NEW TRANSLATIONAL INSIGHTS ON METABOLIC SYNDROME: OBESITY, HYPERTENSION, DIABETES AND BEYOND

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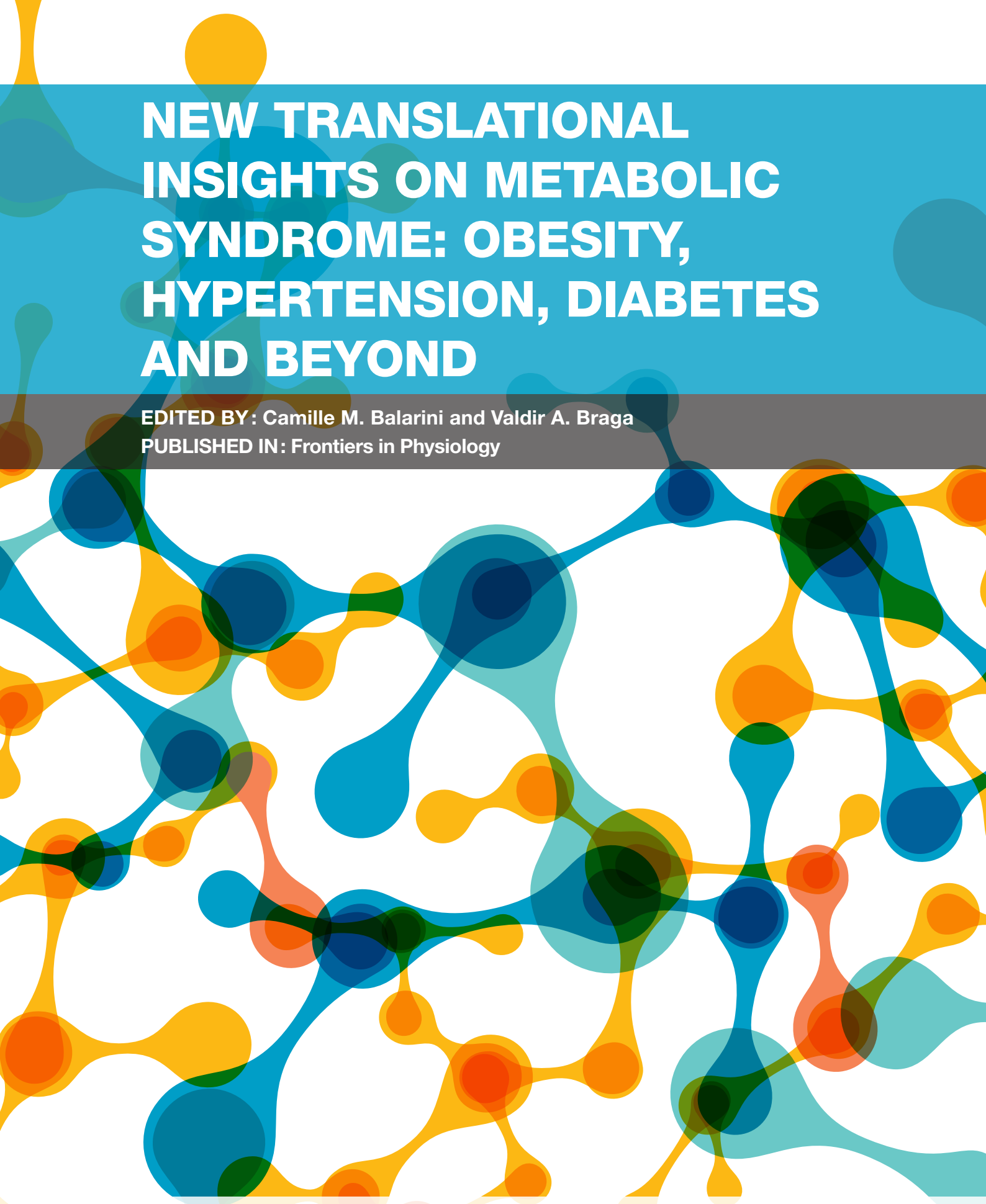

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NEW TRANSLATIONAL INSIGHTS ON METABOLIC SYNDROME: OBESITY, HYPERTENSION, DIABETES AND BEYOND

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Metabolic syndrome (MetS) can be considered as a clustering of several risk factors such as obesity, hypertension, insulin resistance and dyslipidemia, which could lead to the development of diabetes and cardiovascular diseases (CVD). There are several underlying causes for MetS including overweight, physical inactivity and genetic factors. However, the underlying mechanisms that leads to MetS are still poorly understood. Therefore, the aim of this ebook is to provide a space where researchers holding different backgrounds could shed some light onto the pathophysiology of different risk factors involved in MetS, mostly from translational research worldwide.

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EDITORIAL
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Editorial: New Translational Insights on Metabolic Syndrome: Obesity, Hypertension, Diabetes and Beyond

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The Editorial on the Research Topic

New Translational Insights on Metabolic Syndrome: Obesity, Hypertension, Diabetes, and Beyond

Metabolic syndrome (MetS) can be considered as the clustering of several risk factors such as obesity, hypertension, insulin resistance, and dyslipidemia, which could lead to the development of diabetes and cardiovascular diseases (CVD). The criteria for clinical diagnosis of MetS consist of 3 or more of the following: (1) waist circumference >102 cm in men and 88 cm in women; (2) triglycerides >150 mg/dL; (3) HDL <40 mg/dL in men and <50 mg/dL in women; (4) blood pressure ≥ 130/85 mmHg; (5) fasting glucose ≥ 100 mg/dL. Considering that the underlying mechanisms leading to the concurrence of these factors are not yet well-established, the aim of this research topic was to provide a space where researchers holding different backgrounds could shed some light onto the pathophysiology of different risk factors involved in MetS.

The present research topic involves eleven articles including both reviews and original manuscripts. In a translational perspective, the topic includes both clinical and experimental approaches from different research groups located in several countries, which discuss different aspects of MetS such as hypertension, obesity, diabetes, atherosclerosis, and inflammation. At the present moment, almost 3000 article downloads were performed from researchers all over the world. In this editorial, we highlight important insights from these articles leading to a better comprehension of MetS and its complications.

Interestingly, in one aspect of metabolic disorders, the review articles by Costa-Silva et al. and Silva et al. discuss the effects of malnutrition on the establishment of hypertension. Using an *in utero* perspective, Costa-Silva et al. evaluate the effects of maternal malnutrition during pregnancy on sympathetic-respiratory dysfunction that leads to hypertension. It has been shown that maternal low-protein diet during gestation negatively affects organ growth and increases sympathetic tone in the offspring. In addition, Silva et al. focus on post-weaning protein malnutrition and its cardiovascular implications. Authors demonstrated that post-weaning low protein intake impairs cardiovascular reflexes, increases sympathetic tonus, decreases vagal tonus, and enhances arterial blood pressure and heart rate. Both groups agree that increased hypoxia-inducible factor expression in malnutrition is responsible for the enhanced sympathoexcitation arising from chemoreflex activation.

Opposite to malnutrition, obesity was discussed in three independent articles. As highlighted by Del Rio et al., most efforts to understand MetS have been focused on the study of peripheral organ malfunction, while the role of the nervous system on alterations observed in MetS remains poorly understood. Focusing on central mechanisms involved in MetS,

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Moreira et al., Cruz et al., and Del Rio et al., independently discussed the impact of increased sympathetic activity in obesity. Moreira et al. provided us with a substantial review of important MetS components such as obesity, hypertension, insulin resistance, dyslipidemia, and inflammation. The link between obesity and cardiovascular damage may reside on leptin, since this peptide can increase sympathetic renal activity, reactive oxygen species (ROS) in kidney and reduce nitric oxide and that hyperleptinemia is a common feature in obesity. Regarding central nervous system alterations in MetS, Cruz et al. documented that the sympathoexcitation caused by peripheral Ang II-induced ROS formation along the subfornical organ (SFO) and paraventricular nucleus of the hypothalamus (PVN) may be a putative mechanism to explain the metabolic disorders underlying MetS. Lastly, Del Rio et al. bring a different approach and focus their work on glial cells within central nuclei involved in sympathetic control and how these cells could contribute to the pathogenesis of MetS.

Metabolic syndrome is also involved in impairment of glycemic control and end-organ damage. Therefore, Peleli et al. studied the effects of inorganic nitrate on glucose and insulin signaling in an experimental model of MetS (adenosine receptor type A_{2B} knockout mice, $A_{2B}^{-/-}$). Acute nitrate administration to $A_{2B}^{-/-}$ mice resulted in ameliorated glucose tolerance, reduced HOMA-IR and increased plasma nitrate. Authors suggested that impaired AMP-activated kinase (AMPK) activation and increased NADPH oxidase in liver could contribute to MetS in this model, since the ratio of phosphorylated/non-phosphorylated AMPK was reduced and NADPH oxidase activity was increased in liver of $A_{2B}^{-/-}$ mice.

Considering that diabetic nephropathy is an important cause of death among diabetic patients, Gomes et al. proposed that a low-dose of the antioxidant quercetin could improve metabolic parameters and renal function in an experimental model of diabetes and atherosclerosis (apolipoprotein E knockout—$apoE^{-/-}$—diabetic mouse). Furthermore, Arsenijevic et al. propose that a primary reduction in kidney function can be considered the cause of a pro-inflammatory and pro-oxidative status, leading to metabolic disorders.

Lastly, three independent research groups focused their work on the implications of inflammation in different determinants of MetS. Firstly, Freitas Lima et al. discussed the influence of adipokines in insulin resistance and atherosclerosis focusing on adiponectin, TNF-α, IL-6, MCP-1, and leptin. Considering that endothelial dysfunction is a *sine qua non* condition to atherosclerosis development, the comprehension of this phenomenon can create a window of opportunities for preventive measures. Is this regard, Kraemer-Aguiar et al. discussed the hypothesis that, as obesity is a multiple grade disease, an increasing impairment of vascular function would occur from lean to severe obese subjects. Using a different perspective, Cavalcante-Silva et al. discussed the influence of gut microbiota in inflammation and MetS. Authors provide us with a broad review on normal gut microbiota and how it can modulate adiposity, glucose homeostasis, fat accumulation, and other metabolic pathways.

In conclusion, we found that this research topic, devoted to a better understanding of MetS, and its associated comorbidities, was very enlightening. We would to like to thank all authors and reviewers that helped us to provide a quality-based topic.

AUTHOR CONTRIBUTIONS

CB and VB participated in the design of the manuscript, drafted the manuscript, revised it critically and approved the final version.

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MINI REVIEW
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New Insights on the Maternal Diet Induced-Hypertension: Potential Role of the Phenotypic Plasticity and Sympathetic-Respiratory Overactivity

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Systemic arterial hypertension (SAH) is an important risk factor for cardiovascular disease and affects worldwide population. Current environment including life style coupled with genetic programming have been attributed to the rising incidence of hypertension. Besides, environmental conditions during perinatal development such as maternal malnutrition can program changes in the integration among renal, neural, and endocrine system leading to hypertension. This phenomenon is termed phenotypic plasticity and refers to the adjustment of a phenotype in response to environmental stimuli without genetic change, following a novel or unusual input during development. Human and animal studies indicate that fetal exposure to an adverse maternal environment may alter the renal morphology and physiology that contribute to the development of hypertension. Recently, it has been shown that the maternal protein restriction alter the central control of SAH by a mechanism that include respiratory dysfunction and enhanced sympathetic-respiratory coupling at early life, which may contribute to adult hypertension. This review will address the new insights on the maternal diet induced-hypertension that include the potential role of the phenotypic plasticity, specifically the perinatal protein malnutrition, and sympathetic-respiratory overactivity.

Keywords: hypertension, developmental plasticity, perinatal nutrition, respiratory control, protein restriction

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INTRODUCTION

Hypertension is a highly prevalent and significant risk factor for the development of metabolic disease, including coronary heart disease (CHD), stroke, heart failure, aortic, and peripheral arterial disease (Landsberg et al., 2013). The etiology of hypertension includes a complex phenotype that arises from numerous genetic, environmental, behavioral, ethnic, and even social origins (Landsberg et al., 2013). However, it has been observed that the perinatal nutritional milieu during "sensitive" periods of development or in infant affects the normal growth/developing and this may be associated with adult disease (Lucas, 1998; Victora et al., 2008; Wells, 2012). This phenomenon

Abbreviations: SAH, systemic arterial hypertension; BP, Blood pressure; CB, carotid bodies; HF, High frequency; LF, low frequency; VLF, Very low frequency; AP, Arterial pressure; SAP, systemic arterial pressure.

can be understood in the context of phenotypic plasticity. Phenotypic plasticity refers to the ability of an organism to react to an internal and external environmental input with a change in the form, state, movement, or rate of activity without genetic changes (West-Eberhard, 2005).

The association between Systemic arterial hypertension (SAH) and nutritional factors has been studied by a large number of epidemiological and clinical studies (Ashton, 2000; Hemachandra et al., 2006; Antony and Laxmaiah, 2008; Conde and Monteiro, 2014; Parra et al., 2015). Indeed, perinatal malnutrition is associated with the risk of developing cardiovascular disease and co-morbidities in later life including hypertension, metabolic syndrome and diabetes, (Nuyt, 2008; Nuyt and Alexander, 2009). In humans, studies have provided support for the positive association between low birth weight and increased incidence of hypertension (Ravelli et al., 1976; Hales et al., 1991; Sawaya and Roberts, 2003; Sawaya et al., 2004).

It is well established that perinatal malnutrition environmental stimuli can contribute to the programming of subsequent risks of hypertension by mechanisms that include reduced nephron morphology and function, reduced glomerular filtration rate, and dysfunction on the renin-angiotensin-aldosterone system (Nuyt and Alexander, 2009). Recently, studies have highlighted the contribution of the sympathetic-respiratory dysfunctions on the development of the maternal diet induced-hypertension (de Brito Alves et al., 2015). Protein-restricted rats during gestation and lactation showed respiratory dysfunction, which was associated with sympathetic overactivity and enhanced carotid bodies (CB) sensitivity to hypoxia (de Brito Alves et al., 2015; Nanduri and Prabhakar, 2015; Prabhakar et al., 2015). The underlying mechanism may be associated with high levels of hypoxic inducible factor (HIF-1α) in CB peripheral chemoreceptor (Ito et al., 2011, 2012; de Brito Alves et al., 2015). Thus, the aim of this review was to address the new insights about maternal diet induced-hypertension and the concept that perinatal malnutrition may affect the ventilatory and cardiovascular control.

NEW INSIGHTS ON THE PERINATAL ORIGIN OF HYPERTENSION: THE ROLE OF PHENOTYPIC PLASTICITY

One of the best-known attempts to understand the association between early nutrition and SAH is the "thrifty phenotype hypothesis" proposed by Hales and Barker in 1992. This hypothesis is extensively used to consider cardiovascular disease as a "programmed" effect of nutritional restriction during early phases of growth and development, followed by a recovery of the diet during lifespan (Hales and Barker, 1992). This phenomenon can be understood in the context of the phenotypic plasticity (Barker et al., 2005; West-Eberhard, 2005; Labayen et al., 2006; Andersen et al., 2009; Biosca et al., 2011). Phenotypic plasticity is defined as the ability of an organism to react to an environmental stimuli with a adaptive mutual adjustment, without genetic change, among variable aspects of the phenotype, following a novel or unusual input during development (West-Eberhard, 2005). Epigenetic alterations as DNA methylation, histone acetylation and microRNA expression are the molecular basis of the phenotypic plasticity (Wells, 2011). Maternal low-protein diet model during gestation and/or lactation is one of the most extensively studied animal models of phenotypic plasticity (Ozanne and Hales, 2004; Costa-Silva et al., 2009; Falcão-Tebas et al., 2012; Fidalgo et al., 2013; de Brito Alves et al., 2014). An overview about the environmental influence during development on early appearance of SAH is shown in **Figure 1**. Feeding a low-protein diet (8% casein) during gestation followed by the consumption of a normocaloric diet throughout lactation is associated with growth restriction, asymmetric reduction in organ growth, elevated systolic blood pressure, and increased fasting plasma insulin concentrations (Ozanne and Hales, 2004; Fidalgo et al., 2013; de Brito Alves et al., 2014). Recently, it was demonstrated that adult animals subjected to maternal protein restriction presented mainly an increase in the cardiovascular sympathetic tone and increased low frequency (LF) bands of the SAH, suggesting autonomic misbalance, and sympathetic predominance on the cardiovascular system of these animals (Barros et al., 2015).

It is known that the rhythmicity of the sympathetic nervous system can modulate the arterial pressure (AP) and the heart rate at regular frequencies (Tseng et al., 2009). These rhythmic fluctuations in the cardiovascular variables suggest a measurement of cardiovascular autonomic balance (Japundzic-Zigon, 1998). Accordingly, the LF oscillations of the systolic arterial pressure (SAP) are typically enhanced during states of sympathetic activation (Julien, 2006) and are increased in the offspring from dams subjected to protein restriction during perinatal period and may contribute to the development of arterial hypertension in this experimental model (de Brito Alves et al., 2015).

Although the relationship between maternal protein restriction and sympathetic overactivity have been suggested (Johansson et al., 2007; Franco et al., 2008; Barros et al., 2015), less is known about the physiological dysfunctions responsible for producing these effects. In this context, it is described that a baroreflex dysfunction could lead to a sympathetic overactivity and subsequent development of hypertension (Souza et al., 2001; Heusser et al., 2010; Tsyrlin et al., 2013). However, the hypothesis that maternal protein restriction leads to baroreflex dysfunction has not been proved yet.

Nowadays, it is well accepted that perinatal protein malnutrition raise risks of hypertension by mechanisms that include reduced nephron morphology and function, and dysfunction on the renin-angiotensin system (Chen et al., 2010; Siddique et al., 2014). However, other hypotheses have been highlighted considering the role of sympathetic overactivity and the development of hypertension in organisms that suffered perinatal malnutrition. In different models of hypertension, it has been suggested that changes in the generation or modulation of respiratory function can contribute to the development of arterial hypertension (Simms et al., 2009, 2010; Costa-Silva

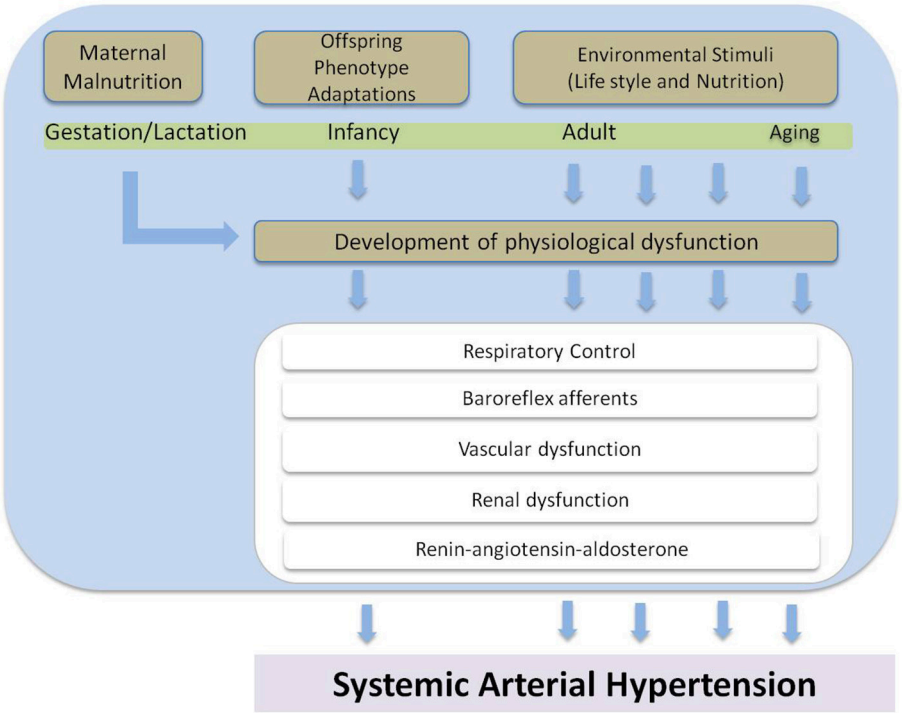

FIGURE 1 | Overview about the environmental influence during development on early appearance of systemic arterial hypertension.

et al., 2012; Moraes et al., 2014). Indeed, respiratory neurons located into the brainstem may modulate the sympathetic nervous system and the levels of arterial pressure by central pathways (Costa-Silva et al., 2009, 2010, 2012; Moraes et al., 2014). These neurons receive strong influences from peripheral respiratory chemoreceptors, located into CB at the aortic and carotid arteries. Activation of these chemoreceptors produces a powerful activation of the cardiorespiratory neuronal network and enhances the sympathetic outflow and respiratory drive, which are essential to cardiovascular and ventilatory stability and for providing a correct O_2 delivery to cells (Costa-Silva et al., 2010, 2012; Moraes et al., 2014). Thus, it has been suggested that CB dysfunction induced by phenotypic plasticity at the early life can lead to autonomic and ventilatory disorders (Nanduri and Prabhakar, 2015; Prabhakar et al., 2015).

Recently, experimental studies showed that maternal protein restriction during pregnancy and lactation leads to relevant short-term effects on the CB sensitivity and respiratory control of the offspring (de Brito Alves et al., 2014, 2015). Maternal protein restriction is able to induce high phrenic burst frequency and amplitude, leading to increased baseline respiratory frequency (up to 28%) and ventilation (up to 40%) (de Brito Alves et al., 2014, 2015). Further, studies *in situ* also observed that these respiratory dysfunctions are associated with enhanced baseline sympathetic activity and amplified ventilatory and sympathetic responses to peripheral chemoreflex activation prior to the establishment of hypertension, and high ventilatory responses to hypoxia (de Brito Alves et al., 2015). Therefore, these data strongly support the hypothesis that protein-restricted rats have respiratory dysfunction, which was associated with sympathetic overactivity and enhanced CB sensitivity to hypoxia. Interestingly, this sympathetic-respiratory overactivity was associated with high levels of hypoxic inducible factor (HIF-1α) in CB peripheral chemoreceptor (de Brito Alves et al., 2015). Increased HIF-1α expression was previously observed in heart and brain from the protein-restricted animals (Ito et al., 2011, 2012) and support the notion that a high expression of this transcriptional factor (cellular response to hypoxia), is associated with enhanced sensory activity of the peripheral chemoreceptors, autonomic dysfunction, sympathetic overactivity, and increased risk of hypertension in the offspring subjected to maternal-protein restriction (**Figure 2**). However, the underlying mechanism involved in the HIF-1α up-regulation in protein-restricted rats is still unclear, but it is hypothesized that epigenetic mechanism produced by DNA methylation could be involved (Altobelli et al., 2013; Prabhakar, 2013; Nanduri and Prabhakar, 2015). Taken together, these studies reinforce the notion that the augmented afferent inputs from the CB (peripheral respiratory chemoreceptors) to brainstem and enhanced sympathetic outflow to the kidney, heart and blood vessels are highlighted as new insights on the maternal diet induced-hypertension, which may lead to increased blood

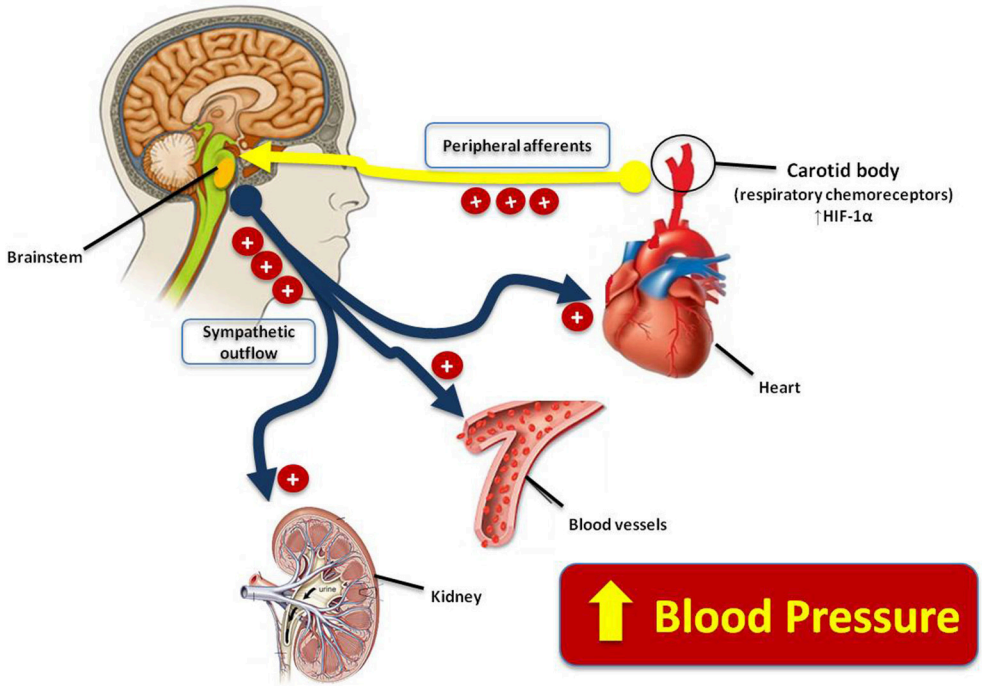

FIGURE 2 | Schematic drawing showing the new insights on the maternal diet induced-hypertension, and the influence of the augmented afferent inputs from the carotid body (peripheral respiratory chemoreceptors) to brainstem and enhanced sympathetic outflow to the kidney, heart and blood vessels, which may lead to increased blood pressure in the adult offspring subjected to maternal protein restriction.

pressure in the adult offspring subjected to maternal protein restriction.

CONCLUSION

The etiology of the SAH is multifactorial involving genetic influences and the physiological integration of cardiovascular, renal, neural, and endocrine systems. Environmental stimuli are also strongly related to the high prevalence of SAH. Recently, it was recognize that perinatal malnutrition is related with the risk of developing metabolic syndrome and hypertension in adult life. The underlying mechanism can be explained in the context of phenotypic plasticity during development that includes adaptive change on the renal morphology and physiology with subsequent arterial hypertension. Moreover, maternal protein restriction may alter the central control of SAH by a mechanism that include enhanced sympathetic-respiratory activities and respiratory dysfunction at early life, which may contribute to adult hypertension. There are experimental evidences that respiratory dysfunction may be associated with both sympathetic overactivity and the high levels of HIF-1α in CB peripheral chemoreceptor.

AUTHOR CONTRIBUTIONS

JC and CL drafted the work and revised critically for important intellectual content; wrote the paper; Final review of the manuscript. JB, VO, MA, KP, AO, IN, JF contributions to the conception of the work; Final review of the manuscript.

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The implication of protein malnutrition on cardiovascular control systems in rats

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The malnutrition in early life is associated with metabolic changes and cardiovascular impairment in adulthood. Deficient protein intake-mediated hypertension has been observed in clinical and experimental studies. In rats, protein malnutrition also increases the blood pressure and enhances heart rate and sympathetic activity. In this review, we discuss the effects of post-weaning protein malnutrition on the resting mean arterial pressure and heart rate and their variabilities, cardiovascular reflexes sensitivity, cardiac autonomic balance, sympathetic and renin-angiotensin activities and neural plasticity during adult life. These insights reveal an interesting prospect on the autonomic modulation underlying the cardiovascular imbalance and provide relevant information on preventing cardiovascular diseases.

Keywords: protein malnutrition, neuroplasticity, sympathetic activity, cardiovascular reflexes, renin-angiotensin system

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Introduction

Malnutrition, an important pathological condition resulting from deficient intake or absorption of macro and/or micronutrients, reaches more than 900 million individuals worldwide and accounts for about 3.5 million deaths of under 5 year-old children (Black et al., 2008). Clinical and experimental researches propose that malnutrition in early life stages is often associated with metabolic and cardiovascular disorders in adult life (Plagemann et al., 2000; Langley-Evans, 2007). From a hypothetical "nutritional programming" point of view, the nutritional deficiency can prompt epigenetic alterations, including the compromising of the autonomic nervous system (ANS), which gives rise to secondary metabolic and cardiovascular disturbances, such as: insulin resistance, coronary disease, and hypertension (Benabe and Martinez-Maldonado, 1993; Lucas, 1998; Barker, 2007). It is not a novelty that low protein intake impairs the cardiovascular homeostasis, increasing the mean arterial pressure (MAP), which is an important cardiovascular risk factor (Handler and Bernheim, 1950; Engen and Swenson, 1969). However, the pathophysiological mechanisms underlying it remain under investigation. The understanding of the cardiovascular changes caused by protein malnutrition comprises two points: understanding the mechanisms that control blood pressure (BP) in this nutritional state, and looking for changes that precede alterations on BP and other variables that influence the cardiovascular homeostasis.

Cardiovascular control involves feedback systems activation, which operates on short and long terms (Shepherd and Mancia, 1986; Dampney, 1994). The short term regulation mechanisms comprise the cardiovascular reflexes. In this regard, peripheral information detected by specific receptors is processed in the central nervous system (CNS) and returns to the periphery by efferent

ANS subdivisions: sympathetic nervous system (SNS) and parasympathetic nervous system, to maintain homeostasis (Machado et al., 1997). The long term regulation mechanisms relate to humoral systems, such as renin-angiotensin system (RAS) whose unbalance contributes to the development/maintenance of high peripheral resistance and vascular hyper-reactivity observed in hypertension (Ferguson and Bains, 1997; Mendoza and Lazartigues, 2015). Both, short- and long-term regulation systems play important roles in physiologic and pathologic conditions. The pathogenesis of cardiovascular diseases (e.g., heart failure, coronary disease, and hypertension) can be associated with unbalanced autonomic cardiovascular regulation, particularly by SNS overactivation (Sinski et al., 2006). Although it has been speculated that sympathetic hyperactivity could be the key alteration, the chronological sequence between impaired sympathetic drive and other abnormalities have not yet been determined (Mancia and Grassi, 2014). Regarding of malnutrition-mediated autonomic cardiovascular disorders, it is essential to identify the mechanisms that are underlying to this process for setting cause-consequence relationship. In this sense, we will discuss the following aspects in the present review: (a) resting MAP and heart rate (HR) and their variabilities, (b) cardiovascular reflexes sensitivity, (c) SNS activity/reactivity, (d) RAS activity, and (e) neural plasticity related to dysfunctional cardiovascular autonomic control observed in the experimental protein malnutrition model.

Post-weaning Protein Malnutrition Experimental Model

Our laboratory has been focused to studying the cardiovascular disorders observed in adult rats submitted to post-weaning protein malnutrition protocol. This model reflects a situation that occurs in underdeveloped countries, where the newborn receives a satisfactory amount of protein by breast feeding, only to see the protein intake reduced after weaning (Rodrigues et al., 2012).

In gestation and weaning periods, females receive regular rat chow and filtered water *ad libitum*. After the weaning period (28 days), male rats are separated from their mothers and kept in grouped cages. Over the next 35 days, rats are fed either a normal or a low protein diet, which make up our two experimental groups: normal protein (NP) and low-protein (LP), respectively. The regular diet contains 15% protein while the low protein diet contained 6%. The diets are isocaloric (422 kcal/100 g of diet) and the salts and vitamins are also similar in both, as described in **Table 1**. Animals are submitted to experiments from 36th up to 41st day (Tropia et al., 2001; Oliveira et al., 2004; Loss et al., 2007; Penitente et al., 2007; Bezerra et al., 2011a,b; Martins et al., 2011a; Rodrigues et al., 2012; Rodrigues-Barbosa et al., 2012; Gomide et al., 2013; Silva et al., 2013).

In order to attest the efficiency of our dietary restriction protocol we measured: body weight, hematocrit, total blood protein, plasma albumin, urea, glucose, and food ingestion in both groups (Tropia et al., 2001; Oliveira et al., 2004; Penitente et al., 2007). The LP group presented lower values in all parameters above mentioned, except for hematocrit percentage, urea concentration and daily food ingestion, which were similar compared to NP group.

TABLE 1 | Composition (g/100 g of diet) of both control and low protein diets (Tropia et al., 2001; Bezerra et al., 2011b).

Components	NP group	LP group
Casein	15	6
Cornstarch	70	79
Soy oil	8	8
Mineral mixture	5	5
Vitamin mixture	1	1
Fiber (Cellulose)	1	1
Energy density, kcal	422	422

The Influence of Post-weaning Low-protein Diet on Resting MAP and HR and their Variabilities

Clinical and experimental studies have being showing, for over five decades, that low protein intake could lead to blood pressure increase, which is one of the most important cardiovascular risk factors (Handler and Bernheim, 1950; Viart, 1978; Sawaya et al., 2005; Martins et al., 2011b; Barros et al., 2015; de Brito Alves et al., 2015). Furthermore, data from our group indicated that cardiovascular reflexes are impaired by protein dietary restriction, see below (Tropia et al., 2001; Loss et al., 2007; Penitente et al., 2007; Bezerra et al., 2011a). Since cardiovascular reflexes are required to modulate MAP and HR (Morais et al., 2015), defective cardiovascular reflex sensitivity is correlated to abnormal variability of these parameters (Oliveira et al., 2004). Studies have also indicated that the risk to cardiovascular complications may depend on BP increase, as well as changes in MAP and HR variabilities (Shaffer et al., 2014; Parati et al., 2015).

MAP variability (MAPV) increase, on short-, mid- or long-term, could predict the development, progression and severity of cardiovascular injury and mortality (Parati et al., 2015). Likewise, HR variability (HRV), which reflects autonomic modulation of the heart (Task Force of European Society of Cardiology, 1996), has been used to identify autonomic changes in pathophysiological cardiovascular complications (e.g., hypertension and heart failure) (Souza et al., 2008; Shaffer et al., 2014). Therefore, in this section, we will discuss the influence of post-weaning low-protein diet on resting MAP and HR and their variabilities.

Low protein diet increases resting MAP and HR, accompanied by an increased variability of both parameters in rats (Oliveira et al., 2004). Among possible mechanisms involved, two stand out: elevated sympathetic activity and/or increased action of vasoactive substances (e.g., angiotensin), which will be better approached in following topics of this review. In this work, the application of advanced acquisition methodology

along with more precise analysis tools concurred to better highlight differences in resting HR and MAP, as expected in cases of elevated sympathetic and RAS activities (Shaffer et al., 2014; Parati et al., 2015). Higher MAPV and HRV observed in malnourished rats were predictable once the gain feedback control loops were also raised (Oliveira et al., 2004). Consequently, little MAP variations may result in HR over corrections due to enhanced cardiovascular reflexes gain feedback mechanisms (Tropia et al., 2001; Penitente et al., 2007; Bezerra et al., 2011a), which is in accordance with our data proposing an enhanced sympathetic tonus in rats submitted to protein deficient dietary (Tropia et al., 2001).

The Influence of Post-weaning Low-protein Diet on Cardiovascular Reflexes Sensitivity

Baroreflex

Baroreflex provides moment-to-moment negative feedback regulation of BP. Carotid sinus and aortic baroreceptors distension generates electrical signals, which are transmitted to the nucleus tractus solitary (NTS), where the first baroreflex synapse, probably using a glutamate as the neurotransmitter, occurs (Talman et al., 1980). Projections from NTS stimulate caudal ventrolateral medulla (CVLM) neurons, which in turn inhibit the rostral ventrolateral medulla (RVLM). Thereby, central sympathetic outflow is suppressed, since RVLM neurons send projections to preganglionic neurons in the intermediolateral column, which comprise the sympathoexcitatory output to the periphery (Schreihofer and Guyenet, 2003; Kumagai et al., 2012). Briefly, a prompt BP increase, which activates baroreflex, enhances the cardiovagal activity and reduces the cardiac and vascular sympathetic activity. This lessens the HR and corrects the BP to appropriate levels. On the order hand, a BP decrease deactivates baroreflex. Therefore, cardiovagal activity is suppressed while cardiac and vascular sympathetic activity is amplified, causing HR elevation and BP adjustment (Vasquez et al., 2012).

The baroreflex control is altered in rats fed a low-protein diet. Protein malnutrition increases the baroreflex activation latency and bradycardia gain evoked by phenylephrine (PHE), without changing the baroreflex deactivation latency and sodium nitroprusside-mediated tachycardia gain (Tropia et al., 2001; Loss et al., 2007).

Regarding the efferent autonomic activity influence on the baroreflex activation in LP rats, the latency was further enhanced and the bradycardic gain remained increased after methyl-atropine intravenous (i.v.) administration. However, after metoprolol i.v. injection, the latency was not affected, but the bradycardic gain decreased (Loss et al., 2007). These data suggest an increased sympathoinhibition and a decreased parasympathetic excitation during baroreflex activation in rats that had a low protein intake.

Considering the baroreflex deactivation in LP rats, the latency increased and the tachycardia gain decreased after methyl-atropine i.v. injection, suggesting an impairment in sympathetic activity modulation. In contrast, after metoprolol injection, these parameters were not affected (Loss et al., 2007). In accordance to another models of malnutrition studies (Herlihy et al., 1992; VanNess et al., 1997; de Belchior et al., 2012), these data indicate that low-protein diet disrupts cardiovascular regulation driven by baroreflex loops.

Chemoreflex

Chemoreflex, another important cardiovascular reflex, comprises peripheral, and central chemoreceptors, mainly located in carotid bodies and brainstem, respectively. The first one is activated mainly by hypoxia while the second one is stimulated fundamentally by hypercapnia (Nurse, 2010; Mansukhani et al., 2014). In this topic, we considered the carotid body chemoreceptor contribution on autonomic control. The carotid body afferents send projections to NTS neurons, which project to RVLM and nucleus ambiguous, respectively, controlling sympathetic and parasympathetic outflows (Schreihofer and Guyenet, 2003; Kumagai et al., 2012; Accorsi-Mendonça and Machado, 2013). Chemoreflex, whose activation stimulates breathing and sympatho/parasympathoexcitatory efferent pathways resulting in pressor and bradycardic responses (Barros et al., 2002), is involved in systemic hypertension onset, since its chronic activity could trigger a sustained MAP rise (Fletcher et al., 2002). In this sense, studies have been performed to address whether the protein malnutrition disrupts the cardiovascular autonomic control driven by chemoreflex pathway.

The carotid body artery ligature, which degenerates chemosensitive cells resulting in impairment of chemoreflex activation, further enhanced baseline MAP and HR in LP rats (Penitente et al., 2007). Previous report, in which carotid body artery ligature reduced baseline MAP in normotensive rats, pointed out the inhibitory effect of chemoreflex response on baroreflex (Franchini and Krieger, 1992). In fact, other results showed that peripheral chemoreflex activation attenuates baroreflex activation (Gu et al., 2007; Yamamoto et al., 2013). However, in our data, carotid body chemoreflex seems to exert stimulatory effect on baroreflex during protein malnutrition, suggesting that such nutritional condition reversed these mechanisms. Thus, specific changes in the central interplay of baroreflex and chemoreflex could justify our findings (Penitente et al., 2007). The reduced parasympathetic and enhanced sympathetic efferent modulation, repeatedly observed in malnourished rats (Benabe and Martinez-Maldonado, 1993; Plagemann et al., 2000; Tropia et al., 2001) also support such hypothesis.

This data is unique by pointing a BP increase as a consequence of chemoreflex response removal in post-weaning protein malnourished rats (Penitente et al., 2007). In this study, PHE-mediated baroreflex activation or baroreflex deactivation by sodium nitroprusside (SNP) i.v. injection produced the expected cardiovascular responses. So, MAP and HR detected changes in ligated-LP rats were not evoked by afferent baroreflex denervation, as a ligature surgery artifact (Penitente et al., 2007). Chemoreflex activation, by potassium cyanide (KCN) i.v. injections, elicits dose-related pressor and bradycardic responses, which are abolished by carotid body arteries ligature. All tested

KCN doses (5, 10, 15, 20, and 40 μg/kg) produced higher decrease in HR, while just the three smaller doses elicited greater pressor responses in malnourished rats, suggesting that low-protein diet increases basal efferent sympathetic tonus (Penitente et al., 2007). Such increased sympathetic activity mediated by malnutrition could be a consequence of following mechanisms: reduction in nitric oxide synthesis (Efron and Barbul, 1999), increases in angiotensin plasma and tissue levels, enhance in angiotensin II mRNA expression (Benabe and Martinez-Maldonado, 1993; Benabe et al., 1993a,b; Tonkiss et al., 1998), as well as enhances in chemoreflex activation and/or peripheral/central chemosensitive responses (Agarwal et al., 1981; de Brito Alves et al., 2015). Supporting the last hypothesis, the hypoxia-inducible factor expression, a transcriptional factor which relates to hypoxia-mediated tissue response and energy availabilities, was enhanced in rats submitted to another protein restriction model, suggesting a sensitization of the carotid peripheral chemoreceptors (de Brito Alves et al., 2015). Moreover, a recent work showed that carotid body chemoreflex activation-mediated pressor response remained elevated even in recovered protein restricted rats (Sá et al., 2014). In view that the chemoreflex pressor response depends on sympathetic activation (Vieira et al., 2012), they proposed that the sympathoexcitation arising from chemoreflex stimulation could remain enhanced in these rats (Sá et al., 2014).

The aforementioned pointed that protein malnutrition enhances cardiovascular responses to carotid chemoreflex activation in conscious rats. As this autonomic imbalance seems to alter the interplay between baroreflex and chemoreflex neuronal mechanisms, it could be considered a risk factor and could set deleterious effects on cardiovascular homeostasis (Penitente et al., 2007).

Bezold–Jarish Reflex

The autonomic control of the circulation also depends on the cardiopulmonary reflexes (Verberne and Guyenet, 1992). The Bezold–Jarisch reflex evoked by unmyelinated cardiopulmonary fibers (C fibers) activation, is characterized by hypotension, bradycardia, and apnea (Thorén, 1979). The C fibers arise from receptors located in the atria, ventricles, aorta and lungs, traveling through the vagus nerve up to the NTS (Kalia and Mesulam, 1980). In fact, besides the central integrative areas, BJR and baroreflex share afferent and efferent cardiovascular pathways, interplaying in a inhibitory manner (Verberne and Guyenet, 1992; Kashihara, 2009). In this sense, in the absence of arterial baroreceptors, the Bezold–Jarisch reflex plays an essential role in the reflex control of circulation, since its responsiveness is enhanced in this condition (Chianca et al., 1997). Considering that post-weaning protein malnutrition disrupts baroreflex and chemoreflex regulation, studies were performed to evaluate its influence on the Bezold-Jarisch reflex.

In a study from our laboratory, Bezold–Jarisch reflex activation, by serotonin injection, induced dose dependent hypotension and bradycardia in NP and LP rats. But, hypotension in LP was higher than in NP in the maximal dose used, whereas bradycardia was greater in all doses tested (Tropia et al., 2001). In a latter study Bezold–Jarisch reflex was activated by phenylbiguanide (5-HT3 serotonin receptor agonist) in NP and LP rats before and after baroreflex denervation (Bezerra et al., 2011a). In this study, protein restriction did not affect the Bezold-Jarisch reflex responses (hypotension and bradycardia). Nevertheless, after baroreflex denervation, such cardiovascular responses were attenuated in malnourished rats. It displayed a reduced BJR responsiveness in LP after baroreceptors removal (Bezerra et al., 2011a).

In conscious rats, hypotension evoked by Bezold–Jarisch reflex activation depends on bradycardia, indicating a plausible role of parasympathetic drive for Bezold–Jarisch reflex-mediated cardiovascular response (Chianca et al., 1997). The relationship between hypotension and bradycardia, evaluated by ΔMAP/ΔHR index, were increased only in animals fed a low protein diet submitted to baroreflex denervation, suggesting that Bezold–Jarisch reflex-evoked sympathetic and parasympathetic responses are, in some way, dissociated in malnourished rats without baroreflex (Bezerra et al., 2011a). In order to verify if that was a specific condition caused by protein malnutrition in baroreflex absence, Bezold–Jarisch reflex was evaluated along with muscarinic blockade in intact NP and LP rats. Our results showed similar pattern response to Bezold–Jarish reflex activation for both groups and proposed that a higher ΔMAP/ΔHR ratio observed in denervated malnourished rats was closely related to the absence of the afferent baroreceptor signals to CNS (Bezerra et al., 2011a). Since the inhibition of baroreflex medullary pathways declines Bezold–Jarisch reflex responsiveness associated with a parallel baroreflex blockade/attenuation (Verberne and Guyenet, 1992), we speculated that the protein malnutrition may result in Bezold–Jarisch reflex higher dependency on the baroreflex at CNS level, so that the absence of the last could lessen the efficacy of the first (Bezerra et al., 2011a). Although more investigations are required for better understanding of this phenomenon, these results strongly indicate that low protein diet changed the interrelation between Bezold–Jarisch reflex and baroreflex required for BP maintenance (Bezerra et al., 2011a).

The Influence of Post-weaning Low-protein Diet on Sympathetic Activity/Reactivity

The initial assessment of heart autonomic activity were preformed using the intrinsic HR and HRV methods (Task Force of European Society of Cardiology, 1996). The data pointed out that low protein diet increases the sympathetic and decreased the parasympathetic tone. Parasympathetic blockade, by a muscarinic receptor antagonist (methylatropine) i.v. injection, increased resting HR in animals fed a normal diet, but not in malnourished rats. This indicates that protein malnutrition could reduce the vagal modulation to HR (Martins et al., 2011a). Sympathetic blockade, by a selective β1-adrenoceptor antagonist (metoprolol) i.v. injection, reduces HR in animals fed a low protein diet but has no effect on animals under a normal diet. This data strongly suggests that a low-protein diet increases the sympathetic efferent activity to the heart

(Martins et al., 2011a). In fact, recent finding corroborates this data showing that protein malnutrition increased sympathetic activity in rats (Barros et al., 2015; de Brito Alves et al., 2015). The double injection of methylatropine and metoprolol reduces HR in malnourished rats, but again has no effect on animals control animals, indicating that malnourished rats have a low intrinsic HR (Martins et al., 2011a). Intrinsic HR can be modified by changes in the centrally mediated sympathetic and/or vagal flow. In addition, intrinsic HR has important role on resting HR providing an important compensatory mechanism to maintain HR in appropriated levels during an autonomic activity unbalance. In this sense, an increase in sympathetic activity along with a decrease in vagal activity leads to intrinsic HR reduction (Machado and Brody, 1989).

Moreover, the cardiac autonomic index, used to measure the sympathetic and parasympathetic activity balance (Goldberger, 1999), was <1 in rats fed a low protein diet, indicating sympathetic dominance in these rats. Interestingly, this index was higher in control than in malnourished rats, suggesting parasympathetic activity dominance in the former (Martins et al., 2011a). This study also demonstrated that the LF/HF ratio was increased in malnourished rats when compared to control rats, in accordance to a recent study, which performed HRV analysis in a different malnutrition model (Barros et al., 2015). These results also pointed to the greater sympathetic activity effect than parasympathetic activity on cardiac autonomic balance in protein malnutrition (Martins et al., 2011a).

Several mechanisms can reflect such autonomic unbalance, including alterations in the synthesis/release of neurotransmitters (Penido et al., 2012) and morphological damage in CNS circuitry recruited in the genesis and/or modulation of autonomic activity (Plagemann et al., 2000; Pinos et al., 2011). It is well established that chronic cardiac sympathetic activation raises the sudden death risk (Schultz and Li, 2007; Pokorný et al., 2011) and malnutrition could trigger cardiovascular disturbances (Sawaya et al., 2005), highlighting the fundamental importance of keeping investigate the autonomic balance in protein-deprived situation.

Among the strong evidence that malnutrition mediates sympathetic overdrive our group evaluated the impact of post-weaning protein malnutrition on the SNS activity. Blockade of α1-adrenoreceptor in malnourished rats caused greater depressor and tachycardia responses when compared to animals fed a regular diet, suggesting an increased vascular sympathetic tone (Tropia et al., 2001). Indeed, a recent work showed that another post-weaning protein malnutrition model, along with BP raise, induces vascular dysfunction, revealed by increases in superoxide anion, nitric oxide, and vascular reactivity of resistance arteries (de Belchior et al., 2012).

Faced with all findings previously discussed, our group considered necessary to assess the SNS responsiveness to malnutrition by a direct methodology. So, we measured renal sympathetic reactivity, directly, during Bezold-Jarisch reflex stimulation in rats submitted to post-weaning protein restriction (Bezerra et al., 2011b). The Bezold-Jarisch reflex activation besides producing hypotension, bradycardia and apnea, reduces renal sympathetic nerve activity in order to exert the homeostatic control of blood volume (Ditting et al., 2006). Although studies showed that Bezold–Jarisch reflex differentially regulates sympathetic drive to different regions, the Bezold–Jarisch reflex activation plays a specific control on renal nerve (Veelken et al., 1993).

Bolus i.v. injection of phenylbiguanide (5 μg/kg) evoked transient drops in renal sympathetic nerve activity of NP and LP rats. However, renal sympathetic reactivity was substantially diminished in malnourished rats (Bezerra et al., 2011b). In this context, considering the ability of renal nerves regulate blood volume and vascular resistance, renal sympathetic overdrive can contribute to the development and progression of cardiovascular disorders (Barrett, 2015). Since we observed similar renal sympathetic nerve activity- mediated MAP and HR drops in NP and LP rats (Bezerra et al., 2011a), changes in peripheral serotonin receptors expression and their effectiveness, as previous described (Chen et al., 1992), could be discarded. However, concerning that low-protein diet modified the cardiovascular reflexes responsiveness (Tropia et al., 2001; Loss et al., 2007; Penitente et al., 2007; Bezerra et al., 2011a,b) and their central pathway have some common medullary structures (Kashihara, 2009), we hypothesized that protein malnutrition may also impairs brain mediated autonomic control, which will be better discussed in the last topic of this review.

The Influence of Post-weaning Low-protein Diet on Renin-angiotesin System Activity

Circulating and local renin-angiotensin system (RAS) components are strictly associated with cardiovascular complications, especially in the development and progression of hypertension. Angiotensin II (Ang II), the main effector of this system, exerts its action on specific receptor isoforms, AT1 and AT2. When Ang II binds the AT1 receptor, it prompts vasoconstriction, cell proliferation, and hypertension (Santos and Ferreira, 2007; Mendoza and Lazartigues, 2015).

Studies have shown that low protein dietary enhances RAS activity, contributing to BP levels increase (Martinez-Maldonado et al., 1993; Benabe et al., 1993a; Goyal et al., 2010). Indeed, protein deprivation in early life increases mRNA expression to numerous RAS components in many tissues (Sangaleti et al., 2004; Goyal et al., 2009). As a result, an interaction between ANS and RAS activation might produce cardiovascular adaptations detected in adult rats submitted to post-weaning protein restriction.

