the responsiveness of arteriole smooth muscle to NO was tested by treating vessels with the NO donor, sodium nitroprusside. Sodium nitroprusside dilated arterioles isolated from control or diabetic animals in a similar concentration-dependent manner (**Figure 5**).

DISCUSSION

In the present study, we found that vascular arginase activity is elevated in streptozotocin-treated diabetic rats and this is associated with a significant increase in the expression of arginase I and II in gracilis muscle arterioles. In addition, we discovered that luminal flow-induced vasodilation is severely compromised in gracilis muscle arterioles isolated from diabetic rats while the vasodilator response to the endothelium-independent agonist, sodium nitroprusside, is preserved in these vessels. We also found that acute *in vitro* pretreatment with arginase inhibitors restores flow-induced vasodilation and abolishes the difference between control and diabetic arterioles. Similarly, acute *in vitro* pretreatment with the arginase and eNOS substrate, L-arginine, but not the inactive D isomer, restores flow-mediated vasodilation and eliminates the difference between the two groups of animals. These findings suggest that increased arginase activity contributes to skeletal muscle arteriolar endothelial dysfunction in type 1 diabetes by restricting the availability of L-arginine.

Diabetes was induced in our study by a single injection of streptozotocin. This glucose moiety selectively destroys the insulin-producing β-cells of the pancreas leading to rapid insulin-deficiency and diabetes. This animal model displays polydipsia, polyuria, hyperglycemia, and weight loss that are similar to the clinical symptoms found in diabetic patients (Wei et al., 2003; Akbarzadeh et al., 2007). In agreement with this, we found that rats treated with streptozotocin develop frank diabetes 4 weeks after treatment as indicated by raised fasting blood glucose and glycated hemoglobin concentrations and loss in body weight. The significant decline in body weight is more pronounced in adult animals in this model and likely reflects the loss of glucose in the urine and an inability to metabolize carbohydrates and a shift to fat metabolism leading to depletion of fat stores (Hoybergs et al., 2008).

Our finding that vascular arginase activity is increased in type 1 diabetic rats is consistent with recent studies in streptozotocin-treated rats and mice where elevated arginase activity was reported in the liver, aorta, kidney, macrophages, retina, and corpora cavernosa (Romero et al., 2008, 2012; Morris et al., 2011; Toque et al., 2011; Sun et al., 2012; Elms et al., 2013). For the first time, we also show that diabetes induces the expression of both arginase I and II in gracilis muscle arterioles. This novel observation contrasts with earlier studies showing that diabetes selectively stimulates

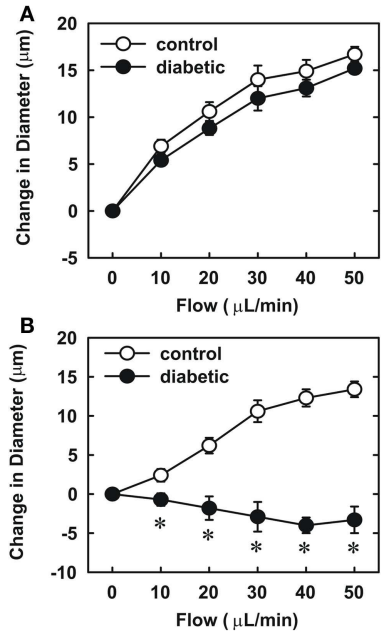

FIGURE 4 | Effect of arginine on flow-dependent increases in internal diameter of gracilis muscle arterioles. Arterioles isolated from control (open symbol) or diabetic (closed symbol) rats were treated *in vitro* with the L-arginine (1 mM) **(A)** or D-arginine (1 mM) **(B)** and then exposed to graded levels of luminal flow. Results are expressed as mean ± SEM ($n = 4$–5). *Statistically significant effect of diabetes ($p < 0.01$).

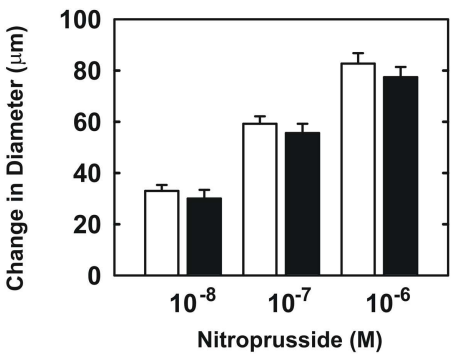

FIGURE 5 | Sodium nitroprusside-mediated increases in internal diameter of gracilis muscle arterioles. Arterioles isolated from control (open symbol) or diabetic (closed symbol) rats were exposed to various concentrations of sodium nitroprusside. Results are expressed as mean ± SEM ($n = 5$).

arginase I or II expression in different tissues, further underscoring the tissue and vessel-dependent pattern of arginase expression (see Durante et al., 2006; Morris, 2009). Interestingly, we previously demonstrated that arginase I and II are also both elevated in rat gracilis muscle arterioles following the development of salt-sensitive hypertension, suggesting that arginase I and II are regulated in a coordinate fashion in these vessels (Johnson et al., 2005). The induction of arginase in diabetes may occur via multiple mechanisms. Since insulin was recently demonstrated to suppress plasma arginase activity in diabetic patients, the reduction or lack of circulating insulin may contribute to the increase in arginase activity in type 1 diabetes (Kashyap et al., 2008). However, hyperglycemia also appears to be involved in the widespread induction of arginase observed in diabetes as exposure of vascular cells to high concentrations of glucose triggers a rise in arginase activity (Durante et al., 2006; Romero et al., 2008). The ability of hyperglycemia to augment arginase activity likely occurs via the RhoA pathway, which can stimulate the induction or activation of both arginase isoforms (Ming et al., 2004; Romero et al., 2008).

We also found that endothelial function is markedly impaired in skeletal muscle arterioles from diabetic rats. Endothelial function was examined using a physiologically relevant stimulus, fluid flow, in first-order gracilis muscle arterioles. We previously reported that flow-mediated vasodilation in gracilis muscle arterioles from normoglycemic rats is completely abolished by eNOS inhibition, indicating that NO is responsible for flow-induced vasodilation in these vessels (Johnson and Johnson, 2008). While arterioles from control rats exhibit pronounced vasodilation in response to luminal flow, flow-induced vasodilation is absent in arterioles isolated from diabetic animals, suggesting that endothelial NO synthesis or bioavailability is dramatically reduced in these vessels. Arterioles from diabetic rats respond as well as arterioles from control animals to the NO donor, sodium nitroprusside, indicating that the sensitivity of arteriolar smooth muscle to NO is unchanged in diabetic animals. Our finding that gracilis muscle arterioles from streptozotocin-treated diabetic rats display a modest vasoconstrictor response to flow has also been reported in mesenteric arteries from these animals and may be linked to diminished NO production (Tribe et al., 1998). To test whether arginase contributes to impaired endothelial function in type 1 diabetes, we employed two distinct arginase inhibitors, L-OHNA and BEC, which are highly potent inhibitors of arginase I and II (Christianson, 2005). We found that acute pretreatment with either L-OHNA or BEC restores flow-induced dilation in arterioles from diabetic rats to levels seen in vessels from control animals. Taken in conjunction with our findings that vascular arginase I and II expression are increased in diabetic arterioles, these results suggest that arginase contributes to gracilis muscle arteriolar NO dysfunction in type 1 diabetes. Our current experimental findings build on previous work in the vasculature of the heart, retina, and corpora cavernosa (Romero et al., 2008, 2012; Toque et al., 2011) and are also supported by a recent clinical study showing arginase inhibition markedly improves endothelium-dependent vasodilation in the forearm of patients with type 2 diabetes and coronary artery disease (Shemyakin et al., 2012). Thus, arginase-mediated endothelial dysfunction may be a characteristic feature of diabetes that encompasses many vascular beds.

Our finding that arginase inhibition restores endothelium-dependent vasodilation of skeletal muscle arterioles from diabetic animals suggests that arginase may modulate L-arginine availability for NO synthesis in diabetes. Consideration of the enzyme kinetics for arginase and eNOS, indicates that arginase can effectively compete with eNOS for substrate L-arginine (Wu and Morris, 1998). Consistent with a role for arginase in depleting substrate for eNOS, vascular L-arginine concentrations

are significantly reduced in streptozotocin-treated diabetic rats (Pieper and Dondlinger, 1997). Moreover, we showed that acute administration of L-arginine mimics the effect of arginase inhibition. L-Arginine restores flow-induced vasodilation in arterioles from diabetic rats whereas the inactive isomer, D-arginine, has no effect. The induction of vascular arginase and the subsequent depletion of L-arginine may also explain the ability of exogenously administered L-arginine to restore endothelial function and NO synthesis in both chemical and genetic animal models of type 1 diabetes (Pieper and Dondlinger, 1997; Pieper et al., 1997; Kohli et al., 2004). Furthermore, arginase-mediated decreases in intracellular L-arginine may negatively impact endothelial function by sensitizing endothelial cells to the endogenous eNOS inhibitor, asymmetric dimethylarginine, which is elevated in streptozotocin-treated rats (Lin et al., 2002). Finally, arginase-mediated depletion of arginine may further compromise endothelial function in diabetes by uncoupling eNOS (Kim et al., 2009).

The ability of arginase to inhibit endothelial function in skeletal muscle arterioles in diabetes is of pathological significance. Skeletal muscle arterioles contribute greatly to peripheral resistance and consequently are major determinants of blood pressure. Thus, arginase-mediated impairment of skeletal muscle arteriolar function may contribute to the development of hypertension in diabetes. Although blood pressure not increased four weeks after streptozotocin administration in our study, a longer duration of diabetes is associated with the development of hypertension in this animal model. Recently, El-Bassossy et al. (2012) reported that diabetic rats develop a significant increase in systolic and diastolic blood pressure 8 weeks following streptozotocin treatment. Notably, administration of arginase inhibitors for the last 6 weeks significantly reduced the developed elevation in diastolic blood pressure in these animals, illustrating a role for arginase in diabetes-associated hypertension. Since skeletal muscle represents a major site of insulin-dependent glucose uptake and utilization, arginase-mediated endothelial dysfunction may limit skeletal muscle perfusion and exacerbate hyperglycemia in diabetes. In support of this proposal, arginase inhibition reduces the rise in serum glucose and advanced glycation end products in type 1 diabetic rats (El-Bassossy et al., 2012), while arginase II deletion improves glucose tolerance and insulin sensitivity in type 2 diabetic mice (Ming et al., 2012). Thus, arginase-mediated endothelial dysfunction of skeletal muscle arterioles may promote the vascular and metabolic derangements observed in diabetes.

In conclusion, the present study identifies arginase as a critical mediator of skeletal muscle arteriolar endothelial dysfunction in diabetes. Flow-induced vasodilation is abolished in gracilis muscle arterioles of streptozotocin-treated rats and this is associated with an increase in arteriolar arginase I and II expression. Moreover, pretreatment of blood vessels with arginase inhibitors or L-arginine fully restores flow-induced vasodilation in arterioles from diabetic rats. These results provide novel insight into the mechanism by which skeletal muscle arteriolar function is compromised in diabetes, and establishes arginase as a potential therapeutic target in treating vascular and metabolic disorders in diabetes.

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Vasomotor regulation of coronary microcirculation by oxidative stress: role of arginase

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Overproduction of reactive oxygen species, i.e., oxidative stress, is associated with the activation of redox signaling pathways linking to inflammatory insults and cardiovascular diseases by impairing endothelial function and consequently blood flow dysregulation due to microvascular dysfunction. This review focuses on the regulation of vasomotor function in the coronary microcirculation by endothelial nitric oxide (NO) during oxidative stress and inflammation related to the activation of L-arginine consuming enzyme arginase. Superoxide produced in the vascular wall compromises vasomotor function by not only scavenging endothelium-derived NO but also inhibiting prostacyclin synthesis due to formation of peroxynitrite. The upregulation of arginase contributes to the deficiency of endothelial NO and microvascular dysfunction in various vascular diseases by initiating or following oxidative stress and inflammation. Hydrogen peroxide, a diffusible and stable oxidizing agent, exerts vasodilator function and plays important roles in the physiological regulation of coronary blood flow. In occlusive coronary ischemia, the release of hydrogen peroxide from the microvasculature helps to restore vasomotor function of coronary collateral microvessels with exercise training. However, excessive production and prolonged exposure of microvessels to hydrogen peroxide impairs NO-mediated endothelial function by reducing L-arginine availability through hydroxyl radical-dependent upregulation of arginase. The redox signaling can be a double-edged sword in the microcirculation, which helps tissue survival in one way by improving vasomotor regulation and elicits oxidative stress and tissue injury in the other way by causing vascular dysfunction. The impact of vascular arginase on the development of vasomotor dysfunction associated with angiotensin II receptor activation, hypertension, ischemia-reperfusion, hypercholesterolemia, and inflammatory insults is discussed.

Keywords: endothelium, superoxide, nitric oxide, inflammation, arterioles, vasodilation

A normal function of the vascular endothelium involving responses to physical (1), chemical (2, 3), and electrical (4, 5) stimuli is essential to maintain microvascular homeostasis and regulate local blood flow by changing vasomotor activity via release of endothelium-derived vasodilators, e.g., nitric oxide (NO), prostacyclin (PGI_2), C-type natriuretic peptide, and hyperpolarizing factors (EDHF). The endothelium also releases vasoconstrictors such as endothelin-1, prostaglandin H/F, thromboxane, and angiotensin. Endothelial dysfunction is one of the earliest markers of vascular abnormalities observed in many cardiovascular diseases associated with oxidative stress due to excessive production of reactive oxygen species (ROS). Redox regulation of proteins by moderate levels of ROS is indispensable for signaling pathways underlying the regulation of subcellular and cellular activity as well as cardiovascular function (6–8). Notably, superoxide and hydrogen peroxide (H_2O_2) are the most common and important ROS involved in the physiological and pathophysiological events (6–8).

Superoxide is produced by several enzyme systems in the cell and it is converted to H_2O_2 by superoxide dismutase. H_2O_2 itself is a potent oxidizing agent that can be converted to hydroxyl radical in the presence of ferric compounds. H_2O_2 can be degraded by catalase to form H_2O and an oxygen molecule. Compared with superoxide, H_2O_2 is stable, lacks charge, has longer half-life, is cell permeable, and can diffuse across longer distances. Therefore, its physical properties are suitable for second-messenger signaling (7, 8). Because a proper delivery of oxygen and nutrients to the tissue is essential for the normal function of an organ, in this review we will discuss the roles of superoxide and H_2O_2 in the physiological and pathophysiological regulation of vasomotor activity of resistance arterioles where blood flow is primarily controlled, with special focus on the coronary microcirculation. The deficiency of endothelium-derived vasodilators such as NO and PGI_2 in relation to oxidative stress and the L-arginine consuming enzyme arginase is discussed.

L-ARGININE, NITRIC OXIDE SYNTHASE, AND ARGINASE

L-Arginine is the precursor for NO synthesis from three different isoforms of NO synthase (NOS). The endothelial NOS (eNOS) is the main isoform contributing directly to the regulation of vasomotor activity. In healthy human adults, it was estimated that 1.2% of arginine flux in the plasma contributes to the formation of NO and about 54% of whole body NO formation is derived from plasma arginine (9), although the fraction of L-arginine flux for NO production in the vasculature is unclear. Experimental

data demonstrate that acute exogenous arginine provision can increase NO production (10, 11) and NO-mediated vasodilation (11, 12) despite the fact that the intracellular arginine level far exceeds the K_m of eNOS (13). It appears that the extracellular L-arginine exerts a significant impact on the synthesis of NO from the membrane-bound eNOS.

Besides NOS, arginase is another major L-arginine consuming enzyme, which converts L-arginine to ornithine and urea. Arginase is expressed most abundantly in the liver for ammonia detoxification via the urea cycle (14). Studies in the cardiovascular system have shown that endothelia (12, 15–18), vascular smooth muscle cells (12, 17, 19), macrophages (20, 21), and red blood cells (22), which do not possess the complete urea cycle enzymes, also express arginase. In humans, about 15% of plasma arginine flux is associated with extrahepatic arginase activity (9). There are two isoforms of arginase. Type 1 arginase (Arg-I) is cytosolic and predominately expressed in the liver. In extrahepatic tissues and cells, a low level of Arg-I expression has also been detected. Type 2, or mitochondrial, arginase (Arg-II) is expressed with low levels in brain, kidney, intestine, red blood cells, and immune cells. Arg-I and -II are derived from distinct genes located on different chromosomes (14) and can be induced or regulated independently by a wide array of agents/factors (23, 24). Although these two arginase isoforms are expressed in a variety of cells, their distribution varies with tissue/organ and cell types (25). In the vasculature, both isoforms of arginase have been identified and their expression is highly regulated for physiological and pathophysiological processes (17) but the relative level of expression may be species dependent (19, 26–28).

Synthesis and release of the vasodilator NO from eNOS, in response to various physiological or pharmacological stimulations, can be related to the substrate bioavailability (10, 11) and thus influence vasomotor activity (11, 12). In this regard, change of protein expression and activity of arginase is expected to have an impact on NO synthesis by affecting the L-arginine level. From the biochemical standpoint, the K_m of arginase for L-arginine in mammals, including humans, is reported to be around 0.5–29 mM (14). Although the K_m of NOS (1–20 μM) (29) is much lower than that of arginase, taking into consideration their V_{max} enzyme activities (1400 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for arginase vs. 900 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for NOS), the arginase is capable of competing with NOS for their substrate arginine (24). Based on the kinetic analysis of these two enzymes, the relative activity of NOS to arginase, in terms of consuming arginine, is diminished with either increasing arginine concentration or decreasing NOS to arginase molar ratio (24). Therefore, arginase activity can exceed NOS activity at higher levels of arginine or at higher arginase to NOS molar concentrations. Interestingly, the competition between NOS and arginase for arginine is more pronounced at lower levels of arginine (24). In terms of functional interpretation, the competitiveness (or importance) of arginase against NOS becomes apparent under conditions with upregulated arginase protein and limited supply of L-arginine.

Interestingly, intravenous administration of arginase causes constriction of cerebral arterioles and enhances platelet aggregation in mice (30), implicating that exogenous arginase may influence endothelial function through attenuation of NO production. However, the direct role of arginase in vasomotor regulation is unclear since the confounding effects from the changes in systemic hemodynamics and neuro-humoral factors cannot be excluded in this *in vivo* preparation. Using an isolated vessel approach, the role of endogenous arginase in vasomotor regulation of NO-mediated vasodilation was demonstrated for the first time in pressurized coronary arterioles (12). It was found that coronary arterioles express Arg-I in both endothelial and smooth muscle cells, and the NO production, as well as NO-mediated vasodilation, is enhanced by inhibiting arginase activity (12). It appears that endogenous arginase plays a counteracting role in the regulation of NO production and thus its associated vasomotor activity. The L-arginine-dependent NO-mediated vasodilation was also observed in various microvascular beds (11, 30–32) including human coronary arterioles (33), suggesting that L-arginine can be a limiting factor for the stimulated NO synthesis in the microcirculation. On the other hand, recent studies on cardiovascular diseases have implicated that upregulation of a specific arginase isoform in the vasculature may contribute to the development of vascular disease linked to L-arginine deficiency and reduced NO production (34, 35), especially under conditions with elevated level of angiotensin II (Ang II), hypertension, and inflammation, all of which are closely associated with oxidative stress (36).

VASOMOTOR REGULATION BY ANGIOTENSIN II

In animal models of hypertension and myocardial hypertrophy, the excessive ROS release associated with renin-angiotensin system activation has been well documented (37, 38). However, the vasomotor action of Ang II in the intact heart is controversial. For example, a decrease (39, 40), an increase (41, 42), or a transient decrease followed by an increase (43, 44) in coronary blood flow by Ang II was reported. Although this inconsistency may be a result of using different animal models or experimental approaches, the complexity of flow regulation in the intact heart may be largely responsible for these diverse findings. Moreover, coronary vasomotor responses are influenced by the neural activity and by the changes in local hemodynamics and cardiac metabolism (44–46). The precise action of Ang II in the coronary microvasculature is difficult to assess in the intact heart because this peptide has direct and indirect actions on these biological factors (43, 44). It is also unclear whether the ROS generated by Ang II can modulate coronary microvascular reactivity in view that enhanced superoxide production by Ang II in endothelial cells is well recognized (47, 48).

Using isolated vessel approaches to eliminate the confounding influences from systemic and local effects inherited in *in vivo* preparations, it was found that Ang II, via activation of its type 1 (AT1) receptors, evokes a moderate vasoconstriction of porcine coronary arterioles (50–80 μm in diameter) at low concentrations (∼1 nM) but a marked vasodilation at higher concentrations (>10 nM) via AT2 receptor activation (49). This vasodilator effect is likely mediated by the released endothelial NO via bradykinin receptor signaling (50). Interestingly, in the human coronary circulation AT2 receptors were found expressed in the microvasculature only (50). Depending upon the concentration used, Ang II appears to exert different vasomotor activities in the coronary microvessels, and thus may explain the inconsistent observations

on coronary flow changes *in vivo*. Moreover, pre-treating the isolated coronary arterioles with a sub-vasomotor concentration of Ang II (0.1 nM) for 60 min caused an elevation of superoxide production in the vessel wall and inhibited NO production and endothelium-dependent, NO-mediated dilation in response to adenosine, a potent metabolic vasodilator in the heart. This inhibitory effect was prevented by AT1 receptor blocker losartan, superoxide scavenger TEMPOL, or NAD(P)H oxidase inhibitor apocynin (49). These microvascular findings indicate that Ang II, at the level without causing vasomotor activity, exerts an adverse effect on NO-mediated vasodilator function via superoxide generated by AT1 receptor-dependent activation of NAD(P)H oxidase. Because acute myocardial ischemia (<60 min) upregulates the cardiac renin-angiotensin system and impairs coronary flow regulation (51, 52), it is speculated that the small elevation of local Ang II at sub-vasomotor levels in the heart during disease states may cause oxidative stress at the local microvascular domain and elicit focal vasoconstriction and myocardial ischemia secondary to the reduced NO bioavailability. In addition to the local vascular spasm, the deficiency of basal NO release from the endothelium, which is subjected to continuous shear stress stimulation, is expected to aggravate ischemic insult by promoting platelet aggregation and thrombosis formation (53, 54) in the microvasculature.

The blunted endothelium-dependent vasorelaxation in aging animals was recently reported to be associated with excessive vascular formation of ROS and upregulation of NAD(P)H oxidase subunits (e.g., Nox-1 and p22-phox), Arg-I, and AT1 and AT2 receptor expression in a manner sensitive to NAD(P)H oxidase inhibition and antioxidants (55). These findings suggest the initiation of vascular dysfunction by oxidative stress linking to Ang II receptors and arginase. However, the role and signaling pathway for Ang II receptor activation leading to NO deficiency in relation to arginase activity and vasomotor regulation is incompletely understood. In cultured bovine aortic endothelial cells, Ang II (0.1 μM, 24 h incubation) was recently shown to increase arginase activity and Arg-I expression through $G\alpha_{12/13}$ protein-coupled AT1 receptor activation (56). The upregulated Arg-I appears to reduce L-arginine bioavailability and hamper NO production. The adverse effect of Arg-I is mediated by the activation of p38 mitogen-activated protein kinase (MAPK) pathways through RhoA/Rho kinase signaling (56). Although the threshold concentration of Ang II necessary for evoking NO deficiency and endothelial dysfunction in the above cell-culture study is unclear, chronic administration of Ang II (42 μg/kg/h, 2 weeks) in the mice was recently shown to impair endothelium-dependent NO-mediated relaxation of a tissue strip from corpus cavernosum (57). In agreement with the findings in cell culture (56), the Ang II-evoked endothelial dysfunction is mediated by the p38 MAPK-dependent upregulation of arginase (57). However, the responsible isozyme is Arg-II rather than Arg-I. Interestingly, inhibition of p38 MAPK not only prevents the effects of Ang II on endothelial function and arginase activity/expression, it also attenuates the increased systemic blood pressure by Ang II.

VASOMOTOR REGULATION IN HYPERTENSION

Hypertension is a major risk factor for coronary artery disease by impairing endothelium-dependent NO-mediated vasodilation (58) in the form of diminished bioavailability of NO, increased Ang II-dependent production of superoxide (59), and decreased endothelial levels of eNOS co-factor tetrahydrobiopterin (BH_4) (60) or substrate L-arginine (61). In some studies, administration of L-arginine has been shown to restore endothelium-dependent vasodilator function in patients with essential hypertension (61) and to normalize coronary hemodynamics (62) and systemic blood pressure with enhanced NO production in hypertensive rats (63, 64). In deoxycorticosterone acetate (DOCA)-salt hypertensive rats, expression and activity of Arg-I protein in the aorta are elevated and correlate positively with blood pressure, suggesting the participation of this enzyme in pathophysiology of arterial hypertension (65). The upregulation of Arg-I in the coronary arteriolar wall was reported to contribute in part to the impairment of endothelial NO production and vasodilation by reducing L-arginine availability in hypertensive pigs (66). In the animal model of genetic (67, 68) or metabolic (69) form of hypertension, chronic inhibition of arginase was recently shown to improve endothelium-dependent vascular function (67–69), reduce cardiac fibrosis (68), prevent vascular remodeling and Arg-I overexpression (68), inhibit insulin-resistance (69), reduce oxidative stress (69), and alleviate hypertension (67–69). Although the evidence for the link of oxidative stress and inflammation to the pathogenesis of hypertension, and vice versa, is well supported in both experimental and clinical studies (70), it is unclear whether the direct elevation of mechanical stress on the vascular wall or the associated oxidative stress and inflammation contribute to the upregulation of vascular arginase during hypertension. Moreover, oxidative stress can probably promote inflammation and, conversely, inflammation *per se* may induce tissue damage and promote oxidative stress. Their individual contributions to the vasomotor dysfunction related to NO deficiency are difficult to define *in vivo* due to the complex and intertwined biological events and multifactorial processes involved in the development of vascular pathophysiology. However, recent studies using cell culture (71–75) and isolated vessel (49, 73, 76–80) approaches suggest that pro-inflammatory factors such as C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), and oxidized low-density lipoprotein (Ox-LDL) are capable of causing endothelial NO deficiency and vasomotor dysfunction through elevated arginase and oxidative stress.

