

ORIGINAL RESEARCH COMMUNICATION

Antioxidants Condition Pleiotropic Vascular Responses to Exogenous H₂O₂: Role of Modulation of Vascular TP Receptors and the Heme Oxygenase System

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Abstract

Aims: Hydrogen peroxide (H₂O₂), a nonradical oxidant, is employed to ascertain the role of redox mechanisms in regulation of vascular tone. Where both dilation and constriction have been reported, we examined the hypothesis that the ability of H₂O₂ to effect vasoconstriction or dilation is conditioned by redox mechanisms and may be modulated by antioxidants. **Results:** Exogenous H₂O₂ (0.1–10.0 μM), dose-dependently reduced the internal diameter of rat renal interlobular and 3rd-order mesenteric arteries ($p < 0.05$). This response was obliterated in arteries pretreated with antioxidants, including tempol, pegylated superoxide dismutase (PEG-SOD), butylated hydroxytoluene (BHT), and biliverdin (BV). However, as opposed to tempol or PEG-SOD, BHT & BV, antioxidants targeting radicals downstream of H₂O₂, also uncovered vasodilation. **Innovations:** Redox-dependent vasoconstriction to H₂O₂ was blocked by inhibitors of cyclooxygenase (COX) (indomethacin-10 μM), thromboxane (TP) synthase (CGS13080-10 μM), and TP receptor antagonist (SQ29548-1 μM). However, H₂O₂ did not increase vascular thromboxane B₂ release; instead, it sensitized the vasculature to a TP agonist, U46619, an effect reversed by PEG-SOD. Antioxidant-conditioned dilatory response to H₂O₂ was accompanied by enhanced vascular heme oxygenase (HO)-dependent carbon monoxide generation and was abolished by HO inhibitors or by HO-1 & 2 antisense oligodeoxynucleotides treatment of SD rats. **Conclusion:** These results demonstrate that H₂O₂ has antioxidant-modifiable pleiotropic vascular effects, where constriction and dilation are brought about in the same vascular segment. H₂O₂-induced oxidative stress increases vascular TP sensitivity and predisposes these arterial segments to constrictor prostanoids. Conversely, vasodilation is reliant upon HO-derived products whose synthesis is stimulated only in the presence of antioxidants targeting radicals downstream of H₂O₂. *Antioxid. Redox Signal.* 18, 471–480.

Introduction

THE TONE OF RESISTANCE arterial vessels is regulated by the interplay of mechanisms promoting vasoconstriction and dilation *via* complex networks of interacting signaling pathways. Reactive oxygen species (ROS) contribute to regulation of vasomotor tone in physiological and pathophysiological settings (26, 46), with superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂), and other ROS displaying constrictor and/or dilator activities (20). H₂O₂ is particularly interesting, because it affects vasoconstriction as well as dilation (1), sometimes in the same vascular preparation (10, 28). These

outcomes are determined by the concentration of H₂O₂, vessel type, and experimental conditions (11, 12).

H₂O₂-induced vasoconstriction has been related to stimulation of vascular smooth muscle thromboxane A₂ (TxA₂)/prostaglandin endoperoxide receptors (thromboxane [TP] receptors) by a product of arachidonic acid metabolism *via* COX (13, 27, 32). It has also been linked to elevation of cytosolic calcium (49) and/or activation of protein kinases (18, 41). Conversely, H₂O₂-induced vasodilation has been associated with activation of guanylate cyclase (33), increasing cellular cAMP levels (16), and stimulation of vascular smooth muscle K⁺ channels (4, 15). Vasodilation has also been linked to