A recent study from our group showed that an interaction between Ang II and the SNS contributes to the BP increase observed in rats fed a low-protein diet (Gomide et al., 2013). Although previous studies have emphasized the specific influence of RAS or SNS on cardiovascular regulation in experimental low-protein dietary condition (Martinez-Maldonado et al., 1993; Martins et al., 2011a), our results are the first to exhibit the interaction between increased RAS and SNS drive as accountable for the BP maintenance in protein malnutrition (Gomide et al., 2013).

Enalapril injection decreases the MAP in LP rats, but does not alter the basal MAP in NP rats. Moreover, such reduction reached lower MAP levels than that observed in NP group (Gomide et al., 2013). This indicates that RAS is important for the small MAP elevation also previously observed in malnourished animals (Oliveira et al., 2004; Loss et al., 2007; Penitente et al., 2007), as well as an essential regulatory mechanism, which could prevent a potential chronically hypotensive state in these animals (Gomide et al., 2013).

Since RAS seems to be involved in the BP regulation in protein-restricted rats, we also conducted experiments to evaluate the role of AT1 receptors on the their BP. Losartan injection also decreased the MAP in LP rats (Gomide et al., 2013), in accordance to previous reports in which losartan by gavage administration during 5 days reduced MAP in rats fed a low-protein diet (Benabe et al., 1993b). Our data revealed that Ang II, acting in AT1 receptors, is an essential factor for the BP maintenance in rats undergoing protein restriction. Interestingly, LP rats presented much lower levels of circulating Ang II than NP rats (Gomide et al., 2013). These data support early results demonstrating that protein restriction reduced both Ang II plasma concentration and plasma renin activity (Fernández-Repollet et al., 1987; Kapoor and Krishna, 1991).

Moreover, increasing doses of Ang II produced smaller MAP raises in LP than NP rats. Given that the basal RAS activity is enhanced in LP rats, it is plausible to suppose that the AT1 receptors are saturated in these animals and, therefore, the crescent Ang II doses administered produced less pronounced effects in LP than in NP group (Gomide et al., 2013). The relation between both poor responsiveness to Ang II and its circulating lower levels is an indicative that the RAS adaptation, pointed as one of the main regulatory system accountable for the high BP observed in rats fed a low-protein diet, is not due to an increase in the Ang II plasma levels, but due to a likely AT1 receptors overexpression in arteries and/or in the CNS (Gomide et al., 2013). In fact, we found increased AT1 receptors expression in the aorta of LP rats. It is important to note that, in the NP group, we did not observe any significant changes in the MAP or HR after enalapril or losartan injections alone (Gomide et al., 2013). These findings are in tune with previous data showing that, with an adequate content-protein diet, the RAS plays no major role in the moment-to-moment maintenance of BP and HR (Ceravolo et al., 2007).

In order to better understand the mechanisms underlying BP control in rats fed a low-protein diet, we evaluated the relative contribution and the possible interaction between RAS and SNS on the BP regulation in these animals. Under AT1 receptor blockade, prazosin infusion further reduced MAP in LP rats, suggesting that Ang II, acting on AT1 receptors, could activate SNS resulting in BP raise (Gomide et al., 2013). It is known that these two systems display positive feedback interplay in CNS and vasculature, in which raised activity of one of them increases the output of the other (Mancia et al., 2006). In addition to this data that shows an increased RAS activity in LP rats, previous data from our laboratory have indicated that these animals also present a higher vasomotor tone probably due to an increased sympathetic efferent activity (Tropia et al., 2001).

As expected, when prazosin was injected before losartan, MAP decreased in both groups. Prazosin-mediated MAP reduction in LP group was analogous to the losartan effect, also indicating the strong AT1 receptors contribution to the BP maintenance in protein restriction condition (Gomide et al., 2013). The following losartan injection further decreased MAP in LP rats. In NP group, prazosin injected alone also reduced MAP. However, such reduction was smaller than in LP rats, confirming that an increased sympathetic drive is required to sustain the raised BP levels after protein restriction. Moreover, both drugs injection, regardless of the order, reduced MAP in NP group, although these responses were smaller than in LP rats (Gomide et al., 2013). This information also converges to the understanding that protein-restricted rats need a higher AT1 receptor activity to maintain an appropriate sympathetic tone to the vascular bed.

To the point, our results displayed that in protein restriction condition, the α1-receptors activation is under strong influence of Ang II acting on AT1 receptors, demonstrating that Ang II is crucial to support the vascular tone driven by the SNS in this situation. RAS (specifically Ang II) and the SNS are both hyperactived, contributing in a complementary manner to maintain the BP levels in LP in order to preserve the cardiovascular system, and maintain sufficient blood supply to the systems. Therefore, the interplay between the RAS and the SNS appears to occur, in the arteries since AT1 receptors expression in the aorta is higher in LP rats (Gomide et al., 2013). However, more investigations are required to reveal if this interaction occurs mainly in the periphery, as suggested by the increased AT1 expression, or whether it is also a consequence of the CNS activation (e.g., by circumventricular organs), which probably increase the sympathetic drive.

The Influence of Post-weaning Low-protein Diet on Neural Plasticity

Neural plasticity, an adaptive process which changes the CNS structure and function during any ontogeny stage, is a result of internal/external environment interactions, or even of neural injuries (Phelps, 1990). According to literature, malnutrition, during critical development periods, reduces the quantity and span of dendritic processes, decreases the synapse/neuron relation (Nordborg, 1978; Díaz-Cintra et al., 1990; Morgane et al., 2002; Cordero et al., 2003; Penido et al., 2012), decreases the thickness myelin sheath and internodal segments (Reddy et al., 1979; Quirk et al., 1995; Cordero et al., 2003), impairs the release and activity of glutamate (Rotta et al., 2003; Penido et al., 2012) and produces morphophysiology alterations in brain regions which are involved in cardiovascular control, such as hypothalamus, hippocampus, frontal cortex, and amygdala (Plagemann et al., 2000; Zhang et al., 2009; Flores et al., 2011; Matos et al., 2011; Pinos et al., 2011). Such neural adaptations could change the electrical conduction system and modify the cardiac autonomic outflow, as was proposed by a recent study, in which cardiovascular responses induced by central injection of α-type scorpion toxin were attenuated in protein restricted rats (Silva et al., 2013).

These observations support our idea that cardiovascular impairment observed in post-weaning malnutrition experimental model might be related to CNS plasticity. In this review, we have already presented data that pointed to a cardiac autonomic dysfunction as a protein malnutrition consequence (Tropia et al., 2001; Oliveira et al., 2004; Loss et al., 2007; Penitente et al., 2007; Bezerra et al., 2011a,b; Martins et al., 2011a; Gomide et al., 2013). In this regard, the impairment of cardiovascular reflexes observed in experimental protein malnutrition, may be triggered by any central plasticity whose magnitude is able to interfere in cardiovascular homeostasis.

Would protein malnutrition be able to change the specific brain nuclei responsiveness to intermittent baroreflex stimulation? In order to investigate this hypothesis, we assessed the expression of neuronal activity marker c-fos protein (immediate-early gene expression) in the paraventricular hypothalamus (PVH); NTS; rostral ventromedial medullary areas (RVMM); raphe pallidus (RPa) and raphe obscurus (Rob); caudal ventrolateral medullary areas (CVLM) and RVLM (Rodrigues-Barbosa et al., 2012).

Baroreflex intermittent activation modifies c-fos expression in the PVH, RPa, medial NTS, and CVLM, independently on the dietary protocol offered to rats. However, in response to baroreflex stimulation, protein restricted dietary protocol influenced the neuronal recruitment pattern in raphe obscurus and in important medullary nuclei of cardiovascular control (rostral and caudal-commissural NTS, RVMM, and RVLM) (Rodrigues-Barbosa et al., 2012).

It is known that RVMM neurons conduct the sympathetic drive to heart and thermogenesis (Cao and Morrison, 2003; Salo et al., 2006; Morrison and Nakamura, 2011; Morrison et al., 2012). Additionally, RVMM neurons activation mediates marked tachycardia (Cao and Morrison, 2003, 2006; Morrison, 2003). Phenylephrine infusion induced neuronal activation within the RVMM of rats fed a low protein diet, but not in the control group, denoting that protein restriction is able to change neuronal recruitment, increasing the resting HR to maintain the cardiac output homeostasis. We also showed a lessened RVLM neuronal activation in LP PHE-infused rats (Rodrigues et al., 2012). The sympathoinhibition triggered by baroreflex activation, essential to preserve the cardiac functionally, results from RVLM neuronal inhibition by CVLM GABAergic input (Guyenet, 2006). Although the CVLM c-fos expression has been similar in NP and LP PHE-infused rats, suggesting comparable recruitment of these nuclei, we did not perform any neurotransmission assay in this region. Therefore, in malnutrition condition, the CVLM GABAergic neurotransmission to RVLM could be impaired and/or RVLM neurons could answer in a different manner to CVLM inhibitory inputs (Rodrigues-Barbosa et al., 2012).

PHE-activated baroreflex also showed differential neural recruitment in NTS of NP and LP rats. In medial NTS, Phe infusions similarly enhanced c-fos expression in both groups, while in rostral and caudal-commissural NTS this expression were higher in LP than NP rats (Rodrigues-Barbosa et al., 2012). These observations support the idea that protein restricted diet could promote differential neural setting in the NTS, a nucleus recognized for receiving and processing afferent cardiovascular information (Guyenet, 2006; Accorsi-Mendonça and Machado, 2013).

Facing to these findings, new assessment of neurochemical plasticity in medullary neurons could become a powerful strategy to deeply understand the autonomic and cardiovascular effects evoked by protein malnutrition.

Based on scarcity of studies about the malnutrition effect on CNS nuclei of cardiovascular control and assuming that: (i) RVLM is an important area for the generation of sympathetic efferent drive, especially to vasomotor tone (Guyenet, 2006; Fontes et al., 2011) and (ii) L-glutamate is the principal excitatory neurotransmitter in this nucleus (Machado et al., 1997), our group also investigated the impact of protein restriction on L-glutamate-mediated pressor response into RVLM (Rodrigues et al., 2012).

Crescent doses of L-glutamate injection into the RVLM evoked smaller pressor responses in LP than NP rats (Rodrigues et al., 2012). Allied to this, protein malnutrition lessened and shifted upward the baroreflex curve, once again indicating the malfunctioning of this reflex in LP rats (Rodrigues et al., 2012). The range of baroreflex gain, which was higher in LP than in NP rats, was in accordance to maximum baroreflex gain. While this occurred at normal levels in NP rats, in LP rats the peak occurred at higher values. In addition, despite of the higher resting HR, the baroreflex activation, by pressor response to glutamate injection in RVLM, produced smaller HR changes in LP when compared to NP rats (Rodrigues et al., 2012). This is another indication that protein malnourished rats present sympathetic efferent overdrive.

All aforementioned observations could be partially explained by damage in glutamate release and/or receptor affinity mediated by protein malnutrition. This would be in concordance to prior studies in the literature which reported that malnutrition, during the first life stages, changes the neurotransmitter concentration in CNS, the neurotransmitter/receptor affinity, the neuronal population, and/or CNS nucleus morphology (Plagemann et al., 2000; Zippel et al., 2003). Although we cannot assure any morphological plasticity, which would characterize and emphasize the changes in the structure of synapses and neurons, our findings point to glutamate neurochemical plasticity. Therefore, post-weaning malnutrition indeed impacts the central mechanisms related to cardiovascular control, particularly considering the glutamate neurotransmission in RVLM—a crucial brainstem nucleus accountable for modulating the sympathetic drive to the cardiovascular system (Rodrigues et al., 2012).

Conclusions

This review aimed at pointing out the protein malnutrition impact on cardiovascular homeostasis, since it: (i) impairs the cardiovascular reflexes sensitivity, (ii) increases resting MAP and HR and their variabilities, (iii) enhances the sympathetic and diminishes the parasympathetic efferent activities to the heart, (iv) raises the vasomotor sympathetic tonus, (v) reduces the renal sympathoinhibition to BJR activation, (vi) increases the RAS activity, and (vii) changes

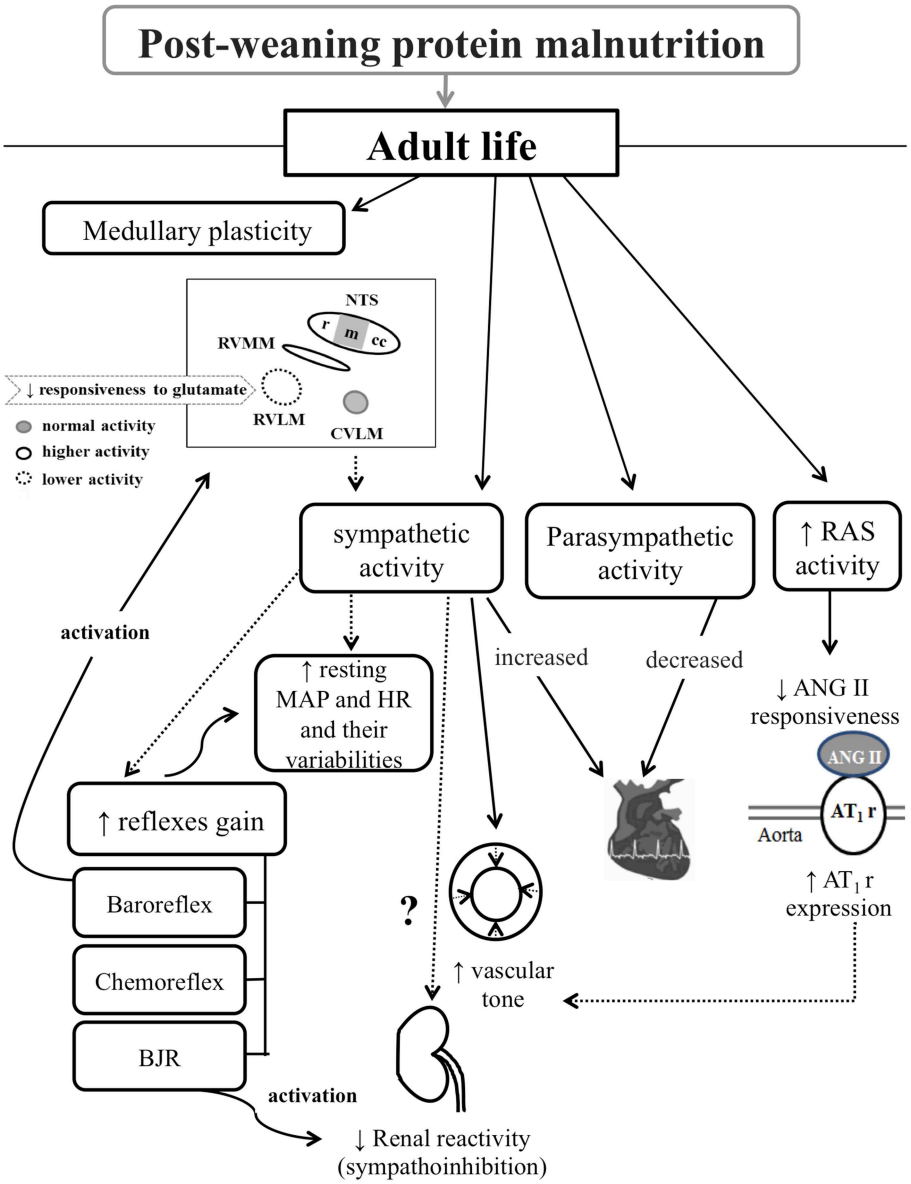

FIGURE 1 | Diagram illustrating the post-weaning protein malnutrition effects on cardiovascular control systems in adult rats. Rectangles and black arrows correspond to data observed in this malnutrition model. Dashed arrows correspond to likely interaction between observed data. From left to right, protein restriction promotes: medullary plasticity, vasomotor and cardiac sympathetic overactivity, decreased cardiac parasympathetic activity, and RAS hyperactivity. The neuroplasticity is characterized by changes in medullary recruitment in response to baroreflex stimulation, and decreased glutamate responsiveness in the RVLM. Such medullary plasticity probably contributes to the sympathetic overactivity. This, in turn, relates to the impaired cardiovascular reflexes sensitivity, increased resting MAP and HR and their variabilities, and plausible shift in baseline renal sympathetic activity in view of the reduced renal sympathoinhibition to BJR activation. The RAS hyperactivity associates with the low ANG II responsiveness and high aortic AT_1r expression, which probably contribute to increased vascular tone. RAS, renin-angiotensin system; MAP, mean arterial pressure; HR, heart rate; BJR, Bezold–Jarish reflex; ANG II, angiotensin II; AT_1r, angiontensin II receptor type 1; NTS, nucleus tractus solitary; rNTS, rostral NTS; mNTS, medial NTS; ccNTS, caudal-commissural NTS; RVMM, rostral ventromedial medullary areas; RVLM, rostral ventrolateral medullary area; and CVLM, caudal ventrolateral medullary area.

the medullary recruitment and glutamate neuromodulation in response to baroreflex stimulation, as outlined in **Figure 1**.

Thus, the present review provides new perspectives on the pathophysiology of metabolic and cardiovascular diseases associated with protein malnutrition.

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MINI REVIEW
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Reactive Oxygen Species in the Paraventricular Nucleus of the Hypothalamus Alter Sympathetic Activity During Metabolic Syndrome

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The paraventricular nucleus of the hypothalamus (PVN) contains heterogeneous populations of neurons involved in autonomic and neuroendocrine regulation. The PVN plays an important role in the sympathoexcitatory response to increasing circulating levels of angiotensin II (Ang-II), which activates AT1 receptors in the circumventricular organs (OCVs), mainly in the subfornical organ (SFO). Circulating Ang-II induces a *de novo* synthesis of Ang-II in SFO neurons projecting to pre-autonomic PVN neurons. Activation of AT1 receptors induces intracellular increases in reactive oxygen species (ROS), leading to increases in sympathetic nerve activity (SNA). Chronic sympathetic nerve activation promotes a series of metabolic disorders that characterizes the metabolic syndrome (MetS): dyslipidemia, hyperinsulinemia, glucose intolerance, hyperleptinemia and elevated plasma hormone levels, such as noradrenaline, glucocorticoids, leptin, insulin, and Ang-II. This review will discuss the contribution of our laboratory and others regarding the sympathoexcitation caused by peripheral Ang-II-induced reactive oxygen species along the subfornical organ and paraventricular nucleus of the hypothalamus. We hypothesize that this mechanism could be involved in metabolic disorders underlying MetS.

Keywords: paraventricular nucleus of the hypothalamus, angiotensin II, oxidative stress, metabolic syndrome

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INTRODUCTION

The sympathoexcitation is linked to various diseases; circulating levels of Ang-II modulate SFO angiotensinergic projection to pre-autonomic PVN neurons, which lead to increases in sympathoexcitatory activity to the spinal cord via direct projections (Koshiya and Guyenet, 1996; Badoer, 2001; Stocker et al., 2004) or indirectly projecting to pre-sympathetic neurons in the RVLM (Koshiya and Guyenet, 1996; Badoer, 2001; Ito et al., 2002; Stocker et al., 2004). Increasing evidence supports the premise that Ang-II in the PVN is involved in pathological conditions originating from sympathoexcitation, such as hypertension, heart failure, diabetes, obesity, and metabolic syndrome (Gutkind et al., 1988; Ito et al., 2002; Oliveira-Sales et al., 2009; Braga et al., 2011). It has also been demonstrated that Ang-II increase reactive oxygen species (ROS) along the subfornical organ–paraventricular nucleus of the hypothalamus–rostral ventrolateral medulla axis [SFO-PVN-RVLM axis (Oliveira-Sales et al., 2008; Braga et al., 2011; Burmeister et al., 2011)].

The main ROS within the central nervous system is superoxide anions (O2−). Within the PVN, superoxide accumulation within the PVN ultimately results in sympathetic overactivity (Oliveira-Sales et al., 2009; Peterson et al., 2009; Burmeister et al., 2011; Cardinale et al., 2012; Campos et al., 2015). The aim of this mini-review is to discuss the role of sympathoexcitation induced by Ang-II-dependent ROS production in the pre-autonomic PVN neurons in modulating the development and/or metabolic disorders that results in the development and/or the maintenance of metabolic syndrome.

Anatomical and Functional Organization of PVN

The paraventricular nucleus of the hypothalamus is anatomically connected to other hypothalamic areas and to the brainstem, playing a pivotal role in several homeostatic responses, being an important integrative nucleus for autonomic and neuroendocrine functions (Swanson and Sawchenko, 1980; Stern, 2001; Cruz et al., 2008; Cruz and Machado, 2009; Reis et al., 2010). Among the PVN functions are regulation of food intake, adipose afferent reflex (AAR), responses to stress, modulation of metabolic rate, thermoregulation, modulation of sympathetic nerve activity, and cardiovascular function (Swanson and Sawchenko, 1980; Stern, 2001; Benarroch, 2005; Cruz et al., 2008; Cruz and Machado, 2009; Reis et al., 2010; Cassaglia et al., 2011; Zsombok et al., 2011; Zhang et al., 2012; Ding et al., 2013; Xiong et al., 2014). The PVN is comprised of magnocellular and parvocellular subnuclei, which have different properties both neurochemically and electrophysiologically (Swanson and Sawchenko, 1980; Stern, 2001). The magnocellular subnucleus projects to the posterior hypophysis and parvocellular subnucleus, which include pre-autonomic neurons, send descending projections to cardiovascular autonomic brainstem nuclei as well as direct projections to the spinal cord (Koshiya and Guyenet, 1996; Badoer, 2001; Stocker et al., 2004; Cruz et al., 2008). Therefore, electrophysiological and functional studies support an essential role for the PVN in central blood pressure control (Cruz and Machado, 2009; Cruz et al., 2010; Busnardo et al., 2013) and sympathetic nerve activity (Koshiya and Guyenet, 1996; Badoer, 2001; Stocker et al., 2004). Our previous studies suggest that parvocellular pre-autonomic neurons modulate baseline blood pressure through activation of glutamatergic, GABAergic, purinergic, nitrergic, and angiotensinergic mechanisms (Chen et al., 2003; Cruz and Machado, 2009; Cruz et al., 2010; Busnardo et al., 2013). Accumulating evidence support the idea that imbalance between paraventricular inhibitory GABAergic and excitatory glutamatergic and/or angiotensinergic neurotransmission in the PVN, contribute to increase the pre-autonomic neuronal drive mediating neurogenic hypertension (Gören et al., 2000; Chen et al., 2003; Li and Pan, 2005; Li et al., 2006; Oliveira-Sales et al., 2009). Magnocellular and parvocellular neurons from PVN express receptors to a wide range of neurotransmitters and neurohormones including leptin, insulin, neuropeptide Y, Ang-II, GABA, glutamate, vasopressin, oxytocin, and noradrenaline (Stanley and Leibowitz, 1984; Saphier and Feldman, 1991; Lenkei et al., 1997; Håkansson and Meister, 1998; Zsombok et al., 2011). Therefore, it is suggested that an imbalance in synaptic function that modulates the pre-autonomic or neurosecretory neuron results in cardiovascular and neuroendocrine dysfunctions that, in turn, contribute to the development and potentiation of sympathoexcitatory response observed in hypertension, heart failure, atherosclerosis, diabetes, and obesity.

SFO-PVN-RVLM Pathway for Circulating Ang-II is Involved in the Cardiovascular Regulation

Some circulating lipophobic substances, incapable of crossing the blood brain barrier (BBB), such as glucose, insulin, leptin, noradrenaline, and angiotensin II have their receptors expressed in neurons of the circunventricular organs (CVOs), which have an incomplete BBB (Lenkei et al., 1997; Boundy and Cincotta, 2000; Braga et al., 2011; Cassaglia et al., 2011; Lob et al., 2013; Prior et al., 2014). One of the major CVOs receiving information from the peripheral circulation is the subfornical organ (SFO). Anatomical and functional evidence suggest that SFO is a pivotal nucleus in modulating pressor and dipsogenic actions of circulating Ang II (Bains et al., 1992; Li and Ferguson, 1993; Sakai et al., 2007; Braga et al., 2011). Genetic and physiological evidence shows that circulating Ang-II is involved in *de novo* synthesis of Ang II within the SFO, which is an integrative mechanism of fluid and cardiovascular homeostasis (Bains et al., 1992; Li and Ferguson, 1993; Sakai et al., 2007; Burmeister et al., 2011). Angiotensin II AT1 and AT2 receptors (AT1R; AT2R) are expressed in neurons and astrocytes of the PVN (Lenkei et al., 1997; Coleman et al., 2009; Oliveira-Sales et al., 2009) and angiotensinergic connections between the SFO and PVN is describe to control drinking and sympathetic nerve activity (Gutkind et al., 1988; Bains et al., 1992; Li and Ferguson, 1993; Anderson et al., 2001; Sakai et al., 2007; Burmeister et al., 2011). In that regard, several studies show that angiotensinergic connections between SFO and PVN are involved in the generation and maintenance of elevated baseline blood pressure in hypertensive rats (Gutkind et al., 1988; Burmeister et al., 2011). For example, studies by Miyakubo et al. (2002) demonstrated that excitatory response elicited by Ang-II in SFO neurons projecting to PVN was higher in spontaneous hypertensive rats (SHR) than in normotensive Wistar Kyoto rats (WKY). The brain Ang-II neurocircuitary also involves pre-autonomic PVN neurons projecting to rostral ventrolateral medulla (RVLM). The RVLM which tonically controls sympathetic vasomotor activity (Guyenet et al., 1989). Studies by Ito et al. (2002) indicate that RVLM vasomotor neurons in SHR, but not in the WKY rats, are tonically excited by PVN driven angiotensin II projections. Furthermore, several studies support the idea that Ang-II along the SFO-PVN-RVLM axis is a significant neuronal pathway involved in the maintenance of neurogenic hypertension (Ito et al., 2002; Oliveira-Sales et al., 2009; Braga et al., 2011).

Ang-II Induced ROS Accumulation Along the SFO-PVN-RVLM Axis Contributing to the Pathogenesis of Hypertension

Accumulating evidence support the idea, that Ang-II-induced oxidative stress within the PVN contributes to the pathogenesis of hypertension. In addition to increasing blood pressure and sympathetic nerve activity, central infusion of Ang-II leads to elevated levels of neurotransmitters (glutamate and norepinephrine), AT1R, pro-inflammatory cytokines, phosphorylated IKKbeta, NF-kappaB subunits, and superoxide in the central nervous system (Erdös et al., 2006; Oliveira-Sales et al., 2009; Peterson et al., 2009; Burmeister et al., 2011).

There is now growing evidence suggesting that inflammation and central Ang-II-induced ROS production are involved in the pathogenesis of neurogenic hypertension. For example, in Ang-II-treated rats, bilateral microinjection of NFkappaB blocker into the PVN induces a local decrease in NFkappaB p65 subunit activity, proinflammatory cytokines, ROS, AT1-R, as well as in blood pressure (Cardinale et al., 2012).

Ang-II acting on the AT1R induces activation of NADPH oxidase through protein kinase C (PKC). The NADPH oxidase is the major source of superoxide anion. This enzyme is composed of catalytic membrane ($gp91^{phox}$ and $p22^{phox}$) and cytoplasmic ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) subunits, which transfer electrons to molecular oxygen, producing reactive oxygen species as superoxide (Chabrashvili et al., 2002; Lassègue and Clempus, 2003).

Oxidative stress is characterized by an imbalance between the production of ROS and antioxidant systems (Betteridge, 2000). Numerous studies support the concept that ROS production is increased in different nuclei in the brainstem and hypothalamus (Oliveira-Sales et al., 2009; Peterson et al., 2009; Braga et al., 2011; Burmeister et al., 2011; Campos et al., 2015). The role of oxidative stress in the development and/or maintenance of neurogenic hypertension has recently been reported in several animal models of hypertension, including the renovascular two-kidney–one-clip model [2K1C (Oliveira-Sales et al., 2008, 2009; Burmeister et al., 2011; de Queiroz et al., 2013)]. Studies by Lob et al. (2013) showed an increase in the superoxide production in the SFO after chronic angiotensin II infusion, which was blunted by SFO-targeted injections of an adenovirus encoding cre-recombinase for reducing of p22 (phox), Nox2, and Nox4 mRNA expression. In addition, studies by Yuan et al. (2013) showed that superoxide dismutase 1 (SOD1), an antioxidant enzyme, was overexpressed in the PVN, attenuating augmented sympathetic activity, and cardiac sympathetic afferent reflex, while improving the myocardial and vascular remodeling in spontaneous hypertensive rats (SHR). The expression of the isoforms Nox1, Nox2, and predominant Nox4 mRNA were found in the PVN under basal conditions. Furthermore, Nox4-generated superoxide within the PVN contributes to the sympathoexcitation and cardiac dysfunction observed in mice that experienced heart failure (Infanger et al., 2010).

It has been suggested that upregulation of ROS in the RVLM and PVN contributes to increased blood pressure and SNA in renovascular hypertensive rats, with ROS preceding the increase in blood pressure in Ang-II-dependent model of hypertension (Kitiyakara and Wilcox, 1998; Botelho-Ono et al., 2011; Burmeister et al., 2011; de Queiroz et al., 2013) mRNA expression studies revealed that AT-1 and NADPH oxidase subunits were greater in the RVLM and PVN in renovascular hypertensive rats (Oliveira-Sales et al., 2009; Campos et al., 2015). In addition, studies by Burmeister et al. (2011) documented that a significant increase in the superoxide production in the PVN of renovascular hypertensive mice leads to activator protein-1 (AP-1) activation, a nuclear transcription factor, resulting in hypertension, while inhibition of AP-1 activity in the prevented renovascular hypertension. Furthermore, microinjection of superoxide dismutase mimetic, 4 hydroxy-2, 2, 6, 6-tetramethyl piperidinoxyl (Tempol) into the RVLM and PVN decreased the mean arterial pressure and renal sympathetic nerve activity in renovascular hypertensive rats, supporting the idea that upregulation of ROS in central cardiovascular areas, such as RVLM and PVN, contributes to elevated arterial pressure and sympathetic activity (Oliveira-Sales et al., 2009). Interestingly, microinjection of an adenovirus (Ad) encoding superoxide dismutase (AdCuZnSOD) in the PVN not only decreased the local superoxide accumulation into the PVN, but also prevented hypertension. Together, these observations led to the proposal (Braga et al., 2011) that the formation of Ang-II-induced ROS along the SFO-PVN-RVLM axis is an important mechanism involved in the pathogenesis of neurogenic hypertension.

Ang-II, Obesity and Diabetes Cross-Talk in the PVN

Diet and lifestyle associated to genetic factors are involved in the development of metabolic syndrome (MetS). Metabolic changes such as dyslipidemia, glucose intolerance, hyperinsulinemia, hyperleptinemia, systemic inflammation, and chronic increase in the SNA, which characterize MetS, also augment the risk of developing diseases such as obesity, diabetes, atherosclerosis, and arterial hypertension. The PVN, as described above, is a key central nucleus participating in the regulation of cardiovascular and sympathetic activity (Koshiya and Guyenet, 1996; Badoer, 2001; Stocker et al., 2004; Cruz et al., 2008). It is involved in the sympathetic overactivity in rats with hypertension (Gören et al., 2000; Chen et al., 2003; Li et al., 2006; Oliveira-Sales et al., 2009), obesity (Xiong et al., 2012; Ding et al., 2013), and insulin resistance [commonly seen in the form of diabetes (Cassaglia et al., 2011; Zhang et al., 2012)]. In addition, several reports suggest that ROS activation contributes to insulin resistance observed in diabetic rats accompanied by obesity and hypertension (Folli et al., 1997; Xiong et al., 2012; Zhang et al., 2012; Cruz et al., 2013; Ding et al., 2013; de Kloet et al., 2013)

It is known that activation of the renin-angiotensin system may lead to insulin resistance in the vasculature (Folli et al., 1997); Ang-II impairs insulin receptor intracellular signaling, inhibiting insulin receptor substrate-1 (IRS-1) phosphorylation and phosphatidylinositol (PI) 3–kinase activation (Folli et al.,

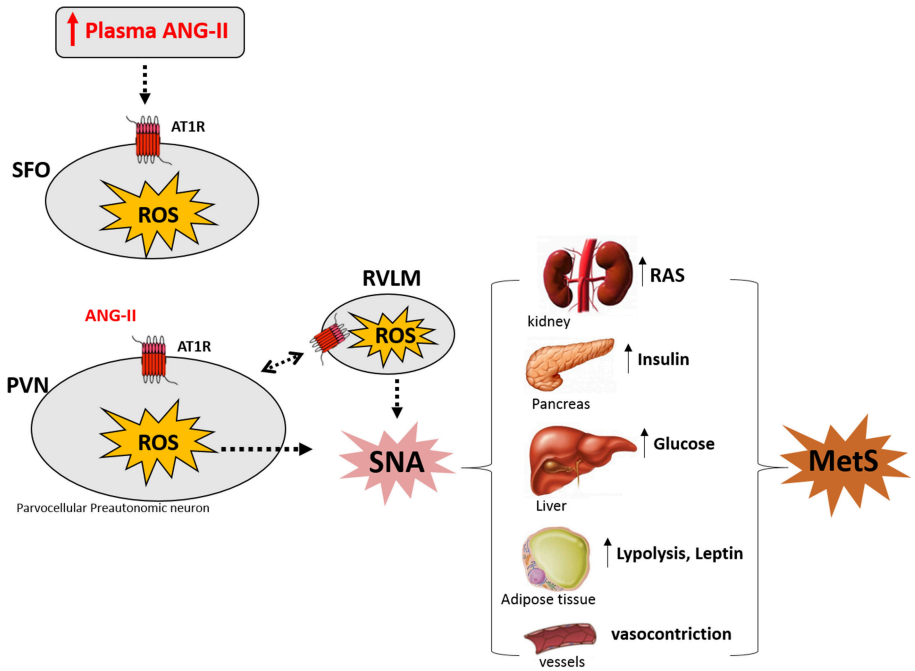

FIGURE 1 | Angiotensin II (Ang-II) induces sympathetic nerve activation (SNA) by increasing reactive oxygen species (ROS) along SFO-PVN axis underlying Metabolic Syndrome (MetS) symptoms (see text). Modified by de Queiroz et al. (2013) and Benarroch (2005). SFO: subfonical organ; PVN, paraventricular nucleus of the hypothalamus; RVLM, rostral ventrolateral medulla; AT1R, AT1 Ang-II receptor; RAS, renin angiotensin system.

1997; Cizmeci and Arkun, 2013). In addition, it has been documented that AT1R receptor expression is increased in the PVN of rats with diabetes and insulin resistance (Zhang et al., 2012; Sun et al., 2014). Furthermore, Ang-II activates NADPH oxidase via AT1 receptors, increasing superoxide anion accumulation in the PVN, thereby contributing to enhanced sympathetic activity in diabetic and insulin resistance rats (Patel et al., 2011; Zhang et al., 2012; Sun et al., 2014).

Sympathetically-mediated interactions between PVN and white adipose tissue via AAR are important for the maintenance of total body fat and energy balance (Xiong et al., 2012; Ding et al., 2013). AAR is increased in obese hypertensive rats (Xiong et al., 2012, 2014; Ding et al., 2013) and inhibition of PVN decreases SNA and mean arterial pressure, while abolishing AAR in hypertensive obese rats (Xiong et al., 2012). Furthermore, studies by Ding et al. (2013) showed that NADPH oxidase-derived superoxide anions in the PVN modulates AAR, while PVN microinjection of tempol decreases baseline renal SNA, blood pressure, and attenuated the AAR. Thus, Ang-II induces ROS in the PVN may be a significant central mechanism modulating AAR. Studies by de Kloet et al. (2013) observed that deletion of AT1 receptors in the PVN not only reduced the local expression of corticotrophin-releasing hormone (CRH), oxytocin, and tumor necrosis factor α (TNF-α, a pro-inflammatory cytokine), but also decreased systolic blood pressure in mice rendered obese by high fat diet. This suggests that AT1 receptors in the PVN regulates the central metabolic changes that promotes metabolic and cardiovascular disorders.

CONCLUSION

In the last years, our laboratory has been investigating the mechanisms underlying neurogenic hypertension and our results strongly suggest that this pathological condition is caused by Ang-II-dependent ROS accumulation along the SFO-PVN-RVLM axis (Peterson et al., 2009; Botelho-Ono et al., 2011; Braga et al., 2011; Burmeister et al., 2011; de Queiroz et al., 2013). Accumulating evidence suggest that hyperactivity of the angiotensin system within the PVN is involved not only in hypertension, but also in diabetes and obesity existing as comorbidities (Oliveira-Sales et al., 2008, 2009; de Kloet et al., 2010, 2013; Braga et al., 2011; Xiong et al., 2012, 2014; Cizmeci and Arkun, 2013; Ding et al., 2013). This mini-review supports the hypothesis illustrated in the **Figure 1** that the increase in the circulating levels of Ang-II activates angiotensinergic neurons in the SFO, which projects to pre-autonomic neurons expressing AT1 receptors in the PVN. The stimulation of AT1 receptors in the PVN and RVLM induces intracellular signals activating NADPH oxidase through protein kinase C. NADPH oxidase activity increases ROS formation, contributing to overactivity of pre-autonomic PVN neurons, resulting in sympathoexcitation through an indirect pathway (angiotensinergic projections to RVLM) and/or directly projections to the spinal cord, thereby mediating increase in plasma renin-angiotensin system, insulin,

glucose, leptin, lipolysis as well as vasoconstriction. All these metabolic changes are involved in the symptoms of MetS.

AUTHOR CONTRIBUTIONS

All authors participated in the design of the manuscript, drafted the manuscript, revised the manuscript critically and approved the final version.

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Neuron-Glia Crosstalk in the Autonomic Nervous System and Its Possible Role in the Progression of Metabolic Syndrome: A New Hypothesis

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Metabolic syndrome (MS) is characterized by the following physiological alterations: increase in abdominal fat, insulin resistance, high concentration of triglycerides, low levels of HDL, high blood pressure, and a generalized inflammatory state. One of the pathophysiological hallmarks of this syndrome is the presence of neurohumoral activation, which involve autonomic imbalance associated to hyperactivation of the sympathetic nervous system. Indeed, enhanced sympathetic drive has been linked to the development of endothelial dysfunction, hypertension, stroke, myocardial infarct, and obstructive sleep apnea. Glial cells, the most abundant cells in the central nervous system, control synaptic transmission, and regulate neuronal function by releasing bioactive molecules called gliotransmitters. Recently, a new family of plasma membrane channels called hemichannels has been described to allow the release of gliotransmitters and modulate neuronal firing rate. Moreover, a growing amount of evidence indicates that uncontrolled hemichannel opening could impair glial cell functions, affecting synaptic transmission and neuronal survival. Given that glial cell functions are disturbed in various metabolic diseases, we hypothesize that progression of MS may relies on hemichannel-dependent impairment of glial-to-neuron communication by a mechanism related to dysfunction of inflammatory response and mitochondrial metabolism of glial cells. In this manuscript, we discuss how glial cells may contribute to the enhanced sympathetic drive observed in MS, and shed light about the possible role of hemichannels in this process.

Keywords: glia, connexins, metabolic syndrome, mitochondria, tripartite synapse, hemichannels

METABOLIC SYNDROME AND AUTONOMIC NERVOUS SYSTEM IMBALANCE

The metabolic syndrome (MS) is a clinical disorder characterized by the common co-occurrence of several physiological alterations, including increased abdominal fat, elevated fasting glucose, high concentration of triglycerides, low levels of HDL and high blood pressure. People suffering MS are

more likely to later developing diabetes mellitus and coronary heart disease, consequently, their life expectancy is reduced (Eckel et al., 2005; Grundy, 2008). This disorder has become a growing health problem that affects millions of people worldwide. Indeed, in United States nearly 50% of people with 60 years or more were estimated to have the metabolic syndrome in 2011–2012 (Aguilar et al., 2015). Up to now, most efforts to understand MS have been focused on the study of peripheral organ malfunction, however, the role of the nervous system on the alterations observed in MS remains unclear.

In the last decade, different groups have proposed that autonomic nervous system (ANS) imbalance may be the hidden factor underlying the progression of different metabolic diseases, including MS (Thayer et al., 2010; Licht et al., 2013; Wulsin et al., 2015). One of the main physiological challenges in the daily life is to keep and maintain the body homeostasis. The ANS conveys sensory afferent information from several territories (e.g., blood vessels, heart, and kidneys) toward nuclei within the central nervous system (CNS), including the medulla and hypothalamus. The sensory inputs are integrated by specific neuronal networks that orchestrate highly coordinated responses to promote adaptive cardiovascular, respiratory, fluid, and energy balance. These functions are complex and requires fine adjustments between the two major branches of the ANS; the sympathetic nervous system (SNS); associated with energy mobilization; and the parasympathetic nervous system (PNS); linked with vegetative and restorative functions. Under physiological conditions, the activities of these branches are in balance. However, when one branch dominates over the other some diseases emerge.

ANS imbalance typically relies on hyperactivation of the SNS and low activity of the PNS, resulting in insulin resistance, altered lipid metabolism, increased blood pressure and endothelial dysfunction (Palatini et al., 2006; Tentolouris et al., 2006; Straznicky et al., 2012; Zucker et al., 2012; Paton et al., 2013; Stern and Filosa, 2013). Indeed, hyperactivation of pre-sympathetic neurons located at the CNS has been pointed out as a key step in the sympatho-excitation observed in MS and further heart failure and diabetes (Li et al., 2008; Zucker et al., 2012; Del Rio et al., 2013; Khoo et al., 2013; Guimaraes et al., 2014; Tremarin et al., 2014; Del Rio, 2015; Moreira et al., 2015; Schlaich et al., 2015). Importantly, evidence from a large cross-sectional study revealed that high sympathetic activity and/or low parasympathetic activity were associated to higher blood pressures, serum triglycerides, serum glucose, and waist circumference (Licht et al., 2010). Neurons that control basal sympathetic activity are located in diverse brain areas, including the paraventricular nucleus of the hypothalamus (PVH), the rostral ventrolateral medulla (RVLM), the spinal cord and the nucleus of the solitary tract (NTS). Among these nuclei, the PVH contains the pre-autonomic neurons that project to the RVLM and spinal cord. At the RVLM, two well-known populations of neurons project toward the spinal cord and other areas, contributing to autonomic regulation (Swanson and Sawchenko, 1983). One population encompassing about of 50–70% of projecting RVLM neurons (C1 group) are glutamatergic but they also synthesize diverse catecholamines, including adrenaline (Guyenet, 2006). Interestingly, non-spinal C1 neurons from the RVLM can innervate the hypothalamus, modulating the excitatory drive to the PVH during baroreceptor activation, a key step in the neural control of circulation (Verberne et al., 1999). Despite the current progress in the field, most of efforts to understand the hyperactivation of SNS during MS have been focused in neurons. Here, we discuss and hypothesize how glial cells and their interaction with neurons at the nuclei that control sympathetic activity could be involved in the pathogenesis and progression of MS.

GENERAL FUNCTIONS OF GLIAL CELLS

In the last two decades, glial cells have emerged as critical players in processing of highly complex information in the CNS. This is particularly true for astrocytes, which create a far-reaching syncytial network that anatomically and functionally communicate neuronal synapses with brain blood vessels (Volterra and Meldolesi, 2005). Through their processes, each astrocyte contact multiple chemical synapses (Oberheim et al., 2006). Thus, astrocytes together with pre- and postsynaptic neuronal structures constitute the "tripartite synapse" (Araque et al., 1999). Embedded in this structure, astrocytes sense neuronal function and respond locally by releasing bioactive molecules termed "gliotransmitters" (e.g., glutamate, ATP, and D-serine) (Perea et al., 2009). In addition, astrocytes also can project specialized terminal processes known as "endfeet" toward capillaries, intracerebral arterioles, and venules; covering about of 99% of abluminal vascular surface (Simard et al., 2003). This complex interaction with neurons and vascular cells facilitate local and long distance astrocytic release of gliotransmitters and vasoactive factors, thereby modulating different neuronal circuits, and networks.

Astrocytes play a crucial role in both gliotransmitter and ionic homeostasis. During high rates of neuronal activity, glutamate and K^+ accumulated in the cleft are taken up by astrocytes through excitatory amino-acid transporters (EAATs) and inwardly rectifying K^+ channels or Na^+/K^+-pumps, respectively (Allaman et al., 2011). Glutamate and K^+ once inside of the astrocytes diffuses to neighboring astrocytes and oligodendrocytes via channels known as gap junction channels (GJCs), a process termed "spatial buffering." Afterwards, glutamate is metabolized to glutamine by glutamine synthetase and released to the extracellular milieu from which it is taken up by neurons and transformed to glutamate or GABA (Allaman et al., 2011). By similar mechanisms astrocytes support metabolic status in neurons. Under physiological conditions, endothelial cells of the blood brain barrier (BBB) take up blood glucose and lactate through GLUT-1 and monocarboxylate transporters (MCTs), respectively. Both lactate and glucose diffuse between adjacent endothelial cells and eventually are taken up by astrocytic and released to the interstitial space (Allaman et al., 2011). Glucose and lactate can diffuse through astrocytes and their gap junctions with neighboring astrocytes to reach relatively distant areas. Glucose can be metabolized to lactate by astrocytes, and both can be released into the extracellular space and taken up by neurons.

Microglia constitute about 5–15% of total cells in the CNS and are essential players of brain innate immune system (Lawson et al., 1990). Originating from peripheral mielomonocytic precursor cells (*fetal macrophages*), microglia populate the brain parenchyma before developmental closure of BBB (Ginhoux et al., 2010). In a healthy brain, microglia exhibit a resting surveillance state (ramified morphology) linked with active exploration of their environment and permanent searching for exogenous or endogenous signals representing a brain threat (Streit, 2001; Kettenmann et al., 2011). When homeostatic balance is altered, resting phenotype of microglia shift to a reactive one, with different degrees of activation depending on nature, intensity and duration of the stimuli (Hanisch, 2002; Block et al., 2007). During brain damage, rather than show a repair-orientated profile, reactive microglia constitute a source of toxic and pro-inflammatory factors (phagocytic morphology), favoring the recruitment of non-resident brain cells involved in the innate and adaptive immune response (Block et al., 2007). In addition to their well-known role on brain immunity and inflammatory response, microglia are now recognized as essential players in the integration and consolidation of neuronal circuits. Various studies have shown that microglia constantly extend toward and retract from synapses, participating in a new range of undiscovered functions, including neuronal surveillance, synapse elimination and regulation of cell death, among others (Tremblay et al., 2010; Schafer et al., 2013; Wake et al., 2013). Indeed, some authors have proposed to shift the current notion of tripartite synapse into a "quad-partite synapse" (Schafer et al., 2013). Interestingly, neurotransmitter release by neurons modifies various aspects of glial cell function, including cellular migration, phagocytosis, intercellular Ca^{2+} wave generation, metabolic coupling, blood flow control and gliotransmitter release among others (Fields and Stevens, 2000; Fields and Stevens-Graham, 2002; Fields and Burnstock, 2006; Inoue et al., 2007). The latter encloses a permanent feedback loop of interactions between neurons and glial cells denominated "neuron-glia crosstalk."

Gliotransmission is part of the basis of "neuron-glia crosstalk" and multiple mechanisms have been described to mediate gliotransmitter release, including the Ca^{2+}-dependent exocytosis (Bezzi et al., 2004; Zhang et al., 2004; Imura et al., 2013), carrier membrane transport (Rossi et al., 2000) and opening of a wide-range of channels encompassing $P2X_7$ channels (Duan et al., 2003; Suadicani et al., 2006; Hamilton et al., 2008), volume-regulated anion channels (Kimelberg et al., 1990; Takano et al., 2005; Rudkouskaya et al., 2008; Lee et al., 2010) and connexin hemichannels (Stout et al., 2002; Ye et al., 2003) (**Figure 1**). Though most studies regarding neuron-glia crosstalk have been focused in gliotransmitter release, in the last decade it has become evident that brain cells can communicate via alternative mechanisms. Among them are those relying on heterotypic glia-to-neuron contacts mediated by homophylic and heterophylic adhesion molecule interactions (Avalos et al., 2009; Sandau et al., 2011) (**Figure 1**). Importantly, vesicles containing molecules/organelles (e.g., exosomes, microparticles, and apoptotic bodies) have resulted in an new unexpected mechanism of brain cell communication, allowing the exchange of gliotransmitters, organelles, genetic information, proteins, and infectious agents between glial cells and neurons (Frühbeis et al., 2013). Direct astrocyte-to-neuron communication not only occur through GJCs (Fróes et al., 1999; Rozental et al., 2001; Dobrenis et al., 2005), but also via intercellular bridges or long cellular extensions called intercellular nanotubes (Wang et al., 2012) (**Figure 1**). In the next section, we focused in a specific route of gliotransmitter communication mediated by single membrane channels called "hemichannels."

HEMICHANNELS AND GLIA-TO-NEURON COMMUNICATION

Hemichannels are composed of six protein subunits called connexins (Cxs). The latter encompass a highly conserved protein family encoded by 21 genes in humans and 20 in mice, with orthologs in other vertebrate species (Söhl and Willecke, 2004). For a long time, the pivotal function attributed to hemichannels was to provide the building blocks of GJCs, permitting direct but selective cytoplasmic continuity for ions and molecular exchange between contacting cells (Sáez et al., 2003). Nevertheless, recent studies have shown that hemichannels in "non-junctional" membranes can allow the permeation of ions and small molecules and thus, provide a diffusional route of exchange between the intra- and extracellular milieu (Sáez et al., 2005). Accordingly, hemichannels allow the cellular release of autocrine and paracrine signaling molecules (e.g., ATP, glutathione, glutamate, D-serine, NAD^+, and PGE_2) to the extracellular milieu, as well as the entry of other important signaling ions and molecules (e.g., Ca^{2+}, cADPR, and glucose) (Retamal et al., 2015). Recently, a new family of three membrane proteins termed pannexins (Panxs 1–3) was shown to form single membrane channels with similar paracrine signaling features of hemichannels (Panchin, 2005). Despite that Cxs and Panxs do not share significant amino acid sequences, both Cx hemichannels and Panx channels (hereinafter referred as Panx for simplicity) exhibit similar membrane topology and oligomerization features.