VASOMOTOR REGULATION BY PRO-INFLAMMATORY FACTORS

The dysfunction of coronary microvascular endothelial cells is closely associated with the development of various inflammatory diseases in the heart (81, 82). The inflammatory marker CRP, which has recently been established as a cardiovascular risk factor, also exhibits adverse effects on endothelium-dependent NO-mediated vasodilator function and NO production in isolated coronary (78) and retinal (83) arterioles by enhancing NAD(P)H oxidase-mediated superoxide production via p38 MAPK activation. Since activation of the endothelial p38 MAPK pathway by oxidative stress also has been documented (84, 85), the positive feedback between p38 MAPK and superoxide production is expected to exacerbate the oxidative insult on the vascular wall. In rats, treatment with human CRP at

concentrations achievable in patients with cardiovascular disease impairs endothelium-dependent vasomotor function linked with uncoupling of eNOS due to reduction in dimerization of the enzyme, as well as inhibition of GTP cyclohydrolase I (GTPCH1), the rate-limiting enzyme in BH_4 biosynthesis, and decrease in BH_4 levels (86). Human CRP also causes the activation of NAD(P)H oxidase resulting in eNOS uncoupling directly or via inhibition of GTPCH1 or oxidation of BH_4 (86). These studies provide the first evidence for the adverse action of human CRP *in vivo* manifested by impairing eNOS-dependent vasodilation and uncoupling of eNOS. Thus, given the importance of CRP-induced pro-oxidative effects and resultant eNOS inhibition, CRP appears to be a key molecule to accentuate endothelial dysfunction and contribute to blood flow dysregulation.

The CRP also displays an adverse effect on arachidonic acid-prostanoid pathways in the endothelium (79). The formation of peroxynitrite from NO (basal release) and superoxide (CRP-stimulated release) in the endothelium appears to compromise PGI_2 production, and thus PGI_2-mediated vasodilation, by inhibiting PGI_2 synthase activity through tyrosine nitration (79). Peroxynitrite also contributes to eNOS uncoupling by oxidizing the co-factor BH_4 (87) and thus reduces NO production. Although there is no evidence at the present time to suggest a direct impact of CRP on vascular arginase expression or activity, the elevation of serum Arg-I has been shown to correlate positively with CRP in asthmatic patients (88). Interestingly, the imbalance in L-arginine metabolism via arginase and NOS has been considered as a unifying element of asthma pathophysiology (89). The upregulation of arginase in the vasculature is expected to compromise endothelial NO with enhanced oxidative stress promoting peroxynitrite formation and hypertension during inflammatory insults in a manner similar to the development of allergic asthma in chronic inflammatory airway diseases (90) with primary and secondary forms of pulmonary hypertension (91–93). The recent report on the close relation between asthma and metabolic syndrome (94), a major risk of cardiovascular disease with dysregulation of L-arginine metabolism (69), supports the emerging role of arginase in the general regulation of NO production and oxidative stress in inflammatory diseases.

Tumor necrosis factor-α is a pro-inflammatory cytokine and an important mediator of cardiovascular complications such as acute myocardial infarction, ischemia-reperfusion injury, atherosclerosis, chronic heart failure, and coronary arterial disease in association with diminished coronary blood flow. Treating the isolated coronary arterioles with a pathological concentration of TNF-α (1 ng/ml, 90 min) caused a significant reduction of NO release, enhanced superoxide production, and c-Jun N-terminal kinase (JNK) phosphorylation in arteriolar endothelial cells and impaired endothelium-dependent dilation to adenosine (77). TNF-α participates in the pathogenesis and progression of myocardial injury induced by ischemia-reperfusion (95). In the model of ischemia-reperfusion injury of porcine coronary arterioles, the upregulation of Arg-I, via *de novo* protein synthesis pathway, causes endothelial dysfunction and NO deficiency (96). Using genetic tools to manipulate TNF-α expression in the mouse, it was found that myocardial ischemia-reperfusion evokes superoxide-dependent endothelial dysfunction and NO deficiency via upregulation of Arg-I, in a manner correlating with TNF-α expression (97). In contrast with the insults elicited by Ang II and CRP, the TNF-α induced oxidative stress and endothelial dysfunction are associated with the activation of ceramide-induced activation of JNK and subsequent production of superoxide via xanthine oxidase (77, 98) rather than the signaling via p38 MAPK-activated NAD(P)H oxidase (49, 78). Recent clinical evidence has shown that arginase blockade improves endothelium-dependent NO-mediated vasodilation in patients with coronary artery disease (99) and increases NO-dependent microvascular perfusion in patients with heart failure (100). Interestingly, the systemic level of Arg-I correlates with the severity of heart failure (100) and Arg-I polymorphisms are associated with myocardial infarction and vascular remodeling (101). The elevated level of Arg-I appears to be a major risk and/or pathogenic factor in developing coronary ischemic disease and vascular pathophysiology.

Experimental studies have shown that the expression of arginase is elevated in a variety of vascular and immune cells with inflammation and oxidative stress (20, 21, 102–104), the conditions that are known to be associated with atherogenesis. Interestingly, L-arginine deficiency coupled to impaired NO-mediated vascular function has been reported in animals (105–108) and humans (109–112) with hypercholesterolemia or atherosclerosis, possibly due to upregulation of arginase in the disease state (34). Furthermore, transgenic mice with overexpression of endothelial Arg-II exhibit increased aortic atherosclerotic lesions (113). In apolipoprotein E deficient mice, the arginase activity of atherosclerotic aorta is significantly elevated (28, 108). In the same mouse model, inhibition of arginase activity or deletion of Arg-II gene alleviates oxidative stress in the endothelium, prevents NO deficiency, and restores endothelial function, suggesting the critical role of Arg-II in triggering ROS-dependent endothelial dysfunction in hypercholesterolemia (114). Since Arg-II blockade reduces superoxide formation via a pathway sensitive to NOS inhibition (114), the uncoupling of eNOS, due to L-arginine deficiency, appears to be involved in the arginase-dependent oxidative stress. It was found that Arg-II activity positively correlates with RhoA protein level in atherosclerotic aortas and that manipulation of RhoA/Rho kinase activity and expression directly affects enzymatic activity of Arg-II (28). In this regard, RhoA/Rho kinase activation is likely responsible for the increased Arg-II activity leading to vascular dysfunction and atheroma formation. Rho kinase activation also contributes to Arg-I-mediated coronary vascular dysfunction in diabetic rats and to NO deficiency induced by hyperglycemia in bovine aortic endothelial cells (115). In the rabbit model of hypercholesterolemia, the expression of both arginase isozymes is elevated in atherosclerotic aortas (27). However, the regulation and role of specific arginase isoforms in disease development remains to be determined.

In the coronary microcirculation, the endothelium-dependent NO-mediated dilation, compared to that mediated by the EDHF and the endothelial prostanoids, is more susceptible to the insult of Ox-LDL (116) than that of native LDL (3). The enhanced superoxide production and reduced L-arginine bioavailability are responsible for the observed endothelial dysfunction of coronary

arterioles (3). In cultured human aortic endothelial cells, Ox-LDL activates lectin-like Ox-LDL receptor-1 (LOX-1) and subsequently increases Arg-II activity/expression and reciprocally inhibits NO production via RhoA/Rho kinase activation (117). Interestingly, the NO deficiency, as well as the increased arginase activity and ROS production, evoked by hypercholesterolemia or Ox-LDL are not observed in endothelial cells absent of LOX-1, suggesting the critical role of LOX-1 in mediating arginase-dependent NO deficiency and oxidative stress (117). The accumulation of superoxide is likely derived from the uncoupled eNOS and NAD(P)H oxidase because blockade of these enzymes attenuates oxidative stress (117). In the intact porcine coronary arterioles, the upregulated Arg-I contributes, in part, to the reduced NO production and impaired endothelium-dependent dilation evoked by Ox-LDL (118). However, it is unclear whether LOX-1 plays a role in this experimental model.

VASOMOTOR REGULATION BY H_2O_2

The elevated level of H_2O_2 has been detected under various pathophysiological conditions, including ischemia-reperfusion, inflammation, hypertension, diabetes, and atherosclerosis. The H_2O_2 can be released from various types of cells, including vascular cells (119, 120) and has been implicated, in some tissues, as an endothelium-derived hyperpolarizing factor exhibiting vasodilator activity (119). Extraluminal administration of H_2O_2 (1–100 μM) elicits concentration-dependent dilation of isolated coronary arterioles in part via an endothelium-dependent mechanism through cyclooxygenase (COX)-1-mediated release of PGE_2 (121). H_2O_2 can also cause smooth muscle hyperpolarization and lead to vasodilation through the opening of calcium-activated potassium channels (121, 122). This vasodilator response plays a role in regulating coronary perfusion by recruiting blood flow to the heart during pressure reduction (i.e., autoregulation) (123) or metabolic activation (i.e., functional hyperemia) (124). Interestingly, in disease states, the vasodilator action of H_2O_2 appears to compensate for the impaired NO-mediated dilation linking to the uncoupling of eNOS with its co-factor BH_4 (125) and to protect ischemia-reperfusion injury in the coronary microcirculation (126). In the pig model of coronary ischemia, the impaired NO-mediated vasodilation in collateral-dependent arterioles distal to chronic coronary occlusion was restored by exercise training (127). The beneficial effect of exercise on coronary arteriolar function was abolished by catalase, suggesting the contribution of H_2O_2 in compensating and restoring endothelium-dependent vasomotor function in the phase of collateral microvessel adaption to myocardial ischemia (127).

On the other hand, H_2O_2 can exert an adverse effect by reducing endothelial release of NO for vasodilation when the endothelium is exposed to a prolonged (e.g., 60 min) elevation of excessive H_2O_2 (e.g., 100 μM) (128). Interestingly, the dilation mechanisms involving the activation of COX, guanylyl cyclase, cytochrome-P450 monooxygenase, and potassium channels are not affected by H_2O_2 (128). Moreover, supplementation of L-arginine or inhibition of arginase restores H_2O_2-impaired vasomotor function, and the adverse effect of H_2O_2 can be prevented by inhibiting hydroxyl radical production (128). It appears that a high intravascular level of H_2O_2 selectively impairs endothelium-dependent NO-mediated dilation of coronary microvessels by reducing L-arginine availability. The formation of hydroxyl radicals leading to Arg-I overexpression is responsible for the adverse effect of H_2O_2 (128). Interestingly, it was recently shown that the oxidative stress elicited by peroxynitrite or H_2O_2 increases Arg-I activity/expression through protein kinase C-mediated activation of RhoA/Rho kinase in bovine aortic endothelial cells (129). It remains unclear whether hydroxyl radicals and protein kinase C contribute to the activation of Rho kinase in intact microvessels.

COX AND ARGINASE IN VASCULAR REGULATION

Although COX activation is known to mediate tissue inflammation and participate in vasomotor regulation (130), its linkage to arginase, another important enzyme related to the inflammation process (89), remains unclear. A recent study has shown that inhibition of arginase improves endothelial function and attenuates vascular COX-2, thromboxane synthase, and PGI_2 synthase activities in the rat model of adjuvant-induced arthritis (131). Thus, arginase activation contributes to the augmentation of inflammatory enzyme activity related to prostanoid synthesis. Interestingly, arginase inhibition improved endothelial function, but it had no effect on the arthritis severity of the animal (131). It appears that this type of inflammatory insult targets vascular arginase and consequently leads to vascular disorder. While COX-2 inhibitors have been shown to reduce tumor growth through arginase inhibition (132, 133), administration of diclofenac, a non-steroidal anti-inflammatory drug against COX-2 (134) and phospholipase A_2 (135), was found to cause tumor suppression via a mechanism related to the inhibition of tumor vascularization (136). Although the expression and activity of arginase in the vasculature was not evaluated in this study, it is speculated that the observed tumor suppression is attributable to the inhibition of vascular arginase since this enzyme has been shown to play an important role in the growth of vascular cells (35, 137–139). Although the direct link between COX and arginase in vasomotor regulation remains to be determined, the finding of the close association between these two enzymes in tumor-promoted angiogenesis (140) and in alleviating chronic hypertension and improving vascular endothelial function and vasomotor activity (68) may provide new direction and insights into this underdeveloped research area.

ARGINASE ISOZYMES AND VASOMOTOR DYSFUNCTION

The arginase inhibitors currently available are not isoform selective and their specificity may be species dependent (17). Therefore, it is difficult to identify the role and function of a specific arginase isoform using pharmacological tools. With above limitations, genetic manipulation of an arginase isoform becomes an important strategy for more precise study of arginase function in a living system. Homozygous deletion of Arg-I is lethal to the animal in the perinatal period (141). In contrast, homozygous deletion of Arg-II in the mice does not cause significant changes in phenotype, except an elevation of plasma level of arginine (142). The observed increase in endothelial NO production and NO-mediated vasorelaxation, in conjunction with reduced vasoconstrictor response, in carotid arteries from Arg-II knockout mice (143) supports the idea that endothelial Arg-II plays a

counteracting role in NO production and the associated vasomotor dysfunction. Deletion of Arg-II gene attenuates vascular disorder (i.e., impaired NO-mediated endothelial function and enhanced sympathetic vasoconstriction) in corpora cavernosal tissue of mice with type 1 diabetes, suggesting the detrimental role of Arg-II in this disease model (144). Arg-II appears to modulate not only vasomotor reactivity but also the physical property of the vascular wall by influencing NOS activity because Arg-II deficient mice exhibit decreased vascular stiffness in a manner sensitive to NOS inhibition (143). On the other hand, selective overexpression of human Arg-II gene in the endothelium causes systemic hypertension, impairs endothelium-dependent NO-mediated vasorelaxation, and promotes atherosclerotic lesions (113). These *in vivo* findings are in agreement with the observed adverse effect of Arg-II on NO-mediated endothelial function in cell culture. Moreover, the experimental data from an Arg-II knockout study indicate that the renal injuries observed in spontaneous or streptozotocin-induced diabetes animals are also mediated by Arg-II (145). However, genetic manipulation of Arg-I (partial deletion) in Arg-II deficient mice shows that upregulation of vascular Arg-I, rather than Arg-II, contributes to the diabetes (type I)-induced endothelial dysfunction, vascular stiffness, and coronary fibrosis (146), in which Rho kinase activation can be responsible for the observed pathophysiology (147). Using the same genetic approach, the detrimental role of Arg-I in mediating blood pressure elevation and vascular endothelial dysfunction was recently reported in the mice subjected to systemic hypertension induced by DOCA-salt (148). In diabetic human patients, Arg-I upregulation appears to be responsible for the impairment of coronary arteriolar dilation to an endothelium-dependent NO-mediated agonist (149).

Surprisingly, a recent study by Huynh et al. showed that Arg-II knockout mice start to display hypertension at 8 weeks old, despite the reduction in vasoconstrictor responsiveness (150). The observed changes in systemic hemodynamics are associated with left ventricular hypertrophy, diastolic dysfunction, and increased sympathetic activity (150). In contrast to the previous report in carotid arteries with Arg-II deletion by Lim et al. (143), the aortic relaxation to an NO-dependent agonist was not significantly enhanced in the Arg-II knockout mice (150), suggesting that the observed reduction in the vasoconstrictor response was not attributable to alterations in NO production. There is no clear explanation to the apparent discrepancies between these two Arg-II knockout studies, especially in the observed global changes in cardiovascular function and vasomotor regulation related to endothelial NO. Nevertheless, the study of Huynh et al. demonstrated a correlation between Arg-II and Rho kinase, suggesting a contribution of downregulation of Rho kinase to the observed reduction in the vasoconstrictor response in Arg-II deficiency (150). This is in agreement with the context that upregulation of arginase in the disease state may enhance Rho kinase activity/expression and consequently alter vasomotor activity because numerous studies have implicated a close association between Rho kinase and arginase in the development of vascular dysfunction (28, 56, 115, 117, 129).

Although recent studies using genetic approaches have provided significant insights into the contribution of specific arginase isozymes in vasomotor regulation in health and disease, the inconsistent results are often reported as discussed above. In view that arginase gene deletion might also alter expression of other genes or activate alternate signaling pathways to confound the consequences of initial gene deletion (151, 152), the interpretation of these results should be cautious. The gene–gene interaction and the development of compensatory and/or decompensatory biological responses, at local or systemic levels, with gene manipulation may contribute to the observed discrepancies, in addition to the variation of involved signaling molecules, age, gender, tissue/organ, species/strain, and experimental conditions.

At the protein level, although Arg-I and -II carry out the same catalytic function, they have different physicochemical characteristics, including immunological cross-reactivity, charge, and subcellular location (14). Because the expression pattern of specific arginase isoforms can be cell/tissue and animal species dependent (17), it is unclear at the present time why and how Arg-I and -II can be targeted differently. Interestingly, recent studies suggest that the catalytic efficiency of arginase can be modulated without altering protein expression (153). It appears that cysteine residues 303 in Arg-I can undergo S-nitrosylation and subsequently increase stability of the arginase trimer and reduce its K_m for arginine (153). This increase in arginase activity can contribute to the endothelial dysfunction and reduced NO bioavailability (153). On the other hand, there is no cysteine in mammalian Arg-II that corresponds to cysteine 303 in Arg-I, suggesting that the post-translational modulation via S-nitrosylation might not occur in Arg-II (17). It is likely that S-nitrosylation elicited by the excessive production of NO during iNOS induction (e.g., inflammation) or by the formation of peroxynitrite during oxidative stress may contribute to a selective activation of Arg-I leading to endothelium-dependent vasomotor dysfunction. Moreover, uric acid has been demonstrated to increase arginase activity by increasing the affinity for arginine (154). This phenomenon is unlikely isoform selective because it is observed in the pulmonary arterial endothelial cell lysates (Arg-II) and rat kidney (Arg-II) and liver (Arg-I) homogenates (154). However, it was recently found that uric acid, at the concentrations reported to affect arginase activity (154), does not alter Arg-II activity in cultured human umbilical vein endothelial cells (155). The explanation for these inconsistent findings on uric acid-arginase interaction remains unclear. Hydroxyl radicals derived from H_2O_2 appear to specifically induce Arg-I expression and lead to endothelial dysfunction in coronary microvessels (128). Interestingly, biochemical studies *in vitro* indicate that Arg-I enzyme activity can be enhanced by hydroxyl radicals (156). Although it has not been demonstrated whether hydroxyl radicals also alter Arg-II activity, the activation of Arg-I, both in protein expression and activity, by oxidative stress (i.e., peroxynitrite and H_2O_2) in cultured endothelial cells also has been reported recently (129). In view that the increase of Arg-I activity (50%) is more than that of protein expression (35%) (129), the direct impact of these insults on arginase enzyme activity *per se* is apparent. Collectively, the above studies suggest the differential activation of arginase isozymes, depending upon the environment and the nature of the stimulation, in addition to the selective regulation of its protein expression in the vasculature. These differential regulation mechanisms may

also contribute to the observed diversity and heterogeneity in involved arginase isoforms in vascular cell, as well as the exerted function, in different tissues, species, and diseases. Further studies on the differential activation of specific arginase isoforms are required.

CONCLUSION AND PERSPECTIVES

Collectively, the NO bioavailability, determined by the synthesis/release and utilization/scavenging at the level of the endothelium, plays an important role in maintaining vascular homeostasis and function, as well as disease development linking to oxidative stress and inflammation. Redox signaling with a low level of ROS released from cardiomyocytes and/or vascular cells displays an indispensable role in maintaining microcirculatory homeostasis by regulating vasomotor activity in response to physiological challenges. The release of H_2O_2 from the vasculature helps to restore vasomotor function by compensating for NO deficiency in coronary collateral microvessels adapted to chronic myocardial ischemia with exercise training. Depending upon the disease model and the pathophysiological insult, the excessive and prolonged production of superoxide, via stress kinase-activated NAD(P)H oxidase or xanthine oxidase, and the subsequent exorbitant formation of H_2O_2, appear to generate oxidative stress and inflammation, which outweighs the benefits of vasoregulation by impairing endothelial function and possibly exhausting vasodilator reserve (**Figure 1**). The status and the balance of redox signaling in the vascular cells and their surrounding parenchymal tissues appear to modulate the vasomotor function of microvessels in health and disease.

The converging evidence suggests that NO-mediated vascular function, including vasomotor activity, can be influenced by the arginase activity in the endothelium and/or its surroundings. The upregulation of arginase, in either protein or activity, contributes to vascular dysfunction in various vascular diseases by initiating or following oxidative stress and inflammation (**Figure 1**). Therefore, therapeutic inhibition of arginase may be useful for disease treatment. However, a global Arg-II deletion can develop hypertension, ventricular hypertrophy, and cardiac dysfunction with age (150). Because these cardiovascular disorders are not present at young age with Arg-II ablation, chronic Arg-II deficiency appears to elicit a series of cardiovascular remodeling (e.g., compensation and decompensation). Moreover, biochemical studies indicate that Arg-I and -II can exhibit different enzyme kinetics for substrate binding and products, as well as different sensitivities and responsiveness toward inhibitors (17, 157). These isozyme-dependent characteristics, in combination with the use of different experimental models and animal species, may complicate the experimental results, interpretations, and conclusions on the effect of arginase inhibition on endothelial function and vasomotor regulation under physiological and pathophysiological conditions. In this regard, the clinical benefits of inhibition of specific arginase isoforms for cardiovascular disease treatment are uncertain and deserve further investigation. It is worth noting that the systemic supplementation of antioxidants showed no benefit but instead promoted possible harmful effects in cardiovascular disease prevention or therapy (158–161). Oxidative stress and inflammation are two sides of the same coin and can be the cause or result of arginase upregulation in the vasculature via diverse signaling mechanisms. Localized manipulation of the redox system and arginase activity in a diseased vessel might be a useful strategy to improve flow regulation and thus enhance oxygen and nutrient delivery for tissue survival and recovery.

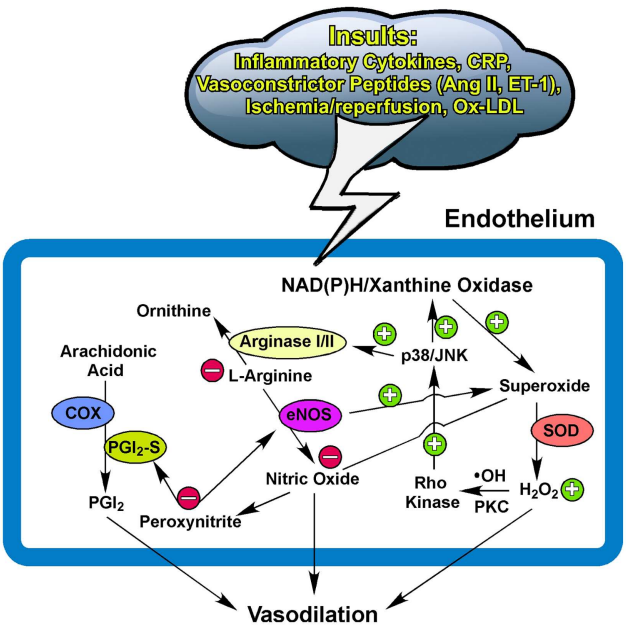

FIGURE 1 | Potential pathways for redox and arginase modulation of vasomotor function. The low level of superoxide and hydrogen peroxide (H_2O_2) is essential for maintaining normal homeostasis of the endothelium to exert vasodilation in response to physiological stimulation. The excessive production of superoxide from the activated NAD(P)H oxidase and/or xanthine oxidase by insults from inflammatory cytokines, C-reactive protein (CRP), ischemia/reperfusion, oxidized low-density lipoprotein (Ox-LDL), or vasoconstrictor peptides such as angiotensin II (Ang II) and endothelin-1 (ET-1) (162–165) scavenges the released nitric oxide and subsequently forms peroxynitrite. The prolonged and elevated production of H_2O_2 from superoxide dismutase (SOD) suppresses NO production by up-regulating vascular arginase via p38 mitogen-activated protein kinase or c-Jun-N-terminal kinase (JNK) signaling following the hydroxyl radical (•OH)- or protein kinase C (PKC)-mediated activation of Rho kinase. The upregulated arginase limits substrate L-arginine availability to nitric oxide synthase (eNOS) for nitric oxide synthesis and also uncouples eNOS to promote superoxide production. The production of nitric oxide from eNOS and prostacyclin (PGI_2) from cyclooxygenase (COX) and PGI_2 synthase (PGI_2-S) is inhibited by peroxynitrite due to uncoupling of eNOS and nitration of PGI_2-S (see text for details). Peroxynitrite also activates arginase and consequently reduces nitric oxide production. These redox events, in company with arginase upregulation, compromise endothelial function and thus augment vascular tone and reduce nitric oxide-mediated arteriolar dilation for blood flow recruitment and regulation. +, enhance/increase/upregulate; −, inhibit/reduce/downregulate.

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Arginase 1: an unexpected mediator of pulmonary capillary barrier dysfunction in models of acute lung injury

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The integrity of epithelial and endothelial barriers in the lower airspaces of the lungs has to be tightly regulated, in order to prevent leakage and to assure efficient gas exchange between the alveoli and capillaries. Both G^- and G^+ bacterial toxins, such as lipopolysaccharide and pneumolysin, respectively, can be released in high concentrations within the pulmonary compartments upon antibiotic treatment of patients suffering from acute respiratory distress syndrome (ARDS) or severe pneumonia. These toxins are able to impair endothelial barrier function, either directly, or indirectly, by induction of pro-inflammatory mediators and neutrophil sequestration. Toxin-induced endothelial hyperpermeability can involve myosin light chain phosphorylation and/or microtubule rearrangement. Endothelial nitric oxide synthase (eNOS) was proposed to be a guardian of basal barrier function, since eNOS knock-out mice display an impaired expression of inter-endothelial junction proteins and as such an increased vascular permeability, as compared to wild type mice. The enzyme arginase, the activity of which can be regulated by the redox status of the cell, exists in two isoforms – arginase 1 (cytosolic) and arginase 2 (mitochondrial) – both of which can be expressed in lung microvascular endothelial cells. Upon activation, arginase competes with eNOS for the substrate L-arginine, as such impairing eNOS-dependent NO generation and promoting reactive oxygen species generation by the enzyme. This mini-review will discuss recent findings regarding the interaction between bacterial toxins and arginase during acute lung injury and will as such address the role of arginase in bacterial toxin-induced pulmonary endothelial barrier dysfunction.

Keywords: pneumonia, capillary leak, pneumolysin, arginase 1, endothelial nitric oxide synthase

INTRODUCTION

Cells require energy to carry out their vital functions. Mitochondrial oxidative phosphorylation is the main pathway through which cellular ATP is generated, provided that an adequate and continuous amount of O_2 is supplied to the mitochondria. This is orchestrated by the microcirculation, which represents the interface between the parenchymal cells in the tissues (the consumers) and the circulatory system (the supplier).

