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GASOTRANSMITTERS: NOVEL REGULATORS OF ION CHANNELS AND TRANSPORTERS

Topic Editors
Wolfgang G. Clauss and Mike Althaus

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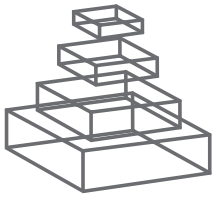

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Rhythm and Sound - Krystyna Spink

Small gaseous molecules, such as nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H2S), are emerging as important regulators of various cellular functions. Especially ion channels and transporters have been demonstrated to represent prominent molecular targets for those gases and are thus key factors in gas-induced cell-signalling. This Research Topic will focus on the interaction of gaseous molecules with ion channels/transporters and will address the following questions:
i) Which ion channels/transporters are regulated by gases?
ii) How are ion channels/transporters mechanistically regulated by gases?
iii) What are cellular and physiological processes, which are triggered by the interaction of gases with ion channels/transporters?

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EDITORIAL
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Gasotransmitters: novel regulators of ion channels and transporters

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More than 25 years ago, it was a big surprise for physiologists that nitric oxide (NO) was identified as the *endothelium derived relaxing factor* which is responsible for endothelium-induced smooth muscle relaxation (Ignarro et al., 1987). Until then, small gaseous molecules were simply regarded as byproducts of cellular metabolism which were unlikely to be of any physiological relevance. The discovery that NO was synthesized by specific enzymes (NO-synthases), upon stimulation by specific, physiologically relevant stimuli (e.g., acetylcholine stimulation of endothelial cells), as well as the fact that it acted on specific cellular targets (e.g., soluble guanylate cyclase), set the course for numerous studies which investigated the physiological roles of gaseous signaling molecules—in other words, *gasotransmitters* (Wang, 2002).

Aside from NO, there are two more gases which subsequently have been identified as gasotransmitters: carbon monoxide (CO) (Wu and Wang, 2005) and hydrogen sulfide (H_2S) (Abe and Kimura, 1996; Wang, 2002). Although the concept of a gas being involved in cellular signaling processes was already accepted since the discovery of NO, the new candidates CO and H_2S were initially met with skepticism—especially since they were well-known for their high toxicity and regarded as environmental chemical threats. However, in concert with the principle of Paracelsus that *"everything is toxic- it just depends on the dose,"* it is nowadays accepted that small amounts of CO and H_2S are of physiological relevance and are even endogenously produced in human cells (Wu and Wang, 2005; Wang, 2012).

All three gasotransmitters are produced in human cells by specific enzymes: NO is produced by NO-synthases (NOS), originating from the amino acid L-arginine. There are three NOS-isoforms, NOS1-3, which have been originally termed neuronal, inducible and endothelial NOS, respectively (Garvin et al., 2011). CO is generated by heme oxygenases (HO) within heme degradation (Wu and Wang, 2005). There are also three heme oxygenases (HO-1-3) (Yoshida et al., 1974; Maines et al., 1986; McCoubrey et al., 1997), including an inducible HO-1 and a constitutively active HO-2. H_2S is mainly produced within the metabolism of L-cysteine by the enzymes cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE or CTH) and 3-mercaptopyruvate sufurtransferase (3-MST) (Wang, 2012). Recently, Kimura's group described an additional pathway for endogenous H_2S production, which involves D-cysteine, 3-MST, and D-amino acid oxidase (Shibuya et al., 2013).

Despite the knowledge of the substrates and specific enzymes which are involved in gasotransmitter production, the precise endogenous concentrations of NO, CO, and H_2S have still not been sufficiently determined. The measurement of endogenous gas concentrations is limited by the accuracy and specificity of currently available methods (Olson, 2013), the reactivity and short half-life of some gases (NO) (Wall et al., 2012), and simply the fact that gases are volatile and rapidly disappear during the preparation procedures of biological samples. As highlighted within this research topic issue, it is crucial—and probably most challenging—to precisely quantify endogenous concentrations of NO, CO, and H_2S (Kimura, 2012; Peers, 2012; Olson, 2013).

Although precise endogenous concentrations of gasotransmitters remain to be determined, numerous studies investigated the physiological effects of those gaseous signaling molecules in almost every organ system [as summarized in excellent review articles such as Olson (2011), Wu and Wang (2005), and Wang (2012)]. Common molecular targets for all gasotransmitters are ion channels and transporters. Changes in the activity of membrane-located ion channels/transporters are involved in the majority of physiological processes which are regulated by gasotransmitters. This allows the gases to specifically act at the interface of cell-environment interactions, electrolyte homeostasis and electrochemical communication—thus allowing the regulation of numerous physiological processes in cells and tissues.

This research topic summarizes currently available data on the regulation of ion channels and transporters by NO, CO, and H_2S. Excellent review and research articles highlight the importance of gasotransmitter/ion channel interactions for vegetative physiology (Althaus, 2012; Peers, 2012; Pouokam and Diener, 2012), neurophysiology (Njie-Mbye et al., 2012; Peers, 2012; Takahashi et al., 2012; Wang et al., 2012), and explain gasotransmitter-induced signaling mechanisms (Wall et al., 2012). Furthermore, opinion articles from pioneers in gasotransmitter research (Kimura, 2012; Peers, 2012; Olson, 2013) give perspectives on important routes which should be followed in this field. With the articles included in this issue we wish to highlight the importance of ion channel/transporter regulation by gaseous signaling molecules and wish to stimulate future research in this exciting area of physiology.

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Oxidative modification of proteins: an emerging mechanism of cell signaling

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There are a wide variety of reactive species which can affect cell function, including reactive oxygen, nitrogen, and lipid species. Some are formed endogenously through enzymatic or non-enzymatic pathways, and others are introduced through diet or environmental exposure. Many of these reactive species can interact with biomolecules and can result in oxidative post-translational modification of proteins. It is well documented that some oxidative modifications cause macromolecular damage and cell death. However, a growing body of evidence suggests that certain classes of reactive species initiate cell signaling by reacting with specific side chains of peptide residues without causing cell death. This process is generally termed "redox signaling," and its role in physiological and pathological processes is a subject of active investigation. This review will give an overview of oxidative protein modification as a mechanism of redox signaling, including types of reactive species and how they modify proteins, examples of modified proteins, and a discussion about the current concepts in this area.

Keywords: redox signaling, thiol, 15-deoxy-prostaglandin J_2 (15d-PGJ_2), electrophile, reactive species

WHAT ARE REACTIVE SPECIES?

"Reactive species" is an umbrella term often used to describe a myriad of small molecules which can participate in chemical reactions. Reactive oxygen species (ROS) include superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and other reactive molecules containing oxygen (Finkel, 2011). Reactive nitrogen species (RNS) are nitrogen-containing molecules including nitric oxide ($NO^{\bullet}$) and higher oxides of $NO^{\bullet}$ such as dinitrogen trioxide (N_2O_3), peroxynitrite ($ONOO^-$), and nitroxyl anion (HNO^-; Hill et al., 2010). Reactive lipid species (RLS) are usually derived from unsaturated lipids and include among others, lipid aldehydes (e.g., 4-hydroxynonenal) and reactive prostaglandins of the A- and J-series (Higdon et al., 2012). While these terms cover a wide variety of molecules, they are a convenient, though often vague, way to describe the action of these types of compounds without implying one specific mediator.

Reactive species can be derived exogenously from the diet and environment as well as endogenously through enzymatic or non-enzymatic processes. Exogenous sources of reactive species have been shown to have both beneficial and harmful cellular effects, since they may either participate in cell signaling or may cause macromolecular damage. For example, dietary compounds such as curcumin in curry, and sulforaphane in cruciferous vegetables (e.g., brussel sprouts) are electrophilic and reactive with protein thiols (Brennan and O'Neill, 1998; Hong et al., 2005). These compounds have been shown to have potent anti-inflammatory properties and potential health benefits (Clarke et al., 2008; Lopez-Lazaro, 2008; Zhao et al., 2011). Other bioactive dietary components such as quercetin which is abundant in fruits, and phytoprostanes in fresh vegetable oils have been shown to be metabolized within the cell to reactive species which are anti-inflammatory (Spencer et al., 2003; Karg et al., 2007). On the other hand, compounds such as acrolein in cigarette smoke and acrylamide in fried foods are associated with predominantly deleterious effects such as depletion of cellular glutathione and neurotoxicity (LoPachin and Barber, 2006; Stevens and Maier, 2008).

In addition, there are endogenous sources of reactive species that have specific roles in physiology. For example, nitric oxide synthase (NOS) produces $NO^{\bullet}$ in a controlled manner to regulate the physiological processes of vasodilation (Gruetter et al., 1979), modulation of mitochondrial respiration (Brookes et al., 2002), and immunodefense (Sakiniene et al., 1997). The overall effect of $NO^{\bullet}$ is determined by the amount of $NO^{\bullet}$ produced and the site of production. Low levels of $NO^{\bullet}$, produced by the endothelial isoform of NOS (eNOS), diffuse to adjacent vascular smooth muscle cells to mediate vasodilation. However, high levels of $NO^{\bullet}$ produced by the inducible NOS isoform (iNOS) during inflammation may cause cell death by inhibition of mitochondrial respiration and other mechanisms (Brown and Borutaite, 2002). Other enzymatic sources of reactive species include xanthine oxidase and

Abbreviations: $O_2^{\bullet -}$, superoxide; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; 4-HNE, 4-hydroxynonenal; ARE/EpRE, antioxidant response element/electrophile response element; BH_4, tetrahydrobiopterin; CICR, Ca^{2+}-induced Ca^{2+} release; H_2O_2, hydrogen peroxide; HNO^-, nitroxyl anion; $HO^{\bullet}$, hydroxyl radical; HO-1, heme oxygenase-1; Keap1; iNrf2, Kelch-like ECH-associated protein 1; N_2O_3, dinitrogen trioxide; $NO^{\bullet}$, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; PGA_1, prostaglandin A_1; PGD_2, prostaglandin D_2; PGIS, prostaglandin I_2 synthase; Prx, peroxiredoxin; RLS, reactive lipid species; RNS, reactive nitrogen species; ROS, reactive oxygen species; RyR2, ryanodine receptor 2; SNAP, S-nitroso-*N*-acetyl penicillamine.

NADPH oxidases. Additionally, electrons from the mitochondrial respiratory chain can univalently reduce oxygen to generate $O_2^{\bullet-}$ (Thannickal and Fanburg, 2000).

Some of the earliest studies in reactive species have centered on $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$, $ONOO^-$. H_2O_2, in particular, is a potent mediator of signal transduction and can mediate oxidation of protein thiols (Rhee et al., 2003). The cellular actions of H_2O_2 are primarily dependent upon the location of production, the concentration of H_2O_2, and presence of H_2O_2 metabolizing proteins (e.g., catalase, peroxiredoxin, glutathione peroxidase, etc.). The expression of H_2O_2 metabolizing proteins is highly regulated with specific isoforms often localized to particular subcellular organelles. Endogenous production of oxidants such as hypochlorous acid (HOCl) and hypobromous acid (HOBr) have also been demonstrated, and their importance in physiology and pathology is becoming more widely recognized since these compounds are thought to play an important role in the antimicrobial response of host immune cells during infection (Weiss et al., 1986; Hurst and Barrette, 1989; Carr et al., 1998).

More recently, the endogenous generation of RLS is emerging an important mechanism of cellular signaling, particularly with respect to inhibiting inflammation, or at higher levels mediating apoptosis. RLS can be derived from oxidation of n-3 or n-6 polyunsaturated lipids which are cleaved from the membrane prior to oxidation. Alternatively, lipids may be oxidized within the membrane giving rise to hydroxy-alkenals and oxidized phospholipids which are active in physiological and/or pathological conditions (Kadl et al., 2004; Catala, 2009). Oxidized lipids can be formed enzymatically (e.g., lipoxygenase, cyclooxygenase), or non-enzymatically via lipid peroxidation or nitration pathways. There are a large number of unique RLS, however, these can typically be grouped according to which functional groups are present. Major classes of reactive lipids include lipid aldehydes, α,β-unsaturated carbonyls, and nitroalkenes [see (Higdon et al., 2012)]. In most cases, reactive lipids are electrophilic and can react with cellular nucleophiles which include certain amino acid side chains. Some reactive lipids contain more than one functional group, which is the case with 4-hydroxynonenal (4-HNE). 4-HNE can participate in Schiff base reactions involving the aldehyde group, and/or Michael addition reactions involving the electrophilic β-carbon [see (Higdon et al., 2012)].

REDOX SIGNALING OR OXIDATIVE DAMAGE?

At the cellular level, the effects of reactive species can range from cell death due to widespread damage to macromolecules to more subtle effects on cell metabolism, morphology, or signaling pathways (Martindale and Holbrook, 2002). The overall impact of a reactive species on cellular function varies with reactive species, but can also be affected by cell type, levels of endogenous antioxidants and antioxidant enzymes, differentiation state, extracellular environment, and many other factors (Jones and Go, 2010). Generally, severe oxidative stresses occur during exposure of cells to radiation, occupational exposures to highly reactive chemicals such as paraquat, and during pathology (Shacter, 2000). In these cases, reactive species can cause DNA base modification, phospholipid damage, and irreversible protein oxidation which can lead to cell death or mutagenesis. However, mild to moderate oxidative stresses occur during normal physiology, exercise, or growth. The reactive species generated during these types of stresses lead to cellular protection, improved metabolism, and resistance to oxidative damage (Higdon et al., 2012).

The difference between cellular signaling and damage by reactive species are governed by a number of properties inherent to the reactive species. Generally, the relative reactivity and specificity are two major properties dictating how reactive species will interact with targets. For the purpose of this review, the term "reactivity" is used to describe the ability and the rate at which reactive species can chemically react with a target. Highly reactive species, such as hydroxyl and alkoxyl radicals, have relatively high reaction rate constants, which in the case of hydroxyl radical is close to the diffusion limit, and therefore will react with and modify targets which are closest to the site of production of these species with little to no specificity (Sies, 1993). Reactivity is derived from the chemical properties of both the reactive species and the target. The term "specificity" refers to the ability of reactive species to make adducts with one class of molecules preferably over another. Reactivity and specificity have an inverse relationship in that, generally, reactive species which are more reactive are also less specific. Thus, highly reactive species usually cannot achieve specificity. The relationship between the reactivity and specificity of selected electrophilic lipid species (electrophiles) is used as an example of this relationship in **Figure 1**. The electrophiles are listed in relative order of reactivity with those on the left having the lowest reactivity and those on the right having the highest (LoPachin et al., 2007). Those reactive electrophiles found on the right of the diagram exhibit less specificity in reactions with nucleophilic targets than those on the left. Importantly, highly reactive compounds are also associated with macromolecular damage and tend to form adducts with protein targets which are relatively abundant, since the probability of a reaction with these proteins is higher. Conversely, reactive species with lower reactivity, as exemplified by the compounds on the left side of **Figure 1**, exhibit more specificity, resulting in the formation of adducts with certain protein residues (e.g., cysteine). As opposed to the highly reactive compounds, these

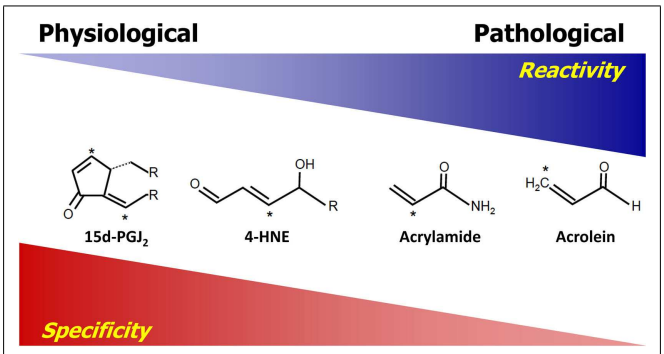

FIGURE 1 | Reactivity versus specificity. The compounds shown are electrophilic and have a very wide spectrum of reactivity. They are listed in order of increasing reactivity from left to right. The relative reactivity is predicted based on previously published reaction rate constants and electrophilicity indexes [see (LoPachin et al., 2007; Rudolph and Freeman, 2009)]. Reactivity is inversely correlated with their specificity in that those on the left are more specific for Cys, which is the most reactive nucleophilic residue in the cell. On the structures listed, the asterisk denotes the electrophilic atom(s) of the compound.

less reactive electrophiles are usually associated with signaling and the modification of a relatively small subset of proteins which is not solely dependent on the abundance of the target and more dependent on the specificity toward a target. We have previously shown that an electrophilic lipid with low reactivity at sublethal doses forms adducts with primarily cysteine residues and alters cell signaling (Levonen et al., 2004; Diers et al., 2010a). Thus, demonstrating that reactive species having low reactivity are more likely to participate in cell signaling than damage (Levonen et al., 2004; Diers et al., 2010a). This concept is also applicable to the reactivity of reactive oxygen and nitrogen species and the relative reactivity of these compounds is reviewed elsewhere (D'Autreaux and Toledano, 2007; Toledo and Augusto, 2012). However, it is important to note that even highly reactive species at low levels may be able to mediate cell signaling, and reactive species with lower reactivity can be damaging at high levels.

Beyond reactivity and specificity, other important factors are involved in determining how reactive species cause specific cellular effects. These include, but are not limited to: (1) site of production, (2) localization, and (3) reactivity of the target. The site of production of the reactive mediator is important with highly reactive species which have limited diffusion distances since these molecules cannot mediate downstream signaling unless the target is nearby (Forman et al., 2004; Pacher et al., 2007). The site of production may also allow for the accumulation of a reactive species, thereby resulting in locally elevated concentrations necessary for signaling. Also, the site of production of those reactive species which are more reactive and therefore less specific is important for dictating a response. Some enzymes produce reactive species in a localized region, such as membrane lipid rafts (Chen et al., 2009). For example, there is evidence that an isoform of the enzyme NADPH oxidase which produces superoxide is located at specialized lipid rafts with other proteins sensitive to superoxide (Jin et al., 2011), thus creating a redox signaling hub. For this reason, it may be speculated that for a reactive species (especially in the case of highly reactive species) to produce a signaling effect, it must either be produced or localized to the "correct" location in order to elicit a specific response. The understanding of location of production of reactive species will most definitely become an increasingly important area of future studies.

Some reactive species are stable enough to diffuse away from the site of production. For example, hydrogen peroxide is relatively stable and can diffuse away from the site of production before reacting (Winterbourn, 2008). Other reactive species such as the electrophilic lipid, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) are very stable, and can diffuse between cells within a tissue to mediate the resolution of inflammation (Gilroy et al., 1999). 15d-PGJ_2 has also been shown to localize to the mitochondrion based on mitochondrial membrane potential (Landar et al., 2006). The reason for this localization is not clear, however, a structurally similar prostaglandin, PGE_2, which is not reactive does not localize to the mitochondrion, suggesting that the localization is dependent on the electrophilic nature of the molecule (Landar et al., 2006). Interestingly, the effects of 15d-PGJ_2 within the mitochondrion may be further enhanced by directing the molecule to the desired compartment using molecular targeting strategies. For example, 15d-PGJ_2 can exhibit protective effects against oxidative stress, however, when this molecule is directed to the mitochondrion by conjugation to a lipophilic cation, its apoptotic signaling is enhanced (Diers et al., 2010b). Since adducts can accumulate over time, the actions of signaling molecules such as 15d-PGJ_2, which function via oxidative post-translational modifications, are determined by the relative rate of production of the species versus the rate of adduct turnover by the cell (Oh et al., 2008).

Lastly, it is worth mentioning that the reactivity of the target is important in determining the effects of a reactive species. It is well-established that some reactive species can react with and damage DNA by chemically adducting to specific bases (LoPachin and Decaprio, 2005). The next section will focus on some of the specific properties of the nucleophilic protein targets. Therefore, for the purpose of this review, we will focus on oxidative modifications of protein residues by low to moderate levels of reactive species which are commonly encountered in pathology and physiology.

EXAMPLES OF OXIDATIVE POST-TRANSLATIONAL MODIFICATION OF PROTEINS

It is becoming widely appreciated that reactive species produced in a controlled manner can covalently adduct to specific amino acids in order to elicit a cellular effect. The most commonly studied amino acids modified by reactive species are tyrosine and cysteine. The endogenous modification of tyrosine by reactive species in biological systems has been a subject of interest since the discovery of protein nitrotyrosine modifications in human atherosclerosis (Beckmann et al., 1994). Most reports on nitrotyrosine involve the use of this modification as a marker of peroxynitrite or other RNS formation, and suggest that this modification is deleterious. In fact, nitrotyrosine has been associated with the development of a number of pathologies including heart failure, atherosclerosis, aging, and hypertension [for review see (Pacher et al., 2007)]. However, nitrotyrosine may also be involved in physiological processes (Pacher et al., 2007). Whether tyrosine nitration is a normal mechanism of redox signaling or a marker of protein damage is currently an area of active investigation, but there is some evidence that low levels of peroxynitrite may cause modification of specific tyrosine residues which demonstrates that not all tyrosyl residues are equally susceptible to modification (Schmidt et al., 2003).

Perhaps, the most well characterized residue involved in redox signaling is cysteine. Cysteine accounts for an estimated 1.9% of residues within proteins, and a small number of these cysteines are known to participate in redox signaling (Go et al., 2011). The thiol group (-SH) on the side chain of cysteine can act as a switch for redox signaling and homeostasis. Because the sulfur atom has multiple oxidation states, the side chain of cysteine is readily oxidized to various products, some of which have specialized functions (Jacob et al., 2003). The protonated form of the thiol group (-SH) is not particularly reactive, but the deprotonated form ($-S^-$), or thiolate anion, is nucleophilic since it is rich in available electrons (LoPachin et al., 2007). For this reason, not all cysteines have thiols which are equally intrinsically reactive. This inherent reactivity is dictated by a number of contributing factors including accessibility and acid dissociation constant (pK_a) of the thiol group. The pK_a of a thiol is defined as the pH at which 50% of that thiol is in the deprotonated state. Thus, as shown in **Figure 2**, at physiological pH (7.4) the reactivity of the thiol can vary widely depending on the pK_a of the thiol. Thiols which have a relatively low pK_a tend to exist in the depronated thiolate anion form, and are thus

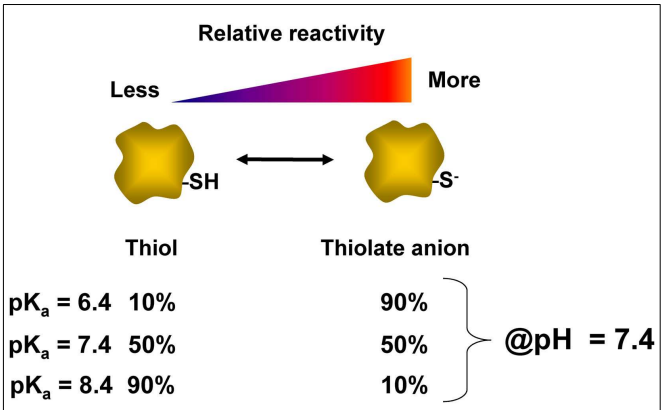

FIGURE 2 | Relationship of thiol pK_a and reactivity. The acid dissociation constant (pK_a) of a thiol group determines the ratio of thiol:thiolate at a given pH. This value varies for each cysteinyl thiol and is influenced by the local protein environment. At a physiological pH of 7.4, the relative percentages of protonated thiol and deprotonated thiolate anion are listed for cysteine residues which have different pK_as of 6.4, 7.4, or 8.4. The thiol with a pK_a of 6.4 is expected to be nearly 90% in the thiolate form at pH 7.4, and is therefore more reactive than thiols with higher pK_a values.

more likely to be modified by a reactive species. For this reason, the pK_a is important in determining the specificity of cysteine modifications by reactive species such as hydrogen peroxide and electrophiles (Rhee et al., 2003; Martyniuk et al., 2011). The thiolate group can participate in reactions with electrophilic reactive species either via nucleophilic substitution reactions or by Michael addition to form covalent adducts. There is also evidence demonstrating site-selective modification of cysteine residues within a single protein by different reactive species (Renedo et al., 2007; Jones, 2010) though characterization of this type of regulation for a broad range of proteins and reactive species has not been reported to date.

The next sections will highlight some specific examples of reactive species modifications organized by type of modification and the functional consequences for each example will be discussed.

TYROSINE NITRATION OF PROSTACYCLIN SYNTHASE

Protein tyrosine nitration occurs with the addition of an NO_2 group to the *ortho* position of the aromatic ring of tyrosine. Tyrosine residues have been shown to be nitrated by at least two multistep mechanisms involving RNS. One pathway involves the formation of a tyrosyl radical which then reacts with a peroxynitrite radical breakdown product, $NO_2^{\bullet}$, to produce 3-nitrotyrosine (for reviews see (Pacher et al., 2007; Peluffo and Radi, 2007). Peroxynitrite is a potent oxidant formed by the reaction of superoxide anion with $NO^{\bullet}$ and is produced during inflammatory processes. Interestingly, the main source of superoxide production in the vasculature during inflammation is from NOS itself. During inflammation, the tetrahydrobiopterin (BH_4) cofactor within the NOS enzyme can become oxidized, thereby uncoupling the enzyme and allowing electrons to reduce molecular oxygen to superoxide. Thus, NOS produces less $NO^{\bullet}$, and ultimately decreases $NO^{\bullet}$ bioavailability (Alp and Channon, 2004). Tyrosine nitration has been of interest during conditions of inflammation by changing the function of target proteins.

Prostaglandin I_2 synthase (PGIS) is one example of a protein which is modified by tyrosine nitration. PGIS is a heme-containing protein which catalyzes the rearrangement of prostaglandin H_2 to prostaglandin I_2 (or prostacyclin). PGIS, also known as CYP8A1, is a member of the cytochrome P450 superfamily and controls vascular tone by regulating the production of prostacyclin in the vasculature. Because prostacyclin is a potent vasodilator, PGIS activity has been shown to play an important role in vascular protection (Wu and Liou, 2005). During times of chronic inflammation and subsequent production of peroxynitrite, the tyrosine residue of PGIS, Tyr430, is nitrated resulting in a decrease in PGIS catalytic activity. This tyrosine nitration, in turn, inhibits prostacyclin-dependent relaxation [for review, see (Zou, 2007)]. Moreover, formation of peroxynitrite also decreases the bioavailability of $NO^{\bullet}$, a key vasodilator, and further promotes vasoconstriction. Though the effects are usually deleterious to vascular function, PGIS represents an example of a protein which is modified by low levels of a reactive species, peroxynitrite (Zou et al., 1999).

CYSTEINE OXIDATION OF PEROXIREDOXIN

Oxidation of cysteines is another widely recognized mechanism of redox signaling (Forman et al., 2004; Winterbourn and Hampton, 2008; Paulsen and Carroll, 2010; Finkel, 2011). Disulfide bond formation has long been known to be important in protein structure and function (Tu and Weissman, 2004), and more recently its role in redox signaling has been demonstrated (Frand et al., 2000; Jones et al., 2004). Currently, there is an interest in oxidation of thiols which results in the addition of oxygen(s) to the sulfur group of the amino acid cysteine. The reaction of hydrogen peroxide (H_2O_2) with the deprotonated cysteinyl thiol of proteins produces an oxidized thiol or sulfenic acid (R-SOH; Poole et al., 2004). A sulfenic acid may be oxidized again to yield a hyperoxidized sulfinic acid cysteine ($R\text{-}SO_2H$). With increasing levels of reactive species, cysteines can further be oxidized to a sulfonic acid ($R\text{-}SO_3H$; Poole et al., 2004). While sulfenic acids are enzymatically reversible by the glutathione and thioredoxin enzyme systems (Berndt et al., 2007), the sulfinic state can only be reversed enzymatically in certain proteins. Sulfonic acid modification is thought to be irreversible and may represent protein damage, rather than signaling.

An example of thiol oxidation is found in the peroxiredoxin (Prx) family of enzymes (Stacey et al., 2012). These proteins contain a reactive cysteinyl thiol in the active site, and the formation of sulfenic acid occurs in their normal catalytic cycle (Rhee et al., 1999, 2005; Stacey et al., 2012). Prxs have the capacity to protect proteins from oxidative damage induced by hydrogen peroxide in a thiol dependent manner. Two cysteine residues, corresponding to Cys47 and Cys170 of yeast Prx are highly conserved. In the presence of hydrogen peroxide, Cys47 gets oxidized to the sulfenic acid intermediate (Cys–SOH). Cys170 is responsible for forming a disulfide linkage with Cys47 (Cys47–S-S–Cys170) in order to resolve the sulfenic acid (Chae et al., 1994). Through a disulfide switching mechanism, the enzyme thioredoxin completes the catalytic cycle by reducing the disulfide bond between Cys47 and Cys170. In addition to H_2O_2, Prx directly reduces peroxynitrite and lipid peroxides (Bryk et al., 2000). Importantly, Prx can be hyperoxidized in the presence of excess hydrogen peroxide to the

sulfinic acid form which inactivates its function. Sulfinic acid formation in many proteins is not reversible, but sulfinic acid in Prx has been shown to be reduced by a unique thiol dependent enzyme, sulfiredoxin (Chang et al., 2004). Thus, Prx plays a crucial role in redox signaling since it regulates peroxide levels, but in turn is regulated by peroxide itself (Rhee et al., 2012).

S-NITROSATION OF RYANODINE RECEPTOR 2

S-Nitrosation is the post-translational modification of a thiol group to form an *S*-nitrosothiol which has the general structure of R-S-N = O (Zhang and Hogg, 2005). While the mechanism for RSNO formation in biological systems remains poorly understood, RSNOs are thought to be downstream mediators of nitric oxide signaling. Because of the critical role of nitric oxide in cardiovascular (patho)physiology, much interest has focused on *S*-nitrosation of proteins involved in excitation-contraction coupling in the heart (Gonzalez et al., 2009). In the beating heart, membrane depolarization causes the opening of L-type voltage-gated channels in the plasma membrane and calcium (Ca^{2+}) influx into cardiac myocytes. This Ca^{2+} influx then triggers the release of Ca^{2+} from the sarcoplasmic reticulum through the opening of ryanodine receptors, a process termed Ca^{2+}-induced Ca^{2+} release (CICR; Fabiato, 1983). Ryanodine receptors as well as other channels involved in CICR (e.g., voltage-gated sodium and potassium channels) have been shown to be targets of *S*-nitrosation (Gonzalez et al., 2009). However, here, we will discuss in detail only the modification of ryanodine receptors.

Ryanodine receptor 2 (RyR2) is the isoform expressed in the heart. The early observation that treatment of RyR2 in reconstituted lipid bilayers with agents which initiate *S*-nitrosation (e.g., *S*-nitroso-*N*-acetyl penicillamine; SNAP) increased the open probability (P_O) of the channel provided one of the first links between nitric oxide signaling and Ca^{2+} handling at the level of sarcoplasmic reticulum. Moreover, this effect was reversed with the addition of thiol reducing agents, further implicating reversible post-translational modification as a mechanism for regulation of RyR2 (Stoyanovsky et al., 1997). RyR2 has 89 cysteine residues per monomeric subunit, and approximately 21 of these are thought to exist as free thiols. Modification of multiple cysteine residues is required for full activation of RyR2 by *S*-nitrosation (Xu et al., 1998). Cardiac ryanodine receptors have also been shown to be constitutively *S*-nitrosated *in vivo* (Sun et al., 2008), and changes in the redox state of RyR2 thiols have been observed in cardiac pathologies (Yano et al., 2005; Terentyev et al., 2008). Examination of ryanodine receptors isolated from canine heart and rabbit skeletal muscle show that these proteins are susceptible to multiple oxidative modifications including *S*-nitrosation, *S*-gluthionylation, and oxidation to form disulfide bonds, and more recent studies demonstrate that ryanodine receptors co-immunoprecipitate with NOS enzymes, implying that ryanodine receptors are spatially linked to an endogenous source of nitric oxide (Barouch et al., 2002; Martinez-Moreno et al., 2005). Taken together, these studies specifically highlight the important role of *S*-nitrosation in mediating downstream nitric oxide-dependent effects on Ca^{2+} handling in the heart and, more generally, point to a mechanism by which nitric oxide regulates cellular events beyond its canonical role in cGMP-dependent responses.

ELECTROPHILIC LIPID MODIFICATION OF KEAP1

The covalent modification of thiol groups of cysteine residues by electrophiles is another example of an oxidative post-translational modification which results in redox signaling (Higdon et al., 2012). Depending on the type of electrophile, adducts may be formed with nucleophilic cysteinyl thiols through Michael addition or through a nucleophilic substitution (S_N2) reaction (Hill et al., 2009). Michael addition involves the formation of an adduct which is equal to the exact mass of the electrophile and nucleophile. Nucleophilic substitution results in the formation of an adduct and a leaving group, such as an iodide or chloride moiety from the electrophile. Cyclopentenone prostaglandins, such as 15d-PGJ_2, are electrophilic lipids which contain an α,β-unsaturated carbonyl group (**Figure 3A**, right hand panel). During adduct formation, the lipid forms a covalent adduct by Michael addition of the electrophilic carbon with the cysteinyl thiol group of the protein. In the case of 15d-PGJ_2, there are actually two β-carbons which are electrophilic (**Figure 3A** right hand panel, denoted by asterisks) due to the presence of two double bonds flanking the carbonyl. However, the β-carbon within the cyclopentenone ring is most commonly found to form adducts with proteins (Uchida and Shibata, 2008). Interestingly, it has been reported that the modification of thiols by electrophilic compounds can be exquisitely site-specific, since different thiols within a single protein can be modified by different cyclopentenone prostaglandins (Gayarre et al., 2005). One well characterized protein modified by electrophilic lipids is the Kelch-like ECH-associated protein 1 (Keap1; Itoh et al., 2004; Levonen et al., 2004), which is involved in the redox regulation of transcription (Brigelius-Flohe and Flohe, 2011).

Keap1 (also known as iNrf2) is normally found in the cellular cytoplasm complexed with the transcription factor Nrf2 (Kaspar et al., 2009). The interaction of Keap1 with Nrf2 under basal conditions directs Nrf2 for degradation by the ubiquitin-proteasomal system (McMahon et al., 2003; Kobayashi et al., 2004). Keap1 cysteine residues, Cys273 and Cys288, have been shown to be modified by 15d-PGJ_2 thereby preventing proteasomal degradation of Nrf2 (Levonen et al., 2004; Yamamoto et al., 2008). Thus, modification of Keap1 allows for accumulation of Nrf2 and promotes its subsequent translocation to the nucleus (Kobayashi et al., 2006) where Nrf2 regulates the transcription of a number of proteins under the control of the antioxidant response element/electrophile response element (ARE/EpRE) such as glutathione synthase and heme oxygenase-1 (HO-1; Levonen et al., 2004; Wakabayashi et al., 2004). In this way, Keap1 is thought to play a critical role in the cellular response to electrophiles (Dinkova-Kostova et al., 2005). Moreover, activation of Nrf2 by electrophiles through this mechanism and the resulting ARE/EpRE-dependent gene transcription has a major impact on the resolution of inflammation (Levonen et al., 2004).