Astrocytes are characterized by their higher expression of Cx30 and Cx43 (Dermietzel et al., 1989; Batter et al., 1992), but other Cxs such as Cx26, Cx40, Cx45, and Cx46 have been detected in a lesser extent (Scemes et al., 1998; Rouach et al., 2008). Astrocytes also express important levels of Panx1 (Iglesias et al., 2009; Santiago et al., 2011), whereas both Cx43 and Panx1 have demonstrated to form functional channels in astrocytes *in vitro* and *in vivo* (Iglesias et al., 2009; Karpuk et al., 2011; Santiago et al., 2011; Orellana et al., 2015). Furthermore, it has been shown that astrocytic hemichannels are permeable to different molecules (Giaume et al., 2013; Montero and Orellana, 2014), thus allowing the release of ATP (Stout et al., 2002; Anderson et al., 2004; Iglesias et al., 2009; Garré et al., 2010), glutamate (Ye et al., 2003), aspartate (Ye et al., 2003), taurine (Stridh et al., 2008), D-serine (Pan et al., 2015), and glutathione (Rana and Dringen, 2007), as well as the uptake of glucose (Retamal et al., 2007). Up to now, only few studies have documented the expression of functional hemichannels in microglia. Cx32 was the first Cx documented able to form hemichannels in microglia. Pioneering observations by Takeuchi and colleagues, showed that

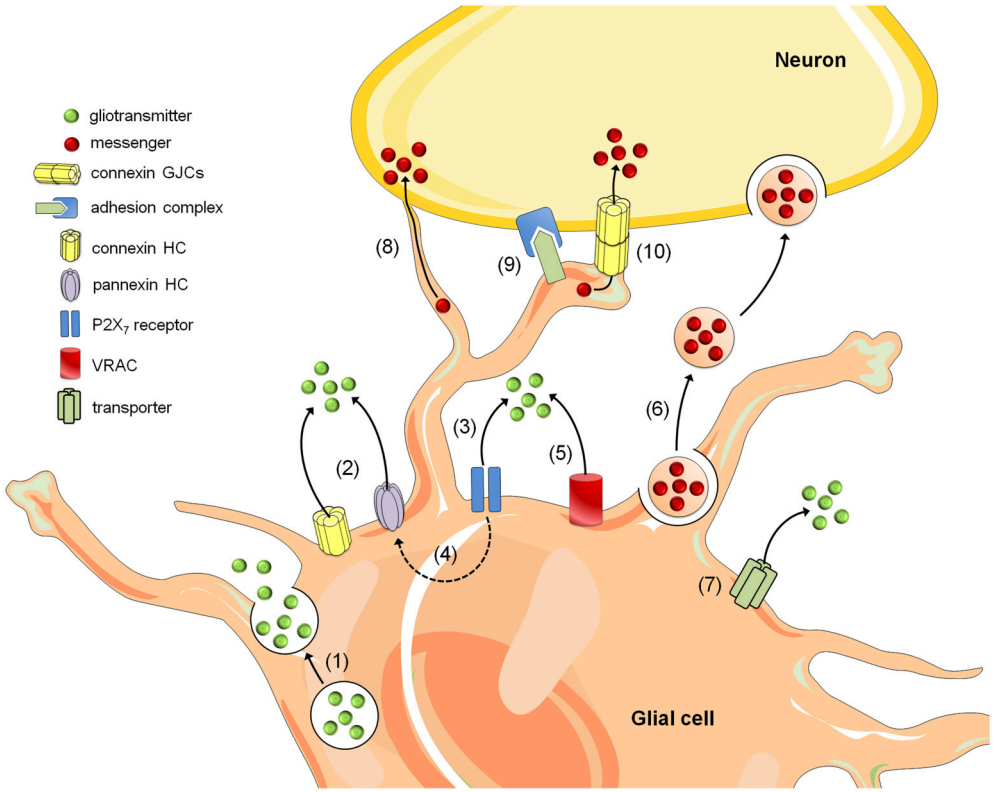

FIGURE 1 | Mechanisms of glia-to-neuron communication. Glial cells release gliotransmitters (e.g., glutamate, D-serine, and ATP) through Ca^{2+}- and SNARE-dependent exocytosis (1) in addition to the release that occurs through alternative non-exocytotic pathways (see below). Depolarization, reductions in extracellular divalent cation concentrations, increases in intracellular Ca^{2+} and posttranslational modifications might result in the opening of connexin and pannexin hemichannels (HCs) and thus allow the release of gliotransmitters (2). Long-lasting activation of $P2X_7$ by ATP might lead to the appearance of large currents and the rapid exchange of large molecules, including the release of gliotransmitters (3). One theory states that $P2X_7$ receptor conductance dilates over the time and thereby allows the passage of large molecules; however, another hypothesis states that ATP activate a second non-selective permeabilization pathway (Baroja-Mazo et al., 2013). Recently, it was shown that Panx1 hemichannels might mediate this permeability for large molecules in astrocytes (4) (Iglesias et al., 2009). Additionally, gliotransmitter release may occur through volume-regulated anion channels (VRAC) (5) and different carriers and/or co-transporters acting normally or in reverse (6) (e.g., excitatory amino-acid transporters, the cystine-glutamate antiporter, and the D-serine/chloride co-transporter). Within the last decade, a growing body of evidence has indicated that glial cells can also communicate with neurons via the release of vesicles (e.g., exosomes, microparticles, and apoptotic bodies), containing different cellular messengers (e.g., mRNA, viruses, and organelles) (7). Adjacent glial cells and neurons can communicate directly through F-actin-based transient tubular connections known as tunneling nanotubes (8), via cell-to-cell contacts between membrane-bound ligand molecules and their receptors (9) or aggregates of intercellular channels known as gap junctions, which allow the exchange of small molecules (10).

TNF-α induces the release of glutamate (Takeuchi et al., 2006), whereas the expression of functional hemichannels formed by Cx43 and Panx1 also has been reported (Orellana et al., 2011a, 2013a).

Which are the major functions of hemichannels in the brain? In the CNS, hemichannels play different physiological roles including ischemic tolerance or preconditioning (Schock et al., 2008), establishment of adhesive interactions (Cotrina et al., 2008), fear memory consolidation (Stehberg et al., 2012), synaptic transmission (Chever et al., 2014a), glucose sensing (Orellana et al., 2012a; Orellana and Stehberg, 2014), chemoreception (Reyes et al., 2014), BBB permeability (De Bock et al., 2011), and neuronal migration (Elias and Kriegstein, 2008). However, an increasing body of evidence has situated hemichannels as potential regulators of starting and maintaining homeostatic imbalances in diverse brain diseases (Orellana et al., 2009, 2012b, 2013b; Davidson et al., 2013; Takeuchi and Suzumura, 2014; Retamal et al., 2015). Indeed, uncontrolled opening of glial cell hemichannels induced trigger an excessive release of gliotransmitters (e.g., ATP, glutamate, and D-serine) that could be neurotoxic for neurons (Orellana et al., 2012b). Recently was demonstrated that astrocytes or microglia stimulated with amyloid-β (Aβ) peptide exhibit increased Cx43 and Panx1 hemichannel-dependent glutamate and ATP release, which results toxic for hippocampal and cortical neurons (Orellana et al., 2011a). Similarly, follow-up work showed that astrocytes pre-treated with conditioned media from Aβ peptide-treated microglia, release neurotoxic amounts of glutamate and ATP via Cx43 hemichannels when subjected to hypoxia in high glucose containing medium (Orellana et al., 2011b). Importantly, the release of both gliotransmitters reduced neuronal survival via activation of

neuronal NMDA/P2X7 receptors and Panx1 (Orellana et al., 2011a,b). How glutamate/ATP affects neuronal hemichannel opening and survival? Most available evidence indicates that neurons express functional hemichannels formed by Panx1 or Cx36 (Thompson et al., 2006; Schock et al., 2008). The opening of Panx1 could occur by protein-protein interactions with activated $P2X_7$ receptors (Iglesias et al., 2008) or through increases in $[Ca^{2+}]_i$ or phosphorylation triggered by activation of $P2X_7$ or NMDA receptors, respectively (Locovei et al., 2006; Weilinger et al., 2012).

RELATIONSHIP BETWEEN GLIAL CELL ACTIVATION AND INFLAMMATION/OXIDATIVE STRESS

In numerous brain disorders, glial cells experience a long-lasting process that underlies striking morphological, molecular and functional changes referred as "reactive gliosis" (Block et al., 2007; Pekny and Pekna, 2014). This phenomenon constitutes a graded, multistage glial cell reaction, which counteracts acute damage, restoring homeostasis and limiting brain parenchyma injury. In this stage, astrocytes show hypertrophy and enlargement of the intermediate filament network via upregulation of the glial fibrillary acidic protein (GFAP) and vimentin and re-expression of nestin (Pekny and Pekna, 2014). In addition, a general impairment of glial cell functions has been described such as altered gliotransmission and Ca^{2+} signaling, disturbed mitochondrial dynamics and antioxidant defense, as well as elevated production of nitric oxide (NO) (Block et al., 2007; Pekny and Pekna, 2014). Although reactive gliosis is an adaptive mechanism for protection, when persistent, it can turn into a detrimental response, leading to uncontrolled production of pro-inflammatory cytokines and reactive oxygen species (ROS), which worsens disease progression. At one end, reactive gliosis leads to neuroinflammation mediated by increased levels of IL-1β, IL-6, TNF-α, IL-3, and TGF-β (Block et al., 2007; Pekny and Pekna, 2014). At the other end, elevated levels of inflammatory mediators further cause the secretion of more cytokines and production of more ROS and reactive nitrogen species (RNS) (Mrak and Griffin, 2005; Rosales-Corral et al., 2010). Therefore, redox metabolism regulates cytokine signaling and vice versa, which in some circumstances could create a vicious cycle between oxidative stress and neuroinflammation (Rosales-Corral et al., 2010).

Glial cells use energy and antioxidant power generated by mitochondria to reduce inflammation and support neuronal health (Quintanilla et al., 2012; von Bernhardi and Eugenín, 2012). These glial beneficial properties have been proposed to be mostly based on the metabolism and fusion and fission dynamics of their mitochondria (Hertz et al., 2007; Stephen et al., 2014). Fusion is necessary to ameliorate stress by mixing components of partially damaged mitochondria, whereas fission helps to produce new mitochondria, but it also serves as quality control by allowing the removal of injured mitochondria, by facilitating apoptosis during significant levels of cellular damage (Westermann, 2010). Importantly, diverse studies have revealed a wide-range of defects in mitochondrial dynamics and bioenergetics in glial cells during inflammation (Brown et al., 1995; Almeida et al., 2001; Motori et al., 2013). In fact, reactive astrocytes exhibit increased glycolytic rate and production of ROS (Brown et al., 1995; Almeida et al., 2001), whereas those exposed to pro-inflammatory cytokines, show mitochondrial fragmentation and reduced mitochondrial respiratory capacity (Brown et al., 1995). Apparently, these effects are produced by activation of Drp-1, a GTPase that control mitochondrial elongation in mammalian cells (Hoekstra et al., 2015). In the same context, treatment with IL-1β increases ROS levels in cultured human astrocytes, an effect which is potentiated by IFN-γ (Sheng et al., 2013). Similarly, microglia exposed to lipopolysaccharide (LPS), a bacterial pro-inflammatory agent, exhibit an impairment in mitochondrial oxygen consumption (Moss and Bates, 2001; Chénais et al., 2002; Volobоueva et al., 2013) and increased ROS production (Volobоueva et al., 2013). Other studies have explored the use of specific mitochondrial antioxidants to reduce mitochondrial injury induced by pro-inflammatory cytokines in glial cells (Park et al., 2015). Inhibition of mitochondrial-ROS production by treatment with Mito-TEMPO, a specific mitochondrial antioxidant (Park et al., 2015), suppressed the production of mitochondrial and intracellular ROS in LPS-stimulated microglia (Park et al., 2015). Interestingly, treatment with Mito-TEMPO significantly prevented the LPS-induced increase in TNF-α, IL-1β, and IL-6 levels found in microglia (Park et al., 2015). Altogether this evidence indicates that mitochondrial failure could be critical to perpetuate the vicious cycle between oxidative stress and neuroinflammation.

REACTIVE GLIOSIS, INFLAMMATION, AND OXIDATIVE STRESS IN THE BRAIN DURING MS

Different studies indicate that most animals subjected to MS models have systemic inflammation and impaired mitochondrial function and redox metabolism (Ando and Fujita, 2009; Litvinova et al., 2015). In the nervous system, animals subjected to different models of MS (e.g., high fat diet, HFD) exhibit an increased number of reactive astrocytes and microglia, as well as elevated levels of pro-inflammatory cytokines, ROS and lipid peroxidation (Thaler et al., 2012; Tomassoni et al., 2013; Gao et al., 2014; Treviño et al., 2015). For instance, 1 day after mice or rats fed a HFD, microglial reactivity increases in the hypothalamus along with the levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1 β) (Gao et al., 2014). Interestingly, these inflammatory and oxidative responses do not depend on weight gain, since obese leptin receptor mutant db/db and melanocortin receptor 4 knockout mice do not show reactive microglia when fed a standard chow diet (Gao et al., 2014). Moreover, IFN-γ and IL-1β levels are significantly increased in Zucker diabetic fatty rats, predominantly in hippocampal regions near to activated astrocytes and microglia (Hwang et al., 2014). On the other hand,

HFD increases brain lipid peroxidation, which is accompanied by increased ROS and mitochondrial depolarization (Ma et al., 2014). In the same animal model, other authors found increased production of NO, lipid peroxidation, impaired glutathione metabolism and mitochondrial failure (Raza et al., 2015). In summary, these data suggest that inflammation and redox imbalance could be key elements in brain dysfunction occurring during MS.

COULD HEMICHANNELS CONTRIBUTE TO HYPERACTIVATION OF SYMPATHETIC NEURONS DURING MS?

Most of the answer to this question relies in how inflammation, oxidative stress and lipids affect the opening of these hemichannels and whether the release of several substances through them could impair normal neuronal excitability (Bennett et al., 2012). Pioneering studies by Takeuchi and colleagues showed that TNF-α treatment could increase the opening of Cx32 hemichannels in microglia, resulting in glutamate release and further neuronal death (Takeuchi et al., 2006). A follow-up study proposed that glutamate released via Cx32 hemichannels play a key role in neuronal damage promoted by experimental autoimmune encephalomyelitis, amyotrophic lateral sclerosis and Alzheimer's disease (Shijie et al., 2009; Takeuchi et al., 2011). On the other hand, Morita and colleagues showed that IL-1β increases astroglial hemichannel opening in culture and brain slices after exposure to a medium lacking divalent cations (Morita et al., 2007). Similarly, IL-1β and TNF-α directly enhances Cx43 hemichannel opening in astrocytes (Retamal et al., 2007); whereas astroglial hemichannel opening evoked by prenatal inflammation is prevented by blocking TNF-α/IL-1β pathways (Avendaño et al., 2015). Different studies have described that IL-1β and TNF-α induce p38 MAPkinase activation in astrocytes (Clerk et al., 1999; Rossa et al., 2006; Mitchell et al., 2007), causing iNOS activation (Gutiérrez-Venegas et al., 2005; Xu et al., 2006) and further NO production (Guan et al., 1997; Badger et al., 1998). Accordingly, upon treatment with NO donors, astrocytes exhibited an increased hemichannel opening associated with S-nitrosylation of Cx43 (Retamal et al., 2006). Similarly, NO is also involved in the increased hemichannel opening and expression of Panx1 observed in neurons subjected to oxygen and glucose deprivation (Zhang et al., 2008). Moreover, opening of hemichannels and microglia subjected to proinflammatory conditions depend on activation of iNOS/NO and p38 MAP kinase pathways (Orellana et al., 2013a; Avendaño et al., 2015). These data suggest that NO could affect the functional state of hemichannels in brain cells exposed to inflammatory conditions, including MS, where iNOS expression is dramatically increased (Ando and Fujita, 2009; Litvinova et al., 2015).

Other focus of attention should be directed to the high levels of fatty acids and different lipids occurring during the progression of MS. HFD induces reactive astrogliosis within days (Calvo-Ochoa et al., 2014; Yeh et al., 2015), which is associated to important changes in hippocampal function and synaptic transmission (Calvo-Ochoa et al., 2014). Recently, Retamal and co-workers showed that unsaturated fatty acids modulate the activity of Cx46 hemichannels in *Xenopus* oocytes (Retamal et al., 2011), whereas linoleic acid also induces Cx43 hemichannel opening in HeLa cells, through a PI3K/Akt/Ca^{2+}-dependent pathway (Figueroa et al., 2013). These data suggest that fatty acids modulate hemichannel opening, which could be an additional and interdependent key step along with inflammation and oxidative stress in the activation of sympathetic neurons during the progression of MS.

Another aspect to take into consideration is the high production of free radicals, ROS and RNS in metabolic disorders. A growing body of studies have described that redox potential modulates hemichannel opening in astrocytes (Retamal, 2014). Pioneering studies by Contreras and colleagues showed that Trolox, a free radical scavenger, blocks hemichannel activity induced by metabolic inhibition in cortical astrocytes (Contreras et al., 2002). Later on, a follow-up work demonstrated that dithiothreitol (DTT), a cysteine-reducing agent, reduced astroglial hemichannel activity observed during ischemia-like conditions (Retamal et al., 2006). Interestingly, the response induced by DTT was mimicked by a cell-permeant reduced glutathione ethyl ester (GSH-EE), but not by the non-permeant GSH, suggesting that intracellular cysteines of Cx43 could be oxidized during brain ischemia, affecting hemichannel function (Retamal et al., 2006). In the context of Cx43 hemichannels, it has been proposed that redox potential could modulate them depending on their phosphorylation status, the latter associated to cell damage (Retamal et al., 2007). Accordingly, in astrocytes under physiological conditions (little Cx43 dephosphorylation), DTT increases hemichannel opening, whereas the opposite occurs in astrocytes exposed to long periods of pathological conditions, including inflammation (conspicuous Cx43 dephosphorylation) (Retamal et al., 2006, 2007; Retamal, 2014). All these data suggest that inflammation, lipids and oxidative stress could be the milestones of hemichannel-dependent glial dysfunction during MS. However, the specific cellular mechanism by how glial cell dysfunction could induce sympathetic neurons hyperactivation during metabolic disorders remains to be elucidated. Astrocytes act as modulators of the synapsis by controlling the neuronal postsynaptic excitability in the NST through release of glutamate (Vance et al., 2015). Moreover, it has been proposed that the interaction between astrocytes and neurons in the hypothalamic paraventricular and supraoptic nuclei, both centers involved in the generation of coordinated neurohumoral responses, influence the autonomic response (Stern and Filosa, 2013). Following this line of thought, the optogenetic activation of astrocytes in the RVLM activates pre-sympathetic neurons in an ATP-dependent manner, thus increasing sympathetic renal nerve activity, arterial blood pressure, and heart rate (Marina et al., 2013). Interestingly, physiological function of astrocytic Cx43 and Panx1 channels include regulation of basal and stimulated excitatory synaptic transmission in the hippocampus (Prochnow et al., 2012; Ardiles et al., 2014; Chever et al., 2014a,b), whereas increased opening of astrocytic Cx43 hemichannels evoked by LPS alters excitatory

synaptic activity (Abudara et al., 2015). Indeed, recently, Stehberg and colleagues showed that gliotransmmiter release through astroglial Cx43 hemichannels modulates the neuronal activity in the amygdala (Stehberg et al., 2012). This work, showed that microinjection of specific Cx43 hemichannel blocking peptides into the rat's basolateral amygdala abolished the fear memory consolidation (Stehberg et al., 2012). Additionally, recent works demonstrate that hemichannels expressed in astrocytes modulates: (i) human neuronal cortex activity during development (Moore et al., 2014), (ii) basal activity of hippocampal neurons in adult mice (Chever et al., 2014a), and (iii) the inhibitory interneuron activity in response to local hyperexcitability (Torres et al., 2012). Thus, nowadays, the role of astroglial Cx43 hemichannels as neuronal modulators emerges as an ongoing concept in the neuroscience field. At this regard, the uncontrolled release of ATP, glutamate or D-serine via glial cell hemichannels (Ye et al., 2003; Takeuchi et al., 2006; Orellana et al., 2011a,b; Pan et al., 2015) could play a crucial role in hyperactivation of sympathetic system. All these gliotransmitters have showed to modulate synaptic transmission in different brain areas, including the SNS (Guyenet, 2006).

We speculate that a moderate uncontrolled hemichannel opening could raise intracellular free Ca^{2+} concentration in glial cells, leading to altered gliotransmitter release. Supporting this idea, a recent study revealed that glutamate release via astroglial Cx43 hemichannels is associated to impaired excitatory synaptic activity in pyramidal neurons in response to Schaffer's collateral stimulation (Abudara et al., 2015). In this context, it was recently demonstrated that astrocytes respond to physiological changes of the pO2 at the brainstem. Thus, when pO2 is decreased, astrocytes release ATP to the extracellular media, increasing pre-sympathetic neurons activity (Angelova et al., 2015). These findings suggest that astrocytes are metabolic sensors at the brainstem and changes in their metabolism could modulate sympathetic activity through the release of gliotransmmiters. In agreement with this idea, it has been shown that ATP released from astrocytes in the RVLM, increase renal nerve activity, arterial blood pressure, and heart rate (Marina et al., 2013).

Given that an increased hemichannel opening could lead to synaptic malfunctioning and, therefore, to worsening some conditions associated to MS, it could be interesting to analyze other alternatives as well. Thus, in addition to the release of gliotransmitters that potentially affect normal neuronal synapses, astrocytes may also release ascorbate through VSOACs and hemichannels (Wilson et al., 2000; Ahmad and Evans, 2002). Neuronal metabolism under physiological and particularly pathological conditions is highly oxidative (Lai, 1992; Coyle and Puttfarcken, 1993). Astrocytes have large intracellular concentrations of antioxidants, which include reduced glutathione and ascorbate (Wilson, 1997). Neurons can take up ascorbate released from astrocytes, oxidizing it to dehydroascorbate (DHA). Then, DHA is released from neurons through facilitative glucose transporters (GLUTs) (Corti et al., 2010), and further imported by astrocytes via GLUTs, where it is reduced back to ascorbate and once again released to the extracellular media by a pathway that is sensitive to VSOAC inhibitors (Wilson, 1997). Thus, astrocytic ascorbate represents a way of membrane electron transport, in which, reducing equivalents derived from astrocytic metabolism are shared with neurons, as antioxidant support (Lane and Lawen, 2009), as well as means of promoting non-transferrin bound iron uptake by astrocytes, which may also play neuroprotective roles (Lane et al., 2010). Accordingly, Corti and co-workers, proposed that the ascorbate released by astrocytes attenuates glutamate-induced excitotoxicity, oxidative stress and acidosis in neurons (Corti et al., 2010).

At the other end, if the hemichannel activity is very high, an excessive release of ascorbate can be expected. In the brain, copper is used for several important physiological processes (Lutsenko et al., 2010; Scheiber et al., 2014). However, changes in copper homeostasis have been correlated to development of some neurodegenerative diseases (Scheiber et al., 2014). This is so because ascorbate can increase copper accumulation in astrocytes (Scheiber et al., 2010a,b), which -at high concentrations- is toxic for them (Bulcke et al., 2015), as well as for neurons (Scheiber et al., 2014). A massive ascorbate release from astrocytes can be associated to an increase of copper associated to neuronal death. Interestingly, in Bulcker's work, they reported that cell loss induced by copper/ascorbate was correlated with increased permeability to propidium iodide, which is a fluorescent dye extensively used to measure hemichannel opening in several cell types (Ebihara et al., 2011; Shahidullah and Delamere, 2014; Mandal et al., 2015). We suggest that moderate increase of hemichannel opening observed during MS could lead to synaptic malfunctioning due to a massive release of glio transmitters, but also can help neuronal survival. In addition, massive hemichannel activity could lead to both neuronal and glial cell death, due to excessive glutamate release and overload of copper in astrocytes. Since this idea has not been confirmed in MS yet, future studies are needed focusing on the role of astrocytes as neuron protectors during MS.

FUTURE DIRECTIONS

Until this point we discussed the possible mechanism that associates glial cell hemichannel opening with the increased sympathetic activation observed during the MS. This hypothesis could plausible if hemichannel opening increases until certain (unknown) level. However, what about if hemichannel activity increase even more? The most obvious suggestion is that neuronal function and synaptic transmission will be compromised, resulting in further production of neuropathies (Retamal et al., 2015). It is well known that metabolic-associated diseases can produce the appearance of neuropathies (Kim and Feldman, 2012; D'Amico and Bertini, 2013). One possibility is that gliotransmitters released from glial cells due to hemichannel opening become neurotoxic, as has been recently demonstrated (Orellana et al., 2011a,b; Avendaño et al., 2015). In summary, we propose that under MS a positive feedback loop can be generated between reactive gliosis, inflammation, mitochondrial dysfunction and hemichannel opening (**Figure 2**). The latter may contribute

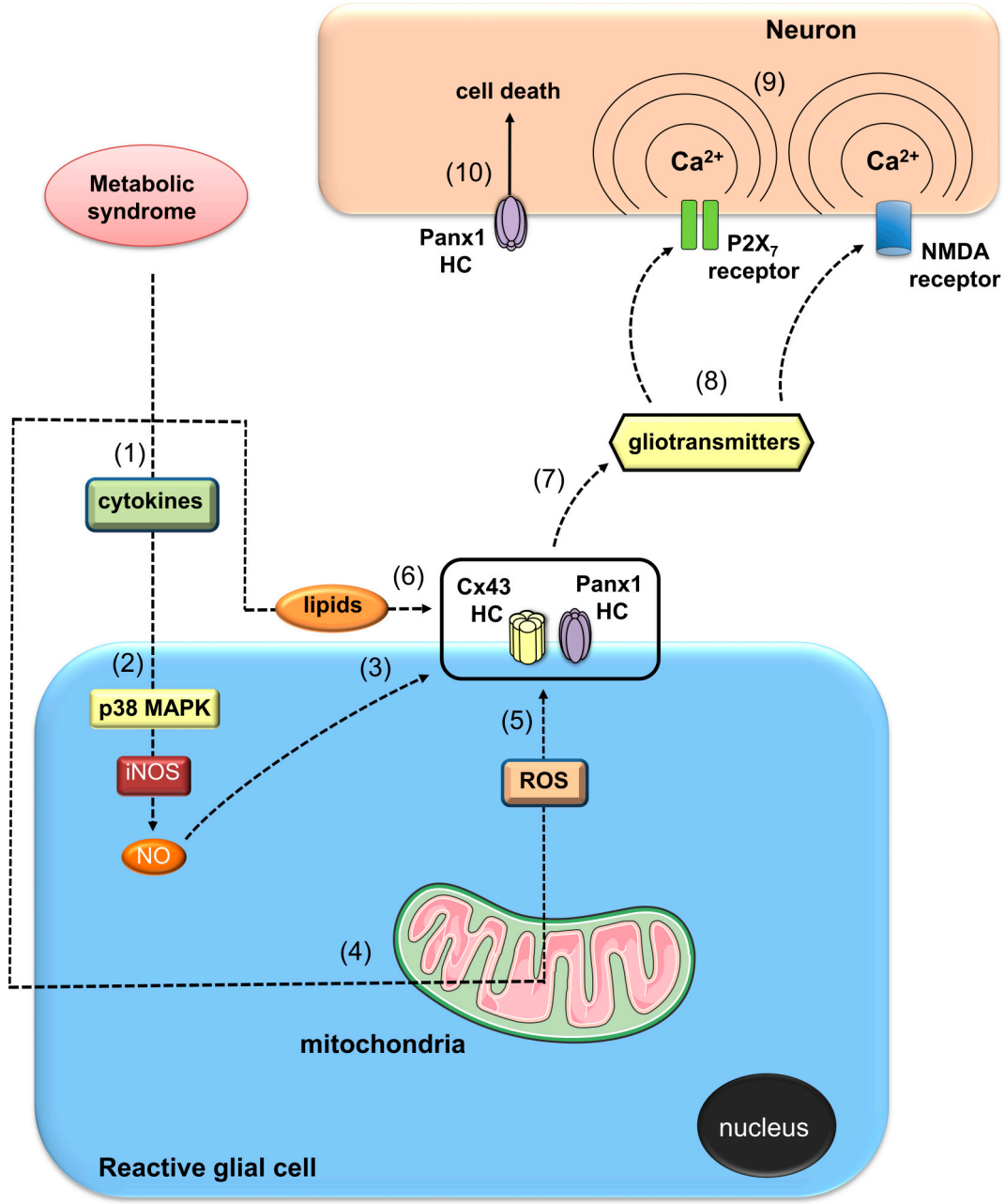

FIGURE 2 | Possible actions of metabolic syndrome on glia-to-neuron communication mediated by hemichannels. Metabolic syndrome (MS) may induce a generalized inflammatory state that could affect the nervous system (1). In this context, autocrine/paracrine release of pro-inflammatory cytokines (e.g., IL-1β and TNF-α) by reactive glial cells could lead to the activation of a p38MAPK/iNOS-dependent pathway and further production of nitric oxide (NO) (2). NO could cause the nitrosilation of Cx43, resulting in opening of Cx43 glial cell hemichannels (3). Alternatively, for an unknown mechanism, NO could increase the activity of Panx1 hemichannels. Along with the systemic inflammatory state, MS impairs mitochondrial function in glial cells, leading to redox potential imbalance and subsequent uncontrolled production of reactive oxygen species (ROS) (4). Modulation of oxidative status of Cx43 and/or Panx1 hemichannels by ROS could increase their activity (5). High levels of triglycerides and fatty acids during the progression of MS could directly enhance the opening of hemichannels in glial cells (6). In addition, paracrine release of gliotransmitters through glial cell hemichannels (e.g., ATP, glutamate, D-serine) (7) could act on neighboring or distant neurons, resulting in the activation of $P2X_7$ and NMDA receptors (8). The latter increase levels of $[Ca^{2+}]_i$, (9) and thereof the activity of neuronal Panx1 channels, resulting in neuronal function impairment and cell death (10).

to the autonomic imbalance at early stages of MS specifically through a glial cell dependent modulation of sympathetic neuron activity in the brainstem. Importantly, as the disease progress, development of neuropathies could take place mainly associated with the neurotoxic consequence of a massive opening of hemichannels.

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REVIEW
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Does the sympathetic nervous system contribute to the pathophysiology of metabolic syndrome?

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The metabolic syndrome (MS), formally known as syndrome X, is a clustering of several risk factors such as obesity, hypertension, insulin resistance, and dislypidemia which could lead to the development of diabetes and cardiovascular diseases (CVD). The frequent changes in the definition and diagnostic criteria of MS are indications of the controversy and the challenges surrounding the understanding of this syndrome among researchers. Obesity and insulin resistance are leading risk factors of MS. Moreover, obesity and hypertension are closely associated to the increase and aggravation of oxidative stress. The recommended treatment of MS frequently involves change of lifestyles to prevent weight gain. MS is not only an important screening tool for the identification of individuals at high risk of CVD and diabetes but also an indicator of suitable treatment. As sympathetic disturbances and oxidative stress are often associated with obesity and hypertension, the present review summarizes the role of sympathetic nervous system and oxidative stress in the MS.

Keywords: obesity, insulin resistance, hypertension, cardiovascular diseases, central nervous system

Introduction

Cardiovascular diseases (CVD) are major causes of morbidity and mortality worldwide (WHO, 2013). The risk factors of CVD include hypertension, hypercholesterolemia, diabetes, genetic predisposition, obesity, sedentary lifestyle, and smoking (WHO, 2013). Unlike other risk factors, smoking and sedentary lifestyle are preventable. The risk factors that cannot be prevented often occur together. The clustering of these risk factors has been studied extensively (Kagota et al., 2010; Salazar et al., 2011; Gyawali et al., 2015). Reaven (1988) observed that insulin-resistant subjects often manifest obesity, high level of low-density lipoprotein (LDL)-cholesterol, low level of high-density lipoprotein (HDL)-cholesterol, fasting hyperglycemia and increased arterial blood pressure. These manifestations with multiple risk factors to the development of CVD were called Syndrome X (Reaven, 1988; Grundy et al., 2004). Later, the Syndrome X was renamed as Metabolic Syndrome (MS). In other words, MS is a clustering of several metabolic changes that affect the organism and often result in CVD.

The prevalence of CVD has increased since the advent of industrial revolution especially in western societies (Gottlieb et al., 2008). The less active lifestyle being promoted by modern transportation system, exclusion of manual jobs with high scale mechanized production and widespread consumption of fat rich food has contributed greatly to the increasing cases of obesity and MS. The consumption of industrial products which are also rich in sugar and salt elicits harmful effects on insulin resistance (Vidonho et al., 2004; Salazar et al., 2011) and blood pressure (Vidonho et al., 2004; He et al., 2008; Moreira et al., 2014). According to Stoian and Stoica (2014), MS patients exhibit lower concentrations of serum phosphate and magnesium as compared to subjects who did not meet the criteria for the diagnosis of this syndrome. Phosphate and magnesium are crucial to carbohydrate metabolism, thus, it is not surprising to suggest that lower level of these ions in MS patients can lead to a decrease in peripheral utilization of glucose as well as the development or aggravation of insulin resistance (Stoian and Stoica, 2014).

Some studies have demonstrated that oxidative and inflammatory stress play an important role in the initiation and progression of CVD (Toshima et al., 2000; Libby et al., 2002). It has been established that obesity could lead to an increase in the circulating markers of oxidative stress and inflammation (Festa et al., 2000; Keaney et al., 2003). As obesity is an important risk factor of MS, the establishment of a definitive relationship between inflammation and oxidative stress has attracted a lot of interest. However, there are still dearth of information in this regard.

In order to unravel the pathophysiology of MS, several definitions and diagnostic parameters have been proposed. The World Health Organization (WHO) defined MS as a group of risk factors of CVD and diabetes (Alberti and Zimmet, 1998; Grundy et al., 2004). WHO considered insulin resistance as the primary cause of MS (Grundy et al., 2004). This diagnostic consideration predicts diabetes in MS. However, the need of a specific test for insulin resistance makes MS diagnostic criteria by WHO less accessible (Grundy et al., 2004). According to WHO, the insulin resistance can be identified through one of the following factors: (i) type 2 diabetes; (ii) impaired fasting glucose; or (iii) impaired glucose intolerance. Besides the insulin resistance, at least two other risk factors are required to diagnose the syndrome (Alberti and Zimmet, 1998; Grundy et al., 2004). Some of the diagnostic criteria for MS are shown in **Table 1**.

TABLE 1 | WHO—Diagnostic criteria for metabolic syndrome.

Factor	Limit levels
High blood pressure	≥140/≥90 mmHg or antihypertensive medication
Plasma triglycerides	≥150 mg/dL (1.7 mM)
HDL cholesterol	
Men	<35 mg/dL (0.9 mM)
Women	<39 mg/dL (1.0 mM)
Body mass index	>30 kg/m^2
Urinary albumin excretion rate	≥20 μg/min

Major Components of Metabolic Syndrome

Abdominal Obesity

Obesity is defined by WHO as an abnormal or excessive fat accumulation that causes health problems (Mendis et al., 2015). The body mass index (BMI) is a parameter being are used to classify overweight and obesity in adults. BMI is calculated using the formula weight/height^2 (kg/m^2). WHO defines a BMI greater than or equal to 30 kg/m^2 as obesity. Although the prevalence of obesity has been doubled worldwide since 1980, it is still a preventable disease. Nowadays, obesity kills more people than underweight globally. In 2014, it was reported that 39% of the adults (≥18 years) were overweight and 13% obese. About 42 million of people under 5 years of age were overweight in 2013 (Mendis et al., 2015).

Basically, obesity is caused by the imbalance between the calories consumed and calories expended. The higher consumption of calories as compared to the calories expended has being associated with weight gain. The accumulation of adipose tissue in the abdominal region causes abdominal obesity. Several studies have demonstrated that abdominal obesity which can be measured by the waist circumference has a strong correlation with the development of CVD (Ferreira and Moisés, 2000; Salazar et al., 2011; Traissac et al., 2015).

Considering the strong relationship among obesity, MS and cardiovascular risk factors, it is important to unravel the autonomic disturbances in obesity. Though the autonomic imbalance is not homogeneous in obesity, some studies demonstrated sympathetic hyperactivity in most part of obese individuals (Lopes and Egan, 2006). In the obese individuals, the sympathetic tone is increased in target organs such as kidney, skeletal muscle and peripheral vessels to elicit hypertension (Esler et al., 2001; Lopes and Egan, 2006).

Hypertension

Hypertension is, by definition, a sustained increase in the arterial blood pressure (WHO, 2013). Nowadays, hypertension and its complications is one of the major cause of death worldwide. Factors such as increase in the sympathetic activity and dysregulation in sodium homeostasis could cause an increase in the arterial blood pressure. Although the pathogenesis of hypertension is still unclear, salt consumption has been implicated as one of the important causes of this disease (da Costa Lima et al., 1997; Ito et al., 1999; Contreras et al., 2000; Sacks et al., 2001; Rodriguez-Iturbe and Vaziri, 2007; He et al., 2008; Moreira et al., 2014). In addition, sedentary lifestyle, obesity and smoking are other factors that contribute to increase in blood pressure (Filho et al., 2008). The long-term increase in arterial blood pressure often affects some organs, like heart and kidneys. The higher the blood pressure, the greater is the resistance needed by heart to function. A higher blood pressure could lead to an increase in the frequency and contractile force. In the long-term, this changes in blood pressure could compromise cardiac function (Zile, 2002; Cingolani et al., 2003; Chen-Izu et al., 2007). According to WHO, hypertension is a systolic blood pressure equal to or above 140 mmHg and/or diastolic blood pressure equal to or above 90 mmHg (WHO, 2013).

Endothelial dysfunction has been identified in hypertension. Endothelium is a cellular layer on the vascular wall that controls the traffic of small and large molecules, and maintains the integrity of vascular wall (Carvalho et al., 2001). The endothelium controls the expansion and contraction of blood vessels by producing local mediators that are involved in vasodilation (endothelium-derived relaxing factors–EDRFs) or vasoconstrictor (endothelium-derived constriction factors–EDCFs) in response to changes in blood flow or vasoactive agents (Carvalho et al., 2001). The endothelium has multiple and important roles in physiological events: (i) it acts as hemodynamic sensor by receiving and transmitting signals from the extracellular matrix and cells; (ii) it produces mediators that interfere with growth, activity, migration and cell death; and (iii) it preserves the adaptive changes to circulatory requirements (Carvalho et al., 2001).

The principal EDRFs are nitric oxide (NO), the endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI2). Angiotensin II (Ang II) and superoxide or reactive oxygen species (ROS) are among the main EDCFs (Kang, 2014). In pathophysiological situations, such as hypertension, an increase of EDCFs occurs. In this condition, study has shown an increase in the release of endothelial derived contractile factors cyclooxygenase (e.g., PGH 2) in response to acetylcholine and angiotensin II in aorta and mesenteric arteries of SHR (Côrtes et al., 1996). This result suggests endothelial dysfunction in hypertension. Under physiological conditions, there is a balance between the release, and production of the most important relaxing and contractile factors. This balance could be changed by attenuation of vasodilatory effect of endothelium. An apparent decrease in vascular endothelial-dependent relaxation is called endothelial dysfunction (Carvalho et al., 2001). The mechanisms involved in endothelial dysfunction as found in hypertension is multifactorial. Preclinical studies have shown an increase in the basal activity of nitric oxide synthase (NOS) and a decrease in the expression and activity of soluble guanylyl cyclase in smooth muscle of spontaneously hypertensive rats (Bauersachs et al., 1998; Kojda et al., 1998). On the other hand, in Dahl rats with salt-sensitive hypertension, there is a decrease in the responsiveness of vascular smooth muscle cells due to a decrease in the eNOS activity and NO production (Luscher and Vanhoutte, 1986; Hayakawa and Raij, 1998). EDHF has been described as one of the principal mediators of endothelium-dependent vasorelaxation in normotensive animals. The contribution of EDHF-mediated relaxation appears significantly greater in small resistance vessels than in large conduit vessels (Félétou and Vanhoutte, 2006). A reduction in the release of EDHF may lead to endothelial dysfunction and subsequently arterial hypertension (Fujii et al., 1992).

The oxygen and oxidative reactions in the body are vital for energy supply and defense against invaders. Under physiological conditions, the enzyme superoxide dismutase is responsible for preventing the formation of reactive species of oxygen such as peroxynitrite (ONOO-) which has a detrimental effects in the body (McIntyre et al., 1999). However, under oxidative stress condition as in MS, a large amount of O_2 reacts with NO to form (ONOO–). This reaction could lead to a significant reduction in the bioavailability of endothelial NO.

Nitric oxide are highly relevant in endothelial dysfunction because in this pathology, production may be reduced due to changes in NOS3 (Nitro oxide synthase type 3) protein as observed in animal models of cardiovascular disease such as SHR, DOCA, diabetic rats (Hink et al., 2001; Sullivan et al., 2002). The reduction in the bioavailability of NO is considered as part of the mechanism for endothelial dysfunction in oxidative stress. NO could reacts with O_2 to cause vasoconstriction, vascular damage and lipid peroxidation (Virdis et al., 2002).

In summary, endothelial dysfunction is characterized by an imbalance in the release of vasoconstrictors and the endothelium-dependent relaxants. Hence, increases in EDCFs are common in pathophysiological condition like hypertension.

Insulin Resistance

The insulin-resistance occurs when the body cells become less sensitive and resistant to insulin. Insulin, an hormone which facilitate glucose absorption, is produced in the beta cells of the pancreas (The IDF consensus worldwide definition of the MS: IDF, 2006). Once the glucose cannot be absorbed by the cells, it remains in the blood and subsequently triggers off the production of more insulin (hyperinsulinaemia reflex). The over production of insulin often wears off beta cells and diminishes its capacity to produce insulin. This condition generally leads to hyperglycaemia and type 2 diabetes (WHO and IDF, 2006; The IDF consensus worldwide definition of the MS: IDF, 2006). The insulin-resistance promotes damage to several insulin-sensitive organs such as liver and kidneys. The hyperinsulinaemia reflex increases the release of triglycerides by liver into the bloodstream and subsequent decrease in the level of HDL cholesterol, increase in the level of small and dense particles of LDL cholesterol (Reaven, 1988, 2003; Yoon et al., 2014). The hyperinsulinaemia reflex can contribute to the pathophysiology of the essential hypertension through an increase in renal water absorption and/or increase in the sympathetic activity (Reaven, 2003; Yoon et al., 2014). The insulin resistance is a major risk factor of type 2 diabetes and atherosclerotic complications such as coronary artery disease, stroke and peripheral arterial disease (Yoon et al., 2014).

Dislypidemia

The dislypidemia is considered as an unbalance serum level of LDL (which increases) and HDL cholesterol particles (which decreases) (Reaven, 1988). As defined by Kaur (2014), dyslipidemia is characterized by spectrum of qualitative lipid abnormalities that reflect perturbations in the structure, metabolism, and biological activities of both atherogenic lipoproteins and antiatherogenic HDL cholesterol. Dislypidemia is frequently associated to insulin resistance. As mentioned above, the hyperinsulinaemia reflex, caused by the insulin resistance, promotes increase in the hepatic release of triglycerides to the blood. This release could decrease the level of HDL cholesterol and increase the level of small and denser particles of LDL cholesterol (Reaven, 1988, 2003; Yoon et al., 2014).

The increase in serum level of small and denser LDL cholesterol particles could lead to the accumulation of triglyceride in the vessels and development of atherosclerosis among other cardiovascular complications (Reaven, 1988, 2003).

Inflammation and Oxidative Stress in Metabolic Syndrome

The increase in oxidative stress and inflammatory state are known to play an important role in the initiation and progression of CVD (Toshima et al., 2000; Libby et al., 2002). Obesity and MS have been associated with increased circulating markers of oxidative stress and inflammation (Festa et al., 2000; Keaney et al., 2003). However, there are still few information on the relationship between MS and inflammation/oxidative stress. The possible link between MS and inflammation is resistance to insulin (RI) (Volp et al., 2008). A defective insulin action in target tissues (muscle, liver, and adipose tissue) could increase chronic inflammation (Dandona et al., 2007). T cells elaborate inflammatory and anti-inflammatory properties of cytokines by stimulating macrophages, endothelial cells and smooth muscle cells (Volp et al., 2008). The key inflammatory cytokines markers include interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), interleukin-1β (IL-1β), CD40, CD40L, and C-reactive protein (Kon et al., 2005; Wu and Wu, 2006). Since the adipocytes cells are the main producers of cytokines proinflammatory, there is a strong relationship between increased secretion and higher levels of cytokines in obese people. This phenomenon increases the risk of developing MS (Vanhala et al., 2006).

IL-6 is a pro-inflammatory cytokine involved in the development of hyperinsulinemia and MS. This cytokine increases lipolysis to release free fatty acids and glycerol while reducing the expression of insulin receptor substrate-1 (IRS-1) and GLUT4 in muscle and liver tissues (Francisco et al., 2006). Though IL-6 is mainly secreted by adipocytes, it is produced by smooth muscle cells, endothelial cells, monocytes and macrophages and may contribute to the development of atherosclerotic lesions through its paracrine, autocrine, and endocrine effect. Studies have correlated the values of serum IL-6 with the waist circumference (Rexrode et al., 2003). People with central obesity are at increased risk to develop MS.

The TNF-α is a cytokine with autocrine, paracrine and endocrine action (Ruan and Lodish, 2003). In obese humans, there is an inverse correlation between TNF-α and glucose metabolism (Winkler et al., 2003). Insulin signaling suppression by the TNF-α reduces the translocation of glucose transporter (GLUT-4) to the membrane and consequently promotes a decrease in insulin-mediated glucose uptake by cells. It is also known that the expression of mRNA and TNF-α secretion are higher in obese person (Hsueh and Law, 2003).

IL-1β is responsible for increase in the expression of endothelial adhesion molecules which facilitate the aggregation of other inflammatory cells in the activated endothelium (Francisco et al., 2006). IL-1β, together with TNF-α, stimulates IL-6 production by smooth muscle cells and increases the expression of macrophages. This process is associated with the progression of inflammatory and atherosclerosis processes (Francisco et al., 2006). IL-18 is a proinflammatory cytokine with pleiotropic action which induces the secretion of the cytokines IL-6, TNF-α, IL-1β and endothelial adhesion molecules (Francisco et al., 2006). This cytokine exerts chemotaxis of human T cells to promote the recruitment into the atherosclerotic plaque. It has been proposed that the IL-18 induces the expression of several matrix metalloproteinases, which may weaken the fibrous cap of injury atherosclerotic (Hung et al., 2005).

The cytokines CD40 and CD40L are expressed by macrophages, lymphocytes T, platelets, endothelial cells and muscle cells flat (Hung et al., 2005). The system CD40/CD40L exerts various pro-inflammatory and pro-thrombotic effects by: (i) stimulating the production of endothelial cells free radicals to antagonize nitric oxide production; (ii) inducing expression of endothelial cells and smooth muscle adhesion molecules; (iii) stimulating the expression of proinflammatory cytokines and chemokines; (iv) inducing tissue factor expression on endothelial and smooth muscle cells to promote an increase in potential thrombogenic plate; and (v) participating in the activation of platelet (Angelico et al., 2006). Data has shown that CD40 which is expressed on the surface of platelets could activate platelet to promote thrombus formation (Angelico et al., 2006).

C-Reactive Protein (CRP) is synthesized by the liver and regulated by cytokines, predominantly IL-6, TNF-α, and IL-1 (Abdellaoui and Al-Khaffaf, 2007). Its levels are increased during acute inflammatory process. Mild increase in the level of CRP also occurs in chronic inflammatory condition such as atherosclerosis. The levels of this protein almost triple in the presence of peripheral vascular disease risk (Abdellaoui and Al-Khaffaf, 2007). It has been shown that MS patients have CRP serum levels significantly higher than people without MS (Bahia et al., 2006).

Macrophages are a heterogeneous population of immune cells that have a range of roles in both the induction and resolution of inflammation (Dey et al., 2015). The pleiotropic responses are coordinated through distinct programs of macrophage activation classified as classical (or M1) and alternative (or M2) activation (Gordon, 2003; Martinez et al., 2008).

It has been shown that the immune response that is activated during inflammation and obesity-induced insulin resistance has the same M1 mechanism (Takeda et al., 2003). In classical activation of macrophages, lipids derived from bacteria bind to Toll-like receptor 4 (TLR4) and activate signaling pathways that induce, for example, the release of NF-kB inflammatory molecules (TNF, IL-6 and 2) (Takeda et al., 2003). In obesity and inflammation, saturated fatty acids activate TLR4 to promote inflammatory responses (Shi et al., 2006; Kim et al., 2007; Nguyen et al., 2007). In fact, acute infusion of lipids into mice potentiates the insulin resistance in both adipose tissue and skeletal muscle in a TLR4-dependent manner (Kim et al., 2007). In addition, other studies have shown that white adipose tissue of obese rats have mainly macrophages with classically activated—M1 phenotype (Bouloumié et al., 2005; Ferrante, 2007). Alternatively, during the resolution of inflammation, the balance of macrophage activation toward an M2 phenotype occurs in order to promote clearance of debris and inhibit the production of inflammatory

mediators to restore tissue homeostasis (Mills et al., 2014). M2 macrophages produce anti-inflammatory cytokines and express endocytic receptors. These cells promote the clearance of apoptotic cells, proliferation and wound healing (Mills et al., 2014). Together, these data suggest a model in which increased flux of saturated fatty acids, as seen in obese states stimulates classical macrophage activation, tissue inflammation, and insulin resistance.

Although the number of classically activated adipose tissue macrophages increases with obesity, adipose tissue of lean animals contains a moderate number of macrophages. The adipose tissue macrophage of lean mice under non-inflammatory conditions express high levels of substances encoded by genes of alternatively activated M2 macrophages that promotes tissue homeostasis and repair (Hung et al., 2005; Vanhala et al., 2006). As suggested by Palaniappan et al. (2003), obesity results in a change in the activation pathway of macrophages.

Van Guilder et al. (2006) evaluated a possible synergistic effect of MS and obesity on the circulating markers of oxidative stress and inflammation. In that study, the authors observed that MS heightens oxidative stress and inflammatory burden in obese adults, thereby suggesting that MS and obesity have a synergistic effect on oxidative stress and inflammation markers (Van Guilder et al., 2006). Furthermore, the authors suggested that the increased oxidative and inflammatory stress may contribute to risk of coronary heart disease and cerebrovascular disease in obese adults with MS (Van Guilder et al., 2006).