In order for gas exchange between the 2^{23} alveoli and the lung capillaries in an adult human lung to occur in an optimal manner, the alveolar-capillary barrier integrity has to be tightly regulated (1). To that purpose, the continuous capillaries found in the lungs are very closely packed together. Since the rate of diffusion of O_2 and CO_2 through the alveolar-capillary barriers is proportional to the exchange area, but inversely proportional to its thickness, no excess leakage of liquid should occur in the interstitium and subsequently in the alveolar space, since this would dramatically impair gas exchange between the alveoli and the pulmonary capillaries. To that purpose, capillary hydrostatic pressure, which is partially defined by the pulmonary venous pressure and vascular tone, has to be kept under control, in order to prevent excess fluid extravasation from the capillaries.

THE ROLE OF ARGINASE DURING LUNG INFLAMMATION: A COMPLICATED MATTER

Arginase, which converts L-arginine into L-ornithine and urea, is not only a key enzyme of the hepatic urea cycle, but is also expressed in extra-hepatic tissues lacking a complete urea cycle. Whereas cytosolic arginase 1 is the predominant isoform in the liver, mitochondrial arginase 2 is mainly expressed in extra-hepatic tissues (2). L-Arginine is not only a substrate for arginase, but can alternatively be converted to NO and L-citrulline by nitric oxide synthases (NOS). As such, arginase's primary biological function in extra-hepatic organs, such as the lungs, lies mainly in the regulation of NO synthesis, by means of competing with NO synthase for the common substrate L-arginine.

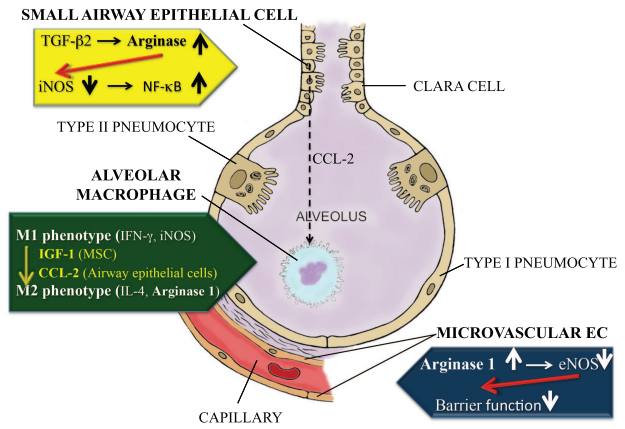

FIGURE 1 | Differential role of arginase in small airway epithelial cells, alveolar macrophages and capillary endothelial cells in the lungs. Activation of arginase in the small airway epithelial cells (yellow panel) leads to a reduction in iNOS mRNA and protein expression, as such reducing NO generation. Since NO suppresses the NFκB response, this culminates in excessive NFκB activation and inflammation. Alveolar macrophages (green panel), upon stimulation with mesenchymal stem cell-derived IGF-1 or small airway epithelial cell-derived CCL2, can convert from a pro-inflammatory M1 phenotype, characterized by IFN-γ production and high iNOS activity, to an M2 phenotype, in which IL-4 generation and arginase 1 activity prevail. Activation of arginase 1 activity in microvascular endothelial cells (blue panel) leads to a reduction in eNOS-derived NO generation, which in turn contributes to a loss of barrier function in the capillary compartment.

Increased arginase activity has been reported in several inflammatory lung diseases, including asthma, chronic obstructive pulmonary disease, cystic fibrosis, and pulmonary hypertension. This suggests a common feature underlying the pathophysiology of these diseases [reviewed in (3)]. As demonstrated in **Figure 1**, both arginase 1 and 2 are constitutively expressed in airway and alveolar epithelial cells, endothelial cells, and alveolar macrophages in the lower airways (4, 5). However, the enzyme's role can be quite different depending on the cell type or the location where its activity is increased.

PRESERVATION OF ARGINASE ACTIVITY BY ANTI-INFLAMMATORY THIOREDOXIN 1

The ubiquitously expressed and evolutionary well-conserved 12 kDa protein thioredoxin was initially thought to be primarily involved in protection against oxidative stress, by means of scavenging reactive oxygen species (ROS) through the interaction with peroxiredoxin and controlling the cellular redox balance (6, 7).

Thioredoxin exists as both a cytoplasmic and an extracellular form. In contrast to thioredoxin 2, thioredoxin 1 has the capacity to function as a chaperone for arginase, as such protecting the enzyme from inhibition by reactive oxygen and nitrogen intermediates and from denaturation by urea and heat. As such, thioredoxin 1 retains arginase in a catalytically active state (8).

Thioredoxin not only reduces oxidative and nitrosative stress, but also suppresses pro-inflammatory cytokine generation (9), reduces leukocyte-endothelial interactions and preserves arginase activity, which in turn blunts deleterious inducible NOS (iNOS) activity. Taken together, all of these activities make thioredoxin a potent and versatile mediator of inflammation (10, 11). Recombinant human thioredoxin was proposed as a therapeutic candidate for the treatment of several inflammatory disorders (12).

In the lungs, the induction of thioredoxin is regarded as an adaptive response against lung inflammation associated with oxidative stress (13). Recent studies have revealed that exogenously administered thioredoxin protects the lungs from acute lung injury induced by influenza virus infection (14). In conclusion, thioredoxin 1 has the capacity to preserve arginase activity in pulmonary cells, as such blunting excessive iNOS activity.

ROLE OF ARGINASE IN ALVEOLAR MACROPHAGES DURING PULMONARY INFECTION AND INFLAMMATION

In alternatively activated alveolar macrophages of the M2 phenotype, arginase activity can limit the consummation of L-arginine by iNOS, as such suppressing the cytotoxic response by these cells (**Figure 1**). Many factors can affect the conversion from the M1 to the M2 phenotype in these cells.

Following *Mycobacterium tuberculosis* infection, alveolar macrophages first become classically (M1) polarized, characterized by an increased expression of IFN-gamma and iNOS (**Figure 1**). However, as the inflammation progresses, they decrease iNOS and IFN-γ expression, but increase IL-4 generation and arginase 1 activity, indicating M2 polarization (15). By contrast, *M. tuberculosis*-induced granuloma-associated macrophages remain M1-polarized throughout the entire process. Azithromycin treatment was shown to have the potential to induce M2 polarization of alveolar macrophages, e.g., in a *Pseudomonas aeruginosa* infection model (16).

The NO-generating capacity and arginase activity of alveolar macrophages also seems to affect susceptibility to infection with *Chlamydia*. Indeed, C57BL/6 mice develop severe pneumonia and poor immunity against *Chlamydia* after moderate respiratory infection, whereas BALB/c mice are protected from the disease and develop a vigorous Th1 response. Infected C57BL/6 macrophages release more iNOS-derived NO than BALB/c macrophages and express lower mRNA concentrations of arginase 2. Reduction of NO production upon incomplete iNOS inhibition abolishes susceptibility of C57BL/6 mice to *Chlamydia*-induced disease. Thus, the quantity of NO released by infected macrophages seems to define pathogenic versus protective macrophage responses to chlamydial infection (17).

In rat alveolar macrophages, bacterial lipopolysaccharide (LPS), released upon antibiotic treatment, was shown to increase both arginase 1 and 2 expression, an effect which could be blunted by glucocorticoids (5). It is important to note that arginase in alveolar macrophages not only contributes to impairment of NO generation, but it can also mediate airway remodeling, as detected in asthma, cystic fibrosis, and COPD, through the increased production of L-proline, a precursor of collagen, and the polyamines putrescine, spermidine, and spermine from L-ornithine (18).

The role of arginase in alveolar macrophages in LPS-induced acute lung injury remains controversial, since a recent study reported that mesenchymal stem cell-conditioned medium mediates the resolution of LPS-induced acute lung injury, by attenuating lung inflammation and promoting a wound

healing/anti-inflammatory M2 macrophage phenotype, characterized by increased arginase 1 activity, at least partially in an insulin growth factor 1 (IGF-1)-dependent manner (19).

ROLE OF ARGINASE IN SMALL AIRWAY AND ALVEOLAR EPITHELIAL CELLS

From the previous paragraph, it is noteworthy that the switch of alveolar macrophages from the M1 to the M2 phenotype can be mediated by other cell types in the lower airways. This is not only the case for mesenchymal stem cells, but also for airway epithelial cells. Indeed, supernatants from chitin-treated airway epithelial cells were shown to induce alternative M2 activation of alveolar macrophages *in vivo*, by means of a CCL2 chemokine-dependent mechanism [**Figure 1**; (20)].

Moreover, TGF-β2 was shown to impact cytokine-induced NO production in primary small airway epithelial cells, by enhancing total arginase activity and reducing iNOS mRNA and protein levels, through a Rho kinase-dependent pathway (21). In a different study, reduction of arginase activity was shown to enhance the cellular content of NO and S-nitrosated proteins in a mouse type II alveolar epithelial cell line (22). As a consequence, TNF- or LPS-stimulated NF-κB DNA binding and transcriptional activity was decreased, in combination with an enhanced S-nitrosation of p50. The NOS inhibitor N-omega-nitro-L-arginine methyl ester (L-NAME) reversed the effects of arginase inhibition on NF-κB, suggesting a causal role for NO in the attenuation of NF-κB induced by arginase suppression. Conversely, overexpression of arginase 1 decreased cellular S-nitrosothiol content, enhanced IκB kinase activity and NF-κB DNA binding and decreased S-nitrosation of p50.

These results point to a regulatory mechanism wherein NF-κB is controlled through arginase-dependent regulation of NO levels, which may impact on chronic inflammatory diseases that are accompanied by NF-κB activation and upregulation of arginases (22, 23).

REGULATION OF CAPILLARY ENDOTHELIAL PERMEABILITY

Endothelial cells form confluent monolayers on the surface of the inner wall of blood vessels. One of their major functions is therefore the separation of blood from underlying tissues, allowing only tightly controlled passage of macromolecules and cells. In the lower airways, where the actual gas exchange between the alveoli and the capillaries only efficiently occurs when barriers are tight (1), confluence is crucial. The adherence of endothelial cells is formed by transmembrane adhesion proteins, which mediate homophilic adhesion and junctional structures.

The transmembrane proteins are linked to specific intracellular partners, which mediate anchorage to the actin cytoskeleton and, as a consequence, stabilize junctions. The changes in the components of the EC cytoskeleton are of critical importance in the determination of the actual shape of the cell. The actin filaments and the phosphorylation/dephosphorylation-controlled actomyosin interactions are dramatically involved in the increase of the vascular permeability. Phosphorylation/dephosphorylation events of cytoskeletal/cytoskeleton-associated proteins also have a regulatory role in endothelial barrier regulation. Ca^{2+}, calmodulin, and myosin light chain kinase (MLCK) were shown to be required components for endothelial cell retraction, whereas myosin phosphatase restores endothelial relaxation (24).

Two main signaling pathways regulate the barrier function, via the inhibition of myosin phosphatase. One of them is the vasoactive agent-induced Rho pathway, which increases the endothelial permeability. Rho Kinase (ROCK) may increase myosin light chain (MLC) phosphorylation indirectly by means of inducing myosin phosphatase inactivation, accumulation of diphospho-MLC, and cell contraction. Another pathway involves the Protein Kinase C (PKC)-potentiated inhibitory protein of 17 kDa (CPI-17), which may also affect isolated protein phosphatase 1c (PP1c) or the holoenzyme form of myosin phosphatase, without dissociating its subunits (25, 26).

External factors, such as certain G^+ bacterial toxins (e.g., pneumolysin and listeriolysin) or pro-inflammatory cytokines, such as TNF, can induce increases in Ca^{2+}-influx in endothelial cells, which can affect the microtubular network dynamics. Increases in intracellular Ca^{2+} can induce disassembly of microtubules (27). The microtubule population in endothelial cells is heterogeneous and can be divided into (1) stable, modified (acetylated), and (2) dynamic microtubules, with the former ones being more stable and thus more resistant to the effects of external factors. It is possible that under conditions compromising vascular endothelial integrity, the stable microtubules may confer stability to the endothelial microtubule network (28). Depolymerization of microtubules can in turn cause disassembly of adherens junction proteins with which they associate, such as VE-cadherin, thus increasing permeability (29).

RESTORING CAPILLARY BARRIER FUNCTION IN PNEUMONIA: A THERAPEUTIC PRIORITY

Childhood pneumonia is the leading single cause of mortality worldwide in children aged less than 5 years (30). Moreover, over four million people develop pneumonia each year in the United States, with over a half a million of them being admitted to a hospital for treatment. Community-acquired pneumonia (CAP) represents a major cause of morbidity and mortality in mainly elderly patients (31, 32). The leading bacterial cause is the Gram-positive bacterium *Streptococcus pneumonia* (pneumococcus), being identified in 30–50% of pneumonia cases. The fatality rate associated with *Streptococcus pneumoniae*, the main etiological agent of severe pneumonia, still approximates 20%, despite the use of potent antibiotics and aggressive intensive-care support (30, 32).

Permeability edema, associated with severe pneumonia, acute lung injury, and the acute respiratory distress syndrome (ARDS), is characterized by a dysfunction of the alveolar-capillary barriers, leading to an infiltration of, e.g., neutrophils and factors contained in the blood into the alveoli. In severe pneumonia, this condition, characterized by capillary endothelial hyperpermeability, can occur days after initiation of antibiotic therapy, thus when tissues are already sterile. Permeability edema in pneumococcal pneumonia correlates with the presence of the bacterial virulence factor pneumolysin (33, 34), a cytoplasmic hemolytic protein released during bacterial autolysis or upon treatment with β-lactam antibiotics (35). Pneumolysin-induced acute lung injury was suggested to result from direct pneumotoxic effects on the alveolar-capillary barrier, rather than from resident or recruited phagocytic cells (36).

Intravascular pneumolysin was shown to cause a significant dose-dependent increase in pulmonary vascular resistance and in lung microvascular permeability. Upon binding of pneumolysin to cholesterol in cell membranes, oligomerization, and pore formation occur, which causes increased intracellular Ca^{2+} levels (37), the initial pivotal signal preceding pathways leading to endothelial cell contraction (38), including MLC-dependent mechanisms and microtubule rearrangement. RhoA and Rho-associated kinase may directly catalyze MLC phosphorylation or act indirectly via inactivation of myosin phosphatase to induce cell contraction and endothelial barrier disruption. In turn, endothelial barrier enhancement is associated with Rac 1-mediated formation of F-actin, increased association of focal adhesion proteins, and enlargement of intercellular adherens junctions. Thus, a precise balance between RhoA- and Rac1-mediated signaling is essential for endothelial barrier regulation.

The Ca^{2+}-dependent PKC isoform, PKC-α, was suggested to play a critical role in initiating endothelial cell contraction and disassembly of VE-cadherin junctions (39–41). The NADPH oxidases, Nox2 and Nox4, are major sources of ROS in endothelial cells and are implicated in redox-sensitive signaling pathways that influence endothelial cytoskeletal organization and permeability (42, 43). Apart from inducing RhoA activation (41), PKC-α activation was also recently shown to upregulate Nox 4 mRNA expression in human endothelial cells (44).

In view of its crucial role in bacterial virulence and its profound effects on the immune system of the host, pneumolysin can be considered as a model toxin for G^+ infection-associated acute lung injury and permeability edema. Since no standard therapy is currently available to treat the pulmonary permeability edema associated with severe pneumonia, the need for novel substances that can improve oxygenation in these patients, by means of barrier restoration is thus very important.

AN IMPORTANT ROLE FOR ARGINASE 1 IN PNEUMOLYSIN-MEDIATED CAPILLARY LEAK

In the larger blood vessels, impaired vasorelaxation capacity can be caused by dysfunctional endothelial nitric oxide synthase (eNOS)-dependent NO generation and increased eNOS-dependent ROS production. Moreover, in acute lung injury, the nitrating agent peroxynitrite, which can be formed by the reaction between ROS and NO in severe pathophysiological situations, can further deteriorate the vascular dysfunction (45). Endothelial NOS dysfunction in large vessel endothelial cells can be induced upon arginase 1 activation (46, 47). Although it is generally recognized that NO is a crucial regulator of vessel vasodilation, its role in the capillaries is less well documented, apart from its anti-adhesive and anti-aggregatory effects on platelets (48).

Maintenance of the endothelial barrier requires a basal level of NO, regulated by eNOS (49). Both the lack of NO and high NO levels destabilize inter-endothelial junctions (50–52). Availability of the semi-essential amino acid L-arginine is required for eNOS activity and NO production and is essential for vascular integrity and function. Recent studies have indicated that increased activity and/or overexpression of the enzyme arginase, which is thought to be dependent on RhoA activation, may play an important role in the availability of L-arginine and thus in the pathogenesis of vascular dysfunction (46, 53). Both arginase 1 and 2 have been found in endothelial cells, with arginase 1 being the dominant isoform. Arginase competes with eNOS for their common substrate, L-arginine, thus reducing the NO-generating capacity of the enzyme. Endothelial NOS catalyzes the two step conversion of substrate L-arginine into NO and utilizes electrons from NADPH to reduce molecular oxygen followed by oxidation of the guanidino N group of arginine to form NO, L-citrulline, and water (54).

It is this repeated activation of molecular oxygen that presents the opportunity for enzymatic missteps and superoxide production from eNOS, in the presence of reduced L-arginine availability, a process that has been termed "eNOS uncoupling" (54, 55). All of these events will culminate in endothelial hyperpermeability.

Our recent studies have shown that treatment of human lung microvascular endothelial cells with a sublytic concentration of pneumolysin, within 2 h significantly increases both arginase 1 and 2 expression, as well as arginase activity (56). This is accompanied by a significant reduction in basal NO generation and hyperpermeability in these cells. Both the barrier function and the NO generation can be partially restored upon treating the cells with the arginase inhibitor (S)-(2-boronoethyl)-L-cysteine (BEC) or with the PKC-α inhibitor Ro32-4032 *in vitro*. PKC-α signals RhoA activation (57), which in turn is involved in the activation of arginase activity in endothelial cells (58, 59).

Moreover, both the arginase inhibitor BEC and the PKC-α inhibitor Ro32-4032 can blunt pneumolysin-induced endothelial hyperpermeability in HL-MVEC *in vitro*. These results are substantiated *in vivo*, since arginase $1^{+/-}$/arginase $2^{-/-}$, but not arginase $1^{+/+}$/arginase $2^{-/-}$ mice (60) are significantly protected from pneumolysin-induced capillary leak (56).

Taken together, these results demonstrate an important role for arginase 1 in pneumolysin-induced barrier dysfunction in HL-MVEC.

MANIPULATING ARGINASE ACTIVITY IN ACUTE LUNG INJURY: HANDLE WITH CARE

The previous paragraph points toward a potential therapeutic potential for arginase inhibitors in the treatment of capillary leak associated with acute lung injury, ARDS, and severe pneumonia. However, as previously discussed, the important role of arginase in the M2 phenotype of alveolar macrophages, which can, e.g., promote clearance of LPS-induced edema inflammation, makes a general inhibition of arginase activity in the lungs during infection problematic. Moreover, direct arginase inhibitors block arginase activity not only in the endothelium but also in other tissues, such as the liver, where its activity is needed in the urea cycle. As such, whereas arginase inhibition in capillary endothelial cells might increase their barrier function, it can potentially also lead to the conversion of alveolar macrophages from the protective M2 to the deleterious inflammatory M1 phenotype, the latter of which is characterized by excessive iNOS-mediated NO generation. Moreover, arginase inhibition in type II alveolar epithelial cells can also cause activation of iNOS and thus excessive NO production, shown to blunt the open probability of the epithelial sodium channel, crucial for alveolar liquid clearance during acute lung injury (61, 62). Furthermore, because arginase is involved in macrophage-mediated anti-bacterial responses and in wound

repair (63), it is possible that substances inhibiting its activation, although conferring resistance to pneumolysin-mediated vascular leak, can interfere with macrophage-mediated innate immunity to pneumococci and wound healing. The same holds true for PKC-α proposed to be important for T-cell proliferation and IFN-γ production (64), and as such can be involved in anti-bacterial defense mechanisms, making that direct PKC-α inhibitors potentially can have negative effects on adaptive immunity.

Therefore, alternative substances interfering with the activation of arginase 1, rather than with its activity *per se*, preferentially in the endothelium, should be identified and investigated more in detail.

Although it is generally assumed that cytokines solely exert their activities upon activating their respective receptors, this does not seem to be true in the case of TNF. Apart from its receptor binding sites, which mediate a plethora of biological activities, ranging from apoptosis to inflammation and proliferation (65, 66), TNF exerts a lectin-like activity, permitting its binding to glycoproteins such as uromodulin, with a $K_D = 10^{-10}$ M (67) uromodulin is a glycoform of Tamm–Horsfall protein, found in the loops of Henle of pregnant women, which was shown to bind the pro-inflammatory cytokines, IL-1β, IL-2, and TNF, proposed as a mechanism to clear excessive levels of these cytokines from the circulation during pregnancy (67, 68).

Since uromodulin-bound TNF was still able to exert cytotoxic effects in L929 fibrosarcoma cells, it was proposed that the lectin-like domain of TNF has to be spatially distinct from its receptor binding sites. Specific oligosaccharides, such as *N*,*N*′-diacetylchitobiose, as well as branched trimannoses, which are known to bind to the lectin-like domain of TNF, are able to inhibit the necrotic activity of TNF in African trypanosomes, but not the cytotoxic activity of TNF in L929 cells. Moreover, lectins with a similar oligosaccharide specificity as TNF, such as Urtica Dioica Agglutinin, but not those with a different specificity, block the trypanolytic effect of TNF (69). Molecular graphics comparisons of tertiary structures of TNF (trypanolytic) and the highly homologous lymphotoxin-α (non-trypanolytic), lead us to propose a dissimilar structure that could be responsible for the lectin-like activity. This structure, which is present at the Tip of the TNF molecule can be mimicked by a circular 17 amino acid peptide, which we called the TIP peptide. Antibodies to this peptide were able to inhibit the trypanolytic activity and moreover, the TIP peptide itself was shown to exert trypanolytic activity (69). Three amino acids, i.e., one threonine and two glutamic acids were shown to be crucial for this activity.

Our recent data have demonstrated that the TIP peptide inhibits pneumolysin-induced PKC-α and arginase activation and restores basal NO generation in human microvascular endothelial cells monolayers. The peptide moreover significantly protects from pneumolysin-induced capillary leak *in vivo* (56). Since the TIP peptide also activates lung liquid clearance upon stimulating epithelial sodium channel function, the latter of which is impaired by pneumolysin (70), it is further being investigated as an alternative to inhibitors of arginase and PKC-α in the treatment of permeability edema. As such, the peptide is being tested in two phase 2a clinical trials in patients with acute lung injury.

GENERAL CONCLUSION

Recent studies have revealed a complex role for arginase during acute lung injury. On the one hand, activation of arginase 1 in alveolar macrophages, as can be induced by airway epithelial cells or mesenchymal stem cells, promotes the protective M2 phenotype in these cells, reducing deleterious iNOS activity. By sharp contrast, an increase in arginase 1 activity in pulmonary microvascular endothelial cells, as can be induced by bacterial toxins, can mediate capillary endothelial hyperpermeability, by means of reducing basal eNOS-dependent NO generation, crucial for the generation of basal barrier function. These findings thus clearly document the difficulty to develop therapies interfering directly with arginase activity during acute lung injury. As such, targeting arginase in specific cell types in the lungs, using, e.g., genetically modified mice with inducible constructs could lead to a better understanding of this complex area.

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Arginase as a mediator of diabetic retinopathy

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We have shown previously that diabetes causes increases in retinal arginase activity that are associated with impairment of endothelial cell (EC)-dependent vasodilation and increased formation of the peroxynitrite biomarker nitrotyrosine. Arginase blockade normalizes vasodilation responses and reduces nitrotyrosine formation, suggesting that overactive arginase contributes to diabetic retinopathy by reducing NO and increasing oxidative stress. We tested this hypothesis by studies in streptozotocin-induced diabetic mice and high glucose (HG) treated retinal ECs. Our results show that arginase activity is increased in both diabetic retinas and HG-treated retinal ECs as compared with the controls. Western blot shows that both arginase isoforms are present in retinal vessels and ECs and arginase I is increased in the diabetic vessels and HG-treated retinal ECs. Nitrate/nitrite levels are significantly increased in diabetic retinas, indicating an increase in total NO products. However, levels of nitrite, an indicator of bioavailable NO, are reduced by diabetes. Imaging analysis of NO formation in retinal sections confirmed decreases in NO formation in diabetic retinas. The decrease in NO is accompanied by increased $O_2^{\cdot-}$ formation and increased leukocyte attachment in retinal vessels. Studies in knockout mice show that arginase gene deletion enhances NO formation, reduces $O_2^{\cdot-}$ and prevents leukostasis in the diabetic retinas. HG treatment of retinal ECs also reduces NO release, increases oxidative stress, increases ICAM-1, and induces EC death. Arginase inhibitor treatment reverses these effects. In conclusion, diabetes- and HG-induced signs of retinal vascular activation and injury are associated with increased arginase activity and expression, decreased bioavailable NO, and increased $O_2^{\cdot-}$ formation. Blockade of the arginase pathway prevents these alterations, suggesting a primary role of arginase in the pathophysiological process.

Keywords: arginase, diabetic retinopathy, high glucose, diabetes, oxidative stress, nitric oxide

INTRODUCTION

Diabetic retinopathy, a microvascular complication of diabetes, is the leading cause of blindness in adults of working age (1). Diabetes-induced retinal vascular alterations include leukostasis, increased permeability, pericyte loss, appearance of acellular capillaries, and pathological angiogenesis (2, 3). The underlying mechanisms are still unclear. However evidence is accumulating that diabetes-induced retinal vascular dysfunction is associated with hyperglycemia-induced increases in formation of superoxide and peroxynitrite (4–8). Studies have shown that diabetes and high glucose (HG)-induced increases in oxidative and nitrosative stress are accompanied by increases in expression and activity of both endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) and that inhibiting NOS activity reduces oxidative and nitrosative stress and prevents early signs of diabetic retinopathy (8–12). However, in spite of the well established link between nitrosative stress and vascular injury in models of diabetes, other work has shown that diabetes impairs ocular hemodynamics by reducing the bioavailability of nitric oxide (NO) (13). NO plays a critical role in maintaining proper tissue blood flow and perfusion, blocking platelet activation and leukocyte adhesion, preventing smooth muscle cell proliferation, and enhancing endothelial cell (EC) survival (14). NO is generated by NO synthase (NOS) from its substrate L-arginine. Acute administration of L-arginine has been shown to increase NO synthesis and restore endothelial-dependent vasodilation in several diseases characterized by vascular dysfunction, including diabetes, hypertension, and heart failure (15, 16), suggesting that decreased L-arginine availability is pivotal to their pathogenesis (17, 18).