COVALENT MODIFICATION AS A MECHANISM OF CELL SIGNALING

As illustrated above, the modification of specific amino acids by reactive species can elicit changes in protein function and thereby mediate redox signaling. Covalent redox signaling differs from classical receptor-ligand mediated signaling in several critical ways. This can be illustrated by comparing structurally related

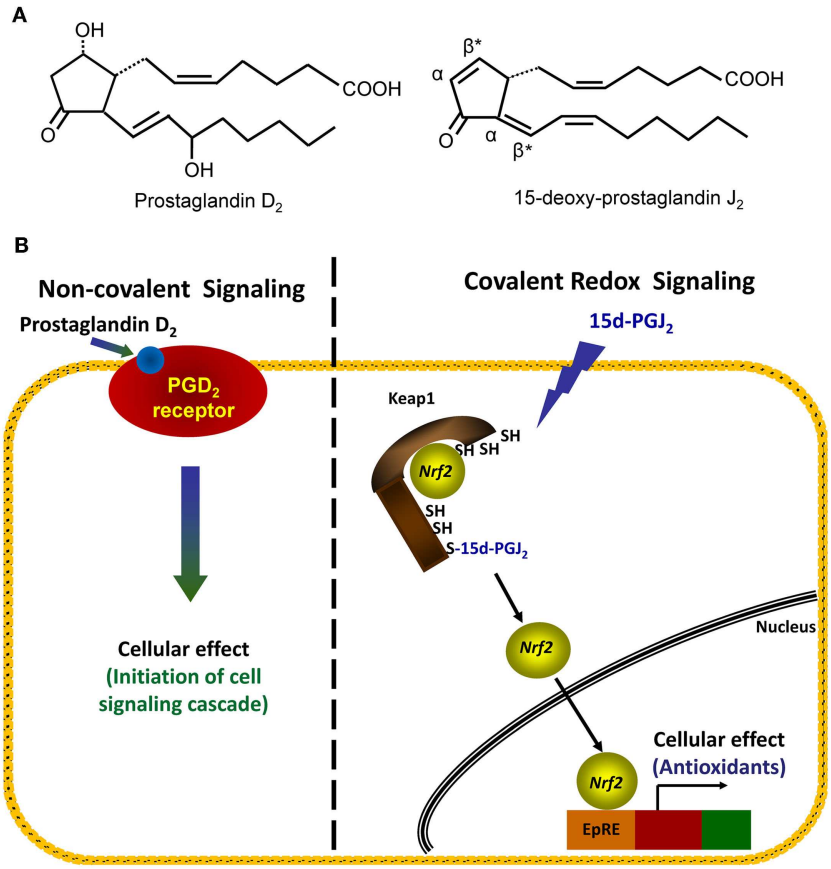

FIGURE 3 | Comparison of non-covalent and covalent signaling molecules. (A) The structures of two prostaglandins, PGD_2 (left structure) and 15d-PGJ_2 (right structure), are shown. Electrophilic carbons which arise from presence of α, β-unsaturated carbonyl functional groups are denoted by asterisks, and α- and β-carbons are indicated. **(B)** Left hand panel: in the non-covalent or classical receptor-ligand model (left hand panel), the ligand PGD_2 is recognized by a specific receptor on the cell membrane. The binding event causes the activation of a signaling cascade which ultimately changes cellular function. Right hand panel: in the covalent signaling model, a reactive species 15d-PGJ_2 directly forms a covalent adduct with select proteins, in this case, the cytosolic repressor protein Keap1. This binding event causes changes in endogenous antioxidant protein transcription via the transcription factor Nrf2.

endogenous lipids: the non-electrophilic prostaglandin PGD_2 and the electrophilic prostaglandin 15d-PGJ_2 (**Figure 3B**). As a classic receptor-mediated ligand, PGD_2 binds reversibly to a G-protein coupled receptor and initiates a signaling cascade. In contrast, 15d-PGJ_2 forms a covalent adduct with its target protein(s) which results in a specific downstream effect (as described above for its effects on the Keap1/Nrf2 system; **Figure 3B**). The signaling of PGD_2 and 15d-PGJ_2, both are dependent on the amount of ligand bound to the "receptor" in order to initiate a signaling cascade. For a non-covalent ligand, such as PGD_2, this is determined by the concentration of ligand present at a given time. However, due to the ability for 15d-PGJ_2 to covalently modify its "receptor(s)," adducts may accumulate over time, even at low concentrations of the ligand. Thus, the steady-state concentration of 15d-PGJ_2 and similar covalent ligands is not necessarily the primary determinant of signaling, but rather the absolute amount of the ligand present over time. For example, we have observed that exposure of endothelial cells *in vitro* to low amounts of a reactive species over a longer period of time will lead to the gradual accumulation of adducts and a level of signaling comparable to an exposure to a higher bolus dose for a shorter period of time (Oh et al., 2008). In this experiment, 20 additions of 0.1 nmol 15d-PGJ_2 over 7 h resulted in similar induction of HO-1 and GSH as a bolus dose of 2 nmol lipid at $t = 0$ h in endothelial cells. Thus, covalent redox signaling molecules may result in sustained signaling at very low levels, and do not need to achieve high concentrations in order to be efficacious. We are only now beginning to appreciate the importance of treatment conditions when comparing reactive species across studies.

DISCUSSION OF THE POTENTIAL FOR COORDINATED CHANGES IN CELLULAR FUNCTION

Another notable characteristic of covalent redox signaling is that, because there is specificity for which proteins are modified, there is often a relatively small group of proteins which are modified simultaneously. Therefore, the downstream effect is likely to be a summation of the effects of the modification of targeted proteins. In addition, covalent redox signaling pathways have been shown to

cross-talk with other signaling pathways, such as phosphorylation cascades and calcium mobilization (Diers et al., 2010a; Klomsiri et al., 2011). Thus, the overall activity of a redox signaling pathway will be dependent on which proteins are specifically modified and the interaction of their coordinated effects.

Our understanding of how reactive species can modulate different cell functions has been significantly strengthened through the use of biotin-tagged electrophilic lipids (e.g., biotin-15d-PGJ_2) to evaluate cell function while concomitantly tracking its protein adducts. 15d-PGJ_2 is particularly interesting since it has been shown to affect cellular functions through pleiotropic mechanisms (Straus and Glass, 2001; Pignatelli et al., 2005). Our studies have shown that 15d-PGJ_2 reacts with a small group of proteins at low levels, and that with increasing exposure, the number of modified proteins and extent of modification also increases (Oh et al., 2008). This is conceptually illustrated in **Figure 4** by a general dose-response of 15d-PGJ_2 on known cellular effects. The cellular effects which we have observed at relatively low, non-toxic levels include inhibition of migration and cytoskeletal alterations (Diers et al., 2010a). With progressively increasing levels of 15d-PGJ_2 modification, up-regulation of HO-1 was observed due to the activation of Keap1/Nrf2 pathway, followed by cell death at the highest levels of lipid (Ricart et al., 2009). Interestingly, at the highest levels of 15d-PGJ_2, HO-1 levels decrease though Keap1 is modified (Ricart et al., 2009), and the reason for this effect remains to be elucidated. However, it is clear that the overall cellular response cannot be explained by the modification of one target protein alone, and is likely due to the modification of multiple proteins. Importantly, the concepts discussed regarding 15d-PGJ_2 should be applicable to other reactive species which may have signaling roles. Importantly, these concepts of redox signaling by reactive lipid, oxygen, and nitrogen species apply to all cell types, but may vary from one cell type to another based on the differences in protein composition and antioxidant pathways specific to that cell type. Appreciating the complexity of these redox signaling systems and understanding how these pathways are dysfunctional during pathology is of utmost importance to this growing field.

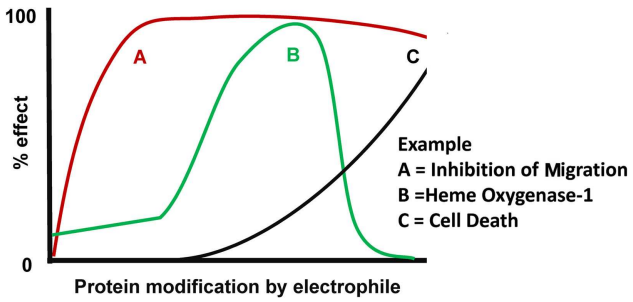

FIGURE 4 | Model of coordinated cellular effects in response to electrophilic protein modification. The overall cellular effects of an electrophile are dependent on the susceptibility of different target proteins to be modified by that electrophile. With low levels of electrophile exposure, post-translational modification of the most sensitive target will elicit a cellular response "A." With moderate electrophile exposure, modification of (an) additional target(s) will result in cellular response "B." Since response A is still present in this example, the combination of responses A and B cause a coordinated change in cell function by the same electrophilic mediator. With relatively high electrophile exposure, modification of a less sensitive target protein will elicit a new cellular response "C." Since response A remains and response B is downregulated, the coordinated change in cell function is distinct from that observed at moderate electrophile doses.

CONCLUSION

As more is learned about oxidative post-translational modifications and redox signaling, we will be able to better piece together ways in which individually targeted modifications form protein networks and orchestrate cellular responses. This will obviously be difficult to study experimentally, but is nevertheless important to understand how a given reactive species may have pleiotropic mechanisms and seemingly incongruent effects on cells, ranging from protection against cell death to induction of apoptosis. There is an urgent need for research strategies which take into account systems biology and embrace the complexity of multiple pathways in order to advance new breakthroughs for drug development. In this vein, the inherent properties of a reactive species can be used to direct the covalent modification of a group of proteins in order to elicit specific cellular responses. In this way, novel redox therapeutics can be designed to generate a sustained and defined cellular effect in a variety of pathological conditions.

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Modulation of ionotropic glutamate receptors and acid-sensing ion channels by nitric oxide

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Ionotropic glutamate receptors (iGluR) are ligand-gated ion channels and are densely expressed in broad areas of mammalian brains. Like iGluRs, acid-sensing ion channels (ASIC) are ligand (H^+)-gated channels and are enriched in brain cells and peripheral sensory neurons. Both ion channels are enriched at excitatory synaptic sites, functionally coupled to each other, and subject to the modulation by a variety of signaling molecules. Central among them is a gasotransmitter, nitric oxide (NO). Available data show that NO activity-dependently modulates iGluRs and ASICs via either a direct or an indirect pathway. The former involves a NO-based and cGMP-independent post-translational modification (S-nitrosylation) of extracellular cysteine residues in channel subunits or channel-interacting proteins. The latter is achieved by NO activation of soluble guanylyl cyclase, which in turn triggers an intracellular cGMP-sensitive cascade to indirectly modulate iGluRs and ASICs. The NO modification is usually dynamic and reversible. Modified channels undergo significant, interrelated changes in biochemistry and electrophysiology. Since NO synthesis is enhanced in various neurological disorders, the NO modulation of iGluRs and ASICs is believed to be directly linked to the pathogenesis of these disorders. This review summarizes the direct and indirect modifications of iGluRs and ASICs by NO and analyzes the role of the NO-iGluR and NO-ASIC coupling in cell signaling and in the pathogenesis of certain related neurological diseases.

Keywords: NMDA, AMPA, ASIC, nitrosylation, cGMP, NO, NOS, gasotransmitter

INTRODUCTION

Ionotropic glutamate receptors (iGluR) are ligand-gated cation ion channels. They are classified into *N*-methyl-D-aspartate receptors (NMDAR), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR), and kainate receptors (Dingledine et al., 1999). These receptors are densely distributed in broad regions of the mammalian central nervous system (CNS). Functional iGluRs are assembled by distinct subunits. Activation of iGluRs by an endogenous ligand, i.e., neurotransmitter glutamate, opens the channel for the Na^+ flow into cells, resulting in depolarization. NMDARs and GluR2-lacking AMPARs also allow Ca^{2+} influx for triggering multifunctional Ca^{2+}-associated signaling pathways. Compared to NMDARs, AMPARs open and close more quickly, and thus mediate most of the fast excitatory synaptic transmission in the CNS. As a major group of excitatory receptors, iGluRs are critical for normal operations of cellular and synaptic activity and plasticity. Malfunction of these ion channels is thus frequently linked to the pathogenesis of a wide range of neurological disorders (Dingledine et al., 1999).

Neuronal cells are sensitive to changing extracellular proton (acid) concentrations. In sensory neurons, an acid-triggered inward ion current was first observed in 1980 (Krishtal and Pidoplichko, 1980). This current was then found to be mediated through the membrane-bound acid-sensing ion channel (ASIC; Waldmann et al., 1997b). ASICs are a distinct family of proton (H^+)-gated, voltage-independent, and cation (Na^+)-selective channels. They are broadly expressed in peripheral sensory neurons and CNS neurons (Waldmann et al., 1997a,b; Alvarez de la Rosa et al., 2003). More specifically, among all ASIC subunits so far identified, ASIC1a, 2a, and 2b are expressed in CNS neurons at a high level, while all other ASICs are expressed in peripheral sensory neurons. In addition, ASICs are seen in non-neuronal cells, such as vascular smooth muscles and bone (Jahr et al., 2005; Grifoni et al., 2008). ASICs are important for maintaining cell homeostasis in response to normal changes in pH signals and are involved in discrete disorders where a dramatic drop in extracellular pH values (acidosis) occurs, such as seen in inflammation, ischemic stroke, traumatic brain injury, and acid nociception (Wemmie et al., 2006; Chu et al., 2011).

Like many other ion channels, iGluRs and ASICs are subject to the modulation by various extracellular and intracellular signals. This modulation is essential for normal channel operations and for adjusting the efficacy and strength of channels to properly respond to a given stimulus. Among important modulators is nitric oxide (NO), a short-lived and plasma membrane diffusible gasotransmitter. Increasing evidence shows that NO can modulate iGluRs and ASICs through a direct (cGMP-independent) or an indirect (cGMP-dependent) pathway (Ahern et al., 2002).

The direct pathway involves a NO-based post-translational modification of iGluR and ASIC proteins, i.e., S-nitrosylation. This biochemical reaction occurs when a nitrosyl group is added to the thiol side-chain of cysteine residues to form *S*-nitrosothiols, leading to changes in tertiary structure and function of modified iGluRs and ASICs (Sen and Snyder, 2010). The indirect pathway starts with NO stimulation of soluble guanylyl cyclase, leading to an increase in the second messenger cGMP production and activation of cGMP-dependent downstream protein kinases (Bredt and Snyder, 1989). This review will discuss current progress in understanding the modulation of iGluRs and ASICs by NO. We reviewed these two types of channels together because (1) they are co-localized in the confined postsynaptic density microdomain (Zha et al., 2006), (2) they are functionally coupled to each other to contribute to neurological disorders involving excessive glutamate release and acidosis (Gao et al., 2005), and (3) they show some common properties in the regulation by NO.

MODULATION OF iGluRs BY NO

NMDARs

The NMDAR is a unique ion channel that is both ligand-gated and voltage-dependent. Co-activation of NMDARs by the endogenous transmitters (glutamate and glycine) and the release of voltage-dependent Mg^{2+} block trigger the opening of ion channels, allowing the inward flow of Na^{+} and small amounts of Ca^{2+} into cells (Dingledine et al., 1999). The Ca^{2+} influx is critical for a variety of NMDAR signaling and function. Functional NMDARs are an assembly of obligatory NR1 and the modulatory NR2 subunits. Among all of the modulatory subunits (A–D), NR2A and NR2B are predominant NMDAR components and are mostly enriched in neurons (Stephenson, 2001).

NMDARs are closely linked to the NO system. Activation of NMDARs consistently increases activity of neuronal nitric oxide synthase (nNOS) in the cytoplasm of neurons. Active nNOS then catalyzes the production of endogenous NO from L-arginine, leading to an increased release of NO from neurons (Garthwaite et al., 1988; Shibuki and Okada, 1991; Bredt and Snyder, 1992). A number of studies *in vitro* and *in vivo* have demonstrated a significant role of NO in the regulation of normal NMDAR function. In cultured neurons, the NO-producing agent 3-morpholino-sydnonimine (SIN-1) inhibited NMDA-induced currents and associated increases in Ca^{2+} influx (Manzoni et al., 1992). Similar results were observed with 1-nitrosopyrrolidine (an NO-containing agent) and NaNO2 (a released form of NO), while NO scavenging by hemoglobin reversed the effect of these agents (Manzoni et al., 1992). Endogenous NOS activity in cultured striatal neurons is also closely linked to NMDAR activity. The natural substrate of NOS, L-arginine, diminished NMDAR activity, which was reversed by the NOS inhibitor L-nitroarginine (Manzoni and Bockaer, 1993). In channel recordings from rat cortical neurons or *Xenopus oocytes*, the NO-generating agent *S*-nitrosocysteine (SNOC) decreased NMDAR channel opening (Lei et al., 1992; Lipton et al., 1998) or NMDA-evoked currents (Omerovic et al., 1995; Choi et al., 2000). Similar to the *in vitro* results, an *in vivo* microdialysis study showed that the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) suppressed the NMDA-stimulated NO release in the rat striatum (Kendrick et al., 1996). Together, *in vitro* and *in vivo* data support an inhibitory modulation of NMDARs by NO under normal conditions. Given that NMDAR stimulation increases NO synthesis, NO and NO-related species serve as key regulators in a use-dependent negative feedback mechanism to prevent excessive NMDAR activation and to maintain the receptor activity at the physiological level (Choi et al., 2000).

The mechanism underlying the NO modulation of NMDARs seems to involve a direct and cGMP-independent pathway. It has been found that NMDARs can be directly modified by a biochemical reaction, S-nitrosylation. S-nitrosylation is an important biological reaction of NO and is a key post-translational modification for multiple ion channels (Hess et al., 2005). Such modification adds a nitrosyl group (NO^{+}) to the thiol side-chain of cysteine residues to form *S*-nitrosothiols, leading to a reversible change in tertiary structure and function of modified proteins (Sen and Snyder, 2010). In dissecting a molecular mechanism for the NO-NMDAR coupling, a single cysteine residue (C399) in the extracellular N terminus of NR2A was found to account for the predominant effect of NO. This cysteine can be physiologically S-nitrosylated after NO^{+} transfer, and site-directed mutation of it to alanine abolished the NO-mediated inhibition of NR1/NR2A receptors (Choi et al., 2000). Thus, C399 S-nitrosylation serves as an important pathway linking NO to NMDARs. Additionally, two pairs of cysteine residues on NR1 (C744, C789) and NR2A (C87, C322) could undergo S-nitrosylation and thereby inhibit NMDARs if they are in the free thiol form (Choi et al., 2000, 2001; Lipton et al., 2002). Of note, nNOS forms a complex with NR1 via a scaffolding protein PSD-95 in the postsynaptic density microdomain (Brenman et al., 1996). Co-localization of these proteins at defined synaptic sites facilitates their direct interactions and enables rapid feedback inhibition of NMDARs via a NO-sensitive mechanism.

NO can also indirectly modulate NMDARs through S-nitrosylating the prime regulators of NMDARs. Serine racemase (SR) is an enzyme which converts L-serine to D-serine, a co-agonist with glutamate at NMDARs. It has been shown that SR is physiologically S-nitrosylated for marked inhibition of the enzyme (Mustafa et al., 2007). This S-nitrosylation was enhanced by NMDAR-activated nNOS. Thus, postsynaptic stimulation of NO formation possibly feeds back to presynaptic cells to S-nitrosylate SR and decrease D-serine availability. This contributes to inhibiting excitatory synaptic transmission and preventing overstimulation of postsynaptic NMDARs. PSD-95 is a principal scaffolding protein at synaptic sites where it determines the number of NMDARs and thus efficiency and strength of the receptor. It also couples NMDARs to nNOS to form signalosomes for local NO production. Like SR, PSD-95 is physiologically S-nitrosylated (Ho et al., 2011). S-nitrosylation occurs at two specific cysteines (C3 and C5). Interestingly, these sites are also subject to palmitoylation, another reversible and cysteine-based post-translational modification which covalently attaches a fatty acid (palmitic acid) to cysteine (Ho et al., 2011). In cerebellar neurons, NO inhibited PSD-95 palmitoylation and decreased synaptic delivery of PSD-95. In heterologous cells, decreased palmitoylation resulted in increased PSD-95 S-nitrosylation (Ho et al., 2011). These data support a model in which NMDARs stimulate NO to regulate

PSD-95 via mutually competitive cysteine modifications. Regulated PSD-95 then reciprocally controls expression and activity of NMDARs.

Normal NMDAR activity is important for cell signaling and synaptic plasticity. Excessive activation of the receptor, however, results in excitotoxity and cell death. To this end, NMDARs are crucial for neuronal survival in various neurodegenerative disorders (Lipton and Rosenberg, 1994). Since NO inhibits excessive stimulation of NMDARs, it could consequently ameliorate NMDAR-mediated neurotoxicity. Of note, both NMDAR and NO activities are upregulated in parallel during neurological disorders such as ischemia, trauma, and inflammation. As such, they both are actively involved in resultant neuronal death under these neurodegenerative conditions (Dawson et al., 1993; Bonfoco et al., 1995). Indeed, hypoxia renders NMDARs exquisitely sensitive to NO-induced S-nitrosylation and inhibition of NMDARs in cortical neurons and *oocytes* (Takahashi et al., 2007). Such enhanced NO inhibition of NMDARs is thought to be neuroprotective and serves as a mechanism to counteract NMDAR-mediated cytotoxicity in hypoxia insults (Lei et al., 1992; Lipton et al., 1993; Choi et al., 2000; Takahashi et al., 2007). In addition, a large increase in NO levels in rat brains was discovered immediately after middle cerebral artery occlusion (Malinski et al., 1993). A higher level of NO, through the NO-mediated vasodilation, can increase cerebral blood flow and oxygen delivery (Toda et al., 2009). This, in concert with the NO inhibition of NMDARs, protects brain tissue during focal ischemia, although neuronal overproduction of NO may elicit neurotoxicity (Dalkara et al., 1994). The inhibitory control of NMDARs by NO has potential clinical implications. Pharmacotherapies targeting nitrosylatable cysteine residues can be developed to prevent NMDAR-mediated neurotoxicity during stroke (Takahashi et al., 2007).

AMPARs

AMPARs are another important glutamate ion channel. Like NMDARs, AMPARs become functional upon a heterotetrameric assembly of four subunits (GluR1–4 or GluRA–D; Dingledine et al., 1999). Most AMPARs contain symmetric "dimer of dimers" of GluR2 and either GluR1, GluR3, or GluR4 (Greger et al., 2007). Activation of these receptors promotes Na^+ influx and induces rapid depolarization. AMPARs open and close quickly and thus carry out the most of fast excitatory synaptic transmission in the CNS.

Little is known about whether AMPAR subunits are direct targets of NO. However, several key AMPAR-interacting proteins that actively control AMPAR trafficking, expression, and function are S-nitrosylated and regulated by NO. *N*-ethylmaleimide sensitive factor (NSF) is an ATPase enriched in neurons. It binds to GluR2 (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998), stabilizing or recycling AMPARs into postsynaptic membranes (Braithwaite et al., 2002; Lee et al., 2002). It was found that synaptic NSF was physiologically S-nitrosylated by endogenous, neuronally derived NO in mouse brain (Huang et al., 2005). NMDAR activation enhanced NSF S-nitrosylation and thereby augmented NSF binding to GluR2 and surface insertion of GluR2. These data reveal a NO-sensitive pathway linking NMDARs to AMPARs. Specifically, NMDARs, through activating associated nNOS, trigger NO formation, which upregulates NSF S-nitrosylation to promote its associations with GluR2 and increase surface expression of GluR2-containing AMPARs (Huang et al., 2005). In this process, PICK1 (protein interacting with C kinase 1), a synaptic anchoring protein binding and retaining GluR2 in intracellular pools, seems to mediate the NSF regulation of GluR2. S-nitrosylated NSF can dissemble the PICK1-GluR2 complex, leading to a release of GluR2 from PICK retention and subsequent surface delivery of GluR2/AMPARs (Hanley et al., 2002; Sossa et al., 2007).

Stargazin is another synaptic protein that links NO to AMPARs. As a founding member of the transmembrane AMPAR regulatory protein (TARP) family, stargazin serves as an auxiliary subunit of all AMPAR subtypes and directly interacts with a large part of AMPARs. As such, stargazin is thought to be a principal determinant of AMPAR surface expression. Indeed, Snyder and co-workers showed that stargazin, like NSF, is physiologically S-nitrosylated at C302 in primary neurons and heterologous cells (Selvakumar et al., 2009). S-nitrosylation of stargazin was positively linked to surface expression of this small tetraspanning membrane protein. S-nitrosylation also increased binding of stargazin to the AMPAR subunit GluR1, causing upregulated surface expression of the AMPAR. NMDAR stimulation increased S-nitrosylation of stargazin and binding of stargazin to AMPARs. Apparently, stargazin, like NSF, is a physiological regulator of AMPARs and a linker between NMDARs and AMPARs. Through a NO-sensitive mechanism, stargazin transmits NMDAR signals to the level of surface expression of AMPARs.

In addition to S-nitrosylation, NO stimulates soluble guanylyl cyclase to form the second messenger cGMP (Bredt and Snyder, 1989). Thus, cGMP and downstream protein kinases could constitute an indirect pathway to regulate AMPARs. A recent study supports this notion (Serulle et al., 2007). In this study, the cGMP-dependent kinase type II (cGKII), a distinct GK isoform located in cellular membranes and broadly expressed in the brain (Francis and Corbin, 1999), was found to directly bind to GluR1. This binding was sensitive to nNOS activity and cGMP. Moreover, the binding enables the kinase to phosphorylate GluR1 at an intracellular serine site (S845). In cultured hippocampal neurons, active cGKII accumulated GluR1 on the plasma membrane at extrasynaptic sites. Blockade of cGKII prevented this accumulation and also an increase in mEPSC frequency and amplitude. Of note, S845 was also phosphorylated by protein kinase A (PKA), which was required for GluR1 synaptic insertion (Esteban et al., 2003). Thus, both cGKII and PKA pathways converge on S845 to regulate its phosphorylation and the phosphorylation-dependent GluR1 insertion into the surface membrane. It is believed that the two pathways operate in concert and in distinct temporospatial manners to modulate synaptic plasticity.

MODULATION OF ASICs BY NO

In addition to glutamate receptor channels, ASICs represent another family of ion channels subjected to the modulation by NO. Seven subunits of ASICs (1a, 1b1, 1b2, 2a, 2b, 3, and 4) have been identified to date, which are encoded by four genes (Price et al., 1996; Lingueglia et al., 1997; Waldmann et al., 1997a,b; Chen et al., 1998; Akopian et al., 2000). A major function of ASICs is to maintain normal cellular activity in response to changing pH

signals. Given the fact that a variety of neurological disorders, including inflammation, ischemic stroke, traumatic brain injury and acid nociception, cause severe acidosis, ASICs are deemed to be activated and play a role in cell death and in the pathogenesis or progression of these diseases (Wemmie et al., 2006; Chu et al., 2011). As a result, ASICs are emerging as new pharmacotherapeutic targets for these illnesses (Wemmie et al., 2006; Xiong et al., 2008; Sluka et al., 2009; Xu and Duan, 2009).

Acid-sensing ion channels are subject to the regulation by various extracellular and intracellular signals (Chu et al., 2011). Among the known regulators is NO. It has been suggested that ASICs are a potential target for direct modulation by NO-dependent S-nitrosylation (Cadiou et al., 2007). This is based on the facts that (1) the channel possesses a large number of cysteine residues on their extracellular domains (Waldmann et al., 1999), and (2) oxidizing agents, 5,5′-dithio-*bis*-(2-nitrobenzoic acid; DTNB), showed a sizable impact on acid-evoked currents in neurons and heterologous cells (Andrey et al., 2005; Chu et al., 2006). In support of this, Cadiou et al. (2007) reported that the NO donor SNAP potentiated proton-gated currents in rat cultured dorsal root ganglion (DRG) neurons and in CHO cells expressing each of ASIC subunits. The SNAP effect was not due to activation of the cGMP pathway because the cGMP analog db-cGMP did not mimic, and the guanylyl cyclase inhibitor ODQ did not block, the SNAP potentiation. The effect of SNAP was thought to be mediated via a direct mechanism involving NO S-nitrosylation at extracellular cysteines of ASICs (Cadiou et al., 2007).

Acid-sensing ion channels are known to participate in mediating acid-evoked pain in humans. This process is believed to be regulated by NO, based on the finding that topical application of an NO donor enhanced acid-evoked pain (Cadiou et al., 2007). Another neurological disorder in which the NO potentiation of ASICs may have a pathophysiological relevance is ischemia. Cerebral ischemia is known to cause metabolic acidosis. This leads to activation of ASICs, especially Ca^{2+} permeable ASIC1a, which increases intracellular Ca^{2+} overload and results in neuronal death (Xiong et al., 2004). NO promoted such neuronal death by potentiating ASICs during mild and moderate acidosis, although in severe acidosis NO synthesis was inhibited and the NO contribution subsided (Jetti et al., 2010).

CONCLUSION

Ionotropic glutamate receptors have long been appreciated to be subject to various types of post-translational modifications which determine expression, trafficking, and function of modified receptors. NO-dependent S-nitrosylation is one of these modifications. Available data show that NMDARs are directly modulated by NO-mediated S-nitrosylation. Direct S-nitrosylation occurs at a cysteine site (C399) in an extracellular N-terminal region of NR2A subunits. In addition to NR2A, NO influences other synaptic proteins that are important for NMDAR activity. Serine racemase which generates D-serine, a co-agonist of NMDARs, is S-nitrosylated. The S-nitrosylation inhibits its enzymatic activity. PSD-95, a scaffolding protein stabilizing NMDARs at synaptic sites and linking nNOS to NMDARs, also undergoes physiological S-nitrosylation, which suppresses synaptic delivery of PSD-95. Apparently, the NO regulation of all three elements (NR2A, serine racemase, and PSD-95) can synergistically generate an inhibitory driving force on NMDARs. Given that NMDARs stimulate NO synthesis, NO forms a negative feedback loop to prevent overstimulation of the receptor.

Direct S-nitrosylation of AMPARs has not been reported. Instead, AMPAR-interacting proteins are S-nitrosylated and regulated by NO. NSF binds to AMPAR GluR2, while stargazin binds to GluR1. NMDAR activation enhances S-nitrosylation of both proteins, facilitating their binding to GluR1/2 and surface expression of AMPARs. In addition to this S-nitrosylation-dependent and cGMP-independent mechanism, the cGMP-dependent pathway also mediates the NO regulation of AMPARs. Like glutamate ion channels, ASICs are modulated by NO. NO is believed to potentiate ASIC activity. This potentiation was not mediated by the cGMP-dependent pathway, but mediated through S-nitrosylation of extracellular cysteines of ASICs. The NO potentiation of ASICs contributes to acid-evoked pain and ischemia-induced neuronal death. It is possible that all NO targets, including NMDARs, AMPARs, ASICs, and their associated proteins, can be co-localized at same subsets of synapses. NO could therefore simultaneously regulate these synaptic proteins and interactions among them to precisely control synaptic transmission. It is anticipated that NO modification biology of ion channels will grow rapidly and more sophisticated NO modification models on glutamate ion channels and ASICs will be characterized *in vivo*. Ultimately, this knowledge will be translated into novel and effective pharmacotherapies for respective neurological disorders.

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TRP channels: sensors and transducers of gasotransmitter signals

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The transient receptor potential (*trp*) gene superfamily encodes cation channels that act as multimodal sensors for a wide variety of stimuli from outside and inside the cell. Upon sensing, they transduce electrical and Ca^{2+} signals *via* their cation channel activities. These functional features of TRP channels allow the body to react and adapt to different forms of environmental changes. Indeed, members of one class of TRP channels have emerged as sensors of gaseous messenger molecules that control various cellular processes. Nitric oxide (NO), a vasoactive gaseous molecule, regulates TRP channels directly *via* cysteine (Cys) S-nitrosylation or indirectly *via* cyclic GMP (cGMP)/protein kinase G (PKG)-dependent phosphorylation. Recent studies have revealed that changes in the availability of molecular oxygen (O_2) also control the activation of TRP channels. Anoxia induced by O_2-glucose deprivation and severe hypoxia (1% O_2) activates TRPM7 and TRPC6, respectively, whereas TRPA1 has recently been identified as a novel sensor of hyperoxia and mild hypoxia (15% O_2) in vagal and sensory neurons. TRPA1 also detects other gaseous molecules such as hydrogen sulfide (H_2S) and carbon dioxide (CO_2). In this review, we focus on how signaling by gaseous molecules is sensed and integrated by TRP channels.

Keywords: TRP channels, gasotransmitter, nitric oxide, oxygen, TRPC5, TRPC6, TRPV1, TRPA1

INTRODUCTION

Transient receptor potential (TRP) proteins are the product of *trp* genes, which were first discovered in *Drosophila melanogaster*, but later found to have homologs in other species. These gene products form cation channels that detect and transduce cellular stimuli into electrical signals (*via* changes in membrane potential) or chemical signals [*via* changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)] (Montell et al., 2002; Clapham, 2003; Voets et al., 2005). TRP proteins are putative six-transmembrane domain polypeptide subunits that assemble into tetramers to form channels (**Figure 1A**). In mammalian systems, TRP channels comprise six related protein subfamilies: TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML (Clapham et al., 2005) (**Figure 1B**). The TRPC homologs form receptor-activated Ca^{2+}-permeable cation channels (RACCs) that, when activated by receptor stimulation, induce phospholipase C (PLC) to hydrolyze phosphatidylinositol-4,5-biphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Zhu et al., 1996; Vazquez et al., 2004). Store-operated channels (SOCs), which are activated by IP_3-induced Ca^{2+} release and depletion of endoplasmic reticulum (ER) Ca^{2+} stores, can also be categorized as RACCs. In contrast, TRPV Ca^{2+}-permeable channels can be functionally defined as thermosensors (Caterina et al., 1997; Clapham, 2003; Patapoutian et al., 2003; Clapham et al., 2005). TRPV1, originally identified as the receptor for the vanilloid compound capsaicin, is responsive to heat (>43°C); proton (H^+) concentration (pH < 5.6); the intrinsic ligand, anandamide; and receptor-driven PLC activity (Clapham et al., 2005). High temperature also activates TRPV2 (>52°C), TRPV3 (>31°C or >39°C), and TRPV4 (>27°C). TRPV5 and TRPV6 comprise a different subfamily because they are activated by $[Ca^{2+}]_i$ (Clapham, 2003; Clapham et al., 2005). The TRPM subfamily is named after melastatin (TRPM1), a tumor suppressor protein isolated in a screen for genes whose level of expression is inversely correlated with the severity of metastatic potential of a melanoma cell line (Duncan et al., 1998), and contains eight mammalian members. TRPM8 channels, in contrast to TRPV channels, are activated by low temperatures (<25°C) and menthol (McKemy et al., 2002; Peier et al., 2002). The sole member of the TRPA subfamily, TRPA1, has a large N-terminal domain with 17 predicted ankyrin repeat (AnkR) domains (Gaudet, 2008). Pungent compounds, such as the allyl isothiocyanate found in mustard oil, trigger TRPA1 activation (Jordt et al., 2004). TRPA1 has been shown to respond to noxious cold (<17°C), although its activation by cold remains controversial (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004; Nagata et al., 2005; Bautista et al., 2006; Kwan et al., 2006; Sawada et al., 2007; Karashima et al., 2009). Thus, TRP channels serve as sensors for a variety of environmental factors. With the exception of TRPM4 and TRPM5 (Nilius, 2007), all of these TRP channels have some Ca^{2+} influx

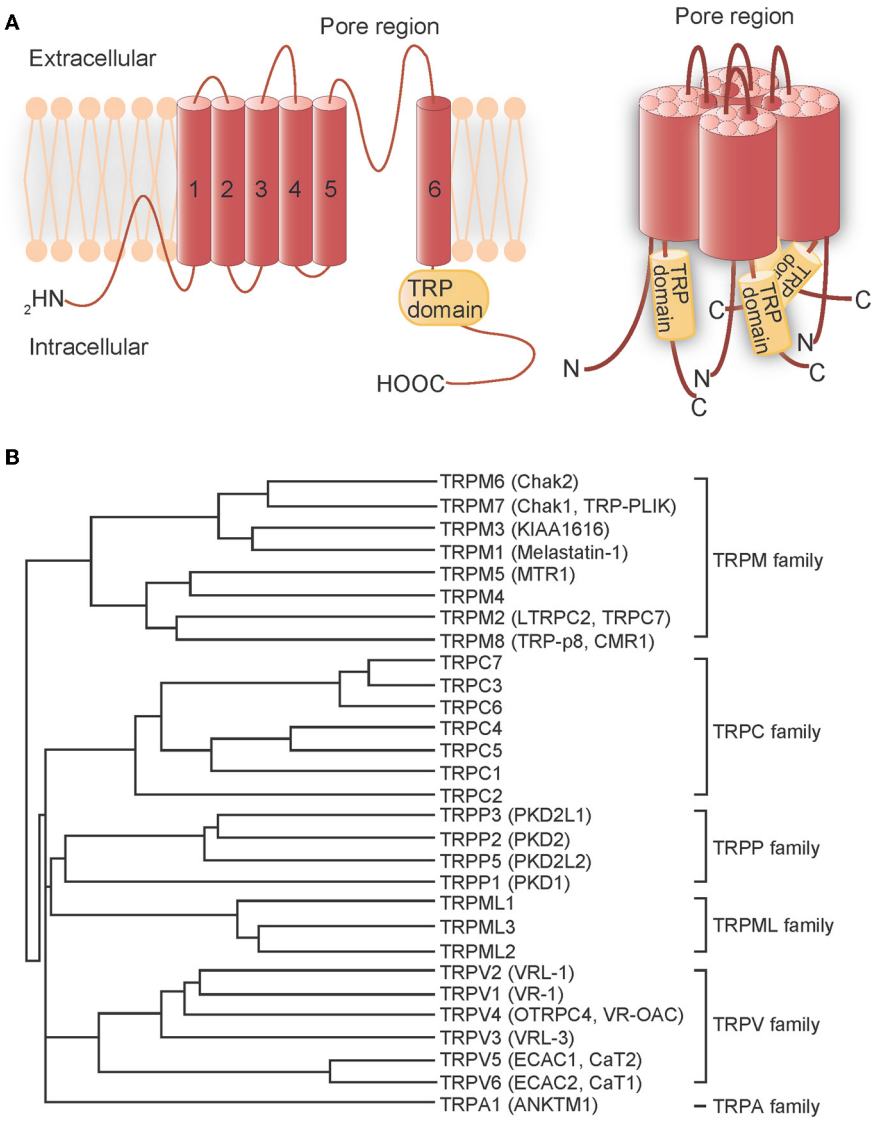

FIGURE 1 | Transmembrane topology and phylogenetic tree of mammalian TRP channels. (A) Transmembrane topology (left) and the quartenary structure of TRP channels (right). The TRP protein has six putative transmembrane domains, a pore region between the fifth and sixth transmembrane domains and a TRP domain in the C-terminal region. The TRP protein assembles into homo-tetramers or hetero-tetramers to form channels. **(B)** Phylogenetic tree of mammalian TRP channels based on their homology.

activity that is a component of their regulation of diverse cellular processes.