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Innovation

Our study examines the vascular effects of exogenous hydrogen peroxide (H_2O_2) in light of its oxidant properties. This radical invokes a constrictive response in resistance arteries, where an antioxidant milieu is not provided. Both constriction and dilation are observed in the same arterial preparation, depending upon the use of antioxidants targeting radicals derived from or leading to H_2O_2 . Where thromboxane sensitization underlies vasoconstriction, increased heme oxygenase (HO)-dependent carbon monoxide (CO) generation causes vasodilation. Notably, radicals downstream to H_2O_2 interfere with its ability to stimulate HO-dependent CO release; targeting these oxidants increases vascular CO and leads to H_2O_2 -induced vasodilation. This study lays the foundation to explore vasoreactivity of endogenous H_2O_2 , in models of chronic oxidative stress where application of broad-spectrum antioxidants should provide further evidence of pleiotropic vascular effects of H_2O_2 .

augmented synthesis of vasodilator mediators, including prostaglandins (16) and endothelium-derived nitric oxide (NO) (50). According to previous studies, whether H_2O_2 promotes vasoconstriction or dilation depends on the functional status of K^+ channels in the target vessels, viz., constriction occurs when vascular smooth muscle K^+ channels are functionally impaired, and dilation happens when they are not (28). The redox status of the vessels influences functionality of vascular K^+ channels (40, 48), as well as of other signaling proteins important for Ca^{2+} -dependent regulation of vasomotor tone (26, 37). Redox mechanisms also modulate the expression of TP receptors (42, 44), the activity heme oxygenase (HO) (21, 23, 29), and the vascular actions of its vasodilatory product—carbon monoxide (CO) (25). Hence, it is plausible that one or more of these redox-controlled vasoregulatory systems condition vasoconstriction or dilation to this nonradical oxidant, H_2O_2 .

We undertook the present study to test the hypothesis that the response of resistance arteries to physiologically relevant

concentrations of H_2O_2 is dictated by the redox status of the preparations. This was achieved *via* activation and/or suppression of redox-modulated vasoactive systems that promote constriction or dilation. First, we contrasted the effect of H_2O_2 on internal diameter (ID) of pressurized rat renal interlobular (RIA) and 3rd order mesenteric artery (MA), in the absence and presence of antioxidants. Second, we connected the redox-dependent constrictor action of H_2O_2 to an associated increase in responsiveness to TP receptor stimulation. Third, we linked the redox-dependent dilator action of H_2O_2 to stimulation of HO-derived CO.

Results

Effect of H_2O_2 on the ID of pressurized arterial vessels: comparison in preparations pretreated and not pretreated with antioxidants

The notion that redox mechanisms influence the response of resistance arteries to H_2O_2 was addressed by contrasting the effects of this oxidant on vascular diameter in preparations exposed and not exposed to agents selected for their ability to create an antioxidant setting. As illustrated in Figure 1, RIA and MA not pretreated with an antioxidant demonstrated a dose-dependent decrease in ID to exogenous H_2O_2 ($p < 0.05$). In contrast, H_2O_2 challenge in RIA and MA pretreated with 1 mM butylated hydroxytoluene (BHT), a general antioxidant with an ability to quench free radicals downstream of H_2O_2 (24), resulted in increment ($p < 0.05$) rather than reduction of ID. H_2O_2 also increased the ID of MA and/or RIA pretreated with desferrioxamine (DES), an iron chelator that by disrupting the Fenton's reaction decreases generation of hydroxyl radical ($OH\cdot$), and subsequent lipid peroxidation (2, 45), dimethyl thiourea (DMTU), $OH\cdot$ scavenger (22), or biliverdin (BV), an antioxidant effective against radicals downstream of H_2O_2 and a known inhibitor of lipid peroxidation (17, 39) (Fig. 2).

Notably, arteries pretreated with pegylated superoxide dismutase (PEG-SOD), an antioxidant that dismutates O_2^- anion to H_2O_2 , or tempol, a SOD mimetic (34), did not respond to exogenous H_2O_2 with an increase of ID. This was observed, even though these pretreatments greatly attenuated