Arginase, an enzyme of the urea cycle, uses L-arginine as substrate to produce urea and ornithine. Studies have shown that excessive activity of arginase limits the supply of L-arginine needed for proper NOS function, which will cause uncoupling of the NOS dimer. Uncoupled NOS will use more molecular oxygen to produce superoxide instead of NO. Superoxide will react rapidly with any available NO to produce the highly toxic and proinflammatory oxidant peroxynitrite. This mechanism has been linked to vascular dysfunction in many diseases including diabetes (19, 20). Moreover, angiotensin II, which is upregulated in the diabetic retina, has been shown to increase arginase expression and activity by a

mechanism involving activation of P38 MAP kinase/Rho kinase pathway (21).

In a model of endotoxin-induced retinal inflammation, we have shown that elevated arginase activity and expression is correlated with decreases in NO formation, increased cytokine release and retinal inflammation (22). Our studies in rodent models of diabetes have shown that impairment of retinal endothelial-dependent vasodilation is mediated by increases in arginase expression/activity (23). In this study, we hypothesized that increases in arginase expression/activity have a role in diabetes-induced retinal vascular activation/injury via a mechanisms involving decreases in bioavailable NO.

MATERIALS AND METHODS

TREATMENT OF MICE

All procedures with animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee (Animal Welfare Assurance no. A3307-01). Experiments were performed with double knockout mice lacking one copy of arginase I and both copies of II ($AI^{+/-}AII^{-/-}$) (24). Deletion of both copies of arginase I is lethal around 10 days postnatal. The knockout mice were provided by Dr. Steven Cederbaum. C57BL6J mice were used as controls. Diabetes was induced by repeated injection (up to four times) of streptozotocin [STZ, 65 mg/kg, dissolved in 0.1 M sodium citrate buffer (pH 4.5), i.p.], once every other day until diabetes was established. Mice with glucose level over 350 mg/dl as determined by a blood glucose meter were considered diabetic. Diabetic and age-matched control mice were used for experiments after 2 months of diabetes.

CELL CULTURE

Bovine retinal ECs (passages 5–9) were incubated in the medium (M199 + 0.2%FBS + 50 μM L-arginine) containing 5.5 mM D-glucose (NG), 25 mM D-glucose (HG) for 1–3 days. For treatment with the arginase inhibitors [*S*-(2-boronoethyl)-L-cysteine (BEC), 2(*S*)-amino-6-boronohexanoic acid (ABH)], cells were treated with BEC (10 μM) or ABH (100 μM) together with HG.

RETINAL VESSEL ISOLATION

Retinas were dissected and placed in water on ice for 1 h followed by treatment with Deoxyribonuclease I (116 U/ml, 25–30 min, Worthington Biochemical Corp., Lakewood, NJ, USA). Vessels were rinsed to remove contaminating neurons and glia and then homogenized. Protein was extracted by RIPA lysis buffer (Millipore, Billerica, MA, USA). The supernatant (each containing 10 μg protein) was mixed with 4× loading buffer. After boiling, the samples were analyzed by western blot as described below. Vessel purity was verified by microscopic examination of the vessel preparations. Retinal vessels from six different animals were combined for each determination and five different replications were prepared for both diabetic and control mice.

WESTERN BLOT

Retinas or ECs were homogenized in a RIPA Lysis Buffer (Millipore) supplied with phosphatase inhibitor cocktail (Roche), 1 mM phenyl methyl sulfonyl fluoride, and protease inhibitor cocktail (Sigma-Aldrich). Twenty micrograms protein samples were subjected to 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a nitrocellulose membrane and the membrane was blocked (5% milk) and incubated with primary antibodies against arginase I (1:500, Santacruz Biotech), arginase II (1:500, Santacruz Biotech), intercellular adhesion molecule 1 (ICAM-1) (1:500, Santacruz Biotech), actin (1:2000, Sigma-Aldrich), and β-tubulin (1:2000, Sigma-Aldrich), followed by horseradish peroxidase-conjugated secondary antibody (1:2000, GE Healthcare Bio-Sciences). Immunoreactive proteins were detected using the enhanced chemiluminescence (ECL) system (GE Healthcare Bio-Sciences).

ARGINASE ACTIVITY

Retinas or ECs were homogenized in ice-cold lysis buffer (50 mM Tris-HCl, 0.1 mM EDTA, and EGTA, pH 7.5) containing protease inhibitors with a pestle. The homogenate was centrifuged at 14,000 *g* for 20 min and the supernatant was removed for enzyme assay. Arginase activity was assayed as previously described (19). Briefly, the enzyme was activated by heating the lysate at 56°C in 25 mM Tris buffer (pH 7.4) containing 5 mM $MnCl_2$. L-Arginine hydrolysis was then conducted by incubating 50 μl of the activated lysate with 50 μl of 0.5 M L-arginine (pH 9.7) at 37°C for 60 min. The reaction was stopped in acid medium. The concentration of urea, which is the end product of L-arginine hydrolysis by arginase, was determined after adding 25 μl of 9% α-isonitrosopropiophenone. Protein concentration in the lysates was determined by a BCA assay (Pierce Biotechnology). Arginase activity was calculated as mmol urea/mg protein and as percent of control.

IMMUNOFLUORESCENCE

Eyes were removed, fixed in 4% paraformaldehyde (overnight, 4°C), washed in PBS and retinas were isolated and cryoprotected in 30% sucrose. Frozen sections (10 μM) were permeabilized in 1% Triton (10 min) and blocked in 10% normal goat serum containing 1% BSA (1 h). Sections were incubated overnight at 4°C in primary antibodies (monoclonal anti-arginase I, 1:200, BD Biosciences; polyclonal anti-arginase II, 1:200, Santacruz Biotechnology; polyclonal CRALBP cellular retinaldehyde binding protein, 1:200, Santacruz Biotechnology) followed by reaction with fluorescein or Texas red conjugated secondary antibodies (Molecular Probes), PBS rinse and mounted with Vectashield (Vector Laboratories).

NITRITE AND NITRATE FORMATION

For *in vivo* studies retinas from WT mice with normal glucose (Con), HG (Db), or from $AI^{+/-}AII^{-/-}$ mice with HG (Db-Ko) were homogenized in PBS and centrifuged at 14000 rpm for 10 min at 4°C and supernatants were collected. The level of nitrite in the supernatants was analyzed using NO-specific chemiluminescence. In brief, samples containing nitrite were refluxed in glacial acetic acid containing sodium iodide. Nitrite is quantitatively reduced to NO under these conditions, which can be quantified by a chemiluminescence detector after reaction with ozone in a Seivers NO analyzer (NOA 280i, GE Analytical Instruments, Boulder, CO, USA). To measure the total level of nitrite plus nitrate, supernatants were incubated with PBS containing nitrate reductase

(0.25 U/ml), NADPH (13 μg/ml), and FAD-Na2 (4 μg/ml) at 30°C for 1 h to reduce nitrate to nitrite. Then the level of nitrite was analyzed using NO-specific chemiluminescence. Protein concentration in the supernatant was determined by BCA assay. The level of nitrite or nitrite plus nitrate was normalized to the protein concentration in the supernatant and calculated as percentage of control. Wild type mice with normal glucose were used as reference. For *in vitro* studies, retinal ECs were treated with 5.5 mM normal glucose (NG), 25 mM HG, or 35 mM glucose for 24 h in M199 medium containing 0.2%FBS and 50 μM L-arginine. Nitrite level in the conditioned media was measured as described in the above procedures for retinal lysates.

NO FORMATION *IN SITU*

The NO fluorescent indicator 4,5-diaminofluorescein diacetate (DAF-2 DA, Calbiochem) was used to assess production of NO in retinal tissue sections. In presence of oxygen, DAF-2 reacts with NO to yield the highly fluorescent product triazolofluorescein, which is monitored using excitation and emission wavelengths of 485 and 538 nm, respectively. Unfixed fresh retinal frozen sections from each group were reacted with DAF-2 DA (10 μM, 15 min in the dark at 37°C). One set was pretreated with the NOS inhibitor L-NAME (1 mM). The slides were washed with Hepes solution (10 mM), covered and a series of nine images from each slide were taken by using AxioVision Imaging System (Zeiss). Fluorescence intensity was quantified using MetaMorph Microscopy Image Analysis Software (Molecular Devices).

DIHYDROETHIDIUM ASSAY FOR SUPEROXIDE FORMATION

To evaluate production of superoxide *in situ* the oxidative fluorescent dye dihydroethidium (DHE) was used as described previously (25, 26). DHE is freely permeable to cells and in the presence of $O_2^{\cdot-}$ is oxidized to ethidium bromide which binds to DNA and fluoresces red. The images were analyzed for reaction intensity by using the MetaMorph Image System (Molecular Devices).

LEUKOCYTE ADHESION

Retinal leukostasis was assayed by labeling the adherent leukocytes using Concanavalin A (Vector Laboratories). This method has been described previously (26).

TUNEL ASSAY

Endothelial cell death was studied using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay using Fluorescein *in situ* cell death detection kit (Millipore) according to the manufacturer's protocol. Fluorescent images were taken and the number of TUNEL positive cells was quantified manually.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Group differences were evaluated by using one way analysis of variance followed by Tukey's *post hoc* test for statistical analysis. $p < 0.05$ was considered significant. Animal studies were performed in groups of 6–18 mice. Tissue culture studies were performed in groups of four to eight cultures and each experiment was repeated in at least twice for cells derived from different primary isolates.

RESULTS

ARGINASE ACTIVITY AND EXPRESSION ARE INCREASED BY DIABETES AND HIGH GLUCOSE

We first examined the effects of diabetes on arginase activity in retinas from STZ-induced diabetic and age-matched control mice (8 weeks). Immunofluorescence analysis showed that both arginase isoforms are expressed in the mouse retina (**Figure 1A**). The two isoforms differed in their distribution pattern. Arginase I was strongly expressed in cells within the ganglion cell layer and inner nuclear layer and in cells that resemble Müller glia (arrowheads). Localization of arginase I in Müller glia was confirmed by double labeling for arginase I and the Müller cell marker cellular retinaldehyde binding protein (CRALBP; **Figure 1B**). Retinal vessels in all groups are also labeled due to cross reactivity between mouse vascular proteins and the anti-mouse secondary antibody. Control studies performed in the $AI^{+/-}AII^{-/-}$ mice showed low levels of arginase I immunoreactivity, consistent with hemizygous deletion of the arginase I gene. Arginase II was strongly expressed in cells of the inner nuclear layer cells as well as in cells in the nerve fiber and inner plexiform layers. Sections from the $AI^{+/-}AII^{-/-}$ mice were negative for arginase II (data not shown). Measurement of arginase activity in retinal tissue extracts using an assay for urea produced by L-arginine hydrolysis confirmed that enzyme activity is significantly increased in the diabetic retinas as compared with the non-diabetic controls (**Figure 1C**).

The above studies showed that diabetes induces increases in retinal arginase activity and suggested that both isoforms are upregulated. However, there are numerous sources of arginase within the retina. To specifically assess the effects of diabetes in increasing arginase in vascular cells, we isolated retinal vessels from diabetic and control retinas and performed Western blot for arginase I and II. Vessels from three groups of six diabetic and control retinas were isolated by isotonic shock and pooled for Western blot analysis. The results showed that the retinal vessels were positive for both isoforms and that arginase I was increased in the vessels from diabetic mice as compared with the controls (**Figure 1D**). Levels of arginase II were similar in vessels from the diabetic and control mice (**Figure 1E**).

In order to determine more specifically the potential effects of diabetes and hyperglycemia on arginase expression/activity in the vascular endothelium, we performed additional experiments using retinal EC treated with HG media (25 mM glucose). L-Arginine hydrolysis assay showed that HG treatment caused a significant increase in arginase activity (∼30%, **Figure 2A**). Western blotting showed that both arginase isoforms are expressed in retinal ECs and that arginase I was increased significantly in the ECs treated with HG as compared with the control ECs (**Figure 2B**). Levels of arginase II were not altered by the HG treatment (**Figure 2C**).

DIABETES OR HIGH GLUCOSE-INDUCED REDUCTIONS IN NO FORMATION ARE RESTORED BY ARGINASE DELETION OR INHIBITION

We next examined the impact of these diabetes- and HG-induced increases in arginase activity on NO formation. These experiments used an NO analyzer to evaluate bioavailable NO vs. total NO products in control and diabetic retina tissue

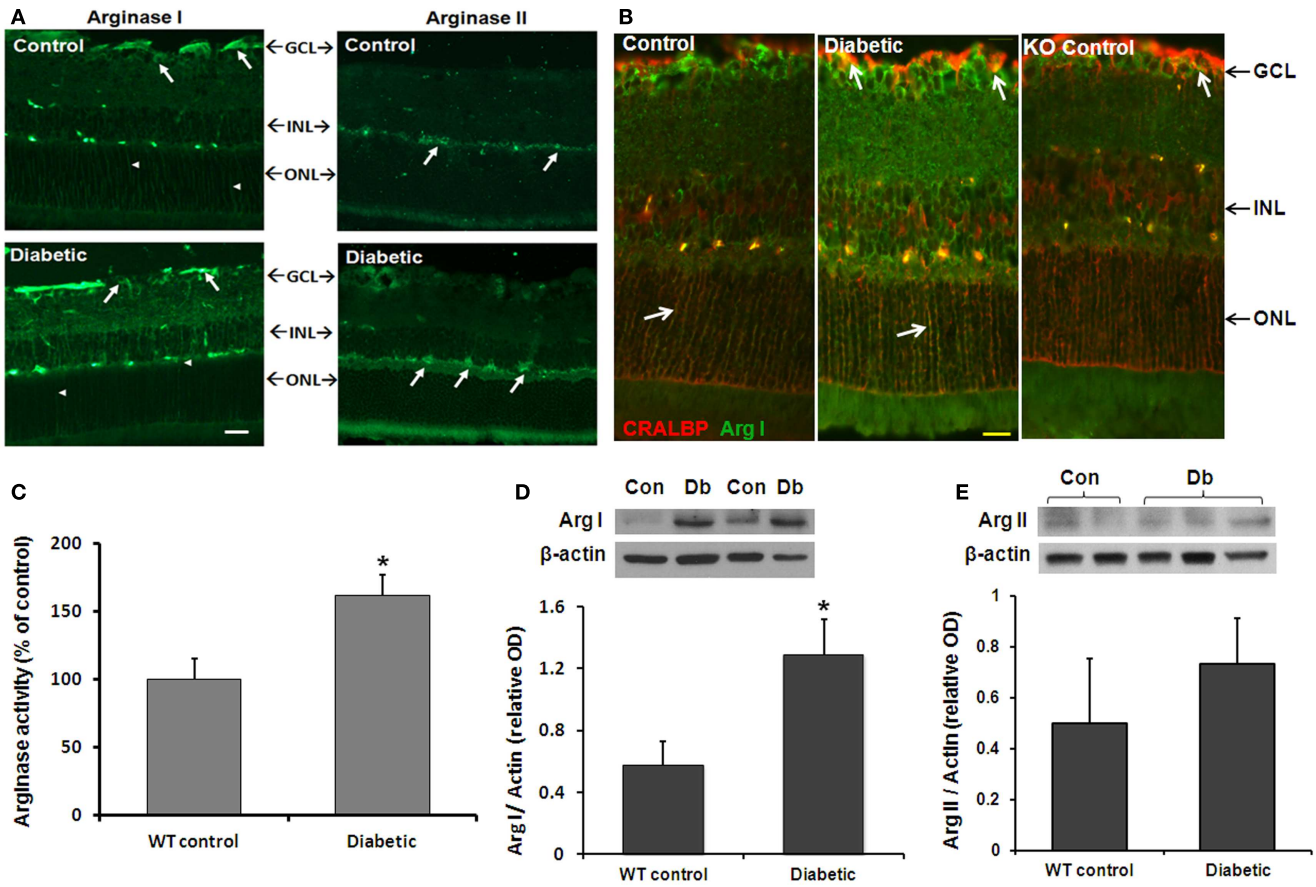

FIGURE 1 | Diabetes-induced increases in retinal arginase protein and activity levels. Mice were rendered diabetic with STZ and sacrificed after 2 months. **(A)** Retinal arginase I and II protein distribution were examined by immunofluorescence imaging. Scale bar = 50 μM. **(B)** Double label of arginase I (green) and cellular retinaldehyde binding protein (red) was used to assess arginase I distribution in retinal Müller cells of the wild type and $AI^{+/-}AII^{-/-}$ (KO) retinas. Scale bar = 20 μM. **(C)** Arginase activity was determined using an assay for urea formation. Relative levels of arginase I **(D)** and arginase II **(E)** protein expression in retinal vascular cells were determined by Western blot analysis of isolated retinal vessels ($n = 6$–30) ($^*p < 0.05$ compared with the non-diabetic control).

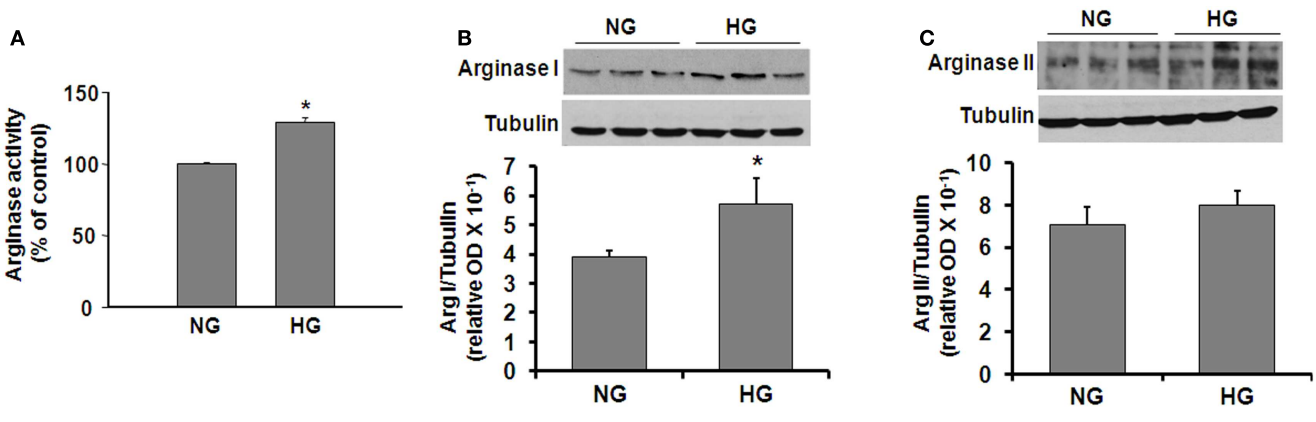

FIGURE 2 | High glucose-induced increases in arginase protein and activity levels in retinal vascular EC. Retinal EC (p5–p9) were incubated in medium (M199 + 0.2% FBS + 50 μM l-arginine) containing 5.5 mM d-glucose (NG) or 25 mM d-glucose (HG) for 24 h. **(A)** Arginase activity in cell lysate was determined by arginase activity assay ($^*p < 0.05$ compared with NG, $n = 4$). **(B,C)** Levels of arginase I and II protein were determined by Western blot analysis ($n = 3$–4) and quantified using ImageJ ($^*p < 0.05$ compared with the NG control).

extracts and HG-treated retinal ECs. In tissue extracts, the total amount of NO is represented by the relative amount of tissue nitrate plus nitrite. Nitrite is the final product of NO auto-oxidation in water or hemoglobin-free media and is an indicator of the amount of bioavailable NO. On the other hand, nitrate formation is promoted in the presence of superoxide. Thus, an increase in nitrate levels serves as an indicator of NOS uncoupling and nitrosative stress. Analysis of total nitrate + nitrite levels showed that STZ-induced diabetes caused a significant increase in the total NO products compared with the controls, whereas nitrite levels were significantly decreased (**Figure 3A**). To examine the potential role of arginase in these alterations, parallel studies were performed in double knockout mice that lack one copy of the gene for arginase I and both copies of arginase II ($AI^{+/-}AII^{-/-}$). The results of this study showed that the diabetes-induced decrease in nitrite was prevented in the $AI^{+/-}AII^{-/-}$ mice (**Figure 3B**), implying the involvement of arginase in the diabetes-induced reductions in bioavailable NO.

The effects of STZ-induced diabetes on tissue distribution of NO were assessed by imaging studies using the NO-specific indicator dye 4,5-diaminofluorescein-2 diacetate (DAF-2 DA). DAF-2 DA reacts with NO to form a green fluorescent product. Fluorescence intensity measurements showed a significant decrease in NO levels in diabetic WT compared to control mice retinas. The DAF-2 DA reaction was predominantly around the blood vessels and in the inner retina and plexiform layers and in the photoreceptor outer segment layer (**Figure 3C**). The signal was completely blocked by pretreatment with the NOS inhibitor L-NAME indicating the specificity of the reaction for NO. Furthermore, the diabetes-induced decrease in NO was abrogated in the $AI^{+/-}AII^{-/-}$ mice, confirming that arginase activity is involved in reducing bioavailable NO levels in the diabetic retina.

To further assess the potential involvement of arginase in altering the function of vascular EC NOS during hyperglycemic conditions, we performed *in vitro* studies using retinal ECs. These experiments showed that the HG-induced increase in arginase activity was accompanied by a significant decrease in

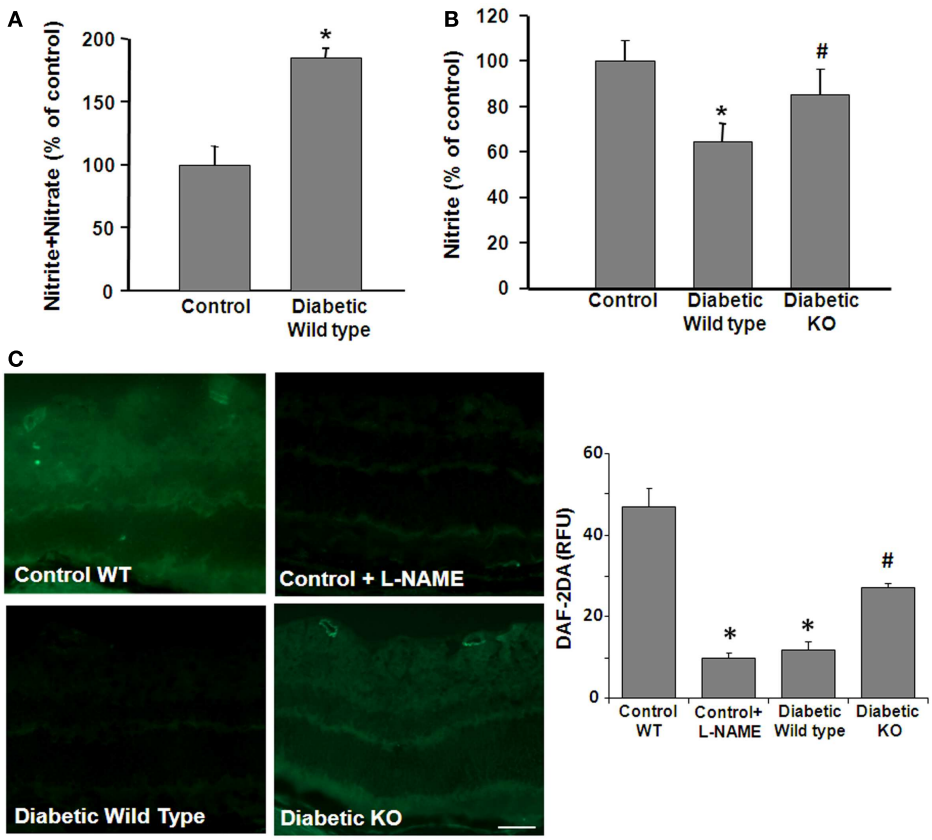

FIGURE 3 | Prevention of diabetes-induced decrease in bioavailable NO by arginase deletion. Retinas from normoglycemic wild type controls, wild type diabetic or diabetic knockout mice (KO) were prepared for analysis of NO formation by using an NO analyzer **(A,B)** and by DAF-2-DA histochemistry **(C)**. **(A)** Total NO content in the diabetic retina was significantly increased compared with the control as shown by measurement of nitrate + nitrite levels. **(B)** The level of bioavailable NO in the wild type diabetic retina was significantly reduced compared with the controls as shown by measurement of nitrite levels. The decrease in nitrite levels was blocked in the diabetic KO mice (*$p < 0.05$, $n = 4$–6). **(C)** NO formation *in situ* was determined by reaction of DAF-2-DA. The DAF-2-DA fluorescent product was significantly diminished in the wildtype diabetic retina as compared with the non-diabetic control. This effect was significantly blunted in the diabetic KO retinas. Pretreatment of the retinal sections with L-NAME (1 mM) markedly reduced formation of the DAF-2-DA product (*$p < 0.05$ vs. control, #$p < 0.05$ vs. diabetic, $n = 4$–6, scale bar = 50 μM).

NO formation as determined by measurement of nitrite in the culture medium using an NO analyzer (**Figure 4**). The HG-induced decline in NO was blocked by treatment of the cultures with the highly specific arginase inhibitor BEC, suggesting that HG-induced activation of arginase is involved in reducing NO production in retinal ECs. All together, these data support the hypothesis that diabetes reduces nitrite levels in the retina even though total NO products are increased and imply that the reduction in nitrite levels is due to increased arginase activity.