Gaseous molecules, such as oxygen (O_2), nitric oxide (NO), carbon monoxide (CO), hydrogen sulfide (H_2S), and carbon dioxide (CO_2), have been shown to play important roles in biological signal transduction. These molecules share several unique physicochemical properties and exert their biological activities through mechanisms distinct from those of other signaling molecules as summarized by Suematsu (2003). Firstly, these molecules are highly membrane-permeable and can readily convey signals in an autocrine, paracrine and/or juxtacrine manner. Secondly, they exert their biological actions *via* a variety of interactions with macromolecules: covalent binding of gases to prosthetic metal complexes in receptor proteins; non-covalent binding to the regulatory subunits of proteins; and space occupancy in and around the protein structure that impedes the access of other gases to the functionally critical protein motifs. Recent evidence suggest that NO, CO, H_2S, and CO_2 function as signaling molecules that also play critical roles in mediating the biological effects of changes in O_2 availability (Semenza and Prabhakar, 2012).

Among the gaseous signaling molecules, NO is the most extensively studied. Its biological significance and the systems by which it is generated were first revealed in the 1980s (Furchgott and Zawadzki, 1980; Palmer et al., 1988; Sakuma et al., 1988) and it is now known to regulate a variety of biological events,

including vascular relaxation and neurotransmission. Conversely, excessive generation of NO and NO-derived reactive nitrogen species (RNS) has been implicated in a number of pathological conditions (Reiter, 2006). Cyclic GMP (cGMP) is the canonical mediator of NO signaling. However, the importance of a cGMP-independent signaling pathway involving protein S-nitrosylation is becoming increasingly recognized (Jaffrey et al., 2001; Hess et al., 2005). S-nitrosylation of cysteine (Cys) is readily reversible with high spatial and temporal specificity. The NADH-dependent oxidoreductase, S-nitrosoglutathione reductase, specifically catalyzes the denitrosylation of S-nitrosoglutathione, by which protein S-nitrosylation is regulated in the cellular equilibrium between S-nitrosylated proteins and S-nitrosoglutathione. Thioredoxin also mediates direct denitrosylation of multiple S-nitrosylated proteins. Thus, the temporal and spatial regulation of S-nitrosylation and denitrosylation confers specificity to NO-based cellular signaling (Benhar et al., 2009).

Ca^{2+} and NO signals are precisely coordinated with each other and converge at two main points (Milbourne and Bygrave, 1995). Firstly, in order to be activated, constitutive NO synthase (NOS) must bind calmodulin (CaM), an event controlled by the level of $[Ca^{2+}]_i$ (Nathan, 1992). An increase in $[Ca^{2+}]_i$ has been shown to activate constitutive NOS, especially in endothelial and neuronal cells, and this results in NO production (Moncada et al., 1997). However, it is still unclear whether activation of endothelial NOS (eNOS) requires specific modes of upstream Ca^{2+}-mobilization, or RACC subtypes formed by particular TRPCs (Hutcheson and Griffith, 1997; Lantoine et al., 1998; Lin et al., 2000; Koyama et al., 2002; Yao and Garland, 2005). In contrast, it is well characterized that Ca^{2+} influx through *N*-methyl-D-aspartate (NMDA) receptors activates neuronal NOS (nNOS) (Dawson et al., 1991) to elicit S-nitrosylation of many synaptic proteins (Jaffrey et al., 2001). The second point of convergence is that NO can directly affect intracellular Ca^{2+} levels by acting on cell surface receptors to promote Ca^{2+} mobilization (Milbourne and Bygrave, 1995). This feedback regulation of Ca^{2+} signaling by NO remains controversial in non-excitable cells (Khan and Hare, 2003), where NO has been reported to regulate Ca^{2+} mobilization pathways (including RACCs) both positively (Volk et al., 1997; Chen et al., 2000; Li et al., 2003) and negatively (Kwan et al., 2000; Dedkova and Blatter, 2002). In contrast, the regulation of Ca^{2+} signaling by NO is better defined in neurons, where NO nitrosylates NR1 and NR2 subunits of NMDA receptors at specific Cys/thiol groups and decreases Ca^{2+} entry *via* this ionotropic receptor (Choi et al., 2000; Jaffrey et al., 2001; Lipton et al., 2002). This negative feedback mechanism is important in precluding overactivation of NMDA receptors. However, NMDA receptors have a very restricted tissue distribution and specific physiological functions, whereas NO signaling *via* protein S-nitrosylation and Ca^{2+} signaling are much more generalized and have a wide array of functions. Therefore, to understand better the molecular mechanisms that link these two key signals, S-nitrosylation targets must be distinguished from the more ubiquitous Ca^{2+}-mobilizing ion channels. As described below, this ultimately provides insights into the activation gating that underlies sensor function of TRP channels. In this review, we focus on how the gaseous signaling is sensed and integrated by TRP channels (**Figure 2**).

CORRELATION BETWEEN RACCS AND NO SIGNALS IN NATIVE TISSUE PREPARATIONS

Tight correlation between native RACCs and NO signaling has been presumed in many types of cells (Xu et al., 1994; Volk et al., 1997; Ma et al., 1999; Chen et al., 2000; van Rossum et al., 2000; Thyagarajan et al., 2001; Li et al., 2003). In pancreatic acini isolated from rat, agonist-mediated Ca^{2+} release from internal stores activates a cellular pool of NOS to generate cGMP, which then modulates Ca^{2+} entry through the plasma membrane (Xu et al., 1994). This mechanism might be responsible for the capacitative nature of Ca^{2+} entry. Interestingly, different concentrations of an NO donor sodium nitroprusside (SNP) revealed that cGMP has a dual effect on Ca^{2+} entry. Increasing cGMP levels by up to 10-fold above that in control cells treated with *N*-nitro-L-arginine, a specific inhibitor of NOS, is associated with activation of Ca^{2+} entry. Further increases in cGMP to levels up to 80-fold above control inhibit Ca^{2+} entry in a concentration-dependent manner. The biphasic effect of cGMP provides the cells with a negative feedback mechanism and inhibits Ca^{2+} entry during periods of high $[Ca^{2+}]_i$, allowing oscillatory behavior in Ca^{2+} entry. In vascular endothelial cells isolated from bovine aorta, exogenous NO gas potentiates Ca^{2+} influx, which markedly increases the sustained phase of $[Ca^{2+}]_i$ elevation and possibly NO production (Chen et al., 2000). In DDT1MF-2 smooth muscle cell line derived from hamster vas deferens and DC-3F Chinese hamster lung fibroblast cell line, Ca^{2+} entry activated by the lipophilic NO donor, GEA3162 [5-amino-3-(3,4-dichlorophenyl)1,2,3,4-oxatriazolium] (25 μM), or the alkylator, N-ethylmaleimide (10 μM), is strongly activated by transient external Ca^{2+} removal that depletes internal Ca^{2+} stores, closely resembling activation of SOC activity in the same cells (Ma et al., 1999). In cortical astrocytes isolated from mice, physiological concentrations of a natural neuromessenger, ATP (10 μM), induces Ca^{2+}-dependent NO production. By promoting Ca^{2+} influx, NO may facilitate the refilling of internal stores that have become partially depleted as a result of Ca^{2+} release during neurotransmitter-induced Ca^{2+} signaling (Li et al., 2003).

Suppressive effects of NO on Ca^{2+} entry have also been demonstrated (Kwan et al., 2000; Dedkova and Blatter, 2002). In vascular endothelial cells, the membrane permeant cGMP analogue 8-Br-cGMP (>300 μM) attenuates SOC activity *via* a protein kinase G (PKG)-dependent mechanism. These results suggest a role of cGMP and PKG in the regulation of Ca^{2+} entry in vascular endothelial cells (Kwan et al., 2000; Dedkova and Blatter, 2002). Contradictory effects of NO on Ca^{2+} influx may depend on the difference of the type of NO donors used for experiments and their concentrations. More importantly, the contradictory effects are at least in part attributable to diversity in the NO susceptibility of Ca^{2+} entry channels and signaling molecules that regulate Ca^{2+} influx. Thus, different ensembles of NO-regulated mechanisms may lead to a wide variety of net Ca^{2+} entry responses in the presence of NO.

REGULATION OF TRP CHANNELS BY NO *VIA* Cys S-NITROSYLATION

Some TRP channels are potently regulated by Cys modifications, including Cys S-nitrosylation by NO in heterologous systems

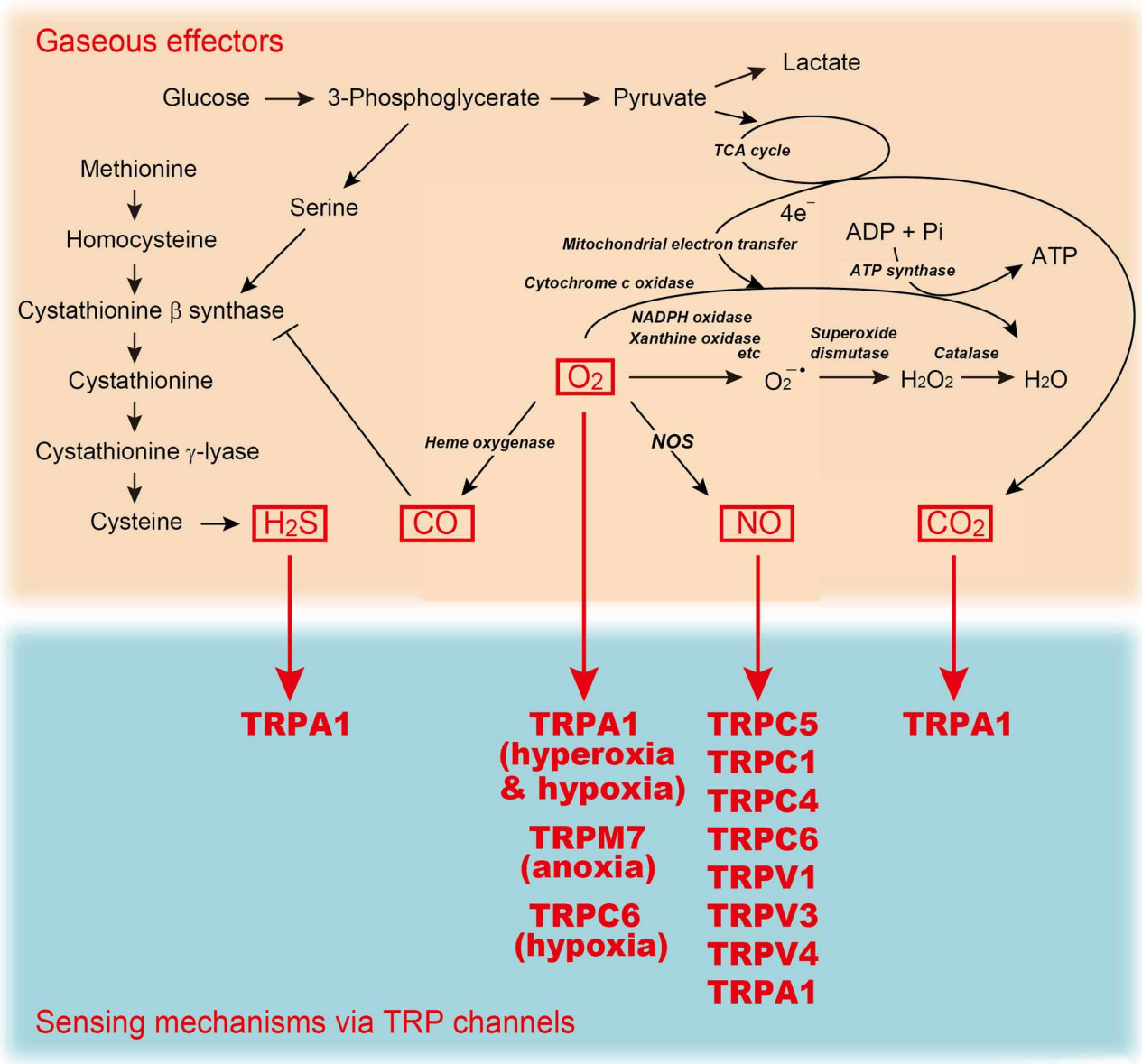

FIGURE 2 | A sensing mechanism for gaseous molecules linked to metabolic pathways. TRP channels mediate sensing mechanism for H_2S, O_2 (anoxia, hypoxia, or hyperoxia), NO, and CO_2.

and bovine aortic endothelial cells (Yoshida et al., 2006). By performing labeling and functional assays with Cys mutants, it has been shown that Cys553 and nearby Cys558 on the N-terminal side of the putative pore-forming region between the fifth and sixth transmembrane domains S5 and S6 are essential for mouse TRPC5 activation in response to an NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP) (300 μM) (**Figure 3**). In *Drosophila melanogaster* Shaker voltage-gated K^+ channels, the activation gate formed by S6 residues near the intracellular entrance of the pore cavity has been identified (del Camino and Yellen, 2001). Considering the longer S5–S6 linkers of TRPC5, the TRPC5 S5–S6 linker with modified Cys553 and Cys558 may be invaginated toward the cytoplasm to reach the S6 activation gate (**Figure 3**). The corresponding Cys sites of TRPC1, TRPC4, TRPV1, TRPV3, and TRPV4 are potential targets of nitrosylation leading to channel activation in heterologous expression systems. Although the differences in maximal $[Ca^{2+}]_i$ responses [Δratio (340/380)] to SNAP, in control cells and cells heterologously expressing either TRPC1 or TRPC4β (a splice isoform of TRPC4) are not statistically significant, a larger fraction (7–9%) of the TRP-expressing cells shows a Δratio (340/380) >0.5 when compared to control cells (2–5%). Cells heterologously co-expressing TRPC4β and TRPC5 give responses to SNAP comparable to those in cells heterologously expressing

TRPC5 alone, whereas cells heterologously co-expressing TRPC1 and TRPC5 give slightly suppressed yet robust responses. Co-immunoprecipitation of TRPC5 with TRPC1 or TRPC4β suggests that heteromultimeric TRPC5/TRPC1 and TRPC5/TRPC4β channels also have NO sensitivity. The thermosensor TRP channels TRPV1, TRPV3, and TRPV4 also show SNAP (300 μM)-induced activation, as predicted from conserved Cys residues in the corresponding regions of these homologs. Indeed, substitutions of two conserved Cys residues in TRPV1 lead to significantly suppressed responses to SNAP and to Cys S-nitrosylation. Notably, the sensitivity of TRPV1 to H^+ and heat is enhanced by SNAP but abolished by the mutations, despite normal surface expression and intact control H^+ and heat responses. Thus, channel activation regulated by nitrosylation is conserved among a number of TRP channels belonging to different subfamilies.

SNAP-activated TRPC5 channels are not entirely inactivated by ascorbate, which reduces S-nitrosothiols to thiols but not disulfides. However, dithiothreitol (DTT), which reduces both S-nitrosothiols and disulfides to thiols, fully suppressed SNAP-activated TRPC5 channel activity, suggesting that both nitrosylation and disulfide bond formation are involved in SNAP-induced TRPC5 activation. In an S-nitrosylation assay (Jaffrey et al., 2001), S-nitrosylation is abolished by mutation of Cys553 in TRPC5 but not by mutation of Cys558. As proposed for the acid–base catalysis of hemoglobin nitrosylation in proteins with high NO sensitivity, basic and acidic amino acids surrounding S-nitrosylated Cys may enhance the nucleophilicity of the sulfhydryl (SH) group, and therefore the S-nitrosylation of this group of proteins (Hess et al., 2005). Charged residues flanking Cys553 and Cys558 may confer modification susceptibility to NO in TRPC5. It is possible that the TRPC5 channel is opened *via* S-nitrosylation of Cys553 and a subsequent nucleophilic attack of nitrosylated Cys553 by the free SH group of Cys558 to form a disulfide bond that stabilizes the open state (**Figure 3**). The NO sensitivity of TRPC5 channels has been disputed by several groups (Xu et al., 2008; Wong et al., 2010). NO sensitivity of TRPC5 may depend on culturing conditions, the way drugs are administered, cell density during measurements, levels of antioxidants or other experimental conditions, molecular and cellular states that may affect the modification state of TRPC5 proteins.

Previous reports have provided important information with respect to the formation in endothelial cells of a TRPC5 "channelsome," a molecular assembly centered upon a channel. As mentioned above, TRPC1 forms heterotetrameric channels with TRPC5 (Strübing et al., 2001) and a protein complex with caveolin-1 in caveolae/lipid raft domains (Lockwich et al., 2000; Bergdahl et al., 2003), which regulate the plasma membrane trafficking of TRPC1 (Brazer et al., 2003). It is therefore possible that TRPC5 forms indirect protein complexes with caveolin-1

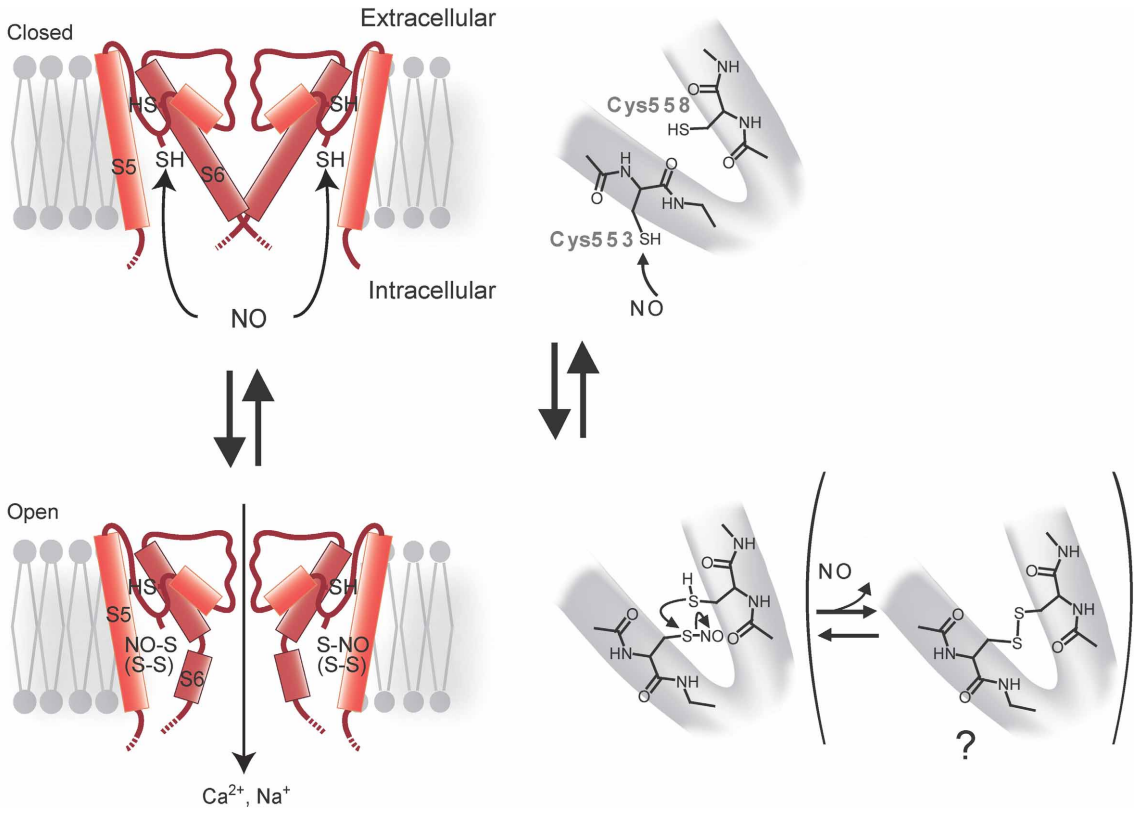

FIGURE 3 | Model for activation of TRPC5 channel by NO. Cys553 is nitrosylated by NO, which triggers TRPC5 channel opening. The free sulfhydryl group of Cys558 nucleophilically attacks nitrosylated Cys553 to form a disulfide bond that stabilizes the open state.

via TRPC1. Indeed, we have found an interaction of TRPC5 with caveolin-1 and eNOS in co-immunoprecipitation experiments as well as by colocalization of TRPC5 with caveolin-1 in heterologous systems and bovine aortic endothelial cells (Mori et al., unpublished data). Among numerous caveolin-associated proteins linked to signaling cascades (Couet et al., 1997; García-Cardeña et al., 1997; Sato et al., 2004; Quest et al., 2008), three isoforms of NOS, including eNOS, have been identified (Kone et al., 2003). The inhibitory association of caveolin is disrupted by the binding of Ca^{2+}-CaM to eNOS, leading to eNOS activation (Ju et al., 1997; Michel et al., 1997a,b; Rizzo et al., 1998; Bernatchez et al., 2005). In addition, eNOS is activated by different kinases, including Akt, protein kinase A (PKA), and protein kinase C (PKC) (García-Cardeña et al., 1996; Fulton et al., 1999; Michell et al., 2001; Boo and Jo, 2003; Heijnen et al., 2004). Based on data from our and other laboratories, we can propose a plausible model to describe the role of the TRPC5 channelsome in regulating receptor-activated NO production in vascular endothelial cells (**Figure 4**). TRPC5 proteins form complexes with vasodilator receptors, G-proteins, PLCβs and eNOS, and are anchored in caveolae by the scaffolding protein, caveolin-1. Upon vasodilator receptor stimulation, TRPC5 is activated by the PLCβ cascade to induce Ca^{2+} influx, which elevates the $[Ca^{2+}]_i$ to form Ca^{2+}-CaM. This then releases eNOS from the inhibitory control of caveolin-1 and leads to an initial NO production that activates TRPC5 channels. Ca^{2+} influx *via* NO-activated TRPC5 channels then induces secondary activation of eNOS to amplify the production of NO, resulting in a positive feedback cycle of receptor-activated Ca^{2+} and NO signaling. This model has been neatly summarized in a short review by Stamler and colleagues (Foster et al., 2006), based largely on our own data (Yoshida et al., 2006).

Recent reports have shown that SNAP (30 μM) and another NO donor (6)-(E)-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide (NOR3) (300 μM) activate human TRPA1 in heterologous systems and mouse TRPA1 in dissociated sensory neurons (Sawada et al., 2008; Takahashi et al., 2008a; Miyamoto et al., 2009). Functional characterization of site-directed Cys

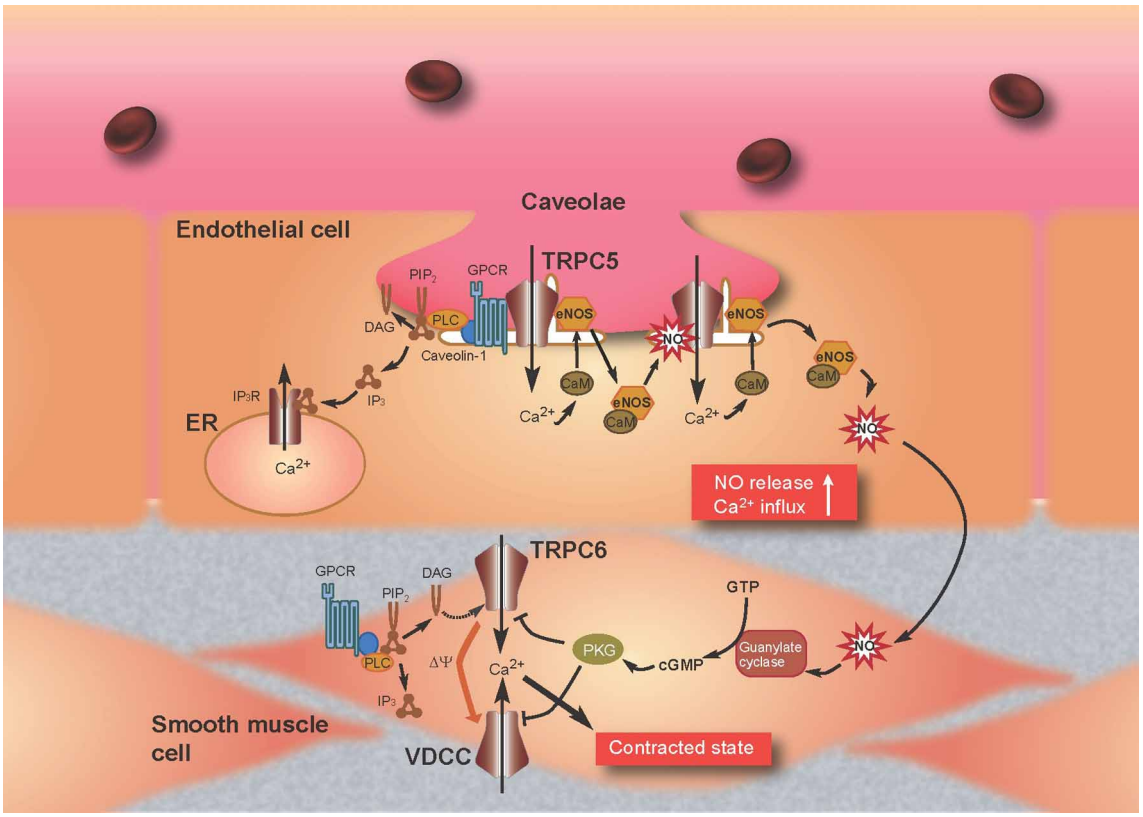

FIGURE 4 | Model for TRPC5-mediated feedback of Ca^{2+} and NO signaling in endothelial cells and attenuation of Ca^{2+} entry through TRPC6 by NO in smooth muscle cells. Stimulation of G protein-coupled receptors (GPCRs) (such as the ATP-activated P2Y receptor) induces Ca^{2+} influx and activation of eNOS as a consequence of binding of Ca^{2+}-CaM and release of eNOS from caveolin-1. TRPC5 undergoes eNOS-dependent S-nitrosylation after GPCR stimulation, resulting in amplified Ca^{2+} entry and secondary activation of eNOS to amplify production of NO. NO diffuses out of endothelial cells into adjacent smooth muscle cells and stimulates the guanylate cyclase, which leads to the activation of PKG in smooth muscle cells. In the most prevailing hypothesis, the magnitude of continuous Ca^{2+} influx through VDCC, which critically determines the contractile status of vascular smooth muscle cells, decreases and increases by membrane hyperpolarization and depolarization, respectively. TRPC6 likely functions as a depolarization ($\Delta\psi$)-inducing channel or a direct Ca^{2+}-entry pathway, activated in response to receptor stimulation. The NO/cGMP/PKG pathway suppresses TRPC6 and VDCC activity to induce relaxation of smooth muscle.

mutants of TRPA1 has demonstrated that Cys421, Cys641, and Cys665 are responsible for human TRPA1 activation by NO (Takahashi et al., 2008a). Cys421, Cys641, and Cys665 are located within the eleventh AnkR domain, seventeenth AnkR domain, and the N-terminal cytoplasmic region between seventeenth AnkR domain and S1, respectively (Gaudet, 2008).

Injection of NOR3 (1.5 mM) in the hind paw after PLC and PKA pathway activation (which sensitizes nociceptors, including TRPV1 and TRPA1), causes nociceptive behavior (Miyamoto et al., 2009). Interestingly, a decrease in nociception is observed in mice lacking both TRPV1 and TRPA1, but not in individual knockout animals (Miyamoto et al., 2009) despite *in vitro* results showing that the NO donor can activate both ion channels. Note that due to solubility issues of NO donors, limitations exist in using NO donors at relatively high concentrations for behavioral experiments (<10-fold the *in vitro* EC_{50}). For capsaicin, injections of capsaicin at >1000-times the *in vitro* EC_{50} are typically used to observe acute pain behavior. It is therefore not surprising that significant acute nocifensive behavior was not observed in terms of NO donors. Another potential explanation for the lack of a phenotype in individual knockout mice is due to functional compensation by each other. Indeed, TRPV1 and TRPA1 have overlapping expression in a subset of DRG neurons (Story et al., 2003) and thereby could potentially interact *via* intracellular signaling *in vivo*. Recently, icilin, an agonist of TRPA1 and TRPM8, has been implicated as a trigger for shaking and hyperthermia which require NO production and NMDA receptor activation (Ding et al., 2008; Werkheiser et al., 2009). In this context, it would be interesting to explore the mechanism of the concerted regulation of Ca^{2+} and NO signaling by the TRP channels, NMDA receptors and nNOS in neurons.

REGULATION OF TRP CHANNELS *VIA* THE NO/cGMP/PKG PATHWAY

One of the principal consequences of activation of the NO/cGMP/PKG cascade in blood vessels is vasorelaxation, which is mediated by phosphorylation of proteins that regulate intracellular Ca^{2+} levels and the sensitivity of the contractile machinery. Several distinct mechanisms have been proposed for the reduction in $[Ca^{2+}]_i$ caused by PKG (Lincoln et al., 2001; Feil et al., 2003). Increased activity of the BK_{Ca} channel following PKG activation has been reported to induce membrane hyperpolarization, thereby decreasing the rate of Ca^{2+} entry into vascular smooth muscle cells (VSMCs) through voltage-dependent Ca^{2+} channels (VDCCs). Phosphorylation of the inositol 1,4,5-trisphosphate receptor (IP_3R)-associated protein, IRAG (IP_3R-associated PKG substrate), is thought to inhibit agonist-induced Ca^{2+} release from internal stores. The possibility has also been suggested that phosphorylation of phospholamban by PKG increases the activity of sarcoplasmic Ca^{2+}-ATPase, facilitating the active transport of Ca^{2+} into internal stores and thereby decreasing $[Ca^{2+}]_i$. In many different types of blood vessel, activation of PKG has been found to inhibit vasoconstrictor-induced Ca^{2+} influx through plasmalemmal pathways other than those employing VDCCs (Karaki et al., 1997). However, little information exists regarding the molecular components of such Ca^{2+} influx pathways and, accordingly, it remains unclear how phosphorylation *via* PKG activation reduces the rate of Ca^{2+} influx.

TRPC6 is a Ca^{2+}-permeable channel regulated negatively by PKG-mediated phosphorylation in the NO/cGMP signaling pathway in heterologous systems and the rat vascular myocytes A7r5 (Takahashi et al., 2008b). Macroscopic and single-channel current recordings using patch clamp techniques have demonstrated that SNAP (100 μM)-induced inhibition of receptor-activated TRPC6 currents is abolished by pharmacological blockade of cGMP/PKG signaling with 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ), 2,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1H-diindolo[1, 2,3-fg:3′,2′,1′-kl] pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid methyl ester (KT5823) or membrane permeable PKG inhibitory peptide (DT3). It is also ablated by site-directed alanine mutation of a PKG phosphorylation site [threonine (Thr) 69] within the N-terminal cytoplasmic region of TRPC6. The critical involvement of Thr69 in PKG phosphorylation is confirmed by ^{32}P-incorporation assays of wild-type and alanine-substitution mutant TRPC6 proteins. Similar NO/cGMP/PKG pathway-mediated negative regulation is also observed for TRPC6-like currents recorded in A7r5 VSMCs. Indeed, vasopressin-evoked membrane depolarization of these cells, which is expected secondarily to activate VDCCs, is significantly slowed and attenuated after application of SNAP (100 μM). The TRPC6 protein is abundantly expressed in various types of VSMCs and has been shown to be a constituent of vasoconstrictor-activated cation channels, which increase Ca^{2+} entry into VSMCs *via* direct Ca^{2+} permeation or secondary activation of a VDCC and/or Na^+-Ca^{2+} exchanger (Inoue et al., 2006; Dietrich et al., 2007; Poburko et al., 2007). Thus, it is highly possible that, in a direct or indirect manner (i.e., *via* changes in membrane potential or an increase in intracellular Na^+ concentration), PKG-mediated mechanisms may work as a universal negative feedback to regulate neurohormonal Ca^{2+} mobilization across the VSMC membrane (**Figure 4**). This mechanism may be physiologically important in vascular tissues where NO is constantly released from vascular endothelial cells or nitrergic nerves.

O_2 SENSING MECHANISMS

Of the gaseous molecules, O_2 is the most well-known to physicians, scientists and laymen alike as an essential physical requirement. O_2 functions primarily as a terminal acceptor of electrons on mitochondrial electron transport. Most of the O_2 consumed in this process is reduced to generate water through the actions of cytochrome oxidase. The remainder is used to generate compounds that exert potent biological actions, including prostaglandins, reactive oxygen species (ROS) and gaseous molecules such as NO and CO (Suematsu et al., 2003). In the last decade, O_2 itself has been increasingly recognized as an important signal molecule that mediates many physiological and pathophysiological processes including proliferation of stem cells, ischemia injury and tumor progression (Csete, 2006; Swartz et al., 2007). Thus, O_2 is not only required for cellular respiration, but also serves as a signaling molecule, and as the essential substrate for the formation of other signaling molecules (**Figure 2**). However, O_2 also exerts toxicity causing aging, respiratory disorders and

eventually death in a high O_2 (hyperoxic) environment. Because of the ambivalent physiological nature of O_2, aerobic life forms must adapt to hyperoxia and low O_2 environment (hypoxia) by sensing O_2 availability and transmitting this information to effector systems.

Cellular responses to changes in O_2 availability can be acute or chronic (López-Barneo et al., 2001). Acute responses rely mainly on O_2-regulated ion channels, which mediate adaptive changes in cell excitability, contractility, and secretory activity (Gonzalez et al., 1994; Neubauer and Sunderram, 2004; Weir et al., 2005). Chronic responses depend on the modulation of transcription factors such as hypoxia-inducible factor (HIF), which determines the expression of numerous genes encoding growth factors, enzymes, and transporters (Semenza and Wang, 1992; Schofield and Ratcliffe, 2004; Webb et al., 2009). O_2-regulated ion channels and transcription factors are part of a widely operating signaling system that helps to provide an appropriate amount of O_2 to the tissues and to protect the cells against toxicity damage due to excess or deficient O_2.

In mammals, the carotid bodies, located near the carotid artery bifurcations, and brainstem catecholaminergic neurons rapidly detect changes in partial O_2 pressure (PO_2) in arterial blood (Gonzalez et al., 1994; Neubauer and Sunderram, 2004). It is known that BK_{Ca}, TASK, and K_V K^+ channels are involved in the mechanism of arterial O_2 sensing in the carotid bodies (Gonzalez et al., 1994; Weir et al., 2005). Hypoxia inhibits K^+ channels through several mechanisms, such as CO production by hemeoxygenase and intracellular ATP depletion that depolarizes glomus cells. This inhibition leads to activation of VDCC, exocytosis, and the excitation of carotid sinus nerves. In contrast, hyperoxia attenuates depolarization and inhibits exocytosis (Gonzalez et al., 1994; Weir et al., 2005). The chemosensory inputs of the carotid sinus nerve are carried toward the medullary centers that regulate the ventilatory pattern. The local O_2 tension is also rapidly detected by other tissues including vagal and sensory neurons in the airway and lungs, chromaffin cells of the fetal adrenal medulla, smooth muscle cells of the pulmonary resistance arteries, cerebral neurons, fetoplacental arteries, systemic arteries, and the doctus arteriosus. However, detection of hypoxia by these tissues remains to be fully characterized. Recently, a class of TRP channels has been demonstrated to act as cell sensors for changes in O_2 availability (Aarts et al., 2003; Weissmann et al., 2006; Takahashi et al., 2011).

ANOXIA-SENSING MEDIATED BY TRPM7 CHANNELS IN THE BRAIN

Excitotoxicity in brain ischemia triggers neuronal death and neurological disability, and yet these are not prevented by antiexcitotoxic therapy (AET) in humans. The failure of AET in the face of a clear role for excitotoxicity in acute neurological disorders is paradoxical (Birmingham, 2002; Ikonomidou and Turski, 2002). In addressing this problem, Aarts et al. have revealed that the TRPM7 channel, termed chanzyme because it possess a channel and α-kinase domain (Runnels et al., 2001; Nadler et al., 2001), is activated by oxygen-glucose deprivation (OGD) through the production of ROS and RNS, permitting Ca^{2+} uptake that further stimulates ROS and TRPM7 activation in heterologous systems and cortical neurons isolated form rats (Aarts et al., 2003). Suppressing TRPM7 expression in rat cortical neurons prevents anoxic neuronal death even in the absence of AET, indicating that TRPM7 is an essential mediator of anoxic death. It is possible that patients enrolled in failed trials studying the use of AET for stroke or traumatic brain injury are selected to have severe injuries (Morris et al., 1999), or that these disorders in humans, by their nature, induce severe ischemia. Therefore, future treatment of such disorders may also need to inhibit TRPM7. Indeed, it has been shown that suppression of hippocampal TRPM7 by intrahippocampal injections of viral vectors bearing shRNA specific for TRPM7 makes neurons resistant to ischemic death after brain ischemia and preserves neuronal morphology and function in rats (Sun et al., 2009). TRPM7 suppression also prevents ischemia-induced deficits in long-term potentiation and preserves performance in fear-associated and spatial-navigational memory tasks. Thus, regional suppression of TRPM7 is feasible, well tolerated and inhibits delayed neuronal death *in vivo* in an animal model.