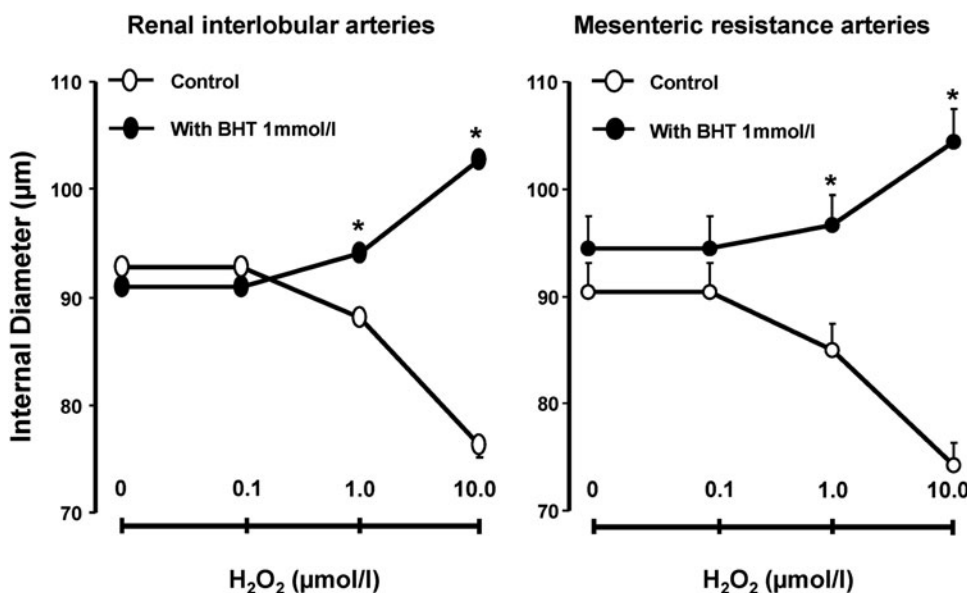


FIG. 1. Effect of H_2O_2 on the ID of renal interlobular and mesenteric resistance arteries, in arteries not exposed and exposed to BHT. Results are means \pm SE, $n = 6$ /group. * $p < 0.05$ versus control. BHT, butylated hydroxytoluene; H_2O_2 , hydrogen peroxide; ID, internal diameter; SE, standard error.

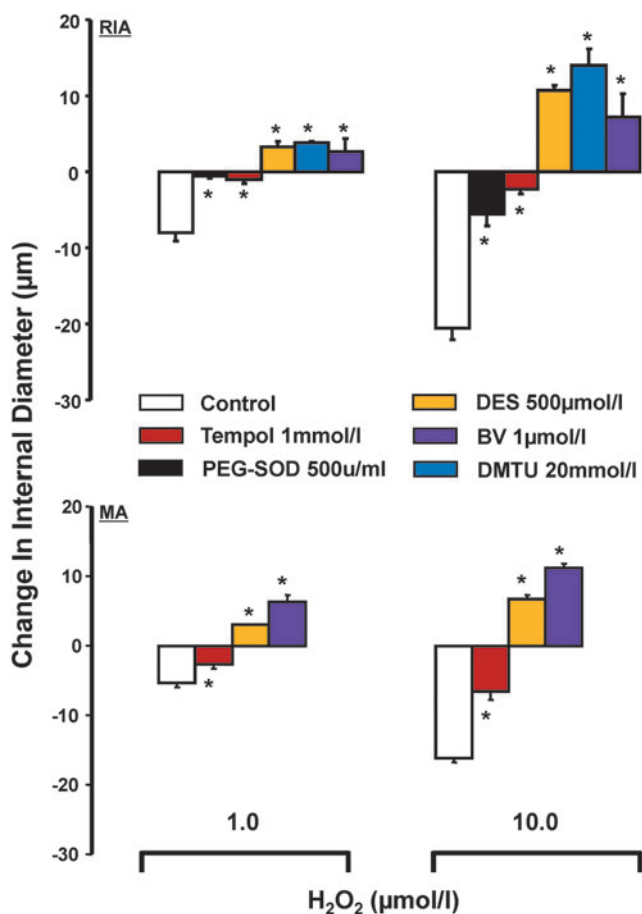


FIG. 2. Effect of H₂O₂ on change in ID, over baseline, of renal interlobular (n : Control-5, PEG-SOD-4, tempol-4, Des-4, DMTU-5, and BV-6) and mesenteric arteries ($n=4$), in arteries not exposed and exposed to antioxidants (as shown). Results are means \pm SE. * $p < 0.05$ versus control. BV, biliverdin; DES, desferroxamine; DMTU, dimethyl thiourea; PEG-SOD, pegylated superoxide dismutase.