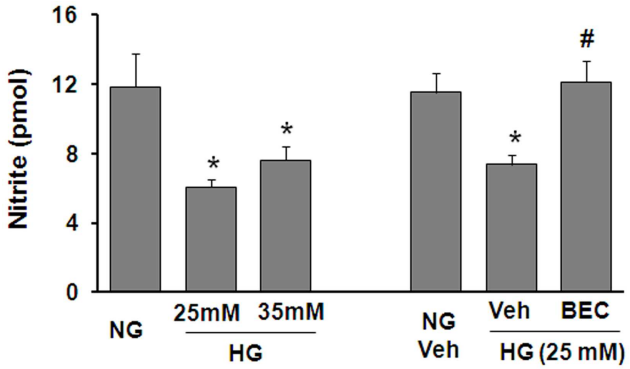

FIGURE 4 | Prevention of high glucose-induced decreases in bioavailable NO levels in retinal endothelial cells by inhibition of arginase. Retinal EC (p5–p9) were incubated in medium (M199 + 0.2%FBS + 50 μM l-arginine) containing 5.5 mM d-glucose (NG), 25 mM d-glucose (HG), or 35 mM d-glucose with or without 10 μM BEC or vehicle (Veh, 0.1% saline) for 24 h. The nitrite level in the medium was determined by NO analyzer (*$p < 0.05$ compared with NG, #$p < 0.05$ vs. Veh, $n = 8$–16).

DIABETES OR HIGH GLUCOSE-INDUCED INCREASES IN OXIDATIVE STRESS LEVELS ARE DIMINISHED BY ARGINASE DELETION/INHIBITION

Increased production of reactive oxygen species (ROS) has been implicated in the pathogenesis of vascular inflammatory diseases involving many organs including the retina (27–29). Uncoupling of NOS serves as an important mechanism of ROS formation in disease. In the present work, we investigated the effect of arginase deletion on ROS production in the diabetic retina. Dihydroethidine (DHE) imaging analysis and quantification of superoxide formation in retinal sections show that superoxide formation was significantly increased in the diabetic retina (**Figure 5**). This effect was markedly blunted by treatment with the NOS inhibitor L-NAME and in the $AI^{+/-}AII^{-/-}$ mice. These results imply that increased arginase activity and uncoupling of NOS are prominently involved in increasing oxidative stress in the diabetic retina. Specificity of the reaction for superoxide was demonstrated by near complete inhibition of the signal by superoxide dismutase (SOD, data not shown).

Tissue culture studies using DHE imaging of retinal ECs also showed a significant increase in superoxide formation following 3 days of HG treatment (**Figure 6**). This increase in superoxide formation was significantly inhibited by treatment of the cells with the arginase inhibitor ABH [2(*S*)-amino-6-boronohexanoic acid] or SOD.

DIABETES-INDUCED INCREASES IN LEUKOSTASIS ARE BLOCKED BY ARGINASE DELETION

An adequate supply of NO is critical for maintaining healthy blood vessels by maintaining appropriate blood flow and inhibiting leukocyte attachment to the vessel walls (30). Therefore, diabetes-induced increases in arginase activity may contribute to vascular injury by increasing leukostasis. To evaluate whether arginase

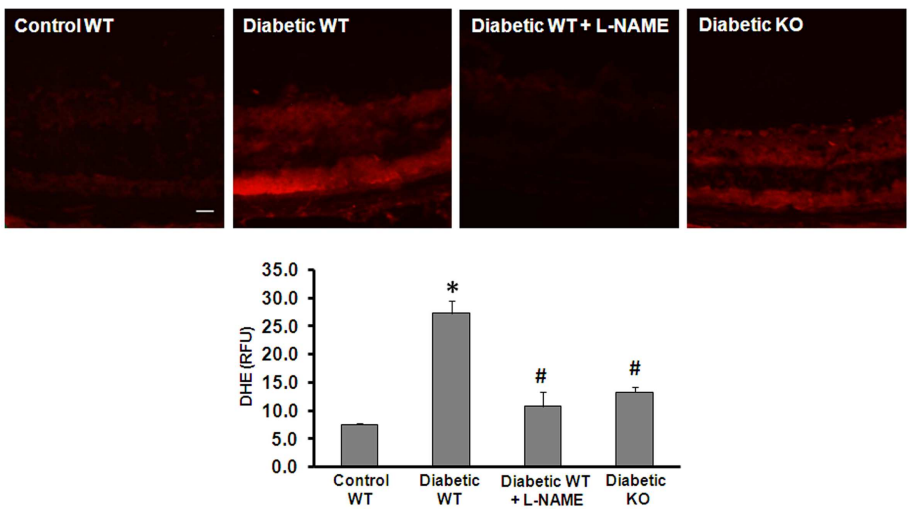

FIGURE 5 | Prevention of diabetes-induced increase in oxidative stress by arginase deletion. DHE imaging was performed using flash frozen retinal sections from wild type control, wild type diabetic, and diabetic $AI^{+/-}AII^{-/-}$ mice (KO). Diabetic retinas display increased production of ROS as compared with the controls. The increased formation of superoxide was blocked by pretreatment of the sections with L-NAME (1 mM). Arginase KO also significantly blunted the diabetes-induced increase in superoxide formation (*$p < 0.05$ vs. control, #$p < 0.05$ vs. diabetic wild type, $n = 6$, scale bar = 50 μM).

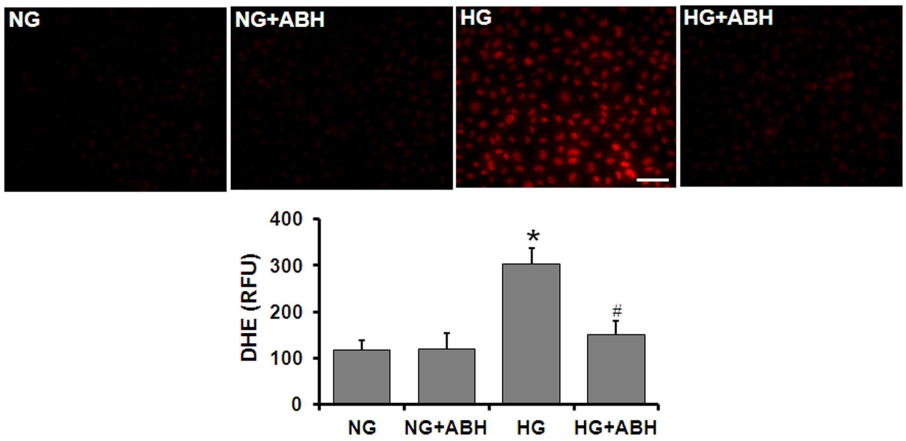

FIGURE 6 | Prevention of high glucose-induced increase in oxidative stress by arginase inhibition. Retinal EC (p5–p9) were incubated in medium (M199 + 0.2%FBS + 50 μM l-arginine) containing 5.5 mM d-glucose (NG) or 25 mM d-glucose (HG) for 3 days. DHE imaging shows a significant increase in superoxide formation in the HG-treated ECs. This effect is markedly blunted by treatment with the arginase inhibitor ABH (*$p < 0.05$ vs. NG, # < 0.05 vs. HG, $n = 4$, scale bar = 50 μM).

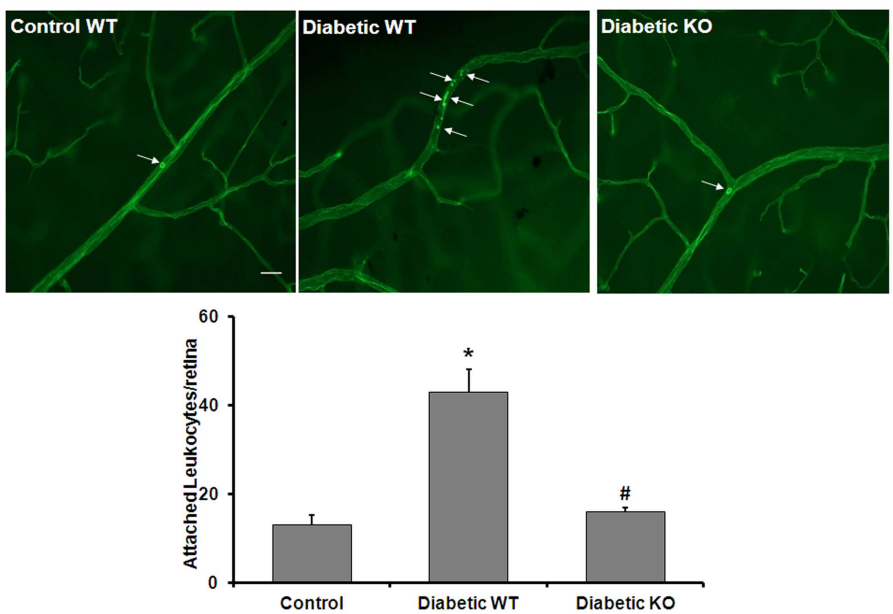

FIGURE 7 | Prevention of diabetes-induced increase in leukocyte adhesion by arginase deletion. Wild type controls, wild type diabetic or arginase $AI^{+/-}AII^{-/-}$ (KO) diabetic mice were perfused through left ventricle with Concanavalin A to label leukocytes attached to the vascular endothelium. The number of attached leukocytes was significantly increased in the wildtype diabetic mice as compared to non-diabetic controls and arginase KO significantly blunted this effect (*$p < 0.05$ vs. control, #$p < 0.05$ vs. diabetic wildtype, $n = 5$, scale bar = 100 μM).

activity plays a role in diabetes-induced retinal vascular activation/injury, we analyzed leukocyte adhesion in the retinal vessels of WT and $AI^{+/-}AII^{-/-}$ diabetic and control mice. Leukostasis was assayed by using concanavalin A to label the adherent leukocytes. The data show that the number of adherent leukocytes was increased significantly in the diabetic retinas compared to control retinas and this increase is abrogated by deletion of arginase (**Figure 7**). This result indicates that increased arginase activity is critically involved in leukocyte-EC attachment in the diabetic retina.

In order to further assess the potential role of HG-induced increases in arginase activity in causing vascular activation and injury, we examined the effects of HG treatment on expression of ICAM-1. ICAM-1 is present in low concentrations in the membranes of normal ECs, but the concentrations increase under conditions of endothelial activation. ICAM-1 is a ligand for the

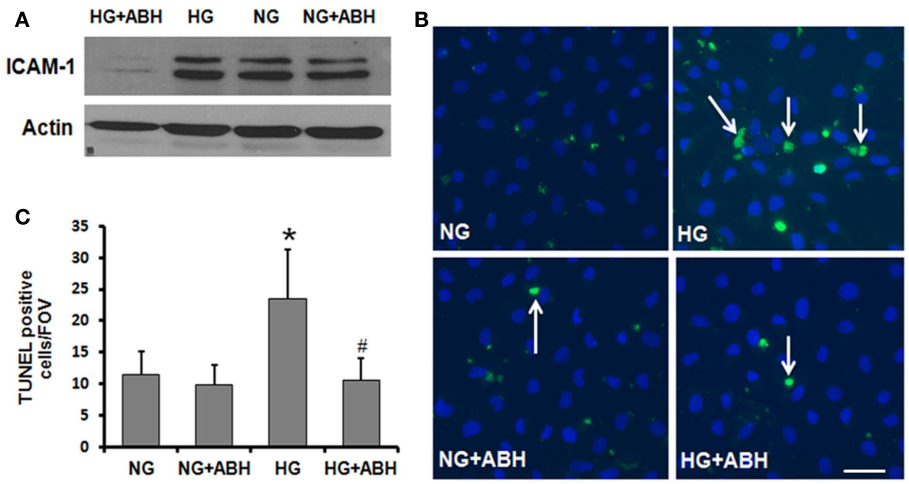

FIGURE 8 | Prevention of high glucose-induced increase in ICAM-1 levels and cell death in retinal ECs by inhibition of arginase. Retinal EC (p5–p9) were incubated in medium (M199 + 0.2%FBS + 50 μM l-arginine) containing 5.5 mM d-glucose (NG) or 25 mM d-glucose (HG) for 3 days. **(A)** Western blotting shows that the high glucose-induced increase in ICAM-1 levels is prevented by treatment with the arginase inhibitor ABH. **(B,C)** TUNEL labeling shows that the high glucose-induced increase in cell death is prevented by treatment with ABH (*$p < 0.05$ vs. NG, #< 0.05 vs. HG, $n = 4$, scale bar = 50 μM).

LFA-1 integrin receptor on leukocytes, which bind to ECs via ICAM-1/LFA-1. Western blotting for ICAM-1 showed that HG treatment of retinal ECs caused upregulation of ICAM-1 and that this effect was abrogated by treatment of the cells with ABH (**Figure 8A**).

HIGH GLUCOSE-INDUCED INCREASES IN CELL DEATH ARE REDUCED BY ARGINASE INHIBITION

We also investigated the potential impact of arginase activation in retinal EC death under HG conditions. TUNEL assay was performed on cells treated with or without the arginase inhibitor ABH. As shown in **Figure 8**, cell death was significantly elevated in HG-treated BRECs compared to NG controls. This increase in cell death was prevented by inhibition of arginase, using ABH treatment (**Figures 8B,C**). These results demonstrate that arginase function is involved in HG-induced EC death.

DISCUSSION

Excessive activity of the urea cycle enzyme arginase has recently emerged as a critical player involved in the development of numerous vascular disease conditions associated with decreased NO bioavailability and endothelial dysfunction including diabetes, aging, ischemia/reperfusion injury, and hypertension (19, 20, 31–34). Diabetic retinopathy is characterized by progressive vascular damage, beginning with oxidative stress, EC dysfunction, reduced blood flow, and leukostasis (3, 35–37). The pathology often progresses with formation of acellular capillaries, development of diabetic macular edema, and/or vitreoretinal neovascularization. Our previous studies have shown that diabetes-induced increases in arginase activity are involved in hyperglycemia-induced impairment of retinal endothelial-dependent vasorelaxation responses (23). Furthermore, the diabetes-induced increase in arginase activity was accompanied by an increase in peroxynitrite formation which was blocked by treatment with ABH [2(*S*)-amino-6-boronohexanoic acid], a highly specific inhibitor of arginase activity. In this study, we determined the impact of arginase activation on signs of diabetic retinopathy in relation to NO bioavailability, oxidative stress, and retinal injury. Our results showed that diabetes or HG-induced increases in arginase expression and activity in retinal vessels and ECs were associated with decreases in NO, increases in ROS formation, leukocyte adherence to the vessel wall, elevated ICAM-1 expression and death of retinal ECs. These changes were prevented in $AI^{+/-}AII^{-/-}$ mice or by treatment with arginase inhibitors, indicating the role of arginase in the pathology.

The effects of diabetes and HG in causing elevation of NOS expression and activity along with increases in oxidative and nitrosative stress in retinas of diabetic animals and retinal ECs are well documented (5, 6, 8, 10, 36). However, this study is the first to show that levels of bioavailable NO are reduced and that this effect is prevented by arginase deletion. Much of the previous work has focused on iNOS as a pathological mediator of retinopathy, emphasizing the damaging effects of iNOS activity and formation of peroxynitrite and other oxidants in relation to vascular EC death and acellular capillary formation in the later stages of diabetic retinopathy (6, 12, 36). Studies have suggested that high levels of NO resulting from upregulation of iNOS expression in retinal glia could be involved in the impairment of retinal blood flow and responses to light during diabetes (38).

Interpretation of NO-mediated toxicity in retinopathy is complicated by the technical difficulty of measuring NO in biological samples. Most studies showing increased "NO levels" in retinal models have used photometric assays to measure total tissue nitrite levels after reduction of nitrate to nitrite. Given that nitrate formation is favored in the presence of excess superoxide (39), the

values obtained in such analyses represent relative levels of total NO produced rather than bioavailable NO. So far, little attention has been given to the impact of diabetes on bioavailable NO in retina. However, it is well understood that when the supply of L-arginine needed for NOS activity is limited, the enzyme will become uncoupled and will use molecular oxygen to produce superoxide which combines rapidly with NO to produce the potent inflammatory and toxic oxidant peroxynitrite. Our current results indicate that the amount of bioavailable NO is substantially diminished by HG or diabetes whereas NOS-dependent formation of superoxide is increased, implying that NOS is uncoupled. Furthermore, the decline in NO is blocked by inhibiting or knocking out arginase, consistent with the proposed action of arginase in causing NOS uncoupling. Our study is the first we are aware of to show that arginase is a key player in diabetes/HG-induced decreases in bioavailable NO.

Studies in a variety of peripheral tissues have shown that endothelial dysfunction resulting from impaired NO synthesis involves arginase. Decreased plasma levels of L-arginine have been reported in diabetic animals and patients (40, 41). In diabetic patients, increased arginase activity has been reported in penile vessels associated with erectile dysfunction (42, 43). Experiments with diabetic rat aorta and coronary vessels showed that diabetes-induced impairment of vasorelaxation to acetylcholine was correlated with increased arginase activity. Treatment of bovine coronary ECs with HG also increased arginase activity and diminished NO production, which was normalized by transfection with arginase I siRNA (19). Here we demonstrate for the first time that diabetes and HG treatment significantly increased arginase activity and arginase I protein expression in both retinal vessels and retinal ECs. Immunolocalization studies showed that the two isoforms differ in their tissue distribution. Arginase I was localized to the ganglion cell layer and inner nuclear layer and processes of Muller glial cells. Arginase II was localized to cells of the inner nuclear layer as well as in the nerve fiber and inner plexiform layers. It is possible that both arginase isoforms contribute to increased arginase activity at specific sites within the retina. However the total amount of arginase II protein was similar in vessels isolated from diabetic and control retinas.

In our studies, diabetic mice lacking one copy of arginase I and both copies of arginase II had increased NO levels, less ROS formation and decreased leukostasis as compared with the wildtype diabetic mice, which highlights the important role of arginase expression in diabetes-induced vascular dysfunction. Further work is needed to determine the specific isoform involved. Depending on specific disease conditions, arginase I or arginase II or both may be involved. Arginase I has been associated with endothelial dysfunction in aging, diabetes, and ischemia (19, 32, 44). Arginase II activity has been implicated in atherosclerosis (45) and in retinal neuronal cell degeneration during oxygen-induced retinopathy (46). Others have shown that arginase I is involved in altering NOS function in aging rat aortas (31, 44). Previous studies in coronary ECs have shown that HG-induced increases in arginase activity can be blocked by transfection of arginase I siRNA (19). Our previous studies of retinal vascular function in diabetic mice showed that diabetes-induced retinal vascular dysfunction is reduced in mice lacking one copy of arginase I (23). In order to identify the arginase isoform and cellular sources responsible for specific pathological changes in diabetic retinopathy, additional studies are needed using cell-specific knockout of arginase I and arginase II.

In view of the well established role of iNOS in retinal injury during diabetes (3), the protective effects of arginase blockade in limiting signs of retinopathy may seem paradoxical. However, it is important to note that NO has multiple protective actions, including blocking platelet aggregation and leukocyte adhesion, inhibiting activation of the proinflammatory transcription factor nuclear factor (NF)-κB and modifying lipids to form nitroalkenes, which are anti-inflammatory (47–50). Therefore, under diabetic conditions, inhibition of arginase could limit retinal injury through increased availability of L-arginine for production of NO. Conversely, the beneficial effect of NOS inhibitor treatment in the diabetic retina may be due in part to blockade of uncoupled NOS. A study using vessels from old rats has shown that inhibition of arginase abrogated the aging-induced decrease in eNOS dimer-to-monomer ratio, an indicator of NOS uncoupling (51) suggesting that arginase inhibition restores eNOS coupling and increases bioavailable NO levels. Our present studies indicate that arginase blockade leads to restoration of bioavailable NO and reductions in ROS levels in both the diabetic retina and HG-treated retinal ECs.

We did not investigate the effects of diabetes on NOS expression and activity in the present study because previous work has shown that eNOS, iNOS, and nNOS are upregulated in the diabetic retina (8, 9, 11, 12). HG treatment of retinal ECs also caused an increase in expression of eNOS (10). Furthermore, these effects were accompanied by increases in oxidative stress and peroxynitrite formation and inhibiting NOS or scavenging peroxynitrite reduced oxidative stress and prevented signs of diabetic retinopathy *in vivo* and *in vitro* (8, 10). Results of preliminary studies in which diabetic mice were treated with the arginase inhibitor ABH showed decreased levels of iNOS protein as compared with the vehicle-treated diabetic mice (data not shown). Further study is required to confirm these results and elucidate the underlying mechanisms.

Further study is also needed to determine the mechanisms by which diabetes and HG increase arginase activity. Based on our current studies and previous work of others, multiple factors are likely to affect arginase activity during diabetes. Increased arginase activity is associated with inflammatory cytokines and oxidative stress, both of which are elevated during diabetes (29, 52). Evidence suggests that there is a link between *S*-nitrosylation and enzyme activation in specific inflammatory milieus. It has been shown that iNOS-derived NO directly *S*-nitrosates and activates arginase I in ECs stimulated with IFNγ/LPS (53). Peroxynitrite and H_2O_2 have been shown to increase arginase activity in ECs, through PKC mediated activation of RhoA/Rho kinase pathway (54, 55).

In conclusion, our data indicate that arginase is a potential therapeutic target for preserving bioavailability of NO, limiting oxidative stress, and preventing early signs of diabetic retinopathy. Whereas the role of overactive arginase in peripheral vascular dysfunction and injury has been a topic of active research, this is the first time it has been mechanistically linked to retinal vascular injury in diabetes.

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Development of novel arginase inhibitors for therapy of endothelial dysfunction

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Endothelial dysfunction and resulting vascular pathology have been identified as an early hallmark of multiple diseases, including diabetes mellitus. One of the major contributors to endothelial dysfunction is a decrease in nitric oxide (NO) bioavailability, impaired NO signaling, and an increase in the amount of reactive oxygen species (ROS). In the endothelium NO is produced by endothelial nitric oxide synthase (eNOS), for which L-arginine is a substrate. Arginase, an enzyme critical in the urea cycle also metabolizes L-arginine, thereby directly competing with eNOS for their common substrate and constraining its bioavailability for eNOS, thereby compromising NO production. Arginase expression and activity is upregulated in many cardiovascular diseases including ischemia reperfusion injury, hypertension, atherosclerosis, and diabetes mellitus. More importantly, since the 1990s, specific arginase inhibitors such as *N*-hydroxy-guanidinium or *N*-hydroxy-nor-L-arginine, and boronic acid derivatives, such as, 2(*S*)-amino-6-boronohexanoic acid, and *S*-(2-boronoethyl)-L-cysteine, that can bridge the binuclear manganese cluster of arginase have been developed. These highly potent and specific inhibitors can now be used to probe arginase function and thereby modulate the redox milieu of the cell by changing the balance between NO and ROS. Inspired by this success, drug discovery programs have recently led to the identification of α–α-disubstituted amino acid based arginase inhibitors [such as (*R*)-2-amino-6-borono-2-(2-(piperidin-1-yl)ethyl)hexanoic acid], that are currently under early investigation as therapeutics. Finally, some investigators concentrate on identification of plant derived compounds with arginase inhibitory capability, such as piceatannol-3′-O-β-D-glucopyranoside (PG). All of these synthesized or naturally derived small molecules may represent novel therapeutics for vascular disease particularly that associated with diabetes.

Keywords: endothelium, endothelial dysfunction, arginase, L-arginine, nitric oxide synthase, reactive oxygen species, diabetes mellitus

NITRIC OXIDE AND ITS REGULATION BY ARGINASE

The endothelium plays a major role in cardiovascular physiology. The intact structure and integrity is vital for endothelial cells in order to fulfill their role separating blood flow from surrounding tissues and ensuring an anti-thrombogenic surface. Previously only known of as a passive barrier between those two, the endothelium is now considered a main hub for regulating vascular tone, hemostasis, immune function, structure, smooth muscle cell proliferation, and migration. The combined amount of surface area of the endothelium can reach up to 350 m^2 in total (1). Endothelial dysfunction has been identified as an early harbinger of multiple diseases and resulting vascular pathology. One of the major contributors to endothelial dysfunction is a decrease in nitric oxide (NO) bioavailability, impaired NO signaling, and an increase in the amount of reactive oxygen species (ROS). NO is not only a potent vasodilator and essential in regulating vascular tone and blood pressure, but it also contributes to the regulation of hemostasis, platelet, and leukocyte adhesion as well as vascular smooth muscle cell proliferation. It is freely diffusible with a half-life of just a few seconds prior to its conversion into nitrates and nitrites that are ultimately excreted. NO is synthesized by nitric oxide synthase (NOS), a family of P450 mono-oxygenase-like enzymes which exist in one of three isoforms: nNOS or NOS-1 (neuronal NOS in the central nervous system, skeletal muscle, and pancreas), iNOS or NOS-2 (inducible NOS in activated macrophages, heart, liver, and smooth muscle cells), and eNOS or NOS-3 (endothelial NOS in the endothelium, brain, and epithelium). In the endothelium NO is produced by eNOS (endothelial NOS), which uses L-arginine as a substrate after activation by either chemical agonists or mechanical forces (shear stress). The process of NO synthesis involves firstly the oxidation of arginine to NG-hydroxy-L-arginine (NHA) using nicotinamide adenine dinucleotide phosphate (NADPH) and O_2 catalyzed by NOS (2). The second step involves the production of NO when NHA is converted to L-citrulline via NOS. The actions of NOS are accelerated by the cofactors flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH4). In the absence of its substrate L-arginine or its cofactor BH4, eNOS uncouples and produces ROS, making it one of the four major enzymes involved in the production of vascular ROS. (The others are xanthine oxidase, NADH/NADPH, the mitochondrial electron transport chain, and eNOS). NOS uncoupling is an important contributor to endothelial dysfunction and plays a crucial role in the cardiovascular phenotype. Arginase, a critical urea cycle enzyme,

also utilizes L-arginine. It thereby directly competes with eNOS for their common substrate L-arginine and constrains its availability to eNOS, compromising NO production and increasing the production of ROS by NOS uncoupling (3–5). Arginase, which is present in two isoforms (arginase I in the liver and arginase II extrahepatic) catalyzes the final step of the urea cycle yielding L-ornithine and urea from L-arginine. Arginase II appears to be the predominant isoform in human endothelial cells (6) and is highly compartmentalized. There appear to be at least three distinct pools of L-arginine that are spatially confined and regulated by different transporters and enzymes (7, 8). Thus, local concentrations of L-arginine in microdomains in which NOS and/or arginase might be located may be limiting for NOS isoforms. This concept of the L-arginine paradox is found in the mammalian organism by which L-arginine concentrations by far exceed K_m values of NOS. Consequently, additional L-arginine should not augment nitric oxide formation. *In vivo* however, increasing the plasma concentration of L-arginine has repeatedly been shown to increase NO production (4). The three existing pools of arginine within the cell are (1) a freely exchangeable pool (pool I) with extracellular L-arginine that is regulated by the cationic transporter (CAT-1) and depleted by exchanging the pool with cationic amino acid lysine, (2) a non-freely exchangeable pool (pool II) with extracellular L-arginine that cannot depleted by L-lysine, and (3) extracellular L-arginine pools (pool III) present in endothelial cells and mitochondria in which arginase II modulates NO synthesis through a non-freely exchangeable L-arginine pool (9). According to recent paradigms, the not freely exchangeable L-arginine pool II is composed of two cytosolic microdomains. The major function of pool IIA appears to be the result of citrulline recycling and conversion to arginine by a combined reaction of argininosuccinate synthetase and argininosuccinate lyase (10). The remaining L-arginine pool IIB, which is mainly used by mitochondria, is composed of L-arginine gained by protein breakdown and cannot be depleted by neutral amino acids such as histidine. Arginase expression and activity is upregulated in many diseases including ischemia reperfusion injury (in the heart, lung, and kidneys), hypertension, atherosclerosis, aging, diabetes mellitus, erectile dysfunction, pulmonary hypertension, and aging. Furthermore it can be induced by lipopolysaccharide (LPS), TNFα, interferon γ, 8-bromo-cGMP, and hypoxia (11–14). It has been shown repeatedly that both arginase isoforms are capable of reciprocally regulating NO production (3, 4, 15). More importantly the development of specific arginase inhibitors like *N*-hydroxy-guanidinium or boronic acid derivatives, such 2(*S*)-amino-6-boronohexanoic acid, and *S*-(2-boronoethyl)-L-cysteine (BEC) can now be used to probe arginase function (16). This development in the 1990s allowed the selective inhibition of arginase in the laboratory and thereby the modulation of the substrate availability for NOS and its end product NO (17–19).