HYPOXIA-SENSING BY TRPC6 CHANNELS IN PULMONARY SMOOTH MUSCLE CELLS

Regional alveolar hypoxia causes local vasoconstriction in the lungs, shifting blood flow from hypoxic to normoxic areas, thereby maintaining gas exchange. This mechanism is known as hypoxic pulmonary vasoconstriction (HPV) (Jeffery and Wanstall, 2001; Weissmann et al., 2001; Schermuly et al., 2005). Disturbances in HPV can cause life-threatening hypoxemia, whereas chronic hypoxia triggers vascular remodeling in the lungs and pulmonary hypertension (Sartori et al., 1999). In studying signaling cascades of this vitally important mechanism, Weissmann et al. have shown that severe hypoxia (1% O_2)-induced cation influx and currents in smooth muscle cells are largely absent in precapillary pulmonary arteries of *Trpc6* knockout mice, although recombinant TRPC6 expressed heterologously cannot be activated by hypoxia (Weissmann et al., 2006). Hypoxia-induced TRPC6 activation in smooth muscle cells is mediated by DAG accumulation probably by activated phospholipases. TRPC6 appears to be a key regulator of acute HPV, because this regulatory mechanism is absent in *Trpc6* knockout mice, whereas the pulmonary vasoconstrictor response to the thromboxane mimetic, U46619, is unchanged. Accordingly, induction of regional hypoventilation results in severe arterial hypoxemia in *Trpc6* knockout mice, but not in wild type mice. Notably, chronic hypoxia-induced pulmonary hypertension is independent of TRPC6 activity. Thus, TRPC6 plays a unique and indispensable role in acute HPV. Manipulation of TRPC6 function may thus offer a therapeutic strategy for the control of pulmonary hemodynamics and gas exchange.

O_2-SENSING BY TRPA1 CHANNEL IN VAGAL AND SENSORY NEURONS

Sensory and vagal afferent neurons, which project nerve endings throughout the body, are thought to detect hypoxia in organs such as the airway, lungs and heart after ischemia, and other conditions of low O_2 supply (Howe et al., 1981; De Sanctis et al., 1991; Longhurst et al., 2001; Gruss et al., 2006). However, the characteristics and mechanisms of hypoxia detection by sensory and vagal

neurons have yet to be fully defined (Longhurst et al., 2001). In terms of hyperoxia, *Caenorhabditis elegans* has been reported to be adept at avoiding hyperoxia due to detection mechanisms in sensory neurons (Gray et al., 2004). Furthermore, insects breathe discontinuously to avoid O_2 toxicity during hyperoxia (Hetz and Bradley, 2005). However, the physiological relevance of hyperoxia detection through sensory systems is less clear in vertebrates, and the delineation of these neuronal hyperoxia-sensing molecular processes in vertebrates remains an exciting area of research.

Systematic evaluation of TRP channels using reactive disulfides with different redox potentials has revealed that TRPA1 can sense O_2 (Takahashi et al., 2011). O_2 sensing by TRPA1 is based upon disparate processes: proline (Pro) hydroxylation by Pro hydroxylases (PHDs), and direct oxidation of Cys residues. In normoxia, PHDs hydroxylate conserved Pro394 within the tenth AnkR domain of TRPA1, inhibiting its activity. In hypoxia, the decrease in O_2 concentration diminishes PHD activity, relieving TRPA1 from the inhibitory action of Pro hydroxylation and leading to its activation. This recovery of TRPA1 activity can be achieved by insertion of fresh, unmodified TRPA1 proteins into the plasma membrane or by dehydroxylation of modified proteins through an unidentified molecular mechanism. In hyperoxia, O_2 activates TPRA1 by oxidizing Cys633, Cys856 or both. Cys633 and Cys856 are located within the seventeenth AnkR domain and the intracellular linker region between S4 and S5, respectively. TRPA1 can assume at least two oxidized forms during hyperoxia: a relatively unstable oxidized state (state 1) readily reversed by glutathione, and a relatively stable oxidized state (state 2). SH groups on the key Cys residues (Cys633 and Cys856) may be modified to sulfenic acid (S-OH) in state 1, and form disulfide bonds (S-S) in state 2. This oxidation mechanism over-rides the inhibition by Pro hydroxylation to activate TRPA1. In mice, disruption of the *Trpa1* gene abolishes hyperoxia- and mild hypoxia (15% O_2)-induced cationic currents in vagal and sensory neurons and thereby impedes enhancement of *in vivo* vagal discharges induced by hyperoxia and hypoxia. The results suggest a new O_2-sensing mechanism mediated by TRPA1 (**Figure 5**).

The vagal nerve conveys sensory information about the state of the organs to the central nervous system, in addition to providing output to the various organs in the body. Enhanced discharges in vagal afferents induce respiratory, cardiac, and vascular responses (Meller and Gebhart, 1992; Longhurst et al., 2001; Kubin et al., 2006). Chemicals encountered in the airway are detected by airway vagal C fibers (Kubin et al., 2006). Recently, TRPA1 has been shown to sense environmental irritants, thus initiating defensive reflexes such as coughing and respiratory depression in the C fibers (Bessac and Jordt, 2008; Bessac et al., 2008; Nassenstein et al., 2008). Notably, TRPA1 activation shows an inverted bell-shaped O_2-dependency curve with a minimum at the PO_2 of 137 mmHg, which is slightly below the atmospheric PO_2 (159 mmHg) (Takahashi et al., 2011). Considering that tracheal PO_2 (149 mmHg) is comparable to atmospheric PO_2 (Cottrell, 1988), it is highly possible that TRPA1 expressed in the trachea is slightly but significantly activated to act as a frontline defense against mild hyperoxia in the atmosphere.

Weather records (McElroy, 2002) suggest that atmospheric PO_2 at sea level ranges between approximately 137 and 170 mmHg. Because our O_2 dependence data reveal that minimal TRPA1 activity at a PO_2 of 137 mmHg is approximately 30% of the maximum activity at a PO_2 of 170 mmHg, so-called normoxia can be hyperoxic in the context of mammalian TRPA1 channels. This is reminiscent of O_2 avoidance in *Caenorhabditis elegans* (Gray et al., 2004) and insects (Hetz and Bradley, 2005).

SENSING OF OTHER GASEOUS MOLECULES BY TRP CHANNELS

H_2S has emerged as a new gaseous modulator of various biological functions including nociception (Szabó, 2007; Gadalla and Snyder, 2010). In rodents, topical application of an H_2S donor, NaHS (1 nmol) produces pain responses through potentiation of T-type Ca^{2+} channels probably in the primary afferents (Kawabata et al., 2007; Matsunami et al., 2009). Endogenous H_2S is also reported to contribute to pain transmission in rat models of formalin-induced inflammation (Lee et al., 2008) and irritable bowel syndrome (Xu et al., 2009).

NaHS (1 mM) activates capsaicin-sensitive sensory neurons in isolated rat urinary bladder (Patacchini et al., 2005). Although the mechanisms underlying the action of H_2S are yet to be clarified, the pharmacological profile of H_2S hints at the involvement of TRPA1. Indeed, Streng et al. have shown that NaHS (1 mM) causes activation of human and mouse TRPA1 in heterologous expression systems, suggesting that TRPA1 is indeed a molecular target for H_2S in the bladder (Streng et al., 2008). In this context, it is interesting to note that bladder inflammation can be triggered by TRPA1 activation (Cox, 1979; McMahon and Abel, 1987) and that potential pathogens, such as *Escherichia coli*, can produce H_2S (Berglin and Carlsson, 1985). Recently, Miyamoto et al. have shown that NaHS (1 μM)-evoked increases in $[Ca^{2+}]_i$ are inhibited by removal of external Ca^{2+} and by a TRPA1-specific inhibitor, HC-030031, suggesting that H_2S stimulates sensory neurons *via* activation of TRPA1 (Miyamoto et al., 2011). Furthermore, the hyperalgesia/allodynia induced in mice by intraplantar administration of NaHS (100 pmol) is significantly suppressed by pre-administration of a TRPA1-specific blocker AP18 and by silencing TRPA1 channels in sensory neurons (Okubo et al., 2012). Thus, H_2S-induced mechanical hyperalgesia and allodynia require activation of TRPA1 channels.

In humans, gaseous CO_2 produces a pungent sensation, as noted by the Scottish philosopher Alexander Bain more than 100 years ago (Cain and Murphy, 1980). A variety of sensory structures and receptors mediate the responses to CO_2 in different organisms (Luo et al., 2009). For example, in flies, gaseous CO_2 is detected by gustatory receptors on the antenna, whereas dissolved CO_2 is detected on the proboscis: CO_2 is either aversive or attractive depending on the sensory structure activated (Suh et al., 2004; Fischler et al., 2007; Jones et al., 2007; Kwon et al., 2007). In mice, ingested CO_2 is sensed by taste receptors in the mouth (Chandrashekar et al., 2009), and blood CO_2 is detected in the brainstem by K^+ channels (Trapp et al., 2008) and in the amygdala by acid-sensing ion channel (ASIC) 1a (Ziemann et al., 2009). Atmospheric levels of CO_2 are detected by guanylyl cyclase D,

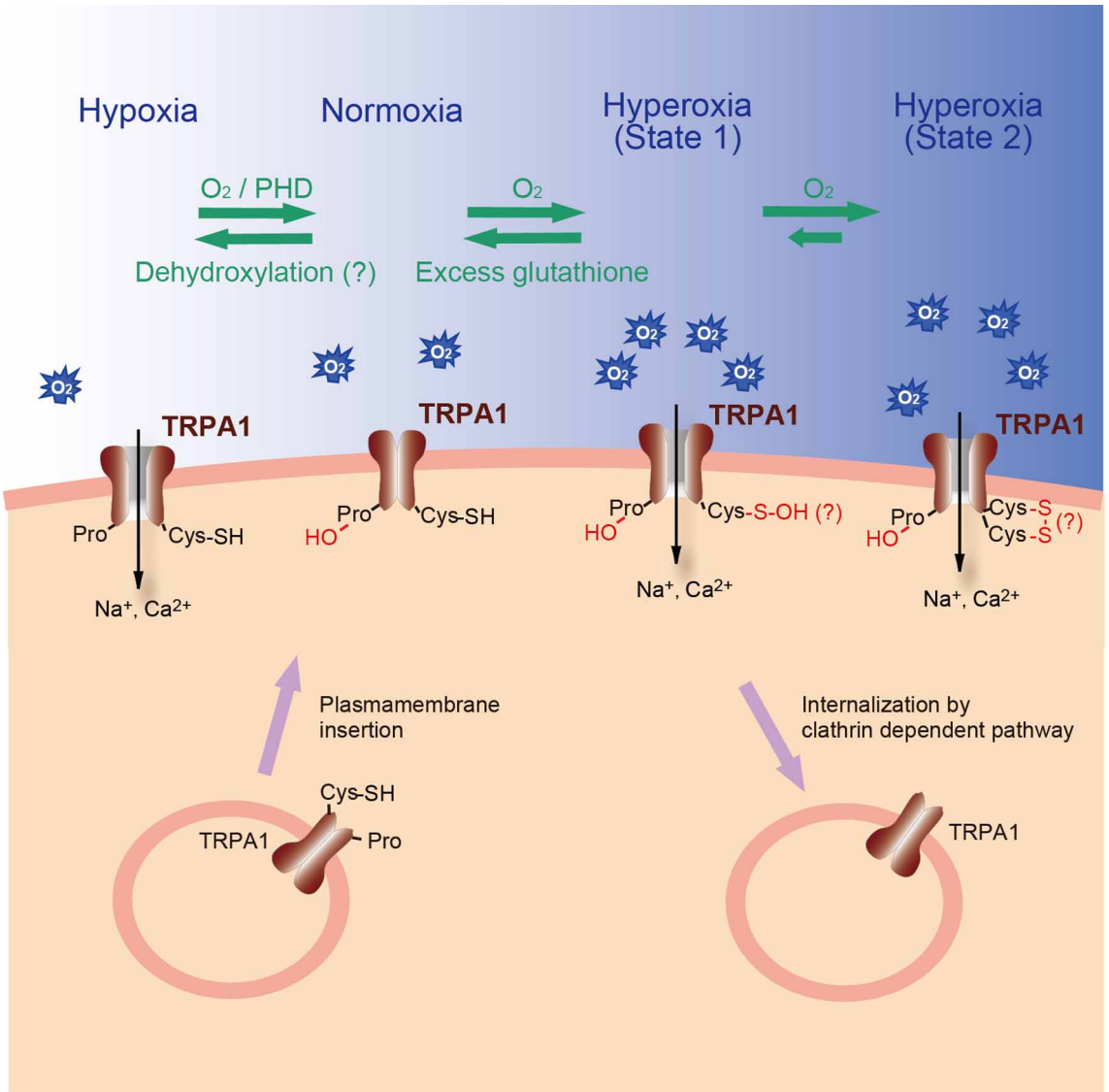

FIGURE 5 | Model for O_2-sensing of TRPA1 channel. PHDs hydroxylate specific Pro residue on the N-terminal AnkR domain of TRPA1 protein in normoxia, whereas a decrease in O_2 concentrations diminishes PHD activity and relieves TRPA1 from the hydroxylation, leading to its activation in hypoxia. The relief can be achieved by insertion of unmodified TRPA1 proteins into the plasma membrane or by dehydroxylation through an unidentified molecular mechanism. In hyperoxia, O_2 oxidizes specific Cys residues, thereby activating TRPA1. TRPA1 may at least take two oxidized state upon hyperoxia: a relatively unstable oxidized state (state 1) readily reversed by glutathione and a relatively stable oxidized state (state 2). Sulfhydryl group(s) of the key Cys residues may be modified to sulfenic acid in the former state of TRPA1, whereas that in the latter state of TRPA1 may form a disulfide bond(s). These oxidation mechanisms over-ride the inhibition by Pro hydroxylation to activate TRPA1.

which is expressed in a subset of olfactory sensory neurons in mice (Luo et al., 2009). However, this latter system is missing in humans and most other primates (Young et al., 2007). It is widely believed that the noxious sensation of CO_2 is due to activation of the trigeminal nerve fibers that innervate the nasal and oral cavities (Silver and Moulton, 1982; Steen et al., 1992). Recently, Wang et al. have shown that CO_2 specifically activates a subpopulation of trigeminal neurons expressing a functional *Trpa1* gene (Wang et al., 2010). CO_2-induced activation of TRPA1 is downstream of intracellular acidification, consistent with our observation that TRPA1 is activated by H^+ (Takahashi et al., 2008a). Thus, TRPA1 makes an important contribution to nociceptive responses to CO_2.

CONCLUSIONS

TRP channels respond to multiple activation triggers and therefore serve as polymodal signal detectors. An important aspect of this multimodal activation of TRP channels is their role in signal integration and amplification. When a TRP channel is activated by downstream or upstream constituents (molecules/proteins/enzymes in a specific signaling cascade), in addition to the primary activation trigger immediately upstream,

the TRP channel plays an important role in forming positive feedback or feed-forward loops in the cascade. For example, in vascular endothelial cells, Ca^{2+} influx *via* NO-activated TRPC5 channels can amplify production of NO by eNOS, resulting in the enhancement of NO production in nearby endothelial cells and NO-dependent relaxation of smooth muscle cells. In smooth muscle cells, NO exerts inhibitory effects on TRPC6 channel activity through PKG-mediated phosphorylation. Together, these mechanisms are capable of ensuring the fidelity of cellular responses and minimizing variation in their magnitude, synchronizing the responses of neighboring cells that comprise functional domains within vascular tissues to reduce blood pressure and maintain local blood flow.

The accumulating evidence summarized above strongly suggests that TRPV1 and TRPA1 are sensors that transduce gaseous signals into electrical signals in sensory and vagal neurons. The chemosensory inputs to these neurons are propagated toward the central nervous system to induce pain sensation or to change ventilatory patterns. However, the roles of Ca^{2+} influx *via* TRPV1 and TRPA1 in controlling Ca^{2+} signaling pathways in neurons remain elusive. Considering that the PHD-HIF pathway is central to chronic hypoxia responses that increase red blood cell mass and stimulate new blood vessel growth (Semenza and Wang, 1992; Webb et al., 2009), it would be interesting to examine the effect of Ca^{2+} influx through O_2-sensitive TRPA1 channels on the functional regulation of PHD and HIF. Studies of TRP channels have been extended dramatically from the simple functional description of single molecules to the holistic analysis and integration of the molecular systems controlled by TRP channels.

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Gasotransmitters: novel regulators of epithelial Na^+ transport?

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The vectorial transport of Na^+ across epithelia is crucial for the maintenance of Na^+ and water homeostasis in organs such as the kidneys, lung, or intestine. Dysregulated Na^+ transport processes are associated with various human diseases such as hypertension, the salt-wasting syndrome pseudohypoaldosteronism type 1, pulmonary edema, cystic fibrosis, or intestinal disorders, which indicate that a precise regulation of epithelial Na^+ transport is essential. Novel regulatory signaling molecules are gasotransmitters. There are currently three known gasotransmitters: nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S). These molecules are endogenously produced in mammalian cells by specific enzymes and have been shown to regulate various physiological processes. There is a growing body of evidence which indicates that gasotransmitters may also regulate Na^+ transport across epithelia. This review will summarize the available data concerning NO, CO, and H_2S dependent regulation of epithelial Na^+ transport processes and will discuss whether or not these mediators can be considered as true physiological regulators of epithelial Na^+ transport biology.

Keywords: Na^+ absorption, electrolyte transport, NO, CO, H_2S, ENaC, transporter, Na^+/K^+-ATPase

EPITHELIAL Na^+ TRANSPORT

The vectorial transport of Na^+ across epithelia of various organs is a crucial event in the maintenance of general salt and water homeostasis in vertebrates. The molecular nature of transepithelial Na^+ transport has been revealed in pioneer studies by Koefoed-Johnsen and Ussing (1958): Na^+ ions enter epithelial cells at the apical membrane of the epithelial cells. The Na^+ uptake mechanism can be Na^+ selective ion channels, such as the amiloride-sensitive epithelial Na^+ channel (ENaC), or Na^+ coupled transporters (**Figure 1**). The chemical driving force for the apical influx of Na^+ is created by the Na^+/K^+-ATPase at the basolateral side of the epithelial cells. This enzyme actively pumps three Na^+ ions out of the cell – in exchange for two K^+ ions. The K^+ ions are recycled and leave the cell via K^+ channels at the basolateral membrane. Furthermore, the K^+-conductance keeps the membrane potential of the epithelial cells negative. Thus, both the Na^+/K^+-ATPase as well as K^+ channels deliver the electrochemical driving force for transepithelial Na^+ transport (**Figure 1**).

Due to the concerted action of Na^+ channels/transporters and the Na^+/K^+-ATPase, there is a net movement of Na^+ ions from the apical to the basolateral side of the epithelium. This results in an osmotic gradient across the epithelium, which eventually drives the transepithelial absorption of water (**Figure 1**). This mechanism is a basic principle for the physiology of various organs: (i) in the kidneys, transepithelial Na^+ and water transport drives the fluid reabsorption from the primary urine and indirectly regulates blood volume and hence blood pressure (Bhalla and Hallows, 2008); (ii) in the lungs, Na^+ and water transport across the pulmonary epithelium regulates the volume and viscosity of the airway lining fluid (Hollenhorst et al., 2011) and keeps alveoli free of fluid (Olver et al., 1986; Hummler et al., 1996; Althaus et al., 2011); (iii) in the intestine, Na^+ transport facilitates uptake of electrolytes and nutrients (e.g., amino acids) as well as water absorption from the chime.

The importance of Na^+ transport processes becomes also evident when one considers pathological conditions which are associated with dysregulated Na^+ transport. (i) In the kidneys, a hereditary form of hypertension, Liddle syndrome, is reasoned by hyperabsorption of Na^+ from the primary urine due to a mutation which leads to an increased number of ENaCs in the plasma membrane (Shimkets et al., 1994; Schild et al., 1995). Conversely, mutations which lead to ENaC hypoactivity cause the salt-wasting syndrome pseudohypoaldosteronism type 1 (Chang et al., 1996). (ii) In the lung, impaired reabsorption of Na^+ and water across pulmonary epithelia can lead to pulmonary edema (Hummler et al., 1996; Althaus et al., 2011), whereas increased Na^+ transport can contribute to cystic fibrosis (CF)-like lung disease (Mall et al., 2004; Azad et al., 2009; Rauh et al., 2010). (iii) In the intestine, an inhibition of Na^+ absorption, e.g., by viral infections, induces diarrhea (Ousingsawat et al., 2011).

These physiological and pathophysiological examples demonstrate that a precise regulation of transepithelial Na^+ transport is crucial for the function of multiple organs. A novel class of regulatory signaling molecules is the class of gasotransmitters (Wang, 2002). Gasotransmitters are endogenously produced as a product of amino acid metabolism and regulate a variety of cell and organ functions. There are currently three molecules which are regarded as gasotransmitters: nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S; Wang, 2002). Recently, there is a growing body of evidence that these molecules may serve as regulators

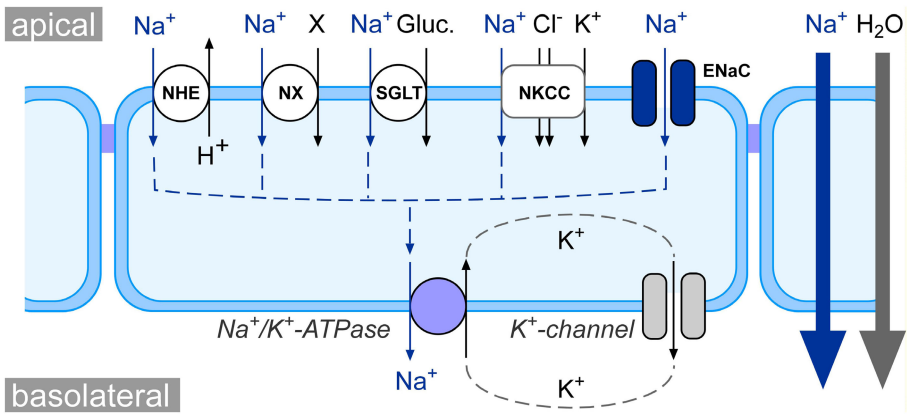

FIGURE 1 | Schematic model of epithelial Na^+ transport physiology. At the apical side of epithelia, Na^+ enters the cell either via Na^+ coupled transporters, such as the Na^+/H^+ exchanger (NHE), Na^+-coupled amino acid or phosphate symporters (NX), Na^+/glucose symporters (SGLT), and $Na^+/K^+/2Cl^-$ cotransporters (NKCC), or via Na^+ selective ion channels such as the epithelial Na^+ channel (ENaC). The electrochemical gradient for apical Na^+ uptake is generated at the basolateral membrane via the activity of the Na^+/K^+-ATPase as well as K^+ channels. The net effect of the concerted interplay between channels/transporters and the Na^+/K^+-ATPase is a movement of Na^+ from the apical to the basolateral side of the epithelium (blue arrows). This creates osmotic forces which drive the absorption of water from the apical to the basolateral compartment (gray arrow).

of epithelial Na^+ transport processes. The following sections will describe the three known gasotransmitters and review their impact on Na^+ transport processes and Na^+ transporting molecules in renal, pulmonary, and intestinal epithelia.

NITRIC OXIDE (NO)

Nitric oxide was the first gasotransmitter that was recognized as a physiological signaling mediator. In 1987, NO was identified to be the "*endothelial derived relaxing factor*" inducing the relaxation of vascular smooth muscle cells (Ignarro et al., 1987). NO is endogenously produced from the amino acid L-arginine by nitric oxide synthases (NOS). There are three NOS isoforms, NOS1 (neuronal NOS), NOS2 (inducible NOS), and NOS3 (endothelial NOS; Garvin et al., 2011). Apart from a classical role as a regulator of smooth muscle tone, NO has also been demonstrated to play an important role in the regulation of transepithelial Na^+ transport.

EFFECTS OF NO ON RENAL EPITHELIAL Na^+ TRANSPORT

NO reduces Na^+ absorption in the kidneys

In the kidneys, NO is generally regarded to facilitate natriuresis and diuresis, which is evident from animal studies using NO-donating molecules. The NO-donor *S*-nitroso-acetylpenicillamine (SNAP) increased urinary Na^+ excretion in anesthetized dogs (Majid et al., 1998). Similarly, the infusion of the NO-donor molecule sodium nitroprusside (SNP) increased natriuresis and diuresis in conscious rats (Grandes et al., 1991). By contrast, the intrarenal arterial infusion of the NO-donor molecule NOC-7 did not affect baseline urinary Na^+ excretion in anesthetized rabbits (Adachi et al., 1997). However, angiotensin 2- and norepinephrine-induced reduction in urinary Na^+ excretion was blocked by NOC-7 in this model (Adachi et al., 1997). These data suggest that NO may block angiotensin 2- or norepinephrine-activated renal transepithelial Na^+ absorption in rabbits. These findings are confirmed in studies with anesthetized rats, where SNP decreased angiotensin 2-stimulated proximal tubular fluid absorption (Eitle et al., 1998). By contrast to the study by Adachi et al. (1997), SNP also decreased basal tubular fluid absorption (Eitle et al., 1998). This discrepancy might be explained by different employed concentrations of SNP (Eitle et al., 1998). These studies indicate that exogenously applied NO-donating drugs increase urinary Na^+ excretion – which is likely the result of a decreased transepithelial Na^+ transport.

Consistent with studies investigating effects of exogenously applied NO, inhibitors of NO synthesis, such as nitro-L-arginine methyl ester (L-NAME), were demonstrated to exert anti-natriuretic and anti-diuretic effects (Lahera et al., 1991, 1993; Ortiz and Garvin, 2002). Furthermore, NOS3 *knock-out* mice had a reduced Na^+ excretion and urinary volume after acute volume load (Perez-Rojas et al., 2010). The treatment of anesthetized rats with cholesterol also blocked urinary Na^+ excretion (Kopkan et al., 2009). There was no such effect in the presence of the NOS inhibitor L-NAME, which suggests that cholesterol prevents NO-mediated natriuresis (Kopkan et al., 2009). Those studies imply that there is a basal tone of NO, which keeps transepithelial Na^+ transport and consequently Na^+ retention in the kidneys low.

NO-mediated regulation of Na^+ transporting molecules along nephrons

Several studies investigated the regulatory impact of NO on ion transporting molecules across nephronic epithelial cells. The focus of the following paragraphs will be on epithelial Na^+ transporting molecules. For effects of NO on other renal ion channels and transporters, the reader is referred to an excellent review by Ortiz and Garvin (2002).

The bulk of the fluid and electrolytes which are filtered at the glomeruli are reabsorbed from the primary urine along the proximal tubule. The reabsorption of Na^+ occurs primarily by Na^+ coupled cotransporters which are located in the apical membrane of the epithelium. The gradient for Na^+ uptake is created by the basolaterally located Na^+/K^+-ATPase. The NO donors SNAP and SNP inhibited the Na^+/H^+-exchanger (NHE) in rabbit

proximal tubules and Caco-2 cells (Roczniak and Burns, 1996; Gill et al., 2002). Furthermore, NO donors also inhibited the Na^+/K^+-ATPase in an opossum proximal tubule cell line (Liang and Knox, 1999). Consistent with this observation, Linas and Repine (1999) demonstrated that endothelial cells can regulate Na^+ transport by proximal tubule epithelial cells via NO synthesis and consequently inhibition of the Na^+/K^+-ATPase. Taken together, NO is thus likely an inhibitor of Na^+ reabsorption in the proximal tubule, which is due to inhibition of NHE as well as the Na^+/K^+-ATPase.

In the thick-ascending limb, the uptake of Na^+ from the primary urine occurs via apical Na^+ transporters, such as the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and NHE, as well as the basolateral Na^+/K^+-ATPase. The NO donors spermine NONOate and nitroglycerin both decreased NHE activity in isolated and perfused rat thick ascending limbs (Garvin and Hong, 1999). Although Spermine NONOate decreased NKCC activity in the same model, the Na^+/K^+-ATPase was not affected (Ortiz et al., 2001). These data indicate that in the thick ascending limb, NO rather interferes with apical transport systems than the basolateral Na^+/K^+-ATPase. The source of NO in thick ascending limbs is likely a production by NOS3, since L-arginine, the substrate for NO production, inhibits thick ascending limb Na^+ transport in NOS1 and NOS2, but not in NOS3 *knock-out* mice (Plato et al., 2000).

Beside the thick ascending limb, there is also Na^+ transport across the thin ascending limb or the descending limb. A putative effect of NO on Na^+ transport activities in these segments, however, has not been investigated so far (Garvin et al., 2011).

In the cortical-collecting duct (CCD), Na^+ is taken up by the epithelium via the concerted action of apically located ENaCs and the basolateral Na^+/K^+-ATPase. Spermine NONOate and nitroglycerin decreased ENaC activity in rat CCD (Stoos et al., 1995). This effect was also apparent for endothelium-derived NO, which decreased ENaC activity (Stoos et al., 1994, 1995). By contrast, Lu et al. (1997) observed an increase in ENaC activity due to NO. This was likely an indirect effect via activation of basolateral K^+ channels (Lu et al., 1997). Thus, whether NO elicits a net-inhibition or activation of ENaC in the CCD remains controversial. Interestingly, there was no effect of NO donors on Na^+/K^+-ATPase activity in the CCD (Stoos et al., 1994, 1995). The reason why NO inhibits the Na^+/K^+-ATPase in the proximal tubule but not in the CCD is unknown. However, this discrepancy might be explained by differences in the oxidative state of epithelial cells, which may counter effects of NO on Na^+ transport (Yu et al., 2007; Helms et al., 2008).

Taken together, the currently available data speak in favor of a NO-mediated inhibition of Na^+ transporting molecules in renal epithelia.

NO-induced signaling events in renal epithelia

The classical mechanism how NO exerts biological effects is the stimulation of soluble guanylate cyclase (sGC). This results in increased cyclic guanosine monophosphate (cGMP) production and downstream effects on protein kinases such as protein kinase G. NO-mediated activation of the sGC/cGMP pathway has been demonstrated to be involved in NO regulation of NHE3 (Roczniak and Burns, 1996; Gill et al., 2002) and the Na^+/K^+-ATPase (Liang and Knox, 1999; Linas and Repine, 1999) in the proximal tubule. These studies showed that NO-induced production of cGMP is crucial for the regulation of Na^+ transport in the proximal tubule. Interestingly, Sasaki et al. (2004) demonstrated that cGMP is produced upon administration of SNAP in proximal tubule cells in humans; however, the resulting decrease in Na^+ uptake by the epithelial cells was blocked by probenecid. This finding suggests that export of cGMP by a probenecid-sensitive organic anion transporter is necessary for the regulation of Na^+ transport (Sasaki et al., 2004). This may be the result of a downstream activation of protein kinase G or Src tyrosine kinase signaling pathways by extracellular cGMP (Jin et al., 2004; Nascimento et al., 2011). The activation of the Src signaling complex is associated with endocytosis of the Na^+/K^+-ATPase as well as the NHE3 in renal cells (Liu et al., 2004, 2005; Oweis et al., 2006; Cai et al., 2008; Nascimento et al., 2011). This would provide a molecular link between NO-mediated cGMP production, cGMP export, and downstream inhibition of Na^+ transport in the proximal tubule.

The general inhibition of Na^+ absorption by NO in the thick-ascending limb is also likely due to cGMP mediated signaling (Ortiz and Garvin, 2001), however, if cGMP and downstream activated mediators specifically decrease NKCC or NHE activity in this segment, remains to be proven.

Aside from the activation of the sGC/cGMP pathway, there are alternative signaling mechanisms that might explain effects of NO on Na^+ transporting molecules. For example, higher oxidative derivates of NO, such as NO_2 or N_2O_3, can interact with cysteine-residues of proteins – a mechanism which is termed S-nitrosylation (Hess et al., 2005). This direct protein-modification can regulate the activity of ion channels and transporters, as for example shown for purified Na^+/K^+-ATPase (Sato et al., 1995). The distinct contribution of S-nitrosylation of Na^+ transporting molecules to NO-mediated interference with general Na^+ transport in renal epithelia, however, is not sufficiently investigated and needs to be elucidated.

Renal sources of NO

An important question which remains is the precise source for NO in the kidneys. Although inhibitors of NOS show obvious effects on renal Na^+ transport (see Section "NO Reduces Na^+ Absorption in the Kidneys"), it is hard to conclude from these studies what is the cellular origin of NO. Furthermore, it is noteworthy that in studies with systemic administration of NOS inhibitors or with *knock-out* mice, a contribution of other physiological parameters, i.e., indirect impact of blood pressure on glomerular filtration and renal function, is difficult to exclude.

Nevertheless, it has been demonstrated that all three NOS isoforms are expressed in nephronic epithelia, which indicates that the epithelial cells are generally capable of producing NO (Cabral and Garvin, 2011). This may imply that NO might be an autocrine regulator of Na^+ transport in renal epithelia.

There is also a cross-talk between the epithelium and other cells, such as endothelial cells or neuronal cells. A study by Linas and Repine (1999) showed that endothelial cells can inhibit the Na^+/K^+-ATPase and Na^+ transport via NO release when co-cultured with proximal tubule epithelial cells. As mentioned earlier, endothelium-derived NO can also regulate ENaC activity in

the CCD (Stoos et al., 1994, 1995). Furthermore, there is evidence that nervous stimulation can regulate Na^+ transport in the kidney. Denervation of the kidney, for example, can abolish the anti-natriuretic effects of L-NAME (Wu et al., 1999; Ortiz and Garvin, 2002). Thus a role for NO as a neurotransmitter with secondary effects on Na^+ transport is also likely.

Taken together, although there are still controversies in the sources of NO, the precise signaling mechanisms as well as targets of action of NO, there is a convincing body of evidence that NO impairs Na^+ transporting molecules in nephronic epithelia and consequently leads to renal natriuresis and diuresis.

EFFECTS OF NO ON PULMONARY EPITHELIAL Na^+ TRANSPORT

General effects of NO on pulmonary Na^+ transport

Consistent with the data from renal epithelia, NO is regarded to inhibit Na^+ transport across the pulmonary epithelium. There are several studies which investigated the effects of NO on lung fluid clearance (which is driven by transepithelial Na^+ transport) in whole-lung models. A study by Nielsen et al. (2000) demonstrated that DETANONOate, a NO-donor, decreased amiloride-sensitive fluid clearance in rabbits, which may indicate a decrease in ENaC-mediated transepithelial Na^+ transport by NO. Further studies investigated the involvement of NO in lung disease-associated impairment of transepithelial Na^+ transport: The instillation of endotoxin, for example, increased lung NO and cGMP levels and inhibited alveolar fluid clearance in rats (Tsubochi et al., 2003). Furthermore, Pittet et al. (2001) demonstrated an increase in NOS2 after hemorrhagic shock in rats, which was associated with a decrease in alveolar fluid clearance. Similarly, hydrostatic pressure increased NO production and consequently decreased fluid reabsorption in isolated rat and mouse lungs (Kaestle et al., 2007).

Aside from the exogenous administration of NO by NO-liberating molecules, these studies imply that endogenous NO production by NOS also leads to a decrease in pulmonary epithelial Na^+ transport (Pittet et al., 2001; Tsubochi et al., 2003; Kaestle et al., 2007). Consistent with these studies, inhibition of NOS2 decreased NO production and increased amiloride-sensitive Na^+ currents in a human airway epithelial cell line (H441; Song et al., 2009). These data generally suggest an inhibitory effect of NO on pulmonary epithelial Na^+ transport. By contrast, NOS2 *knock-out* mice exhibited a decreased amiloride-sensitive alveolar fluid clearance (Hardiman et al., 2001), which is likely due to a reduced protein expression of the α and γ subunits of ENaC (Hardiman et al., 2004). Although indirect effects of a long-term NOS inhibition on Na^+ transport cannot be excluded (see section about renal physiology), it might be hypothesized that there are different physiological reactions due to acute or long-term changes in pulmonary NO content.

Thus there are various studies available which demonstrate an inhibition of fluid clearance by NO and consequently suggest an impairment of Na^+ transport across pulmonary epithelia – especially in the distal lung regions.

Aside from the distal lung epithelia, Na^+ transport plays also an important role in the regulation of airway surface liquid and thus airway physiology (Hollenhorst et al., 2011). The administration of NOS2 inhibitors increased amiloride-sensitive nasal potential difference in mice (Kelley and Drumm, 1998; Elmer et al., 1999), indicating that endogenously produced NO keeps Na^+ transport across nasal epithelia low. By contrast, Rückes-Nilges et al. (2000) did not detect effects of the NO donors SNP and spermine NONOate on Na^+ transport by cultured primary human nasal epithelial cells. The reason for this discrepancy is unknown.

A hyperabsorption of Na^+ across the airway epithelia can promote CF-like lung disease (Mall et al., 2004). Interestingly, NOS2 expression is lower in CF compared to non-CF airway epithelia (Kelley and Drumm, 1998; Moeller et al., 2006). Furthermore, the NO concentration in exhaled air is inversely correlated with transepithelial potential difference in patients with CF (Texereau et al., 2005). This might suggest a regulatory mechanism for Na^+ hyperabsorption across CF epithelia due to lack of tonic inhibition by endogenously produced NO.