Several studies have related the adipose tissue to the elevated markers of oxidative stress and inflammation once the expression and secretion of such markers increase in proportion to adiposity (Mohamed-Ali et al., 1998; Bertin et al., 2000; Kern et al., 2001; Van Guilder et al., 2006). However, as observed by Van Guilder et al. (2006), the adiposity alone do not seems to be the primary cause of oxidative stress and inflammation once the markers are increased only in the obese individuals diagnosed with MS.

In addition, an increase in glucose metabolism has been implicated in oxidative stress—MS, relation (Furukawa et al., 2004; Tangvarasittichai, 2015). During the metabolism of glucose (glycolysis and tricarboxylic acid—TCA cycle), the electron donors NADH (nicotinamide adenine dinucleotide) and FADH2 (flavin adenine dinucleotide) are generated (Tangvarasittichai, 2015). In the case of over nutrition or obesity, a large amount of glucose is oxidized in such a way that an increase in the generation of NADH and FADH2 in the mitocondrial electron transport chain subsequently result in an increase in superoxide generation (Tangvarasittichai, 2015). Furthermore, the increase in free fatty acid and acetyl coenzyme A (CoA) oxidation (due to the excessive of free fatty acids) in TCA cycle generate more molecules of NADH and FADH2 to be oxidized, and overproduction ROS (Furukawa et al., 2004; Tangvarasittichai, 2015). Moreover, NADPH oxidase is involved in fatty acids—ROS generation in adipocytes once the treatment with NADPH oxidase inhibitor blocks the ROS generation (Furukawa et al., 2004; Tangvarasittichai, 2015).

Like oxidative stress, inflammatory state is also an important feature of MS. Inflammation is one the manifestations of oxidative stress (Roebuck, 1999; Tangvarasittichai, 2015). Festa et al. (2000) demonstrated that chronic subclinical inflammation is a key feature of the MS and components of MS (dyslipidemia, abdominal obesity, and hypertension) increase in parallel to the increasing levels of plasma CRP in non-diabetic individuals. It is possible that chronic inflammation triggers MS (Festa et al., 2000), overnutrition, cytokine hypersecretion, insulin resistance, and diabetes in predisposed individuals (Festa et al., 2000). Festa et al. (2000) also suggested that the decrease in insulin sensitivity may lead to increase in CRP expression by counteracting the physiological effect of insulin on hepatic protein synthesis (Campos and Baumann, 1992; Festa et al., 2000).

Although there are many studies focusing on oxidative stress, inflammation and MS (Campos and Baumann, 1992; Roebuck, 1999; Festa et al., 2000; Furukawa et al., 2004; Tangvarasittichai, 2015), there is still no clear consensus about the causal relationship among the triad oxidative stress—inflammation—MS.

The Sympathetic Nervous System and the Metabolic Syndrome

The sympathetic nervous system (SNS) is an arm of the autonomic nervous system which plays vital role in the regulatory mechanisms of blood pressure, sodium balance and maintenance of homeostatic state. The SNS is fundamental in the control of daily energy expenditure through the regulation of resting metabolic rate and thermogenesis in response to physiological stimuli, changing energy states, food intake, carbohydrate consumption and hyperinsulinemia (Thorp and Schlaich, 2015). Furthermore, the activation of sympathetic nerves in target organs like liver, pancreas, skeletal muscle, and adipose tissue can elicit acute catabolic responses (i.e., glycogenolysis and lipolysis) (Thorp and Schlaich, 2015).

Over activation of SNS is strongly associated with two components of the MS, i.e., obesity and hypertension (Tentolouris et al., 2006). In fact, enhanced SNS activation exerts unfavorable effects like cardiac hypertrophy, arterial remodeling, and endothelial dysfunction on the cardiovascular system (Grassi and Seravalle, 2006). Increase in sympathetic activity enhances systemic and regional norepinephrine spillover and elevate resting heart rate. This condition has been linked to hypertension, obesity, and insulin resistance (Mancia et al., 2007). Furthermore, it has been shown that high levels of fasting insulin, an index of insulin resistance, were positively associated with the low-to-high frequency (LF/HF) ratio of the heart rate variability (HRV)—an index of the sympathovagal balance at the heart level (Emdin et al., 2001).

In view of the strong relationship among obesity, MS and the development of cardiovascular risk factors, it is important to elucidate autonomic disturbances that occur in obese individuals. As cited by Hall et al. (2000), there are two lines of evidences about the central nervous system involvement in obesity-induced hypertension: (i) increase in sympathetic activity in obese as compared to lean subjects; and (ii) attenuation of obesity-related hypertension through pharmacological blockade of adrenergic activity (Landsberg and Krieger, 1989; Grassi et al., 1995;

Hall et al., 2000). Though the autonomic disorders are not homogeneous in obesity, some studies have demonstrated that most individuals exhibit sympathetic hyperactivity (Lopes and Egan, 2006). Both baroreflex sensitivity (BRS) and impaired HRV in obese women (Skrapari et al., 2007). Esler et al. (2001) demonstrated that the sympathetic tone in obese individuals is increase in some target organs like kidney, skeletal muscle and vessels. Sympathetic hyperactivity in obesity indicates that obesity impairs renal-pressure natriuresis, increases renal tubular sodium reabsorption and causes hypertension (Kassab et al., 1995; Hall et al., 2000).

It is known that sympathetic disturbances are directly related to increase in arterial blood pressure (Tan et al., 2010; Oliveira-Sales et al., 2014). In humans, several features confirm typical increase in sympathetic tone as observed in obesity. Various studies have demonstrated increased blood pressure and serum catecholamine levels in obese individuals. The loss of weight is associated to the decrease in plasma concentration norepinephrine (Tuck, 1992; Lopes and Egan, 2006). Obese hypertensive children show increase in sympathetic nerve activity (Rocchini et al., 1989; Lopes and Egan, 2006). However, in these patients, a low salt diet (or hyposodic diet) is capable of promoting a decrease in arterial pressure (Rocchini et al., 1989; Lopes and Egan, 2006). These studies point out the fact that sympathetic hyperactivity is related to sodium retention and increase of arterial blood pressure in obese children (Lopes and Egan, 2006). Furthermore, muscular sympathetic nerve activity (MSNA) is increased in obese (normotensive and hypertensive) as compared to non-obese normotensive individuals (Grassi et al., 2000). Moreover, it has been shown that the MSNA and the plasmatic norepinephrine are reduced and the BRS is increased after weight loss in normotensive obese individuals (Grassi et al., 1998; Lopes and Egan, 2006). The SNS response is blunted in obese subjects despite the fact that plasma insulin levels were almost 45% higher in the obese as compared to the lean patients (Tentolouris et al., 2003).

The heart rate variations are a visible effect of the autonomic influences on the heart in cases of emotional stress. An inability to sustain varying heart rate is an important risk factor to the development of CVD (Hemingway et al., 2001; Brunner et al., 2002). The study of Brunner et al. (2002) demonstrated a relative sympathetic dominance and a lower vagal tone to the heart in MS cases, thereby indicating unbalance sympathovagal in those individuals. Jamerson et al. (1993) demonstrated an inverse relationship between sympathetic vascular tone and the insulin-mediated cellular consumption of glucose. Thus, it is not surprising to speculate that the increased SNA as observed in obese individuals increases the vascular constriction and impairs the glucose transportation into the cells (Grassi et al., 2000; Lopes and Egan, 2006). Previous studies with obese models have implicated vascular constriction in insulin-resistance (Laakso et al., 1990; Lopes and Egan, 2006). Specific alpha-adrenergic vasoconstriction seems to be more malefic on glucose consumption than the angiotensin-induced vasoconstriction (Jamerson et al., 1993; Lopes and Egan, 2006). This assumption suggests, once again, sympathetic influence on glucose metabolism.

In patients with type 2 diabetes mellitus (T2DM), MS has approximately 70% of prevalence rate (Monami et al., 2007; Bianchi et al., 2008). The basic mechanism involved in the pathogenesis of T2DM is the insulin resistance. The insulin resistance, in turn, is strongly associated with sympathovagal imbalance. Furthermore, many data suggest the involvement of increased SNS activity in insulin resistance (Mancia et al., 2007). Epidemiological studies have found a correlation between insulin resistance and hypertension (Modan and Halkin, 1991; Skyler et al., 1995). In patients with type 1 diabetes mellitus (T1DM), the hypertension is usually developed after the onset of nephropathy and it is associated with rennin-angiotensin induced SNS activation (Perin et al., 2001). In contrary, the prevalence of hypertension in patients with T2DM is extremely common (Perin et al., 2001). Thus, it can be assumed that Insulin resistance and hypertension as observed in the MS are closely linked with sympathetic overactivation (Frontoni et al., 2005).

The SNS activity plays a crucial role in the regulation of circulation and blood pressure (Fisher and Paton, 2011; Zubcevic et al., 2011). Sympathetic vasomotor and cardiac neural activities are induced by the sympathetic preganglionic neurons in the spinal cord. These neurons receive tonic excitatory drive from pre-sympathetic networks within the brainstem and hypothalamus (Dampney, 1994; Guyenet et al., 1996; Dampney et al., 2002; Madden and Sved, 2003). Increased in SNS activity has been linked to the pathogenesis of hypertension in humans with essential hypertension (Abboud, 1984; Mancia et al., 1999; Esler et al., 2001; Guyenet, 2006). Sympathetic overload is implicated in the pathogenesis and/or deterioration of essential hypertension through the modification of heart rate, cardiac output, peripheral vascular resistance and renal sodium retention (Grassi and Seravalle, 2006). The study of sympathetic nerve firing rate in hypertensive patients has shown sympathetic overactivity in young, middle-aged, and elderly hypertensive (Grassi et al., 2000). Some studies with essential hypertensive patients have plasmatic overflow of norepinephrine. This overflow indicates an increase in the activation of sympathetic outflow to the heart, kidneys and cerebrovascular circulation of these individuals (Esler et al., 1991; Mancia et al., 2007). These observations are evidences that some target organs are negatively affected by increased blood pressure (Grassi et al., 2007). Moreover, increase in heart rate has been observed in subjects with MS as compared to those without MS (Mancia et al., 2007). In this way, the physical inactivity can be associated with obesity as well as to the increase in cardiac sympathetic drive in the MS.

The SNS disturbances are closely related to all the main features of the MS. Although SNS participation in the pathophysiology of MS is clear, it is difficult to determine whether the metabolic changes are responsible for sympathetic disturbances or vice versa.

Role of Leptin in the Elevation of Sympathetic Activity

Obesity, an important risk factor for the development of MS, is characterized by the increase in size and number of adipocytes

(cells that produce adipokines, Martínez-Martínez et al., 2014). Leptin is one of the adipokines that has been postulated as a link between obesity and cardiovascular damage (Martínez-Martínez et al., 2014). Leptin is a peptide synthesized and secreted from white adipose tissue (Head et al., 2014) in addition to other sources like placenta, stomach, and heart (Zeidan et al., 2011). Leptin expression can be induced by obesity, insulin and TNF-α. This adipokine has been implicated in numerous physiological functions such as immune response and reproduction (Frühbeck, 2002; Mark, 2013). However, its main action is related to glucose homeostasis and regulation of appetite. This hormone provides feedback to the CNS on the status of peripheral energy reserves (Rahmouni, 2010).

Plasma leptin concentrations are significantly elevated in several rodent and human models of obesity in a proportional manner to adiposity (Magni et al., 2000). Although circulating levels of leptin rise in obesity, these individuals are thought to be leptin resistant due to lack of satiation (Magni et al., 2000). It is known that systemic infusion of leptin increased renal sympathetic nerve activity (RSNA) and supplies (Haynes et al., 1997). Central infusion of this peptide increases blood pressure (Shek et al., 1998). Head et al. (2014) recently demonstrated that central infusion of leptin antagonist in obese rabbits is able to return blood pressure to their basal levels (Head et al., 2014). These findings may contribute to understand an obesity-induced hypertension. Furthermore, recent studies have demonstrated that antagonism of central leptin receptor, LepR, caused a reduction in BP and HR in hypertensive mice (Tumer et al., 2007). The cardiovascular effects of leptin administration are not exclusively due to its central action. In fact, it has been shown that systemically administration of leptin antibodies elicited similar changes as compared to central administration (Tumer et al., 2007).

In an animal model of non-obese animals with hyperleptinemia, leptin increased systolic blood pressure and promoted an increase in intrarenal and systemic oxidative stress (Beltowski et al., 2004). In these model, the increase in ROS cause inactivation of nitric oxide. This effect can explain the leptin-associated hypertension in this model (Beltowski et al., 2004; Martínez-Martínez et al., 2014). Renal sodium retention has also been implicated in hyperleptinemia hypertension (Martin et al., 2008). Beltowski et al. (2004) have demonstrated that hyperleptinemia decreases urinary sodium excretion to promote volume retention and consequent increase in arterial blood pressure. Other harmful effects such as atherosclerosis (Gruen et al., 2007), inflammation, thrombosis and cardiac myocyte hypertrophy (Northcott et al., 2012) have been associated with leptin. Based on these observations, it is reasonable to suggest that pharmacological approaches targeting leptin's effects could represent a potentially useful therapeutic strategy for the treatment of obesity-associated hypertension among other cardiovascular disease.

Treatment of Metabolic Syndrome

Since MS is a clustering of dysfunctions, different treatment strategy has been adopted. WHO suggested treatments focusing on insulin resistance as first-line therapy. A more active lifestyle (which could lead to the weight loss) could be a promising therapy as it decreases insulin resistance. Since there are direct relationships between sedentary lifestyle and cardiovascular risk factors, the benefits of physical exercises on the prognosis of the MS are clear (Lakka et al., 2003; Rennie et al., 2003). In fact, translational studies demonstrate lower insulin levels and increased insulin sensitivity in athletes as compared to match-aged sedentary individuals (Nuutila et al., 1994; Roberts et al., 2013). Eriksson et al. (1997) demonstrated that one single session of exercise increase the glucose offer mediated by insulin in healthy and insulin resistant obese and type 2 diabetes individuals. Chronic exercise improves insulin sensibility in healthy, non-diabetic obese, type 1 and 2 diabetic individuals (Eriksson et al., 1997). Also, the use of drugs like metformin and thiazolidinediones (insulin sensitizers) have also been prescribed for insulin resistance treatment (Einhorn et al., 2003; Grundy et al., 2004).

As previously mentioned, the serum phosphate and magnesium is reduced in MS patients (Stoian and Stoica, 2014). The compensatory hyperinsulinemia can promote decrease of serum phosphate and magnesium, a vicious cycle which contributes to the pathophysiology of MS (Stoian and Stoica, 2014). Hence, a pharmacological treatment that restores the level of these ions could be beneficial (Stoian and Stoica, 2014) to MS patients.

Final Considerations

The definition and diagnostic criteria of MS are still controversial for obvious reasons. The divergent opinions on the diagnosis of MS among health institutions constitute challenges to its treatment and identification of individuals at high risk of CVD and diabetes. The diagram presented in the **Figure 1** below summarizes the complex relationship between some of the MS components.

A clinical diagnosis of MS is critical as it affects therapeutic strategy. A multidisciplinary approach including lifestyle changes, pharmacological and surgical approaches could be helpful toward the management of this syndrome. Physical inactivity, insulin resistance, advance age, hormonal factors (androgens and corticosteroids), and diets rich in fats which promote abdominal obesity or adiposity have been consistently identified as major risk factors of MS. Atherogenic dyslipidemia, elevated blood pressure, smoking, elevated glucose, prothrombotic, and proinflammatory state are cardiovascular risk factors that accompany MS. Change in lifestyle and anti-obesity drugs among others could engender effective prevention or treatment of MS. Although, lifestyle changes remain first-line therapy for the improvement of all the underlying metabolic risk factors, cases of unsuccessful lifestyle modification therapy can be substituted with anti-obesity treatment. Lifestyle therapy could dampen MS progression at every stage. This kind of therapy does not treat each risk factor in isolation but rather target multiple risk factors simultaneously. Although lifestyle therapy may not modify any given risk factor as much as drug, its benefit lies in the fact that it produces moderate reduction in all metabolic risk factors. In most situations, drug therapies might be required in the case of worsening condition of MS.

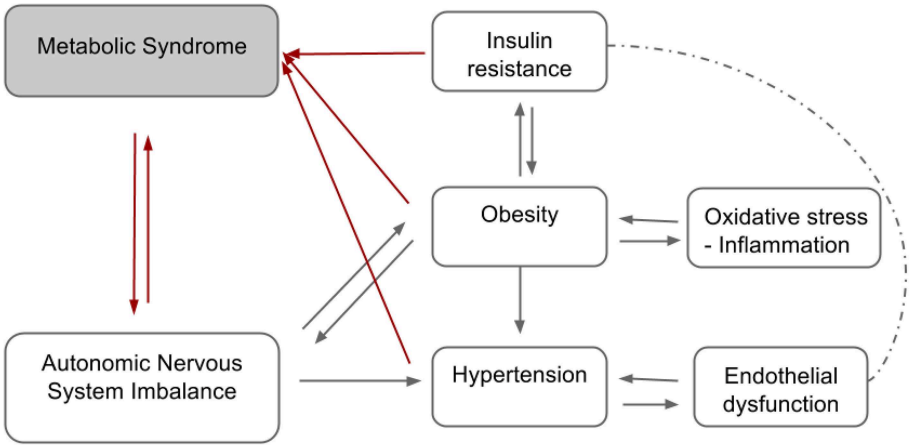

FIGURE 1 | Hypothetical diagram summarizing the relationship between the components of the metabolic syndrome.

The effectiveness of drugs targeting individual risk components of MS separately is still uncertain. This approach could lead to aggressive use of medications at the expense of lifestyle therapy. The ineffectiveness of some available drugs for the treatment of MS has been compounded by the impractical approach of simultaneous prescription and administration of all the drugs that could modify all of the risk factors. Current efforts being made to combine drugs into a single capsule that targets multiple risk factors seems ingenious as it could reduce the burden of polypharmacy. A single drug that can affect multiple metabolic risk factors simultaneously and provide health benefit to MS patient seems promising. Drugs that act as angiotensin and adrenergic (alpha and beta) receptors blockers and peroxisome proliferator–activator receptor-γ agonism could lower blood pressure, vascular and cardiac sympathetic tone as well as plasmatic glucose simultaneously. Though new drug development programme looks interesting, better understanding of MS, improved diagnostic criteria and treatment strategy is key to future clinical practice.

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In adenosine A_{2B} knockouts acute treatment with inorganic nitrate improves glucose disposal, oxidative stress, and AMPK signaling in the liver

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Rationale: Accumulating studies suggest that nitric oxide (NO) deficiency and oxidative stress are central pathological mechanisms in type 2 diabetes (T2D). Recent findings demonstrate therapeutic effects by boosting the nitrate-nitrite-NO pathway, which is an alternative pathway for NO formation. This study aimed at investigating the acute effects of inorganic nitrate on glucose and insulin signaling in adenosine A_{2B} receptor knockout mice ($A_{2B}^{-/-}$), a genetic mouse model of impaired metabolic regulation.

Methods: Acute effects of nitrate treatment were investigated in aged wild-type (WT) and $A_{2B}^{-/-}$ mice. One hour after injection with nitrate (0.1 mmol/kg, i.p.) or placebo, metabolic regulation was evaluated by intraperitoneal glucose and insulin tolerance tests. NADPH oxidase-mediated superoxide production and AMPK phosphorylation were measured in livers obtained from non-treated or glucose-treated mice, with or without prior nitrate injection. Plasma was used to determine insulin resistance (HOMA-IR) and NO signaling.

Results: $A_{2B}^{-/-}$ displayed increased body weight, reduced glucose clearance, and attenuated overall insulin responses compared with age-matched WT mice. Nitrate treatment increased circulating levels of nitrate, nitrite and cGMP in the $A_{2B}^{-/-}$, and improved glucose clearance. In WT mice, however, nitrate treatment did not influence glucose clearance. HOMA-IR increased following glucose injection in the $A_{2B}^{-/-}$, but remained at basal levels in mice pretreated with nitrate. NADPH oxidase activity in livers from $A_{2B}^{-/-}$, but not WT mice, was reduced by nitrate treatment. Livers from $A_{2B}^{-/-}$ displayed reduced AMPK phosphorylation compared with WT mice, and this was increased by nitrate treatment. Finally, injection with the anti-diabetic agent metformin induced similar therapeutic effects in the $A_{2B}^{-/-}$ as observed with nitrate.

Conclusion: The $A_{2B}^{-/-}$ mouse is a genetic mouse model of metabolic syndrome. Acute treatment with nitrate improved the metabolic profile in it, at least partly via reduction in oxidative stress and improved AMPK signaling in the liver.

Keywords: insulin resistance, metabolic syndrome, NADPH oxidase, nitric oxide, nitrite, superoxide, obesity, type 2 diabetes

Introduction

Metabolic syndrome, which worsens during aging and obesity, is a cluster of biochemical and physiological abnormalities that increase the risk of developing cardiovascular disease and type 2 diabetes (T2D) (Eckel et al., 2005; Carlström, 2011). Reduced nitric oxide (NO) production from endothelial nitric oxide synthase (eNOS) and augmented oxidative stress are proposed to be central events in metabolic syndrome (Litvinova et al., 2015). In the past decade, an alternative pathway for NO formation has been described where inorganic nitrate is serially reduced to nitrite and then NO and other bioactive nitrogen oxides (Lundberg et al., 2008, 2009, 2011). We have shown that several features of metabolic syndrome present in aged eNOS-deficient mice can be reversed by dietary supplementation with inorganic nitrate (Carlström et al., 2010). A recent study showed that chronic nitrite supplementation through increased phosphorylation of the skeletal muscle AMP activated kinase (AMPK) improved some metabolic syndrome components in a model of obesity (Singamsetty et al., 2015). Moreover, chronic treatment with nitrate attenuates oxidative stress and high blood pressure in models of renal and cardiovascular disease (Carlström et al., 2011a; Gao et al., 2015).

Adenosine is another important regulator of metabolism, and signaling via its different receptor subtypes, A_1, A_{2A}, A_{2B}, and A_3, has also gained a lot of interest (Chen et al., 2013). In a recent publication we demonstrated that abrogation of adenosine A_1 signaling improves metabolic regulation in aged mice by modulating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and immune responses (Yang et al., 2015a). Besides the A_1 receptor, signaling via both A_{2A} and A_{2B} receptors play important roles in modulating glucose homeostasis and fat mass (Johnston-Cox et al., 2012; Gnad et al., 2014). Another recent study suggested gene deletion of adenosine A_{2B} receptor as a suitable model for metabolic syndrome (Csóka et al., 2014). The authors showed that A_{2B} knockout mice ($A_{2B}^{-/-}$), fed a regular chow, displayed increased body weight and fat mass, impaired glucose and insulin homeostasis, together with dysregulated insulin, adipokine, triglyceride, and cholesterol metabolism compared with wild-type (WT) control mice. Food consumption was similar between genotypes, but daily walking time was reduced in the $A_{2B}^{-/-}$ mice. Moreover, Johnston-Cox et al. reported that a high fat diet (HFD) aggravated the abnormal metabolic phenotype in $A_{2B}^{-/-}$, whereas Csoka and colleagues did not observe this.

The current study aimed at investigating the acute effects of inorganic nitrate treatment on metabolic functions in aged A_{2B} receptor knockout mice ($A_{2B}^{-/-}$). Considering previous findings about nitrate- or nitrite-mediated modulation of both NADPH oxidase (Montenegro et al., 2011; Carlström et al., 2011a; Gao et al., 2015; Yang et al., 2015b) and AMPK (Kamga Pride et al., 2014; Singamsetty et al., 2015), we hypothesized that nitrate could improve abnormal metabolic functions during aging and increased fat mass by increasing AMPK activation and moderating oxidative stress. We observed improved metabolic regulation in the $A_{2B}^{-/-}$ mice after nitrate treatment and this was indeed associated with decreased NADPH oxidase-derived superoxide production in the liver, possibly mediated via restored AMPK activation.

Materials and Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee (IACUC) in Stockholm, and performed according to the National Institutes of Health guidelines for the conduct of experiments in animals. Experiments were conducted on aged (12–16 months) adenosine A_{2B} receptor gene-deleted and WT mice from heterozygous breeding pairs. $A_{2B}^{-/-}$ mice (a gift from professor M. Sitkovsky at Northwestern University, Boston, Mass) were backcrossed 11 times to a C57BL/6J background at Northwestern University. Both sexes were used, with equal distribution for all experimental series. Mice were housed in temperature-controlled rooms with 12 h light/dark cycles and received a standard rodent chow (4% fat, R34, Lactamin AB, Kimstad, Sweden) and tap water *ad libitum*. An overview of the experimental protocol is shown in **Figure 1**.

Intraperitoneal Glucose, Insulin, and Pyruvate Tolerance Tests

Glucose tolerance tests (IPGTT) were performed following 6 h of fasting, as described previously (Yang et al., 2015a). Inorganic nitrate ($NaNO_3$; 0.1 mmol/kg body weight) or placebo (NaCl, 0.1 mmol/kg body weight) was administered intraperitoneally 60 min prior to the tolerance tests. In a human (70 kg) this dose of nitrate corresponds to around 450 mg; an amount found in a single serving of a nitrate rich vegetable such as spinach, beetroot, or lettuce (Weitzberg and Lundberg, 2013). A bolus of D-glucose or pyruvate was injected (2 g/kg body weight; 30% in saline) and tail blood was sampled at 0, 15, 30, 60, and 120 min. Plasma glucose was determined using a portable glucose meter (FreeStyle Lite, Abbot Diabetes Care Inc, CA). In a cohort of $A_{2B}^{-/-}$ mice we also investigated the effects on glucose disposal with the anti-diabetic drug metformin. Metformin (0.1 mmol/kg body weight) or placebo (NaCl, 0.1 mmol/kg body weight) was administered intraperitoneally 60 min prior to the IPGTT. Homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated at baseline, at 60 min after injection with nitrate or placebo, and again 30 min after injection with glucose.

In order to investigate the acute effects of nitrate in a model with more pronounced obesity, IPGTT were performed as described above in WT mice given a HFD (34.9% fat, D12492, Research Diets Inc., New Brunswick, NJ) for 14 months (Supplementary Material).

Intraperitoneal insulin tolerance tests (IPITT) were performed similarly to IPGTT without fasting. A bolus of insulin (0.75 IE/kg body weight; Novorapid 100 IE/ml, Novo Nordisk A/S, Denmark) was injected (0.2 IE/ml in saline) and blood samples were obtained for plasma glucose measurements.

Plasma Analysis

Insulin content was measured using ELISAs purchased from Mercodia (Uppsala, Sweden). Plasma samples containing IBMX

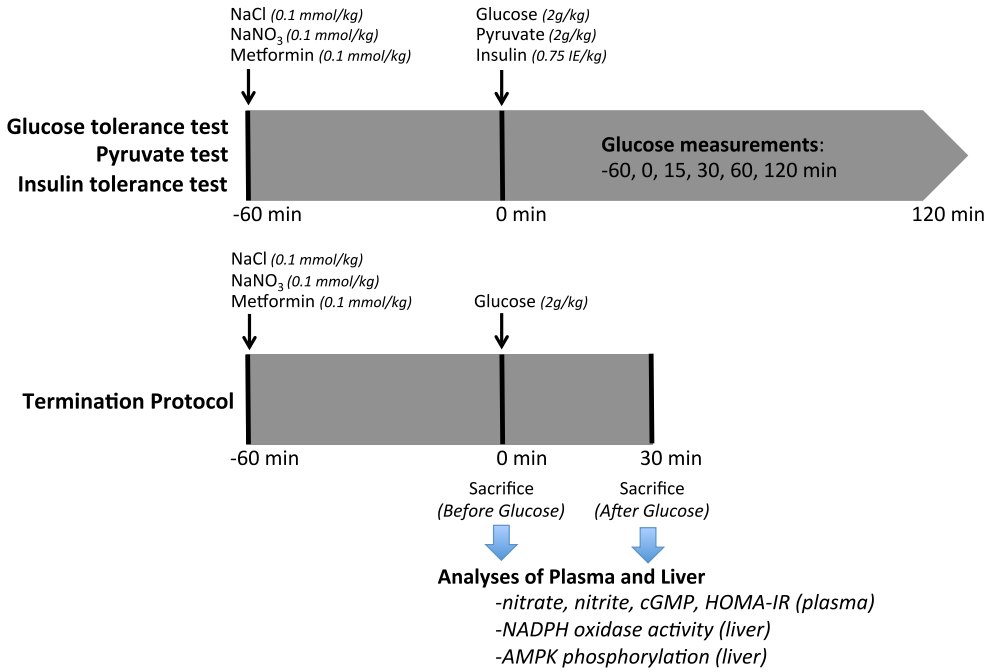

FIGURE 1 | Experimental Protocol. Diagram of the experimental procedures on aged WT or A_{2B}KO mice undergoing intraperitoneal glucose (IPGTT), pyruvate or insulin (IPITT) tolerance tests. In the termination series, plasma and liver were collected at 0 or 30 min. NaCl, sodium chloride; $NaNO_3$, sodium nitrate; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test.

(10 μM) were analyzed for cGMP using ELISA method (EIA system; GE Healthcare). All kits were run according to manufacturers' instructions. Nitrite and nitrate were analyzed by HPLC (ENO-20) and autosampler (840, EiCom, Kyoto, Japan), as described previously (Carlström et al., 2010; Hezel et al., 2015). The plasma samples were extracted using methanol (1:2) and then centrifuged for 10 min (4°C; 10.000 g), separated by reverse phase/ion exchange chromatography followed by nitrate reduction to nitrite by cadmium and reduced copper. The nitrite was then derivatized using Griess reagent to form diazo compounds and analyzed by detection at 540 nm.

NADPH Oxidase Activity

NADPH oxidase-mediated superoxide formation was detected by lucigenin-dependent chemiluminescence assay (Carlstrom et al., 2013; Yang et al., 2015a). Livers were separately homogenized and used for subsequent activity measurement.

Western Blotting of AMPK

Livers obtained from mice under (1) basal condition, (2) after pretreatment with placebo, nitrate or metformin, and (3) after stimulation with glucose were weighed and homogenized using 0.5 mm zirconium oxide beads (Bullet Blender™, Next Advance, Inc., Stockholm, Sweden) in 2.5 volumes of lysis buffer containing 10 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA, 60 mM N-octyl glucoside, 1% Triton X-100, protease, and phosphatase inhibitor cocktails (Sigma-Aldrich, Stockholm, Sweden). After centrifugation and protein quantification of the soluble fraction (Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Solna, Sweden), equal amounts of protein were separated by SDS-PAGE followed by transfer to a PVDF membrane (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in Tween-containing TBS, incubated with specific primary antibody for phosphorylated AMPK (Thr172; Cell signaling/BioNordika, Stockholm, Sweden) and anti-rabbit secondary antibody (horseradish peroxidase-conjugated goat antibody to rabbit IgG, Santa Cruz, Heidelberg, Germany). To detect total AMPK, Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific™, Göteborg, Sweden) was applied followed by blocking and re-probing the membranes with primary antibody for AMPK (Cell Signaling/BioNordika) and anti-rabbit secondary antibody. Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad), intensities were quantified using densitometry (Image Lab 5.2.1 software, Bio-Rad) and results are reported as relative optical density of the specific proteins.

Statistical Analysis

Values are presented as means ± SEM. Single comparisons between normally distributed parameters were tested for significance using the Student's paired or unpaired *t*-test as appropriate. For multiple group comparisons, One-Way ANOVA followed by Bonferroni's *post-hoc* test was used to allow for more than one comparison with the same variable. Statistical significance was defined as $p < 0.05$.

Results

Animal Characteristics

Body weight was significantly higher in aged (12–16 months) $A_{2B}^{-/-}$ (36.5 ± 0.8 g; $n = 42$) compared with age-matched WT mice (32.5 ± 0.9 g; $n = 40$), and plasma glucose levels in non-fasting mice were also higher in the $A_{2B}^{-/-}$ mice (9.5 ± 0.4 vs. 7.6 ± 0.2 mmol/L). WT mice fed with HFD for 14 months were more obese (body weight; 60.6 ± 3.3 g; $n = 10$) compared with the aged-matched mice on a regular chow ($P < 0.05$).

Glucose Tolerance Tests

To investigate the ability of acute inorganic nitrate treatment to modulate the metabolic phenotype in aged $A_{2B}^{-/-}$ mice we performed glucose tolerance tests in aged $A_{2B}^{-/-}$ and WT mice 1 h after nitrate injection ($NaNO_3$; 0.1 mmol/kg body weight). Fasting blood glucose levels were similar in $A_{2B}^{-/-}$ and WT mice and nitrate treatment had no influence on glucose clearance in WT mice (**Figure 2A**). Interestingly, the impaired glucose tolerance in $A_{2B}^{-/-}$ mice compared to WT was significantly improved after acute nitrate treatment (**Figure 2B**). Administration of the anti-diabetic drug metformin to the $A_{2B}^{-/-}$ mice resulted in an even more pronounced increase in glucose tolerance (**Figure 2C**). Similar to that observed in $A_{2B}^{-/-}$ mice, acute treatment with nitrate also improved glucose disposal (AUC 1493 ± 91 vs. 1779 ± 118; $n = 10$) in HFD-treated mice (Supplementary Material).

Pyruvate Tolerance Test

We probed whether nitrate influences gluconeogenesis in $A_{2B}^{-/-}$ mice, which could contribute to the production and clearance of glucose. To this end, the administration of the gluconeogenic substrate precursor pyruvate showed that there was no difference in glucose production in mice with nitrate pretreatment. Hence, nitrate had no significant impact on the gluconeogenesis pathway. However, upon glucose production (after 60 min), the clearance rates of glucose were again significantly faster in nitrate treated $A_{2B}^{-/-}$ mice compared to placebo group (**Figure 2D**; 120 min), confirming a promotion in glucose clearance upon acute treatment with inorganic nitrate.

Insulin Tolerance Tests and HOMA-IR

Insulin sensitivity after insulin injection (IPITT) was lower in $A_{2B}^{-/-}$ mice (**Figure 3B**) compared to WT (**Figure 3A**) resulting

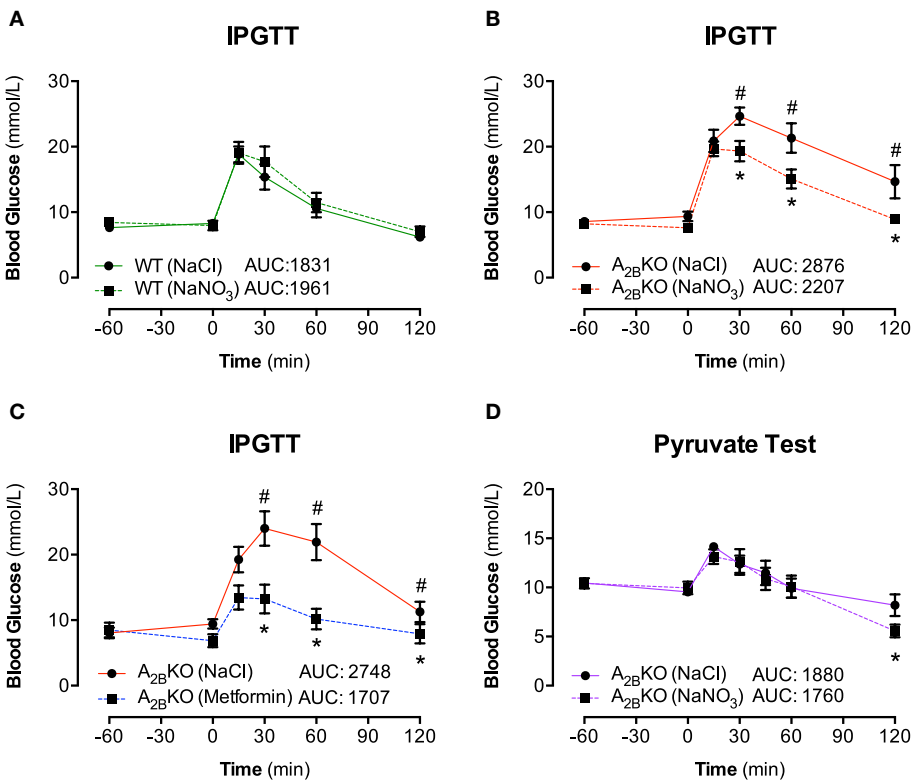

FIGURE 2 | IPGTT and Pyruvate test. The effect of inorganic nitrate on glucose tolerance was determined by measuring plasma glucose levels in WT mice **(A)** and A_{2B}KO mice **(B)** after placebo (NaCl) or nitrate injection ($NaNO_3$). Glucose levels after injection of metformin were determined in A_{2B}KO mice to investigate the effect of the anti-diabetic drug in mice with metabolic syndrome **(C)**. The impaired glucose tolerance in A_{2B}KO mice compared to WT could be significantly improved both with $NaNO_3$ and with metformin. Finally, to assess any potential effect of nitrate on gluconeogenesis, a pyruvate tolerance test was performed **(D)**. The total AUC (mmol/L/min) for the 0–120 min period was calculated. Values are mean ± SEM, $n = 10$–16/group. $^*p < 0.05$ vs. A_{2B}KO (NaCl); $^\#p < 0.05$ vs. WT.

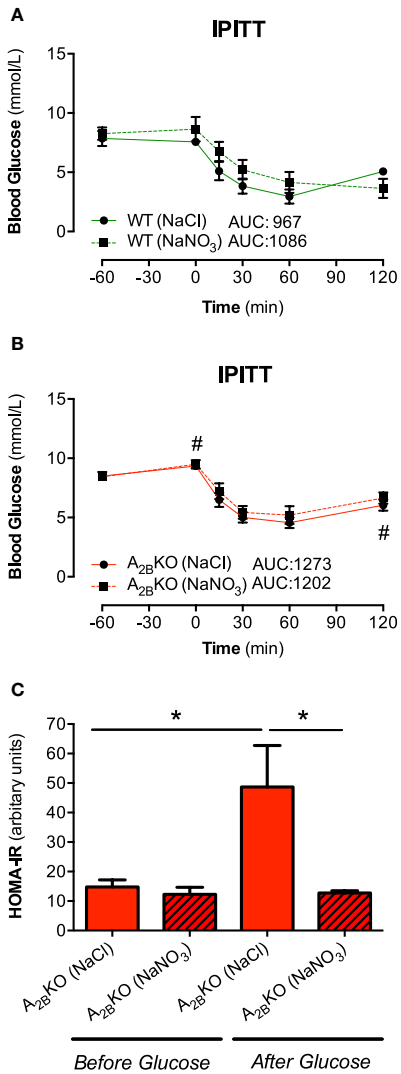

FIGURE 3 | IPITT and HOMA-IR. Plasma glucose levels in response to insulin injection were not influenced by nitrate treatment in WT **(A)** and A_{2B}KO mice **(B)**. HOMA-IR, a measure of insulin resistance, increased following glucose injection in A_{2B}KO mice, which was prevented by prior nitrate treatment **(C)**. Values are mean ± SEM, $n = 10$–16/group. $^{\#}p < 0.05$ vs. WT; $^{*}p < 0.05$ among the indicated groups.

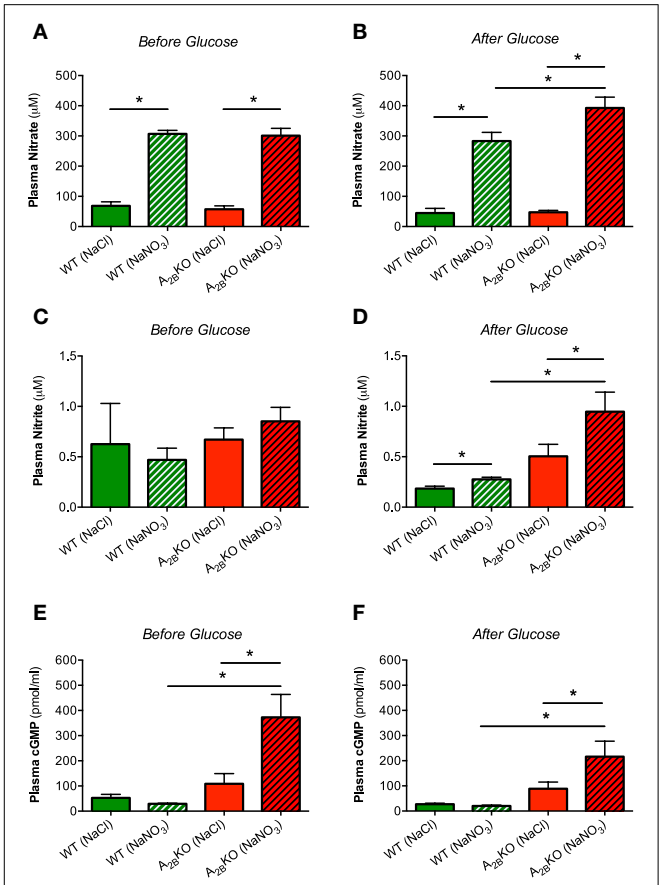

FIGURE 4 | Nitrate, nitrite and cGMP in plasma. Treatment with nitrate significantly increased plasma levels of nitrate in both genotypes before **(A)** and after glucose injection **(B)**. Plasma nitrite levels were significantly increased only in nitrate treated A_{2B}KO mice after glucose **(C, D)**. In A_{2B}KO mice, nitrate supplementation caused a significant increase of plasma cGMP levels both before **(E)** and after glucose injection **(F)**. Values are mean ± SEM, $n = 6$–14/group. $^{*}p < 0.05$ among the indicated groups.

in higher AUC. Glucose clearance did not differ between placebo or nitrate treated animals of both genotypes. In addition, insulin resistance (expressed as HOMA-IR) during fasting condition was similar in both genotypes but increased following glucose injection in the $A_{2B}^{-/-}$ mice (**Figure 3C**). This could be prevented by prior injection with nitrate.

Nitrate, Nitrite, and cGMP in Plasma

Nitrate treatment increased plasma nitrate levels in both WT and $A_{2B}^{-/-}$ mice as expected (**Figure 4A**) and this increase was even higher in $A_{2B}^{-/-}$ mice compared to WT after glucose injection (**Figure 4B**). Plasma nitrite levels were not different between groups during fasting (**Figure 4C**) but after glucose injection, $A_{2B}^{-/-}$ mice treated with nitrate showed significantly higher nitrite levels compared to WT and also $A_{2B}^{-/-}$ placebo group (**Figure 4D**). The second messenger cGMP, a central downstream NO signaling target, was not influenced by nitrate or glucose in WT mice (**Figures 4E,F**). However, in $A_{2B}^{-/-}$ mice treatment with nitrate resulted in a significant increase in plasma cGMP levels compared to the placebo group and WT mice.

NADPH Oxidase Activity in the Liver

NADPH oxidase-derived superoxide production in liver homogenates from $A_{2B}^{-/-}$ mice was significantly higher compared to WT mice (**Figure 5A**). Interestingly, nitrate treatment as well as injection of metformin significantly reduced superoxide production whereas nitrate had no effect on NADPH oxidase activity in WT mice. The same beneficial effects of

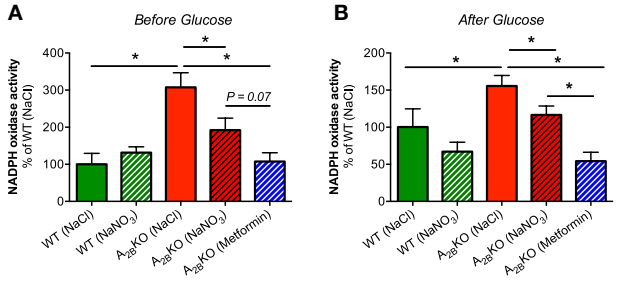

FIGURE 5 | NADPH oxidase activity in the liver. NADPH oxidase-derived superoxide formation was measured with lucigenin-dependent chemiluminescence signal in liver homogenates derived from A_{2B}KO and WT mice before **(A)** and after glucose injection **(B)**. A significantly increased NADPH oxidase activity was observed in A_{2B}KO compared to WT, which could be diminished by prior injection of nitrate or metformin. Values are mean ± SEM, $n = 6$–14/group. $^*p < 0.05$ among the indicated groups.

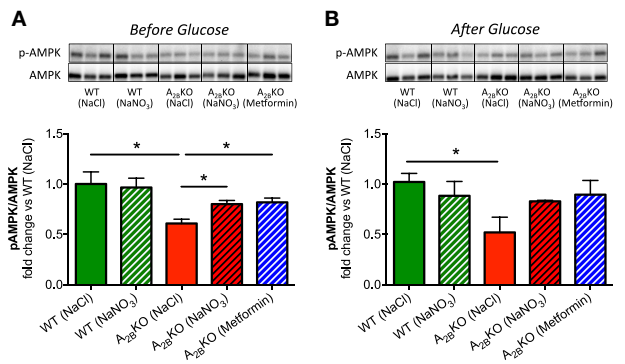

FIGURE 6 | AMPK regulation in the liver. Western blot was used to determine AMPK expression and phosphorylation in liver. Before **(A)** and after glucose injection **(B)**, A_{2B}KO mice showed significantly lower AMPK activation compared to WT. This could be improved by prior injection of nitrate or metformin. Three representative samples per group obtained from different gels are shown together with densitometric quantification, presented as ratio p-AMPK/AMPK. Values are mean ± SEM, $n = 4$/group. $^*p < 0.05$ among the indicated groups.

nitrate and metformin were observed after glucose injection (**Figure 5B**).

AMPK Regulation in the Liver

Expression and phosphorylation levels of AMPK were assessed in liver tissue derived from all animal groups. The ratio of phosphorylated AMPK to total AMPK, as a measure of AMPK activation, was significantly lower in $A_{2B}^{-/-}$ mice compared to WT (**Figures 6A,B**). This could be partially rescued by treatment with nitrate or metformin. As was the case with the NADPH oxidase activity, AMPK activation could also be improved with nitrate or metformin after glucose injection.

Discussion

Aged $A_{2B}^{-/-}$ Mice Present Characteristics of the Metabolic Syndrome

We confirmed previous studies (Johnston-Cox et al., 2012; Csóka et al., 2014) showing that aged $A_{2B}^{-/-}$ mice present several features of the metabolic syndrome; they are more obese than WT mice, display hyperglycemia and poor glucose clearance.

Impaired Liver AMPK Activation Could Contribute to the Metabolic Dysregulation in Aged $A_{2B}^{-/-}$ Mice

The mechanisms leading to the development of this impaired metabolic regulation in the $A_{2B}^{-/-}$ mice are still being investigated and there are many unanswered questions. Major contributing factors are elevated hepatic inflammation and IRS-2 expression (Johnston-Cox et al., 2012) and augmented classical macrophage activation in the adipose tissue (Csóka et al., 2014). In this study we focused mainly on the liver since it is an organ of high importance in glucose metabolism and T2D development (Bechmann et al., 2012) and has not been extensively investigated in $A_{2B}^{-/-}$ mice. AMPK is an important intracellular energy sensor and one of the key players in maintaining liver glucose homeostasis (Viana et al., 2006; Wang et al., 2012), which is often downregulated under hyperglycemic conditions (Kraegen et al., 2006). The ability to activate liver AMPK is a major reason why the anti-diabetic drugs metformin and 5-amino-4-imidazolecarboxamide riboside (AICAR) were developed and used as treatments (Towler and Hardie, 2007). In our study we show for the first time that aged $A_{2B}^{-/-}$ mice present lower phosphorylation levels of liver AMPK compared to normoglycemic WT mice of similar age. In agreement with that, the AMPK activator metformin elevated liver AMPK phosphorylation levels together with a remarkable improvement of glucose clearance. Thus, one may speculate that impaired AMPK activation is one of the factors contributing to metabolic dysfunction in this animal model.

Elevated Liver NADPH Oxidase Activity Could Contribute to the Metabolic Dysregulated Phenotype of the Aged $A_{2B}^{-/-}$ Mice

Another key regulator of liver glucose uptake and metabolism is the enzyme family of NADPH oxidases. It is well known that elevated levels of NADPH oxidase-derived superoxide in the liver diminish glucose uptake and contribute to the development of hyperglycemia (Guichard et al., 2008). To our knowledge, this is the first study showing that $A_{2B}^{-/-}$ mice present higher levels of liver NADPH oxidase activity compared to WT, both before and after glucose treatment. Therefore, the investigation of pharmacological interventions to target liver NADPH oxidases may be of great interest for the improvement of the metabolic phenotype when the A_{2B} receptors are ablated.

AMPK and NADPH Oxidases are Closely Linked

Several studies have indicated that pharmacological activation of AMPK can reduce NADPH oxidase activity and expression

in various target organs and cells like liver (Adachi and Brenner, 2008), cardiomyocytes (Balteau et al., 2014), podocytes (Piwkowska et al., 2010), and human umbilical vein endothelial cells (Ceolotto et al., 2007). One mechanism leading to reduced production reactive oxygen species (ROS) might be via upregulated mRNA expression of the antioxidant enzymes SOD2 and catalase, as it was seen in activated hepatic stellate cells treated with AMPK activators AICAR or metformin (Adachi and Brenner, 2008). Another mechanism of how activated AMPK can inhibit NADPH oxidase activity was shown in activated human neutrophils where AMPK activation with AICAR prevented phosphorylation and membrane translocation of the cytosolic NADPH oxidase subunit p47phox, which are both crucial to the enzyme activation (Alba et al., 2004). However, it is still unknown if this inhibition of NADPH oxidases and ROS production by p-AMPK can lead to improved glucose clearance. Speaking in favor of this concept, activation of AMPK by inorganic nitrate or metformin in our study was clearly associated with both reduced NADPH oxidase activity and improved glucose tolerance in $A_{2B}^{-/-}$ mice. Functional AMPK may therefore be important as an early warning system for oxidative stress to trigger compensatory antioxidant effects, which in turn improve glucose uptake. Future studies are needed to investigate if inorganic nitrate exerts its beneficial effect via upregulation of antioxidant enzymes, prevention of p47phox phosphorylation and translocation or another mechanism.