ARGINASE STRUCTURE, ENZYMATIC FUNCTION, AND INHIBITOR DESIGN

The first step toward the generation of arginase inhibitors was the determination of the crystal structure of arginase and its active site. Dr. Christianson and his laboratory team from the University of Pennsylvania first demonstrated the binuclear manganese cluster required for catalysis at the active side of rat arginase using X-ray crystallography (20). Successive studies determined the structures of human arginase I (21) and human arginase II (22), both of which contain almost identical metal clusters and active site configurations, this similarity makes it very difficult to develop inhibitors that are specific for one arginase isoform. At the active site, L-ornithine and urea are formed by the collapse of a tetrahedral intermediate that forms after the addition of a hydroxide ion to the L-arginine guanidinium group in the binuclear manganese cluster (**Figures 1A,B**).

The first group of arginase inhibitors consisted of the boronic acid analogs of L-arginine (2)*S*-amino-6-hexanoic acid (ABH) and *S*-2-BEC both of which inhibit the catalytic activity of arginase (16, 23, 24). As both contain trigonal planar boronic acid moieties instead of a trigonal planar guanidinium group, found in L-arginine, binding to the active site of arginase results in a nucleophilic attack of the boron atoms by the metal-bridging ion, resulting in a tetrahedral boronate ion (18). This reaction is identical to the creation of a tetrahedral intermediate by nucleophilic attack of hydroxide ions at the guanidinium group of L-arginine and has been confirmed by crystallographic structure determination (18, 22, 24) (**Figures 1C,D**). The ability of the boronic side chains of ABH and BEC to bind the active side chain of arginase is 50,000 times stronger than the binding of comparable amino acids, aldehyde, or tetrahedral sulfonamide, both of which mimic the tetrahedral intermediate in the arginase mechanism (22, 25). ABH [$K_i = 0.11\,\mu M$ for arginase I and $K_i = 0.25\,\mu M$ (at pH of 7.5) for arginase II (26, 27)] and BEC [$K_i = 0.4$–$0.6\,\mu M$ for arginase I and $K_i = 0.31\,\mu M$ (at pH of 7.5) for arginase II (18)] are therefore specific inhibitors of arginase as they are closely matched to the metal-bridging hydroxide ion in the active site of arginase.

Another category of arginase inhibitors, that is mainly represented by *N*-hydroxy-L-arginine (NOHA) and *N*-hydroxy-nor-L-arginine (nor-NOHA), is characterized by *N*-hydroxy-guanidinium side chains (25, 28–30). Analysis of the enzyme structure by X-ray crystallography reveals that both NOHA and nor-NOHA inhibit arginase by displacing the metal-bridging hydroxide ion of arginase with their *N*-hydroxy group (31). Based on this mechanism, both amino acids inhibit arginase activity with nor-NOHA being a more potent inhibitor ($K_i = 500$ mM for nor-NOHA vs. $K_i = 10\,\mu M$ for NOHA) (28, 30) and with both being less specific than the boronic acid derivates BEC and ABH [for nor-NOHA the K_i values for arginase I and arginase II are 500 and 50 nM, respectively (32)].

Recent efforts now concentrate on expanding the range of arginase inhibitors based on a structure based design program, translating ABH's mechanism of action into new compounds (33, 34). Identifying the α-position of ABH as a target for site substitution, a tertiary amine linked via a two-carbon chain improves the ability of ABH to inhibit both arginase I and arginase II (35). X-ray crystallography demonstrates a close contact between nitrogen and the carboxylic side chain of Asp 181 (arginase I) and Asp 200 (arginase II) at the active site (**Figure 2**) (35). This has led to the discovery of (*R*)-2-amino-6-borono-2-(2-(piperidin-1-yl)ethyl)hexanoic acid (compound 9) a small

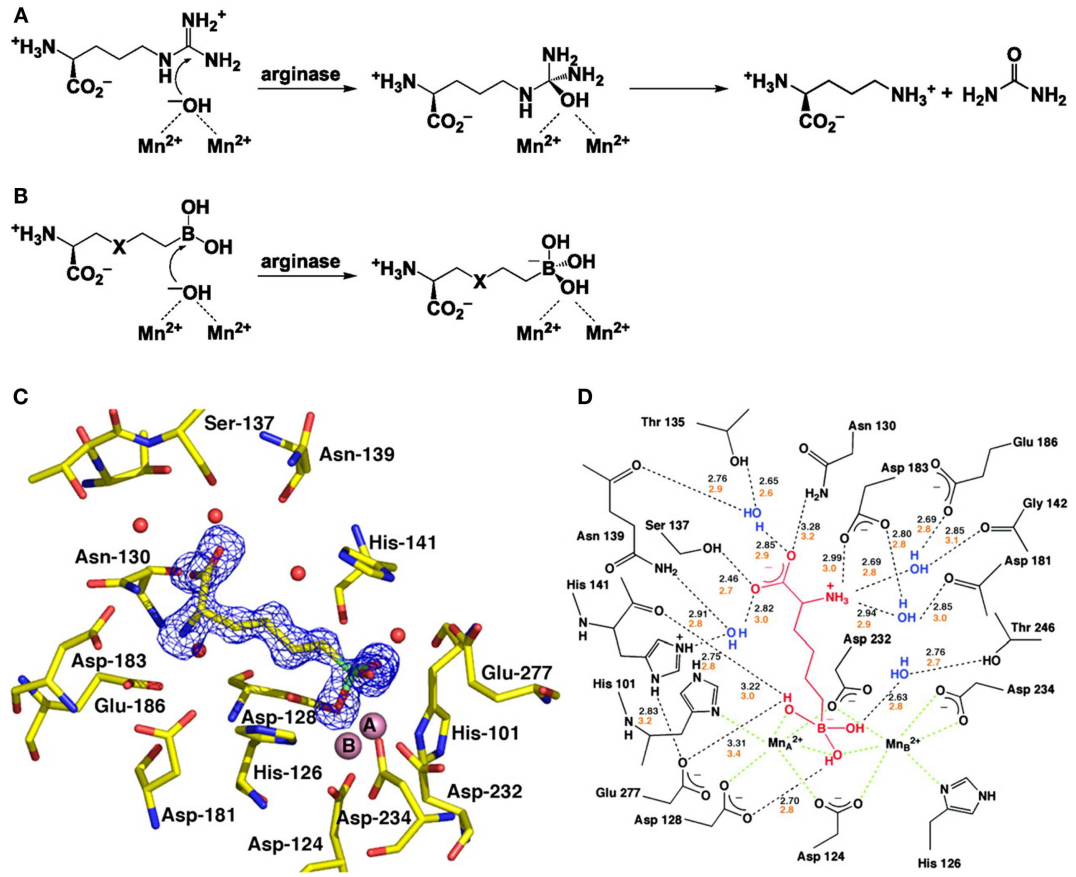

FIGURE 1 | Structure and function of arginase and the interaction with BEC. **(A)** The formation of L-ornithine and urea from L-arginine by arginase. **(B)** The reaction of the boronic acid analogs of L-arginine, 2(*S*)-amino-6-hexanoic acid (ABH) (X representing CH2) and *S*-(2-boronoethyl)-L-cysteine (BEC) (X representing S). **(C)** Electron density map of ABH bound to human arginase I. **(D)** A schematic showing the enzyme-inhibitor hydrogen bond (black dashed lines) and metal coordination interactions (green dashed lines). With kind permission from Santhanam et al. (55).

molecule that has shown efficacy in the attenuation of myocardial reperfusion injury (33). Compound 9 contains a piperidine linked to the α-carbon by a two-carbon aliphatic chain at the α-position. This results in the formation of new through-water hydrogen bonding interaction with Asp 181 and Asp 183 (arginase I), providing a roughly sixfold increase in potency compared to ABH. Co-crystallizing compound 9 with arginase II yields a similar, albeit weaker interaction of the through-water contacts between the piperidine ring nitrogen atom and Asp 200 and Asp 202 (arginase II: IC50 509 nM vs. arginase I: IC50 223 nM).

In a parallel approach to finding new and improved inhibitors of arginase, some investigators have concentrated on characterizing a plant derived compound with the ability to inhibit arginase (36, 37). It has been demonstrated that piceatannol-3′-*O*-β-D-glucopyranoside (PG), an important component of rhubarb extract has, antioxidant effects (38), Woo et al. tested the ability of this extract to act as an arginase inhibitor (36). They were able to demonstrate that PG inhibits arginase I and arginase II activity and increased nitric oxide production in a dose-dependent manner. In their experiments PG proved to be a non-specific arginase inhibitor with an IC50 value of 11.22 μM (arginase I) and 11.06 μM (arginase II) respectively (36). Furthermore they were able to extend their studies by demonstrating that PG improves endothelial dysfunction via eNOS activation in a rodent model of hyperlipidemia (39).

This search for a new plant derived arginase II specific inhibitor has very recently been extended by screening hundreds of plant extracts for potential targets (37). This investigation yielded a methanol extract of *Scutellaria indica*, that has the ability inhibit arginase II. Following multiple additional fractionations, and repeated column chromatography, the group was able to isolate eight different compounds from the extract. One of the compounds (compound 1, flavan type) has been previously unknown while the remaining seven compounds (compound 2–8) have been described earlier. Arginase II activity was inhibited by two of the eight compounds (compound number 3 and 5) with an IC50 of 25.1 and 11.6 μM, respectively (37). They authors did not test the capability of the extract or compounds to inhibit arginase I, nor did they investigate the underlying mechanism of arginase II inhibition.

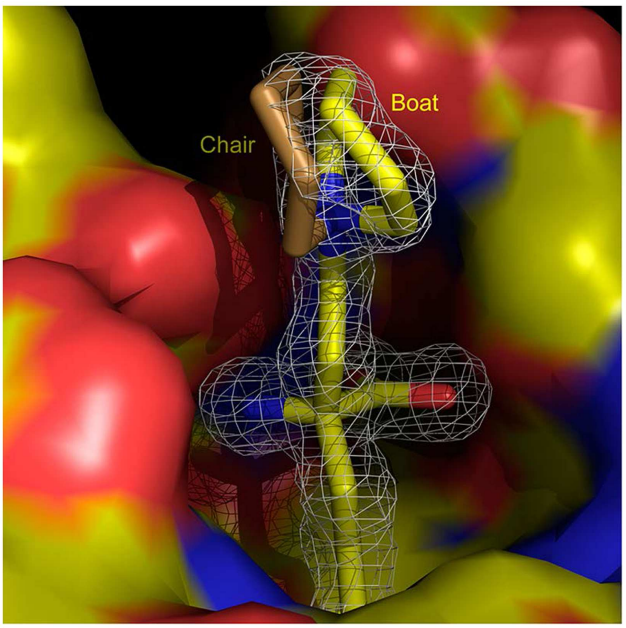

FIGURE 2 | (*R*)-2-amino-6-borono-2-(2-(piperidin-1-yl)ethyl)hexanoic acid) and arginase 1. Structure of (*R*)-2-amino-6-borono-2-(2-(piperidin-1-yl) ethyl)hexanoic acid) at the active site of arginase I (surface colored according to charge), shown superimposed with a difference map calculated without the inhibitor model contoured at 3 RMS (white contours). With kind permission from Ref. (33).

IMPLICATIONS OF INHIBITORS IN DIABETES MELLITUS

Diabetes mellitus has long been shown to be a disease tightly associated with endothelial dysfunction (40). Recent data suggests that metabolic degradation of L-arginine is directly involved in the L-arginine enhanced insulin-stimulated glycogen synthesis (41). Furthermore, hyperglycemia and the hemoglobin A1C levels correlate to arginase activity, with arginase activity being increased in type 2 diabetic subjects with impaired NOS activity (42–44). These higher arginase activity levels could be a result of reduced insulin action and increased protein catabolic processes in diabetic subjects (45). Consequently, insulin treatment reverses increased arginase activity and mRNA levels to close to control values (46). In addition to the effect of arginase in the endothelium of diabetic patients, arginase is also present and active in human islets cells of the pancreas, where arginase activity regulates the generation of NO (47).

The molecular mechanism of glucose-induced upregulation of arginase activity appears to involve small G proteins. In fact, the Rho kinase inhibitor Y-27632 as well as a HMG-coenzyme reductase inhibitor (statin) blunt the upregulation of the enzyme as well as ROS production under these conditions. Therefore, statins, which are known to inhibit the Rho/Rock pathway, reduce vascular events in patients with diabetes in part by a mechanism that involves inhibition of arginase activation (48, 49). Moreover, studies show that diabetes-induced impairment of vasorelaxation is correlated with increases in ROS, arginase activity, and arginase expression in the aorta. A treatment regime with simvastatin or L-citrulline is able to blunt these effects and acute treatment of diabetic coronary arteries with arginase inhibitors has been shown to reverse the impaired vasodilation to acetylcholine (50). This is likely due to the upregulation of arginase I in coronary arterioles of diabetic patients, which contributes to reduced NO production and consequently diminished vasodilation (51). Thus, endothelial dysfunction in diabetes may be caused, at least partially, by reduced L-arginine availability for eNOS. Given both preclinical data from animal models, early but provocative human data, as well as potent small molecule inhibitor drug candidates, arginase promises to be an exciting, novel target for therapy in diabetic vasculopathy, a scourge for which there is currently little effective treatment. However caution is advised in selectively inhibiting arginase isoforms in macrophages. The inflammatory phenotype M1 macrophages (Th1 immune response) mainly expresses arginase II, while the profibrotic and repair phenotype M2 (alternatively activated macrophage, Th2 cytokine response) mainly expresses arginase I (52). Therefore selective inhibition of arginase I might lead to an expansion of the M1 phenotype, which could aggravate iNOS mediated inflammatory effects (53), while selective arginase II inhibition might enhance the profibrotic response of alternatively activated ornithine producing macrophages with potential deleterious effects on vessels and other organs (54).

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L-citrulline protects from kidney damage in type 1 diabetic mice

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Rationale: Diabetic nephropathy (DN) is a major cause of end-stage renal disease, associated with endothelial dysfunction. Chronic supplementation of L-arginine (L-arg), the substrate for endothelial nitric oxide synthase (eNOS), failed to improve vascular function. L-Citrulline (L-cit) supplementation not only increases L-arg synthesis, but also inhibits cytosolic arginase I, a competitor of eNOS for the use of L-arg, in the vasculature.

Aims: To investigate whether L-cit treatment reduces DN in streptozotocin (STZ)-induced type 1 diabetes (T1D) in mice and rats and to study its effects on arginase II (ArgII) function, the main renal isoform.

Methods: STZ-C57BL6 mice received L-cit or vehicle supplemented in the drinking water. For comparative analysis, diabetic ArgII knock out mice and L-cit-treated STZ-rats were evaluated.

Results: L-Citrulline exerted protective effects in kidneys of STZ-rats, and markedly reduced urinary albumin excretion, tubulo-interstitial fibrosis, and kidney hypertrophy, observed in untreated diabetic mice. Intriguingly, L-cit treatment was accompanied by a sustained elevation of tubular ArgII at 16 weeks and significantly enhanced plasma levels of the anti-inflammatory cytokine IL-10. Diabetic ArgII knock out mice showed greater blood urea nitrogen levels, hypertrophy, and dilated tubules than diabetic wild type (WT) mice. Despite a marked reduction in collagen deposition in ArgII knock out mice, their albuminuria was not significantly different from diabetic WT animals. L-Cit also restored nitric oxide/reactive oxygen species balance and barrier function in high glucose-treated monolayers of human glomerular endothelial cells. Moreover, L-cit also has the ability to establish an anti-inflammatory profile, characterized by increased IL-10 and reduced IL-1β and IL-12(p70) generation in the human proximal tubular cells.

Conclusion: L-Citrulline supplementation established an anti-inflammatory profile and significantly preserved the nephron function during T1D.

Keywords: arginase, L-citrulline, glomerulosclerosis, diabetic nephropathy, IL-10

INTRODUCTION

Patients with Type 1 diabetes (T1D) have a considerably worse long-term prognosis than individuals without diabetes, due to the high incidence of cardiovascular disease and end-stage renal disease (ESRD). Diabetic nephropathy (DN), the leading cause of chronic kidney disease in the United States, is responsible for up to 40% of all ESRD cases (1). Since conventional or recently proposed therapies toward DN are still under ongoing investigation, or lack major efficacy, the search for novel targets involved in diabetes-induced renal damage is of primary importance.

It is now generally recognized that dysfunction of endothelial nitric oxide synthase (eNOS) contributes to vascular pathology in diabetes. An important cause of impaired endothelial nitric oxide (NO) production is the reduced availability of the eNOS substrate

L-arginine (L-arg). Patients with diabetes and cardiovascular disease were shown to benefit from acute L-arg supplementation (2), but chronic L-arg therapy caused adverse effects (3).

Oral L-citrulline (L-cit, precursor of L-arg) increases circulating levels of L-arg and augments NO-dependent signaling (4, 5), not only by means of increasing L-arg synthesis but also by decreasing L-arg catabolism (6). The latter activity occurs due to L-cit's capacity to allosterically inhibit arginase I (ArgI), an enzyme which can impair eNOS function (7, 8). As such, this dual effect of L-cit makes it a suitable supplemental amino acid to provide sufficient L-arg for proper eNOS function. In this regard, L-cit has been shown to prevent coronary vascular dysfunction in diabetic rats (8), with concomitant reduction of endothelial ArgI activity, which was also recently shown to contribute to coronary endothelial dysfunction in patients with diabetes mellitus (9) and in diabetic mice (10).

The effects of L-cit on vascular endothelial function may also positively influence the endothelial glycocalyx, thus contributing to glomerular barrier preservation (11, 12). However, L-cit supplementation has been neither evaluated in a model of diabetic kidney disease, nor its effects on renal arginase. In the kidneys, arginase II (ArgII) is the only isoform expressed in mouse and humans (13). ArgII is present in the proximal tubules (PT) and in the inner medullary collecting ducts (14) and plays an important role in renal physiology and homeostasis (15). Arginase metabolizes L-arg to urea and ornithine. Whereas urea has a key role in the urinary concentrating mechanism (16), ornithine is the substrate for the ornithine/polyamine and ornithine/proline pathways. Both of these pathways play an important role in kidney physiology and pathology (17–19). Indeed, production of polyamines enhances progression of the cell cycle and is associated with cell survival (20). Proline, on the other hand, is a precursor needed for collagen synthesis (21). Thus, although these mechanisms are important to maintain kidney function, they may also contribute to kidney hypertrophy and glomerulosclerosis of diabetes. Up-regulation of renal ArgII, proposed to be a mediator of DN, may play a role in these processes (22). However, L-cit supplementation to newborn rats was accompanied by enhanced ArgII expression in lungs, but it still protected from pulmonary hypertension (23).

In this study, we determined whether L-cit supplementation to streptozotocin (STZ)-diabetic rodents blunts the development of DN, and whether L-cit has an effect on renal ArgII.

MATERIALS AND METHODS

ANIMALS AND DIABETIC MODEL

Experiments were performed with C57BL/6 wild type (WT) mice (Jackson Laboratories, Bar Harbor, ME, USA), or ArgII homozygous knockout mice on a C57BL/6 background (24, 25). Ten-week old male mice (18–20 g) were rendered diabetic with intraperitoneal injections of STZ (65 mg/kg) (Sigma Aldrich, St. Louis, MO, USA), on alternating days for up to four injections (10). A group of control (vehicle) and diabetic mice were treated with L-cit (50 mg kg^{-1} day^{-1}, supplemented in drinking water) (8). Animals were housed in individual cages. The L-cit dose was adjusted to each animal according to the daily water intake. Mice were studied after 2 and 16 weeks with diabetes. In addition, male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC, USA), weighing between 225 and 250 g, were rendered diabetic with a single dose of STZ (50 mg/kg, intraperitoneally). A group of diabetic rats (≥350 mg/dl) was treated with L-cit, as indicated above. Rats were studied after 8 weeks with diabetes. Animals had free access to food and water throughout the study. All animals received humane care in compliance with federal laws and institutional guidelines at Georgia Regents University.

MEASUREMENT OF KIDNEY HYPERTROPHY

Determination of kidney to body weight ratio was used as a measure of kidney hypertrophy. The left kidney was removed, decapsulated, placed on tissue paper for 1 min, and weighed.

ANALYTICAL METHODS

Mouse urinary albumin excretion (UAE), and rat proteinuria were determined after 24 h urine collection, using an ELISA kit (AssayPro, St. Charles, MO, USA), and a protein assay kit (BCA Pierce, Rockford, IL, USA), respectively. Blood glucose levels were measured by the Alpha Trak-Blood glucose monitoring system (Abbott Laboratories, St. Clara, CA, USA). Plasma urea levels were measured by colorimetric determination of urea at 540 nm in the presence of α-Isonitrosopropiophenone (α-ISPF, 9% in ethanol) (Sigma Aldrich, St. Louis, MO, USA). Results were expressed as milligram per deciliter of blood urea nitrogen (BUN). Mouse plasma samples, separated from heparinized whole-blood, were used for the measurement of 32 cytokines and chemokines, using a magnetic bead-based multiplex assay, as described in Ref. (26) (32 Multiplex MCYTOMAG-70K assay, EMD Millipore).

TISSUE HISTOLOGY

After being excised and decapsulated, mouse kidneys were immersed in 10% formalin for 24 h, embedded in paraffin and sectioned at 4 μm thickness. Sections were deparaffinized in xylene, rehydrated through graded ethanols to water, and stained with periodic acid Schiff (PAS) for morphology evaluation. Picro-Sirius red was used to stain for tissue collagen. Rat kidneys were frozen in liquid nitrogen, and cryosections (5 μm) were air-dried for 30 min. Cryosections stained with Picro-sirius red were processed as previously described in Ref. (27). All PAS and Picro-sirius red-stained sections were visualized on a computer connected to a light microscope (AxioVision; Carl Zeiss Meditec, Inc.). Quantitative analysis of collagen was performed on photomicrographs of kidney sections by using specific software (Image J). Seven to ten non-overlapping fields per section were analyzed for each animal. Tissue collagen content was assessed by a fibrosis index (%) that indicated the ratio of the mean sirius red-stained area to the mean whole area of the section, calculated as the mean of the fibrosis indexes for each section for each animal.

RENAL ARGINASE ACTIVITY

Renal arginase activity (RAA) was measured in kidney cortex homogenized in ice-cold lysis buffer (50 mmol/L Tris-HCl, 0.1 mmol/L EDTA and EGTA, pH 7.5) at 1:4 (wt:vol) ratio, containing protease inhibitors. The homogenate was centrifuged at 14,000 × *g* for 20 min. The supernatant was removed for enzyme assay using a colorimetric determination of urea production from L-arg, as previously described in Ref. (28). Samples were assayed in triplicate. Values were corrected by adjusting for protein concentration in the homogenate and expressed as nanomole urea per milligram protein per hour. Additional corrections were made

after subtracting basal levels of urea obtained from each sample of kidney cortex homogenates in the absence of $MnCl_2$ and of L-arg.

WESTERN BLOT ANALYSIS

Mouse and rat frozen kidney cortex were pulverized and homogenized in RIPA lysis buffer (EMD Millipore, Billerica, MA, USA), containing protease and phosphatase inhibitor cocktails (Sigma Aldrich, St. Louis, MO, USA). Soluble protein extracts from tissue homogenates were subjected to SDS-PAGE electrophoresis, transferred to polyvinylidene fluoride membranes and reacted with anti-ArgII primary antibody (1:500, Santa Cruz Biotechnology, St. Cruz, CA, USA), at 4°C overnight. Subsequently, the bound antibody was detected by donkey anti-rabbit horseradish peroxidase-labeled secondary antibody (1:6,000, GE Healthcare, Pittsburgh, PA, USA), and visualized with ECL substrate (Amersham, Buckinghamshire, UK). Membranes were then stripped and re-probed with anti-GAPDH (Santa Cruz Biotechnology, St. Cruz, CA, USA) to assess level of protein loading. Protein expression was determined using densitometry analysis of films.

IMMUNOHISTOCHEMISTRY

Immunohistochemical detection of ArgII was performed in deparaffinized and rehydrated mouse kidney sections by means of light microscopy studies. Briefly, antigen retrieval was performed by immersing the slides in 0.01 M citrate buffer (pH 6.0), at 95°C for 30 min in a water bath. Endogenous biotin and peroxidase activity were blocked before staining, by using commercial avidin/biotin and peroxidase kits, respectively (Vector Laboratories, Burlingame, CA, USA). Slides were then incubated for 1 h with primary antibody against ArgII (1:500). The primary antibody was localized using the VECTASTAIN ABC-Elite peroxidase detection system (Vector Laboratories, Burlingame, CA, USA). Primary antibody against kidney injury molecule 1 (KIM-1) (1:500, R&D Systems, Minneapolis, MN, USA), followed by anti-goat secondary antibody (1:6,000, Invitrogen, Grand Island, NY, USA), were used for immunofluorescent staining of rat frozen sections. Nuclei were counterstained with DAPI. All sections were examined by two different researchers in a blinded manner. The number of tubules that exhibited positive red fluorescent staining to KIM-1 was counted per field. Five to seven fields were examined in each kidney section. Sections of each kidney were processed in parallel with the appropriate negative control tissue, processed with omission of the primary antibody in the staining procedure.