the ability of H₂O₂ to effect decrease of vascular diameter, as observed in vehicle-pretreated controls (Fig. 2). Accordingly, the response of resistance arteries to exogenous H₂O₂ appears conditioned by their redox status: constriction *versus* blunted constriction or dilation, respectively, in vascular preparations not afforded and afforded protection from oxidative stress.

Relative to this point, complementary experiments documented that H₂O₂ (10 μ M) increase O₂⁻ levels as revealed by lucigenin chemiluminescence (from 66.9 \pm 10.9 CPM/ μ g protein to 87.5 \pm 7.7 CPM/ μ g protein, $n=7$, $p < 0.05$). Vessels exposed to H₂O₂ also displayed increased levels of TBARS (from 433.9 \pm 168.8 to 1892.3 \pm 498.0 nmoles/mg protein, $n=4$, $p < 0.05$), an index of lipid peroxidation and oxidative stress (35). This increase was blunted in vessels concurrently pretreated with BHT (234.3 \pm 76.2 *vs.* 138.4 \pm 15.6 nmoles/mg protein, $n=4$), BV (185.7 \pm 56.1 *vs.* 214.9 \pm 89.1 nmoles/mg protein, $n=4$), tempol (103.5 \pm 7.52 *vs.* 190.2 \pm 26.1 nmoles/mg protein, $n=4$), PEG-SOD (96.6 \pm 4.1 *vs.* 144.7 \pm 15.0 nmoles/mg protein, $n=3$), or DES (321.8 \pm 162.3 *vs.* 207.5 \pm 42.3 nmoles/mg protein, $n=3$), whereas arterial vessels exposed to H₂O₂ did not display any significant change in endogenous SOD activity (control: 331.3 \pm 68.6 *vs.* H₂O₂: 281.7 \pm 71.4 U/mg protein).

Effect of H₂O₂ on the ID of pressurized arterial vessels: comparison in preparations with and without endothelium

To investigate contribution of the vascular endothelium to the redox-dependent vascular actions of H₂O₂, RIAs, denuded and not denuded of endothelium were contrasted in terms of responsiveness to H₂O₂, in the absence and presence of BHT. As shown in Figure 3A, endothelium removal did not affect H₂O₂-induced vasoconstriction in vessels without antioxidant pretreatment. On the other hand, as depicted in Figure 3B, H₂O₂-induced increase of vascular ID was blunted by endothelium removal in arteries pretreated with BHT. Accordingly, antioxidant-conditioned H₂O₂-induced vasodilation is endothelium dependent, whereas constriction of vessels without antioxidant pretreatment is not.

Redox-dependent H₂O₂-induced vascular constriction: contribution of TP receptor activation

Earlier reports have linked oxidative stress to mechanisms of vasoconstriction involving TP receptor stimulation (13, 43). The notion that redox-dependent H₂O₂-induced vasoconstriction relies on TP receptor activation was