Effects of NO on Na^+ transporting molecules in pulmonary epithelia

Consistent with the effects of NO on whole-lung Na^+ and fluid clearance, the application of NO-donating molecules decreased amiloride-sensitive Na^+ absorption by cultured rat alveolar type 2 cells (Guo et al., 1998) as well as the human airway epithelial cell line H441 (Althaus et al., 2010). In both studies, a cGMP-independent inhibition of ENaC as well as the Na^+/K^+-ATPase was demonstrated (Guo et al., 1998; Althaus et al., 2010). By contrast, a patch-clamp study on rat alveolar type 2 cells described a cGMP-dependent inhibition of Na^+ channel activity due to NO (Jain et al., 1998). The discrepancies between the studies by Jain et al. (1998) and Guo et al. (1998) might be explained by different culture conditions which were demonstrated to affect the phenotype of Na^+ channels in alveolar epithelial cells (Jain et al., 2001). Especially the supplement or non-supplement of dexamethasone, in order to induce Na^+ transport, or the culture at air/liquid interface, has large effects on transepithelial Na^+ transport properties (Althaus et al., 2010). The inhibitory effect of NO on Na^+ channel activity was also confirmed in patch-clamp studies on lung slices from rat, although this effect was solely apparent on type 2, but not on type 1 alveolar epithelial cells (Helms et al., 2008). The authors explained this observation by higher levels of reactive oxygen species, such as superoxide anions (O_2^-), in type 1 cells. O_2^- might interact with NO and thereby counter inhibitory effects of NO on type 1 cells (Helms et al., 2008).

Nevertheless, although NO-mediated signaling mechanisms vary, the described studies demonstrate a general inhibition of Na^+ channels by exogenous NO administration in pulmonary epithelia. By contrast to studies on renal epithelia, potential effects of NO on Na^+-coupled transporters in lung epithelia is, to the author's knowledge, currently unknown.

Pulmonary sources of NO

Direct evidence for endogenous NO synthesis in lungs is the fact that NO can be detected in the exhaled air of humans (Khatri et al., 2001; Texereau et al., 2005). However, the major cellular source of this exhaled NO has not been identified to date.

The expression of NOS has been shown in various pulmonary epithelial cells (Kelley and Drumm, 1998; Ermert et al., 2002; Moeller et al., 2006; Song et al., 2009). In addition, alveolar macrophages express all three NOS isoforms (Ermert et al., 2002). Furthermore, LPS-activated macrophages reduced

epithelial amiloride-sensitive Na^+ absorption when co-cultured with fetal rat distal lung epithelial cells (Ding et al., 1998). Endothelial cells also contain NOS1 (Ermert et al., 2002; Lührs et al., 2002), NOS3 (Ermert et al., 2002), and can express NOS2 upon stimulation by exposure to endotoxin (Ermert et al., 2002). These data demonstrate that NO might not only serve as an autocrine regulator of Na^+ transport in epithelial cells, but may also originate from other cells and tissues, such as macrophages or the pulmonary vasculature.

Taken together, NO is endogenously produced in lung tissues. Although NO-mediated signaling mechanisms vary, the available data suggest an inhibition of Na^+ transport across pulmonary epithelia due to impaired activity of Na^+ channels and the Na^+/K^+-ATPase.

EFFECTS OF NO ON INTESTINAL EPITHELIAL Na^+ TRANSPORT

In the intestine, transepithelial Na^+ transport drives the absorption of organic substances as well as the absorption of water from the chime. The absorption of amino acids, glucose, or phosphate occurs mainly in the duodenum and jejunum by Na^+-coupled symporters, whereas in the ileum, Na^+ is taken up by the epithelial cells via NHEs. Na^+ absorption in the colon occurs via Na^+ selective ion channels (such as ENaC) in most-species in an aldosterone-dependent way.

There are various studies on the effects of NO on electrolyte and water transport across intestinal epithelia and both pro-secretory as well as pro-absorptive actions of NO have been reported (Izzo et al., 1998). However, under physiological conditions, the available studies speak in favor of a NO-dependent pro-absorptive intestinal tone (Izzo et al., 1998). At this point the reader is referred to the excellent review by Izzo et al. (1998).

Although basic effects of NO on electrolyte and fluid transport in intestinal epithelia have been intensively investigated, there are surprisingly few studies on the regulation of distinct Na^+ transporting ion channels and transporters by NO.

Consistent with a pro-absorptive role of NO, treatment of rat small intestinal epithelial cells (IEC-18) with L-NAME decreased the activity of the Na^+ glucose cotransporter SGLT1 (Coon et al., 2008), suggesting that endogenous NO would simulate SGLT1.

By contrast, the treatment of IEC-18 cells with L-NAME stimulated NHE3 by enhanced protein expression (Coon et al., 2008). The stimulation of NHE3 by L-NAME was also observed on rabbit ileum (Coon et al., 2007). These data suggest that constitutively produced NO inhibits NHE3 in the small intestine. Consistent with these studies, the exposure of Caco-2 cells, a human colon adenocarcinoma cell line, to NO-donating molecules inhibited NHE3 activity via cGMP-dependent mechanisms (Gill et al., 2002).

Furthermore, an increase in Na^+/K^+-ATPase activity was observed after treatment of IEC-18 cells with L-NAME (Coon et al., 2008), which would suggest an inhibitory action of NO on this enzyme. Furthermore, the NO-donor SNAP decreased Na^+/K^+-ATPase activity in rat IEC-6 cells (Suzuki et al., 2005).

Those data indicate that, depending on the intestinal segment and distinct Na^+ transporting molecules, NO might have net pro- or anti-absorptive effects on electrolyte and Na^+ transport across intestinal epithelia. Furthermore it is important to point out that the described studies on Na^+ transporting molecules investigated effects of NO on epithelial cells – independently of neuronal regulation. A neuronal input – using NO as a transmitter – might superimpose local effects on Na^+ transporting molecules and might also account for different net-effects that are attributed to NO.

NO REGULATION OF Na^+ TRANSPORT: CONCLUSIONS

There is a convincing body of evidence that speaks in favor of an inhibitory role for NO in the regulation of Na^+ transport in renal and pulmonary epithelia. NO is endogenously produced in the kidneys or lungs, affects Na^+ transporting molecules via cellular signaling cascades and eventually regulates organ physiology, such as natriuresis/diuresis or Na^+-driven alveolar fluid clearance.

By contrast, it is difficult to make a decisive conclusion about a pro- or anti-absorptive role for NO in intestinal epithelia. NO-mediated effects in the intestine seem to be dependent on differences between intestinal segments, species, as well as concentrations of NO (Izzo et al., 1998). Furthermore, NO-mediated signaling mechanisms vary, dependent on the amount of NO, and consequently cGMP, or the availability of other oxidative species and the formation (or quenching) of higher oxidative derivates of NO. The heterogeneity of NO effects in the intestine might therefore not necessarily be contradictory, especially given the fact that the inner milieu of the intestine is more variable (depending on the nutritional status or intestinal flora) when compared to kidneys or lungs.

CARBON MONOXIDE (CO)

Although generally regarded as a highly toxic gas, carbon monoxide (CO) has been recognized as a physiological cellular signaling mediator. CO is endogenously produced in the human body as a product of heme metabolism and is generated by heme-degrading enzymes referred to as heme oxygenases (Ryter et al., 2006). There are two distinct forms of heme oxygenases: heme oxygenase 1 (Hmox1) an inducible form, and constitutively expressed heme oxygenase 2 (Hmox2; Maines et al., 1986). Originally, CO has been considered simply as a by-product of heme degradation by heme oxygenases, however, in the past decade, evidence accumulated to suggest that CO produced by heme oxygenases serves as a regulated cellular signaling molecule. This has been demonstrated in multiple studies addressing the cellular effects of exogenous administration of physiologically relevant doses of CO. By this means, it was demonstrated that CO has potent cyto- and tissue-protective properties due to its implication in anti-apoptotic, anti-inflammatory, and anti-proliferative signaling mechanisms (Ryter et al., 2006; Motterlini and Otterbein, 2010). In addition, there is recently a growing body of evidence that CO might be a regulator of ion channels/transporters and consequently epithelial ion transport processes.

EFFECTS OF CO ON RENAL EPITHELIAL Na^+ TRANSPORT

Both heme oxygenase isoforms are expressed in the kidney (Csongradi et al., 2012) and endogenous production of CO has been demonstrated in the kidney (Jackson et al., 2011).

Similar to NO, CO is also an activator of sGC, although with less potency (Ma et al., 2007). Given that NO regulates Na^+ transport mainly by sGC/cGMP mediated signaling mechanisms, it is

likely that CO also affects Na^+ transporting molecules in epithelia. The first hint toward this hypothesis was provided by Nathanson et al. (1995). These authors demonstrated a CO-induced activation of neuronal Na^+/K^+-ATPase by cGMP-dependent mechanisms (Nathanson et al., 1995). An activation of the Na^+/K^+-ATPase by CO in epithelia would consequently enhance transepithelial Na^+ transport. Consistent with this idea, the inhibition of heme oxygenases by chromium mesoporphyrin (CrMP) and thus endogenous CO production, decreased the absorption of Na^+ in the loop of Henle of microperfused rat kidney tubules (Wang et al., 2003). Furthermore, CO was shown to activate ENaC in a M1 mouse kidney CCD cell line (Wang et al., 2009).

By contrast, chronic induction of heme oxygenases by cobalt-protoporphyrin resulted in increased urinary Na^+ excretion in cirrhotic rats (Di Pascoli et al., 2011), which would suggest an inhibition of renal epithelial Na^+ absorption by CO. Another study has shown that the inhibition of heme oxygenase, and CO production with CrMP leads to a decreased urinary Na^+ excretion in rats (Jackson et al., 2011), which is likely due to enhanced Na^+ absorption. Those studies would speak in favor of an inhibitory effect of CO on transepithelial Na^+ absorption in the kidney.

Interestingly, the studies by Wang et al. (2003) and Jackson et al. (2011) describe oppositional effects of heme oxygenase inhibition on urinary Na^+ excretion – despite using the same species, the same inhibitor and without observing effects on glomerular filtration rate. It may be speculated that there are differences between systemic administration of inhibitors (Wang et al., 2003) versus microperfusion studies (Jackson et al., 2011), which would indicate spatiotemporal differences in CO-mediated signaling events. Based on the available studies, whether CO is an activator or inhibitor of transepithelial Na^+ absorption in the kidney is yet to be determined.

EFFECTS OF CO ON PULMONARY EPITHELIAL Na^+ TRANSPORT

Concerning a putative role of CO on pulmonary Na^+ transport, we have previously investigated the effects of CO administration in isolated, ventilated, and perfused rabbit lungs (Althaus et al., 2009). In this model, CO, applied as a gas or by the donor molecule CO-releasing molecule 3 (CORM-3), led to a decreased amiloride-sensitive transepithelial Na^+ transport as measured by radioactive $^{22}Na^+$ clearance and resulted in an increased alveolar lining fluid volume. Thus, CO impaired Na^+ transport as well as fluid clearance in rabbit lungs. This effect has been attributed to impaired amiloride-sensitive Na^+ transport across pulmonary epithelial cells, as indicated by transepithelial Ussing chamber studies using cultured H441 monolayers as well as primary isolated alveolar type 2 cells from the rat. In contrast to NO which affected both the activity of Na^+ channels and the Na^+/K^+-ATPase (Althaus et al., 2010), CO did not impair Na^+/K^+-ATPase activity, but did inhibit amiloride-sensitive ENaC (Althaus et al., 2009). This finding is also in contrast to the study by Wang et al. (2009), who observed an increased activity of ENaC due to CO. This discrepancy is interesting, especially since both in M1 cells, and H441 cells, the major Na^+ conductance is an ENaC-typical ∼5–10 pS channel (Althaus et al., 2009; Wang et al., 2009). The effect of CO on ENaC in H441 cells likely involves histidine residues since CO-effects can be mimicked and abolished by the histidine-modifying agent diethyl pyrocarbonate (Althaus et al., 2009). As pointed out by Wilkinson and Kemp (2011), there are differences in the histidine contents of human and mouse ENaCs. Those contradictory findings might therefore be explained by taxonomical differences. Furthermore it is important to point out that it is difficult to compare findings from excised patch-clamp experiments (Wang et al., 2009) with intact epithelial monolayers or isolated organ studies (Althaus et al., 2009), as additional regulatory CO-sensitive mediators might be present in intact cells.

These studies indicate that exogenously applied CO affects Na^+ transport in the pulmonary epithelium. However, if CO is endogenously produced in the lung and affects Na^+ transport is an important question that needs to be addressed. Constitutively expressed Hmox2 is expressed at low levels in lung epithelial cells (Roth et al., 2009). Furthermore, alveolar macrophages express both heme oxygenase isoforms (Maestrelli et al., 2001). In addition, pulmonary endothelial cells express constitutive Hmox2 (Roth et al., 2009). Thus, several cell types in the lung contain the enzyme system required for the generation of CO. Similar to NO, CO can also be detected in the exhaled air of humans (Khatri et al., 2001). Thus, CO is indeed endogenously produced in the lung. However, a putative link between CO production and epithelial Na^+ transport needs to be demonstrated.

EFFECTS OF CO ON INTESTINAL EPITHELIAL Na^+ TRANSPORT

In the intestine, CO might also serve as a regulator of epithelial ion transport processes since the expression of heme oxygenases has been demonstrated and the administration of CO can activate a Cl^- secretion across colonic epithelia (Pouokam et al., 2011; Steidle and Diener, 2011). However, whether or not CO also influences Na^+ absorption in the intestine, as well as other epithelia, is currently unknown.

CO REGULATION OF Na^+ TRANSPORT: CONCLUSIONS

It was demonstrated that CO is able to affect Na^+ transport processes across various epithelia. In the kidneys, both pro- and anti- Na^+-absorptive effects of CO have been described. In the lung, exogenous administration of CO decreased Na^+ transport and fluid clearance, however, a regulatory contribution of endogenously synthesized CO remains to be demonstrated. Thus, there is promising data available which indicate that CO might be a putative novel regulator of epithelial Na^+ transport physiology. However, there are important questions remaining: How is endogenous CO-production regulated? What are the stimulators of CO production? How do concentrations of CO employed in experimental studies reflect endogenously produced levels of CO? These questions need to be answered before a decisive conclusion that CO is a physiological regulator of epithelial Na^+ transport physiology can be drawn.

HYDROGEN SULFIDE (H_2S)

Hydrogen sulfide (H_2S) is a well known environmental threat with the typical odor of rotten eggs. However, similar to NO and CO, H_2S has been suggested to represent the third biologically active gasotransmitter (Wang, 2002). H_2S is endogenously produced by mammalian cells from the amino acid L-cysteine by mainly two enzymes: cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE; Stipanuk and Beck, 1982; Wang, 2002). CSE *knock-out*

mice have reduced H_2S levels in serum, aorta, and heart and increased systolic blood pressure (Yang et al., 2008), which convincingly showed for the first time a physiological role for H_2S. Although H_2S has been shown to interact with various ion channels (Tang et al., 2010), putative effects of H_2S on Na^+ transporting molecules are hardly investigated.

EFFECTS OF H_2S ON RENAL EPITHELIAL Na^+ TRANSPORT

Despite the fact that H_2S is produced by CBS/CSE in the kidney (Xu et al., 2009; Aminzadeh and Vaziri, 2012), up to today there are no studies on the impact of H_2S on Na^+ transport processes across renal epithelia. However, recently it has been shown that H_2S can activate AMP-activated protein kinase (AMPK) in rat glomerular epithelial cells (Lee et al., 2012). Since AMPK is an important regulator of the Na^+/K^+-ATPase and ENaC (Carattino et al., 2005; Woollhead et al., 2007; Albert et al., 2008; Mace et al., 2008), it might be speculated that H_2S-mediated AMPK activation affects renal Na^+ absorption. This hypothesis remains to be investigated.

EFFECTS OF H_2S ON PULMONARY EPITHELIAL Na^+ TRANSPORT

A first hint that H_2S might affect Na^+ transport processes across lung epithelia comes from pathological observations on patients which had prolonged exposure to this gas. One of their symptoms is, amongst others, pulmonary edema (Cordasco and Stone, 1973). Based on the link between alveolar fluid clearance and Na^+ transport across the alveolar epithelium (Althaus et al., 2011), it might be speculated that H_2S poisoning leads to fluid accumulation in the airspaces due to impairment of transepithelial Na^+ and, consequently, water reabsorption. Consistent with this idea, our laboratory has recently shown that the administration of the H_2S liberating molecule NaHS decreased amiloride-sensitive Na^+ transport across the H441 lung epithelial cell line as well as native tracheal preparations of pigs and mice (Althaus et al., 2012). This was not the result of a direct impairment of Na^+ transporting molecules, but rather occurred indirectly via H_2S-mediated inhibition of basolateral K^+ channels and consequently impairment of Na^+/K^+-ATPase activity (Althaus et al., 2012). An impaired activity of the Na^+/K^+-ATPase due to NaHS has also been reported for rat colonic epithelia; although this study did not investigate transepithelial Na^+ transport processes (Pouokam and Diener, 2011). In general, an impaired transepithelial Na^+ transport by H_2S in the lung may provide a molecular explanation for edema development in patients with H_2S poisoning. However, whether or not H_2S is also an endogenous, physiological regulator of pulmonary epithelial Na^+ transport, needs to be addressed. A first step toward the answer to this question is the fact that H_2S generating enzymes are expressed in rat lungs (Madden et al., 2012) and H_2S production has been shown in lung tissue homogenates of mammals (Olson et al., 2010). If such endogenously produced H_2S affects pulmonary Na^+ transport is a question that remains to be answered.

EFFECTS OF H_2S ON INTESTINAL Na^+ TRANSPORT

In the intestine, production of H_2S has also been shown in rat ileum (Zhao et al., 2003). Additionally, the H_2S generating enzymes CBS and CSE are expressed in the colon and inhibition of these enzymes affects transepithelial ion transport (Schicho et al., 2006; Hennig and Diener, 2009). Furthermore, the application of NaHS induces a Cl^- secretion across colonic epithelia (Schicho et al., 2006; Hennig and Diener, 2009). Putative effects of H_2S on intestinal Na^+ absorption, however, remain unknown.

H_2S REGULATION OF Na^+ TRANSPORT: CONCLUSIONS

Although H_2S is an emerging signaling molecule in organs such as kidneys, lung, or the intestine, its effects on epithelial Na^+ transport processes in those organs is largely unknown. Interestingly, a major target for H_2S are different types of K^+ channels (Telezhkin et al., 2009; Tang et al., 2010). K^+ channels play a crucial role in epithelial ion transport physiology since these channels regulate the membrane potential and thus electrochemical gradients which are necessary for ion fluxes. For example, we and others have shown that modulation of K^+ channel activity in the basolateral membrane of pulmonary epithelia indirectly affects transepithelial Na^+ transport processes (Greenwood et al., 2009; Althaus et al., 2012). Therefore it may be speculated that H_2S likely affects ion and Na^+ transport processes across various other epithelia by interference with K^+ channel activity.

Beside the elucidation of effects of H_2S on Na^+ transport processes, a major point that needs to be addressed is the question if H_2S is a physiological regulator of epithelial transport. Physiological concentrations of H_2S are hard to determine and still controversial (Olson, 2011). The future challenges will be the answer to the question what the real physiological concentrations of H_2S are and if such concentrations are necessary or sufficient to maintain or affect epithelial Na^+ transport processes.

SUMMARY AND PERSPECTIVES

There are various studies available which demonstrate that the gasotransmitters NO, CO, and H_2S affect epithelial Na^+ transport processes. However, before one can decisively consider any gasotransmitter as a true physiological regulator of Na^+ transport, the following criteria need to be fulfilled:

(1) The transmitter needs to be generated by specific enzymes either directly in the epithelium or in associated tissues, such as endothelium, nerve fibers, or other specific cells.
(2) The production of the transmitter needs to be either tonic or stimulated by specific, physiologically relevant inducers.
(3) The endogenously produced transmitter needs to regulate specific protein targets (Na^+ transporting molecules), either directly or by cellular signaling cascades.

Although these criteria seem to be quite straightforward, it is not easy to address them in experimental studies. This is reasoned by the facts that (i) it is difficult to measure physiological concentrations of the transmitters and (ii) consequently it is not easy to decide whether transmitter concentrations, when applied as gas or by donating molecules, are in a physiological range.

So far, NO is the only gasotransmitter which sufficiently fulfills the mentioned criteria. This is evident from studies demonstrating that NO is produced by distinct cell types, either tonic or stimulated, and can regulate specific Na^+ transporting molecules in epithelia via cellular signaling mechanisms, such as activation of the sGC/cGMP pathway.

Additionally, there is a growing body of evidence that the gasotransmitters CO and H_2S can influence epithelial Na^+ transport processes by affecting distinct Na^+ transporting molecules in epithelia. However, especially the first two criteria concerning a physiological role of CO and H_2S have not been sufficiently addressed. The future challenge will be the investigation of the role of endogenously produced CO/H_2S, the identification of stimulators of their synthesis and finally the elucidation of their impact on Na^+ transport processes across epithelia.

Taken together, gasotransmitters are emerging as an important class of signaling molecules which might be identified as novel regulators of epithelial Na^+ transport processes in the future. The investigation of their physiology and putative dysregulation in pathophysiological situations is an exciting field which eventually may expand our understanding of Na^+ transport biology in health and disease.

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Modulation of ion transport across rat distal colon by cysteine

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The aim of this study was to identify the actions of stimulation of endogenous production of H_2S by cysteine, the substrate for the two H_2S-producing enzymes, cystathionine-β-synthase and cystathionine-γ-lyase, on ion transport across rat distal colon. Changes in short-circuit current (Isc) induced by cysteine were measured in Ussing chambers. Free cysteine caused a concentration-dependent, transient fall in Isc, which was sensitive to amino-oxyacetate and β-cyano-L-alanine, i.e., inhibitors of H_2S-producing enzymes. In contrast, Na cysteinate evoked a biphasic change in Isc, i.e., an initial fall followed by a secondary increase, which was also reduced by these enzyme inhibitors. All responses were dependent on the presence of Cl^- and inhibited by bumetanide, suggesting that free cysteine induces an inhibition of transcellular Cl^- secretion, whereas Na cysteinate – after a transient inhibitory phase – activates anion secretion. The assumed reason for this discrepancy is a fall in the cytosolic pH induced by free cysteine, but not by Na cysteinate, as observed in isolated colonic crypts loaded with the pH-sensitive dye, BCECF. Intracellular acidification is known to inhibit epithelial K^+ channels. Indeed, after preinhibition of basolateral K^+ channels with tetrapentylammonium or Ba^{2+}, the negative Isc induced by free cysteine was reduced significantly. In consequence, stimulation of endogenous H_2S production by Na cysteinate causes, after a short inhibitory response, a delayed activation of anion secretion, which is missing in the case of free cysteine, probably due to the cytosolic acidification. In contrast, diallyl trisulfide, which is intracellularly converted to H_2S, only evoked a monophasic increase in Isc without the initial fall observed with Na cysteinate. Consequently, time course and amount of produced H_2S seem to strongly influence the functional response of the colonic epithelium evoked by this gasotransmitter.

Keywords: Cl^- secretion, cysteine, cytosolic pH, electrolyte transport, H_2S, rat colon

INTRODUCTION

Colonic ion transport is not only controlled by classical neurotransmitters or hormones (for review see Binder and Sandle, 1994), but is also influenced by gasotransmitters such as nitric oxide (Toda and Herman, 2005), carbon monoxide (Steidle and Diener, 2011), or hydrogen sulfide (Schicho et al., 2006; Hennig and Diener, 2009). The latter gas is produced from the amino acid cysteine via the enzymes cystathionine-β-synthase and cystathionine-γ-lyase (Wang, 2002; Martin et al., 2010). Both enzymes are found within enteric ganglia of guinea-pig and human colon (Schicho et al., 2006) as well as in smooth muscle layers and the epithelium of rat colon (Hennig and Diener, 2009). Plasma levels of H_2S are reported in the range of 50–160 $\mu mol \cdot l^{-1}$ (Zhao et al., 2003). The local concentration within the intestinal wall is unknown, but the production rate of H_2S has been measured for rat ileum to be in the range of 12 $nmol \cdot min^{-1} \cdot g^{-1}$ tissue (Zhao et al., 2003). Furthermore, there is an upregulation of H_2S production during experimental colitis in rats (Wallace et al., 2009), so that rat colon is an interesting model to investigate the modulation of ion transport by this gasotransmitter.

Hydrogen sulfide, which can be released from a donor molecule such as NaHS (Lee et al., 2006), evokes a Cl^- secretion in guinea-pig and human colon. In these tissues, the primary action site of this H_2S donor are enteric neurons, where H_2S is thought to act at capsaicin-sensitive cation channels of the type transient receptor potential vanilloid receptor 1 (TRPV1; Schicho et al., 2006). The consequence is a release of substance P and an activation of secretomotor submucosal neurons, which finally induce epithelial anion secretion (Krueger et al., 2010). This is supported by the observation that NaHS does not evoke anion secretion in the human colonic cell line, T84 (Schicho et al., 2006). Another mechanism of action of NaHS was observed in rat colon. In this tissue, NaHS evokes a triphasic change in Isc, which is a measure of net ion movement across the epithelium. An initial increase in Isc (mediated by Cl^- secretion) was followed by a transient fall (assumed to represent a transient K^+ secretion), before the Isc finally rose again to a long-lasting Cl^- secretory response. Partial resistance against the neurotoxin, tetrodotoxin, and inhibition by glibenclamide, which acts as blocker of ATP-sensitive K^+ channels (Cook and Quast, 1990), as well as tetrapentylammonium, known as inhibitor of Ca^{2+}-dependent K^+ channels (Maguire et al., 1999), indicated an action at epithelial K^+ channels (Hennig and Diener, 2009). Direct epithelial actions of H_2S released from NaHS were observed in experiments at isolated colonic crypts loaded with

the Ca^{2+}-sensitive fluorescent dye, fura-2, where NaHS evoked a biphasic change in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), i.e., an initial decrease followed by a secondary increase (Pouokam and Diener, 2011). Consequently, there seem to be pronounced species differences in the mechanism of action of NaHS.

In the literature, there is a controversy about beneficial and/or adverse effects of H_2S on intestinal functions. The above mentioned *in vitro* studies, although differing in the presumed mechanisms of action, indicate a prosecretory action of this putative gasotransmitter, which – *in vivo* – would aggravate gastrointestinal symptoms such as diarrhoe, e.g., during inflammatory bowel disease. Indeed, H_2S exerts proinflammatory actions in a mouse model of acute pancreatitis (Tamizhselvi et al., 2007). Further negative actions of H_2S on gastrointestinal integrity have been deduced from the observation that inhibition of H_2S synthesis by propargylglycine protects rats from ethanol-induced gastritis (Chávez-Piña et al., 2010). In contrast, other experimental evidence clearly demonstrates beneficial effects of this gasotransmitter. Exogenous H_2S exerts an antiinflammatory and antinociceptive action during synovitis in rats (Ekundi-Valentim et al., 2010). The H_2S-releasing derivative of mesalamine, ATB-429, is highly effective as therapeutic in a model of murine colitis (Fiorucci et al., 2007). Further protective actions of hydrogen sulfide are observed in the heart, where this gasotransmitter protects against ischemia/reperfusion damage (Ji et al., 2008). In contrast to the model of ethanol-induced gastritis (Chávez-Piña et al., 2010), where the beneficial effect of blockade of H_2S synthesis suggests proinflammatory actions of H_2S, the gastric damage caused by inhibitors of cyclooxygenases has been found to be reduced when using a cyclooxygenase inhibitor which simultaneously releases H_2S (Wallace et al., 2010). As in some studies both inhibition of H_2S synthesis as well as its stimulation by cysteine, the precursor for H_2S production, exert a similar response (Chávez-Piña et al., 2010), one reason for this discrepancy in the literature might be that the effect evoked by H_2S may differ in situations, where endogenous enzymes probably produce relative low concentrations of this gasotransmitter, and situations, in which exogenous donor molecules might release higher concentrations of H_2S.

Consequently, in the present study we investigated changes in ion transport across rat colon, in which we had previously characterized the effect of the exogenous H_2S donor, NaHS (Hennig and Diener, 2009; Pouokam and Diener, 2011), induced by cysteine as precursor of endogenous H_2S formation within the tissue. Ussing chamber experiments and experiments with the pH-sensitive dye, BCECF, were used in order to identify the mechanisms involved.

MATERIALS AND METHODS

SOLUTIONS

The standard solution for the Ussing chamber experiments was a buffer solution containing (mmol·l^{-1}): NaCl 107, KCl 4.5, $NaHCO_3$ 25, Na_2HPO_4 1.8, NaH_2PO_4 0.2, $CaCl_2$ 1.25, $MgSO_4$·1 and glucose 12. The solution was gassed with carbogen (5% CO_2 in 95% O_2, vol·vol^{-1}); pH was 7.4. For the Cl^--free buffer, NaCl and KCl were equimolarly substituted by Na gluconate and K gluconate, respectively.

For the experiments carried out with isolated crypts, the following buffers were used. The EDTA (ethylenediamino-tetraacetic acid) solution for the isolation contained (mmol·l^{-1}): NaCl 107, KCl 4.5, $NaHCO_3$ 25, Na_2HPO_4 1.8, NaH_2PO_4 0.2, glucose 12.2, EDTA 10 and 1 g·l^{-1} bovine serum albumin (BSA). It was gassed with carbogen; pH was adjusted by *tris*-base (*tris*(hydroxymethyl)-aminomethane) to 7.4. The isolated crypts were stored in a high potassium Tyrode solution consisting of (mmol·l^{-1}): K gluconate 100, KCl 30, HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid) 10, NaCl 20, $MgCl_2$ 1, $CaCl_2$ 1.25, glucose 12.2, sodium pyruvate 5, and 1 g·l^{-1} BSA; pH was 7.4. For superfusion of the isolated crypts during the imaging experiments, the following buffer was used (in mmol·l^{-1}): NaCl 140, KCl 5.4, $CaCl_2$ 1.25, $MgSO_4$ 1, HEPES 10, glucose 12.2. pH was 7.4.

TISSUE PREPARATION AND CRYPT ISOLATION

Wistar rats of both sexes were used with a weight of 180–240 g. The animals had free access to water and a standard rat diet until the day of the experiment. Animals were killed by a blow on the head followed by exsanguination (approved by Regierungspräsidium Gießen, Gießen, Germany). The serosa and muscularis propria were stripped away to obtain a mucosa–submucosa preparation of the distal colon. The distal colon was distinguished from the proximal colon by the absence of palm leaf-like striae (Lindström et al., 1979). Briefly, the colon was placed on a small plastic rod with a diameter of 5 mm. A circular incision was made near the anal end with a blunt scalpel and the serosa together with the lamina propria were gently removed in a proximal direction. Two segments of the distal colon of each rat were prepared. In general, one tissue served to measure the control response evoked by cysteine and the other to measure the response in the presence of a putative inhibitor (see below). There was no obvious segment dependence in the Isc evoked by cysteine between the early and the late distal colon (data not shown).

For the isolation of intact crypts, the mucosa–submucosa was fixed on a plastic holder with tissue adhesive and transferred for about 7 min to the EDTA solution. The mucosa was vibrated once for 30 s in order to obtain crypts. They were collected in a high-K^+ gluconate Tyrode buffer (Böhme et al., 1991).

SHORT-CIRCUIT CURRENT MEASUREMENT

The mucosa–submucosa preparation was fixed in a modified Ussing chamber, bathed with a volume of 3.5 ml on each side of the mucosa. The tissue was incubated at 37°C and short-circuited by a computer-controlled voltage-clamp device (Ingenieur Büro für Mess- und Datentechnik Mussler, Aachen, Germany) with correction for solution resistance. Tissue conductance (Gt) was measured every min by the voltage deviation induced by a current pulse (±50 μA, duration 200 ms) under open-circuit conditions. Short-circuit current (Isc) was continuously recorded on a chart-recorder. Isc is expressed as $\mu Eq \cdot h^{-1} \cdot cm^{-2}$, i.e., the flux of a monovalent ion per time and area, with $1\,\mu Eq \cdot h^{-1} \cdot cm^{-2} = 26.9\,\mu A \cdot cm^{-2}$.

In general, one tissue served to measure the control responses evoked by at least two administrations of cysteine. The other tissue was pretreated with putative inhibitors before the second cysteine administration. If the inhibitor had to be administered in

a solvent, the control tissue was pretreated with the solvent, too. After each cysteine administration, the serosal compartment was washed three times with five times the chamber volume.

IMAGING EXPERIMENTS

Relative changes in the cytosolic pH were measured using BCECF [2′,7′-*bis*(carboxyethyl)-5,6-carboxyfluorescein/acetomethoxy methyl; Life Technologies, Darmstadt, Germany], a pH-sensitive fluorescent dye (Rink et al., 1982). The crypts were pipetted into the experimental chamber with a volume of about 3 ml. They were attached to the glass bottom of the chamber with the aid of poly-L-lysine (0.1 $g \cdot l^{-1}$; Biochrom, Berlin, Germany). The crypts were incubated for 45 min with 8 $\mu mol \cdot l^{-1}$ BCECF/acetoxymethylester (AM). Then the dye ester not taken up by the cells was washed away. The preparation was superfused hydrostatically throughout the experiment with 140 $mmol \cdot l^{-1}$ NaCl Tyrode. Perfusion rate was about 1 $ml \cdot min^{-1}$.

Changes in the cytosolic pH values were monitored as changes in the BCECF ratio (*R*; emission at an excitation wave length of 500 nm divided by the emission at an excitation wave length of 450 nm). For each experiment, it was waited until a stable baseline had developed before cysteine or any other substance was administered. Changes in the BCECF ratio induced by free cysteine or Na cysteinate are expressed as change in relation to this baseline just prior administration of cysteine or the corresponding substance (Δ BCECF ratio). Experiments were carried out on an inverted microscope (Olympus IX-50; Olympus, Hamburg, Germany) equipped with an epifluorescence set-up and an image analysis system (Till Photonics, Martinsried, Germany). Several regions of interest, each with the size of about one cell, were placed over an individual crypt. The emission above 520 nm was measured from the regions of interest. Data were sampled at 0.2 Hz. The baseline in the fluorescence ratio of BCECF was measured for several minutes before drugs were administered.

DRUGS

BCECF/AM and diallyl trisulfide (Cayman, Ann Harbor, USA) were dissolved in dimethylsulfoxide (DMSO; final maximal concentration 0.3 $ml \cdot l^{-1}$). Bumetanide and forskolin were dissolved in ethanol (final maximal concentration 2.5 $ml \cdot l^{-1}$). Tetrodotoxin was dissolved in 2×10^{-2} $mol \cdot l^{-1}$ citrate buffer. Amino-oxyacetate (AOA), $BaCl_2$, carbachol, β-cyano-L-alanine (CLA), L-cysteine, GYY 4137 (*p*-methoxyphenyl)morpholino-phosphinodithioic acid; Cayman, Ann Harbor, USA), L-methionine, and tetrapentylammonium (TPeA) chloride were dissolved in aqueous stock solutions. Na cysteinate was prepared by dissolving free cysteine equimolarly in 1 $mol \cdot l^{-1}$ NaOH. Charybdotoxin was dissolved in an aqueous stock solution containing 1 $g \cdot l^{-1}$ BSA. If not indicated differently, drugs were from Sigma, Taufkirchen, Germany.

STATISTICS

Values are given as means ± 1 SEM. In the case that means of several groups had to be compared, an analysis of variance was performed followed by *post hoc* test of Tukey. For the comparison of two groups, either a Student's *t*-test or a Mann Whitney *U*-test was applied. An *F*-test decided which test method had to be used. Both paired and unpaired two-tailed Student's *t*-tests were applied as appropriate. $P < 0.05$ was considered to be statistically significant.

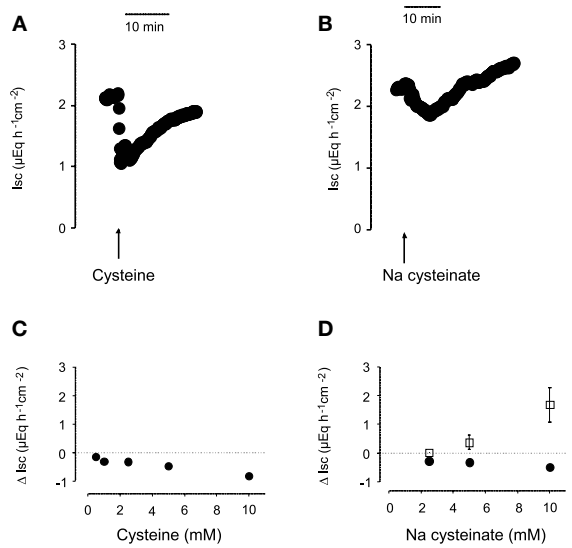

FIGURE 1 | Time course of the monophasic Isc response to free cysteine [(A), 10^{-2} $mol \cdot l^{-1}$ at the serosal side; indicated by the arrow] and of the biphasic response evoked by Na cysteinate [(B), 10^{-2} $mol \cdot l^{-1}$ at the serosal side; indicated by the arrow]. (**C,D**) Concentration-dependent initial decrease (filled circles) and secondary increase (filled squares) in Isc induced by serosal administration of free cysteine **(C)** or Na cysteinate **(D)** in mucosa–submucosa preparations of rat distal colon. The data are given as change of Isc (Δ Isc) compared to baseline (indicated by the dashed line) just prior administration of the amino acid. Each administration of the amino acid in **(C,D)** was followed by three times washing the serosal compartment with fresh, cysteine-free buffer solution. Values are given as means (symbols) ± SEM (bars), $n = 8$. The decrease in Isc was statistically significant ($P < 0.05$, paired *t*-test) for all concentrations of free cysteine or Na cysteinate tested, whereas the increase in Isc evoked by Na cysteinate reached statistical significance only for the highest concentration tested (10^{-2} $mol \cdot l^{-1}$).