The Nitrate-Nitrite-NO Pathway is Upregulated in the Aged $A_{2B}^{-/-}$ Mice

We and other groups have previously shown that long-term treatment with inorganic nitrate or nitrite improves glucose clearance, insulin sensitivity, and reduces visceral fat levels during aging and obesity (Carlström et al., 2010; Hezel et al., 2015; Singamsetty et al., 2015). However, the underlying mechanisms of how inorganic nitrate and nitrite mediate their beneficial effects remain to be elucidated. In the current study we investigated if acute treatment with inorganic nitrate can exert similar effects as observed with long-term supplementation. Interestingly, nitrate treatment significantly increased the plasma levels of nitrite and cGMP only in $A_{2B}^{-/-}$ mice, but not in the WT, and the increase in plasma nitrate levels was higher in $A_{2B}^{-/-}$ mice compared with WT after glucose load. Since the baseline levels of nitrate, nitrite and cGMP were similar between WT and $A_{2B}^{-/-}$ it seems that the nitrate-nitrite-NO pathway is sensitized in the absence of A_{2B} receptors. Activation of the A_2 receptors has been linked with increased NOS activation, and therefore it is likely that the NOS function is compromised in the A_{2B} knockouts. There are publications showing that A_2 receptor signaling is associated with higher NO production in the renal microvasculature (Carlström et al., 2011b) and in the liver during ischemia/reperfusion injury (Peralta et al., 1999). Moreover, A_2 receptor activation, especially of the type 2B, leads to higher NO production in coronary artery endothelial cells (Olanrewaju and Mustafa, 2000) and enhances vasorelaxation in mouse aorta (Ansari et al., 2007). Another mechanism potentiating the action of nitrate in the $A_{2B}^{-/-}$ mice but not in WT might be via the higher superoxide levels. Several studies in redox biology suggest that stimulating NO production or antioxidant systems are more potent when there are higher levels of ROS and in particular superoxide (Wink et al., 2001; Silva et al., 2012; Araujo and Wilcox, 2014). Since activation of A_{2B} receptors can facilitate NO production and mice lacking these receptors already present higher levels of liver superoxide one could speculate that this oxidative stress leads to a more prominent and faster activation of the alternative nitrate-nitrite-NO pathway.

The Nitrate-Nitrite-NO Pathway in T2D: Clinical and Experimental Data

In recent bibliography there are several experimental *in vivo* and *in vitro* studies showing favorable effects of inorganic nitrate and nitrite in T2D (Bahadoran et al., 2015). The proposed mechanisms involve compensation for disturbed eNOS-derived NO generation (Carlström et al., 2010), improved antioxidant capacity (Khalifi et al., 2015), and increased pancreatic islet blood flow and insulin secretion (Nyström et al., 2012). Moreover, nitrate/nitrite-mediated NO production may also improve insulin resistance and glucose uptake by activation of glucose transporter 4 (GLUT4) (Jiang et al., 2014; Ohtake et al., 2015). Apart from the experimental reports some clinical studies have been conducted. So far there is evidence that inorganic nitrate or nitrite could have beneficial effects on overweight or slight obese patients or in T2D despite no clear correlation between T2D and plasma or urinary levels of nitrate and nitrite (Bahadoran et al., 2015). Joris et al. showed that supplementation with beetroot juice, which is high in inorganic nitrate, improved postpranial endothelial function in slight overweight or obese men (Joris and Mensink, 2013). In patients with T2D, a single dose of inorganic nitrate was suggested to lower basal plasma glucose and improve oral glucose insulin sensitivity index, however no improvement in glucose tolerance was observed (Cermak et al., 2015). In another small study in patients with T2D, Gilchrist and colleagues did not observe improvement in endothelial function or insulin sensitivity (Gilchrist et al., 2013), but their findings suggested that dietary nitrate could improve cognitive function in diabetic patients (Gilchrist et al., 2014). Taken together, despite several studies reporting beneficial properties with inorganic nitrate and nitrite, the data from small size clinical studies are still contradictory and clearly show the need for a carefully conducted large-scale, long-term follow up trial in patients.

In summary, the present study demonstrates an important influence of acute inorganic nitrate treatment in modulating metabolic functions. In aged A_{2B} receptor knockout mice, characterized by metabolic syndrome, inorganic nitrate improved their glucose clearance and this was associated with increased AMPK activation and reduced NADPH oxidase activity in the liver. Similar favorable effects of acute nitrate on glucose disposal was also observed in HFD-treated obese WT mice. Intriguingly, the dose of nitrate was similar to what is found in a single serving of a green leafy vegetable; the predominant dietary source of nitrate. These findings suggest that the beneficial effects of inorganic nitrate act not only long-term but also acutely and future studies should be

aimed at determining the therapeutic value of dietary nitrate supplementation in patients with metabolic disease.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2015.00222

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The protective effects of oral low-dose quercetin on diabetic nephropathy in hypercholesterolemic mice

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Aims: Diabetic nephropathy (DN) is one of the most important causes of chronic renal disease, and the incidence of DN is increasing worldwide. Considering our previous report (Gomes et al., 2014) indicating that chronic treatment with oral low-dose quercetin (10 mg/Kg) demonstrated anti-oxidative, anti-apoptotic and renoprotective effects in the C57BL/6J model of DN, we investigated whether this flavonoid could also have beneficial effects in concurrent DN and spontaneous atherosclerosis using the apolipoprotein E-deficient mouse (apoE$^{-/-}$).

Methods: Streptozotocin was used to induce diabetes (100 mg/kg/day, 3 days) in male apoE$^{-/-}$ mice (8 week-old). After 6 weeks, the mice were randomly separated into DQ: diabetic apoE$^{-/-}$ mice treated with quercetin (10 mg/kg/day, 4 weeks, $n = 8$), DV: diabetic ApoE$^{-/-}$ mice treated with vehicle ($n = 8$) and ND: non-treated non-diabetic mice ($n = 8$).

Results: Quercetin treatment diminished polyuria (∼30%; $p < 0.05$), glycemia (∼25%, $p < 0.05$), normalized the hypertriglyceridemia. Moreover, this bioflavonoid diminished creatininemia (∼30%, $p < 0.01$) and reduced proteinuria but not to normal levels. We also observed protective effects on the renal structural changes, including normalization of the index of glomerulosclerosis and kidney weight/body weight.

Conclusions: Our data revealed that quercetin treatment significantly reduced DN in hypercholesterolemic mice by inducing biochemical changes (decrease in glucose and triglycerides serum levels) and reduction of glomerulosclerosis. Thus, this study highlights the relevance of quercetin as an alternative therapeutic option for DN, including in diabetes associated with dyslipidemia.

Keywords: quercetin, apoE, diabetes, streptozotocin, atherosclerosis, nephropathy

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Introduction

Diabetic nephropathy (DN) is the major cause of chronic renal disease in industrialized nations and is linked with a significant increase in cardiovascular morbi-mortality (Foggensteiner et al., 2001; Ahmad, 2015; Donate-Correa et al., 2015). It occurs because of an interaction between both genetic and environmental factors in diabetic individuals, such as genetic pre-disposition, sedentary lifestyle, hypertension, persistent hyperglycemia and dyslipidemia (Lassila et al., 2004; Matheus et al., 2013; Ahmad, 2015). By different routes, all these factors can contribute directly and/or indirectly to an abnormal balance between reactive oxygen species (ROS) production and its antioxidant mechanisms aggravating the pathogenesis of DN (Lassila et al., 2004; Xu et al., 2006; Duran-Salgado and Rubio-Guerra, 2014; Gorin and Wauquier, 2015; Lv et al., 2015).

In recent decades, although the use of animal models has provided new insights into understanding the pathogenesis, diagnosis and treatment of nephropathy (Balakumar et al., 2008), most of the models employed do not associate comorbidities, limiting the extrapolation of these studies to humans. In an attempt to combine the effects of two severe clinical risk factors (dyslipidemia and diabetes) for renal disease, we used the hyperlipidemic diabetic apolipoprotein E-deficient (apoE$^{-/-}$) mouse in our study. Recent data indicate that when this animal is administered streptozotocin (STZ), a toxin widely used to induce experimental diabetes (Like and Rossini, 1976; Vessal et al., 2003), it develops accelerated hypercholesterolemia/atherosclerosis (Candido et al., 2004; Vedantham et al., 2011) and nephropathy (Wen et al., 2002; Lassila et al., 2004; Xu et al., 2006).

Considering that only partial renoprotection from DN is achieved by current standard therapies (e.g., by the inhibition of the renin-angiotensin-aldosterone system), the search for alternative, effective and safer therapeutic approaches is an interesting goal. In this context, recent findings from our laboratory (Gomes et al., 2014) demonstrate that an orally administered low-dose of the antioxidant quercetin (10 mg/Kg), a bioflavonoid ubiquitously contained in vegetables and fruits (Kawabata et al., 2015), exhibits metabolic, anti-oxidative, anti-apoptotic and renoprotective effects in the C57BL/6J mouse model of DN. In parallel, others have found cardiovascular protection from quercetin in the ApoE$^{-/-}$ mouse model (Lara-Guzman et al., 2012; Ulasova et al., 2013). In light of these evidences, we tested the hypothesis that, due to its antioxidant properties, quercetin treatment could improve metabolic parameters and renal function in the diabetic apoE$^{-/-}$ mouse model.

Materials and Methods

Animals

The apoE$^{-/-}$ male mice (8 week-old, $n = 24$) were obtained from the animal facilities of the Laboratory of Translational Physiology, at the Federal University of Espirito Santo, Brazil. The mice were fed a normal laboratory chow diet (Labina®) and water *ad libitum* until the time of the experiments. The animals were housed at 22°C, 50% humidity with a 12 h-light/12 h-dark cycle. All of the procedures were conducted in accordance with of the institutional guidelines for animal research, and the protocols were previously certified by the Institutional Ethics Committee for Use of Animals (Protocol # 013/2010).

Experimental Protocol

Diabetes was induced by three daily intraperitoneal injections of streptozotocin (STZ, Boehringer Mannheim, Mannheim, Germany) at a dose of 100 mg/kg diluted in citrate buffer solution (10 mM, pH 4.5). Non-diabetic apoE$^{-/-}$ mice were administered the vehicle citrate buffer and served as controls. One week after the STZ injection, the glycemia was measured using blood samples (tail vein) obtained from mice after 6 h of inanition. The inclusion criteria were those animals that 1 week after STZ injection exhibited hyperglycemia (>250 mg/dL), when it was confirmed at least in two independent moments (success rate was approximately 65%). After 6 weeks, the animals were randomized to receive vehicle (soy oil, DV, $n = 8$) or oral quercetin (DQ, $n = 8$; Sigma, St. Louis, MO, USA) at a dosage of 10 mg/kg per day orally for 4 weeks, based on our prior study (Gomes et al., 2014) and others (Ajay et al., 2006; Machha and Mustafa, 2005).

Metabolic and Biochemical Parameters

The body weight of all the animals was measured weekly. At week 4, the mice were adapted to 24-h in individual metabolic cages. Thereafter, a known quantity of food and water were positioned in the feeder and the drinking bottles, respectively. After 24 h, we measured the volume of water and amount of chow remaining in the cages. Urine volume was measured and protein concentration was determined by the Bradford method (Bradford, 1976). Finally, animals received a lethal dose of thiopental (Cristalia, Sao Paulo, Brazil, 200 mg/kg, i.p.) after 6 h of inanition in the morning. The blood samples were collected using the retro-orbital sinus of the mouse as a source of venous blood for all measurements, with exception of the determination of glycemia, which was trough the tail venipuncture. The biochemical analysis of glucose, triglycerides, cholesterol, creatinine, urea and uric acid measurements were performed by colorimetric kits. Animals were perfused with cold PBS (pH 7.4, 0.1 mol/L) through the left ventricle. Creatinine clearance was calculated using serum and urine creatinine levels and urine flow through the standardized formula: [urine creatinine concentration (mg/dL) × 24 h urine volume (μL)]/[serum creatinine concentration (mg/dL) 1440 min].

Kidney Histology

After perfusion of the animal, the kidneys were carefully fixed with Duboscq solution (aqueous solution of 0.4% picric acid, 54% ethanol, 27% formaldehyde, and 7% acetic acid), weighed and managed for histological and morphometric analysis. The samples were dehydrated in increasing concentrations of alcohol and finally mounted in paraffin blocks. Thereafter, the kidneys were sliced using a microtome into 3-μm-thick cross-sections with hematoxylin-eosin staining. Images were obtained with video camera (VKC150, Hitachi, Tokyo, Japan) connected to a microscope (AX70, Olympus, Center Valley, PA, USA). The

mean glomerular tuft area of each kidney was obtained by calculating the mean value of 30 individual glomeruli measured by Image J software (version 1.33u, Public Domain). Masson's trichrome staining was used to quantify glomerulosclerosis. A total of 30 glomeruli were used to calculate the percentage of the stained area for each kidney using the Image J program (Public Domain Image Processing Program, National Institutes of Health, Bethesda, MD).

Statistical Analysis

The data are presented as the mean ± SEM. The normality of the variables was tested by Kolmogorov-Smirnov. The statistical analysis was performed using One-Way analysis of variance (ANOVA) followed by the Tukey's *post-hoc* test using Prism software (Prism 6, GraphPad Software, Inc., San Diego, CA, USA). The level of significance was set at $p < 0.05$.

Results

The Effects of Quercetin on Metabolic Parameters

Figure 1 summarizes the data obtained through metabolic cages (food intake, water intake, and urine volume) and the body weight gain in the three groups studied. DV mice showed hyperphagia ($p < 0.05$, **Figure 1A**) and polydipsia ($p < 0.05$, **Figure 1B**) when compared with ND mice and no effect of quercetin was observed on these parameters. Interestingly, DV mice showed polyuria ($p < 0.05$), which was reduced by approximately 30% of DQ mice ($p < 0.05$) (**Figure 1C**). Body weight was statistically similar in the three groups at the beginning of the protocol, but as shown in **Figure 1D**, over the 2-week period, only the DV mice showed reductions in body weight, in contrast to the ND mice and DQ mice ($p < 0.05$), which showed significant increases in body weight.

Effects of Quercetin on Biochemical Parameters

As summarized in **Figure 2**, DV mice exhibited a significant augmentation in glycemia (2.5-fold), triglycerides (1.9-fold) and total cholesterol (2.3-fold) when compared with control ND mice ($p < 0.05$). The treatment of diabetic apoE$^{-/-}$ mice with quercetin caused significant attenuation of plasma glucose (∼25%) and abolished the hypertriglyceridemia ($p < 0.05$); however, this dose of quercetin did not reverse the hypercholesterolemia.

Effects of Quercetin on Kidney Functional and Morphometric Parameters

Figure 3 shows the mean values of the traditional renal function biomarkers. As expected, DV mice exhibited significantly high plasma concentrations of creatinine (**Figure 3A**), urea (**Figure 3B**), uric acid (**Figure 3D**), and impairment of renal clearance (**Figure 3C**) compared with ND animals ($p < 0.05$). In DQ mice, plasma creatinine and clearance returned to baseline levels ($p < 0.05$, **Figure 3C**). In addition, quercetin did not modify the high plasma both urea and uric acid ($p > 0.05$). Proteinuria was significantly increased (4.4-fold, $p < 0.05$) in the DV mice compared to the ND mice ($p < 0.01$, **Figure 3E**). Treatment with quercetin showed a tendency to reduce proteinuria (∼15%), but the levels were still significantly higher than those of the ND mice.

Diabetes was related to an augment of ∼35% in the kidney weight/body weight ratio when compared with ND mice ($p <$

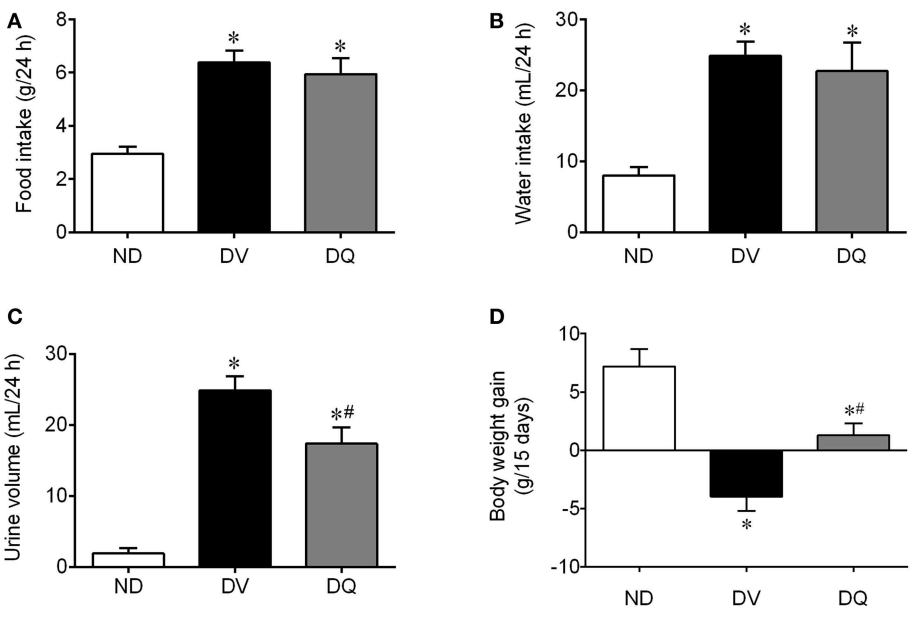

FIGURE 1 | Food and water intake, urine volume and body weight gain in diabetic apoE$^{-/-}$ mice treated with quercetin (DQ) compared with diabetic apoE$^{-/-}$ mice administered vehicle (DV) compared with non-diabetic apoE$^{-/-}$ (ND) mice. Values are the means ± SEM for $n = 6$–8 mice per group. $^{*}p < 0.05$ vs. ND, $^{\#}p < 0.05$ vs. DV.

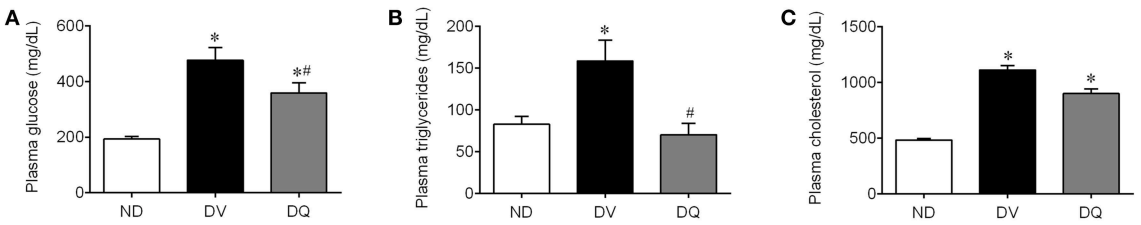

FIGURE 2 | Total plasma glucose (A), triglycerides (B), and cholesterol (C) in diabetic apoE$^{-/-}$ mice treated with quercetin (DQ) compared with diabetic apoE$^{-/-}$ mice administered vehicle (DV) compared with non-diabetic apoE$^{-/-}$ (ND) mice. Values are the means ± SEM for $n = 6$–8 mice per group. $^{*}p < 0.05$ vs. ND, $^{\#}p < 0.05$ vs. DV.

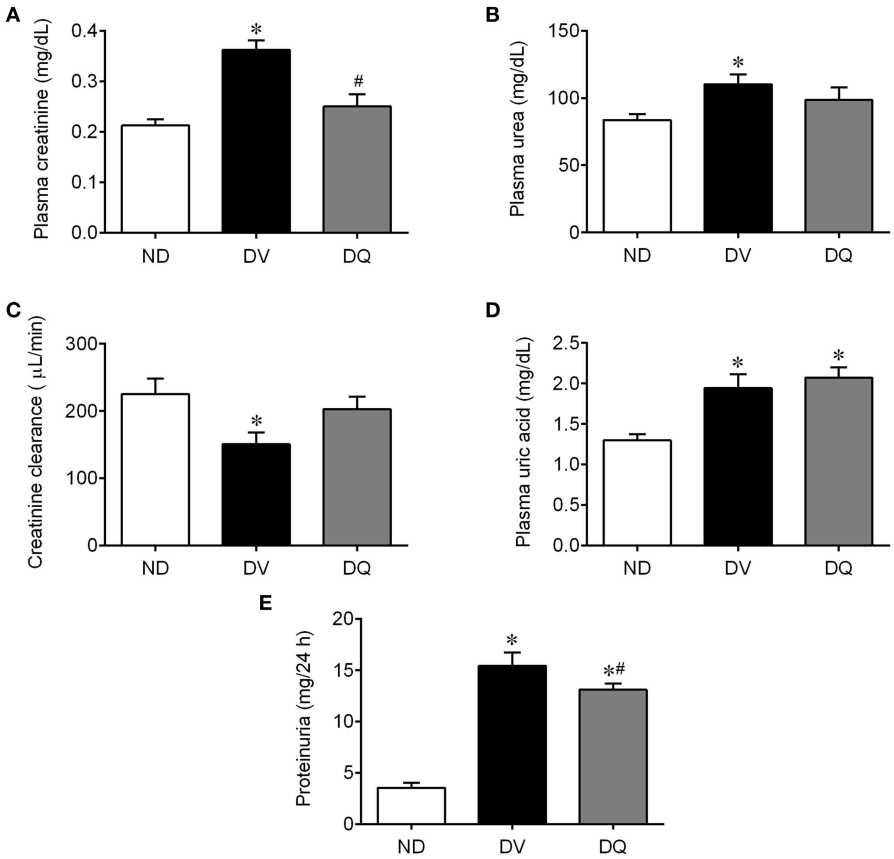

FIGURE 3 | Plasma creatinine (A), urea (B), creatinine clearance (C), uric acid (D), and proteinuria (E) in diabetic apoE$^{-/-}$ mice treated with quercetin (DQ) compared with diabetic apoE$^{-/-}$ mice administered vehicle (DV) compared with non-diabetic apoE$^{-/-}$ (ND) mice. Values are the means ± SEM for $n = 6$–8 mice per group. $^{*}p < 0.05$ vs. ND, $^{\#}p < 0.05$ vs. DV.

0.05), whereas quercetin reversed this consequence of diabetes in the apoE$^{-/-}$ mice ($p < 0.05$, **Figure 4A**). As illustrated in the typical microscopy images (**Figure 4D**), the glomerulosclerosis, which was characterized by glomerular hyperplasia and by deposition of extracellular matrix in the mesangium, was more prominent in the DV mice, than in the ND mice, and quercetin showed a favorable effect on this condition. More specifically, the analysis of glomerulosclerosis demonstrated a significant increase of approximately 50% when compared with ND mice ($p < 0.05$), and quercetin abolished this glomerular injury (**Figure 4B**). Additionally, the mean glomerular tuft area of each kidney revealed an increase of approximately 40% compared to those of ND mice ($p < 0.05$), and DQ had a tendency to attenuate this glomerular injury (**Figure 4C**).

Discussion

Recent data from our laboratory showed that oral low-dose quercetin ameliorated the consequences of hyperglycemia-induced ROS overproduction in the kidney

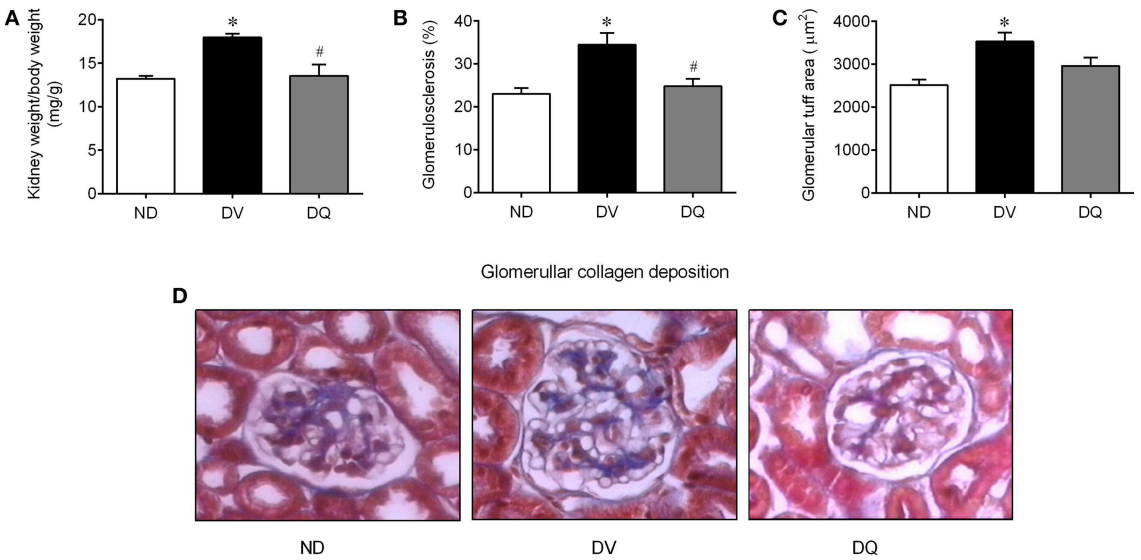

FIGURE 4 | Kidney weight (wt)/body weight ratio (A), glomerulosclerosis (B), glomerular tuff area (C) in diabetic apoE$^{-/-}$ mice treated with quercetin (DQ) compared with diabetic apoE$^{-/-}$ mice administered vehicle (DV) compared with non-diabetic apoE$^{-/-}$ (ND) mice. Photomicrographs **(D)** are representative glomerular sections (magnification of 400x), stained with Masson's trichrome. Values are the means ± SEM for $n = 6$–8 mice per group. $^{*}p < 0.05$ vs. ND, $^{\#}p < 0.05$ vs. DV.

in wild-type diabetic mice (Gomes et al., 2014), the most common genetic background for gene-modified mice (Haug et al., 2013). The novelty of this study is that the same dose of this bioflavonoid was capable of reducing the classical signs of diabetes and attenuated the progression of nephropathy in diabetic-induced apoE$^{-/-}$ mice. These data are reinforced by a reduction in hyperglycemia, hypertriglyceridemia, azotemia, proteinuria and the diminution of mesangial matrix expansion in the kidneys of diabetic apoE$^{-/-}$ mice.

Although there are limitations to the experimental diabetic mouse model compared to humans (Wu and Huan, 2007; Müller et al., 2012), STZ-induced diabetic ApoE$^{-/-}$ mouse is an interesting model for exploring new therapeutic options for diabetes-associated dyslipidemia and renal injury. First, the diabetic condition in this model is preserved for many weeks, which allowed us long periods of treatment with quercetin. Second, the diabetic status is not refractory to medical interventions (Wu and Huan, 2007), which was evidenced in our study in the antidiabetic protection observed with administration of this bioflavonoid. Moreover, in order to avoid interference with the direct nephrotoxic effects of STZ, the experiments were performed after 6 weeks to avoid acute renal injury bias (Ortega et al., 2013; Gomes et al., 2014).

As in other STZ models, STZ-induced diabetic ApoE$^{-/-}$ mice showed damaged β cells that compromised the secretory capacity of insulin (Like and Rossini, 1976; Wu and Yan, 2015). Consequently, this atherosclerotic model exhibits the expected progressive signs of the disease, such as hyperglycemia, polyuria, polydipsia, polyphagia, proteinuria and the decline of renal function, similar to those in C57BL/6J mice (Gomes et al., 2014). Based on the 5 stages of the clinical classification of DN and by the sum of these characteristics (Mogensen et al., 1983; Jerums et al., 2009), we consider this model to correspond to stage 4 clinical classification because the animals exhibited a diminished creatinine clearance and proteinuria similar to that observed in C57BL/6J mice (Gomes et al., 2014). Exceptionally, only the loss of body weight was more evident when compared to their respective genetic background, probably due to the lack of apoE. Pendse et al. (2009) demonstrated that the absence of this apolipoprotein contributes directly to the suppression of body weight gain and consequent fat accumulation in apoE$^{-/-}$ mice, which corroborates our hypothesis.

For more than almost 20 years, it has been known that oxidative stress plays a crucial role in the development of diabetic complications (Baynes, 1991; Wright et al., 2006; Alam et al., 2014). In this context, the search for nontoxic natural antioxidant compounds to prevent oxidative damage in experimental models of diabetes (Wang et al., 2012) and in diabetic patients (Valensi et al., 2005; Lobo et al., 2010; Sunarwidhi et al., 2014) has been intensified in recent years. Typically, the best candidates are molecules that exhibit high antioxidant activity, long half-lives (Sesink et al., 2001; Manach et al., 2005), and high mitochondrial permeability (Ortega and García, 2009) and are able to suppress pro-oxidant enzymes and stimulate antioxidant enzymes (Bouayed and Bohn, 2010). Interestingly, quercetin exhibits all of these advantages (Sanders et al., 2001; Ortega and García, 2009; Gomes et al., 2014). Similarly, recent studies from our group (Gomes et al., 2014) and other groups (Pereira Braga et al., 2013) have demonstrated that this bioflavonoid diminishes ROS bioavailability through distinct pathways: (1) by the chelation of metals, (2) by neutralizing lipid peroxyl radicals, (3) by interacting directly with O_2^- during

initiation and (4) by increasing the activity of glutathione peroxidase/reductase/transferase, superoxide dismutase and catalase (Oršolic et al., 2011; Alam et al., 2014).

Although the antioxidative benefits of quercetin are well-established in diabetic experimental models (Kobori et al., 2009; Oršolic et al., 2011; Kanter et al., 2012), other effects still require further investigation (Youl et al., 2010; Gomes et al., 2014). Interestingly, our results demonstrated for the first time that quercetin attenuates hyperglycemia in a mouse model of dyslipidemia and diabetes, as observed recently by others in diabetic rats (Kanter et al., 2012), in Balb/C mice (Kobori et al., 2009) and by us in diabetic C57BL/6J mice (Gomes et al., 2014). The beneficial effect of quercetin on glycemia may work through different mechanisms, such as through the stimulation of glucose influx via GLUT4 (Alam et al., 2014; Xu et al., 2014) and via augmented glucokinase activity and, consequently, the increase in glucose liver uptake, inhibiting hepatic glycogenolysis and gluconeogenesis (Alam et al., 2014). Moreover, it has been shown that quercetin can inhibit α-glucosidase (Ishikawa et al., 2007; Kim et al., 2011) and the intestinal glucose transporter GLUT2 (Kwon et al., 2007), reducing the absorption of monosaccharides in the small intestine. Because the low dose of quercetin we used has been associated with intrinsic low bioavailability, interference with the absorption of monosaccharides seems reasonable (Gomes et al., 2014) and is consistent with the findings of Galindo et al. (2012), who showed a better effect when compared to administration via the intraperitoneal route. However, we cannot exclude the protective role of quercetin in Langerhans β-cells from damage on improving insulin production in STZ models, as observed by others (Vessal et al., 2003; Kim et al., 2011). Independent of this mechanism, the attenuation of chronic hyperglycemia reduces damage to a number of cell types through several pathways, such as the augmented formation of advanced glycation end-products (AGEs) and its respective receptor, polyol pathway flux, the overactivity of the hexosamine pathway, activation of protein kinase C (PKC) isoforms and even mitochondrial dysfunction (Wright et al., 2006; Giacco and Brownlee, 2010; Alam et al., 2014), which attenuates progressive damage to major target organs.

Although treatment with quercetin ameliorated the reduction in body weight gain and polyuria, it probably prevented reductions in body weight gain and polyuria. This effect may be justified as a consequence of better glycemic control, with a reduction of the compensatory lipolytic response and consequent normalization of triglyceridemia without modifying the hypercholesterolemia, as recently observed by our group (Gomes et al., 2014) and others (Ozcelik et al., 2011). Furthermore, we cannot reject the possibility of a modification in the non-HDL/HDL ratio, which maintains invariable total serum cholesterol levels (Negi et al., 2013; Gomes et al., 2014).

In a previous study, we have shown that apoE$^{-/-}$ mice exhibit early impaired renal function when compared with normocholesterolemic C57 mice (Balarini et al., 2011). Now, using the experimental model of DN aggravated by hyperlipidemia, we observed signs of renal glomerular injury, which could be justified by azotemia with reduced creatinine clearance associated with the histological assessment. Moreover, the glomerular tuft size was exacerbated in diabetic apoE$^{-/-}$ mice, indicating an initial diabetes-induced renal injury, which is consistent with the literature (Xu et al., 2006; Menini et al., 2015). For the first time, our study demonstrates that treatment with quercetin ameliorated the glomerulosclerosis and recovered the kidney weight/body weight ratio. However, we emphasize that this latter finding should not be interpreted as an occurrence of renal hypertrophy because we observed that the diabetic animals exhibited lower body weight. Additionally, this bioflavonoid also exhibited marked beneficial effects on renal function as indicated by the significant decrease of creatininemia, restoration of the clearance of creatinine and tended to reduce the proteinuria in diabetic apoE$^{-/-}$ mice. The non-modification of the uremia and uric acid parameters may be justified by the following: (1) an intense purine and amino acid catabolism (respectively) in this induced diabetic experimental model (Gomes et al., 2014) and (2) by more glomerular sensitivity to oxidative injuries than other nephron segments (Schena and Gesualdo, 2005; Gomes et al., 2014), favoring the amelioration of renal filtration that we observed in the present study. All of these renoprotective effects of quercetin could be explained by direct benefits such as the vasorelaxant effect in vascular tissues recently described (Schena and Gesualdo, 2005; Lodi et al., 2009; Galindo et al., 2012), in addition to indirect effects such as its hypoglycemic/anti-dyslipidemic actions (Lassila et al., 2004) and the reduction of ROS formation (Gomes et al., 2014). Likewise, we cannot reject that quercetin can also positively modulate the functional activities of endothelial progenitor cells (EPCs) in vascular and kidney repair after damage, as observed recently *in vitro* by Zhao et al. (2014), offering new insights into antidiabetic therapies.

In conclusion, we have demonstrated that an oral administered low-dose of quercetin exhibits antidiabetic and renoprotective effects in a mouse model of concurrent apoE$^{-/-}$-induced hypercholesterolemia and STZ-induced DN. Although further studies are needed to reveal the intrinsic mechanisms involved, this bioflavonoid is a potential nutraceutical alternative to prevent and/or treat renal dysfunction caused by diabetes and dyslipidemia as shown in the present study.

Author Contributions

Conception and design of the experiments: IG, MP, AG, and EV. Collection, analysis and interpretation of the data: IG, MS, BC, EV, and TP. Drafting or revising the article critically for intellectual content: SM, TP, and EV.

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Uninephrectomy in rats on a fixed food intake results in adipose tissue lipolysis implicating spleen cytokines

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The role of mild kidney dysfunction in altering lipid metabolism and promoting inflammation was investigated in uninephrectomized rats (UniNX) compared to Sham-operated controls rats. The impact of UniNX was studied 1, 2, and 4 weeks after UniNX under mild food restriction at 90% of *ad libitum* intake to ensure the same caloric intake in both groups. UniNX resulted in the reduction of fat pad weight. UniNX was associated with increased circulating levels of beta-hydroxybutyrate and glycerol, as well as increased fat pad mRNA of hormone sensitive lipase and adipose triglyceride lipase, suggesting enhanced lipolysis. No decrease in fat pad lipogenesis as assessed by fatty acid synthase activity was observed. Circulating hormones known to regulate lipolysis such as leptin, T3, ghrelin, insulin, corticosterone, angiotensin 1, and angiotensin 2 were not different between the two groups. In contrast, a select group of circulating lipolytic cytokines, including interferon-gamma and granulocyte macrophage–colony stimulating factor, were increased after UniNX. These cytokine levels were elevated in the spleen, but decreased in the kidney, liver, and fat pads. This could be explained by anti-inflammatory factors SIRT1, a member of the sirtuins, and the farnesoid x receptor (FXR), which were decreased in the spleen but elevated in the kidney, liver, and fat pads (inguinal and epididymal). Our study suggests that UniNX induces adipose tissue lipolysis in response to increased levels of a subset of lipolytic cytokines of splenic origin.

Keywords: uninephrectomy, lipolysis, body composition, cytokines, spleen

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Introduction

Disease conditions such as the metabolic syndrome, diabetes, obesity, inflammation and infection, are often associated with diminished kidney function. It is generally believed that this reduction in kidney function is a consequence of the progression of the disease. Recent evidence in both humans and animal models suggests that a primary reduction in kidney function may also play a role in altering metabolism (Odamaki et al., 1999; Zhao et al., 2011) inflammation and oxidative stress (Zheng et al., 2011) and hence in the pathogenesis of the disease.

Previous studies of the consequences of uninephrectomy (UniNX) in Sprague Dawley rats have shown that there was no difference in body weight and no evident changes in metabolic profile

Abbreviations: ASP, acylation stimulating protein; ATGL, adipose triglyceride lipase; FXR, farnesoid x receptor; GM-CSF, granulocyte-macrophage colony stimulating factor; HSL, hormone sensitive lipase; IFNγ, interferon-gamma; UniNX, uninephrectomy.

and tissue pathology up to 3 months. Afterwards, pathologies start to appear, in particular deterioration of kidney function, fatty infiltration into various tissues (Zhao et al., 2008, 2011) and the progressive development of glucose intolerance (Sui et al., 2007). However, the mechanisms underlying these temporal changes from subtle changes to chronic severe changes in metabolic and immune regulation are not clearly defined. Angiotensin may play a role in metabolic and immune changes observed in kidney disease (Amorena et al., 2001; Deferrari et al., 2002), but its contribution in early reduced kidney function remains to be determined.

More recently, studies have shown that other factors regulating metabolism and inflammation are modified by diminished kidney function in humans (Wu et al., 2006; Spoto et al., 2012) and in rodent UniNX models (Gai et al., 2014b), including the sirtuin SIRT1, farnesoid X receptor (FXR), inflammation and complement factors. Activation of SIRT1 and FXR can counter the metabolic syndrome by acting on lipid and glucose metabolism. We and others have recently shown that bile salts and their receptor FXR are modified by UniNX (Penno et al., 2013; Gai et al., 2014a,b; Chin et al., 2015). It has also been reported that SIRT1 may regulate FXR activity (Liu et al., 2014).

Only a few studies have investigated the role of cytokines in UniNX-induced metabolic changes (Mak et al., 2006; Zhang et al., 2014). However, recent studies in mice suggest that cytokines and their signaling pathways are altered by UniNX (Zheng et al., 2011; Gai et al., 2014b). In a more severe form of reduced kidney function, 5/6 nephrectomy, cytokines have been shown to play a role in pathology (Gao et al., 2011). The role of cytokines in metabolic disease especially concerning lipid metabolism is complex as the dose administered of the cytokine is important; at different doses different phenotypes can occur (Feingold et al., 1992; Khovidhunkit et al., 2004). Furthermore, the source of cytokines in kidney disease (Spoto et al., 2012) may not be the same as in obesity or metabolic disease where adipose tissues are believed to be a major source (Fruhbeck et al., 2001). In other inflammation/infection models, other tissues such as spleen and liver can be a major source of cytokines (Arsenijevic et al., 1998; Park et al., 2010).

It has been shown in chronic human kidney disease that there is an association between circulating cytokines and body weight (Pecoits-Filho et al., 2002). At both extremes of body weight perturbations, obesity and cachexia, it has been shown that cytokines can alter body composition and metabolic pathways. These pathways include protein, lipid and glucose metabolism (Johnson, 1997). Cytokines can act directly on tissue or indirectly via the brain to affect tissue metabolism (Johnson, 1997; Sanchez-Lasheras et al., 2010).

In pilot studies we found that UniNX decreased fat pad weight and increased certain circulating cytokines. We therefore conducted studies to investigate whether UniNX induces changes in body composition, in particular body fat pad lipolysis and lipogenesis, under conditions of fixed food intake (90% of *ad libitum* intake) and whether those changes are associated with selected hormones or cytokines. We also investigated the tissue source of lipolytic cytokines and whether anti-inflammatory tissue regulators FXR/SIRT1 were modified in tissues.

Methods

Animal Preparation and Experimental Protocol

Animal Model

Male Sprague Dawley rats were purchased from Elevage Janvier (Le Genest-St-Isle, France) at 5 weeks of age with an average weight of 160 g/rat. They were placed individually in cages and given pellet food *ad libitum*. After a 1 week acclimation period, rats were either sham operated or uninephrectomized (UniNX) by removal of the left kidney. One day prior to surgery a group of eight non operated rats were sacrificed (day 0 group).

Surgery

Rats were first anesthetized with sevoflurane and then placed on a heated mat. The left flank was shaved and swabbed with polyvidone-iodine (Braunoderm, Braun). Anesthesia and analgesics were given i.p.: medetomidine hydrochloride (Domitor) 150 μg/kg, Midazolam (Dormicum) 2 mg/kg, fentanyl 5 μg/kg and to awake by atipamezole hydrochloride (Antisedan) 0.75 mg/kg, Sarmazenil (Sarmasol) 0.2 mg/kg, Naloxone (Narcan) 120 μg/kg. A small incision was made in the left flank to gain access to the left kidney. The kidney was ligated with non-absorbable thread (Ethilon 11 4-0, Johnson–Johnson) and was then cut loose with surgical scissors. The incision sites were sutured with absorbable thread (Vicryl 3-0 Johnson–Johnson) and metal Michel suture clips (Provet, Switzerland) were applied to close the wound. Metal clips were removed after 14 days. Post-operation analgesic treatment with buprenorphine 0.05 mg/kg s.c. was given 2X/day for 3 days to Sham and UniNX animals.

Diet

Most of the studies analyzing the impact of UniNX have been done under *ad libitum* fed conditions. We chose instead to put the rats under a fixed food intake (90% of ad lib fed diet) to ensure the same caloric intake between sham and UniNX rats. *Ad lib* feeding results in uncontrolled levels of nutrition, which can influence metabolites, hormones, inflammation and oxidative stress, parameters of interest (Diamond, 1990). Dietary intake differences can also influence other variables such as locomotor activity and metabolic rate (Leveille and O'Hea, 1967). Therefore, fixed intake obviates some of these confounding factors encountered in *ad libitum* experiments. This fixed intake approach has previously been used successfully to study the mechanisms underlying body composition regulation during catch-up growth and energy balance in young Sprague–Dawley male rats (Summermatter et al., 2009). After surgery animals were given a fixed intake of normal chow paste. Dry food powder (Maintenance diet composed of 23.5% protein, 12.9% fat, and 63.6% carbohydrates as percentage of metabolisable energy: Cat. No. 3433, Provimi-Kliba, Cossonay, Switzerland) was mixed with an equal amount of tap water and was prepared daily (equivalent to 90 kcal/rat) and given in food cups.

Experimental Protocol

Rats were kept in individual cages and had free access to water. The environmental temperature was maintained at 22 ± 1°C

in a room with a 12 h light/dark cycle (light 7.00 a.m.–7.00 p.m.). Body weight was measured daily before feeding (9.00–11.00 a.m.). Operated rats were sacrificed at 1, 2, and 4 weeks after surgery. At each time point, eight sham rats and eight UniNX rats were sacrificed for collection of blood, tissue samples and animal carcasses. Rats were anesthetized with ketamine (70 mg/kg) for sacrifice, then decapitated for immediate blood collection. Animals were placed on ice for collection of peritoneal macrophage using pyrogen free phosphate saline buffer (see below) and small pieces of tissues were collected for analysis. All experimental protocols were approved by the Ethical Committee of the Veterinary Office of Fribourg, Switzerland.

Body Composition

For body composition analysis the rats were killed by decapitation. The skull, thorax and abdominal cavity were incised and the gut was cleaned of undigested food. The carcasses were dried in an oven maintained at 60°C for 2 weeks, after which they were homogenized. Carcass fat content was measured by the Soxhlet fat extraction method using petroleum-ether (Entenman, 1957). Body water content was determined by subtracting the weight of the animal after the 2 weeks in the oven to the weight prior to this. The fat free dry mass (FFDM) was calculated as the fat mass subtracted from the dry homogenate.

Blood Parameters

Blood was collected on ice (in EDTA- or heparin-coated tubes) and centrifuged at 4°C at 3000 rpm in a microcentrifuge. Serum and plasma were then kept at -20°C until analyzed. For a complete list of metabolites, hormones analyzed and the provenance of kits, see **Table 1**.

TABLE 1 | Assay kits for metabolites, hormones, and cytokines.

Assay kit	Kit name/Cat. No.	Company
METABOLITE ASSAY KITS FOR USE ON SYSTEM ROCHE/HITACHI ANALYSER COBAS C501		
Urea	UREAL kit	1
Triglycerides	TRGL kit	1
Cholesterol	Total cholesterol CHOL2 kit	1
HDL	High density lipoprotein HDLC3 kit	1
METABOLITE PLATE ASSAY KITS		
Free fatty acid assay kit	Cat. No. K612-100	2
β-Hydroxybutyrate assay kit	Cat. No. K623-100	2
Glycerol assay kit	Cat. No. K630-100	2
HORMONE ELISA OR EIA		
Aldosterone	EIA kit Cat. No. 10034377	3
Leptin	EIA kit for Mouse/rat Cat. No. A05176	3
Corticosterone	EIA kit Cat. No. 5006553	3
Ghrelin	EIA kit Cat. No. EK-031-31	4
Angiotensin-1	EIA kit Cat. No. EKE-002-01	4
Angiotensin-2	EIA kit Cat. No. EK-002-12	4
T3 total	ELISA kit Cat. No.90060	5
Insulin	EIA kit Cat. No. 07BC1005	6
CYTOKINE ELISA KITS AND OTHER KITS		
Erythropoietin	ELISA Cat. No. DY959	7
Interleukin(IL)1α	ELISA Cat. No. DY500	7
IL1β	ELISA Cat. No. RLB00	7
IL1RA	ELISA Cat. No. DY480	7
Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)	ELISA Cat. No. DY518	7
C-reactive protein (CRP)	ELISA Cat. No. DY1744	7
IL4	ELISA Cat. No. BMS628MST	8
IL6	ELISA Cat. No. BMS625	8
IL10	ELISA Cat. No. BMS629	8
Tumor Necrosis factor (TNFα)	ELISA Cat. No. 88-7340	8
Interferon-gamma (IFNγ)	ELISA Cat. No. BMS621	8
Acylation Stimulating Protein (ASP)	ELISA Cat. No.MBS728340	9
Serum Neopterin	ELISA Cat. No. RE59321	10
Cystatin-C	Immunoassay Cat. No. KK-CYC	11

1, Roche, Switzerland; 2, Biovision, Milpitas, CA, 95035, USA; 3, Cayman Ann Arbor, Michigan, 48108, USA; 4, Phoenix Europe, D-76133, Karlsruhe, Germany; 5, Crystal Chem, IL, USA; 6, MP Biomedicals Europe, Illkirch, 67402, France; 7, R&D, Abingdon OX14 3NB, UK; 8, eBioscience – San Diego, CA 92121, USA; 9, MyBiosource – San Diego, CA 92195, USA; 10, IBL Toronto, ON, M3J 2N5, Canada; 11, Buhlmann Basel Switzerland.

TABLE 2 | RT-PCR primers.

Primers name, sequence, and original source of sequences
Adipose triglyceride lipase (**ATGL)**sense 5-TGTGGCCTCATTCCTCCTAC-3, antisense 5-AGCCCTGTTTGCACATCTCT-3 (Palou et al., 2009)
Hormone sensitive lipase (**HSL)**sense 5-TCACGCTACATAAAGGCTGCT-3, antisense 5-AGTTCCCTCTTTACGGGTGG-3 (Palou et al., 2009)
CD36 sense 5-GTCCTGCCTGTGTGA-3, antisense 5-GCTCAAAGATGCTCCATTG-3 (Palou et al., 2009)
Cyclophilin sense 5-TCAGGGCTCTTGAAGTCCC-3, antisense 5-CAGAAAATCACAGCAGCCAAC-3 as reference control (Summermatter et al., 2009)

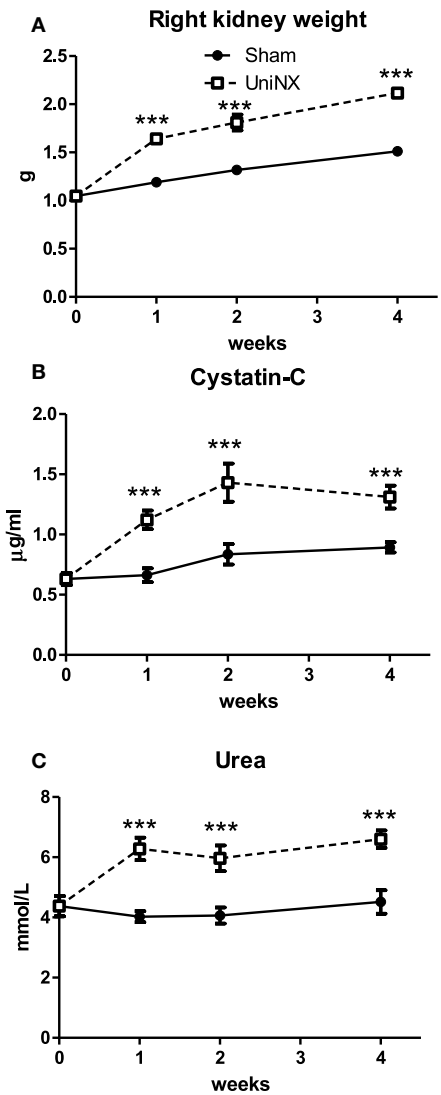

FIGURE 1 | Evolution over 4 weeks of the weight of the remaining right kidney of UniNX and of the right kidney of Sham animals (A). Evolution of plasma indicators of kidney function Cystatin-C **(B)** and Urea **(C)**. Values are means ± SE; $n = 8$/group. $^{***}P < 0.001$ corresponds to Sham vs. UniNX.

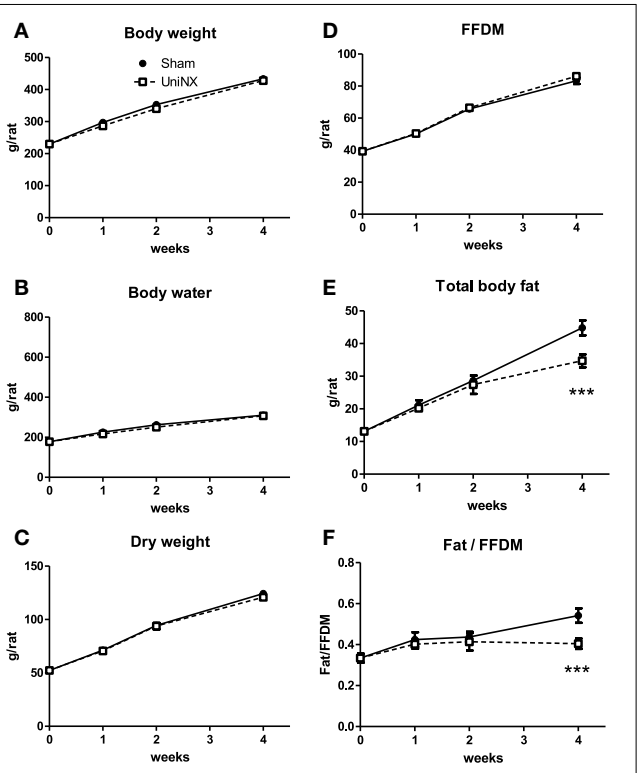

FIGURE 2 | Evolution over 4 weeks of body weight (A), body water (B), dry weight (C), total body fat (D), fat free dry mass–FFDM (E), and the ratio of body fat/FFDM (F) in Sham operated controls and UniNX rats. Values are means ± SE; $n = 8$/group. $^{***}P < 0.001$ corresponds to Sham vs. UniNX.