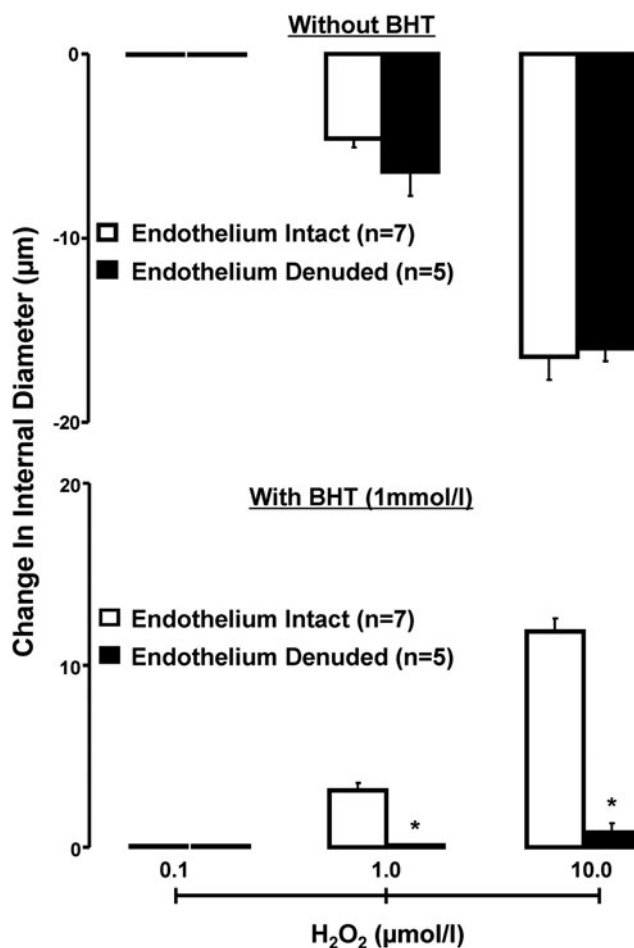


FIG. 3. Effect of H₂O₂ on change in ID, over baseline, of renal interlobular in the presence or in the absence of endothelium, in arteries not exposed and exposed to BHT (1 mM). Results are means \pm SE. * $p < 0.05$ versus endothelium intact.

addressed by comparing the constrictor action of the oxidant in pressurized RIA pretreated and not pretreated with indomethacin—to inhibit COX-dependent synthesis of constrictor prostanoids, CGS-13080—to selectively inhibit thromboxane synthase, or SQ29548, to effect blockade of TP receptors. As depicted in Figure 4A, the reduction of ID elicited by H_2O_2 in arteries not pretreated with antioxidants was blunted ($p < 0.05$) in preparations bathed in Krebs' buffer (KB) containing indomethacin, CGS-13080, or SQ29548. These observations suggest dependence of vasoconstrictor action of H_2O_2 on a TP receptor agonist manufactured *via* a pathway involving COX and thromboxane synthase activities, most likely TxA_2 . However, data presented in Figure 4B show that vascular thromboxane B_2 (TxB_2) synthesis, an estimate of TxA_2 , is neither stimulated by H_2O_2 nor suppressed by BHT or tempol. Yet, as illustrated in Figure 4C, the sensitivity of pressurized RIA to the constrictor action of a synthetic agonist for TP receptor, U46619, was significantly enhanced in vessels pretreated with H_2O_2 (EC_{50} values: 0.63 ± 0.07 nM in controls *vs.* 0.17 ± 0.08 nM with H_2O_2 , $p < 0.05$). This sensitizing action of H_2O_2 was not observed in arteries pretreated with PEG-SOD (EC_{50} values: 0.49 ± 0.05 nM in PEG-SOD *vs.* 0.47 ± 0.04 nM with PEG-SOD + H_2O_2 , $p < 0.05$), suggesting that its expression requires a pro-oxidant setting. Accordingly, the constrictor action of H_2O_2 in renal arteries without antioxidant protection is linked to TP receptor activation, relying on a mechanism involving augmented sensitivity of the vessels to TP receptor stimulation rather than increased vascular production of TxA_2 . That pretreatment with H_2O_2 does not sensitize RIA to the constrictor action of α -1 adrenergic receptor stimulation with phenylephrine (EC_{50} values: 9.3 ± 0.99 and 10.7 ± 2.2 nM, in arteries with and without H_2O_2 pretreatment, respectively) is keeping in line with the possi-

bility that the sensitizing action of H_2O_2 on TP receptor agonist-induced vasoconstriction is specific.