RESULTS

EFFECT OF CYSTEINE ON SHORT-CIRCUIT CURRENT

L-Cysteine, when administered to the serosal side of the tissue, induced a prompt decrease in Isc (see, e.g., **Figure 1A** for time course). The decrease in Isc was concentration-dependent (**Figure 1C**). A significant fall in baseline Isc was evoked by the lowest concentration used (5×10^{-4} $mol \cdot l^{-1}$ at the serosal side) and was maximal at a concentration of 10^{-2} $mol \cdot l^{-1}$ at the serosal side. Most of the further experiments were performed with an intermediate concentration (5×10^{-3} $mol \cdot l^{-1}$) of cysteine. The decrease in Isc was not mimicked by another S-containing amino acid, L-methionine. L-Methionine, when administered at a concentration of 5×10^{-3} $mol \cdot l^{-1}$ (at the serosal side), did not evoke a significant change in Isc ($n = 8$; data not shown). Cysteine and methionine are known to block K^+ channels of the type TREK-1 (Park et al., 2005). The failure of methionine to mimic the cysteine response indicates that such a mechanism (or non-specific osmotic effects) cannot be the reason for the change in Isc induced by cysteine.

A potential problem in the administration of free cysteine is the fact that this amino acid, despite the high buffer capacity of the buffer solution used, caused a fall in the pH of this solution, which might (and indeed does, see below) cause an acidification of the intracellular milieu (see cytosolic pH measurements below). Therefore, in a second series of experiments, cysteine was neutralized by NaOH. When administered as Na cysteinate, the time course of the Isc response evoked by the amino acid changed: an initial fall, which developed slower compared to the corresponding response evoked by free cysteine, was followed by a secondary increase above the baseline, which was not observed in the case of free cysteine (**Figure 1B**). Both phases exhibited a clear concentration dependence (**Figure 1D**). When Na cysteinate (5×10^{-3} mol·l^{-1}) was administered to the mucosal side, there was no significant change in Isc ($n = 5$, data not shown).

The effect of free cysteine (2.5×10^{-3} mol·l^{-1} at the serosal side) on Isc was significantly reduced, when the tissue was pretreated with a combination of amino-oxyacetate (5×10^{-3} mol·l^{-1} at the serosal side), an inhibitor of the enzyme cystathionine-β-synthase, and β-cyano-L-alanine (5×10^{-3} mol·l^{-1} at the serosal side), an inhibitor of the enzyme cystathionine-γ-lyase (for references to both inhibitors, see Zhao et al., 2003). In the presence of these inhibitors, cysteine only induced a fall in Isc of -0.17 ± 0.044 μEq·h^{-1}·cm^{-2} compared to -0.41 ± 0.090 μEq·h^{-1}·cm^{-2} in tissues which were – for osmotic reasons – pretreated with mannitol (10^{-2} mol·l^{-1} at the serosal side) instead of the two enzyme inhibitors ($n = 6$, $P < 0.05$; **Table 1**). When a higher concentration of cysteine (5×10^{-3} mol·l^{-1} at the serosal side) was used, the reduction in the cysteine response by these two blockers lost statistical significance. This observation would be consistent with the assumption of a competitive action of the two inhibitors on the H_2S-producing enzymes; a mechanism of inhibition, which has indeed been shown for β-cyano-L-alanine (Pfeffer and Ressler, 1967). Also both phases of the Isc response evoked by Na cysteinate were reduced by the enzyme inhibitor combination. In the presence of the inhibitors of H_2S-producing enzyme, Na cysteinate (10^{-2} mol·l^{-1} at the serosal side) evoked an initial fall in Isc of only -0.16 ± 0.029 μEq·h^{-1}·cm^{-2} ($n = 8$) compared to -0.46 ± 0.10 μEq·h^{-1}·cm^{-2} in the absence of the inhibitors ($n = 8$, $P < 0.05$). The secondary increase in Isc evoked by Na cysteinate was reduced from 1.05 ± 0.49 μEq·h^{-1}·cm^{-2} ($n = 8$) under control conditions to 0.50 ± 0.14 μEq·h^{-1}·cm^{-2} ($n = 8$) in the presence of the inhibitors, although the latter difference did not reach statistical significance due to the large variation of the control response.

Table 1 | Effect of free cysteine in the presence of inhibitors of H_2S synthesizing enzymes.

	Δ Isc (μEq·h^{-1}·cm^{-2})	
	+ AOA/CLA	**− AOA/CLA**
Free cysteine 2.5×10^{-3} mol·l^{-1}	-0.17 ± 0.044*	-0.41 ± 0.090
Free cysteine 5×10^{-3} mol·l^{-1}	-0.37 ± 0.088	-0.49 ± 0.094

*Effect of free cysteine (at the serosal side) in the absence (–; right column) or presence (+; middle column) of a combination of amino-oxyacetate (AOA, 5×10^{-3} mol·l^{-1} at the serosal side; cystathionine-β-synthase blocker) and β-cyano-L-alanine (CLA, 5×10^{-3} mol·l^{-1} at the serosal side, cystathionine-γ-lyase blocker). The control tissues (-AOA/CLA) were treated with mannitol (10^{-2} mol·l^{-1} at the serosal side). Data are given as change of Isc (Δ Isc) compared to baseline just prior administration of the respective cysteine concentration and are means ± SEM, n = 6. *P < 0.05 versus response to the same concentration of cysteine in the absence of the two enzyme inhibitors.*

Table 2 | Effect of free cysteine after inhibition of Cl^- secretion.

	Δ Isc (μEq·h^{-1}·cm^{-2}) evoked by free cysteine	
	Control	**±Inhibitor**
Cl^- free	-0.87 ± 0.14^a	-0.17 ± 0.036^b
With Cl^-	-0.78 ± 0.15^a	-0.74 ± 0.19^a
With bumetanide	-0.94 ± 0.19^a	-0.42 ± 0.10^b
Without bumetanide	-0.95 ± 0.19^a	-0.86 ± 0.21^a

The effect of free cysteine (5×10^{-3} mol·l^{-1} at the serosal side) on Isc was tested in the absence of any inhibitors (control), then in the presence or absence of the respective inhibitor (or replacement of Cl^- with the impermeable anion gluconate). The first two rows give the response to free cysteine in the absence or presence of Cl^- ion in the buffer, whereas in the subsequent rows bumetanide (10^{-4} mol·l^{-1} at the serosal side) was used as inhibitor. Data are given as change of Isc (Δ Isc) compared to baseline just prior administration of the respective cysteine administration and are means ± SEM, n = 7. Different letters (a, b) indicate statistically homogenous groups (analysis of variances followed by post hoc test of Tukey).

The negative Isc induced by cysteine did not show a desensitization. When free cysteine (5×10^{-3} mol·l^{-1} at the serosal side) was administered two times to the same tissue with a washing procedure between the individual administrations (see Materials and Methods), there was no significant decrease in the Isc response evoked by this amino acid (see, e.g., **Tables 2** and **4**, control responses). The same was observed for the biphasic change in Isc induced by Na cysteinate, which could be repetitively evoked at the same tissue without a reduction in either the initial fall nor in the final increase in Isc (see **Table 3**, control responses). In contrast, there was even a tendency (especially for Na cysteinate) that the second administration of the amino acid evoked larger changes in Isc than the first administration; a phenomenon, however, which was not studied further here.

Consequently, for all inhibitor experiments designed to clarify the mechanism of action of cysteine the following protocol was used. The response to cysteine was first measured in each tissue under control conditions, i.e., in the absence of other drugs. After a washing step, the response evoked by cysteine was measured in the presence of a putative inhibitor.

A CHANGE IN Cl^- SECRETION UNDERLIES THE CYSTEINE-INDUCED Isc

The negative Isc induced by free cysteine can, in principle, be caused by two mechanisms: an inhibition of spontaneous Cl^- secretion, which is responsible for the generation of the positive baseline Isc in rat colon (see, e.g., Strabel and Diener, 1995),

Table 3 | Effect of Na cysteinate after inhibition of Cl^- secretion.

	Δ Isc ($\mu Eq \cdot h^{-1} \cdot cm^{-2}$) evoked by Na cysteinate			
	Control		± Inhibitor	
	Initial decrease	Peak	Initial decrease	Peak
Cl^- free	-0.59 ± 0.16^{a}	$0.24 \pm 0.16^{a,b}$	-0.17 ± 0.015^{a}	-0.03 ± 0.041^{a}
With + Cl^-	-0.81 ± 0.26^{a}	-0.11 ± 0.25^{a}	-0.59 ± 0.16^{a}	0.72 ± 0.26^{b}
With bumetanide	$-0.49 \pm 0.25^{a,b}$	0.23 ± 0.21^{b}	-0.23 ± 0.016^{a}	-0.021 ± 0.090^{a}
Without bumetanide	$-0.68 \pm 0.13^{a,b}$	0.37 ± 0.22^{a}	-1.12 ± 0.16^{b}	0.85 ± 0.61^{a}

The effect of Na cysteinate (10^{-2} mol·l^{-1} at the serosal side) on Isc was tested in the absence of any inhibitors (control), then in the presence or absence of the respective inhibitor (or replacement of Cl^- with the impermeable anion gluconate). The first two rows give the response to Na cysteinate in the absence or presence of Cl^- ion in the buffer, whereas in the subsequent rows bumetanide (10^{-4} mol·l^{-1} at the serosal side) was used as inhibitor. Data are given as change of Isc (Δ Isc) compared to baseline just prior administration of the respective Na cysteinate administration and are means ± SEM, n = 7–8. Different letters (a, b) indicate statistically homogenous groups (analysis of variances followed by post hoc test of Tukey).

or the induction of the secretion of cations, i.e., the induction of K^+ secretion (see, e.g., Hörger et al., 1998). The negative Isc evoked by free cysteine was strongly dependent on the presence of Cl^-. In the presence of Cl^-, cysteine (5×10^{-3} mol·l^{-1} at the serosal side) evoked a fall in Isc of $-0.87 \pm 0.14\ \mu Eq \cdot h^{-1} \cdot cm^{-2}$. This change in Isc was reduced by about 80%, when the amino acid was administered to the same tissue after substitution of Cl^- by the impermeant anion, gluconate, on both sides of the tissue (**Table 2**). Similarly, both the initial fall as well as the secondary increase in Isc evoked by Na cysteinate (10^{-2} mol·l^{-1} at the serosal side) were significantly reduced in the absence of Cl^- (**Table 3**).

A significant inhibition of the cysteine-evoked Isc was also observed, when the Na^+–K^+–2 Cl^--cotransporter, the dominant Cl^--loading mechanism in the basolateral membrane necessary to maintain transepithelial Cl^- secretion (Kaplan et al., 1996), was blocked with bumetanide (10^{-4} mol·l^{-1} at the serosal side; **Figure 2** and **Table 2**). Bumetanide inhibited the biphasic change in Isc induced by Na cysteinate (10^{-2} mol·l^{-1} at the serosal side) to a similar extent (**Table 3**).

The further experiments, which served to elucidate the potential role of changes in K^+ transport in the negative Isc evoked by the amino acid were performed only with free cysteine in order to avoid any contamination of the measured currents with the secondary increase in Isc, i.e., the parallel induction of Cl^- secretion observed with Na cysteinate. In contrast to two maneuvers interfering with Cl^- secretion (i.e., anion substitution and inhibition of the Na^+–K^+–2 Cl^--cotransporter), inhibition of apical K^+ channels, which are a prerequisite for colonic K^+ secretion (SØrensen et al., 2010), did not change the cysteine response. None of the K^+ channel blockers tested (for references to these K^+ channel blockers, see Cook and Quast, 1990) had any significant effect on the Isc evoked by free cysteine. Neither Ba^{2+} (10^{-2} mol·l^{-1} at the mucosal side), a broad K^+ channel blocker affecting many types of K^+ channels, nor tetrapentylammonium (10^{-4} mol·l^{-1} at the mucosal side), a blocker with some preference for Ca^{2+}-dependent K^+ channels (Maguire et al., 1999), nor charybdotoxin (2×10^{-7} mol·l^{-1} at the mucosal side), a selective blocker of large-conductance (BK_{Ca}) Ca^{2+}-dependent K^+ channels (Berkefeld et al., 2010), had any significant effect on the

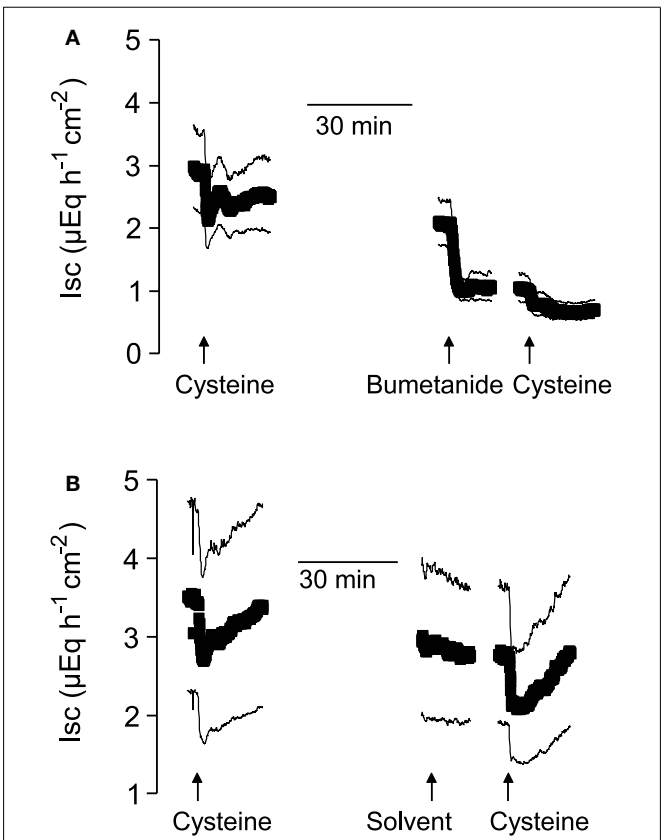

FIGURE 2 | After suppression of Cl^- secretion with bumetanide, the action of cysteine is prevented. Shown are the effects of two administrations of free cysteine (5×10^{-3} mol·l^{-1} at the serosal side; indicated by the arrows) in mucosa–submucosa preparations of rat distal colon. The line interruptions are caused by omission of current artifacts induced by washing the serosal compartment three times with five times the chamber volume, before the next administration of cysteine followed. The effect of cysteine was first tested in the absence of any inhibitors and then in the presence **(A)** or absence **(B)** of bumetanide (10^{-4} mol·l^{-1} at the serosal side). The solvent for bumetanide (ethanol) was administered to the tissues which were not treated with bumetanide. Values are means (symbols) ± SEM (lines), $n = 7$. For statistics, see **Table 2**.

negative Isc induced by free cysteine (**Table 4**). Consequently, these data allow the conclusion that the negative Isc evoked by cysteine is caused by an inhibition of spontaneous anion secretion and not due to an activation of transepithelial K^+ secretion.

This conclusion was corroborated by experiments in which K^+ channel blockers were administered to the serosal side of the tissue. A sufficient basolateral K^+ conductance is necessary to maintain basal membrane potential as driving force for Cl^- exit across apical Cl^- channels (Strabel and Diener, 1995). Blockade of basolateral K^+ channels with Ba^{2+} (10^{-2} mol·l^{-1} at the serosal side) or tetrapentylammonium (10^{-4} mol·l^{-1} at the serosal side) significantly inhibited the negative Isc evoked by free cysteine. In contrast, charybdotoxin (2×10^{-7} mol·l^{-1} at the serosal side) was ineffective (**Table 4**). In these series of experiments with K^+ channel blockers, a clear tendency for increased cysteine effects was observed in the experiments designed to measure the effect of Ba^{2+}. The reason for this discrepancy to the other experimental series may be that these experiments were performed in HCO_3^--free, HEPES buffered Tyrode solution (in order to avoid precipitation of Ba^{2+} as carbonate or sulfate salt). This phenomenon, however, was not studied further.

Table 4 | Effect of free cysteine after blockade of K^+ channels.

	Δ Isc (μEq·h^{-1}·cm^{-2}) evoked free cysteine	
	Control	**± Inhibitor**
With serosal Ba^{2+}	-1.30 ± 0.17^a	-0.78 ± 0.10^b
Without serosal Ba^{2+}	-1.57 ± 0.19^a	-2.02 ± 0.40^a
With serosal TPeA	-0.58 ± 0.12^a	-0.18 ± 0.039^b
Without serosal TPeA	-0.82 ± 0.13^a	-1.14 ± 0.28^a
With serosal CTX	-1.01 ± 0.11^a	-0.86 ± 0.13^a
Without serosal CTX	-0.81 ± 0.1^a	-0.62 ± 0.098^a
With mucosal Ba^{2+}	-0.89 ± 0.082^a	$-1.64 \pm 0.30^{a,b}$
Without mucosal Ba^{2+}	-0.80 ± 0.078^a	-1.30 ± 0.098^b
With mucosal TPeA	-0.56 ± 0.10^a	-0.63 ± 0.13^a
Without mucosal TPeA	-0.61 ± 0.067^a	-0.59 ± 0.093^a
With mucosal CTX	-0.79 ± 0.096^a	$-0.56 \pm 0.046^{a,b}$
Without mucosal CTX	-0.78 ± 0.087^a	-0.75 ± 0.15^a

The effect of free cysteine (5×10^{-3} mol·l^{-1} at the serosal side) on Isc was tested in the absence of any inhibitors (control) and then in the presence or absence of the respective inhibitor. The following K^+ channel blockers were applied either at the serosal or the mucosal side: Ba^{2+} (10^{-2} mol·l^{-1}), tetrapentylammonium (TPeA; 10^{-4} mol·l^{-1}), and charybdotoxin (CTX; 2.10^{-7} mol·l^{-1}). The experiments with Ba^{2+} were performed in HCO_3^--free Tyrode buffer solution in order to avoid precipitation of Ba carbonate. The reason for the apparent enhancement of the cysteine response in this buffer is unknown. Data are given as change of Isc (Δ Isc) compared to baseline just prior administration of the respective cysteine administration and are means ± SEM, n = 7–8. Different letters (a, b) indicate statistically homogenous groups (analysis of variances followed by post hoc test of Tukey).

DO ENTERIC NEURONS PARTICIPATE IN THE RESPONSE TO CYSTEINE?

Immunohistochemical experiments have demonstrated that the H_2S-producing enzymes, cystathionine-β-synthase and cystathionine-γ-lyase, are expressed within the epithelium, but also in muscle cells and neurons (Schicho et al., 2006; Hennig and Diener, 2009). Consequently, it seemed to be of interest whether enteric neurons might mediate the response evoked by cysteine. The effect of free cysteine (5×10^{-3} mol·l^{-1} at the serosal side) on Isc was unaffected when neuronal activity was blocked with tetrodotoxin (10^{-6} mol·l^{-1} at the serosal side), a blocker of voltage-dependent neuronal Na^+ channels (**Table 5**). The same resistance against tetrodotoxin was observed with Na cysteinate (5×10^{-3} mol·l^{-1} at the serosal side; **Table 5**). Consequently, cysteine obviously does not act via modulation of the activity of secretomotor submucosal neurons present in the mucosa–submucosa preparations used in this study.

EFFECTS OF CYSTEINE ON CYTOSOLIC pH

L-Cysteine is a weak acid, which causes – despite the relative high buffer capacity of the HCO_3^-/phosphate buffer used, an acidification of the extracellular solution, which might affect transepithelial transport. Administration of free cysteine in a concentration of 2.5×10^{-3} mol·l^{-1} or 5×10^{-3} mol·l^{-1} induced an acute fall in pH to 7.03 or 6.82, respectively. In order to find out whether a proton release might occur intracellularly after cellular uptake of cysteine, experiments were carried out with BCECF-loaded

Table 5 | Effect of cysteine after neuronal blockade.

	Control		± Inhibitor	
	Initial decrease	**Peak**	**Initial decrease**	**Peak**
Δ Isc (μEq·h^{-1}·cm^{-2}) EVOKED BY FREE CYSTEINE				
With tetrodotoxin	-0.56 ± 0.11^a	–	-0.56 ± 0.16^a	–
Without tetrodotoxin	-0.45 ± 0.069^a	–	-0.47 ± 0.078^a	–
Δ Isc (μEq·h^{-1}·cm^{-2}) EVOKED BY Na CYSTEINATE				
With tetrodotoxin	-0.317 ± 0.071^a	0.180 ± 0.100^a	-0.249 ± 0.055^a	0.390 ± 0.122^b
Without tetrodotoxin	-0.308 ± 0.075^a	0.064 ± 0.077^a	-0.305 ± 0.132^a	0.782 ± 0.207^b

The effect of free cysteine (5×10^{-3} mol·l^{-1} at the serosal side, upper two rows) and Na cysteinate (5×10^{-3} mol·l^{-1} at the serosal side, lower two rows) on Isc was tested in the absence of any inhibitors (control) and then in the presence or absence of tetrodotoxin (10^{-6} mol·l^{-1} at the serosal side). Data are given as change of Isc (Δ Isc) compared to baseline just prior administration of the respective cysteine administration and are means ± SEM, n = 6. Different letters (a, b) indicate statistically homogenous groups (analysis of variances followed by post hoc test of Tukey).

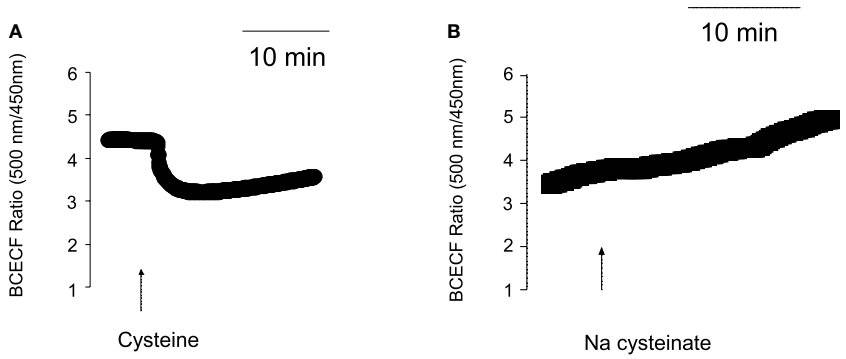

FIGURE 3 | (A) Cysteine, but not Na cysteinate induces a cytosolic acidification. Free cysteine (10^{-2} mol·l^{-1}; indicated by the arrow) induced a fall in the BCECF ratio signal. Values are given as means (symbols) ± SEM (lines), $n = 72$. **(B)** The response was not mimicked by Na cysteinate (10^{-2} mol·l^{-1}; indicated by the arrow). Values are given as means (symbols) ± SEM (lines), $n = 97$. In both cases, the ratio signal (emission at an excitation wave length of 500 nm divided by the emission at an excitation wave length of 450 nm) was measured at isolated colonic crypts.

isolated crypts. The fluorescence ratio signal of this dye decreases, when the cytosolic pH falls.

In deed, free cysteine (10^{-2} mol·l^{-1}) induced a prompt decrease in the BCECF ratio signal indicating cytosolic acidification (**Figure 3**). This fall was followed by a slow recovery, probably due to cellular pH counter-regulation. Such a phenomenon was not observed with Na cysteinate (10^{-2} mol·l^{-1}; **Figure 3B**). The sensitivity of different types of K^+ channels to cellular acidification is well known, also from isolated rat colonic crypts (Diener and Scharrer, 1994), and might be responsible for the different effects evoked by free cysteine in comparison to Na cysteinate (see Discussion). In deed, when the serosal buffer solution was acidified by administration of HCl to similar values as reached by administration of free cysteine, this caused a prompt fall in Isc of $0.95 \pm 0.22\,\mu$Eq·h^{-1}·cm^{-2} ($n = 7$, $P < 0.05$ versus baseline) in the case of an acidification to a pH of 7.0 and of $0.96 \pm 0.13\,\mu$Eq·h^{-1}·cm^{-2} ($n = 7$, $P < 0.05$ versus baseline) in the case of an acidification to a pH of 6.8. In no case, however, a secondary rise in Isc was observed as it was the case when Na cysteinate was administered.

COMPARISON WITH OTHER H_2S DONORS

The divergent response in Isc evoked by Na cysteinate, which induces a biphasic change in Isc, compared to NaHS, which stimulates a triphasic change in Isc, suggests that the speed of H_2S release might affect the biological effect at the colonic epithelium. In order to test this hypothesis in more detail, two other H_2S-releasing molecules were used (for references to these donors, see Martelli et al., 2011). GYY 4137 (5×10^{-5} mol·l^{-1} at the serosal side), a very slow H_2S-releasing molecule, did not evoke any increase in Isc. Instead, a slow fall in Isc was observed which appeared to be faster than the usual time-dependent decrease in Isc observed over prolonged time-periods in mucosa–submucosa preparations of rat distal colon (**Figure 4A**). In contrast, diallyl trisulfide, a compound found in garlic, which is converted intracellularly to H_2S by reaction with glutathione, evoked a monophasic increase in Isc in all concentrations tested (up to 1.5×10^{-4} mol·l^{-1} at the serosal side). When administered in a concentration of 1.5×10^{-5} mol·l^{-1} at the serosal side, diallyl trisulfide evoked an increase of $0.89 \pm 0.22\,\mu$Eq·h^{-1}·cm^{-2} above baseline ($P < 0.05$, $n = 7$; **Figure 4B**). In contrast, a roughly equieffective concentration of the fast H_2S donor, NaHS (2.5×10^{-3} mol·l^{-1} at the serosal side), evoked the typical multiphasic change in Isc as reported earlier (Hennig and Diener, 2009), i.e., a quick increase in Isc by $1.06 \pm 0.14\,\mu$Eq·h^{-1}·cm^{-2} above baseline ($P < 0.05$, $n = 7$), followed by a transient fall and finally a secondary increase of $1.60 \pm 0.22\,\mu$Eq·h^{-1}·cm^{-2} above baseline ($P < 0.05$, $n = 7$; **Figure 4B**). Consequently, three different H_2S-releasing drugs evoked different patterns of Isc responses across the colonic mucosa.

INTERACTIONS WITH SECRETAGOGUES

As cysteine modulates basal anion secretion (**Figure 1**), it seemed to be of interest to study whether this activator of endogenous H_2S synthesis might be able to interfere with the response of the epithelium to secretagogues. Ca^{2+}-dependent secretagogues such as the stable acetylcholine derivate, carbachol, typically induce a strong, but only transient increase in Isc (see, e.g., Strabel and Diener, 1995). Therefore, the effect of Na cysteinate on the carbachol response was measured by pretreatment with this amino acid. In the absence of Na cysteinate, carbachol evoked an increase in Isc of $9.11 \pm 1.62\,\mu$Eq·h^{-1}·cm^{-2} ($n = 6$), which only amounted to $6.84 \pm 1.83\,\mu$Eq·h^{-1}·cm^{-2} in the presence of Na cysteinate (5×10^{-5} mol·l^{-1} at the serosal side, $n = 6$, **Figures 5A,B**), although this difference did not reach statistical significance. Similar experiments were performed with free cysteine. In the absence of free cysteine, carbachol (5×10^{-5} mol·l^{-1} at the serosal side) evoked an increase in Isc above baseline which amounted to $7.66 \pm 0.63\,\mu$Eq·h^{-1}·cm^{-2} ($n = 6$). In contrast, after pretreating the tissue with free cysteine (5×10^{-3} mol·l^{-1} at the serosal side), Isc increased by only $4.20 \pm 1.02\,\mu$Eq·h^{-1}·cm^{-2} ($n = 7$, $P < 0.05$ versus response to carbachol in the absence of free cysteine).

In contrast to Ca^{2+}-dependent secretagogues, stimulation of the cAMP-pathway with forskolin, an activator of adenylate cyclase(s) (Seamon and Daly, 1981), leads to a stable increase in Isc (see **Figure 5D**), so that cysteine could be administered

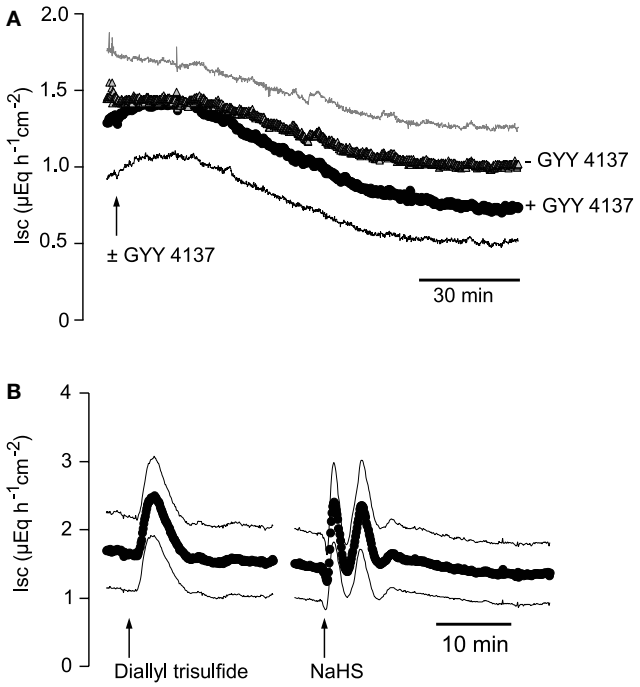

FIGURE 4 | (A) Comparison of the Isc evoked by different H_2S donors. The very slow H_2S-releasing molecule, GYY 4137 (5×10^{-5} mol·l^{-1} at the serosal side; arrow; black symbols and curve marked with + GYY 4137), did not evoke an increase in Isc in mucosa–submucosa preparations of rat distal colon. In contrast, a slow decrease in Isc, which had the tendency (not statistically significant) to be more pronounced compared to time-dependent control experiments (– GYY 4137) not pretreated with GYY 4137 (gray symbols) was observed. **(B)** The slow H_2S donor, diallyl trisulfide (1.5×10^{-5} mol·l^{-1} at the serosal side; left arrow), causes a transient, monophasic increase in Isc, whereas the fast H_2S donor, NaHS, administered in an equieffective concentration (2.5×10^{-3} mol·l^{-1} at the serosal side; right arrow), induced a triphasic change in Isc. Values are means (symbols) ± SEM (lines), $n = 5$–7. In the case of the experiments with GYY 4137, for graphical reasons for the time-dependent control experiments (– GYY 4137) only +SEM (gray line) and for the group treated with GYY 4137 (+ GY 4137) only −SEM (black line) are shown. For statistics, see text.

during the plateau phase of the current induced by forskolin (5×10^{-6} mol·l^{-1} at the mucosal and the serosal side). Under these conditions, Na cysteinate only evoked a transient and small decrease in Isc (**Figure 5C**). A similar response was observed with free cysteine. For example, administration of free cysteine in a concentration of 5×10^{-3} mol·l^{-1} (at the serosal side) induced a transient decrease of the forskolin-induced Isc by -0.55 ± 0.028 μEq·h^{-1}·cm^{-2} ($n = 7$), which is not larger than the effect of cysteine on baseline Isc in the absence of forskolin (see e.g. control series in **Table 2** and **Table 4**). So similar as it was observed with another gasotransmitter, carbon monoxide (Steidle and Diener, 2011), which when given alone evokes anion secretion but reduces the carbachol-induced Isc, also hydrogen sulfide impairs Ca^{2+}-dependent secretion, probably because both gasotransmitters interfere with intracellular Ca^{2+} signaling (Hennig and Diener, 2009; Pouokam and Diener, 2011; Steidle and Diener, 2011).

DISCUSSION

Hydrogen sulfide is recognized as a neurotransmitter in the enteric nervous system, i.e., the central regulator responsible for the control of most gastrointestinal functions (Jimenez, 2010). Expression of cystathionine-β-synthase and cystathionine-γ-lyase, the key enzymes for H_2S production (Wang, 2002), in smooth muscle and in epithelial cells from rat colon suggests additional paracrine actions of this gaseous molecule (Hennig and Diener, 2009). As stated in the Introduction, the biological role of H_2S during inflammatory processes is discussed controversially because this gasotransmitter has been reported to aggravate (see, e.g., Chávez-Piña et al., 2010) as well as to ameliorate (see, e.g., Fiorucci et al., 2007; Wallace et al., 2009) inflammatory processes. The observation that the exogenous H_2S donor NaHS evokes a strong Cl^- secretion across the colon of different species *in vitro* (Schicho et al., 2006; Hennig and Diener, 2009), i.e., would finally induce secretory diarrhoe *in vivo*, is – at first glance – in contradiction to the observed therapeutic effect of a H_2S-releasing mesalamine-derivative (ATB-429) in a model of mouse colitis (Fiorucci et al., 2007). Interestingly, a similar controversy about pro/antiinflammatory or proabsorptive/prosecretory actions exists for a second gasotransmitter, NO (see, e.g., Schirgi-Degen and Beubler, 1995; Schultheiss et al., 2002), suggesting that depending on differences in time, location, or amplitude of the production of these gasotransmitters the physiological response may differ in a fundamental way.

The present results demonstrate that cysteine (especially when administered as Na cysteinate), the precursor for endogenous H_2S synthesis, has a biphasic effect on anion secretion: an initial decrease followed by prolonged increase in Isc above the former baseline (**Figure 1B**). This second phase, i.e., a rise above the former baseline, is missing in the case of free cysteine (**Figure 1A**). Most probably the intracellular acidification, which is evoked by free cysteine, but not by Na cysteinate (**Figure 3**), is responsible for the missing ability of the presumed stimulation of H_2S synthesis to induce anion secretion (see below). Plasma cysteine concentrations in human blood are in the range of 0.3 mmol·l^{-1} (Van den Brandhof et al., 2001). So the concentrations used in the present study are clearly above physiological level. However, our aim was not mimic the physiological situation, but to stimulate maximally endogenous H_2S production, which is shown here by the amino-oxyacetate- and β-cyano-L-alanine-sensitive changes in Isc.

Both phases of the response to Na cysteinate, i.e., the initial fall as well as the secondary rise in Isc, were inhibited in the presence of bumetanide or after substitution of Cl^- by an impermeant anion confirming the modulation of Cl^- secretion by Na cysteinate (**Table 3**). This response differs from the change in Isc evoked by the exogenous H_2S donor, NaHS, which evokes a polyphasic Cl^- secretion in the same tissue (Hennig and Diener, 2009). Theoretically, the negative Isc induced by cysteine might also be caused by the activation of K^+ secretion, as both an inhibition of anion secretion as well as a stimulation of cation secretion exert the same effect on the transepithelial potential difference, i.e., the serosal side gets less positive. However, none of the K^+ channel blockers tested (Ba^{2+}, tetrapentylammonium, or charybdotoxin),

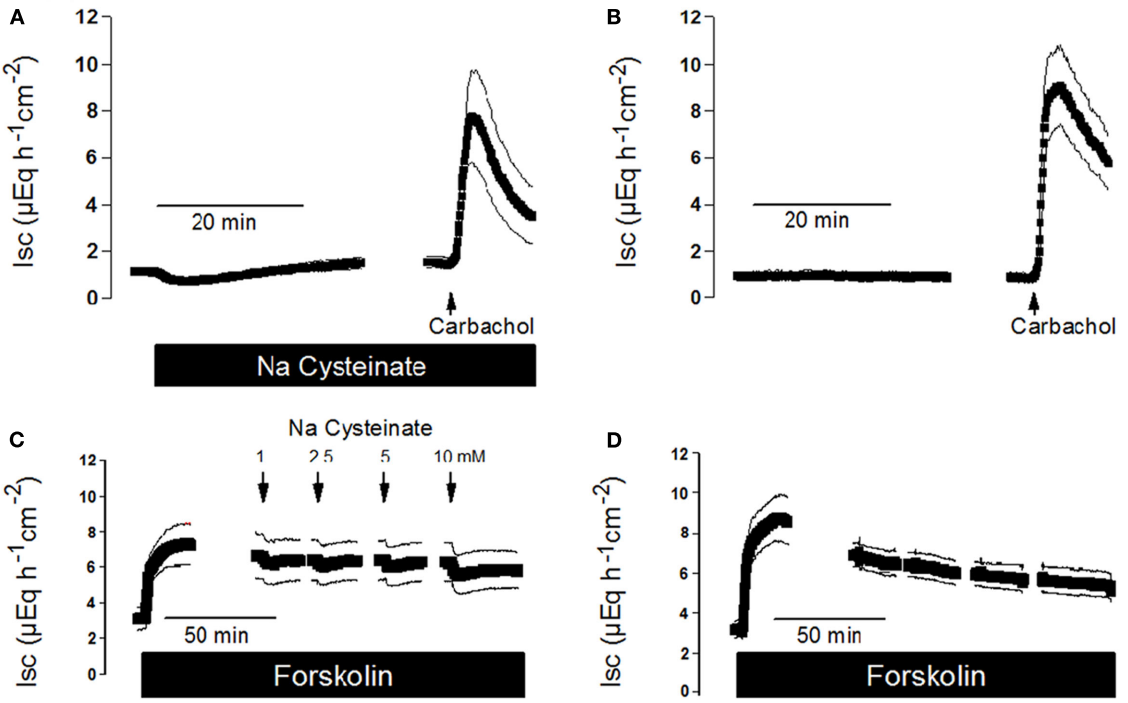

FIGURE 5 | (A) Interaction of Na cysteinate with the Isc evoked by secretagogues. Pretreatment with Na cysteinate (5×10^{-3} mol·l^{-1} at the serosal side; black bar) slightly reduced the increase in Isc evoked by carbachol (5×10^{-5} mol·l^{-1} at the serosal side; arrow) in mucosa–submucosa preparations of rat distal colon compared to a time-dependent control not treated with Na cysteinate **(B)**. **(C)** Na cysteinate (10^{-3} to 10^{-2} mol·l^{-1} at the serosal side; arrows) caused transient reductions in the Isc evoked by forskolin (5×10^{-6} mol·l^{-1} at the mucosal and the serosal side; black bar) compared to a time-dependent control not treated with Na cysteinate **(D)**. Line interruptions are caused by omission of time intervals of 5–10 min in order to synchronize the tracings of individual records to the administration of drugs. Values are means (symbols) ± SEM (lines), $n = 6$. For statistics, see text.

when administered at the mucosal side in order to block apical K^+ channels mediating apical efflux of K^+ during potassium secretion, had any effect on the decrease in Isc induced by free cysteine suggesting that an induction of K^+ secretion is not involved in the effect of cysteine (**Table 4**).