RT-PCR in Epididymal/Inguinal White Adipose Tissue (EWAT/IWAT) and Liver

Total RNA was isolated as previously described (Arsenijevic et al., 1997). The RNA was then treated with DNase, after which it was reverse transcribed (Promega). Thereafter we ran a RT-PCR (iQ cycler Bio-Rad). Each sample was normalized to its cyclophilin value. For the list of primers used and their sources, see **Table 2**. Samples were incubated in the iCycler instrument (BioRad, iCycler iQ, Version 3.1.7050) for an initial denaturation at 95°C for 3 min, followed by 40 cycles of amplification. Each cycle consisted of 95°C for 10 s, 60 or 62°C for 45 s, and finally 95, 55, and 95°C for 1 min each. Green I fluorescence emission was determined after each cycle. The relative amount of each mRNA was quantified by using the iCycler software. Amplification of specific transcripts was confirmed by melting curve profiles generated at the end of each run. Cyclophilin was used as the control for each study and the relative quantification for a given gene was normalized to cyclophilin mRNA values. Note that as representative of subcutaneous white adipose tissue (SWAT)

we used inguinal fat (IWAT) for PCR, western blot and other analysis.

Western Blot Analysis

Western blots on protein extracts from pulverized tissue were performed as previously described (De Bilbao et al., 2009). Protein samples were loaded at 30 μg/20 μl, after migration proteins were transferred by semi-dry transfer (De Bilbao et al., 2009). Membranes were pre-incubated with 1% casein (Vectorlab), then incubated 2 h with primary antibody uncoupling protein-1 (UCP1) dilution 1/5000 (cat. no. UCP11, Alpha Diagnostics), SIRT1 dilution 1/200 (sc-19857, Santa Cruz), FXR 1/200 dilution (sc-13063, Santa Cruz), and beta-actin dilution 1/1000 (Cat No. 4970—Cell Signaling). Secondary antibody LI-COR anti-rabbit (1/15000) or anti-goat (1/15000) were used to detect bands (De Bilbao et al., 2009). The signals were visualized with the use of Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany).

Lipogenic Enzyme Activity Assays

Fatty acid synthase (FAS) activity was measured according to a method described by Penicaud et al. (1991). The frozen white adipose tissue pads were homogenized on ice in four volumes of freshly prepared polyethylene glycol buffer, pH 7.3 (100 mmol/l KH_2PO_4, 5 mmol/l EDTA, and 1.5 mg/ml glutathione in reduced form). After centrifugation, these extracts were assayed using 15 μl of extract in 1.7 ml of FAS buffer (50 mmol/l K-phosphate stock solution, pH 6.8, and 0.1 mg/ml NADPH) and using a spectrophotometer set at 340 nm and 37°C. The readings were performed by sequentially adding 15 μl of extract in 1.7 ml of FAS buffer to the cuvettes, followed by 10 μl of 7.5 mmol/l acetyl-CoA, and followed by 10 μl of 8 mg/ml malonyl-CoA.

Circulating Cytokines and Markers of Immune Activation

Rat serum ELISA assays for interleukin (IL)1α, IL1β, IL1Receptor Antagonist (IL1RA), erythropoietin (EPO) and C-reactive protein (CRP) were purchased from R&D, Abingdon, OX14 3NB, UK. Other cytokine ELISA kits were purchased from eBioscience, San Diego, CA 92121, USA, including IL4, IL6, IL10, tumour necrosis factor alpha (TNFα), interferon-gamma (IFNγ) and granulocyte macrophage colony stimulating factor (GM-CSF). Acylation stimulating protein (ASP) was measured by ELISA from MyBiosource. Serum Neopterin, a by-product specific of IFNγ activated macrophages, was also determined by ELISA (IBL Toronto, ON, M3J 2N5, Canada). For a complete list of cytokines and provenance see **Table 1**.

Cytokine Levels in Tissues

Tissue cytokine determination was performed, as previously described (Arsenijevic et al., 2006), on tissues from week 4 post UniNX. Briefly, 100 mg of tissue were homogenized with 600 μl

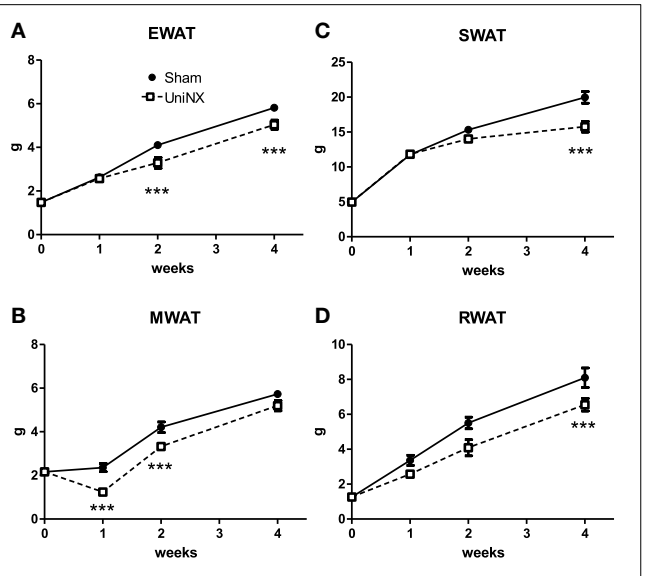

FIGURE 3 | Evolution over 4 weeks of fat pad weight, epididymal–EWAT (A), mesenteric–MWAT (B), subcutaneous–SWAT (C), and retroperitoneal–RWAT (D) in Sham operated and UniNX rats. Values are means ± SE; $n = 8$/group. $^{***}P < 0.001$ corresponds to Sham vs. UniNX.

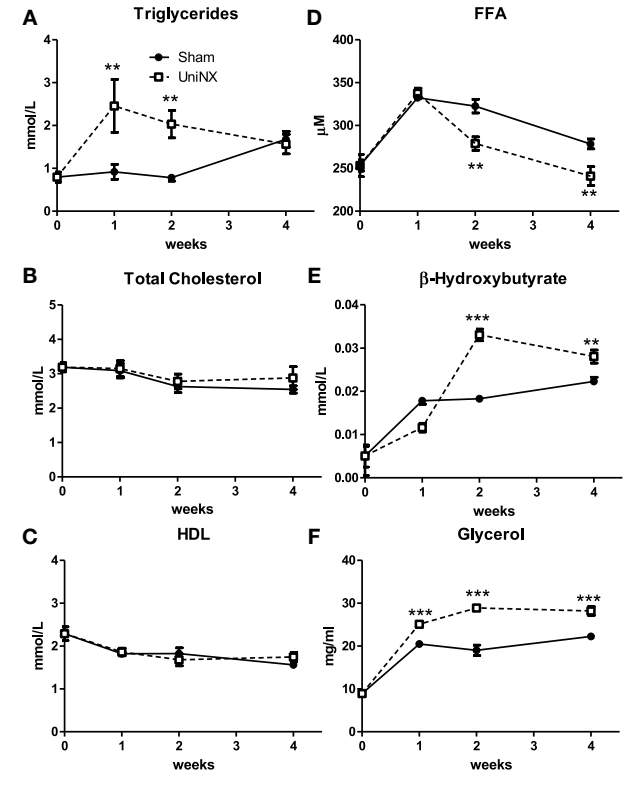

FIGURE 4 | Evolution over 4 weeks of triglycerides (A), total cholesterol (B), high density lipoprotein–HDL (C), free fat acids–FFA (D), β-hydroxybutyrate (E), and glycerol (F) in Sham operated and UniNX rats. Values are means ± SE; $n = 8$/group. $^{**}P < 0.01$. $^{***}P < 0.001$ corresponds to Sham vs. UniNX.

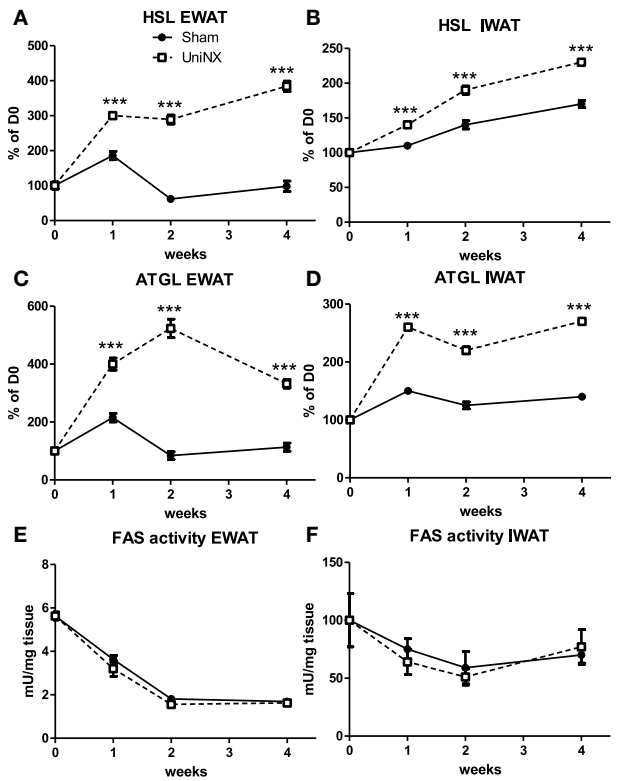

FIGURE 5 | Evolution over 4 weeks of mRNA levels of hormone sensitive lipase–HSL, adipose triglyceride lipase–ATGL, and fatty acid synthase–FAS activity in epididymal fat pad–EWAT (A,C,E) and inguinal fat pad–IWAT (B,D,F) in Sham operated and UniNX rats. Values are means ± SE; $n = 8$/group. $^{***}P < 0.001$ corresponds to Sham vs. UniNX.

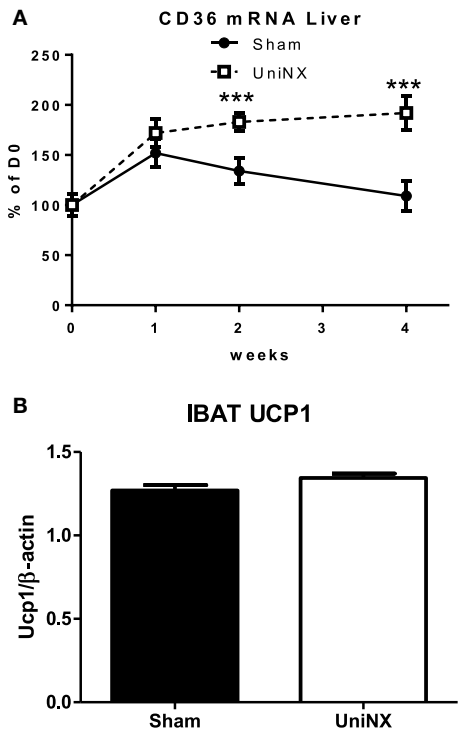

FIGURE 6 | Evolution over 4 weeks of liver free fatty acid transporter CD36 mRNA in Sham operated and UniNX rats (A). Interscapular brown adipose tissue (IBAT) UCP1 protein levels from western blot on the 4th week in Sham operated and UniNX rats **(B)**. Values are means ± SE; $n = 8$/group. $^{***}P < 0.001$ corresponds to Sham vs. UniNX.

of 1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) in RPMI-1640 medium without Phenol red (R7509, Gibco) with a polytron homogenizer (Nakane et al., 1992). The supernatant was collected and frozen at −20°C (Arsenijevic et al., 2006). Cytokines mentioned above were assayed using immunoassay kits, as described previously (Arsenijevic et al., 2006).

Macrophage Intracellular ROS Production

Reactive oxygen species (ROS) production was measured from isolated macrophages by measuring their ability to reduce nitro blue tetrazolium. Peritoneal macrophage layers in 96 well plates were isolated from peritoneal cavity of Sham and UniNX ($n = 8$) with ice cold pyrogen free phosphate buffered saline (PBS). After being centrifuged and washed with PBS three times macrophages were counted and plated at 100,000 per well and let to adhere to plates by incubating at 37°C for 30 min. After this period a solution of nitro blue tetrazolium with 5% glucose in PBS was incubated for a further 3 h at 37°C. The supernatant was removed and gently washed with PBS 3 times. Cells were then fixed with 70% methanol and allowed to dry. Formazan was solubilized with 2 M KOH and dimethyl sulphoxide. The absorbance was determined at 630 nm (Arsenijevic et al., 2000).

Data Analysis

All data are presented as means ± SE. Statistical analysis were performed using Kruskal–Wallis One-Way non-parametrical ANOVA or Mann-Whitney (non-parametrical) for 2 sample comparisons. A value of $p < 0.05$ was considered as significant. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Results

Uninephrectomy Effect on Kidney Function

Left nephrectomy resulted in hypertrophy of the remaining right kidney (**Figure 1A**), which was 38% heavier than the right Sham kidney on week 4. A mild reduction in kidney function is reflected by the increased plasma Cystatin-C and urea levels (**Figures 1B,C**).

Body Weight, Body Composition, and Organ Weight

Over the 4 week period, there was no significant difference in body weight between the UniNX and Sham groups (**Figure 2A**). However, UniNX animals had a tendency to weigh less than Sham animals. Body composition analysis showed that body water, dry body weight and FFDM (**Figures 2B–D**) did not differ significantly between the two groups over the 4 week

period. Total body fat was only reduced significantly by week 4 in the UniNX group (**Figure 2E**), which was reflected in the fat to FFDM ratio (**Figure 2F**). Fat pad weights were significantly decreased during the course of the 4 weeks. In general, the UniNX group had significantly lower epididymal, mesenteric, subcutaneous and retroperitoneal fat pads than Sham counterparts (**Figures 3A–D**). These significant decreases in fat mass were not associated with a significant increase in FFDM although there was a tendency for FFDM to be higher in the UniNX rats. This small increase may be explained by increases in non-muscle tissues such as spleen (Sham 1.12 ± 0.14 g/rat and UniNX 1.44 ± 0.15 $p < 0.05$) and gastrointestinal tract (for intestines—Sham 7.25 ± 0.83 g/rat vs. UniNX 8.50 ± 0.76 g/rat $p < 0.01$; stomach—Sham 1.52 ± 0.17 g/rat and UniNX 1.82 ± 0.17 g/rat $p < 0.01$). No significant differences between Sham and UniNX were seen for the liver weights.

Blood Lipid Metabolites

Plasma triglyceride concentrations showed a transient increase after UniNX, declining after 1 week so that by week 4 UniNX triglycerides were similar to Sham levels (**Figure 4A**). Total blood cholesterol and high density lipoprotein (HDL) levels were not significantly different between Sham and UniNX groups (**Figures 4B,C**). However, from week 2 to week 4, free fatty acids in the UniNX group were reduced compared to the Sham group (**Figure 4D**). Blood β-hydroxybutyrate, a product of fatty acid oxidation, showed a marked increase from week 2 to week 4 (**Figure 4E**). Circulating glycerol, a product of lipolysis, was persistently elevated over the 4 weeks (**Figure 4F**).

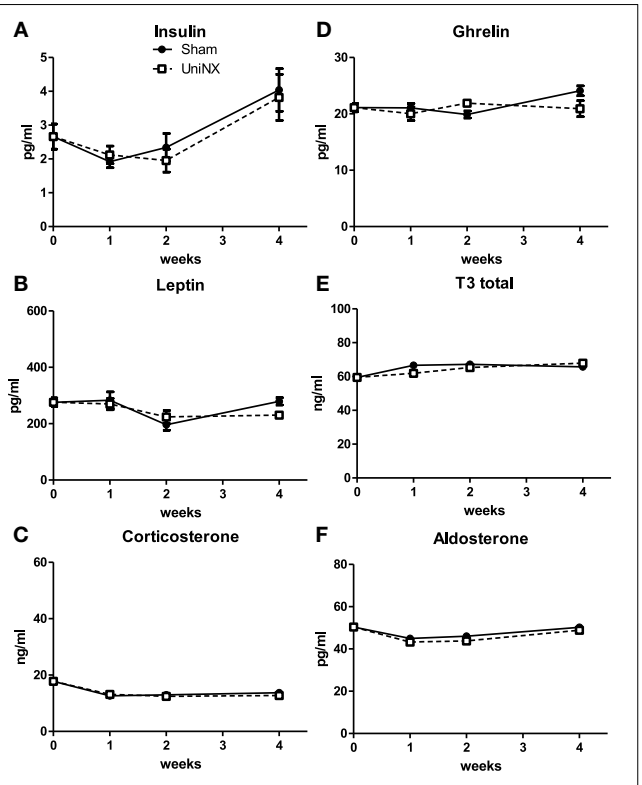

FIGURE 7 | Evolution over 4 weeks of plasma insulin (A), leptin (B), corticosterone (C), ghrelin (D), total T3 (E), and aldosterone (F) in Sham operated and UniNX rats. Values are means ± SE; $n = 8$/group.

Lipid Metabolism Assessment by RT-PCR and Western Blots in Tissues

Hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) mRNA (**Figures 5A–D**) were elevated in the EWAT and IWAT fat pads over the 4 weeks in the UniNX group. Fatty acid synthesis as determined by FAS activities were similar in Sham and UniNX groups (**Figures 5E,F**) in EWAT and IWAT fat pads. The free fatty acid transporter CD36 mRNA in the liver was higher from week 2 to week 4 in UniNX animals than Sham (**Figure 6A**). In addition, we also observed increased CD36 mRNA in selected UniNX tissues (by 142% in the kidney and by 79% in the gastrocnemius muscle). Interscapular brown adipose tissue (IBAT) thermogenic uncoupling protein 1 (UCP1 protein levels) showed no differences between Sham and UniNX (**Figure 6B**) at 4 weeks.

Blood Hormones

Blood hormones insulin, leptin, corticosterone, ghrelin, T3, and aldosterone (**Figures 7A–F**) were not significantly different between Sham and UniNX animals over the 4 weeks. We also observed that on week 4, UniNX and Sham groups showed similar levels of circulating angiotensin 1 (Sham: 4.4 ± 0.2 ng/ml and UniNX: 4.2 ± 0.2 ng/ml) and angiotensin 2 (Sham: 6.5 ± 0.3 ng/ml and UniNX: 6.2 ± 0.2 ng/ml).

Serum and Tissue Cytokines

IL1α, IL1β, IL1RA, IL6, IL10, ASP, CRP, EPO, TNFα, GM-CSF, IFNγ, and neopterin were measured in serum after UniNX. Serum IL1α, GM-CSF, EPO, IFNγ, and ASP were all higher in UniNX than in Sham controls from week 1 to week 4 (**Figure 8**). Neopterin, a specific indicator of IFNγ activated macrophages, was also higher over the 4 week period in the UniNX group than in Sham controls. CRP, an indicator of liver inflammation state, was lower in the UniNX group from week 2 to week 4 (**Figure 8**).

Four selected cytokines (TNFα, IL6, GM-CSF, and IFNγ) were measured in various tissues at week 4, as shown in **Figure 9**. In most tissues, UniNX decreased tissue cytokine protein levels compared to the Sham group. The only tested UniNX tissue that showed a marked increase in the cytokines was the spleen. Peritoneal macrophage ROS production was doubled in UniNX rats (**Figure 9**), reflecting immune activation.

Tissue SIRT1 and FXR Protein Levels

Since SIRT1/FXR have anti-inflammatory properties it was decided to determine whether their levels were modified by UniNX. On week 4 SIRT1 and FXR proteins levels in IWAT, EWAT, kidney, and liver were higher in UniNX animals than in Sham controls. In sharp contrast, SIRT1 and FXR were lower in UniNX spleen (**Figure 10**).

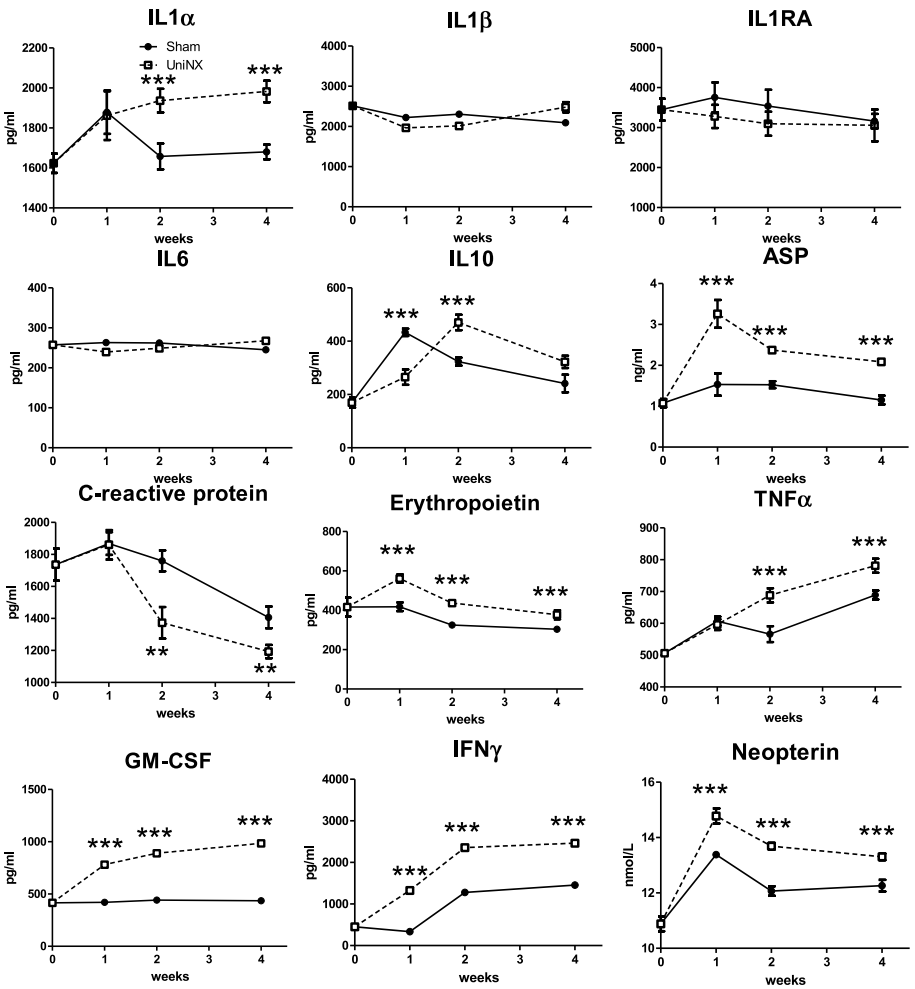

FIGURE 8 | Evolution over 4 weeks of serum IL1α, IL1β, IL1RA, IL6, IL10, ASP, C-reactive protein, erythropoietin, TNFα, GM-CSF, IFNγ, and Neopterin in Sham operated and UniNX rats. Values are means ± SE; $n = 8$/group. $^{**}P < 0.01$, $^{***}P < 0.001$ corresponds to Sham vs. UniNX.

Discussion

Compared to Sham controls, UniNX in young male rats resulted in a mild reduction in kidney function as judged by the chronic elevation of circulating Cystatin-C and urea over a 4 week period. No significant differences in body weight were observed between Sham and UniNX groups. However, UniNX reduced fat pad weight, and this decrease was also evident in total body fat content as determined by body composition analysis 4 weeks post UniNX. The causes of decreased fat pad weight could not be attributed to differences in food intake since we used fixed intake feeding; in addition it could not be explained by reduced FAS as no difference in the activity of this enzyme was found between the two groups in inguinal and epididymal fat pads. Since UCP1 protein levels were not different between the two groups, increased brown adipose tissue thermogenesis does not appear to be involved in the lower body fat content following UniNX.

Analysis of plasma lipid metabolites revealed that glycerol was chronically elevated over the 4 weeks after UniNX, suggesting enhanced lipolysis after UniNX. Indeed, increased ATGL and HSL lipase mRNA levels were found in IWAT and EWAT. Although one may have expected that circulating triglycerides and fatty acids increased in plasma associated with the increased lipolysis, these were not observed, possibly because of increased lipid clearance. Indeed, fatty acid transporter CD36 mRNA was elevated in the liver and in selected tissues (kidney and gastrocnemius). This may explain, at least in part, the previously reported findings (Zhao et al., 2008, 2011) that UniNX led to excessive fatty infiltration and lipid accumulation in tissues (as determined by histology), albeit at time points greater than 3–6 months. Since we did not observe fatty infiltration in liver and kidney in our shorter duration study of 4 weeks, intracellular lipids are likely handled in a different manner in our time frame. They may be metabolized more rapidly, but not completely oxidized as is suggested by the higher circulating

β-hydroxybutyrate and lack of increased UCP1 in brown adipose tissue.

Our data showed that circulating levels of hormones that regulate energy expenditure and body fat, such as leptin, T3, insulin, ghrelin, angiotensin 1 and angiotensin 2, were not significantly different between the two groups over the 4 week period. Hence, these hormones are unlikely to explain the activation of lipolysis (i.e., elevated circulating glycerol levels, increased fat pad lipases ATGL and HSL levels). Similar lack of differences in hormones have been found in UniNX in ad lib standard chow-fed rats in the first 6 months after UniNX (Zhao et al., 2011).

Other potential candidates for increasing lipolysis are cytokines. Of the increased circulating cytokines, IFNγ is of particular interest since its elevation induces both lipolysis and increases circulating ketone bodies *in vivo* (Khovidhunkit et al., 2004), which is what we observe in the UniNX group. Furthermore, our *in vivo* data reveal increased circulating neopterin and increased macrophage ROS production, which are both IFNγ-dependent. IFNγ has also been shown to increase lipid metabolism *in vitro* in adipocytes (Waite et al., 2001), kidney mesangial cell culture (Hao et al., 2013) and in whole animals studies (Feingold et al., 1992). We showed that other circulating cytokines known to induce lipolysis such as ASP, TNFα, and IL1α were also elevated. GM-CSF and erythropoietin have not been shown to directly mediate lipolysis but they can clearly regulate body weight and fat in rodent models (Reed et al., 2005; Lee et al., 2008; Meng et al., 2013; Alnaeeli et al., 2014).

Interestingly we showed that UniNX resulted in anti-inflammatory state in most tissues and this was associated with reduced cytokines in tissues such as liver, kidney and fat pads. Recently, it has been shown that in mouse UniNX models, tissues including fat pads showed a reduced inflammatory state (Sui et al., 2010; Chin et al., 2015). In our study in contrast, IFNγ and GM-CSF protein levels were increased in the UniNX spleen, suggesting that the increased circulating levels of these cytokines may arise from the spleen. A role for cytokine production by the spleen after kidney removal has been shown in mice (Andres-Hernando et al., 2012). Furthermore, nephrectomy can activate immune cells in the spleen (Lukacs-Kornek et al., 2008). In human kidney donors, the activation of cytokine signaling pathways through STATs and SOCS has been shown to occur (Xu et al., 2014).

Since we had previously shown increases in bile salts following UniNX, we chose to investigate whether bile salt receptor FXR (Penno et al., 2013; Gai et al., 2014a) and its potential regulator

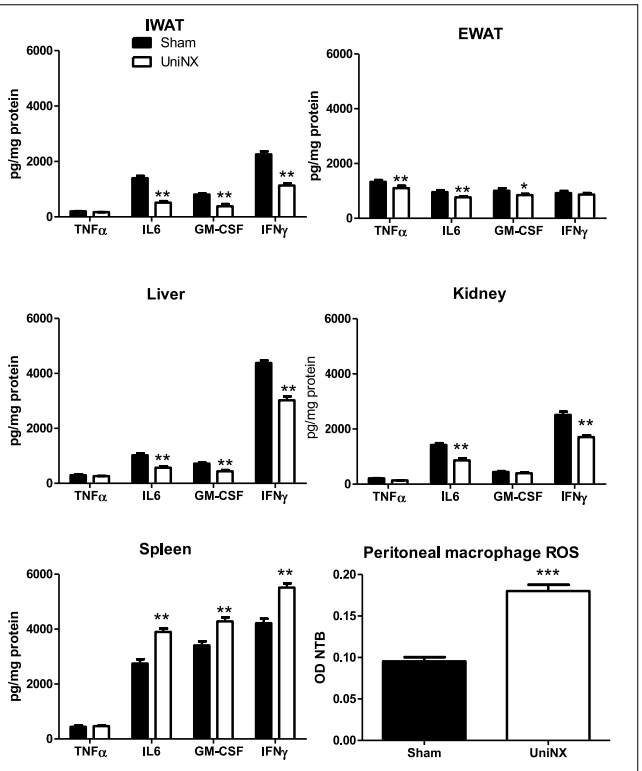

FIGURE 9 | TNFα, IL6, GM-CSF, and IFNγ cytokine levels in inguinal fat pad–IWAT, epididymal fat pad–EWAT, kidney, liver, and spleen on the 4th week in Sham operated and UniNX rats. Peritoneal macrophage reactive oxygen production capacity as determined by measurement of nitro blue tetrazolium. Values are means ± SE; $n = 8$/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ corresponds to Sham vs. UniNX.

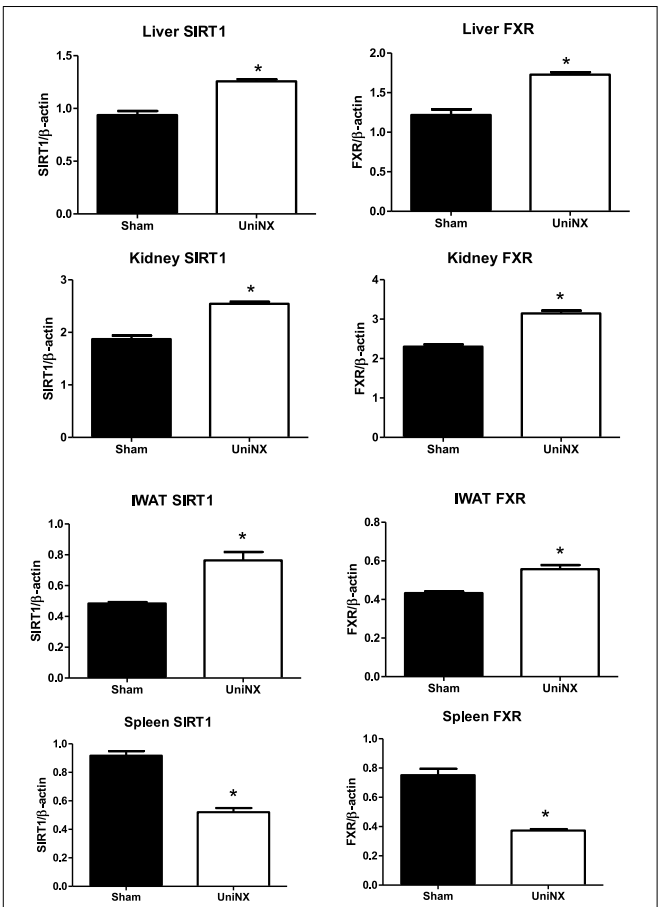

FIGURE 10 | SIRT1 and FXR protein levels in liver, kidney, IWAT, kidney, and spleen on the 4th week in Sham operated and UniNX rats. Values are means ± SE; $n = 8$/group. $^{*}P < 0.01$ corresponds to Sham vs. UniNX.

SIRT1 (Garcia-Rodriguez et al., 2014) were altered in various tissues. Here we show that both were modified in tissues by UniNX. These two factors can regulate not only metabolism but also inflammation. We observed an inverse relationship between tissue cytokine levels and tissue anti-inflammatory FXR/SIRT1 protein levels. The higher the tissue cytokines (in spleen), the lower the FXR/SIRT1 protein levels, and conversely the lower the cytokines (in adipose tissue, liver, kidney), the higher the FXR/SIRT1 levels. Although we have previously shown that the bile salt receptor FXR at the mRNA level showed a tendency to be elevated in the liver (Gai et al., 2014a), we now provide evidence that UniNX may increase FXR protein levels in liver, kidney and IWAT. Whether these increases represent the active form of the FXR warrants further studies. Interestingly SIRT1 may affect activity of various other signaling pathways by modifying the acetylated state of regulatory proteins including STATs (Liu et al., 2014). The age-dependent fatty infiltration of tissue could also potentially be attributed to decreased SIRT1, which is known to be down-regulated with age and is considered responsible for age-related metabolic changes (Kitada et al., 2013). It would therefore be of interest to determine whether these age effects of UniNX pathological fat infiltration and increased tissue inflammation are associated with decreases in SIRT1. The increases in FXR and SIRT1 levels found here in non-immune tissues also support our findings of the leaner phenotype following UniNX.

In summary, our study shows that, under conditions of a fixed intake of normal chow, young male rats that have undergone UniNX had lower body fat. This was associated with enhanced lipolysis and was paralleled by increases in subsets of circulating cytokines rather than changes in circulating hormones levels. Of the measured cytokines, IFNγ appear to be the best candidate for explaining body composition changes after UniNX, based on our *in vivo* physiological activation of IFNγ (increased circulating neopterin, β-hydroxybutyrate and increased macrophage ROS production). Further studies are required to determine whether these cytokines, and especially IFNγ, are acting directly on peripheral tissue or indirectly via the brain. Support for kidney–brain interactions have been shown to occur in chronic kidney disease induced by 5/6 nephrectomy (Mak et al., 2006; Cheung and Mak, 2012) and which results in wasting/cachexia. Altered body composition with loss of body fat and lean mass (Cheung et al., 2008; Cheung and Mak, 2012) after 5/6 nephrectomy implicates cytokines and central melanocortin 4 receptor (MC4R) pathways. However, the neuronal circuits involved, and whether these neurons have receptors for cytokines, remain to be demonstrated.

Author Contributions

Conceived and designed the experiments: JM, AD, DA. Performed the experiments: DA, JC. Analyzed the data: DA, JM, AD. Wrote paper: DA, JM, AD. Edited manuscript: DA, JM, AD.

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REVIEW
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Adipokines, diabetes and atherosclerosis: an inflammatory association

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Cardiovascular diseases can be considered the most important cause of death in diabetic population and diabetes can in turn increase the risk of cardiovascular events. Inflammation process is currently recognized as responsible for the development and maintenance of diverse chronic diseases, including diabetes and atherosclerosis. Considering that adipose tissue is an important source of adipokines, which may present anti and proinflammatory effects, the aim of this review is to explore the role of the main adipokines in the pathophysiology of diabetes and atherosclerosis, highlighting the therapeutic options that could arise from the manipulation of these signaling pathways both in humans and in translational models.

Keywords: adipokines, diabetes, atherosclerosis, adiponectin, TNFα, IL-6, MCP-1, leptin

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INTRODUCTION

Diabetes mellitus (DM) is characterized by insufficient production of insulin (type 1) or, more commonly, inefficient insulin signaling pathways (type 2), a state known as insulin resistance (IR) (International Diabetes Federation, 2013; American Diabetes Association, 2014). Cardiovascular diseases (CVD) are the most important cause of death in the diabetic population (Skyler et al., 2009; American Diabetes Association, 2014). Obesity, a global health problem, is characterized by overproduction of inflammatory adipokines by adipose tissue and this may be the link between obesity, CVD and diabetes (Ohman et al., 2008).

Adipokines can be defined as a group of over 600 bioactive molecules produced by adipose tissue that acts as paracrine and endocrine hormones (Blüher, 2014). These molecules are important in the regulation of diverse processes including appetite and satiety, fat distribution, inflammation, blood pressure, hemostasis and endothelial function. They act in different organs including adipose tissue itself, brain, liver, muscle and vessels (Blüher, 2009, 2014; Lehr et al., 2012; Van de Voorde et al., 2013). These adipokines include mainly adiponectin, leptin, tumor necrosis factor alpha (TNFα), osteoprotegerin, interleukin 6 (IL-6), resistin, interleukin 1 (IL-1), apelin, visfatin, monocyte chemotactic protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), retinol binding protein 4 (RBP4) and others (Van de Voorde et al., 2013; Blüher, 2014; Fisman and Tenenbaum, 2014). The pattern of secretion of adipokines can reflect adipose tissue function and this pattern is important to establish the individual risk to develop metabolic and cardiovascular comorbidities of obesity (Blüher, 2009, 2014). When adipose tissue inflammation and dysfunction are established, adipokine secretion is significantly changed toward a diabetogenic,

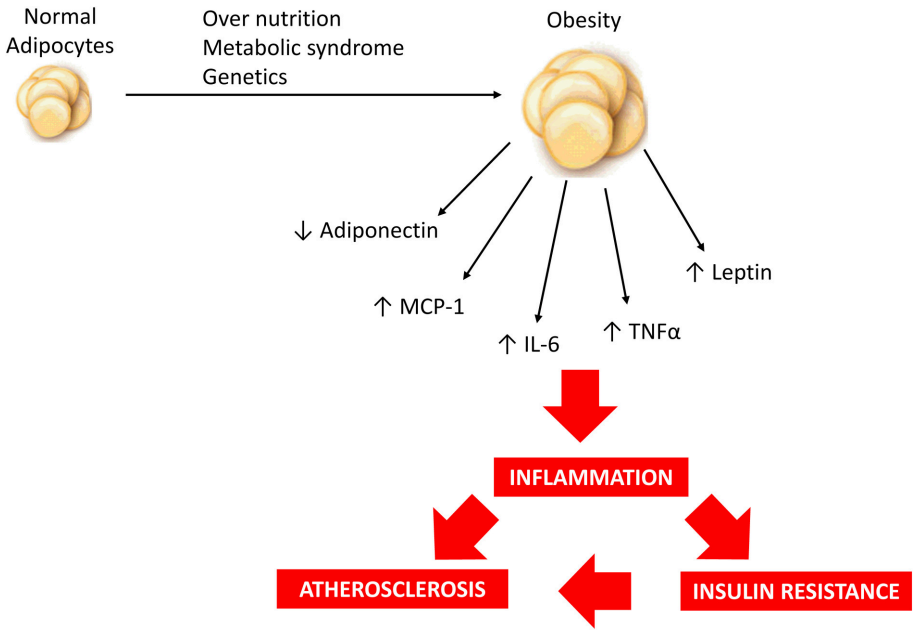

FIGURE 1 | Over nutrition, metabolic syndrome and/or genetic predisposition may contribute to obesity development and modulate adipokine profile resulting in a low-grade inflammatory state which is associated with increased risk of insulin resistance and atherosclerosis.

proinflammatory and atherogenic pattern (Blüher, 2009, 2014), as represented in **Figure 1**. The nature of obesity-induced inflammation is different from other inflammatory situations such as infections or autoimmune diseases. Considering that obesity is a chronic condition, it produces a low-grade activation of innate immune system that affects homeostasis over time (Lumeng and Saltiel, 2011). It is important to highlight that adipose tissue macrophages (ATM) can also be considered as important sources of proinflammatory cytokines (Xu et al., 2015).

Although diabetes and other cardiovascular complications such as atherosclerosis have increasing importance in modern societies, the high mortality due to these diseases reveals that there are insufficient treatment options. Considering that, in the present review we provide an overview on the involvement of the main pro and anti-inflammatory adipokines in diabetes and atherosclerosis and discuss the therapeutic alternatives that could arise from the manipulation of these signaling pathways.

ADIPOKINES AND DIABETES

Insulin resistance (IR) is known to be an important factor underlying the pathogenesis of type 2 diabetes and it usually precedes the onset of this disease (Xu et al., 2015). It occurs in several tissues including liver, muscle and adipose tissue (Lee and Lee, 2014). Cytokines released by adipose tissue are involved in initiating and promoting a proinflammatory status, contributing to IR (Timar et al., 2014). Moreover, these molecules are involved in regulation of insulin sensitivity and secretion (Blüher, 2014). Thus, the impaired adipokine production observed in obesity contributes to diabetes pathogenesis. In metabolic syndrome (MS), adipocytes secrete factors that reduce insulin-mediated glucose uptake (including free fatty acids and proinflammatory cytokines) (Havel, 2002; Timar et al., 2014). The interaction if the main adipokines discussed in the present work to promote insulin resistance are illustrated in **Figure 2**.

Modulation of Insulin Sensitivity by Adiponectin

Adiponectin links visceral adiposity, IR, and atherosclerosis (Swarbrick and Havel, 2008). Unlike other adipokines, circulating concentration of adiponectin is inversely proportional to adiposity and low adiponectin levels predict the development of DM and CVD (Arita et al., 1999; Kadowaki et al., 2006). Moreover, strategies known to help delay or prevent DM and CVD like low-calorie, high-unsaturated fat diet and/or exercise are associated with increased circulating adiponectin levels (Esposito et al., 2003; Lim et al., 2014). Thus, adiponectin may contribute to the prevention of these diseases (Lim et al., 2014), although its use as a marker of cardiovascular risk is still controversial, as further discussed. Adiponectin production is primarily determined by adipocyte size and insulin sensitivity. It was observed that larger and insulin-resistant adipocytes produce less adiponectin (Bahceci et al., 2007; Swarbrick and Havel, 2008). Other cells and tissues can also secrete adiponectin, including cardiomyocytes (Waki et al., 2005; Huang et al., 2009; Caselli et al., 2014).

Independent groups revealed the insulin-sensitizing effect of adiponectin (Berg et al., 2001; Yamauchi et al., 2001; Kadowaki et al., 2006; Wascher et al., 2011). These researchers took the first steps to unravel the mechanisms involving adipokines

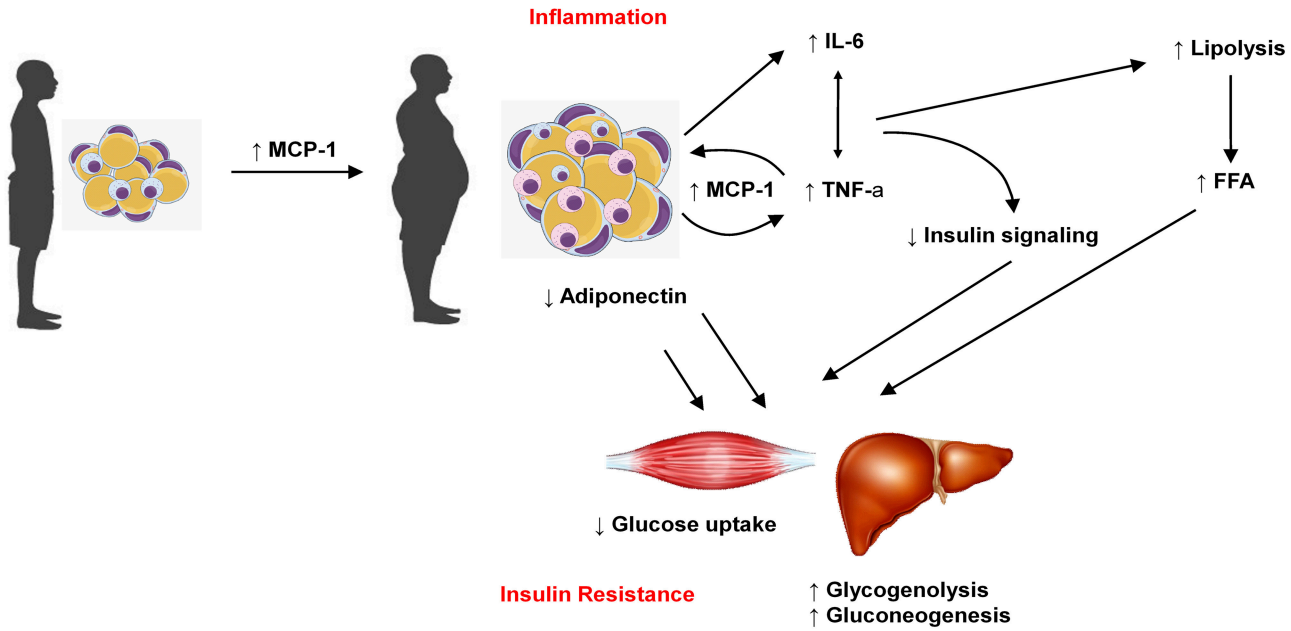

FIGURE 2 | Increase in adipose tissue mass is related to increase in MCP-1, which favors macrophages migration to adipose tissue and the production of other adipokines such as IL-6 and TNFα. On the other hand, obese adipose tissue produces less adiponectin. Taken together, these features reduce glucose uptake in muscle cells and favors glycogenolisis and gluconeogenesis in liver, which contribute to insulin resistance and diabetes.

and insulin resistance. Yamauchi et al. observed that the replenishment of adiponectin significantly ameliorates high-fat diet-induced insulin resistance and hypertriglyceridemia. They proposed that adiponectin is an insulin-sensitizing adipokine (Yamauchi et al., 2001). It has been described that an acute increase in the concentration of circulating adiponectin triggers a transient decrease in basal glucose level by inhibiting both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production in both wild-type and type 2 diabetic mice, suggesting that adiponectin sensitizes the body to insulin (Berg et al., 2001; Wascher et al., 2011). It was reported that a proteolytic cleavage product of adiponectin, which structurally resembles globular adiponectin, increases fatty-acid oxidation in muscle, decreases plasma glucose and causes weight loss in mice (Fruebis et al., 2001).

Adiponectin Receptors

Adiponectin interacts with two different transmembrane receptors: AdipoR1 (expressed ubiquitously and at a high level in skeletal muscle) and AdipoR2 (expressed predominantly in the liver) (Yamauchi et al., 2003a; Scheid and Sweeney, 2014). It was detected that the deletion of both AdipoR1 and AdipoR2 in mice led to increased lipid accumulation in various tissues, IR and glucose intolerance. AdipoR1 deletion results in lack of adiponectin-stimulated adenosine monophosphate-activated protein kinase (AMPK) activation (Yamauchi et al., 2007). When AdipoR2 is deleted, the principal signaling defect occurs in peroxisome proliferator-activated receptor alpha (PPARα) signaling (Yamauchi et al., 2007) AdipoR1$^{-/-}$ mice exhibited decreased glucose tolerance and defective AMPK activation (Scheid and Sweeney, 2014). On the other hand, cultured myotubes from obese diabetic participants showed increased levels of AdipoR1 relative to lean controls (Holmes et al., 2011). Jang and colleagues reported that AdipoR2 levels are significantly lower in DM participants than in lean controls. These findings suggest that circulating levels of adiponectin and expression of AdipoR genes play an important role in the regulation of skeletal muscle insulin action (Chen et al., 2005; Jang et al., 2008; Holmes et al., 2011), although the exact effect of adiponectin in type 1 or type 2 receptors in different CVD is still under investigation. Okada-Iwabu and collaborators observed that orally active AdipoR agonists (AdipoRON) presented similar effects to adiponectin via AdipoR1 and 2 in both liver and skeletal muscle of diabetic mouse model, suggesting that adiponectin receptors could be a promising therapeutic target for the oral treatment of DM (Okada-Iwabu et al., 2013). Adiponectin receptors are also expressed in pancreatic β-cells and their expression is increased by exposure to free fatty acids, suggesting that adiponectin and its receptors are also involved in insulin secretion (Lim et al., 2014).

It is already established that AdipoR1 activates AMPK, promoting glucose uptake in muscle cells via translocation of GLUT4 transporters to cellular membrane (Fisman and Tenenbaum, 2014). Simultaneously, it blocks gluconeogenesis by inhibiting the hepatic enzyme phosphoenolpyruvate carboxylase, inhibits the synthesis of fatty acids and stimulates their oxidation (Kadowaki et al., 2006; Scheid and Sweeney, 2014). Moreover, adiponectin also increases fatty-acid combustion and energy consumption via activation of AdipoR2 through PPARα and PPARγ activation, which leads to glucose uptake and decreased

triglyceride content in the liver and skeletal muscle, contributing to *in vivo* insulin sensitivity (Kumada et al., 2004; Kadowaki et al., 2006; Lim et al., 2014). Interestingly, AMPK can directly increase insulin sensitivity by stimulating the phosphorylation of peroxisome proliferator-activated receptor-γ co-activator 1 α (PGC-1α), a transcription co-activator that plays a critical role in the biosynthesis of mitochondria and oxidative phosphorylation. This reveals a cross-talk between the two different adiponectin receptors (Jäger et al., 2007; Scheid and Sweeney, 2014). Finally, adiponectin also enhances insulin sensitivity indirectly by increasing hepatic insulin receptor substrate 1 (IRS-1) expression via a macrophage-derived IL-6-dependent pathway. Thus, these multiple actions confer to adiponectin a key role in ensuring an effective protection against the development of IR (Awazawa et al., 2011; Fisman and Tenenbaum, 2014). In summary, adiponectin has insulin-sensitizing and cardiovascular-protective effects. These properties may help explain the inverse association between circulating adiponectin level and CVD, DM and obesity.

TNFα-induced Insulin Resistance

The mechanistic link between obesity, DM, and adipose tissue inflammation was first proposed based on the finding that the level of the proinflammatory adipokine TNFα was increased in adipose tissue of obese rodents and humans and that its blockage led to improvement in insulin sensitivity (Hotamisligil et al., 1993). Subsequently, macrophages were found to infiltrate into adipose tissue of obese mice and humans and nearly 40–50% of total cells are F4/80-expressing macrophages in mice. These cells were also the major source of TNFα in adipose tissue (Weisberg et al., 2003; Cildir et al., 2013). The interaction between TNFα and its receptors, TNFR1 and TNFR2, mediates apoptosis, IR, lipolysis, inhibition of insulin-stimulated glucose transport and inhibition of insulin receptor autophosphorylation (Blüher, 2009; Cildir et al., 2013; Palomer et al., 2013). In adipocytes, TNFα reduces the secretion of adiponectin, induces IR and favors atherogenic dyslipidemia due to the reduction in GLUT4 expression, reduction in lipoprotein lipase (LPL) activity and increasing in expression of hormone-sensitive lipase (Cildir et al., 2013). TNFα impairs insulin signaling in adipocytes and hepatocytes through activation of stress-related protein kinases, as JNK-1, and activation of the IKKB/NF-κB pathway (Hirosumi et al., 2002; Arkan et al., 2005; Tarantino and Caputi, 2011) In addition, TNFα stimulates inhibitory phosphorylation of the serine residues of IRS-1, which is recognized as the major pathway in IR, corroborating the link between inflammation, obesity and IR (Wellen and Hotamisligil, 2005).