Redox-dependent H_2O_2 -induced vascular dilation: contribution of HO-derived CO

The hypothesis that redox status conditions the response of resistance arteries to H_2O_2 *via* mechanisms involving vascular production of HO-derived CO was addressed by examining the effect of exogenous H_2O_2 on CO released from isolated small artery segments incubated in oxygenated KB containing and not containing antioxidant agents. Complementary studies were conducted in pressurized RIA bathed in KB containing antioxidants, to contrast the ability of H_2O_2 to elicit vasodilation in preparations exposed and not exposed to an inhibitor of HO activity. Dilatory responsiveness to H_2O_2 also was studied in RIA taken from rats pretreated with antisense oligodeoxynucleotides (AS-ODN) targeting HO-1 and HO-2 or with the corresponding scrambled oligodeoxynucleotides (SAS-ODN).

Figure 5 depicts the effect of H_2O_2 on release of CO from MA without and with antioxidant pretreatment, in the absence and presence of the HO inhibitor chromium mesoporphyrin (CrMP). In preparations not exposed to CrMP, H_2O_2 enhanced ($p < 0.05$) the release of CO from arteries pretreated with BHT or BV, although it failed to do so in vessels without antioxidant pretreatment or pretreated with tempol. H_2O_2 also induced CO release from MA pretreated with DES ($500 \mu M$) (from 135.5 ± 16.1 to 227.7 ± 39.0 pmol/mg protein/h, $n = 7$, $p < 0.05$, but not from arteries pretreated with PEG-SOD (163.2 ± 26.1 *vs.* 156.4 ± 47.6 pmol/mg protein/h, $n = 6$). Release of CO from arteries exposed to CrMP was diminished ($p < 0.05$) by about 50%, and did not increase by further challenge with