HUMAN GLOMERULAR ENDOTHELIAL CELL CULTURE

Human glomerular endothelial cells (Lonza, Walkersville, MD, USA) were grown in complete CSC medium, and maintained at 37°C in a humidified 5% CO_2 incubator. Cells were used between passages four and six for the experiments. Treatment of cells with normal (5.5 mM, NG) or high [25 mM, high glucose (HG)] D-glucose-supplemented medium was performed in basic CSC medium. As control for the osmotic effect of high D-glucose, L-glucose was added to the basic endothelial medium. Pre-treatment of HGEC with L-cit (1 mM) was performed by adding the amino acid 2 h prior to adding HG or iso-osmotic control. HGEC were cultured under NG or HG conditions for either 24 h or 14 days, before they were used for experiments.

MITOCHONDRIAL SUPEROXIDE

Human glomerular endothelial cells were seeded in 0.2% gelatin-coated four well slide chambers at 1×10^5 cells per well, and allowed to reach confluence. Then cells were exposed to HG for 24 h as described above, with or without pre-treatment with L-cit. At the end of incubation, MitoSOX (Invitrogen) 5.0 μM was added to the cells and incubated further for 10 min at 37°C in 5% CO_2 atmosphere, according to manufacturing instructions. Subsequently, cells were washed in hanks balanced salt solution (HBSS, with Ca/Mg) and used for confocal microscopy imaging. The digital images were taken by an inverted confocal laser scanning microscope LSM Pascal (Zeiss, Germany), with an excitation/emission of 510/580 nm. Images were captured using 40× oil immersion objective lens.

NITRIC OXIDE METABOLITE

Human glomerular endothelial cell were seeded at 1×10^5 cells per well in 24-well plates. Confluent quiescent cell monolayers were exposed to HG or proper iso-osmotic control for 24 h. L-Cit (1 mM) was applied 2 h prior to HG. Exposure was terminated by removal of the supernatant. Fresh basic CSC medium was replaced and cells incubated for additional 30 min. Supernatant was then removed, subsequently centrifuged and stored at −80°C for NO analysis. Cell supernatants containing nitrite $\left(NO_2^-\right)$ the stable breakdown product of NO in aqueous medium were refluxed in glacial acetic acid containing sodium iodide. NO_2^- is quantitatively reduced to NO under these conditions, which can be quantified by a chemiluminescence detector in a NO analyzer (Sievers) as described in Ref. (8).

PERMEABILITY ASSAY OF HGEC MONOLAYERS

Human glomerular endothelial cell monolayer permeability to high molecular mass proteins was assayed by using 2,000-kDa FITC-dextran, based on the Transwell model (EMD Millipore). For this, HGEC were seeded on collagen-coated Transwells at a density of 1×10^5 cells per well in 250 μl of CSC growth medium. The inserts were placed into 24-well plates containing 500 μl of medium. Upon reaching confluence, HGEC were exposed to HG as described above, with or without pre-treatment with L-cit. Transendothelial passage of dextran was determined after 14 days of incubation in HG media as described previously (12). Briefly, medium was aspirated and 150 μl of FITC-dextran was added into the insert and incubated for 3 h. The insert was then removed, and 100 μl of medium was collected from the bottom chamber and transferred to a black 96-well plate. The fluorescent density of samples was analyzed on a Paradigm Microplate Fluorometer (Beckman-Coulter) at 485 nm excitation and 530 nm emission wavelengths.

HUMAN PROXIMAL TUBULAR EPITHELIAL CELL CULTURE

Human proximal tubular epithelial cell (huPTEC) (Lifeline Cell Technology, Frederick, MD, USA) were grown in the commercial RenaLife medium, and maintained at 37°C in a humidified 5% CO_2 incubator. Cells were used between passages two and four for the experiments. Treatment of cells with normal (5.5 mM, NG) or high (25 mM, HG) D-glucose-supplemented medium was performed in six-well plates and maintained

for 7 days, before they were used for experiments. As control for the osmotic effect of high D-glucose, L-glucose was added to the culture medium. Pre-treatment of huPTEC with L-cit (1 mM) was performed by adding the amino acid 2 h prior to adding HG or iso-osmotic control, with or without concurrent pre-treatment with a neutralizing anti-human IL-10 antibody (5 μg/ml, R&D Systems). Upon completion of treatment, culture medium supernatants were collected, centrifuged, and freeze at −80°C until use for cytokine measurement. Cells were lysed in RIPA buffer and protein extracts were loaded for Western blot analysis of ArgII as described for tissue extracts.

MULTIPLEX HUMAN CYTOKINE/CHEMOKINE MEASUREMENT

A panel of 13 pro-inflammatory cytokines [interferon-γ (IFN-γ), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, TNF, and granulocyte-monocyte colony-stimulating factor (GM-CSF)] was assessed in triplicates in 50 μl cultured medium supernatants from cultured primary huPTEC, using a highly sensitive magnetic beads-based kit (MILLIPLEX MAP High Sensitivity Human Cytokine Panel – Premixed 13 Plex, EMD Millipore) (29). This assay has a high sensitivity, typically with a detection limit in the range from 0.01 to 0.48 ng/l.

IMMUNOFLUORESCENCE STAINING OF CULTURED huPTEC

Cells were seeded in slide chambers at 1×10^5 cells per well. When cells reached about 75–80% confluence, HG was added for 1 week as described above, with or without pre-treatment with L-cit. Upon completion of treatment, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min. Then, a blocking solution (1X PBS/5% normal goat serum/0.3% Triton™ X-100) was applied to the attached cells in the slide chambers for 1 h, prior to addition of anti-caspase 6 antibody (1:800, Cell Signaling, Boston, MA, USA) for incubation overnight at 4°C. Cells were washed twice with PBS and incubated with a fluorochrome-conjugated secondary antibody (1:400, Cy5 goat anti-rabbit, Jackson ImmunoResearch). DAPI was used for nuclear staining. For non-specific binding (negative control) the primary antibody was omitted. Images were collected with fluorescent microscopy. Fluorescence intensity measurements were performed in nuclei, normalized to DAPI nuclei area, and corrected by subtraction of background from negative controls.

STATISTICAL ANALYSIS

All data were expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA with a Tukey post test. In some experiments, statistical differences were determined by a Student's *t*-test. A p value of <0.05 was considered statistically significant.

RESULTS

BLOOD GLUCOSE, WATER CONSUMPTION, URINE VOLUME, BODY WEIGHT, KIDNEY WEIGHT, AND BUN IN MICE

All diabetic groups had elevated blood glucose levels and increased daily water intake and urinary volume excretion, both at 2 (**Table 1**) and 16 weeks (**Table 2**) of the disease vs. respective non-diabetic controls. The kidney hypertrophy and wasting of body mass detected in untreated diabetic WT mice was not observed in L-cit-treated mice, despite significant hyperglycemia (**Tables 1** and **2**). Intriguingly, although ArgII has been proposed to be a mediator of DN (22), we observed a significant greater kidney size and BUN levels in the ArgII knock out mice, as compared to diabetic WT animals (**Table 2**). These results indicate that L-cit does not affect blood glucose levels in the diabetic state, but prevents body weight loss and kidney hypertrophy. In addition, the results observed in the ArgII knock out mice suggest that the lack of ArgII enhances diabetes-induced kidney hypertrophy and may accelerate the decay of kidney function in diabetic mice.

RENAL ARGINASE ACTIVITY AND ArgII PROTEIN LEVELS

At 2 weeks, RAA was elevated in untreated diabetic WT mice by 8.6-fold over control. By contrast, L-cit-treated diabetic WT mice showed only twofold elevated RAA levels over control values (**Figure 1A**). The marked elevation of RAA, observed at 2 weeks in untreated diabetic WT mice, declined by 16 weeks to a level of ~2.4-fold over respective control. At that time period, diabetic WT mice treated with L-cit showed a rise in RAA of 3.8-fold over control (**Figure 1B**). These results indicate that diabetes strongly induces arginase activity in renal tissues, and that long-term supplementation of L-cit does not prevent this effect. The absence of the ArgII gene in both control and diabetic ArgII knock out mice, resulted in RAA values below control WT mice by 0.2- and 0.3-fold, respectively. These low levels of arginase activity could be due to the presence of vascular and blood cell-derived ArgI.

Western blot analysis of protein extracts from kidney cortex homogenates of untreated diabetic WT mice showed levels

Table 1 | Biochemical and physical characteristics of study groups after 2 weeks. Effect of L-cit supplementation.

	Blood glucose (mg/dl)	Water intake (ml/day)	Urine volume (ml/day)	Body weight (g)	K/BW ratio
Control	103.8 ± 8.2	7 ± 0.9	1.55 ± 0.2	23 ± 0.7	5.97 ± 0.4
Diabetic	460.3 ± 71.7[a]	19.5 ± 2.4[b]	14.5 ± 1.4[c]	19 ± 0.5[d]	8.53 ± 0.03[d]
L-Cit-Con	132.3 ± 11.1	7.2 ± 0.6	1.4 ± 0.4	26 ± 1.3	6.39 ± 0.4
L-Cit-Diab	465.8 ± 96.4[a]	17.5 ± 1.1[b]	9.6 ± 2.4[c]	25 ± 0.4	5.85 ± 0.1

K/BW, kidney/body weight ratio; control, untreated control mice; diabetic, untreated diabetic mice; L-cit-Con, L-cit-treated control mice; L-cit-Diab, L-cit-treated diabetic mice. Values are expressed as mean ± SEM.

[a] $p < 0.001$ vs. control groups; [b] $p < 0.01$ vs. control groups; [c] $p < 0.05$ vs. control groups; [d] $p < 0.01$ vs. all groups.

Table 2 | Biochemical and physical characteristics of study groups after 16 weeks. Effect of L-cit supplementation and ArgII deletion.

	Blood glucose (mg/dl)	Water intake (ml/day)	Urine volume (ml/day)	Body weight (g)	K/BW ratio	BUN (mg/dl)
Control	148.8 ± 20.1	7.3 ± 1.1	2.2 ± 0.3	28 ± 0.3	6.80 ± 0.5	10.52 ± 0.5
Diabetic	546.2 ± 19.7[a]	19.3 ± 2.2[a]	19.2 ± 1.7[c]	21 ± 0.2[d]	9 ± 0.4[g]	12.03 ± 0.5
L-Cit-Con	119 ± 6.8	7.2 ± 0.6	1.6 ± 0.3	31 ± 0.8[f]	6.86 ± 0.3	14.24 ± 0.8
L-Cit-Diab	518.3 ± 27[a]	26.8 ± 2.1[b]	20.9 ± 2.3[c]	26 ± 0.5[e]	6.93 ± 0.4	13.36 ± 0.33
C ArgII KO	133.7 ± 18.9	6.9 ± 0.5	2 ± 0.3	21 ± 1.8	8.13 ± 0.3[h]	15 ± 2.5
D ArgII KO	554.8 ± 54.5[a]	30.3 ± 3.6[b]	28.2 ± 1.9[c]	20 ± 0.6	11.45 ± 0.8[i]	17.07 ± 1.3[b]

K/BW, kidney/body weight ratio; BUN, blood urea nitrogen; control, untreated control WT mice; diabetic, untreated diabetic WT mice; L-cit-Con, L-cit-treated control WT mice; L-cit-Diab, L-cit-treated diabetic WT mice; C ArgII KO, control ArgII knock out mice; D ArgII KO, diabetic ArgII knock out mice. Values are expressed as mean ± SEM.

[a] $p < 0.01$ vs. all control groups; [b] $p < 0.05$ vs. all groups; [c] $p < 0.05$ vs. all control groups; [d] $p < 0.001$ vs. control and L-cit-Con; [e] $p < 0.001$ vs. diabetic; [f] $p < 0.001$ vs. L-cit-Diab; [g] $p < 0.01$ vs. control and Lcit-Con; [h] $p < 0.05$ vs. control; [i] $p < 0.01$ vs. all groups.

of ArgII protein that were increased up to 10-fold over control at 2 weeks (**Figure 1C**). ArgII protein in tissues from L-cit-treated diabetic WT mice were ~fivefold higher than in controls (**Figure 1C**). Conversely, upon progression of diabetes to 16 weeks, the highest levels of ArgII protein were observed in L-cit-treated diabetic WT mice (**Figure 1D**). ArgII was neither detected in control nor in diabetic ArgII knock out mice (**Figure 1D**). These results indicate that the induction of arginase activity observed in kidney cortex of diabetic mice is due to increased protein levels of ArgII. L-Cit does not prevent diabetes-induced ArgII up-regulation, and may even have an additive effect upon long-term supplementation.

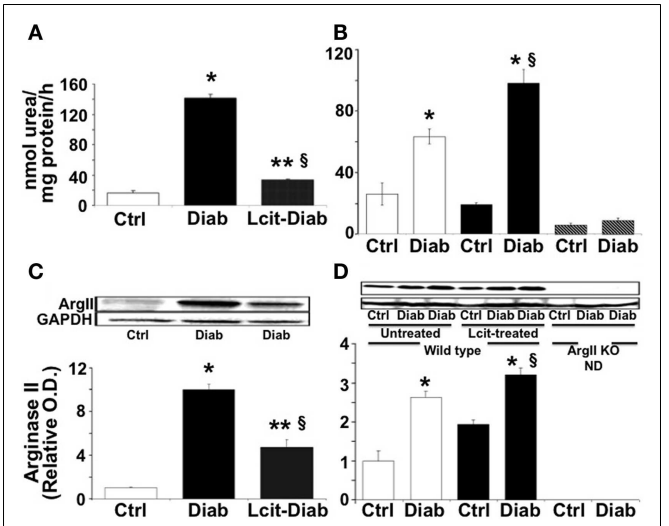

FIGURE 1 | Renal arginase. Kidney cortex isolated from mice after 2 **(A–C)** and 16 **(B–D)** weeks of diabetes was homogenized in lysis buffer. Arginase activity **(A,B)** was assayed using a colorimetric determination of urea production from L-arginine. Relative levels of arginase II protein expression **(C,D)** were determined by western blot analysis. Ctrl, control; Diab, diabetic; WT, wild type; ArgII KO, ArgII knock out; ND, not detected. Values are expressed as mean ± SEM, $n = 4$–6. **(A–C)** $*p < 0.001$ vs. Ctrl, $^{\S}p < 0.01$ vs. Ctrl, $**p < 0.001$ vs. Diab. **(B–D)** □, Untreated WT; ■, L-cit-treated WT; ▩, ArgII KO. $*p < 0.01$ vs. untreated and L-cit-treated Ctrl WT, and ArgII KO groups, $^{\S}p < 0.05$ vs. untreated Diab WT.

IMMUNOHISTOCHEMISTRY

Diffuse ArgII immunoreactivity was observed in cells of the urinary pole of the Bowman's capsule, and of the PT of untreated control WT mouse kidneys. Enhanced tubular ArgII staining was detected in untreated diabetic WT mice after 16 weeks of the disease (**Figure 2A**). L-Cit-treated diabetic WT mice also demonstrated increased ArgII immunoreactivity in cortical tubular segments, while maintaining a more conserved epithelial morphology (**Figure 2A**). No positive staining was observed in either the ArgII knock out mouse kidneys (**Figure 2B**), or in tissue sections stained in parallel with omission of primary antibody (**Figure 2C**).

URINARY ALBUMIN EXCRETION

Urinary albumin excretion was significantly elevated above control in untreated diabetic WT mice as early as 2 weeks, but this effect was markedly blunted upon L-cit treatment (untreated diabetic: 811.43 ± 161.04 μg/ml vs. control: 97.73 ± 29.6 μg/ml, and L-cit-treated diabetic: 138.47 ± 47.3 μg/ml, $p < 0.05$). This preventive effect of L-cit on urinary albumin leakage was observed for up to 16 weeks, while non-treated diabetic WT mice maintained elevated UAE at that time point (**Figure 3**). Urine samples from diabetic ArgII knock out mice showed a trend to reduced albumin excretion, as compared to non-treated diabetic WT mice (**Figure 3**). These data thus indicate that L-cit may be protective toward diabetes-induced glomerular barrier dysfunction and/or impairment of proximal tubular protein uptake.

RENAL HISTOLOGY

Histological examinations of PAS-stained kidney sections of untreated diabetic WT mice at 16 weeks revealed glomerular hypertrophy, Bowman's capsule thickening and periglomerulosclerosis, in comparison to control mouse kidneys (**Figures 4A,B**). The PT showed hypertrophy and markedly thickened and wrinkled basement membranes. Interstitial expansion and focal areas of hypercellularity were also observed. Treatment of diabetic WT mice with L-cit markedly ameliorated all diabetes-induced alterations in the kidney. Intriguingly, we observed a marked dilatation of cortical tubules, focal blebbing of the luminal edge of the cells and detachment in the kidneys from diabetic

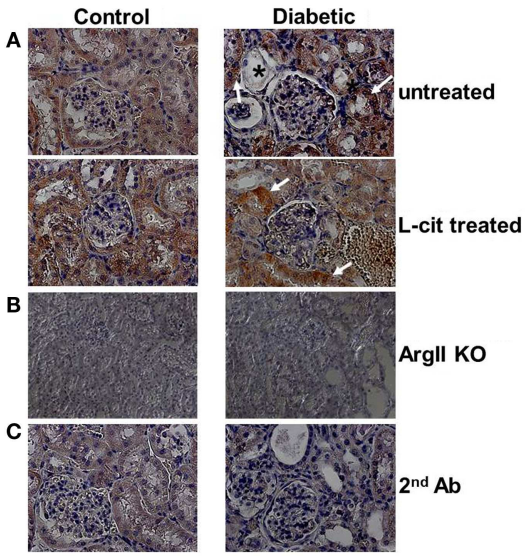

FIGURE 2 | Immunohistochemistry of ArgII in paraffin-embedded mouse kidney sections. Representative photographs of paraffin-embedded kidney sections showing immunolocalization of ArgII (brown color; 40× immersion oil). **(A)** Cortical tubular segments in untreated diabetic mice show higher stain density than untreated control mice, after 16 weeks (white arrows). Atrophic tubules with widened lumina and flattened tubular cells, or with altered epithelial morphology, show reduced ArgII staining (*). L-Cit-treated diabetic mice show also enhanced ArgII stain intensity (white arrows). Kidney segments of control and diabetic ArgII knock out mice **(B)**, or tubules stained with secondary antibody alone **(C)** were completely negative for ArgII immunostaining.

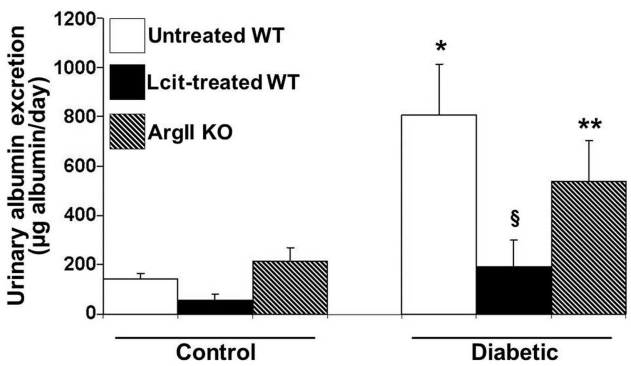

FIGURE 3 | Urinary albumin excretion (UAE) in mice after 16 weeks. Mice were placed in metabolic cages. Urine samples were collected for 24 h to determine albumin excretion. Values are expressed as mean ± SEM, $n = 5$–9. WT, wild type; ArgII KO, ArgII knock out. *$p < 0.001$ vs. untreated, L-cit-treated, and ArgII KO control, §$p < 0.05$ vs. untreated diabetic WT, **$p < 0.05$ vs. untreated and L-cit-treated control WT.

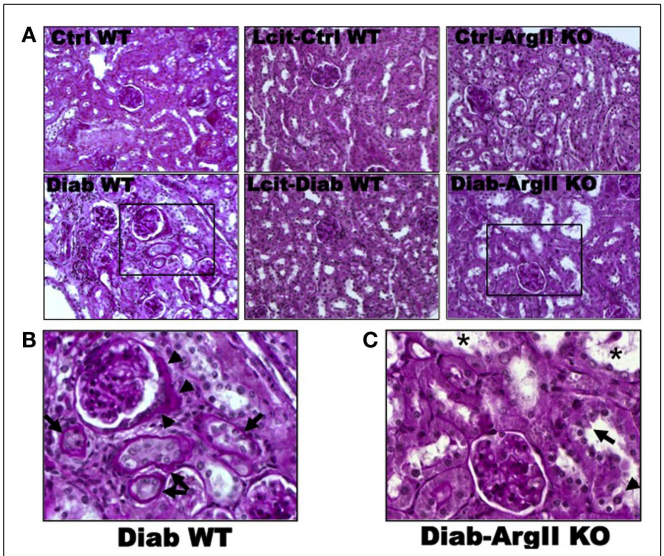

FIGURE 4 | Periodic acid Schiff-stained paraffin-embedded kidney sections. **(A)** Representative photomicrographs of PAS-stained kidney sections from: untreated (Cont WT) and L-cit-treated (Lcit-Cont WT) control wild type, and control ArgII knock out (Ctrl-ArgII KO) mice (upper). Untreated (Diab WT) and L-cit-treated (Lcit-Diab WT) diabetic wild type, and diabetic ArgII knock out (Diab-ArgII KO) mice (lower) at 16 weeks (20×). Morphological alterations of a selected area (rectangle) observed in Diab WT and Diab-ArgII KO kidneys are shown at higher magnification (40× immersion oil) in: **(B)** a hypertrophied glomerulus with thickening of Bowman's capsule is observed (arrow heads). Surrounding tubules show markedly thickened and wrinkled tubular basement membranes, and partial disruption of brush border (arrows). Interstitial expansion and hypercellularity around the glomerulus and surrounding tubules are also observed. These effects were markedly attenuated by L-cit treatment in diabetic mice. **(C)** Marked dilatation of cortical tubules (*), focal blebbing of the luminal edge (arrow), and focal detachment (arrow head) of epithelial cells in a proximal tubule.

ArgII knock out mice (**Figures 4A,C**). However, no thickening of tubular basement membrane was observed in this group.

As visualized in **Figure 5A**, picro-sirius red staining showed an enhancement of peri-glomerular and peritubular-interstitial collagen deposits in kidneys of WT diabetic mice at 16 weeks, as compared to control mice. This effect was reduced in L-cit-supplemented WT diabetic mice (**Figures 5A,B**). Interestingly, induction of diabetes by STZ in ArgII knock out mice did not result in enhanced collagen deposits, as compared to diabetic WT mice (**Figures 5A,B**).

ANTI-INFLAMMATORY EFFECT OF L-CIT IN DIABETIC MICE

Because diabetes is considered a chronic inflammatory state (30), we examined the effect of L-cit supplementation on plasma cytokine levels in diabetic mice at the end of the experiment. We found enhanced levels of the pro-inflammatory cytokines TNF and IL-6 in the diabetic animals, with the former being significantly different when compared to control mice (**Figure 6**). Strikingly, the level of the anti-inflammatory cytokine IL-10 was significantly enhanced in plasma of L-cit-treated diabetic mice (**Figure 6**). We also found significantly enhanced levels of the pro-inflammatory chemokine MIP-2 in diabetic vs. control mice (ctrl: $0.2 + 0.02$ pg/ml; STZ: 328.7 ± 2.6 pg/ml, $n = 3$, $p < 0.001$ vs. ctrl). However, there was a significant reduction of MIP-2 upon L-cit supplementation to diabetic mice (L-cit/STZ: 311.6 ± 3.1 pg/ml, $n = 3$, $p < 0.05$ vs. STZ, $p < 0.001$ vs. ctrl). These results thus

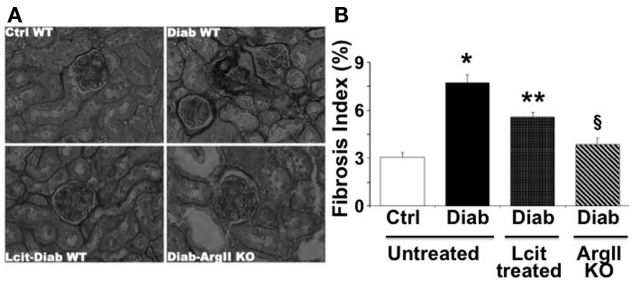

FIGURE 5 | Picro-sirius red staining of paraffin-embedded kidney sections. **(A)** Representative photomicrographs of picro-sirius red-stained kidney sections from untreated control wild type (Ctrl WT), untreated diabetic wild type (Diab WT), L-cit-treated diabetic wild type (Lcit-Diab WT), and diabetic ArgII knock out (Diab-ArgII KO) mice at 16 weeks (RGB image using *Image J*). Increased collagen deposits (dark color) are observed in Diab WT. This effect was significantly reduced in Lcit-Diab WT, and in Diab-ArgII KO mice. **(B)** Fibrosis index (%) of tissue collagen content that indicates the ratio of the mean picro-sirius red-stained area to the mean whole area of the section. Values are mean ± SEM. $*p < 0.001$ vs. untreated Ctrl WT, $**p < 0.01$ vs. untreated Ctrl and Diab WT, $^{§}p < 0.001$ vs. untreated Diab WT.

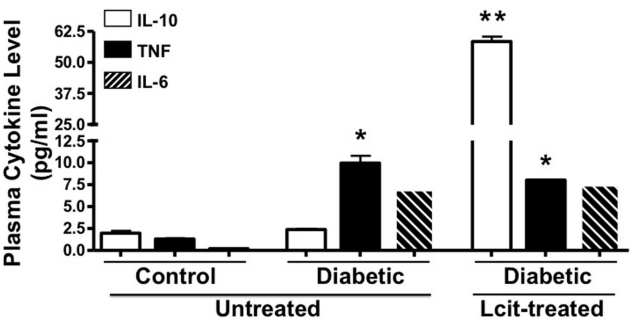

FIGURE 6 | Plasma cytokine level. Mouse plasma samples were used for cytokine and chemokine measurement, using a magnetic bead-based multiplex assay. L-Cit induced an anti-inflammatory response in diabetic mice, as observed by significant increased levels of IL-10. Values are mean ± SEM. $*p < 0.001$ vs. untreated control mice, $**p < 0.001$ vs. untreated control and diabetic mice.

indicate that L-cit treatment increases the anti-inflammatory response in STZ-treated diabetic mice.