TNFα antagonism is efficient to treat patients with chronic inflammatory conditions such as rheumatoid arthritis. However, studies using anti-TNF therapies did not show significant improvement in insulin sensitivity. In obese Zucker rats, anti-TNF treatment had no effect on insulin sensitivity or lipid profile (López-Soriano et al., 1997; Cildir et al., 2013). Controversially, in some rodent studies, administration of TNFα antibodies resulted in inhibited inflammatory activity, improved fatty liver disease, protection against diet-induced obesity and IR (Li et al., 2003; Liang et al., 2008; Blüher, 2009). Unfortunately, the promising results from some animal studies using anti-TNF treatment in metabolic diseases were not successful clinically (Blüher, 2009). In a recent study, chronic TNFα neutralization by infliximab led to improvement in inflammatory status but did not ameliorate IR nor endothelial function in insulin-resistant volunteers (Wascher et al., 2011). Therefore, the effect of TNFα neutralization on insulin sensitivity in patients with DM needs to be further evaluated to open the perspective of new pharmacological targets.

Interleukin-6 and Insulin Resistance

Interleukin-6 (IL-6) can be considered an adipokine since it is released by adipocytes from obese individuals, which occurs in a size-dependent manner (i.e., larger adipocytes release greater amounts of IL-6) and links obesity to a state of low-grade inflammation (Skurk et al., 2007; Schuett et al., 2009). The IL-6 family includes a range of diverse molecules such as IL-6 itself, IL-11, IL-27, IL-31, and others (Schuett et al., 2009; Scheller et al., 2011). These molecules have a common feature of containing two signal transducing receptor subunits, which one of them is glycoprotein 130 (gp130) (Schuett et al., 2009; Rose-John et al., 2015). Interestingly, only a few defined cell types (e.g., hepatocytes, monocytes, neutrophils and inactive T- and B-lymphocytes) express the specific IL-6 receptor (IL-6R) and can, therefore, respond to IL-6 classic signaling pathways (Schuett et al., 2009). However, a soluble form of IL-6R (sIL-6R) can be released due to shedding by A disintegrin and metalloprotease 17 (ADAM17). Interleukin-6 can bind to sIL-6R and activate gp130 in cells which do not former express IL-6R, a process known as trans-signaling (Matthews et al., 2003; Schuett et al., 2009; Scheller et al., 2011). This explains the wide range of effects elicited by this adipokine in different cell types. The exact metabolic role of IL-6 is still controversial because it has multiple functions, including tissue-specific effects on glucose metabolism and insulin signaling (Sabio and Davis, 2010). It appears to present dual functions, depending on the target tissue (liver or muscle), the duration of stimulus (acute vs. chronic) or the source of the cytokine (adipose tissue or skeletal muscle) (Schuett et al., 2009; Piya et al., 2013).

Chronically-elevated IL-6 has been described to be related to metabolic disorders such as obesity and IR (Franckhauser et al., 2008; Schuett et al., 2009). Circulating levels of IL-6 have been reported to be positively associated with MS, IR and diabetes (Ferreira-Hermosillo et al., 2015; Sindhu et al., 2015). Also, IL-6 mediates, at least in part, hepatic insulin resistance due to impairment of insulin receptor and IRS-1 phosphorylation (Sabio and Davis, 2010; Piya et al., 2013). It appears that the effect of IL-6 in hepatic control of insulin sensitivity and glucose tolerance is mediated by IL-6 classic rather than trans-signaling pathway (Scheller et al., 2011). This idea is reinforced by the fact that liver expresses IL-6R and that a long-term IL-6 trans-signaling inhibition in mice revealed no unfavorable metabolic effects (Schuett et al., 2009). Chronically-elevated IL-6 levels also lead to impaired insulin-mediated glucose uptake by muscle cells (Hassan et al., 2014). On the other hand, during exercise, acutely elevated IL-6 produced by skeletal muscle increases glucose uptake and AMPK-mediated fatty acid oxidation in these cells (Schuett et al., 2009; Piya et al., 2013). Considering the dual roles played by IL-6 on insulin sensitivity in diverse tissues and

that these effects depend on different times of exposition and signaling pathways, further studies are necessary to ensure the safety of blocking IL-6 pathways as a pharmacological target to treat diabetes.

MCP-1 and Insulin Resistance

Monocyte chemoattractant protein 1 (MCP-1) (also referred as chemokine C-C motif ligand 2, CCL2) is involved in leucocyte recruitment to inflammation sites. The effects of MCP-1 in recruiting monocytes, T lymphocytes and natural killer cells are dependent of the C-C motif chemokine receptor (CCR2), since the use of a specific antagonist for this receptor attenuates obesity-induced macrophage accumulation (Charo and Taubman, 2004; Weisberg et al., 2006; Gonzalez-Quesada and Frangogiannis, 2009). The interaction of MCP-1 with its receptor, CCR2, is considered pivotal for the recruitment of adipose tissue macrophages (ATMs) and the development of obesity-induced insulin resistance, although ATM recruitment can occur independently from MCP-1/CCR2 signaling (Xu et al., 2015). Adipocytes are an important source of MCP-1 and it causes adipose tissue inflammation even in the absence of macrophages (Sindhu et al., 2015). Adipose-derived MCP-1 is critical in exacerbating insulin resistance in adipose tissue of obese individuals (Uchida et al., 2012).

Interestingly, although chronic stress leads to atrophy of adipose tissue with a reduction in cell size, it also induces a low-grade inflammation status similar to obesity-related phenotype. In this model, MCP-1 is involved in the establishment of IR and a prothrombotic state (Uchida et al., 2012). Mice deficient in MCP-1 or the CCR2 are protected against high fat diet-induced IR (Weisberg et al., 2006). Although it was demonstrated that MCP-1/CCR2 signaling is important for obesity-induced insulin resistance and that the use of a CCR2 antagonist can ameliorate this condition without affecting macrophage infiltration into adipose tissue, it is difficult to state if IR is induced by MCP-1 *per se* or if it depends on recruited macrophages that release other cytokines (Kanda et al., 2006; Panee, 2012). Monocytes recruited into adipose tissue by CCR2 activation also secrete TNFα, IL-6 and MCP-1, which enhances the amplification cascade and favors continuous adipose tissue inflammation and IR through autocrine and paracrine interactions between monocytes and adipocytes (Uchida et al., 2012). Nevertheless, it is clear that MCP-1 links obesity to IR and macrophage infiltration into adipose tissue (Kanda et al., 2006). Importantly, MCP-1 is involved in diabetic nephropathy. Under stimulation of a high glucose concentration, advanced glycation end-products, oxidatively modified lipoproteins and angiotensin II, MCP-1 is expressed in mesangial cells, leading to glomerulosclerosis (Yadav et al., 2010).

Leptin and Insulin Resistance

The implications of leptin in cardiovascular diseases has been studied since its first description in the classical paper by Zhang et al. (1994). Leptin is an adipose tissue-specific adipokine, known as a key molecule that regulates appetite, energy expenditure, behavior and glucose metabolism (Amitani et al., 2013; Adya et al., 2015). It crosses blood-brain barrier and, in hypothalamus, it acts in specific receptors to decrease appetite and increase energy expenditure (Koh et al., 2012). Also, it inhibits neuropeptide Y neurons (Elmquist et al., 1999). Leptin plasma concentration increases in proportion to body fat mass (Amitani et al., 2013). This adipokine acts on target cells through transmembrane receptors, which exist in 6 different isoforms (from Ob-Ra to Ob-Rf) (Koh et al., 2008; Adya et al., 2015). In obesity, despite increased leptin levels, a dysregulation of energy balance is observed, suggesting that obese people are resistant to leptin (Seufert et al., 2004; Koh et al., 2008). According to the concept of selective leptin resistance introduced by Mark and colleagues in 2002, only the anorectic effect of leptin is imbalanced, whereas other activities are maintained (Mark et al., 2002; Koh et al., 2012). It is important to highlight that exogenous leptin is efficient in promoting weight loss in obese humans and mice genetic deficient in leptin but not in diet-induced obesity (Blüher, 2014).

Leptin exerts an important role in regulation of glucose homeostasis, independent of its actions on food intake or body weight (Jung and Choi, 2014). Pancreatic β cells express leptin receptors and leptin inhibits insulin biosynthesis and secretion (**Figure 3**). There is a feedback loop where insulin stimulates leptin secretion from adipose tissue (Amitani et al., 2013) and leptin is decreased in low insulin states (Ahima and Flier, 2000). Several pathways are involved in leptin-induced inhibition of insulin secretion: suppression of preproinsulin mRNA, inhibition of GLP-1-induced insulin production, impairment of glucose transport via GLUT 2, regulation of ATP-sensitive potassium channels, inhibition of cAMP/PKA pathway, which regulates calcium channels and exocytosis (Seufert et al., 2004; Marroquí et al., 2012; Amitani et al., 2013). In skeletal muscle, leptin can impair GLUT 4 translocation, which contributes to insulin resistance (**Figure 3**) (Thorp and Schlaich, 2015). It was demonstrated that insulin resistance is associated with elevated plasma leptin levels (Segal et al., 1996). Thus, hyperleptinemia can be considering another critical link between obesity and insulin resistance. Given that leptin levels are increased in obesity and that, due to selective leptin resistance, its proinflammatory and insulin desensitizing effects are maintained, body weight reduction is important in diabetic patients as a strategy to preserve insulin efficacy.

ADIPOKINES AND ATHEROSCLEROSIS

Atherosclerosis can be defined as a chronic and progressive disease characterized by an inflammatory response of arterial wall to injuries promoted by risk factors such as dyslipidemia, diabetes, hypertension and others (Ross, 1999). The concept that atherosclerosis is an inflammatory disease is not new, since the inflammatory nature of atherosclerotic plaque was already described by Virchow in 1858 (Virchow, 1858, 1989). Although hypercholesterolemia figures among the most important risk factors for atherogenesis, nowadays it is well established that atherosclerosis is not only the accumulation of fat in arterial walls but is also a complex process involving both innate and adaptive immune processes (Ross, 1999; Hansson et al., 2002). In brief, atherogenic process initiates in sites where

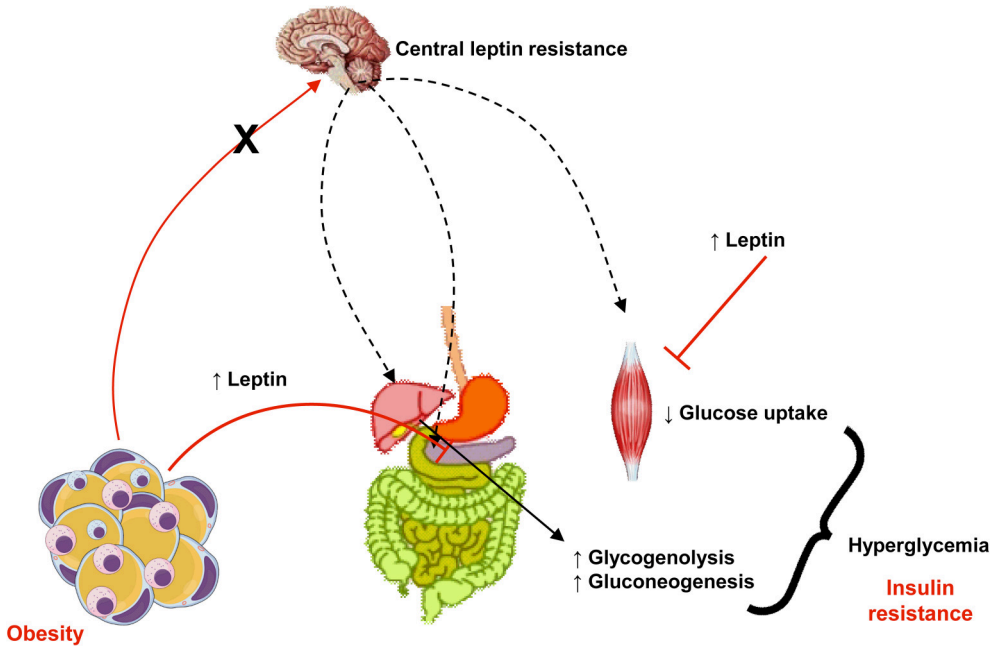

FIGURE 3 | In obesity, despite increased levels of leptin, central actions of this adipokine that control appetite, body weight, and energy expenditure are impaired (a phenomena known as leptin resistance, represented in the figure by the crossed red arrow from adipose tissue to the brain and black arrows). Otherwise, peripheral actions of leptin to reduce insulin secretion in pancreas and its signaling pathways is muscle (represented in the figure by plain arrows from adipose tissue to pancreas and muscle) culminates in insulin resistance, hyperglycemia and diabetes.

endothelium is submitted to shear stress (i.e., aortic root, aortic arch, superior mesenteric artery, and renal arteries). In these sites, endothelial dysfunction is observed and the permeability of the intimal layer is altered, favoring the migration of low density lipoprotein particles (LDL) to sub endothelial space (Tabas et al., 2007). Once endothelium has been activated by risk factors, it expresses adhesion molecules such as E-selectin, vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1), which attracts leukocytes. They adhere to endothelial lumen and migrate through vascular wall to the media. There, these cells express scavenger receptors and phagocyte oxidized LDL (oxLDL) turning into foam cells (Stephen et al., 2010). Growth factors and cytokines released by inflammatory cells contribute to the formation of a fibrous cap of smooth muscle and extracellular matrix around the lipid core, which compromises vascular lumen (Ross, 1999; Lusis, 2000; Hansson et al., 2002; Libby et al., 2010). Thus, it is clear that any factor involved in modulation of inflammatory response can influence atheroma development. Inflammatory process is not only involved in progression of atherosclerosis but is also responsible for acute thrombotic complications due to plaque rupture (Kumada et al., 2004), which may represent the major problematic event associated with atherosclerosis. Many adipokines can induce angiogenesis, which has deleterious effects on atheroma as the proliferation and migration of endothelial cells can lead to plaque destabilization and rupture (Van de Voorde et al., 2013). The adipokines effects on atherogenesis are illustrated in **Figure 4**.

Adiponectin and Atherosclerosis

Adiponectin can influence several steps in atheroma formation, from endothelial dysfunction to plaque rupture (Zhu et al., 2008; Lindgren et al., 2013). Accordingly to the "response to injury" theory of atherogenesis proposed by Ross (1999), the first step toward atheroma formation is an injury to endothelial wall. Considering that adiponectin can diminish endothelial response to mechanical injury (Fisman and Tenenbaum, 2014), it is clear that this adipokine present protective role in atherosclerosis. Hypercholesterolemia, the main risk factor to atheroma formation, can reduce endothelial progenitor cells (EPC) number and function (Dussault et al., 2009). Adiponectin was shown to recover EPC number and function, favoring endothelial repair (Huang et al., 2011; Issan et al., 2012; Fisman and Tenenbaum, 2014).

It has already been shown that adiponectin can inhibit the expression of VCAM-1, ICAM-1 and E-selectin by the endothelium (Ouchi et al., 2000), the initial phase of leukocyte migration through arterial wall. Adiponectin can modulate macrophage phenotype from the activated macrophage to an anti-inflammatory phenotype (Ouchi et al., 1999; Kumada et al., 2004), inhibiting its transformation into foam cell. Moreover, it can also reduce intracellular cholesteryl ester content, suppress TNFα production and stimulate the production of IL-10, which present anti-inflammatory features (Ouchi et al., 1999). In 2001, Ouchi and colleagues demonstrated that adiponectin is capable of diminishing the expression of class A scavenger receptors in macrophages, resulting in

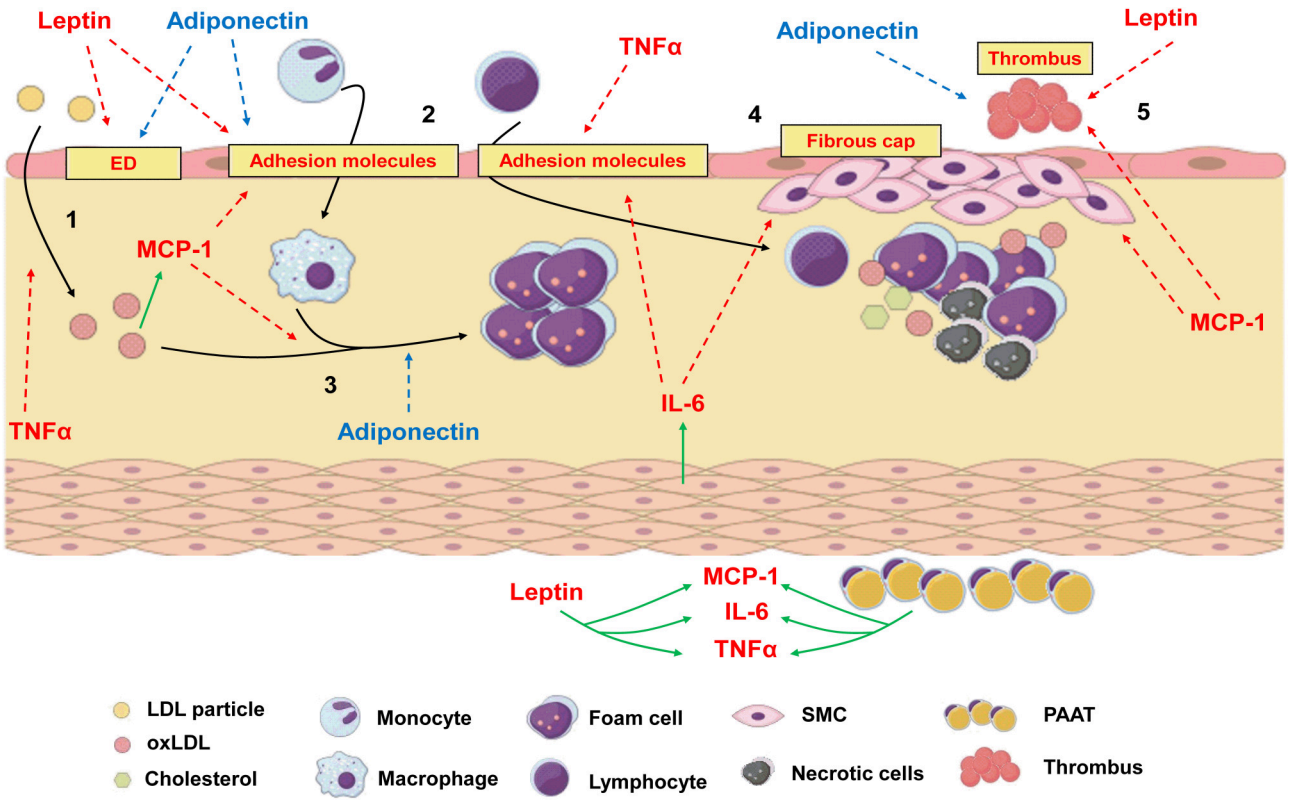

FIGURE 4 | The process of atherogenesis can be influenced by diverse adipokines.1: Endothelial dysfunction (ED) and transmigration of LDL particles to subendothelial space can be worsen by leptin and TNFα. Adiponectin, which is reduced in obesity, recovers endothelial function. **2:** Once in subendothelial space LDL is oxidized (oxLDL), which is positively related to MCP-1 levels. Leptin, IL-6, MCP-1 and TNFα increase the expression of adhesion molecules in endothelium and increase leucocyte transmigration. **3:** Monocytes turn into macrophages under the stimulus of MCP-1 and phagocytes oxLDL, turning into foam cells. Adiponectin inhibits phagocytosis of oxLDL and foam cell formation. **4**: IL-6 can be produced by local smooth muscle cells (SMC) under the stimulus of angiotensin II. Along with MCP-1, it increases recruitment and proliferation of SMC and extracellular matrix deposition to form a fibrous cap around a lipid-rich necrotic core. **5**: Due to stimulation of matrix metalloproteinases and prothrombotic molecules, MCP-1 and leptin favors plaque rupture and thrombus formation, while adiponectin inhibits thrombosis. Periadventitial adipose tissue (PAAT) and leptin induce the production of proinflammatory adipokines. Red arrows represent proinflammatory pathways which are stimulated during obesity and contribute to atherogenesis. Blue arrows represent anti-inflammatory pathways which are inhibited during obesity since adiponectin levels are low. Green arrows represent production or stimulation of adipokine secretion.

inhibition of foam cell transformation. In addition, it induced cholesterol efflux from macrophages due to upregulation of ATP-binding cassette transporter (ABCA1) (Ouchi et al., 2001; Tsubakio-Yamamoto et al., 2008). It has been shown that adiponectin is capable to increase the expression of tissue inhibitor of metalloproteinase 1 (TIMP1), protecting against plaque rupture and thrombotic events (Kumada et al., 2004).

Although adiponectin can be considered as an anti-inflammatory adipokine, some studies have indicated that its level is related more closely to the degree of insulin resistance than to the degree of adiposity in humans (Weyer et al., 2001; Ohman et al., 2008) and that the relationship between adiponectin concentration and CVD is still controversial (Weyer et al., 2001). One possible explanation is the different forms of adiponectin found in plasma and their diverse biological effects. Adiponectin can be present as a trimer, with anti-inflammatory properties, as a trimer-dimer or as a large multimeric structure, which present proinflammatory effect. In this context, the percentage of the different isoforms observed in diverse pathophysiological conditions could be responsible for discrepant observations (Kim et al., 2012; Hao et al., 2013). Another possible explanation is that adiponectin acts differently depending on the receptor activated. This adipokine can mediate its effects via AdipoR1 and AdipoR2, respectively. In 2013, Lindgren and colleagues demonstrated that crossbreeding apolipoprotein E knockout mice (apoE$^{-/-}$) and AdipoR2$^{-/-}$ animals can generate a lineage which presents smaller plaque area in brachiocephalic artery, suggesting that the activation of this receptor has proatherogenic effect, despite no differences in plasma lipid profile (Lindgren et al., 2013). However, it was described that overexpression of adiponectin protects against atherosclerosis in apoE$^{-/-}$ mice (Yamauchi et al., 2003b). Some authors defend that adiponectin can be used as a marker of cardiovascular risk, once it correlates negatively with coronary artery disease (Ouchi et al., 1999), although

the diverse actions of this adipokine and different responses depending on which receptor is activated make it still a controversial issue. Nevertheless, it is generally well accepted that hypoadiponectinemia (<4 μg/mL) is associated with a variety of diseases, including atherosclerosis, DM, hypertension and others, although hyperadiponectinemia can be associated with increased renal and pulmonary diseases (Kishida et al., 2014).

TNFα and Atherosclerosis

Although it was initially suggested that the main source of TNFα in obesity were adipocytes, it is now well recognized that M1 macrophages infiltrated in adipose tissue are responsible for increased levels of this cytokine (Arkan et al., 2005; Solinas et al., 2007; Galic et al., 2010; Nakamura et al., 2014). Still, TNFα was the first adipokine suggested to represent a link between obesity, inflammation and diabetes (Hotamisligil et al., 1993; Galic et al., 2010).

Similarly to adiponectin, TNFα is considered to be involved in all aspects regarding atheroma formation, although it presents proinflammatory properties. In endothelial cells, TNFα induces the activation of proinflammatory, procoagulant and proliferative genes (Ohta et al., 2005; Xiao et al., 2009; Ntaios et al., 2013; Nakamura et al., 2014; Steyers and Miller, 2014). Considering that endothelial dysfunction can be defined as an unbalance in the production of vasoconstrictors and vasodilators, pro and anti-inflammatory substances, inhibitor and stimulator factors and pro and anti-coagulators (Rubanyi, 1993), the production of TNFα during obesity can be considered as an inductor of endothelial dysfunction (Kobayasi et al., 2010; Steyers and Miller, 2014). Endothelial dysfunction is the first event in atherogenesis pathway (Ross, 1999) and the induction of this stat by TNFα is, at least in part, responsible for the increased incidence of atherosclerosis-related events in obese patients. Another marker of endothelial dysfunction is the inability of acetylcholine to induce endothelium-dependent relaxation in vessel preparations *in vitro* (Balarini et al., 2013) and this was also induced by TNFα (Wang et al., 1994) since it reduces endothelial nitric oxide synthase (eNOS) expression and activity (Steyers and Miller, 2014). Vasocrine signaling by TNFα derived from periadventitial adipose tissue (PAAT) is also responsible for decreased NO production and endothelial dysfunction (Yudkin et al., 2005; Ronti et al., 2006).

Another aspect of atherosclerosis-related inflammation that is induced by TNFα is the alteration in endothelial permeability. This adipokine can increase the expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) by endothelium and alter endothelial cell morphology, augmenting its permeability not only to immune cells but also to small particles like LDL (Marcos-Ramiro et al., 2014; Steyers and Miller, 2014). Interestingly, Zhang and colleagues demonstrated that TNFα is capable to induce transcytosis of LDL at the first stages of atherosclerosis development through a mechanism dependent of nuclear factor kappa B (NF-κB) and peroxisome proliferator-activated receptor gamma (PPAR-γ) crosstalk (Zhang et al., 2014). The activation of endothelium and increasing in expression of adhesion molecules by TNFα can be reduced by adiponectin (Van de Voorde et al., 2013).

TNFα is first synthesized as a transmembrane protein and then is turned into is soluble form through the cleavage by ADAM-17 (A disintegrin A metalloproteinase 17), which increased activity is related to ischemia, heart failure, atherosclerosis, diabetes and hypertension (Peschon et al., 1998; Menghini et al., 2013; Xia et al., 2013; Speck et al., 2015). There is only one endogen inhibitor of ADAM-17, known as tissue inhibitor of metalloproteinase 3 (TIMP3), which activity is reduced in obesity, atherosclerosis, diabetes and insulin resistance (Chavey et al., 2003; Cardellini et al., 2009, 2011; Menghini et al., 2013). This results in increased ADAM17 action and augmented TNFα.

Adipokines can also be considered important clinical biomarkers and pharmacologic targets to treat atherosclerosis. Blood level of TNFα was associated with coronary heart disease in elderly, serving as a biomarker for CVD risk (Cesari et al., 2003). The inhibition of TNFα-induced signaling pathways that lead to LDL transcytosis was efficient in reducing atherosclerosis in experimental model (Zhang et al., 2014) as well as silencing of TNFα-encoding gene (Brånén et al., 2004). Apart from decreasing plasma cholesterol, simvastatin reduced leptin, and TNFα, increased adiponectin levels and decreased TNFα-induced apoptosis of endothelial progenitor cells (EPC), which contributes to clinical effectiveness of this class of drugs (Du et al., 2014; Krysiak et al., 2014). This highlights that strategies aimed to modulate inflammatory actions elicited by TNFα could be promising therapeutic options to treat atherosclerosis.

IL-6 and Atherosclerosis

Interleukin 6 (IL-6) is an important adipokin secreted by adipocytes. However, it can also be released by smooth muscle cells under the influence of angiotensin II (Libby, 2002). Tikellis and colleagues demonstrated that feeding apoE$^{-/-}$ mouse with a low salt diet activated the renin-angiotensin-aldosterone system (RAAS) and increased IL-6 in serum and aorta (Tikellis et al., 2012). As previously discussed, IL-6 can present different effects depending on the signaling pathway activated (classic or trans-signaling), the duration of the stimulus and the source of the cytokine (Schuett et al., 2009; Piya et al., 2013). It seems that pro- and anti-inflammatory effects of IL-6 depends on ADAM17 activation and the balance between the activation of classic and trans-signaling cascades (Scheller et al., 2011). Considering that in atherosclerosis ADAM17 is overactive (Speck et al., 2015), IL-6 is expected to present a proinflammatory role in this disease.

In endothelial cells, IL-6 trans-signaling is responsible for the up regulation of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin and the control of lymphocytes trafficking (Chen et al., 2006; Scheller et al., 2011), which in turn favors an proatherogenic phenotype. Also, this adipokine contributes to the differentiation of monocytes into macrophages (Chomarat et al., 2000). Interestingly, Speck and colleagues observed that the antiatherogenic effect of fish oil is due to reduction in ADAM17 activity. The consequent decreased release of endothelial adhesion molecules would contribute to endothelial barrier improvement. Moreover, authors found a reduction in sIL-6R in animals that received fish oil (Speck et al., 2015), which reinforces the role played by IL-6 trans-signaling in

early stages of atherosclerosis development. Additionally, IL-6 is negatively correlated with EPC number in patients with rheumatoid arthritis. These patients present increased morbidity and mortality attributable to accelerated atherosclerosis and develop endothelial dysfunction and EPC impaired function even at young age (Herbrig et al., 2006). IL-6 can also influence the production of other cytokines. It was reported that the increase in C-reactive protein (CRP), a marker of acute inflammation, is rather produced by the liver under the influence of IL-6 than a direct product of adipose cells (Bays, 2009). It was described that IL-6 can stimulate the production of matrix metalloproteinases, which contribute to plaque vulnerability/rupture and arterial remodeling (Watanabe and Ikeda, 2004; Schuett et al., 2009).

Blocking IL-6 effects using neutralizing monoclonal antibodies to treat atherosclerosis is controversial. In patients with lymphoproliferative disorder it was associated with an increase in body weight, hypertriglyceridemia and hypercholesterolemia (Nishimoto et al., 2005), which could favor the development of atherosclerotic plaques. Although inhibition of IL-6 with tocilizumab (an antibody that binds to both soluble and membrane bound IL-6R) has been reported to improve endothelial function and reduce arterial stiffness (Protogerou et al., 2011), it also increases LDL-cholesterol (Ridker and Lüscher, 2014). Thus, anti-IL-6 therapies are still considered a double-edged sword in atherosclerosis management. On the other hand, indirect approaches can reduce inflammatory actions of IL-6. Statin therapy was reported to reduce the IL-6-induced production of CRP and MCP-1, important inflammatory markers (Rodriguez et al., 2012). Blocking renin-angiotensin system (RAAS) with an inhibitor of angiotensin converting enzyme (ACE) reduced IL-6 and inflammation markers in atherosclerosis experimental model (Tikellis et al., 2012).

MCP-1 and Atherosclerosis

The release of MCP-1 by endothelial cells, smooth muscle cells, T cells, monocytes, macrophages and foam cells perpetuates inflammation and lipid accumulation in atheroma (Tabata et al., 2003; Lin et al., 2014), although experimentally this depends on a high cholesterol diet (Namiki et al., 2002). Also, adipocyte-derived MCP-1 is overexpressed in obesity, in proportion of adiposity (Weisberg et al., 2006). In early atheroma formation, MCP-1 can be considered the link between oxLDL and foam cell recruitment to vessel wall whereas oxLDL (but not native LDL) induce MCP-1 production (Cushing et al., 1990). Apart from migration of monocytes/macrophages, MCP-1 also controls its differentiation into foam cells. During this process, the number of LDL receptors decrease while the number of scavenger receptors (responsible for phagocytosis of oxLDL) increases (Stephen et al., 2010). It was reported that MCP-1 induce the expression of scavenger receptors on monocytes through extracellular signal-regulated kinase (ERK) (Tabata et al., 2003). In summary, MCP-1 can be considered a key molecule in the regulation of oxLDL phagocytosis/foam cell formation sequence. Interestingly, Hashizume and Mihara showed that oxLDL-induced MCP-1 was augmented by IL-6 and TNFα and that this mechanism is also involved in the induction of scavenger receptors by IL-6 and TNFα, which creates a self-perpetuating and amplifying cycle of inflammation and atherogenesis and suggests that IL-6 and TNFα participate in atherogenesis process also via oxLDL/MCP-1 induction (Hashizume and Mihara, 2012; Uchida et al., 2012). During fibrous cap formation around the lipid core, MCP-1 participates in smooth muscle cells (SMC) proliferation and activation (Gonzalez-Quesada and Frangogiannis, 2009).

MCP-1 is involved not only in the initial phase of atherosclerosis development but also in the final fatal complication of atherosclerotic plaque rupture and thrombosis. In endothelial cells, MCP-1 can induce the secretion of matrix metalloproteinases (Werle et al., 2002; Gonzalez-Quesada and Frangogiannis, 2009), which is crucial for plaque disruption. In addition, it was described that MCP-1 contributes to thrombin generation, thrombus formation and upregulation of tissue factor and plasminogen activation inhibitor-1 (PAI-1) (Charo and Taubman, 2004; Gonzalez-Quesada and Frangogiannis, 2009; Uchida et al., 2012).

Gonzalez-Quesada and Frangogiannis state that the effects of MCP-1 inhibition after myocardial infarction should be carefully evaluated because the suppression of this adipokine could delay the phagocytosis of dead cardiomyocytes and extend the injury extension (Gonzalez-Quesada and Frangogiannis, 2009). In this context, similarly to IL-6, indirect approaches that decrease not only MCP-1 but also other inflammatory markers are of interest. Statins were shown to decrease MCP-1, IL-6, IL-8 in hypercholesterolemic patients (Rezaie-Majd et al., 2002), although this might be dependent on treatment duration since that high doses of atorvastatin during 5 days did not modified inflammatory markers, including MCP-1, in aorta of high fat-feeding apoE$^{-/-}$ mice (Ekstrand et al., 2015). Nevertheless, MCP-1 inhibition resulted in decreased TNFα, IL-6, tissue factor and PAI-1 in an inflammation model induced by stress in mice (Uchida et al., 2012), suggesting a beneficial effect not only in inflammation but also in the prothrombotic state in the presence of atherosclerosis.

Leptin and Atherosclerosis

Unlike other adipokines, which are produced from different sources, leptin is mainly produced by adipocytes and plasma levels are positively correlated with white adipose tissue mass (Scotece et al., 2012). It regulates energy balance and metabolism both centrally and peripherally (Koh et al., 2012). In cardiovascular system, blood vessels and cardiomyocytes express the specific leptin receptor (named Ob-R, which presents 6 isoforms) and its actions are potentially proatherogenic, prothrombotic and angiogenic (Koh et al., 2012; Scotece et al., 2012; Adya et al., 2015). Interestingly, in obese individuals only the anorectic effect of leptin is impaired, whereas other effects are maintained (a phenomenon known as selective leptin resistance) (Mark et al., 2002; Singh et al., 2010; Adya et al., 2015) thus hyperleptinemia contributes to atherogenesis in these patients.

Hyperleptinemia is associated to impairment of NO-dependent vasorelaxation, increase in oxidative stress as well as increase in endothelin (a potent vasoconstrictor) (Yamagishi et al., 2001; Adya et al., 2015; Husain, 2015). All these features are markers of endothelial dysfunction, the first step in atherogenesis. It was described that leptin increases NADPH

oxidase expression and activity (Dong et al., 2006; Schroeter et al., 2012). Moreover, it increases the expression of type-1 angiotensin II receptor (AT1R) in smooth muscle cells (Zeidan et al., 2005). It is well established that angiotensin II increases oxidative stress through AT1R-dependent activation of NADPH oxidase (Braga et al., 2011). Thus, leptin potentiates deleterious angiotensin II effects in vascular function, which is potentially dangerous in hypertensive obese patients. Conversely, angiotensin II increases leptin synthesis (Koh et al., 2012), generating a self-perpetuating cycle of excessive oxidative stress and vascular dysfunction.

In initial phase of atheroma formation, leptin plays a crucial role in inflammatory pathways. It increases the secretion of TNFα, IL-6, and MCP-1 (Yamagishi et al., 2001; Koh et al., 2012) important inflammatory molecules as previously discussed. The expression of adhesion molecules such as VCAM-1, ICAM-1, and E-selectin are also increased by leptin (Adya et al., 2015). This favors monocytes attraction and migration through endothelial wall. During atheroma formation, leptin is also involved growth and migration of SMC (Zeidan et al., 2005; Husain, 2015). Leptin also induces a prothrombotic state once it enhances platelets activation and aggregation, thrombus formation and PAI-1 expression (Beltowski, 2006; Singh et al., 2010; Husain, 2015). Plaque rupture is an important event that usually precedes thrombus formation and leptin is involved in plaque rupture since it induces the production of MMP (Li et al., 2005; Adya et al., 2015).

Hyperleptinemia is related to acute cardiovascular events independent of traditional risk factors (Koh et al., 2012). Leptin treatment was described to be efficient in reducing weight in leptin-deficient obese mice and humans, but this effect was small in diet-induced obesity (Blüher, 2014), probably due to the selective leptin resistance previously mentioned. Although leptin may be involved in inflammation response in certainly conditions, inhibition of inflammatory cytokines such as TNFα did not modified leptin levels (Scotece et al., 2012). On the other hand, considering the synergism between angiotensin II and leptin, antihypertensive therapies may decrease leptin levels. Umeda and colleagues demonstrated that inhibition of AT1R decreased leptin in adipose tissue (Umeda et al., 2003). Also, inhibition of angiotensin II synthesis decreased leptin (Cassis et al., 2004). These results highlight the importance of blood pressure control in obese hypertensive patients, especially using RAAS-antagonists.

Periadventitial Adipose Tissue

Considering that patients with autoimmune diseases have increased risk for atherosclerosis, inflammation in different sites can be involved in atherogenesis, possibly due to the generation of cytokines and other factors that can be released into the circulation (Hahn et al., 2007; Rosenfeld, 2013). Medium and large arteries, where atherosclerotic plaques develop, are surrounded by periadventitial adipose tissue (PAAT), which provides chemical messengers and vasoactive mediators into the bloodstream and function as a paracrine organ (Mattu and Randeva, 2013; Chaldakov et al., 2014). Even though the view of atherosclerosis has been mainly focused on intimal lesions and luminal loss, it is likely that other components of vascular wall are involved in this inflammatory process (Chaldakov et al., 2014). In this regard, inflamed PAAT can be considered as an important source of pro and anti-inflammatory adipokines which contribute to plaque formation and stabilization. In the heart, adventitial lymphocytic inflammation is related to epicardial fat metabolism. It was observed that, in epicardial fat harvest during coronary bypass, there was an increase in proinflammatory markers (as IL-6, MCP-1, and TNFα) when compared to abdominal fat (Mazurek et al., 2003; Tavora et al., 2010). This confirms the relation between PAAT and atherosclerosis. Moreover, inflamed fat from other sources can also be responsible, at least in part, for atherogenesis. It was already shown that that inflammatory visceral fat accelerated atherosclerosis in apoE$^{-/-}$, possibly due to increase in MCP-1 since a pharmacological approach capable of reducing MCP-1 was efficient in reducing atherosclerosis in this model (Ohman et al., 2008).

CONCLUSION

In summary, it is now well established that adipose tissue can be considered a source of diverse molecules, which play important roles in the body homeostasis. Obese adipose tissue can induce a state of low-grade inflammation due to secretion of proinflammatory adipokines and the reduced secretion of anti-inflammatory ones. In this brief review we highlighted the participation of the main adipokines in insulin resistance, diabetes and atherosclerosis. The comprehension of molecular pathways involved in the mechanism of action of these molecules created the possibility for clinical and translational studies aiming to provide new therapeutic interventions. Patients who suffer from chronic inflammatory diseases present increased risk of diabetes and atherosclerosis. However, the use of anti-inflammatory therapies to treat these conditions is still controversial and often the results are inferior to the expected. On the other hand, indirect approaches which culminate in reduction of adipokines secretion or signaling seems to be promising. Nevertheless, considering that obesity is a manipulable risk factor which is often related to individual life style, an important approach to prevent CVD and diabetes is still the alteration of bad alimentary habits and reduction in body weight.

AUTHOR CONTRIBUTIONS

All authors participated in the design of the manuscript, drafted the manuscript, revised the manuscript critically and approved the final version.

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Increment of body mass index is positively correlated with worsening of endothelium-dependent and independent changes in forearm blood flow

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Obesity is associated with the impairment of endothelial function leading to the initiation of the atherosclerotic process. As obesity is a multiple grade disease, we have hypothesized that an increasing impairment of endothelial and vascular smooth muscle cell functions occurs from lean subjects to severe obese ones, creating a window of opportunities for preventive measures. Thus, the present study was carried out to investigate the grade of obesity in which endothelial dysfunction can be detected and if there is an increasing impairment of endothelial and vascular smooth muscle cell functions as body mass index increases. According to body mass index, subjects were allocated into five groups: Lean controls ($n = 9$); Overweight ($n = 11$); Obese class I ($n = 26$); Obese class II ($n = 15$); Obese class III ($n = 19$). Endothelial and vascular smooth muscle cell functions were evaluated measuring forearm blood flow responses to increasing intra-arterial infusions of acetylcholine and sodium nitroprusside using venous occlusion plethysmography. We observed that forearm blood flow was progressively impaired from lean controls to severe obese and found no significant differences between Lean controls and Overweight groups. Known determinants of endothelial dysfunction, such as inflammatory response, insulin resistance, and diagnosis of metabolic syndrome, did not correlate with forearm blood flow response to vasodilators. Moreover, several risk factors for atherosclerosis were excluded as independent predictors after confounder-adjusted analysis. Our data suggests that obesity *per se* could be sufficient to promote impairment of vascular reactivity, that obesity class I is the first grade of obesity in which endothelial dysfunction can be detected, and that body mass index positively correlates with the worsening of endothelium-dependent and independent changes in forearm blood flow.

Keywords: obesity, overweight, endothelial function, vascular reactivity, venous occlusion plethysmography, forearm blood flow

Introduction

Obesity is the worldwide leading metabolic disease with progressively increasing prevalence in developed and developing countries. Due to the rising trend in its prevalence, by the year 2030, if no actions are taken against this threat, the number of obese adults is projected to be around 600 million to 1 billion individuals (Kelly et al., 2008). This alarming situation made the World Health Organization refers to obesity as a global epidemic (Formiguera and Cantón, 2004).

Besides being an important clinical and public health burden in itself, obesity represents an important risk factor for atherosclerosis-related cardiovascular diseases, such as coronary artery disease. Indeed, some studies have indicated that obesity is associated with the impairment of endothelial function, one of the earliest markers of the atherosclerotic process (Hashimoto et al., 1998; de Jongh et al., 2004; Anderson, 2007; Yeboah et al., 2007). In this pathophysiological model of atherosclerotic disease, before the development of clinically overt atherosclerosis, arterial wall changes are limited to impaired endothelial function (Hashimoto et al., 2000).

As obesity is a multiple grade disease, we have hypothesized that an increasing impairment of endothelial and vascular smooth muscle cell functions occurs from lean subjects to severe obese ones, creating a window of opportunities for preventive measures. In fact, previous studies support the idea of the existence of a reversible early stage of atherosclerosis (Hashimoto et al., 2000). Unfortunately, although our current knowledge offers a potential explanation for the relationship between obesity and atherosclerosis, it still lacks the grade of obesity in which endothelial dysfunction begins. Thus, the present study was carried out to investigate the grade of obesity in which endothelial dysfunction can be detected and if there is an increasing impairment of endothelial and vascular smooth muscle cell functions as body mass index increases. Additionally, in order to perform a confounder-adjusted analysis we have tried to correlate our findings with known determinants of endothelial dysfunction, such as markers of inflammatory response, insulin resistance, and diagnosis of metabolic syndrome.

Materials and Methods

Subjects

Eighty women, aged 18–30 years old, with sedentary lifestyle were enrolled in this study. These subjects were consecutively recruited from Rio de Janeiro State University's outpatient clinic and from community volunteers. A structured interview, complete physical examination, and laboratory tests were performed to exclude subjects with diseases other than obesity, hypertension, hyperlipidemia, insulin resistance, and metabolic syndrome. Patients with glucose intolerance, diabetes mellitus, smoking habit, regular drinking habit, regular use of medications, and clinical manifestations of atherosclerosis were intentionally excluded due to known changes of vascular function.

The Ethics Committee of Pedro Ernesto University Hospital (Rio de Janeiro State University, Rio de Janeiro, Brazil) approved the study design and protocol and, after receiving a thorough explanation of the study, each subject gave written informed consent before enrollment.

Study Design

Each subject made two visits (on two consecutive days) to the Laboratory for Clinical and Experimental Research in Vascular Biology at Rio de Janeiro State University. In the morning of the first clinical visit, they were subjected to physical examination, anthropometric measurements, and laboratory tests. Blood and urine sampling was performed after 10–12 h overnight fast to measure plasma glucose, lipid profile, insulin, and other biochemical and inflammatory parameters. After fasting blood sampling, all subjects underwent a 2-h 75-g oral anhydrous glucose tolerance test to exclude glucose intolerance or diabetes (according to American Diabetes Association criteria, American Diabetes Association, 2014). In the morning of the second clinical visit, venous occlusion plethysmography was performed after 8–12 h overnight fast to assess endothelial function and vascular reactivity.

Physical Examination, Anthropometric Measurements, and Laboratory Tests

Arterial blood pressure was measured twice in supine position with 5-min interval between measurements, using an automated apparatus (LifeWindow LW6000, Digicare Biomedical Technology, West Palm Beach, FL, USA). A trained examiner collected all anthropometric measurements in duplicate: waist was measured at its smallest point with a relaxed abdomen, hip at the widest part of gluteal region, height using a vertical bar stadiometer, and weight using a digital scale (Filizola, São Paulo, SP, Brazil). Body mass index (BMI) was defined as the ratio between weight in Kg and squared height in meters.

All laboratory measurements were performed in duplicate. Plasma glucose level was assayed by the glucose oxidase method and serum insulin level by electrochemiluminescence. Serum non-esterified fatty acids (NEFA), serum total cholesterol, and serum triglyceride levels were measured enzymatically. Serum high-density lipoprotein (HDL) cholesterol level was measured by the heparin-Ca^{2+}/Ni^{2+} precipitation method. Serum C-reactive protein was measured by immunoturbidimetry. Urinary 8-isoprostane, serum interleukin-6 (IL-6), serum leptin, serum adiponectin, and serum resistin were assayed by ELISA. 8-isoprostane was measured in urine due to methodological problems with its serum measurement.

Low-density lipoprotein (LDL) cholesterol level was calculated according to Friedwald equation. In order to evaluate the insulin resistance status, the Homeostasis Model Assessment (HOMA-IR) was calculated according to the equation: HOMA-IR = [Plasma Glucose (in mmol/L) × Insulin (in μUI/mL)/22.5], using fasting levels.

Abbreviations: Ach, acetylcholine; BMI, body mass index; FBF, forearm blood flow; HDL, high-density lipoprotein; HOMA-IR, Homeostasis Model Assessment-Insulin resistance; IL-6, interleukin-6; LDL, low-density lipoprotein; NEFA, non-esterified fatty acids; SNP, sodium nitroprusside.

Metabolic Syndrome Diagnosis

Metabolic syndrome was diagnosed according to NCEP-ATPIII criteria (National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), 2002), which defines metabolic syndrome when three or more of the following criteria are present: 1, waist circumference $\geq$88 cm; 2, triglycerides $\geq$150 mg/dL; 3, HDL $<$50 mg/dL; 4, fasting plasma glucose concentration $\geq$100 mg/dL; 5, arterial blood pressure $\geq$130/85 mmHg.

Venous Occlusion Plethysmography

Studies were carried out in a quiet temperature-controlled room (21 $\pm$ 1°C) with the subjects in supine position. Forearm blood flow (FBF), in mL.100 mL^{-1} forearm volume.min^{-1}, was measured by mercury-in-silastic strain gauge venous occlusion plethysmograph (Hokanson EC6, D.E. Hokanson, Bellevue, WA, USA) placed in the point of maximal circumference of the non-dominant forearm, which was maintained at the level of the heart. Drugs or normal saline were continuously infused at 1.0 mL.min^{-1} into the ipsilateral brachial artery through a 27 SWG needle introduced under local anesthesia (1 mL of 1% lidocaine). After arterial needle insertion, subjects were allowed to rest for 30 min before any flow measurement.

Endothelial and vascular smooth muscle cell functions, as depicted by vascular reactivity, were evaluated measuring FBF responses to intra-arterial infusion of three increasing doses of endothelium-dependent [acetylcholine (Ach) 7.5, 15, and 30 μg.min^{-1}] and independent [sodium nitroprusside (SNP) 2, 4, 8 μg.min^{-1}] vasodilators, in this order. Each dose was infused for 5 min and FBF was recorded during the last 2 min of infusion. Prior to initiation of Ach and SNP, normal saline was infused for 20 min, and then blood flow measurements were taken to establish resting control values (baseline). This allowed an interval of 20 min between Ach and SNP infusions.

During the recording periods, hand circulation was excluded by inflating a wrist cuff to suprasystolic pressure 1 min before flow measurement. The upper arm congesting cuffs were intermittently inflated to 40 mmHg for 10 s in 15 s cycles to temporally occlude venous return, producing five flow measurements (slopes). Venous occlusion plethysmography apparatus was connected to an analog-to-digital converter (PowerLab 8/35, AD Instruments, Castle Hill, Australia) and data were recorded directly onto computer for later analysis with LabChart Pro 8 software (AD Instruments, Castle Hill, Australia). The first flow measurement was always excluded and the mean of the remaining four measurements in each recording period was used for data analysis. Responses to each dose of Ach or SNP were summed and analyzed as Cumulative FBF, which represents an integrated measurement of the cumulative response to the progressive doses of each vasodilator.

During FBF measurement, arterial blood pressure was measured in contralateral arm using a semiautomated oscillometric device (LifeWindow LW6000, Digicare Biomedical Technology, West Palm Beach, FL, USA).

Statistical Analysis

Results are expressed as means $\pm$ standard deviation of the mean (SD) for each group, unless otherwise noted. Statistical comparisons of normally distributed variables were performed using One-Way ANOVA, whereas Kruskal-Wallis test was used for other variables. When appropriate, an adequate test was used for *post-hoc* analysis: Bonferroni method or Dunn's multiple-comparisons. We also have performed univariate and multiple regression analysis of cumulative FBF responses to intra-arterial infusion of vasodilators in relation to other study variables or set of variables in order to find significant correlations and to perform confounder-adjusted analysis. Spearman correlation coefficients and p-values were calculated for univariate analysis. All statistical analyses were performed using Stata 10.1 (StataCorp, College Station, TX, USA) and GraphPad Prism 6.03 (GraphPad Software, La Jolla, CA, USA) and the significance level was set as $p < 0.05$ for a two-tailed test.

Results

According to BMI, subjects were allocated into five groups for data analysis: 1, Lean controls ($n = 9$; BMI = 22.2 $\pm$ 1.3 $kg.m^{-2}$); 2, Overweight ($n = 11$; BMI = 27.5 $\pm$ 1.4 $kg.m^{-2}$); 3, Obese class I ($n = 26$; BMI = 32.3 $\pm$ 1.4 $kg.m^{-2}$); 4, Obese class II ($n = 15$; BMI = 37.6 $\pm$ 1.6 $kg.m^{-2}$); 5, Obese class III ($n = 19$; BMI = 44.0 $\pm$ 2.4 $kg.m^{-2}$). Clinical, anthropometric, and laboratory characteristics of groups are presented in **Table 1**.