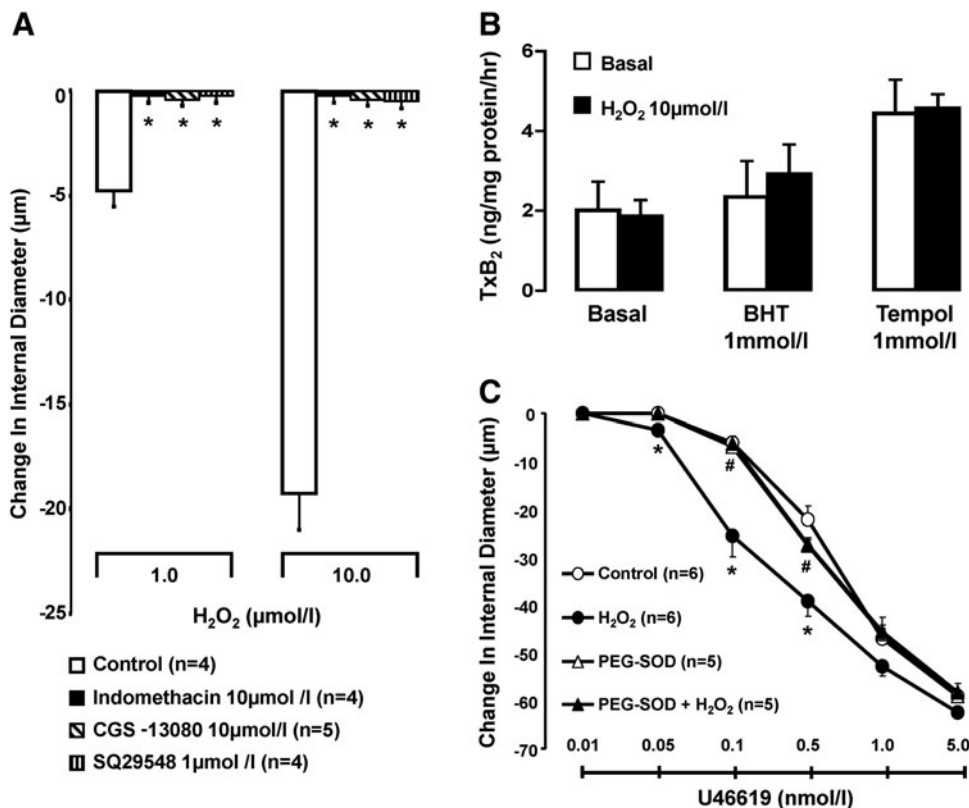


FIG. 4. Vascular effects of H_2O_2 in the absence or presence of modulators of the COX- TxA_2 -TP receptor pathway. (A) Effect of H_2O_2 on change in ID, over baseline, of renal interlobular arteries, in arteries not exposed and exposed to treatments as shown. Results are means \pm SE. * $p < 0.05$ versus control. (B) Effect of H_2O_2 on vascular TxB_2 synthesis in renal interlobular arteries, in the arteries exposed and not exposed to BHT or tempol; Results are means \pm SE. (C) Effect of H_2O_2 on concentration-response curve of a TP agonist (U46619), in arteries concurrently exposed or not exposed to pegylated SOD. Results are means \pm SE; * $p < 0.05$ versus vehicle; # $p < 0.05$ versus H_2O_2 . EC_{50} is presented in the text. TP, thromboxane; TxB_2 , thromboxane B_2 .

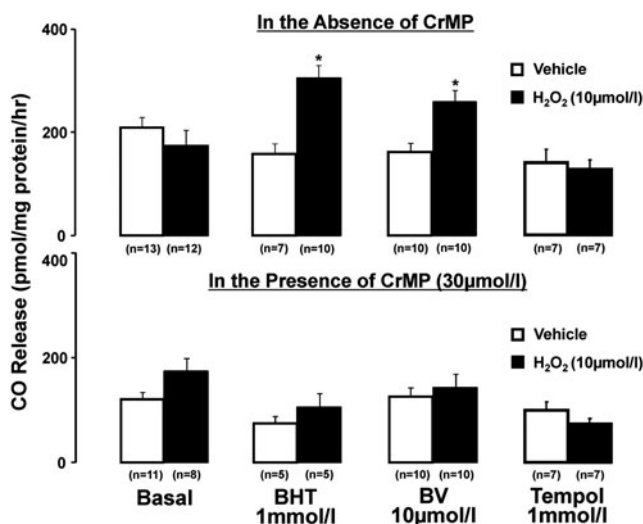


FIG. 5. Effect of H₂O₂ on vascular CO generation in arteries not exposed or concurrently exposed to the HO inhibitor, CrMP. Experiments conducted in the absence or presence of antioxidants, as shown. Results are means ± SE. **p* < 0.05 versus respective vehicle in the presence of antioxidant, either BHT or BV. CO, carbon monoxide; CrMP, chromium mesoporphyrin; HO, heme oxygenase.