EFFECTS OF L-CIT IN DIABETIC STZ-RATS

Since C57BL6 mice develop only a moderate nephropathy upon STZ-treatment, we have also evaluated the effect of L-cit treatment in a more sensitive rodent model of STZ-induced diabetes, i.e., the rat. STZ-diabetic rats had increased daily proteinuria, as compared to non-diabetic control rats. However, L-cit treatment prevented this effect (**Figure 7A**). In addition, kidneys from untreated diabetic STZ-rats showed characteristic features of human DN, as observed by substantial collagen deposits of intraglomerular and peritubular distribution (**Figures 7B,C**). These effects were reduced in L-cit-supplemented STZ-rats (**Figures 7B,C**). These findings were accompanied by an elevation of renal ArgII protein

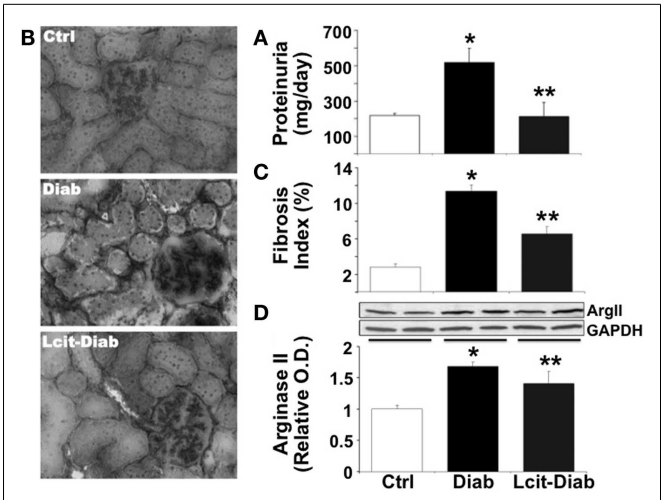

FIGURE 7 | Effect of l-cit on kidney pathology in STZ-rats. Urine and renal tissues from untreated control (Ctrl), untreated diabetic (Diab), and L-cit-treated diabetic (Lcit-Diab) rats were evaluated. **(A)** Rats were placed in metabolic cages for 24 h urine collection. Total protein excretion was measured in urine. Lcit-Diab rats had a significant reduction of proteinuria when compared to Diab rats. $*p < 0.05$ vs. Ctrl, $**p < 0.05$ vs. Diab. **(B)** Representative photomicrographs of frozen sections from rat kidneys stained with picro-sirius red, show enhanced collagen deposits (dark color, RGB image using *Image J*) in untreated diabetic rats. This effect was markedly blunted in L-cit-treated diabetic rats. **(C)** Fibrosis index (%) of tissue collagen content that indicates the ratio of the mean picro-sirius red-stained area to the mean whole area of the section. $*p < 0.001$ vs. Ctrl, $**p < 0.001$ vs. Diab. **(D)** Renal ArgII protein expression determined by densitometric analysis of immunoblots performed with protein extracts from rat kidney cortex ($n = 4$, representative blot is shown). $*p < 0.001$ vs. Ctrl rats, $**p < 0.05$ vs. Ctrl rats.

levels in both, untreated and L-cit-treated diabetic STZ-rats, when compared to control non-diabetic rats (**Figure 7D**).

RENAL EXPRESSION OF KIDNEY INJURY MOLECULE 1

Kidney injury molecule 1 is a relevant biomarker of renal tubular damage that has been found to be associated with albuminuria in the early stage of nephropathy in diabetic patients (31), and with the progression of DN in experimental models (32). Therefore, we evaluated the expression of KIM-1 in kidneys of diabetic rats with or without L-cit supplementation. While renal tissues of control non-diabetic rats were negative for KIM-1 immunostaining, numerous tubular segments in the cortex and in the outer strip of the outer medulla were intensely stained in diabetic rats (**Figure 8A**). L-Cit-treated diabetic rats showed fewer positive tubules than untreated diabetic rats. An objective score of the number of positive tubules per field is shown in **Figure 8B**.

EFFECT OF L-CIT IN HUMAN GLOMERULAR ENDOTHELIAL CELLS EXPOSED TO HIGH GLUCOSE

Since HG-induced reactive oxygen species (ROS) generation is known to impair endothelial-derived NO production, we evaluated the effect of L-cit on NO production in HGECs exposed to HG. L-Cit pre-treatment of HGECs prevented the impaired NO production observed under exposure to HG for 24 h (**Figure 9A**). This effect correlated with a marked attenuation of mitochondrial

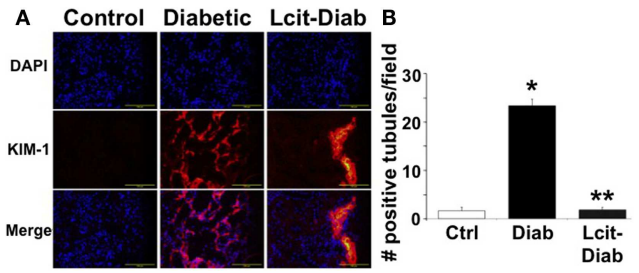

FIGURE 8 | KIM-1 immunofluorescence staining. Kidney injury molecule 1 (KIM-1) antibody was used for immunofluorescent staining (red) of frozen sections from control (Ctrl), untreated diabetic (Diab), and L-cit-treated diabetic (Lcit-Diab) rats. Nuclei were counterstained with DAPI (blue). **(A)** Representative photomicrographs show negative staining for KIM-1 in control kidneys. Conversely, an increased number of tubules positively stained for KIM-1 is observed in kidneys of untreated diabetic rats. The number of positive tubules was dramatically decreased in kidneys of L-cit-treated diabetic rats. **(B)** Staining score determined by counting the number of positive tubules per field, using 40× magnification lens. Five to seven fields were examined in each kidney section ($n = 3$–4). *$p < 0.001$ vs. Ctrl, **$p < 0.001$ vs. Diab.

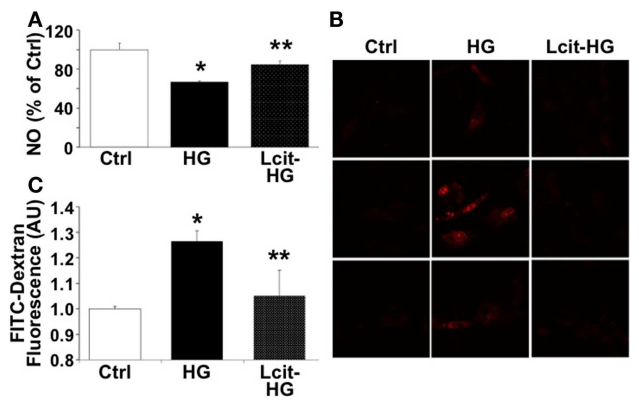

FIGURE 9 | Effect of L-cit on human glomerular endothelial cells exposed to high glucose. HGECs were cultured in basic CSC medium containing 25 mM D-glucose (HG) with or without 1 M L-cit for 24 h **(A,B)** or 14 days **(C)**. L-Glucose (19.5 mM) was added to 5.5 mM D-glucose present in basic CSC medium, and used as iso-osmotic control (Ctrl). **(A)** The nitrite level (picomole) in the medium was determined by NO analyzer and expressed as percent of control (*$p < 0.01$ vs. Ctrl, **$p < 0.05$ vs. HG, $n = 3$). **(B)** HGECs were seeded onto gelatin-coated slide chambers. After completion of treatments, MitoSOX (a marker of mitochondrial superoxide, 5.0 μM) was added to the cells and incubated further for 10 min. Cells were washed and used for confocal microscopy imaging. Representative images show increase in mitochondrial MitoSOX fluorescence following treatment with HG. This effect was markedly reduced by concurrent L-cit treatment. **(C)** Transendothelial passage of FITC-dextran was used to determine permeability of HGEC monolayer, seeded onto collagen-coated Transwells. Fluorescent density of samples was analyzed on a paradigm microplate fluorometer (*$p < 0.01$ vs. Ctrl, **$p < 0.05$ vs. HG, $n = 4$).

superoxide generation, as opposed to the increase in mitochondrial red fluorescence intensity of MitoSOX in confocal microscopic images observed in HG-treated cells (**Figure 9B**).

Increasing evidence suggests that a NO/ROS imbalance causes endothelial barrier dysfunction (11). We therefore examined the effect of L-cit on HG-induced loss of barrier function in HGEC monolayers, by means of assessing their permeability to FITC-dextran. As shown in **Figure 9C**, HG (25 mM) significantly increased permeability of HGEC monolayers to FITC-dextran, but pre-treatment of the monolayers with L-cit (1 mM) conferred a significant protection from HG-induced hyperpermeability. These results suggest that L-cit protects glomerular barrier function at least in part by preserving glomerular endothelial NO synthase (NOS) function, and by reducing ROS generation under hyperglycemic insult.

EFFECT OF L-CIT IN HUMAN PROXIMAL TUBULAR EPITHELIAL CELLS EXPOSED TO HIGH GLUCOSE

Proximal tubular cells are capable of generating IL-10 (33). Therefore, we investigated the effect of L-cit on cytokine production in huPTECs exposed to HG. huPTEC cultured under HG-supplemented medium in the presence of L-cit for 1 week, produced significantly enhanced levels of the anti-inflammatory cytokine IL-10, as compared to cells treated with HG alone (**Figure 10A**). This effect was accompanied by a significant reduction of levels of the pro-inflammatory cytokines IL-12 (p70) and IL-1β, the generation of which is increased in cells cultured under HG-supplemented medium without L-cit co-treatment. Addition of a neutralizing antibody against IL-10 to huPTEC cultured under HG in the presence of L-cit, significantly abolished the reduction of IL-12 (p70). In addition, elevation of IL-10 was accompanied by significant elevated protein levels of ArgII, an effect that was partially reduced when anti-IL-10 antibody was added along with L-cit to the HG-supplemented medium (**Figure 10B**). These data indicate that elevation of ArgII in huPTEC in culture is a marker of the anti-inflammatory actions of L-cit through its IL-10-inducing capacity.

We also examined the activation of the apoptotic marker caspase 6, which was shown to be involved in PTEC apoptosis during nephropathy (34). We observed an increased nuclear translocation of caspase 6 in huPTEC exposed to HG. L-Cit significantly blunted this effect of HG at least partially in an IL-10-dependent manner, since concurrent treatment of huPTEC with a neutralizing IL-10 significantly prevented the reduction in caspase 6 nuclear translocation by L-cit (**Figure 10C**). This indicates that the observed caspase 6 activation was linked to a pro-inflammatory cytokine, the generation of which was inhibited by IL-10.

DISCUSSION

Hyperglycemia, which activates several reactions, including oxidative stress and chronic or subclinical inflammation, is clearly recognized as the primary player in diabetic endothelial dysfunction and DN (35–37).

It is now generally recognized that an important cause of impaired endothelial NO production, characteristic of diabetic endothelial dysfunction, is reduced availability of the eNOS substrate L-arg. Despite diverse data from studies assessing plasma amino acid levels in diabetic patients with or without chronic kidney disease (38, 39), patients with diabetes and cardiovascular disease were shown to benefit only from acute (2), but not from chronic (3) L-arg supplementation.

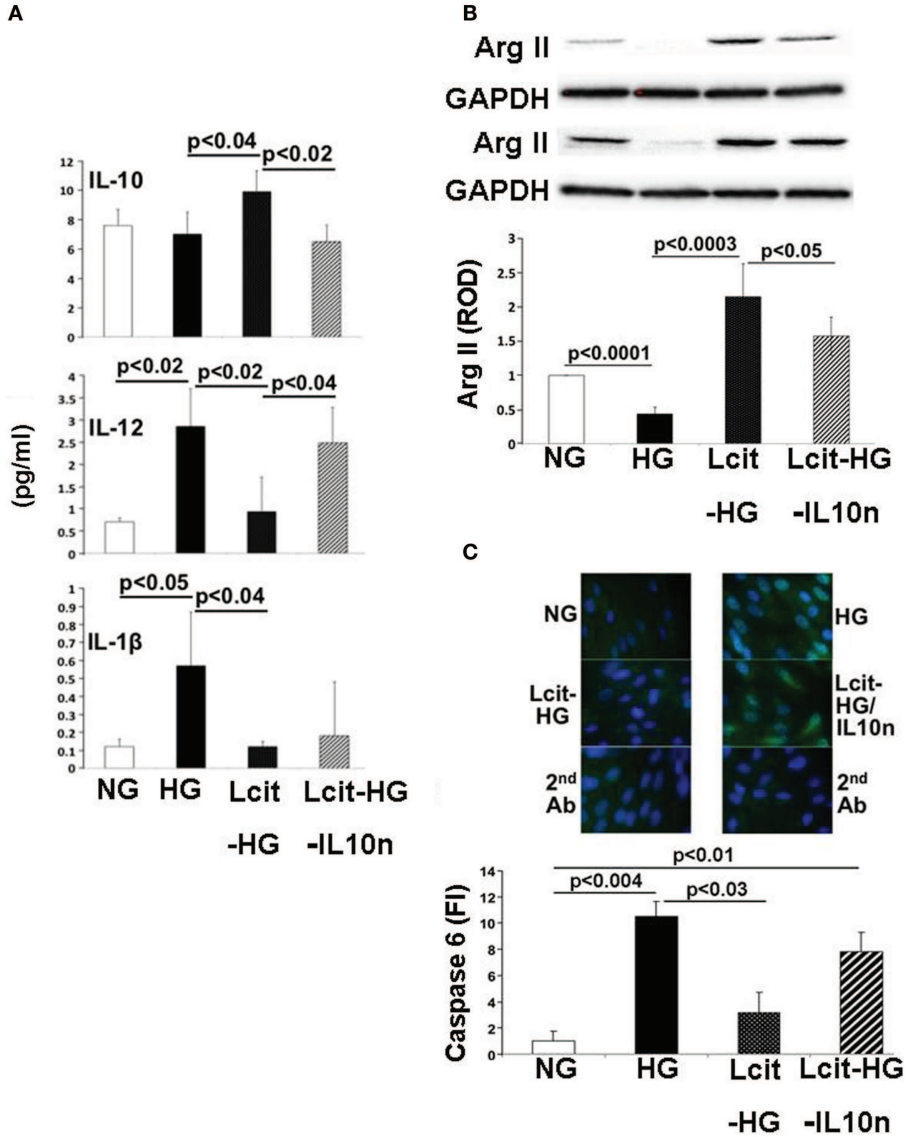

FIGURE 10 | Effect of L-cit in human proximal tubular epithelial cells (huPTEC) exposed to high glucose (HG). huPTEC were cultured in Renalife medium containing 25 mM D-glucose (HG) for 7 days. L-Glucose (17.8 mM) was added to 7.2 mM D-glucose present in medium, and used as iso-osmotic control (NG). Pre-treatment of huPTEC with L-citrulline (L-cit, 1 mM) was performed by adding the amino acid 2 h prior to adding HG or iso-osmotic control, with or without a neutralizing anti-IL-10 antibody (IL10n, 5 μg/ml). **(A)** The cytokine level (picogram per milliliter) in the medium was determined by using a commercial magnetic beads-based human cytokine kit. **(B)** Representative blot (upper) and densitometric analysis of blots (lower) show arginase II levels from protein extracts of huPTEC. A decreased in arginase II expression is observed following treatment with HG. This effect was markedly reduced by concurrent L-cit treatment, while addition of anti-IL-10 antibody (IL10n) along with L-cit partially prevented L-cit-induced elevation of arginase II. **(C)** huPTEC were seeded onto slide chambers. After completion of treatments, cells were immunostained using caspase 6 as primary antibody, followed by Cy5-conjugated goat anti-rabbit secondary antibody. DAPI was used for nuclear staining. Representative images (upper) and microscopy analysis of nuclear fluorescence intensity (lower) show an increase nuclear localization of caspase 6 (green fluorescence) following treatment with HG. This effect was markedly reduced by concurrent L-cit treatment, while the effect was abolished by addition of neutralizing anti-IL-10 antibody (IL10n) along with L-cit.

Conversely, we and others have been shown that oral L-cit (precursor of L-arg) augments NO-dependent signaling, not only by means of increasing L-arg synthesis, but also by decreasing L-arg catabolism, as such increasing circulating L-arg levels (4–6). However, the effects of L-cit on the development of diabetic kidney damage have not been studied. Therefore, in this study, we assessed the actions of supplemental L-cit in a murine model of DN. Our data demonstrate that oral L-cit supplementation protects diabetic STZ-mice from the sustained elevation of UAE, as observed in untreated mice at 16 weeks of the disease. This protective effect of L-cit occurs despite significant hyperglycemia. We also observed similar benefits conferred by L-cit in a more aggressive

model of DN in STZ-rats, which also showed reduced proteinuria after 8 weeks of treatment.

Diabetic urinary albumin leakage involves several mechanisms, including proximal tubular injury (40, 41) and disruption of the glomerular barrier (42). The relevance of the glomerular endothelium in the maintenance of barrier function has only been recently recognized (12). While endothelial NO generation contributes to endothelial glycocalyx and barrier preservation (11, 43, 44), an increase in the ROS/NO ratio causes disruption of the glycocalyx, resulting in enhanced albumin permeability (45, 46).

We have found a reduction in mitochondrial ROS generation, combined with a restoration of NO production in HGECs treated with L-cit before exposure to HG-supplemented medium. This effect may thus at least partially account for the reduced glomerular albumin leakage we have found in the diabetic animals supplemented with L-cit. In support of this is the reduced permeability to FITC-dextran of HGEC monolayers exposed to HG and concurrently treated with L-cit.

We did not assess either constitutive (endothelial and neuronal) or inducible NOS expression in the kidneys of our diabetic animals, because the three NOS isoforms have been described to be differentially altered in DN (47, 48). Indeed, the discrepant results on NOS expression and NO involvement in diabetic pathology have been evaluated in other diabetic complications (49, 50).

Our current results, along with previous work, support the notion that bioavailability of NO is reduced in the diabetic vessels (8, 51, 52). Therefore, adding L-cit to current therapies may lead to a safe and efficacious option to improve vascular diabetic complications. Moreover, due to the significant role of NO in the regulation of insulin release from pancreatic β-cells (53), L-cit may also be useful as a potential insulinotropic agent. However, the effect of L-cit on pancreatic β-cell function requires further studies.

Several studies have also suggested a role for endothelial NO in suppressing fibrotic pathways in different organs and pathologies associated with diabetes and other diseases (54, 55). As such, the protective effect of L-cit on eNOS function may have led to the reduction in kidney fibrosis, as observed in our study in diabetic mice and rats after 16 and 8 weeks of diabetes, respectively.

The protective effects of L-cit toward UAE and kidney fibrosis were observed despite a sustained elevation of ArgII protein levels in the renal cortex. ArgII protein was significantly elevated in L-cit treated diabetic mice and rats over control and untreated diabetic animals at the end of the study, when protection on kidney pathology was more evident. Interestingly, the protective effects of L-cit administration in other pathologies have also been shown to be accompanied by an enhanced tissue expression of ArgII (23).

Our findings prompt the question about the role of ArgII for tubular function in diabetes. ArgII is present in the mitochondria of PT, as well as of inner medullary collecting ducts (17) and provides L-ornithine for the synthesis of polyamines (56). The cellular balance of polyamines is necessary for DNA stabilization and replication (57), as well as for the maintenance of PT integrity and function (58).

Damage of PT under the insult of HG levels, especially in patients with poor glycemic control (59), requires an extensive repair process, either by regeneration of de-differentiated surviving cells (60) or by proliferation and differentiation of stem cells (61). It has been recently demonstrated that spermidine enhances epithelial stem cell function (62). Thus, adequate polyamine levels may allow the PT to resume normal functions, and L-cit may facilitate this process by providing more L-arg for ArgII function.

In addition, up-regulation of mitochondrial ArgII in diabetic PT may represent a stress response to an increased energy demand in this actively reabsorptive segment of the nephron. Arginase-derived ornithine in the mitochondria may be converted to L-glutamate that enters the tricarboxylic acid cycle as oxoglutarate (63). L-cit could as such provide precursors to maintain the energetic metabolism of PT via mitochondrial ArgII. Diabetic kidneys from L-cit-treated rats clearly showed a reduced number of positive tubules for KIM-1 expression, a marker of proximal tubular damage. This effect may also be associated with an improvement of proteinuria (64) observed in L-cit treated diabetic rats.

The results of our comparative studies between WT and ArgII knock out mice partially differ with a recent report by others (22). Despite a trend to reduced levels of albuminuria in diabetic ArgII knock out mice, the reduction was not significantly different from untreated WT diabetic mice. Differences between the Morris study and ours likely arise from the significantly greater age of our mice. Indeed, we observed a severe dilation and morphological alterations of cortical tubules, as well as greater BUN levels in ArgII knock out mice, as compared to untreated diabetic WT mice. These findings suggest that with advanced age, lack of ArgII may limit tubular repair and may accelerate the decay in glomerular filtration rate observed in the diabetic condition. Other reported mechanisms may also apply for tubular damage in ArgII knock out mice (65, 66). To that purpose, it would be interesting to determine in future studies, whether L-cit supplementation to ArgII knock out mice prevents diabetes-induced tubular damage and enhancement of BUN levels.

Intriguingly, collagen deposition in kidneys isolated from diabetic ArgII knock out mice was not different from the one observed in control WT mice. A limited availability of the precursor proline, provided by the ArgII/ornithine aminotransferase pathway, may be the cause of reduced renal collagen synthesis/crosslink in this group, which indicates that ArgII has an important contribution to renal collagen content. However, the cost-effect of specific ArgII inhibition in advanced stages of diabetic animal models remains to be investigated.

In addition to the previous findings, L-cit treatment to diabetic mice prevented body wasting even in the absence of blood glycemic control. Type 1 diabetic patients under poor glycemic control, common in low income or un-insured patients in the United States or in under-developed countries (67) exhibit detrimentally low intracellular energy metabolism and significant weight loss, leading to chronic fatigue and general body weakness. L-Cit may protect against diabetic muscle wasting via nutritional support, providing the precursor for creatine synthesis (68, 69).

A substantial benefit conferred by L-cit supplementation is the significant elevation of the anti-inflammatory cytokine IL-10. It has been recently recognized that common inflammatory factors play a role in both type 1 and 2 diabetic pathology (30, 70), which has important therapeutic implications (71–73). IL-10 has

been shown to selectively induce ArgII expression in macrophages (74) and to also attenuate a pro-inflammatory cytokine expression and iNOS-derived NO production in human and mouse monocyte/macrophage cells in the presence of apoptotic cells (75). Apoptosis of PT epithelial cells is a feature of the hyperglycemic insult in DN, and activated PT epithelial cells, as an alternative to macrophages, are able to phagocytose neighboring apoptotic cells (76). Moreover, PT epithelial cells play an important role in anti-inflammatory mechanisms within the tubulointerstitium during renal injuries (77, 78) and are capable of generating their own production of IL-10 (33).

Although we did not measure local kidney tissue or urinary levels of IL-10 in our study, but rather in plasma, it is nevertheless likely that both local and systemic anti-inflammatory mechanisms may take place under the setting of L-cit supplementation, since this treatment was accompanied by enhanced ArgII expression in PT, and by increased plasma levels of IL-10. In support of our findings *in vivo*, we observed that huPTECs cultured under HG-supplemented medium in the presence of L-cit for 1 week, produced significantly enhanced levels of the anti-inflammatory cytokine IL-10, as compared to cells treated with HG alone. This is a prominent feature of L-cit's actions on huPTEC, which may be of high significance in the context of current clinical trials aimed to limit inflammation in diabetic patients, and to reduce progression of DN toward ESRD. In correspondence with our observations that L-cit increases IL-10 generation, it has been recently shown that *Citrullus lanatus* (Watermelon), a rich source of L-cit, was beneficial in a murine inflammatory disease model, by means of increasing plasma levels of IL-10 (79).

The enhanced production of the anti-inflammatory cytokine IL-10 by L-cit in HG-treated huPTEC was accompanied by a reduction of levels of the pro-inflammatory cytokines IL-12 (p70) and IL-1β both of which were induced above basal levels by HG in cells not treated with L-cit. *In vivo*, these pro-inflammatory cytokines may establish the settings for a crosstalk between tubular cells and surrounding infiltrating leukocytes, to amplify the inflammatory milieu of diabetic kidneys. As such, L-cit-induced IL-10 generation may be important in limiting inflammation in the kidney.

In accordance with our findings on ArgII expression in kidneys of mice at late stages of diabetes, cultured huPTEC under HG condition had a reduced expression of ArgII protein levels. L-Cit significantly enhanced ArgII in HG-treated huPTEC, an effect that was reduced by co-administration of IL-10 neutralizing antibody with L-cit. These results indicate that up-regulation of ArgII in PT is a marker of the anti-inflammatory actions of IL-10 on renal tubules.

We could detect an increased level of nuclear localization of the executioner caspase 6, a mediator of apoptosis, in huPTEC exposed to HG levels. L-Cit blunted this effect of HG at least partially in an IL-10-dependent manner. This indicates that the observed caspase 6 activation was linked to a pro-inflammatory cytokine, the generation of which was inhibited by IL-10. Although more research is needed to unravel what cytokines are responsible for the caspase 6 activation in HG-treated huPTEC, an interesting candidate could be IL-1, which was shown to be increased by HG in huPTEC in our experiments and whose generation was blunted by L-cit. IL-1 was shown to induce Fas ligand generation, a potent inducer of apoptosis in renal tubular cells (80).

In conclusion, our study demonstrates that L-cit supplementation is protective to the nephron function. L-Cit not only reduces UAE and prevents collagen deposits in the kidneys of diabetic animals, but also establishes the settings for an anti-inflammatory response in the PT, with the potential to direct the immune response toward an anti-inflammatory profile in monocyte/macrophages as well. These observations are substantiated by the elevation of tubular ArgII expression, and of plasma levels of IL-10.

It remains to be established whether L-cit sustains tubular mitochondrial function, by providing precursors *via* ArgII and whether this effect is linked to repair processes of the proximal nephron under the hyperglycemic insult. As such, this work lays the foundation for a broader investigation of the effects of L-cit supplementation on local vs. systemic IL-10 generation, which may have important therapeutic applicability in diabetic patients.

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