When compared with lean subjects, obese class I, II, and III ones showed greater systolic blood pressure, serum insulin levels, HOMA-IR, and leptin levels but lower HDL-cholesterol and adiponectin levels. IL-6 was significantly greater in Obese class II and III groups. Obese class III group had a significantly greater percentage of subjects with metabolic syndrome.

Venous Occlusion Plethysmography

Baseline FBF did not significantly differ between groups (**Table 2**). Ach and SNP intra-arterial infusions were associated with FBF improvements in all groups, but when compared with lean subjects, significant impairment of vascular reactivity was observed in obese class I, II, and III ones (**Table 2**). A lesser cumulative FBF response to intra-arterial infusion of Ach was observed in obese class I, II, and III subjects when compared with lean and overweight ones (**Figure 1A**). However, no significant differences were found between lean, overweight, obese class I, and obese class II subjects when cumulative FBF response to intra-arterial infusion of SNP was analyzed (**Figure 1B**). Comparisons between the three obese groups showed greater impairment of vascular reactivity in obese class III subjects (**Table 2** and **Figure 1**). No significant differences were found between Lean controls and Overweight groups.

On univariate analysis BMI ($r = -0.69$; $p < 0.0001$), systolic blood pressure ($r = -0.25$; $p = 0.03$), waist circumference ($r = -0.58$; $p < 0.0001$), hip circumference ($r = -0.64$; $p < 0.0001$), leptin levels ($r = -0.54$; $p < 0.0001$), and adiponectin levels ($r = 0.47$; $p = 0.0001$) showed significant correlation with the cumulative FBF responses to intra-arterial infusion of Ach, whereas BMI ($r = -0.54$; $p < 0.0001$), systolic blood pressure

TABLE 1 | Clinical, anthropometric, and laboratory characteristics of groups.

	Lean controls	Overweight	Obese class I	Obese class II	Obese class III
Age (years)	22.7 ± 3.3	26.2 ± 3.2	25.0 ± 3.2	25.5 ± 3.4	25.9 ± 3.1
Weight (kg)	55.4 ± 5.5#	73.5 ± 5.3#	83.0 ± 9.1#	96.0 ± 10.0#	113.9 ± 13.2#
Waist (cm)	66.8 ± 4.6#	85.9 ± 6.6#	95.2 ± 8.1#	102.1 ± 8.3#	111.9 ± 9.5#
Hip (cm)	94.7 ± 6.9	104.6 ± 6.9	112.5 ± 6.0*	122.0 ± 7.6*	132.6 ± 10.5*
Systolic blood pressure (mmHg)	108.9 ± 9.0	118.8 ± 13.7	128.3 ± 10.8*	129.1 ± 12.4*	133.0 ± 15.8*
Diastolic blood pressure (mmHg)	68.7 ± 10.1	76.2 ± 10.9	76.1 ± 8.6	76.3 ± 6.5	77.1 ± 10.2
Fasting plasma glucose (mmol.L^{-1})	4.6 ± 0.2	4.8 ± 0.2	4.9 ± 0.2	4.8 ± 0.2	4.9 ± 0.1
Serum insulin (μUI.mL^{-1})	5.7 ± 2.7	11.5 ± 5.6	15.5 ± 8.3*	20.3 ± 10.5*	22.3 ± 10.4*
HOMA-IR	1.2 ± 0.5	2.6 ± 1.2	3.4 ± 2.0*	4.5 ± 2.5*	4.8 ± 2.3*
Serum total cholesterol (mg.dL^{-1})	180.9 ± 22.9	174.3 ± 34.6	196.5 ± 27.4	168.3 ± 22.6	184.1 ± 35.0
LDL-cholesterol (mg.dL^{-1})	95.2 ± 23.6	99.7 ± 29.1	116.3 ± 26.4	97.3 ± 17.7	110.8 ± 30.5
Serum HDL-cholesterol (mg.dL^{-1})	67.9 ± 12.9	52.2 ± 13.1	53.3 ± 14.4	48.7 ± 10.5*	46.7 ± 13.3*
Serum triglycerides (mg.dL^{-1})	74.0 ± 25.0	92.5 ± 30.2	134.3 ± 72.6	111.5 ± 52.1	132.6 ± 67.5
Serum leptin (pg.mL^{-1})	11, 163 ± 5217	23, 086 ± 6190	35, 266 ± 20, 105*	53, 534 ± 17, 602*	49, 847 ± 23, 798*
Serum adiponectin (ng.mL^{-1})	13, 205 ± 2660	7965 ± 6996	5789 ± 2154*	4401 ± 1848*	5275 ± 2339*
Serum resistin (ng.mL^{-1})	8.5 ± 3.1	7.5 ± 2.6	7.0 ± 2.9	7.9 ± 3.8	8.4 ± 5.8
Serum NEFA (mmol.L^{-1})	0.4 ± 0.2	0.7 ± 0.1	0.7 ± 0.3	0.8 ± 0.2*	0.7 ± 0.2
Serum C-reactive protein (mg.dL^{-1})	0.4 ± 0.3	0.4 ± 0.3	0.9 ± 0.7	1.0 ± 0.7	0.7 ± 0.5
Serum IL-6 (pg.mL^{-1})	1.4 ± 0.2	2.0 ± 0.9	1.9 ± 0.8	3.6 ± 2.4*	2.9 ± 1.2*
Urinary 8-isoprostane (pg/μmol creatinine)	77.6 ± 18.7	106.6 ± 65.6	114.7 ± 103.4	131.7 ± 107.8	148.0 ± 145.6
Percentage of subjects with metabolic syndrome diagnosis (%)	0%	0%	42%	27%	63%*

*Results are expressed as means ± SD for each group. *$p < 0.05$ as compared with Lean controls group. #$p < 0.05$ as compared with any other group.*

TABLE 2 | Forearm blood flow (FBF) at baseline and after all three doses of Ach or SNP.

	Lean controls	Overweight	Obese class I	Obese class II	Obese class III
FBF prior to Ach infusion	1.5 ± 0.4	1.7 ± 0.4	2.1 ± 0.6	1.9 ± 0.5	1.7 ± 0.5
FBF after Ach infusion	10.9 ± 2.9	9.4 ± 3.0	6.6 ± 1.5*	5.9 ± 1.5*	3.7 ± 1.1#
FBF prior to SNP infusion	1.6 ± 0.6	1.9 ± 0.2	2.1 ± 0.7	1.8 ± 0.7	2.0 ± 0.7
FBF after SNP infusion	9.6 ± 1.8	9.3 ± 1.6	7.6 ± 2.2*	6.7 ± 1.9*	5.1 ± 1.5#

*Results are expressed as means ± SD for each group. FBF is expressed in mL.100 mL^{-1} forearm volume.min^{-1}. *$p < 0.05$ as compared with Lean controls group. #$p < 0.05$ as compared with any other group.*

($r = -0.35$; $p = 0.002$), waist circumference ($r = -0.41$; $p = 0.0002$), hip circumference ($r = -0.51$; $p < 0.0001$), and leptin levels ($r = -0.53$; $p < 0.0001$) showed significant correlation with the cumulative FBF responses to intra-arterial infusion of SNP. After confounder-adjustment, on multiple regression analysis only BMI showed significant correlation with the cumulative FBF responses to intra-arterial infusion of vasodilators ($R^2 = 0.47$ for Ach and 0.28 for SNP). Spearman correlation coefficients for univariate analysis of BMI data are presented in **Figure 2**.

Arterial blood pressure level evolution during vasodilators infusions showed no systemic effects of drugs nor significant differences between groups (data not shown).

Discussion

This study showed that vascular reactivity to intra-arterial infusions of endothelium-dependent (Ach) and independent (SNP) vasodilators, was greater in lean subjects than in obese ones. Several studies have already indicated that obesity is associated with the impairment of endothelial function (Hashimoto et al., 1998; de Jongh et al., 2004; Anderson, 2007; Yeboah et al., 2007), and that an inflammatory state associated with the release of a variety of cytokines and cytokine-like substances, such as leptin and resistin, is among the mechanisms that underlie the resultant microvascular dysfunction (Singer and Granger, 2007). An altered sympathetic neuronal responsiveness leading to diminished nitric-oxide dilation has also been suggested (Vollenweider et al., 1994). Our new finding was that an increasing impairment of endothelium-dependent and independent changes in FBF could be observed as BMI increases. Statistically, not only univariate analysis but also multiple regression analysis confirmed that BMI is an independent variable relating to cumulative FBF response to vasodilators.

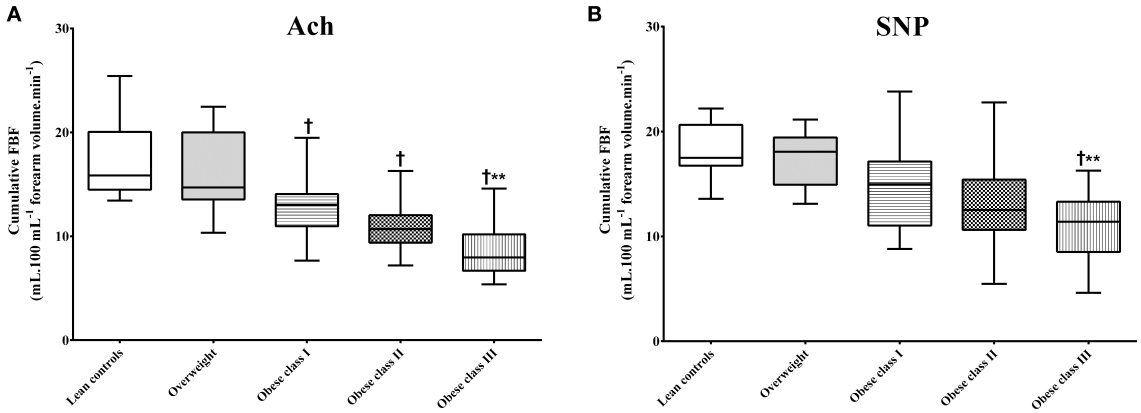

FIGURE 1 | Cumulative forearm blood flow (FBF) after intra-arterial infusions of Ach or SNP. (A,B) Show the cumulative FBFs derived from the sum of responses to each dose of Ach or SNP. $^{\dagger}p < 0.05$ as compared with Lean controls or Overweight groups. $^{**}p < 0.05$ as compared with Obese class I group.

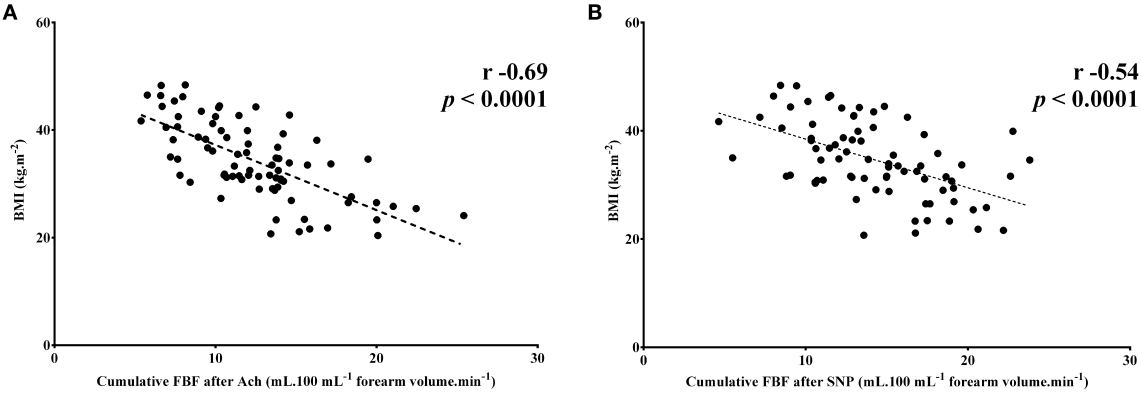

FIGURE 2 | Scatter plots of significant correlations between cumulative forearm blood flow (FBF) responses to intra-arterial infusion of vasodilators and body mass index (BMI). (A,B) Note that the less the BMI is, the better the cumulative response to the progressive doses of vasodilators are. Spearman correlation coefficient and *p*-values for univariate analysis are shown for each correlation. $n = 80$.

Other important finding of our study was that no statistical differences were observed in vascular smooth muscle cell function between lean, overweight, obese class I, and obese class II subjects (considering cumulative SNP response data) and in both endothelial and smooth muscle cell function between lean subjects and overweight ones. Together, these findings support the hypotheses that obesity class I is the first grade of obesity in which endothelial dysfunction can be detected, that arterial wall damages progress in direct relationship with BMI, and that in initial stages of obesity (obesity class I and II) before the development of overt atherosclerosis with vascular smooth muscle cell function impairment, arterial wall changes are limited to impaired endothelial function. Of note, it has already been shown that implementation of strategies targeting the reduction of risk factors related to atherosclerotic disease resulted in improvements of impaired endothelial function (Celermajer et al., 1993; Vogel, 1999; de Kleijn et al., 2001; Gokce et al., 2002; Taddei et al., 2002), allowing us to hypothesize about the existence of a "potentially reversible stage of atherosclerosis" when preventive measures could be taken. Unfortunately, despite considerable amount of evidence that impaired endothelial function can be improved by risk reduction strategies, no consensus exists that the reversal of endothelial dysfunction is associated with a reduction in cardiovascular events (Taddei et al., 2002).

Interestingly, known determinants of endothelial dysfunction, such as inflammatory response, insulin resistance (using HOMA-IR as a surrogate marker), and diagnosis of metabolic syndrome, did not correlate with FBF response to vasodilators in our study. Moreover, several risk factors for atherosclerosis were excluded as independent predictors after confounder-adjusted analysis. Our results indicated that obesity *per se* could be sufficient to promote impairment of endothelial function and vascular wall reactivity. The selection of young subjects and exclusion of those with diabetes mellitus, smoking habit, regular use of medications, and clinical manifestations of atherosclerosis, imposing age and disease restrictions to our study design may have helped in dealing with confounding variables, clarifying the role of obesity.

Finally, we have chosen to measure FBF with venous occlusion plethysmography method given its minimally invasive characteristic and our previous experience with this methodology (de Aguiar et al., 2006). Furthermore, systemic administration of vasoactive drugs may lead to central effects, hormonal responses, changes in sympathetic output, and alterations in blood pressure that make changes in forearm blood flow difficult to interpret (Benjamin et al., 1995). Thus, another advantage of our *in vivo* study model is the possibility to elevate flow locally by intra-arterial infusions of sub-systemic doses of vasodilators (often 100–1000 times lower than a systemically effective dose), obviating any confounding systemic effects on drug response. Drugs are infused through a fine needle placed in the brachial artery allowing the study of the direct vascular effects, without affecting systemic parameters. There is extensive worldwide experience with this technique and it is considered quite safe (Benjamin et al., 1995).

In conclusion, the present study demonstrated that obesity class I is the first grade of obesity in which endothelial dysfunction can be detected, that BMI is an independent variable relating to FBF response to intra-arterial infusions of vasodilators, existing an increasing impairment of such response when BMI increases, and that the worsening of vascular response to vasodilators is limited to an impaired endothelium-dependent response in initial stages of obesity.

Limitations and Perspectives

Our study has some limitations. First, fitting BMI as a predictor in our linear model resulted in low R-squared coefficients, allowing us to hypothesize that other non-measured/studied predictors are present. Those predictors could help to explain part of the variations in vascular reactivity to intra-arterial infusions of vasodilators fitting better linear models. Second, the observational design of our study does not allow further inferences about the reversal of endothelial dysfunction with implementation of preventive measures. Third, although our inclusion/exclusion criteria may have helped in dealing with confounding, it may have decreased the external validity of our findings. Finally, we cannot exclude that the relatively small and unbalanced number of subjects per group may have biased our findings. Further studies are needed to elucidate these issues.

Author Contributions

LK designed the study, recruited patients, performed the experiments, collected and analyzed the data, and critically revised the final version of the manuscript; MM analyzed the data and wrote the final version of the manuscript; DB designed the study, performed the experiments, and collected the data; RL performed the experiments and collected the data; MS performed the experiments and collected the data; MB analyzed the data and wrote the final version of the manuscript; NV designed the study, performed the experiments, and collected and analyzed the data; EB designed the study and critically revised the final version of the manuscript. All authors read and approved the final version of the manuscript.

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REVIEW
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Obesity-Driven Gut Microbiota Inflammatory Pathways to Metabolic Syndrome

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The intimate interplay between immune system, metabolism, and gut microbiota plays an important role in controlling metabolic homeostasis and possible obesity development. Obesity involves impairment of immune response affecting both innate and adaptive immunity. The main factors involved in the relationship of obesity with inflammation have not been completely elucidated. On the other hand, gut microbiota, via innate immune receptors, has emerged as one of the key factors regulating events triggering acute inflammation associated with obesity and metabolic syndrome. Inflammatory disorders lead to several signaling transduction pathways activation, inflammatory cytokine, chemokine production and cell migration, which in turn cause metabolic dysfunction. Inflamed adipose tissue, with increased macrophages infiltration, is associated with impaired preadipocyte development and differentiation to mature adipose cells, leading to ectopic lipid accumulation and insulin resistance. This review focuses on the relationship between obesity and inflammation, which is essential to understand the pathological mechanisms governing metabolic syndrome.

Keywords: adipose tissue, cytokines, gut microbiota, immune system, toll-like receptors

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GUT MICROBIOTA ROLE IN OBESITY

Obesity has increased alarmingly worldwide, promoting mortality and morbidity (Mitchell and Shaw, 2015). Overweight and obesity are commonly associated with accumulated abdominal visceral fat and can be related to psycho-sociological behavioral disorders (Burdette and Hillb, 2008; Jauch-Chara and Oltmanns, 2014). Fat gain and adipose tissue inflammation, resulted from excessive caloric intake and reduced energy expenditure, lead to positive energy balance and can contribute to metabolic syndrome (Trayhurn, 2005; Emanuela et al., 2012; DeMarco et al., 2014). Besides, chronic stress and gut microbiota deregulation can affect obesity development (McGill, 2014).

Human microbiota, made up of bacteria, archaeas, viruses and unicellular eukaryotes, represents more than 10^{14} microbial cells/humam, which live peacefully in our body (Sekirov et al., 2010). These microbes are found in our skin, genitourinary, respiratory and gastrointestinal tracts. Gut microbiota represents over than 7×10^{13} microbial cells/human, but its composition can be altered throughout life, including changes in gene expression (Walsh et al., 2014).

There are over 50 bacterial phyla, but the human gut microbiota is dominated mostly by the Bacteroidetes and the Firmicutes (Schloss and Handelsman, 2004; Sekirov et al., 2010). Gut specific microbial phyla, species and strains of humans and other animals are related to gene expression alterations observed in obesity (Ley et al., 2005; Turnbaugh et al., 2008; Fujimura et al., 2010; Clarke et al., 2012; Cotillard et al., 2013; de Theije et al., 2014). It has been demonstrated that obesity is associated with reduced bacterial diversity and modified representation of bacterial genes and metabolic pathways (Turnbaugh et al., 2009). Furthermore, Turnbaugh et al. (2006) provide evidences that gut microbiota in obese mice have an increased ability for energy harvest from the diet. In this work, colonization of germ-free mice with caecal microbiota harvested from obese donors results in a significant total body fat gain.

Probiotics (e.g., many bacterial strains of the *Lactobacillus* and *Bifidobacterium* genera), when administered in adequate amounts, induces health-beneficial effects, representing a novel anti-obesity mechanism (Raoult, 2009; Aronsson et al., 2010; Kadook et al., 2010). Studies demonstrated that *Lactobacillus* treatment reduces fat accumulation and pro-inflammatory cytokines in adipose tissue (Park et al., 2013; Yoo et al., 2013; Miyoshi et al., 2014; Ukibe et al., 2015). *Lactobacillus* strain (*L. plantarum*) anti-inflammatory effect was also observed in intestinal inflammation rat model, mostly by NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) inhibition (Štofilová et al., 2015). Similar results were also observed in endotoxin- and metabolic-related inflammatory process in rats (Vilahur et al., 2015). However, in diabetic and non-diabetic individuals, oral supplementation with another *Lactobacillus* strain (i.e., *L. acidophilus*) did not affect systemic inflammatory response (Andreasen et al., 2010). These opposite results could be related to differences in *Lactobacillus* strains or even to different experimental models.

Lactobacillus effect on fat storage may involve upregulation of circulating lipoprotein lipase inhibitor, angiopoietin-like 4 protein (ANGPTL4), which controls triglyceride deposition into adipocytes (Aronsson et al., 2010). In addition, probiotics treatment can modulate gut flora composition, which in turn enhance metabolic functions to prevent overweight and obesity (Park et al., 2013; Yadav et al., 2013). Moreover, obese mice antibiotics treatment is also capable to reduce adiposity and adipose tissue inflammation, which reinforce the benefits of gut microbiota regulation (Tremaroli and Bäckhed, 2012).

Gastrointestinal microbiota also interferes with carbohydrate, lipid and amino acid metabolism (Hooper et al., 2002), complementing our own human metabolic apparatus (Bäckhed et al., 2004, 2007; Cani and Delzenne, 2009; Rabot et al., 2010). Thus, human gut microbiota can regulate many metabolic pathways, including bile acids biotransformation, which involves deconjugation, dehydroxylation, and reconjugation reactions (Ridlon et al., 2014). Gut microbiota components, such as bacterial bile salt hydrolases and bacterial 7α-dehydroxylase, can control these reactions and, thus, maintain bile acids pool size and composition (Ridlon et al., 2006). It has been demonstrated that bile acids have both direct antimicrobial effects on gut microbes and indirect effects through FXR (farnesoid X receptor)-induced antimicrobial peptides (Inagaki et al., 2006). This antimicrobial effect promoted by bile acids prevent mucosal injury in the small intestine and other injuries caused by excessive bacterial proliferation (Hofmann and Eckmann, 2006; Merritt and Donaldson, 2009). It was also described that reduced bile acid levels in the gut are associated with bacterial overgrowth and inflammation. However, some bacteria, such as *Alistipes, Bilophila,* and *Bacteroides*, are bile acids tolerant, which could lead to other symbiotic microbes suppression (David et al., 2014).

Bile acids can also regulate adiposity and glucose homeostasis. Studies demonstrated that nuclear receptor FXR deficiency leads to mass adipose tissue reduced levels (Cariou et al., 2006; Prawitt et al., 2011). On the other hand, FXR absence has different effects on glucose homeostasis in lean and obese mice. $FXR^{-/-}$ lean mice presents impaired glucose tolerance and insulin resistance (Cariou et al., 2006; Ma et al., 2006), while obese mice (murine models of genetic and diet-induced obesity) presents glucose homeostasis improvement (Prawitt et al., 2011). This difference can be explained by bile acids action in other receptors, such as TGR5 (also known as G protein bile acid receptor-1), since Thomas et al. (2009) showed that TGR5 activation results in the maintenance of glucose homeostasis and insulin sensitivity in obese mice.

Furthermore, gut microbiota plays a physiological role in host immune system development [e.g., gut-associated lymphoid tissue (GALT) development] (Bäckhed et al., 2005; Willing et al., 2010; Guinane and Cotter, 2013) and immune tolerance modulation (Bailey et al., 2005; Vael and Desager, 2009; Martin et al., 2010; Belkaid and Hand, 2014). In addition, gut microbiota modulates other important intestinal functions such as angiogenesis and epithelium function (Hooper et al., 2001). Epithelial (e.g., enterocytes and goblet cells) and endocrine cells provide an interplay between the host and its own gut microbiota via receptors such as toll-like receptors (TLRs; Lotz et al., 2003; Kelly et al., 2004; Hornef and Bogdan, 2005; Shibolet and Podolsky, 2007; Wells et al., 2011; Pott and Hornef, 2012). After TLR activation, pro-inflammatory molecules can be produced in the gut microbiota and impair host metabolism, which in turn can further cause adipose inflammation and obesity (Sanz and Moya-Pérez, 2014).

Additionally, gut homeostasis is related to other innate immune receptors, such as nucleotide-binding oligomerization domain (NOD) like receptors (NLR; Zambetti and Mortellaro, 2014). This family of cytosolic receptors includes NOD1/2 and NLRPs (NLR family, pyrin-domain-containing proteins). After activation, NLRP forms signaling complexes called inflammasomes, which generate active forms of the inflammatory cytokine IL-1β and IL-18. Some different inflammasome subtypes have been described such as NLRP1, NLRP3, NLRP6, NLRC4, AIM2 (Latz et al., 2013). Studies have demonstrated that NLRC4 inflammasome is involved in mucosal protection against infections (Sellin et al., 2014; Nordlander et al., 2014), while NLRP6 and NLRP3 are associated with gut microbiota homeostasis (Elinav et al., 2011; Hirota et al., 2011; Wlodarska et al., 2014). Inflammasomes and gut homeostasis interaction is substantially detailed by Sellin et al. (2015) and Zambetti and Mortellaro (2014).

INTERPLAY BETWEEN INFLAMMATION AND OBESITY

Inflammation is a tightly controlled physiological process that is orchestrated by immune system (Ashley et al., 2012), but is also regulated by other systems, such as endocrine (de Vasconcelos et al., 2011; Leite et al., 2015; Ren et al., 2015) and nervous system (Martelli et al., 2014; Bassi et al., 2015). Despite the protective body response represented by inflammation, deregulated, or excessive immune response can lead to several chronic diseases such as hypertension (Mirhafez et al., 2014), Alzheimer (Takeda et al., 2014), and obesity (Khan et al., 2014). The classical acute inflammatory process includes five cardinal signals: redness, heat, swelling, pain, and, eventually, loss of function (Medzhitov, 2010). These macroscopic signals are reflex of vascular (e.g., vascular permeability) and cellular (e.g., leukocytes migration) alterations during inflammation (Medzhitov, 2008). However, inflammatory response in obesity has some particular features (Gregor and Hotamisligil, 2011). Obesity involves immune response impairment affecting both innate and adaptive immunity. However, the mechanisms involved in the relationship between obesity and inflammation have not been completely elucidated (Sanz and Moya-Pérez, 2014).

Obesity is related to inflamed adipose tissue and increased local cell infiltration (Gregor and Hotamisligil, 2011). Different cell types contribute to adipose tissue inflammation, among these cells monocytes/macrophages play a critical role in this process (Cinti et al., 2005; Subramanian and Ferrante, 2009; Ferrante, 2013). Yoshimura et al. (2015) demonstrated that obese young adults have increased number of leukocytes, mostly monocytes, when compared with non-obese individuals. Also, elevated monocytes level is positively correlated with visceral subcutaneous fat as well as with body fat mass. Peripheral blood of obese women presents an elevated inflammatory monocytes amount (Ziegler-Heitbrock, 2007; Krinninger et al., 2014). In addition, Poitou et al. (2011) also demonstrated that inflammatory monocytes are increased in obese individuals and fat body loss is associated with significant decrease of these cells.

Once within tissues, monocytes differentiate in M1 or M2 polarized macrophages (Dalmas et al., 2011). The first type is classified in pro-inflammatory cell which expresses inducible nitric oxide synthase and pro-inflammatory cytokines (e.g., IL-6 and TNF-α), while M2 macrophages express arginase (Arg1) and the anti-inflammatory cytokine IL-10. In lean individuals, M2 macrophage predominates in adipose tissue unlike in obese individuals; wherein M1 macrophages are mostly present (Kraakman et al., 2014). Macrophages of high-fat diet fed mice display autophagy impairment, a cytoprotective response to different stimulus, which leads to M1 polarization (Liu et al., 2015).

In obese individuals, monocytes up-regulate chemokine receptor type 2 (CCR2) and thus they migrate toward adipose tissue. Despite the natural ligand of this receptor, the chemokine CCL2 (also as known as MCP-1), plays an important role in adipose tissue macrophage recruitment (Kanda et al., 2006), other studies demonstrated that CCL2 is not critical for macrophage infiltration into adipose tissue (Inouye et al., 2007; Kirk et al., 2008). These findings can be related to macrophage recruitment toward adipose tissue by other chemokine, such as CXCL12 and CXCL14, as demonstrated by Kim et al. (2014) and Nara et al. (2007), respectively. Furthermore, the chemokine CCL5 (also as known as RANTES) and its receptors CCR5 are also important in this macrophage migration process (Keophiphath et al., 2010; Kitade et al., 2012).

Additionally, CCR2 modulates other parameters than macrophage recruitments. High-fat diet fed mice with genetic CCR2 deficiency present food intake reduction and lower obesity development (Weisberg et al., 2006). In addition, obese $CCR2^{-/-}$ mice have an increased adipose tissue eosinophil number and high levels of IL-4 and IL-13, cytokines which lead to M2 macrophage polarization (Bolus et al., 2015).

Not only migration, but also macrophage proliferation contributes to adipose tissue inflammation. Amano et al. (2014) showed that obese mice increased macrophage proliferation, especially in visceral adipose tissue. Moreover, they showed that CCL2 stimulates adipose tissue macrophage proliferation.

Adipose tissue macrophages are source of inflammatory cytokines in obese individuals. Between these cytokines, IL-6 displays pleiotropic role in metabolism and obesity. Sárvári et al. (2015) demonstrated that macrophages engulf portions of adipocytes *in vitro* leading to NF-κB activation and IL-6 secretion. In addition, Kraakman et al. (2015) related pro-inflammatory action to IL-6 *trans*-signaling, a process where IL-6 binds a soluble receptor to trigger inflammation. In this work, they demonstrated that this IL-6 signaling induces macrophage recruitment to adipose tissue.

IL-6 can also induce C reactive protein (CRP) liver production, which is associated to complement activation, phagocytosis and cytokines production (Deban et al., 2009; Du Clos, 2013). In obese individuals, CRP is elevated, demonstrating a state of active immune response and inflammation in these subjects (Shaharyar et al., 2015; Yoshimura et al., 2015). On the other hand, Ma et al. (2015), using a different model, showed that sustained IL-6 gene expression in obese mice reduces body weight loss, fatty liver and insulin resistance. Additionally, it was evidenced that IL-6 supports M2 polarization, an anti-inflammatory cell, by sensitizing macrophages to IL-4 (Mauer et al., 2014). Despite its variable effects, these findings demonstrate IL-6 critical role of in obese individuals.

Although macrophages infiltration is considered a hallmark of adipose tissue inflammation, other cells of the immune system display a fundamental role (Sell et al., 2012). In fact, some studies demonstrated that neutrophil migration into adipose tissue, as well as in classical acute inflammation, occurs after 3 days of high-fat diet in mice (Elgazar-Carmon et al., 2008; Talukdar et al., 2012). In addition, Xu et al. (2015) demonstrated an increased peripheral blood neutrophil percentage in obese young male.

Several types of lymphocytes interact with other cells in adipose tissue environment to enhance or decrease inflammatory response. Interactions between macrophages and $CD4^+$ T cell via MHC class II is required for adipose tissue inflammation and for obesity-induced insulin resistance (Cho et al., 2014). $CD4^+$ T cell could polarize to different subtypes of lymphocytes,

TABLE 1 | Role of lymphoid origin cells in obesity-related inflammation.

Lymphoid subsets cells	Role in obesity
Th17	Increased in obese individuals (Winer et al., 2009). IL-17A, a Th17 key cytokine, up-regulates IL-6, IL-8, and PGE2 levels in adipocytes (Shin et al., 2009).
Th22	Increased in obese individuals (Zhao et al., 2014). Unclear role.
NK cell	Contributes to M1 macrophage polarization (Wensveen et al., 2015).
iNKT	Induce M2 macrophage polarization and control Treg proliferation (Lynch et al., 2015).
ILC2s (group 2 innate lymphoid cells)	Control obesity development by inducing caloric expenditure (Brestoff et al., 2014).

namely Th1, Th2, Th17, regulatory T (Treg) cells, and other types of cells (Luckheeram et al., 2012). Despite all these subtypes of cells are related to obesity and metabolic syndrome, pro-inflammatory Th1 and Th17 predominate over Treg and Th2 during adipose tissue inflammation (Sell et al., 2012; McLaughlin et al., 2014).

High-fat diet fed mice present Th1 polarized and IFN-γ production predominance, which occurs after macrophage recruitment (Strissel et al., 2010). IFN-γ expression displays a regulatory role in adipose tissue inflammation, since its absence reduces TNF-α and CCL-2 mRNA expression and macrophage adipose tissue accumulation (Rocha et al., 2008). Interestingly, T-box transcription factor (T-bet) absence, a key factor to development of Th1 cell, leads to obesity possibly by IL-6 up-regulation (Kim et al., 2013). In **Table 1**, we summarize other types of lymphoid cells involved in obesity-related inflammation.

Immune cells need to sense foreign structures to develop an immunological response. Particularly, innate immune cells use pattern recognition receptors (PRR) to recognize specific pathogen or damaged molecules (Janeway and Medzhitov, 2002). Between these receptors, toll-like receptors are structurally and functionally well-defined (Kawai and Akira, 2010), and are related to obesity.

TOLL-LIKE RECEPTORS (TLR) AND OBESITY

TLRs (toll-like receptors) can recognize pathogen-associated molecular patterns (PAMPs) of microorganisms, which are not conserved in eukaryotes. This recognition triggers immune system activation, setting up innate immune response (Kawai and Akira, 2010). These receptors were initially identified in the fruit fly *Drosophila melanogaster*, first being associated with its embryonic development. Later on, its role on pathogens detection and immune response was described (Lemaitre et al., 1996; Williams et al., 1997). Janeway and his collaborators identified the first toll homolog in humans, the TLR4 (Medzhitov et al., 1997). In mammals, there are 12 members from TLRs family, but only TLR1-TLR10 function is known (Akira et al., 2006).

TLRs location is important to grant the access to the ligand. The majority of plasma membrane TLRs recognizes microbial membranes components, such as proteins, lipoproteins and lipids; while intracellular TLRs are able to recognize nucleic acids of microorganism (Werling and Jungi, 2003). TLRs can recognize a broad variety of PAMPs derived from many classes of microorganisms such as parasites, fungi, viruses and bacteria (Medzhitov, 2007). These PAMPs include many molecules including β-glucan, found on fungus, both viral RNA and DNA, and also a huge quantity of elements derived from bacteria (e.g., lipopeptides, peptidoglycan, lipoteichoic acid, and lipopolysaccharide (LPS; Aderem and Ulevitch, 2000).

Despite the fact that TLRs recognize a variety of PAMPs, each TLR can only recognize a limited group of patterns and, therefore, has a determined specificity for their ligands (Beutler, 2003). TLR4 is the LPS receptor (Poltorak et al., 1998a,b). TLR2 was found to recognize bacterial peptidoglycan and lipopeptide (Takeuchi et al., 1999). TLR5 is able to recognize flagellin, a protein derived from bacterial flagella (Hayashi et al., 2001). TRL3 is associated to the identification of double-stranded RNA molecules (Alexopoulou et al., 2001). TLR7 can recognize RNA molecules, especially small interfering RNAs (Hornung et al., 2005). TLR8 is similar to TLR7 and recognize viral ssRNA. Finally, TLR9 is associated with the recognition of non-methylated bacterial DNA (Hemmi et al., 2000). Together, all these receptors are able to recognize a broad variety of microorganisms and promote activation of the NF-κB, which is responsible for synthesis of inflammatory mediators (Lee et al., 2012).

TLRs are specially expressed in hematopoietic cells, including immune system cells. However, its expression was already confirmed in other kind of cells such as adipocytes (Kanczkowski et al., 2008). Therefore, these receptors can act promoting interplay between the innate immune system and metabolism (Fresno et al., 2011). Studies conducted on the role of TLRs on adipose tissue suggest that all subtypes of TLRs can be found in this tissue. (Hwa et al., 2006; Pietsch et al., 2006; Poulain-Godefroy and Froguel, 2007; Vitseva et al., 2008). Nevertheless, initially only TLR2 and TLR4 were functional in human adipocytes (Bès-Houtmann et al., 2007), but lately TLR5 activation was evidenced (Pekkala et al., 2015). It was described that TLR2, TLR4, or TLR5 deficiency have a major role on obesity development (Fresno et al., 2011).

It was described that TLR2 activation can be triggered by saturated fatty acids (SFAs; Lee et al., 2001, 2003). During endotoxemia, TLR2 is also activated by bacterial peptidoglycan from the intestines (Cani et al., 2008). Moreover, TLR2 absence decreases expression of inflammatory mediators and macrophages infiltration in white adipose tissue (WAT). Also, other studies demonstrated that TLR2 reduced levels protects against obesity and inflammation (Himes and Smith, 2010; Davis et al., 2011). Together, these data reveal a certain importance regarding TLR2 role in obesity. In addition, the role played by TLR5 in obesity is not well-established. It was recently found that TLR5 signaling in adipose tissue could corroborate to obesity, inflammation and metabolic alterations. Additionally, it was reported that TLR5 activation leads to ERK1/2 (extracellular

signal-regulated kinase) phosphorylation and adipocytes insulin signaling inhibition (Pekkala et al., 2015).

Both obesity and metabolic syndrome are characterized by inflammatory responses, triggered by adipose tissue disruption mediated TLR signaling (Pekkala et al., 2015). After activation, individual TLRs recruit TIR (Toll/IL-1 receptor) domain-containing adaptors members such as MyD88 (Myeloid differentiation primary response gene 88), TRIF (TIR-domain-containing adapter-inducing interferon-β), TIRAP/MAL (Toll-interleukin 1 receptor domain containing adaptor protein/MyD88 adapter-like) or TRAM (TRIF-related adaptor molecule). However, MyD88 is used by all TLRs to activate NF-κB and MAPKs (mitogen-activated protein kinases) for the induction of inflammatory cytokine genes (Kawasaki and Kawai, 2014).

TLR4 AND CELL SIGNALING PROTEINS: TARGETS TO OBESITY AND ITS COMPLICATIONS

Obese patients express high levels of TLR4 (Reyna et al., 2008). TLR4 activation, which occurs in obesity, can be activated by gut microbial patterns, such as LPS, to promote inflammatory mediators production (Kim et al, 2012). In addition, TLR4 can also mediate the pro-inflammatory effect of SFAs, often found at high levels in plasma of obese individuals (Lee et al., 2001; Shi et al., 2006; Dasu and Jialal, 2010). Many studies demonstrated that decreased TLR4 expression protects from obesity development, adipose tissue inflammation and insulin resistance (Shi et al., 2006; Suganami et al., 2007; Tsukumo et al., 2007; Davis et al., 2008; de Mello et al., 2008). A similar effect was observed using anti-TLR4 antibodies (Milanski et al., 2009). In TLR4 deficient mice, adipose tissue inflammation reduction could be explained by M2 macrophage polarization (Orr et al., 2012).

Studies suggest that obesity TLR4 signaling essentially depends on MyD88 expression and up-regulated NF-κB activity, with IL-6 and TNF-α pro-inflammatory cytokines increased expression (Fresno et al., 2011). Despite this classical signaling pathway, new insights about TLR4 signaling are emerging. In fact, Luo et al. (2014) demonstrated that small GTPase Rab8a and phosphatidylinositol 3-kinase γ (PI3Kγ) act as regulators of cytokines production, decreasing pro-inflammatory cytokines and increasing anti-inflammatory cytokines. These effects are mediated by Akt/mTOR signaling. The protein kinase mTOR restrains the pro-inflammatory cytokines production by NF-κB inhibition, while the anti-inflammatory cytokine (i.e., IL-10) are enhanced by STAT3 activation (Weichhart et al., 2008). Thus, the TLR4 signaling can regulate the inflammatory response by modulating different transcriptions factors.

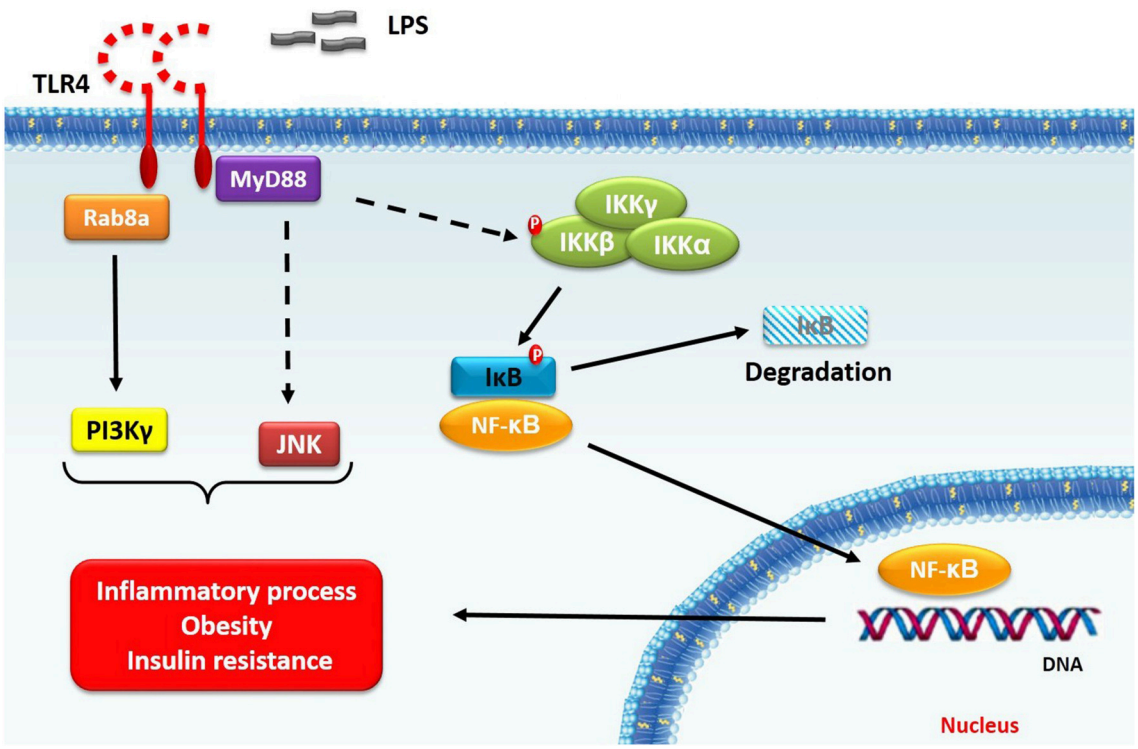

FIGURE 1 | TLR4 signaling in obesity. TLR4 activation (i.e., after LPS stimulus) leads to signal transduction, which involves IKK-β–NF-κB classical pathway. After stimulation, MyD88 is recruited to TLR4 receptor to mediate downstream signaling, including IKK-β phosphorylation. Once activated, IKK-β phosphorylates IκB protein, which, in turn, release NF-κB complex. Besides this pathway, TLR4 signaling also results in PI3Kγ and JNK activation. Taken together, these signaling proteins play a fundamental role in inflammation, obesity and insulin resistance relationship. Note: dashed arrows indicate that other signaling intermediates are required.

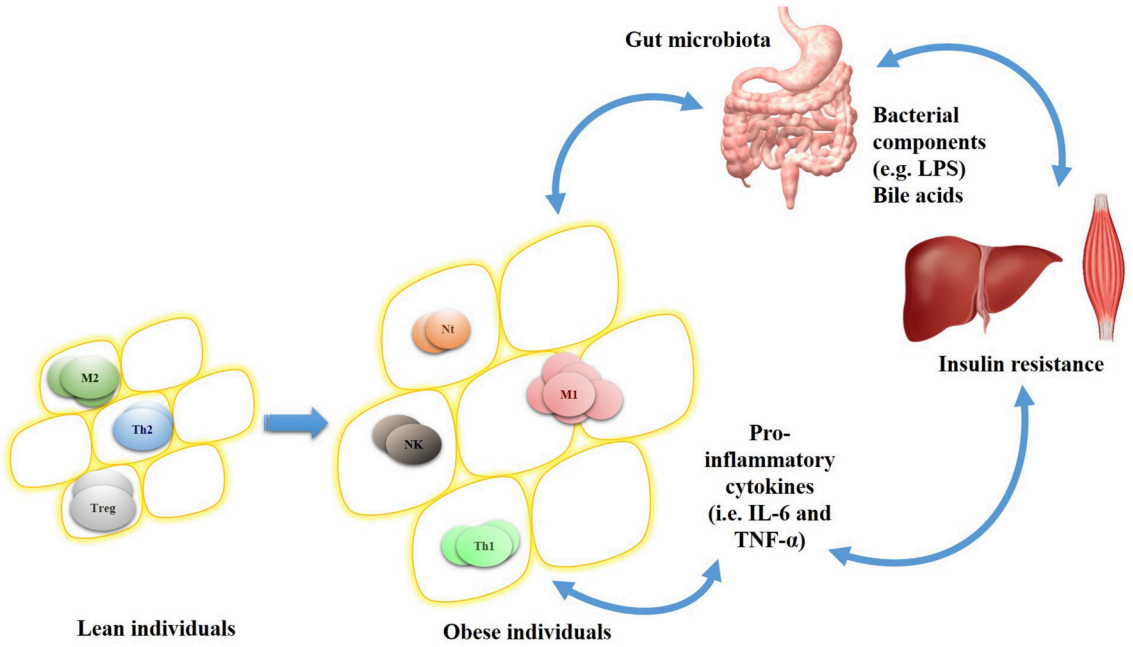

FIGURE 2 | Adipocytes-infiltrating immune cell profile in lean and obese individuals and relationship between gut microbiota, insulin sensitive organs, and inflammation. In lean individuals, adipocytes cells (yellow circles) are infiltrated by anti-inflammatory cells (e.g., M2 macrophage and regulatory T cell [Treg]) and helper T lymphocyte 2 (Th2). On the other hand, obese individuals have hypertrophied adipocytes associated with pro-inflammatory cells (e.g., M1 macrophage and neutrophils [Nt]), NK and Th1 lymphocyte, which altogether induces pro-inflammatory mediators release. This inflammatory cell infiltration is influenced by the cytokines produced locally and also by host-gut microbiota interactions (e.g., bile acids and LPS influence), which in turn are directly associated to obesity and its complications (i.e., insulin resistance).

Obesity leads to an increase in IKK-β–NF-κB signaling, a primary regulator of inflammatory response, in the liver. This phenomenon is related to fatty liver accumulation, which activates IKK-β–NF-κB, resulting in pro-inflammatory cytokines and insulin resistance (Cai et al., 2005). In addition, myeloid cells IKK-β absence improves systemic insulin sensitivity (Arkan et al., 2005). Hypothalamic neurons IKK-β–NF-κB axis is also involved in obesity and insulin resistance (Zhang et al., 2008). This pathway is a target to non-acetylated salicylates drugs, which can emerge as a new treatment to glucose reduction in diabetic patients (Rumore and Kim, 2010). Furthermore, the IKKε deficiency protects from obesity, inflammation and insulin resistance (Chiang et al., 2009; Olefsky, 2009).

Other signaling protein is related to obesity (Hirosumi et al., 2002) and insulin resistance is cJun NH2-terminal kinase (JNK; Nguyen et al., 2005), a stress-responsive MAPK. Han et al. (2013) demonstrated that high-fat diet fed mice with JNK-deficient macrophages remains insulin-sensitive. However, these animals still develop obesity. On the other hand, Solinas et al. (2007) showed that JNK absence in non-hematopoietic cells reduces fat gain, possibly by increasing metabolic rate, besides insulin sensitivity improvement. JNK is also important to obesity induced-inflammation, since its deletion reduces M1 macrophage polarization, adipose tissue infiltration by macrophages and inflammatory cytokines levels (Solinas et al., 2007; Han et al., 2013). Between these cytokines, IL-6 is implicated to insulin resistance. Perry et al. (2015) demonstrated that macrophage IL-6 production via JNK pathway promotes lipolysis in white adipose tissue, which in turn are related to hepatic glucose increase production. Besides JNK peripheral role in obesity, studies provide evidences that JNK deficiency in the central nervous system, mostly of hypothalamic–pituitary axis, improves insulin sensitivity and reduces body mass (Belgardt et al., 2010; Sabio et al., 2010).

Between signaling proteins involved in inflammation and obesity, PI3K has emerged as an obesity treatment target (Wymann and Solinas, 2013; Perino et al., 2014). This class of enzymes catalyze the phosphorylation of inositol phospholipids to generate molecular messengers (Hawkins and Stephens, 2015). PI3Kβ and PI3Kγ isoforms inhibition are implicated in fat mass reduction by promoting increased energy expenditure in mice (Perino et al., 2014). Additionally, blockade of PI3Kγ reduces pro-inflammatory macrophages infiltration into adipose tissue (Kobayashi et al., 2011). In fact, different receptors stimulation (e.g., G protein-coupled or tyrosine kinases receptors) induces PI3Kγ activation, which promotes integrin α4β1 activation in myeloid cells, a fundamental step in cell migration (Schmid et al., 2011).

Furthermore, PI3Kγ inhibition is also related to ameliorate obesity complications, mostly improving systemic insulin sensitivity (Becattini et al., 2011; Kobayashi et al., 2011). In this regard, TLR4/PI3Kγ axis is important not only for immune cells, but also for non-immune cells. Hepatocytes TLR4 absence, but

not in myeloid cells, improved glucose tolerance and enhanced insulin sensitivity. Besides that, it also attenuates inflammatory response (Jia et al., 2014). Becattini et al. (2011) showed that PI3Kγ activity within non-hematopoietic cells promotes insulin resistance in high-fat diet fed mice. However, the relationship between TLR4 and cell signaling proteins (summarized in **Figure 1**), obesity and metabolic syndrome is not completely established.

CONCLUSIONS AND PERSPECTIVES

Although several pathophysiological studies of metabolic syndrome and obesity were reported, little has been done about translational research in this field. In this regard, gut microbiota emerges with a key role in these disorders by interacting with host metabolism (i.e., bile acid biotransformation) or by promoting immune responses (i.e., TLR activation and cytokines production). Hence, gut microbiota-driven inflammation may promote the activation of the signal transducers IKKβ, JNK, and PI3Kγ which in turn control obesity development, adipose tissue inflammation and insulin resistance. Further studies may consider the relationship between gut microbiota, immune system and obesity (**Figure 2**) as a novel scope for disorders prevention and health maintenance. This comprehension will allow the development of new specific targets and integrated strategies to modulate gut microbiota in order to improve or even treat metabolic syndrome and obesity.

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