In contrast, two of these K^+ channel blockers, i.e., Ba^{2+} and tetrapentylammonium, when administered at the serosal side in order to block basolateral K^+ channels, reversibly inhibited the Isc response evoked by free cysteine (**Table 4**). This observation would fit to a mechanism, in which free cysteine reduces basolateral K^+ conductance. This K^+ conductance, which is dependent on the number of K^+ channels, their single channel conductance and their open probability, keeps the membrane potential at hyperpolarized values. If the K^+ channels involved are already inhibited by one of the K^+ channel blockers, this action of cysteine is no more possible. In the case of free cysteine, the observed cytosolic acidification (**Figure 3A**) might – at first glance – offer a reasonable explanation for the inhibition of basal Cl^- secretion by free cysteine, as epithelial K^+ conductance is known to be sensitive to a fall in cytosolic pH (Diener and Scharrer, 1994). Any inhibition of K^+ conductance, will reduce the driving force for Cl^- exit across the apical membrane via apical anion channels and thereby lead to an inhibition of transepithelial anion secretion.

This acidification and the presumed inhibition of cellular K^+ conductance is probably the reason why the second, prosecretory response evoked by the pH-neutral form of the amino acid, Na cysteinate, is missing in the case of free cysteine. Nevertheless, a fall in pH cannot be the reason for the initial inhibition in basal anion secretion evoked by Na cysteinate (see fall in Isc, e.g., in **Figure 1B**), as Na cysteinate does not affect cytosolic pH in the epithelial cells (**Figure 3B**). As this fall in Isc as well as the increase in Isc evoked by Na cysteinate was sensitive to inhibition of H_2S-synthesizing enzymes (see Results), these results suggest that the functional response induced by this gasotransmitter depends on the velocity of the H_2S release and/or the local concentration reached. This is supported by the comparison with compounds known to release H_2S at different velocity. Whereas the very slowly releasing compound GYY 4137 never induced an increase in Isc, but only evoked a prolonged fall in baseline Isc (**Figure 4A**), diallyl trisulfide only increased Isc, and the rapidly releasing molecule NaHS polyphasically changed the current across the colonic epithelium (**Figure 4B**). Consequently, the local concentration of H_2S seems to critically determine whether this gasotransmitter exerts a prosecretory or an antisecretory action in the gastrointestinal mucosa.

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Hydrogen sulfide: role in ion channel and transporter modulation in the eye

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Hydrogen sulfide (H_2S), a colorless gas with a characteristic smell of rotten eggs, has been portrayed for decades as a toxic environmental pollutant. Since evidence of its basal production in mammalian tissues a decade ago, H_2S has attracted substantial interest as a potential inorganic gaseous mediator with biological importance in cellular functions. Current research suggests that, next to its counterparts nitric oxide and carbon monoxide, H_2S is an important multifunctional signaling molecule with pivotal regulatory roles in various physiological and pathophysiological processes as diverse as learning and memory, modulation of synaptic activities, cell survival, inflammation, and maintenance of vascular tone in the central nervous and cardiovascular systems. In contrast, there are few reports of a regulatory role of H_2S in the eye. Accumulating reports on the pharmacological role of H_2S in ocular tissues indicate the existence of a functional trans-sulfuration pathway and a potential physiological role for H_2S as a gaseous neuromodulator in the eye. Thus, understanding the role of H_2S in vision-related processes is imperative to our expanding knowledge of this molecule as a gaseous mediator in ocular tissues. This review aims to provide a comprehensive and current understanding of the potential role of H_2S as a signaling molecule in the eye. This objective is achieved by discussing the involvement of H_2S in the regulation of (1) ion channels such as calcium (L-type, T-type, and intracellular stores), potassium (K_{ATP} and small conductance channels) and chloride channels, (2) glutamate transporters such as EAAT1/GLAST and the L-cystine/glutamate antiporter. The role of H_2S as an important mediator in cellular functions and physiological processes that are triggered by its interaction with ion channels/transporters in the eye will also be discussed.

Keywords: cystine/glutamate antiporter, EAAT/GLAST transporter, hydrogen sulfide, ion channels, ocular tissues, cysteine transporter

INTRODUCTION

The potential role of hydrogen sulfide (H_2S) as a regulatory mediator has stimulated a surge of interest in its biological significance in cellular functions in the human body. This colorless gas, known for decades only as a toxic environmental pollutant has been found to be produced in substantial amounts in mammalian tissues. The endogenous production of H_2S in mammalian tissues is dependent on the activity of two pyridoxal-5′-phosphate dependent-enzymes, cystathionine β-synthase (CBS; EC 4.2.1.22) and cystathionine γ-lyase (CSE; EC 4.4.1.1). Both CBS and CSE are enzymes of the trans-sulfuration pathway that inter-converts L-methionine and L-cysteine but can also use L-cysteine as an alternative substrate to form H_2S (Stipanuk and Beck, 1982; Erickson et al., 1990; Swaroop et al., 1992). Recently, a newly identified enzyme, 3-mercaptopyruvate sulfurtransferase (3MST), has been reported to be involved in the production of H_2S (Shibuya et al., 2009a,b). Current biomedical research suggest that H_2S is an important gasotransmitter in mammals, and is involved in several physiological and pathophysiological processes as diverse as learning and memory, inflammation, and the regulation of blood pressure (Abe and Kimura, 1996; Lowicka and Beltowski, 2007). In the cardiovascular system, H_2S has been shown to play a pivotal role in maintenance of vascular tone (Hosoki et al., 1997; Zhao et al., 2001; Cheng et al., 2004; Webb et al., 2008) whereas in the central nervous system (CNS) this gas was found to exert a neuroprotective role on neurons and exhibit neurotransmitter-like function in the modulation of synaptic activities (Zhao et al., 2001; Kimura, 2002; Kimura et al., 2005, 2006; Szabo, 2007; Qu et al., 2008; Webb et al., 2008). Many of the cellular effects of H_2S in the vasculature and brain have been reported to be mediated by ion channels and transporters.

There is ample evidence that H_2S targets different ion channels to modulate varied physiological functions. Extensive studies in the vasculature and CNS demonstrate that H_2S interacts with ion channels such as ATP-sensitive potassium (K_{ATP}) channels, calcium (Ca^{2+}) and chloride (Cl^-) channels to regulate vascular tone, and exert its neurotransmitter and neuroprotective-like properties (Kimura and Kimura, 2004; Tang et al., 2010). In addition, there is evidence that the neuromodulatory role of H_2S in cellular functions and physiological processes are triggered by

its interaction with several transporter systems. H_2S has been reported to enhance the activity of transporters, thereby facilitating the release of antioxidants that are essential for neuronal protection against excitotoxic damage (Lu et al., 2008; Kulkarni et al., 2009; Kimura, 2011a,b). Furthemore, through its interaction with transporters, H_2S plays an important role in maintaining the redox balance and thus serves both as a neuroprotectant and neuromodulator.

In contrast to the central nervous and cardiovascular systems, there are few reports of the involvement of H_2S in the regulation of ion channels and transporters in the eye. Given the important modulatory effects of H_2S on different ion channels and transporter systems in cellular functions and disease conditions in the central nervous and cardiovascular systems, there is a great need for studies centered on the potential role of H_2S as a signaling molecule in ocular tissues. Indeed, we have evidence that H_2S can induce pharmacological effects in mammalian ocular tissues, alter sympathetic and glutamatergic neurotransmission, and play a regulatory role in signal transduction processes in the eye (Monjok et al., 2008; Opere et al., 2009; Njie-Mbye et al., 2010; Ohia et al., 2010). The presence of CBS and CSE, the biosynthetic enzymes for H_2S have also been reported in several ocular tissues, (Persa et al., 2006; Pong et al., 2007) indicating the existence of a functional trans-sulfuration pathway and a potential physiological role for H_2S as a gaseous neuromodulator in the eye. Understanding the regulatory role of H_2S in ion channel and transporter modulation in the eye is critical to our expanding knowledge of this gasotransmitter in ocular neuropathies. In this review article we will discuss the interaction of H_2S with different types of ion channels and transporter systems found in the eye. Our attention will be particularly devoted on the role of H_2S as a molecule able to trigger cell signaling in ocular tissues.

HYDROGEN SULFIDE AND THE EYE

Evidence from literature supports the presence of a functional trans-sulfuration pathway and a potential physiological/pharmacological relevance for H_2S in the mammalian eye (De et al., 1974; Persa et al., 2006; Pong et al., 2007; Kulkarni et al., 2011). CBS and CSE, the primary enzymes of the trans-sulfuration pathway have been localized in mammalian ocular tissues (De et al., 1974; Persa et al., 2006; Pong et al., 2007). Moreover, deficiency of CBS has been linked to ocular disorders such as lens dislocation, retina degeneration, retinal detachment and acute glaucoma, (Kraus and Kozich, 2001) suggesting a physiological significance for this pathway in ocular tissues. Further support for a physiological relevance of H_2S in mammalian ocular tissues was provided by us when we demonstrated the endogenous production of H_2S in bovine ocular tissues (Kulkarni et al., 2011). Interestingly, the magnitude of H_2S content corresponded to the reported expression of CBS and CSE enzymes in ocular tissues (De et al., 1974; Persa et al., 2006; Pong et al., 2007). In bovine retina, both CSE and CBS antagonists, propargyglycine (PAG), and aminooxyacetic acid (AOA) attenuated while the CBS stimulator, S-adenosyl-L-methionine (SAM) enhanced endogenous production of H_2S, (Kulkarni et al., 2011) corroborating the involvement of these trans-sulfuration pathway enzymes in the production of H_2S in retina. In addition to its *in situ* production, there is evidence supporting a pharmacological role for this gasotransmitter in mammalian ocular tissues (**Figure 1**). In the anterior uvea, we observed an inhibitory action of H_2S (using sodium hydrosulfide, NaHS, and/or sodium sulfide, Na_2S as donors) on both electrically evoked [^{3}H]NE (norepinephrine) release and endogenous catecholamine concentrations in porcine iris-ciliary body in a concentration-dependent manner (Kulkarni et al., 2009). The inhibitory action of H_2S donors on NE release was reversed by CBS and CSE antagonists, AOA and PAG respectively, suggesting that H_2S attenuates sympathetic neurotransmission from isolated porcine anterior uvea by an effect that is partially dependent on its intramural biosynthesis. Moreover, H_2S donors may exert their inhibitory action on sympathetic neurotransmission by a direct effect of this gasotransmitter on endogenous neurotransmitter release (Kulkarni et al., 2009). In another study, H_2S donors exhibited an inhibitory action on carbachol-induced tone in isolated porcine irides that was dependent on endogenous production of prostanoids and the biosynthesis of H_2S by CBS (Monjok et al., 2008). Whereas the nitric oxide (NO) synthase inhibitor, N (G)-nitro-L- arginine methyl ester (L-NAME) had no effect, the K_{ATP} channel inhibitor, glibenclamide (100 and 300 μM), blocked relaxations induced by NaHS, suggesting the involvement of K_{ATP} channels on the H_2S on response in the anterior uvea (Monjok et al., 2008). In porcine irides, we observed an inhibitory action of L-cysteine (H_2S substrate) that was dependent upon the endogenous production of H_2S by CBS and CSE and was mediated by prostanoids and K_{ATP} channels (Ohia et al., 2010). Taken together, these data support a pharmacological role for H_2S in the anterior uvea. So far, the potential therapeutic implications of the action of H_2S in these tissues have not been fully elucidated. In preliminary studies, H_2S donors reduced intraocular pressure (IOP) in normotensive rabbits (*Ohia et al., US Patent #8,092,838, Jan 10, 2012*). Similarly, the H_2S-hybrid molecule ACS67 significantly reduced IOP in glaucomatous rabbits (Perrino et al., 2009) suggesting a potential application for H_2S in the regulation of IOP. In spite of these findings, the exact role of the trans-sulfuration pathway in the anterior uvea and the mechanisms by which H_2S regulates IOP remain unknown and merit further investigation.

In addition to the anterior uvea, pharmacological actions have been reported for H_2S in mammalian retina as well (**Figure 1**). H_2S donors inhibited amino acid neurotransmission from both isolated bovine and porcine retina by an effect that was dependent, at least in part, on intramural biosynthesis of H_2S (Opere et al., 2009). Moreover, the gasotransmitter enhanced cyclic AMP production in bovine and porcine isolated neural retina and retinal pigment epithelial (RPE)-J cells by mechanisms that were dependent on biosynthesis of H_2S by CBS and CSE and partially dependent on activation of the K_{ATP} channels (Njie-Mbye et al., 2010, 2012). Because an increase in retinal glutamate concentrations has been linked to retinal excitotoxicty, the ability of H_2S to reduce glutamate release suggest a potential neuroprotective action in retinal neurons. Several investigators have since confirmed the neuroprotective effect of H_2S in retina (Biermann et al., 2011; Mikami et al., 2011). Indeed, H_2S donors protected mice retinal neurons from light-induced degeneration

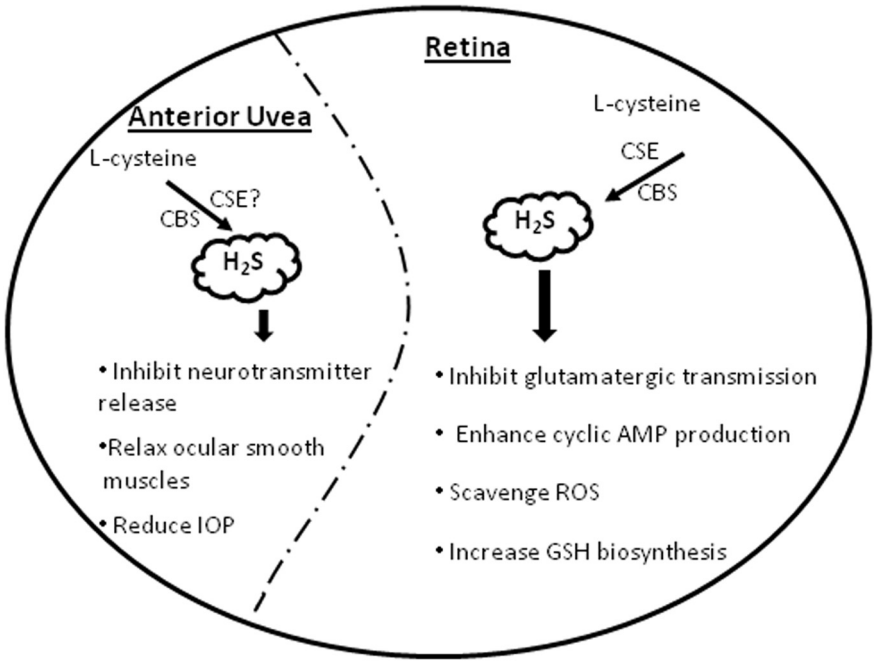

FIGURE 1 | A schematic representation summarizing the physiological and pharmacological effects of H_2S in the eye. GSH = glutathione, ROS = Reactive oxygen species, IOP = intraocular pressure.

(Mikami et al., 2011). Similarly, H_2S preconditioning conferred to protection of rat retina exposed to ischemia reperfusion injury (Biermann et al., 2011). The H_2S-hybrid, ACS67 increased reduced glutathione levels, suggesting a potential neuroprotective role for this H_2S-donor (Osborne et al., 2010). It is now apparent that H_2S plays a dual role in biological tissues, being cytotoxic at higher and cytoprotective at lower concentrations of the gas (Martelli et al., 2010). The latter action, which has been demonstrated in various cell types and neurons, (Kimura et al., 2006; Sivarajah et al., 2006; Elrod et al., 2007) is partially ascribed to its ability to scavenge several reactive oxygen species (e.g., such as superoxide radical anion, hydrogen peroxide, peroxynitrie and hypochlorite) and increase GSH biosynthesis (Martelli et al., 2010). Several questions remain to be addressed, such as the role of molecular targets of H_2S such as K_{ATP} channels in its neuroprotective action of H_2S; integration of the trans-sulfuration pathway in retinal neurotransmitter pathways; interaction of H_2S and transporters and other ion channels in the eye. Based upon the known pharmaocological role and protective mechanisms of H_2S in biological systems, it is conceivable that H_2S could find a significant application in ocular neuropathies, thereby opening up new molecular targets for management of ocular diseases.

REGULATION OF ION CHANNELS BY H_2S IN THE EYE

CALCIUM CHANNELS (Ca^{2+}) IN OCULAR TISSUES

Calcium (Ca^{2+}) is an essential ion that is involved in the regulation of several processes in the body such as signal transduction pathways, contraction, secretion, blood coagulation, gene expression, apoptosis, necrosis, cell division, and endocytocis (Williams, 1974; Shuttleworth, 1997; Berridge, 2005; Carafoli, 2005; Wimmers et al., 2007). Within the cell, free intracellular $[Ca^{2+}]_I$ content is tightly regulated at about 100 nM to maintain a steep inwardly directed concentration and electrochemical gradients across the cell membrane by an interplay of several Ca^{2+} channels, pumps, transporters, buffering systems and intracellular storage organelles (Bogeski et al., 2011). Several ion channels facilitate transmission of $[Ca^{2+}]$ ions across the membranes: the voltage-gated calcium channels (CaV), transient receptor potential (TRP) ion channels, transmitter-gated Ca^{2+} permeant ion channels and the store operated Ca^{2+} entry (SOCE) and Ca^{2+} released-activated Ca^{2+} (CRAC/Orai) channels (Bogeski et al., 2011). Although various potential molecular targets for calcium channels have been identified, only the L-type voltage-activated calcium channels have found wide therapeutic beneficial application. There is evidence in the eye for the existence of a calcium transport system. Ca^{2+} has been reported to play a key role in mammalian lens physiology and pathology. Excessive levels of Ca^{2+} have been implicated in cortical cataract and there is presence of Ca^{2+} linked receptors in the lens (Rhodes and Sanderson, 2009). Voltage-gated Ca^{2+} channels: transient (T-type) and dihydropyridine-sensitive long-lasting (L-type) channels, have been reported to be expressed in muller cells of the retina (Puro and Mano, 1991; Puro et al., 1996; Bringmann et al., 2000). The retinal pigment epithelium (RPE) has also been reported to expresses voltage- and ligand-gated Ca^{2+}conducting channels (Wimmers et al., 2007). These channels

act as regulators of secretory activity, and thus contribute to RPE function. Changes in Ca^{2+} channel function, or activity has been shown to lead to degenerative diseases of the retina (Wimmers et al., 2007).

EFFECTS OF H_2S ON CALCIUM CHANNELS (Ca^{2+}) IN OCULAR TISSUES

Despite the implication of Ca^{2+} in ocular physiology and pathology, there is a great need for studies centered on the regulatory role of H_2S and its interaction with calcium channels. Only one study to date, has addressed the possible interaction of H_2S and Ca^{2+} channels in the eye. In this study the authors' report that the production of H_2S in retinal neurons is regulated by intracellular Ca^{2+}, (Mikami et al., 2011) and in turn H_2S can suppress Ca^{2+} channels by activating vacuolar type H^+-ATPase (V-ATPase). Furthermore, the study also demonstrated that H_2S can suppress the elevation of Ca^{2+} in photoreceptor cells by activating V-ATPase in horizontal cells and thus maintain Ca^{2+} homeostasis. From these observations, the authors conclude that H_2S protects photoreceptor cells from the insult caused by excessive levels of light. Clearly results from this study provides a new insight into the regulation of H_2S production and the modulatory interaction of H_2S and Ca^{2+} channels in retinal transmission. In addition, the study postulates a cytoprotective effect of H_2S on retinal neurons and provides a basis for the therapeutic target for retinal degeneration. Increasing knowledge about the properties of Ca^{2+}channels in ocular tissuess especially the retina will not only provide a new understanding of ocular function but could also provide a better understanding of the role of H_2S in ocular health and vision.

POTASSIUM (K^+) CHANNELS IN OCULAR TISSUES

Potassium ion (K^+) channel family represents one of the most prominent and ubiquitous ion channels in living organisms where their physiological role range from regulation of the action potentials in excitable cells to regulation of transepithelial transport processes, intracellular pH, cell survival and growth factor secretion in non-excitable cells (Ashcroft and Gribble, 1999; Bauer et al., 1999; MacDonald and Wheeler, 2003; Warth, 2003; Masi et al., 2005). K^+ channel family consists of four subfamilies, the inwardly rectifying K^+ (K_{ir})-channels, voltage-gated K^+ channels, Ca^{2+}-activated K^+ channels, and two-pore or leak K^+-channels that are classified based upon number of transmembrane domains and electrophysiological properties. In the eye, K^+ channels play central roles in maintaining ion, fluid balance and membrane potential. Several K^+ channel subtypes such as voltage-gated K^+ (Kv) channels and 4-aminopyridine (4-AP)-sensitive K^+ channels are expressed in mammalian corneal epithelial cells (Rae, 1985; Rae et al., 1990; Rae and Farrugia, 1992). Studies have shown that changes in K^+ channel activity modulate essential corneal epithelial functions needed for tissue homeostasis (Wolosin and Candia, 1987; Klyce and Wong, 1977). Furthermore, emerging evidence suggest that K^+ channels play a crucial role in controlling apoptosis and proliferation in corneal epithelial cells (Lu et al., 2003; Roderick et al., 2003). Three major potassium currents (an outwardly rectifying current, an inwardly rectifying current, and a calcium-activated current) have been characterized in several mammalian lens epithelial cells (Rae, 1986; Cooper et al., 1991). These potassium conductances are essential for the maintenance of lens volume and transparency. Inwardly rectifying potassium (Kir) channel was reported to be highly expressed in bovine and human trabecular meshwork cells, (Llobet et al., 2001) as well as muller glial cells of the retina (Kofuji et al., 2002). K^+ channels (Kv11; ether à-go-go related gene; erg) belonging to the family of voltage-gated K^+ channels are present in mouse and human retina with the most abundant expression in rod bipolar cells. These channels are also found in the inner and outer plexiform layer, inner segments of photoreceptors, as well as the retina pigment epithelium (Cordeiro et al., 2011). These channels are vital for the control of the membrane potential in retinal neurons. Given the importance of K^+ channel modulation in ocular tissues, evidence of an interaction between H_2S and these channels in the eye is imperative for understanding the role of H_2S as a signaling molecule in ocular functions.

EFFECTS OF H_2S ON POTASSIUM (K^+) CHANNELS IN OCULAR TISSUES

The pharmacological effects of H_2S in the vasculature and brain, has been reported to involve K^+ channels. To the best of our knowledge there are no studies in the literature pertaining to the effects of H_2S on K^+ channels in the eye, except for those generated from our laboratory. In previous studies we have demonstrated that the pharmacological effects of H_2S (using H_2S–releasing compounds) in ocular tissues are partly mediated by K_{ATP} channels (Monjok et al., 2008; Kulkarni et al., 2009; Opere et al., 2009; Ohia et al., 2010). With the use of specific channel blockers, we report that H_2S interacts with K_{ATP} channels to relax ocular smooth muscle, and alter sympathetic and glutamergic neurotransmission in the anterior uvea and retina (Monjok et al., 2008; Kulkarni et al., 2009; Opere et al., 2009; Ohia et al., 2010). Furthermore, we recently show that K_{ATP} channels are involved in the regulatory role of H_2S in signal transduction processes in retina pigment epithelium cells (Njie-Mbye et al., 2012). It is reported that K^+ channels play vital roles in cellular functions including vascular tone regulation, mediating neurotransmitter release, and neuroprotection in cardiovascular and CNSs (Yamada and Inagaki, 2005). Thus it is tempting to speculate a physiological role of H_2S in ocular tissues that involves the activation of K^+ channels.

CHLORIDE (Cl^-) CHANNELS IN OCULAR TISSUES

Chloride (Cl^-) is one of the most prominent anions in the body that is involved in the regulation of a variety of important physiological and cellular functions such as volume homeostasis, organic solute transport, cell migration, cell proliferation, cell differentiation, and apoptosis. Unlike most physiological ions whose levels are tightly regulated within a limited range, the resting Cl^- ion concentration varies in different mammalian cell types and in developing cells (Wimmers et al., 2007). Cl^- conductance across membranes is facilitated by several pumps and co-transporters that are localized in plasma membranes and membranes of intracellular organelles. For example, chloride influx is facilitated by the $Na^+/K^+/Cl^-$ co-transporters, Cl^-/HCO_3-exchangers, and $Na^+/-Cl^-$ co-transporters while efflux is achieved by the cell $K^+/-Cl^-$co-transporters and Na^+-dependent Cl^-/ HCO_3 – exchanger. Other channels and transporters expressed in intracellular

membranes as well as Cl^- -binding proteins regulate intravesicular pH and Cl^- concentration (Duran et al., 2010). Several channels mediate passive flow of Cl^- ions across membranes. With exception of the transmitter-gated GABA and glycine receptors, these Cl^- channels are broadly classified into five subfamilies, the voltage-sensitive ClC subfamily, calcium-activated channels, high- (maxi) conductance channels, the cystic fibrosis transmembrane conductance regulator (CFTR) and volume-regulated channels (Verkman and Galietta, 2009). So far, only the voltage-sensitive ClC subfamily, CFTR and the transmitter-gated channels have been well described. In general, Cl^- channels are fairly non-selective for inorganic ions. Dysfunctional Cl^- channels have been linked to channelopathies such as myotonia congenita and cystic fibrocis (Duran et al., 2010). Cl^- channels are more abundantly expressed in the anterior segment of the eye due to Cl^- being the principal anion of aqueous humor secretion. Studies show that chloride efflux plays an important role in aqueous humor production and chloride channels present in the ocular ciliary epithelium are involved in aqueous humor homeostasis. Chloride currents have been reported to be present in bovine non-pigmented ciliary epithelium (NPE) and in transformed cultured human NPE (Chen and Sears, 1997). High-(maxi) conductance chloride channels are expressed in ciliary pigmented epithelial (PE) cells, (Do et al., 2004) whilst cAMP-activated Cl^- channels are present in the basolateral membrane of nonpigmented (NPE) ciliary epithelium (Edelman et al., 1995). CFTR is functionally expressed in corneal and conjunctival epithelium, corneal endothelium, and RPE (Shiue et al., 2002; Sun and Bonanno, 2002; Turner et al., 2002; Blaug et al., 2003; Levin and Verkman, 2005; Reigada and Mitchell, 2005). CFTR expression patterns in these tissues suggest the involvement of these chloride channels in regulation of tear film volume, corneal hydration and transparency, aqueous humor volume and IOP, and subretinal compartment size and ionic composition. In the retina, several Cl^- transporter and channels including the $Na^+/K^+/Cl^-$ cotransporters, CFTR, and the voltage-sensitive ClC subfamily were reported to be highly expressed in the pigment epithelial layer (Zhang et al., 2011).

EFFECT OF H_2S ON CHLORIDE (Cl^-) CHANNELS IN OCULAR TISSUES

The activation of Cl^- channels by H_2S in the CNS has been shown as a protective mechanism for neurons from oxytosis (Tang et al., 2010). Electrophysiological evidence demonstrates that H_2S interacts with Cl- channels in the vasculature (Tang et al., 2010). To the best of our knowledge there are no studies reporting the interaction of H_2S with Cl^- channels in the eye. The observation of the presence of chloride channels in ocular tissues especially in the anterior uvea, coupled with evidence of channel activation by H_2S in non-ocular tissues, suggest possible regulation of chloride fluxes by H_2S in the eye with neuroprotective consequences and IOP lowering effects.

REGULATION OF TRANSPORTERS BY H_2S IN THE EYE

EXCITATORY AMINO ACID TRANSPORTER/GLUTAMATE ASPARTATE TRANSPORTER (EAAT/GLAST) IN OCULAR TISSUES

Glutamate is the major excitatory neurotransmitter in the mammalian CNS. Under normal physiological conditions, extracellular glutamate is tightly regulated (resting levels $\leq 1\,\mu M$) by five distinct excitatory amino acid transporters, EAAT1 (glutamate/aspartate transporter [GLAST]); EAAT2 (glutamate carrier [GLT-1]), EAAT3 (excitatory amino acid carrier 1 [EAAC1]), EAAT4 and EAAT5 (Zerangue and Kavanaugh, 1996; Levy et al., 1998). Excessive extracellular glutamate is known to lead to excitotoxicty in neuronal tissues. Thus, EAAT transporters play the essential role of rapidly terminating synaptic transmission, maintaining low ambient extracellular glutamate while simultaneously conserving neuronal glutamate for reuse via the glutamate-glutamine cycling system (Copenhagen et al., 2002; Zou and Crews, 2005). Glutamate transporter uptake activity is accompanied by a net inward movement of positive ions ($3Na^+$:$1H^+$ co-transport versus K^+ counter-transport) (Kanner, 2006) and could be coupled to Na, K-ATPase pump (Rose et al., 2009). Glutamate transporters exhibit differential distribution in different tissues. In the mammalian retina, using immunocytochemical studies, EAAT1 (GLAST) has been shown to be localized in Muller cells (Rauen et al., 1996; Pow and Barnett, 1999). EAAT2 have been identified in cone photoreceptor and bipolar cells (Rauen and Kanner, 1994) while EAAT3 (EAAC1) in inner retinal neurons (Rauen et al., 1996). EAAT4 is localized in retinal astrocytes (Ward et al., 2004) and EAAT5 in photoreceptors, bipolar cells and in some muller cells (Arriza et al., 1997; Pow and Barnett, 2000). Interestingly, EAAT5 is found exclusively in retina (Pow and Barnett, 2000). The sodium-dependent glutamate–aspartate transporter (GLAST or EAAT1) is the major glutamate transporter in muller cells. This glutamate transporter (EAAT1/GLAST) maintains extracellular glutamate at a low level to ensure a high signal-to-noise ratio for glutamatergic neurotransmission and thus shield neurons from excitotoxic damage. To the best of our knowledge there are no studies till date that have examined the effect of H_2S on glutamate transporter in ocular tissues. Only one study, by our laboratory has demonstrated that H_2S donors caused an attenuation of glutamatergic transmission in mammalian retinae (Opere et al., 2009). Although the exact mechanism of action is not clear, it is feasible that H_2S donors could decrease glutamatergic transmission in mammalian retinae due to involvement of EAAT.

CYSTINE/GLUTAMATE ANTIPORTER (SYSTEM X_C^-) IN OCULAR TISSUES

The cystine/glutamate antiporter (System x_C^-) is responsible for the Na^+ independent electroneutral exchange of cystine and glutamate. It is a member of the heteromeric amino acid transporter family which is composed of a heavy subunit and a corresponding light subunit linked by a disulfide bridge (Lim and Donaldson, 2011). System x_C^- is regulated by extra- and intracellular gradients of glutamate which drives the import of cystine coupled to export of glutamate (Fiorucci et al., 2006; Lowicka and Beltowski, 2007). System x_C^- has two major functions. First, it mediates cellular uptake of cystine for the maintenance of intracellular levels of glutathione, essential for protection of cells from oxidative stress. Second, it is instrumental in maintaining the redox balance between extracellular cystine and cysteine (Lo et al., 2008). In the eye, system x_C^- has been identified in veterbrate lens) (Lim et al., 2005) and different parts of the mamamlian retina including

the retinal endothelial cells, outer plexiform of retina, muller cells, retinal pigment cells and retinal ganglion (Kato et al., 1993; Bridges et al., 2001; Hosoya et al., 2002; Tomi et al., 2002; Dun et al., 2006). Oxidative damage of proteins is believed to underlie several major eye diseases such as age related nuclear (ARN) cataract, age related macular degeneration (AMD) and diabetic retinopathy (Lo et al., 2008). GSH, a major and potent antioxidant in the cells may prevent or slow down the progression of such diseases by protecting the thiol groups of proteins and minimizing oxidation-induced protein aggregation formation. However, oxidative stress alters rate of conversion of cysteine to glutathione and leads to depletion of glutathione levels (Fiorucci et al., 2006). System x_C^- plays an important role in maintaining elevated intracellular levels of glutathione and serves as a potential therapeutic target for a number of ocular diseases.

EFFECTS OF H_2S ON CYSTINE/GLUTAMATE ANTIPORTER (SYSTEM X_C^-) IN OCULAR TISSUES

In the CNS, H_2S demonstrates cytoprotective effect by protecting neurons and astrocytes, major type of glial cells from oxidative stress (Kimura, 2011a,b). H_2S enhances the activity of system x_C^- and thus significantly increases the transport of cystine into neurons to increase the levels of substrate cysteine, for glutathione synthesis. Even in the presence of glutamate, H_2S significantly reverses the inhibition of cystine transport by glutamate (Kimura and Kimura, 2004). Based on the conclusions from these studies (Kimura and Kimura, 2004; Kimura, 2011a,b) in CNS, it will be interesting to investigate the effect of H_2S on system x_C^- transporter in astrocytes and muller cells in retina under oxidative stress. Since, muller cells are the primary sites of glutathione localization in the retina, and the retina is extremely vulnerable to oxidative stress, understanding the function of H_2S on system x_C^- in muller cells could play a pivotal role in protecting the retina from a variety of retinal diseases, such as diabetic retinopathy, age-related macular degeneration, and glaucoma. In the eye, there is evidence from few studies that demonstrate an increase in GSH production, following application of H_2S releasing drugs such as ACS67, ACS1. Although the mechanism of action is not clear, the authors suggest that intracellular cysteine levels are enhanced indirectly to form GSH by H_2S stimulation of glutamate/cystine antiporters (Sparatore et al., 2009; Perrino et al., 2009; Osborne et al., 2010). Moreover, as mentioned above, a study by Opere and Ohia (1997) had demonstrated the inhibitory action of H_2S donors on glutamatergic transmission in mammalian retinae. So, it is plausible that H_2S donors can render there cytoprotective effects by upregulating system x_C^- transporter in the retina and thereby increasing the production of glutathione. As the exact mechanism of action needs to be investigated, there is very demanding need to understand the potential role of H_2S on the system x_C^- transporter in the eye.

CYSTEINE TRANSPORTER IN OCULAR TISSUES

Cysteine transporters are widely distributed in various cell types including the muller cells of retina. Cysteine transporters readily import cysteine into the cell for direct conversion to glutathione

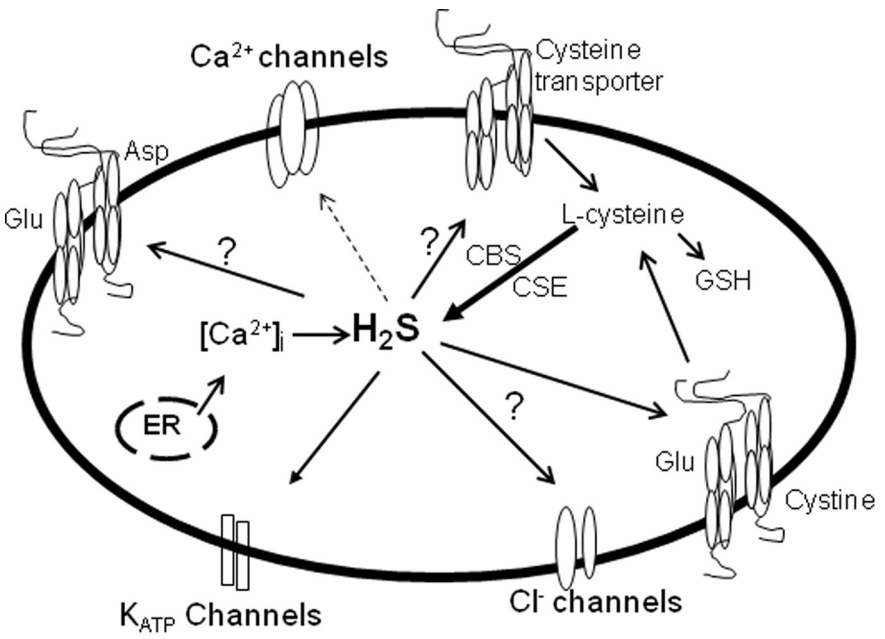

FIGURE 2 | The effects of H_2S on ion channels and transporters in the eye. Solid arrow, stimulatory; dotted arrow, inhibitory. Studies on the effects of ion channels and transporters in the eye and/or their interaction with H_2S is lacking. H_2S which is formed by CBS and CSE activities, stimulates K_{ATP} channels and the cystine/glutamate antiporter thereby regulating ocular smooth muscle relaxation, neurotransmitter release and oxidant/antioxidant balance (solid arrow). The production of H_2S can be regulated by intracellular Ca^{2+}, and in turn H_2S can suppress Ca^{2+} channels to exert its neuronal effects (dotted arrow). Whether H_2S activates or inhibits glutamate aspartate transporter, cysteine transporter and Cl^- channels remains to be determined.

(Bringmann et al., 2009; Mathai et al., 2009; Kimura, 2010). So far only one study by Kimura (2010) on brain cortex has shown the effect of H_2S on cysteine transporters. The study demonstrates that H_2S (acting as a reducing agent) reduces cystine into cysteine in the extracellular space and makes cells efficiently transport cysteine into cells for GSH production. Moreover authors state that, the contribution of cysteine transport to the production of GSH is much greater than that of cystine transport. This production of GSH is further enhanced by H_2S under conditions of oxidative stress caused by excessive glutamate toxicity. To the best of our knowledge there is a lack of studies on cysteine transporters in the eye and no evidence of the effect of H_2S on these transporters in ocular tissues. Although the production of H_2S in the eye is not well understood as there is only one study, from our laboratory showing that this gasotransmitter is endogenously produced in ocular tissues (Kulkarni et al., 2011), it is possible that the release of H_2S may regulate the transport of cysteine in ocular tissues, thus facilitating the production of GSH. This increase in the levels of GSH by H_2S may contribute to the potential protective effect of H_2S.

CONCLUDING REMARKS

Current evidence suggests that ion channels and transporters are present in ocular tissues and are involved in the regulation of vital cellular functions related to vision processes (**Figure 2**). However, the interaction of these signaling cascades with H_2S in the eye is lacking. Clearly, there exists ample evidence that portrays the critical role H_2S plays in physiological and pathophysiological processes in the human body. Furthermore, there is enough data that demonstrates that H_2S targets different ion channels and transporters to modulate varied physiological functions in the central nervous and cardiovascular systems. Our current knowledge of such interactions in these systems should help facilitate research targeted on investigating the neuromodulatory role of H_2S in the eye and its interaction with ion channels and transporters that are play pivotal roles in the preservation of vision. Indeed, future studies are warranted to examine the pharmacological effects of H_2S on different types of ion channels and transporters in ocular tissues. Altered effects of H_2S on ion channels and transporters, under different pathophysiological conditions in the eye also calls for intensive investigation.

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Metabolic turnover of hydrogen sulfide

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More than 15 years have passed since hydrogen sulfide (H_2S) emerged as a biological signaling molecule – initially, in the nervous and vascular systems through the modulation of *N*-methyl D-aspartate (NMDA) receptors and the activation of transmembrane receptor potential (TRP)- and ATP-dependent K^+ (K_{ATP})-channels (Abe and Kimura, 1996; Hosoki et al., 1997; Dello Russo et al., 2000; Zhao et al., 2001; Teague et al., 2002; Nagai et al., 2004; Streng et al., 2008), and later, in nearly every organ system (Predmore and Lefer, 2010). H_2S was found to play a role as a cytoprotectant in the nervous system (Kimura and Kimura, 2004; Whiteman et al., 2004); this finding led to the discovery of the cardioprotective effect of H_2S (Elrod et al., 2007) (**Figure 1**). Previous studies have also described that H_2S plays several roles: as a regulator of insulin release, inflammation, and angiogenesis, and as an oxygen (O_2) sensor (Li et al., 2005; Yang et al., 2005; Kaneko et al., 2006; Olson et al., 2006; Zanardo et al., 2006; Cai et al., 2007; Papapetropoulos et al., 2009).

Previous studies report measurement of endogenous concentrations of sulfide by methods involving high concentrations of acids; therefore, contamination by free H_2S released from acid-labile sulfur resulted in an overestimate of the free H_2S levels (50–160 μM; Goodwin et al., 1989; Warenycia et al., 1989; Savage and Gould, 1990). A sulfur/silver electrode has frequently been used for the measurement of sulfide concentrations in biological samples. The electrode measures the level of S^{2-}, and a pKa value of 13.9 results in the replacement of cysteine sulfide groups in proteins with hydroxyl groups, thereby releasing H_2S from proteins. Because tissue and blood samples contain abundant proteins, this method estimates erroneously high concentrations of sulfide (Whitfield et al., 2008). Recently, the basal or steady state endogenous concentrations of H_2S have been re-evaluated using methods that avoid release of contaminant H_2S from proteins; these methods give concentration estimates of 20 nM to a few micromolar in tissue and blood samples (Furne et al., 2008; Ishigami et al., 2009; Wintner et al., 2010).

It is necessary to determine the active state concentrations of H_2S. At least three factors influence H_2S concentration: (1) rate of H_2S production, (2) rate of H_2S metabolism, and (3) storage of H_2S as bound sulfane sulfur and its associated release.

RATE OF H_2S PRODUCTION

H_2S production by three enzymes has been studied extensively; these enzymes are cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST; Stipanuk and Beck, 1982; Chiku et al., 2009; Shibuya et al., 2009a,b; Singh et al., 2009) (**Figure 1**). CBS and CSE metabolize cysteine and/or homocysteine to release H_2S, whereas 3MST produces H_2S from 3-mercaptopyruvate (3MP), which is produced by the action of cysteine aminotransferase (CAT) on cysteine and α-ketoglutarate (Cooper, 1983; Shibuya et al., 2009a,b). 3MST requires cofactors to reduce a persulfide intermediate generated between a cysteine residue of 3MST and a sulfide provided by 3MP. We recently found that thioredoxin and dihydrolipoic acid (DHLA) are endogenous reducing cofactors that facilitate H_2S release from 3MST (Mikami et al., 2011a). We also found that the 3MST/CAT pathway is regulated by Ca^{2+} (Mikami et al., 2011b; Mikami and Kimura, 2012) and that the activity of CBS is enhanced by *S*-adenosyl methionine (SAM; Abe and Kimura, 1996). Thus, the physiological stimuli that alter the intracellular levels of Ca^{2+} and SAM should be investigated in order to clarify the regulation of H_2S production.

RATE OF H_2S METABOLISM

H_2S is metabolized in the mitochondria, via the sulfide oxidation pathway (Hildebrandt and Grieshaber, 2008). The first step is catalyzed by a membrane-bound sulfide:quinone oxidoreductase (SQR), which oxidizes H_2S to persulfide. SQRs were initially identified in invertebrates, and their mammalian counterpart SQRs were later discovered (Theissen et al., 2003; Theissen and Martin, 2008). In the presence of O_2 and water, sulfur dioxygenase oxidizes persulfide to sulfite, which is combined with another persulfide molecule by sulfur transferase rhodanese in order to producing thiosulfate (**Figure 1**). Because H_2S consumption by a dioxygenase is high in the presence of O_2 (Furne et al., 2008), high H_2S production is offset by rapid H_2S clearance under aerobic conditions, accounting for very low basal levels of H_2S. Small deviations in the rates of H_2S production and clearance may lead to rapid and several-fold changes in the H_2S levels (Vitvitsky et al., 2012).

STORAGE OF H_2S AS BOUND SULFANE SULFUR AND ITS ASSOCIATED RELEASE

In addition to enzymatic regulation of H_2S levels, H_2S may be stored in proteins as bound sulfane sulfur that is divalent sulfur bound mostly to sulfur of cysteine residues. (Ishigami et al., 2009) (**Figure 1**). Exogenously applied H_2S is absorbed and stored as bound sulfane sulfur, and the rate of absorption varies according to tissue types. Cells expressing 3MST and CAT have higher levels of bound sulfane sulfur than control cells (Shibuya et al., 2009a,b). In contrast, cells expressing 3MST mutants – that lack the ability to produce H_2S – retain control-like levels of bound sulfane sulfur. Therefore, H_2S produced by enzymes is stored in cells as bound sulfane sulfur. Bound sulfane sulfur releases H_2S under reducing conditions. In the presence of major cellular reducing substances, glutathione, cysteine, and dihydrolipoic acid (DHLA) at their physiologic concentrations, H_2S is released from lysates of cultured neurons and astrocytes at pH 8.0–8.4 (Ishigami et al., 2009; Mikami et al., 2011a). When neurons are excited, sodium ions enter and potassium ions exit from cells, resulting in high potassium concentrations in the extracellular environment, which

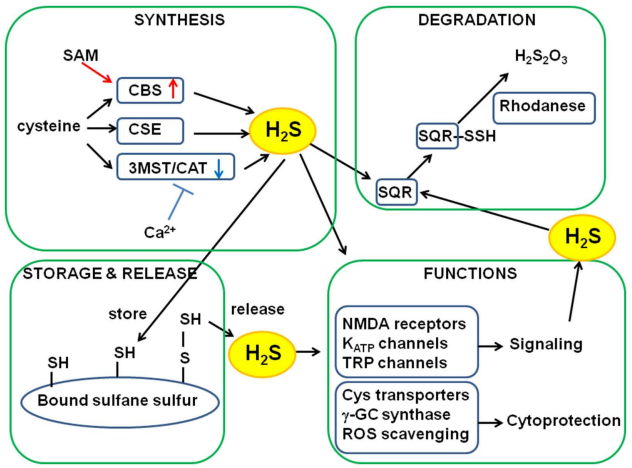

FIGURE 1 | Production, metabolism, storage, and functions of H_2S.

depolarizes the membrane of surrounding astrocytes. To recover from depolarization, Na^+ / HCO_3^- cotransporters are activated in astrocytes (Brookes and Turner, 1994). The entrance of HCO_3^- causes alkalinization. Approximately 10% of the astrocytes shifted their intracellular pH to 8.4, which can induce bound sulfane sulfur to release H_2S (Ishigami et al., 2009).

The concentrations of H_2S can vary locally in restricted areas of cells, while the reported values are those averaged over whole tissues, using tissue homogenates or blood samples (Furne et al., 2008; Ishigami et al., 2009; Wintner et al., 2010). Such local variations in H_2S concentrations must be determined. Recently developed H_2S-sensitive fluorescence probes are able to estimate local H_2S levels in live cells (Lippert et al., 2011; Liu et al., 2011; Peng et al., 2011; Qian et al., 2011; Sasakura et al., 2011). However, the probes bind to H_2S irreversibly; the detectable limit for H_2S is approximately 5–30 μM, i.e., values higher than the basal or steady state concentrations; and the time to reach maximum sensitivity is between 3 min and 1 h. The development of probes able to detect rapid changes in H_2S concentrations is awaited.

The metabolic balance between the production and clearance of H_2S has a considerable effect on its endogenous concentrations. In addition, because of bound sulfane sulfur, which releases and absorbs H_2S, the H_2S concentrations change more rapidly and extensively. The discrepancy between the endogenous basal concentrations and the effective-concentrations of exogenously applied H_2S has been discussed frequently. Some effects of H_2S may be elicited by its entry into cells through the plasma membrane. However, the rate of permeation and how freely and rapidly H_2S diffuses within the cytosol have not been determined. We also need to clarify the H_2S concentrations reached when cells are stimulated and the associated mechanism.

In conclusion, the basal or steady state levels of H_2S have been re-evaluated and found to be much lower than those previously reported. There is a difference between the steady state- and the effective-concentrations of H_2S. Therefore, it is urgent to determine the local concentrations of H_2S achieved when cells are stimulated.

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Hydrogen sulfide: both feet on the gas and none on the brake?

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I remember as a neophyte academician in the early 80's the excitement and at times heated discussion generated by nitric oxide (NO). How could this toxic and relatively unheard-of gas possibly be a regulatory molecule? Even its discovery was the result of a laboratory mistake (Furchgott, 1995). As with any revolutionary idea, there were both zealous aficionados, naysayers and folks in between that "just wanted more proof." As a result, it took nearly 10 years for this field to reach its log growth phase (**Figure 1A**); the fact that the number of yearly publications has now leveled off at approximately 7000 per year is probably more attributable to resource limitations than to enthusiasm. The physiological relevance of carbon monoxide (CO) was realized shortly after (Wu and Wang, 2005) but again there was a similar lag between discovery and acceptability as the scientific community tried to separate toxicology and pharmacology from physiology (**Figure 1B**).

Hydrogen sulfide (H_2S) was the third gas to be elevated to signaling status, i.e., a "gasotransmitter" (Wang, 2002). H_2S has the same toxic pedigree as NO and CO (Guidotti, 2010) but it has not received the degree of initial skepticism that accompanied the latter two. As shown in **Figure 1C**, following the initial demonstrations of H_2S signaling in the nervous and cardiovascular systems by Kimura's group (Abe and Kimura, 1996; Hosoki et al., 1997) the lag-time for H_2S to catch on in the scientific community took about as long as the time for these two seminal papers to circulate. Within 3 years the log phase of H_2S publications had begun, and it continues to this day.

It is not surprising that H_2S was greeted with enthusiasm; gaseous signaling molecules were no longer novel, the implications of toxicity arising from excess signal were obvious, and a precedent for therapeutic potential (and profit) were firmly established. Add to this the fact that exogenous H_2S appears to affect virtually every physiological system, it has potentially more biochemical diversity than the other gases, journals are eager for reviews (132 in 2011 and 93 as of the time of this writing in 2012), and away we go. The thought that the Emperor may not be wearing any clothes, and may even have a few warts, seems to be lost in the gala of the parade to the publisher and the patent office.

This is not to belittle the considerable potential of H_2S research, there have been momentous gains, and undoubtedly more to come. Exogenous H_2S affects virtually every organ system and physiological process to which it has been applied. Many of the effects of H_2S are supported by compounds that are thought to inhibit or augment endogenous H_2S production (See reviews; Caliendo et al., 2010; Kimura, 2010; Szabo, 2010; Olson, 2012b; Wang, 2012). The most notable among these are the cardiovascular system, where H_2S mediates systemic vasodilation and pulmonary vasoconstriction, angiogenesis, oxygen sensing and is cardioprotective, the nervous system where H_2S affects neuronal signaling, contributes to involved retinal function and may be involved in degenerative diseases, and the gastrointestinal (GI) tract where it contributes to signaling, insulin release, and metabolism. H_2S appears to be anti-inflammatory in most tissues, although it may be inflammatory in some. Contrary to common thought, H_2S itself does not readily react with reactive oxygen species (ROS) but it does stimulate glutathione production and augment reducing equivalents in the central nervous system (Kimura et al., 2010). Even novel signaling mechanisms whereby H_2S interacts with the reduced (sulfhydration) or oxidized sulfur of cysteine to activate or inactivate proteins have been described (Mustafa et al., 2009; Tao et al., 2012). As well-illustrated in this issue, it is also becoming increasingly evident that the many of the biological effects of H_2S are mediated directly through H_2S interactions with ion channels (see also; Tang et al., 2010; Munaron et al., 2012; Peers et al., 2012).

Sulfide salts, such as NaHS and Na_2S, have been historically used to rapidly generate H_2S but this occurs at an uncontrolled rate and their purity may be problematic. These salts are gradually being replaced by the next generation of compounds that slowly release H_2S. The clinical applicability these true H_2S "releasing" drugs is now becoming evident and they offer additional advantages in that they can be combined with other, unrelated drugs and provide a multi-focal therapeutic approach (Caliendo et al., 2010; Olson, 2011; Kashfi and Olson, 2012). Already, H_2S-releasing drugs have been combined with a variety of compounds including virtually all non-steroidal anti-inflammatory drugs (NSAIDs), sildenafil, levodopa, the anti-glaucoma drug lantanoprost, and the angiotensin-1 receptor antagonist losartan to name a few. The ability of some of these H_2S-releasing drugs to uniquely counter the adverse effects of aspirin and other NSAIDs on the GI tract or to treat other inflammatory process such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and acute and chronic joint pain are nearing or already in initial clinical trials. A third generation of compounds that can be combined with drugs

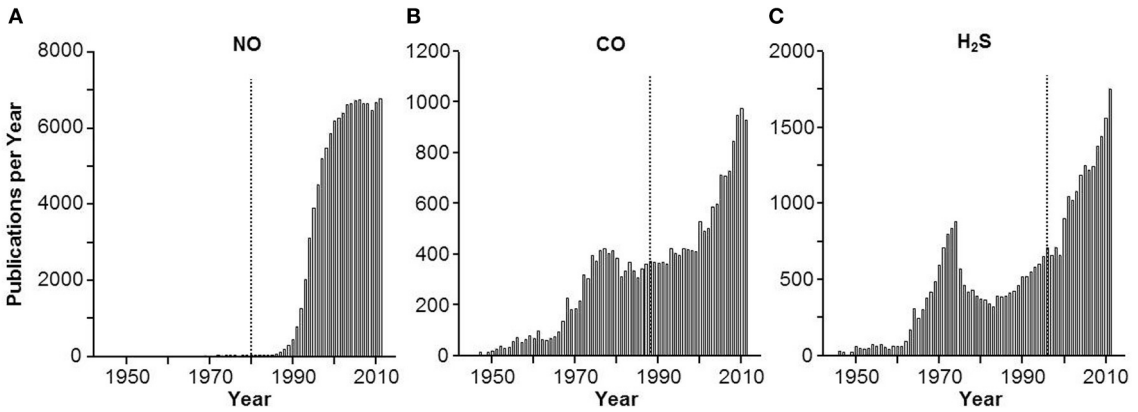

FIGURE 1 | Frequency histogram of the number of yearly publications on (A) nitric oxide (NO), (B) carbon monoxide (CO) and (C) hydrogen sulfide H_2S. Vertical lines indicate approximate year of the first description of a biological effect of the gas. Approximately 10 years passed before NO and CO caught on as biologically relevant signal molecules, acceptance of H_2S was much quicker.

such as NSAIDs and release both H_2S and NO, the so-called "NOSH" compounds may further increase potency and versatility; preliminary studies suggest they may be especially effective in the treatment of cancer (Kashfi and Olson, 2012).

A better understanding of H_2S biochemistry and metabolism has also lead to promising therapy (Viscomi et al., 2010; Drousiotou et al., 2011). Ethylmalonic encephalopathy (EE) is an autosomal recessive deletion in the ETHE1 gene that encodes a mitochondrial sulfur dioxygenase. EE is typically fatal in the first decade of life. Patients with EE present with greatly elevated urinary and tissue thiosulfate concentrations and increased tissue H_2S, presumably due to the inability of cells to efficiently metabolize H_2S. This condition has recently been treated by oral administration of the bactericide metronidazole, to inhibit H_2S production by colonic bacteria, and *N*-acetylcysteine. The latter acts as a membrane-permeable precursor of reduced glutathione (GSH) and appears to combine with H_2S to form a non-toxic persulfide, GSSH. Targeting another mitochondrial enzyme, sulfur quinone oxidoreductase (SQR), which catalyzes the first step in H_2S oxidation, may be a future therapeutic option (Linden et al., 2010).

The intent of this opinion is not to be a nayser, but just to request more proof that while we are going full-speed ahead we are also going in the right direction and not out of control. Clearly, we don't need proof that exogenous H_2S activates/inhibits numerous biological systems, or that these systems can be modulated by fiddling with H_2S metabolism, or that H_2S has specific molecular targets, there are at least 225 reviews to substantiate these. However, we do need to critically examine a number of practical and technical issues. These include the promiscuity of many "selective" inhibitors of H_2S biosynthesis, separating physiological from pharmacological effects of H_2S, dosing issues, and measuring H_2S in tissues and blood. Obviously, all are related; I will focus on the latter two.

H_2S is a gas and we need some justification for ignoring its ephemeral nature; the half-life of H_2S in open containers is a few scant minutes, even less when aerated (DeLeon et al., 2011). When we expose cells and tissues to H_2S for hours and days in tissue culture or organ baths what are we looking at? Is this why we need to expose tissues to excessive H_2S concentrations that can't possibly exist naturally? And where do we separate physiology from toxicology?

More importantly, however, we really need proof that what we are measuring in tissues and blood is actually H_2S. This has profound implications in perhaps the most exciting avenue of H_2S research, its therapeutic potential. My biggest concern is that if we do not do this right it can have disastrous consequences resulting in missed diagnoses and inappropriate therapies. I will give three examples, one where a simple calculation can be used to tell us if tissue H_2S values are realistic, a second that will (hopefully) red-flag plasma and blood H_2S measurements and a third that serves as an excellent example of how critical thinking can put the brake on a potentially dangerous clinical application.

First, lets look at tissue H_2S concentrations. Most studies report tissue H_2S concentrations anywhere from 1 nmol/mg protein to up to values well in excess of 10 μmol/mg protein. As I have previously calculated (Olson, 2012a) if one assumes a cell is 15% protein and 70% water then 1 μmol H_2S/mg protein would result in a cytosolic concentration of 214 mmol/l! Even 1 nmol/mg protein in a cell is still be more than five times the toxic level. How can these values be so high? There are at least two reasons. First, most methods require incubating the tissue for 30 min or more under anoxic conditions. These conditions prevent normal H_2S oxidation while H_2S production continues unabated. Second, the most commonly used methods, methylene blue, monobromobimane and ion-selective electrodes (ISE) measure more than just H_2S; and it's generally not clear what many of these other molecules are (Olson, 2012a). The alkaline antioxident buffer used with the ISE generates H_2S from cysteine in serum albumin (Whitfield et al., 2008).

Even more troubling are H_2S measurements in plasma and blood. H_2S is commonly measured in plasma using the above methods or in headspace gas (typically after equilibration for 30 min or more

under anoxic conditions) and concentrations from 1–30 μmol/l are common, although some go as high as 300 μmol/l. These are unrealistic because of the reasons described above, and as so elegantly shown by Furne et al. (2008), the human nose can detect 1 μmol/l; blood obviously doesn't smell like rotten eggs. While these erroneous values are a detraction in laboratory experiments, they are potentially dangerous when used diagnostically. And the latter is becoming more common. I offer a simple challenge to any laboratory that is interested in, or currently measuring H_2S in blood; in addition to making your standard curve in buffer, make another standard curve in whole blood and compare the two. I guarantee the values obtained in the plasma will not correlate with buffer values and, in fact, there will be no measurable H_2S in the former because red blood cells avidly remove H_2S (Whitfield et al., 2008).

There is considerable, and alarming interest in using blood H_2S measurements for clinical diagnosis and a variety of pathological conditions have been correlated with altered plasma H_2S. Here I will give a single example. Goslar et al. (2011) used the standard methylene blue method to measure total plasma sulfide in patients admitted to the intensive care unit (ICU) with various forms of shock. Presuming plasma pH is 7.4 this would be ∼20% H_2S gas; in acidosis the percent H_2S gas will increase. Goslar et al. (2011) reported that plasma sulfide was lower in survivors than non-survivors (13 vs. 32 μM) and they concluded that plasma sulfide could be used as a predictor of mortality in the ICU. This concept was critically tested in a rat model of hemorrhagic shock by Van de Louw and Haouzi (2012) who found no evidence for such a correlation and, in fact, the methylene blue method measured plasma turbidity, not sulfide.

This Opinion is not meant to discourage research in this exciting field but to encourage critical thinking about how we are generating information and interpreting it. Clearly, these are exciting times. However, it may be necessary now and then to apply the brakes on exuberance to keep things from getting out of control. These points are further detailed in several recent reviews (Olson, 2009, 2011, 2012a; Linden et al., 2010).

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Modulation of ion channels and transporters by carbon monoxide: causes for concern?

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INTRODUCTION

Heme oxygenases, particularly the inducible heme oxygenase-1 (HO-1), are the subject of intensive research since they show great promise as cytoprotective agents. These enzymes degrade heme to generate biliverdin, free iron, and carbon monoxide (CO), and this reaction appears to be crucial in a number of diverse biological systems. Decreasing free heme in itself is beneficial [for example in sepsis and infections (Gozzelino et al., 2010)], but much attention is also paid to the products of heme degradation as biologically active agents with therapeutic potential (Motterlini and Otterbein, 2010; Wegiel et al., 2012). Indeed, the physiological roles and potential of CO in particular (applied either by inhalation, or via CO releasing molecules; CORMs) are currently topics of intense research, with clinical trials currently evaluating its safety in human subjects and its usefulness in treating a variety of disorders (http://clinicaltrials.gov/ct2/search, *"carbon monoxide"*) (Wu and Wang, 2005; Durante et al., 2006; Kim et al., 2006; Ryter et al., 2006).

In this current climate of hopeful promise for CO-based therapies, it is easy to lose sight of the fact that it is a highly toxic gas: CO poisoning accounts for more than 50% of all fatal poisonings worldwide (Meredith and Vale, 1988; Cobb and Etzel, 1991; Varon et al., 1999). Although the number of fatalities arising from acute exposure may be considered relatively low, chronic exposure can much more commonly produce neurological and cardiovascular damage (Von Burg, 1999; Gandini et al., 2001; Omaye, 2002; Prockop and Chichkova, 2007), particularly in the aging population, and symptoms are often difficult to diagnose (Harper and Croft-Baker, 2004). Appropriately, therefore, much caution is taken as clinical trials progress and as our awareness of the biological actions of CO continues to develop.

In recent years, ion channels (and, more recently, transporters) have emerged as major targets for modulation by CO (Peers, 2011; Wilkinson and Kemp, 2011; Peers and Steele, 2012). Intriguingly, modulation of some channels by CO may contribute to its beneficial actions, yet the sensitivity of other channels to CO may account, at least in part, for some of its deleterious actions (summarized in **Figure 1**). In this article, I draw upon some recent examples of ion channel/transporter modulation by CO in the cardiovascular and central nervous systems in order to compare the beneficial and deleterious cellular effects of CO, and to examine whether we should be concerned about the therapeutic index of CO.

CARDIOVASCULAR EFFECTS OF CO

In the cardiovascular system, HO-1 (most commonly via its production of CO) exerts multiple beneficial effects. In addition to its known antihypertensive actions, it appears to be involved in numerous vascular diseases. Paradoxically, it suppresses vascular smooth muscle proliferation, yet augments endothelial proliferation, both of which can be considered beneficial. Suppression of smooth muscle proliferation is important in combating development of vessel thickening associated with vascular injury and grafting, and also in the progression of atherosclerosis [reviewed by Barbagallo et al. (2012)]. Stimulation of endothelial proliferation is dependent on vascular endothelial growth factor (VEGF) production, and is required for angiogenesis: HO-1 induction or CO exposure promotes endothelial VEGF production, proliferation, migration, and neovascularization (Jozkowicz et al., 2003).

In the heart and coronary vasculature, HO-1 expression can be increased by various stress factors including myocardial infarction or ischemia/reperfusion (I/R) (Lakkisto et al., 2002). The importance of HO-1 is well illustrated by studies employing transgenic mice: for example, in heterozygote HO-$1^{+/-}$ KO mice I/R caused significantly greater cardiac damage than wild-type mice (Yoshida et al., 2001). In HO-$1^{-/-}$ mice, chronic hypoxia (which normally up-regulates HO-1) caused significantly greater right ventricular hypertrophy, oxidative damage, and pulmonary hypertension (Yet et al., 1999). Furthermore, cardiac-specific overexpression of HO-1 strongly protects against I/R damage (Yet et al., 2001) and in a coronary ligation model of heart failure (Wang et al., 2010).

Although HO-1 is clearly protective, the relative contributions of heme reduction or biliverdin, Fe^{2+} and CO production are not fully elucidated. However, there is much evidence to support a key role for CO in HO-1-mediated cardioprotection. For example, Wang et al. (2010) reported that CORM-3 protected the myocardium against adverse remodeling following coronary ligation to a degree comparable with over-expression of HO-1. Indeed, CORMs have provided much support for the idea that HO-1 is cardioprotective via CO production. For example, infusion of CORM-3 during the reperfusion phase of I/R challenges reduced myocardial damage *in vitro* and *in vivo* (Clark et al., 2003; Guo et al., 2004), and CORM-3 can have a positive inotropic effect (Musameh et al., 2006).

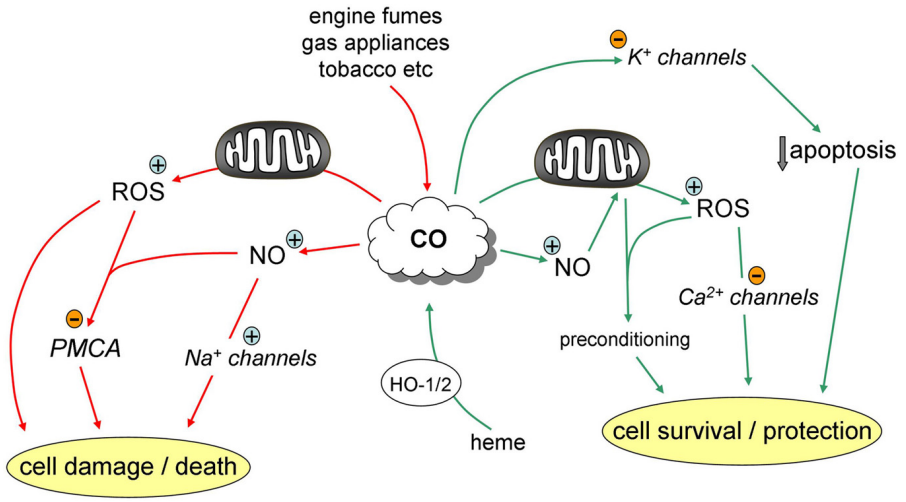

FIGURE 1 | Pathways contributing to the protective (green) and deleterious (red) effects of CO.

As part of a study aimed at identifying potential mechanisms accounting for the protective effects of CO in the myocardium, we discovered that CO could inhibit the L-type Ca^{2+} current (Scragg et al., 2008). This effect was mediated by a CO-induced increase in ROS production from mitochondria, which modulated the channel via key cysteine residues located in the intracellular C-terminal domain of the α subunit. Inhibition of L-type Ca^{2+} channels would be predicted to reduce the energy demands of the myocardium, and hence could be considered as protective. However, when we subsequently examined the effects of CO and CO donors on single cardiac myocytes, the dominant effect was for CO to be pro-arrhythmic (Dallas et al., 2012): early after-depolarization events were detected via patch-clamp and Ca^{2+} imaging. These effects were attributable to CO-induced increases in NO levels, which led to an augmentation of the late component of the Na^+ current via direct nitrosylation of Nav1.5. Crucially, rats exposed to 500 ppm CO displayed ECG abnormalities consistent with these effects, and when they were exposed to CO following isoprenaline injection, ventricular fibrillation, and death was observed. All effects were reversed by ranolazine, a known inhibitor of the late Na^+ current (Dallas et al., 2012).

NEUROLOGICAL EFFECTS OF CO

The protective effects of CO in the central nervous system have also been demonstrated in a number of model systems. For example, CO inhalation (up to 250 ppm) provides clear beneficial effects against the damage of I/R brain injury and ischemic stroke (Wang et al., 2011; Zeynalov and Dore, 2009). Furthermore, pre-exposure to CO (also at 250 ppm) provided pre-conditioning protection against neurological damage (specifically, neuronal apoptosis) in a pig model of deep hypothermic circulatory arrest, which is a widely used technique in surgical treatment of heart defects (Mahan et al., 2012).

At the cellular level, pretreatment of primary cultures of cerebellar granule neurons with 250 ppm CO provided protection against apoptosis induced by oxidative stress or excitotoxic levels of exogenous glutamate (Vieira et al., 2008). Interestingly, protection appeared to be comparable to the pre-conditioning associated with sub-lethal ischemia, where neurons become more resistant to otherwise lethal ischemic challenges by prior exposure to a less severe challenge. Vieira et al. reported that protection afforded by CO involved activation of guanylate cyclase and subsequent activation of mitochondrial ATP-sensitive K^+ channels. Importantly, they also reported increased intracellular ROS levels and stimulation of NO formation in response to CO exposure (Vieira et al., 2008). The increase in ROS formation is a key pre-conditioning step, since it triggers up-regulation of protective protein expression, including HO-1.

We subsequently provided another mechanism to account for neuroprotective effects of CO. According to a number of studies from Aizenman and colleagues (Redman et al., 2007; Pal et al., 2003, 2006), a key early stage in stress-induced neuronal apoptosis is the rapid insertion into the plasma membrane of K^+ channels, particularly Kv2.1, which leads to loss of cytoplasmic K^+ and the initiation of apoptotic signaling. Consistent with this hypothesis, we found that over-expression of Kv2.1 in HEK293 cells increased susceptibility to oxidative apoptotic stimuli, but that this could be prevented by CO i.e., CO provided protection against apoptosis specifically in Kv2.1 expressing cells (Dallas et al., 2011). Furthermore, CO directly inhibited Kv2.1 channels, and the currents arising from the "surge" of K^+ channels in response to apoptotic stimuli. Importantly, these effects were reproduced in primary cultures of hippocampal neurons (Dallas et al., 2011). Interestingly, a recent study demonstrated that the CO donor, CORM-3, provided a degree of neuroprotection against a collagenase injection model of hemorrhagic stroke if administered in advance, but under other circumstances could exacerbate the associated damage (Yabluchanskiy et al., 2012).

The above described data indicate that CO may provide neuroprotection via multiple mechanisms. However, stimulation of both ROS and NO formation (implicated

in some protective pathways) is not without risk, in part because it can lead to formation of the highly damaging ROS species peroxynitrite ($ONOO^-$). Indeed, we reported recently that such effects of CO may account for some of the deleterious neurological effects of CO poisoning: we monitored Ca^{2+} homeostasis in human neuroblastoma (SH-SY5Y) cells and noted that exposure of cells to the CO donor CORM-2 caused an apparent increase in both voltage gated Ca^{2+} entry and prolonged receptor-mediated rises of $[Ca^{2+}]_i$ arising from mobilization of Ca^{2+} from endoplasmic reticulum stores and subsequent capacitative Ca^{2+} entry. These potentially deleterious effects of CO were abolished either by an antioxidant or by inhibition of NO formation with L-NAME (Hettiarachchi et al., 2012). NO donors alone were unable to mimic these actions of CO, indicating that both NO and ROS were required for CO to disrupt Ca^{2+} signaling. A rise of $ONOO^-$ levels was detected via APF fluorescence, and the $ONOO^-$ scavenger FeTPPs also inhibited the effects of CO. Mechanistically, we reasoned that for CO to disrupt so many Ca^{2+} signaling pathways it most likely acted via modulation of a common target protein, and we identified a CO-dependent down regulation of the plasmalemmal Ca^{2+} ATPase (PMCA) in both SH-SY5Y cells and also in whole brain homogenates from rats exposed to 1000 or 3000 ppm CO for 40 mins (Hettiarachchi et al., 2012).

A QUESTION OF CONCENTRATION?

The example studies cited here raise the question of whether beneficial effects of CO differ from deleterious effects (shown schematically in **Figure 1**) simply because of concentration, or for some other, unidentified reasons. This is not a straightforward issue to resolve from the current literature, since CO is applied by various means (inhalation, or via CORMs), and final concentrations at the intended sites of action are difficult or impossible to determine accurately. Although the potential for neurological damage arising from loss of the PMCA was observed only at high (>1000 ppm) levels of inhaled CO *in vivo*, the cellular effects on PMCA were manifest using CORM-2 at 10–30 μM. Such concentrations are comparable to other reported effects of CO/CORMs which have been regarded, rightly or wrongly, as beneficial/physiological effects. Most electrophysiological studies have employed CORMs at concentrations of 1–100 μM (Peers, 2011; Wilkinson and Kemp, 2011), often at 30 μM or lower. Perhaps most alarmingly, the pro-arrhythmic actions of CO mediated by induction of the late Na^+ current (Dallas et al., 2012) were observed when animals inhaled 500 ppm (or cells were exposed to 20–30 μM CORM-2 or -3). Such levels have been reported in heavy traffic or as a consequence of exposure to cigarette smoke (Reboul et al., 2012), and clinical trials are under way employing 250 ppm (see earlier). Clearly, as discussed by Reboul et al. (2012), the duration of exposure to CO is critical in determining whether its outcome is beneficial or detrimental, but as new targets for modulation become realized, it is becoming clear that a better understanding (or, better, a means of determination) of CO concentration at its sites of action is needed before we can describe effects of CO observed *in vitro* as potentially beneficial or detrimental. Such information is important in the progression of CO therapy.

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