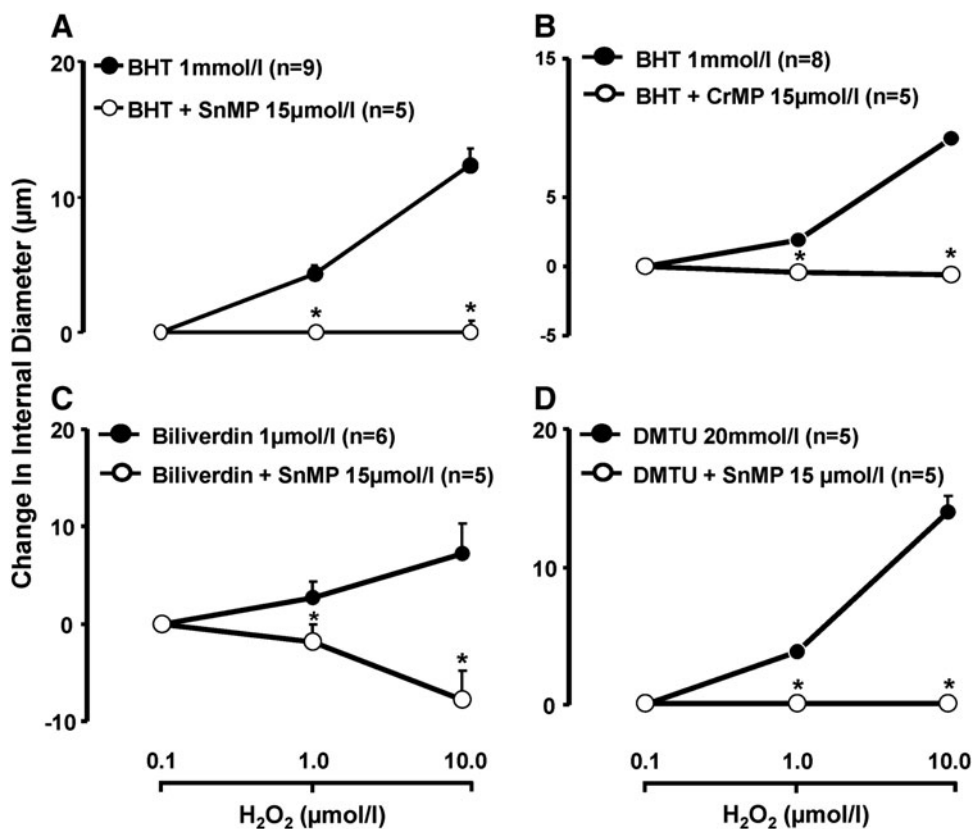
H₂O₂, either in the absence or presence of antioxidants (Fig. 5). That H₂O₂-induced CO release from arteries pretreated with BHT or BV was prevented by CrMP implies that the oxidant promotes release of only HO-derived CO. Relevant to this point, the release of CO-induced by H₂O₂ from MA pretreated

with BHT is not increased in endothelium-denuded preparations (47.4 ± 11.0 vs. 67.7 ± 8.5 pmol/mg protein/h, *n* = 6). It is also noteworthy that estimates of HO activity in arterial homogenates revealed no stimulatory effect of H₂O₂, either in the absence (729 ± 41 vs. H₂O₂: 817 ± 78 pmol/mg protein/h, *n* = 7) or in the presence of the antioxidant BV (807 ± 72 vs. 887 ± 83 pmol/mg protein/h, *n* = 4). Hence, estimates of HO activity in cell-free vascular homogenates are not necessarily a reflection of HO-derived CO release from intact vessels afforded antioxidant protection.

Complementary studies also revealed no effect of H₂O₂ on HO-1 or HO-2 protein expression, in arteries incubated for 90 min in oxygenated KB containing 1 mM BHT (HO-1/β-actin ratio: 0.56 ± 0.14 vs. 0.54 ± 0.03, without and with H₂O₂ (10 μM), respectively; HO-2/β-actin ratio: 0.62 ± 0.10 vs. 0.60 ± 0.04, without and with H₂O₂, respectively). Accordingly, the action of H₂O₂ to promote vascular release of HO-derived CO appears not to depend on enhanced HO isoform protein expression.

Figure 6 illustrates the result of experiments comparing the effect of H₂O₂ on the ID of pressurized RIA, bathed in KB containing an antioxidant (BHT, BV, or DMTU), with and without concurrent treatment with an inhibitor of HO, CrMP, or stannous mesoporphyrin (SnMP). The ability of H₂O₂ to increase the ID of such vessels was blunted by pretreatment with CrMP or SnMP. The aforementioned observations linking H₂O₂-induced vasodilation to release of HO-derived CO were corroborated in a study examining the effect of H₂O₂ on the ID of BHT- (1 mM) treated RIA taken from rats pretreated with AS-ODN, or corresponding SAS-ODN, targeting HO-1 and HO-2. Shown in Figure 7A–C, respectively, arteries from rats pretreated with HO-1 and HO-2 AS-ODN in

FIG. 6. Antioxidant-conditioned vasodilation to H₂O₂ in the absence or in the presence of HO inhibitor. Effect of H₂O₂ on change in ID, over baseline, of renal interlobular (A, C, D) or mesenteric resistance arteries (B), in arteries exposed to various antioxidants, and not exposed or concurrently exposed to HO inhibitors. Results are means ± SE. **p* < 0.05 versus without HO inhibitor.



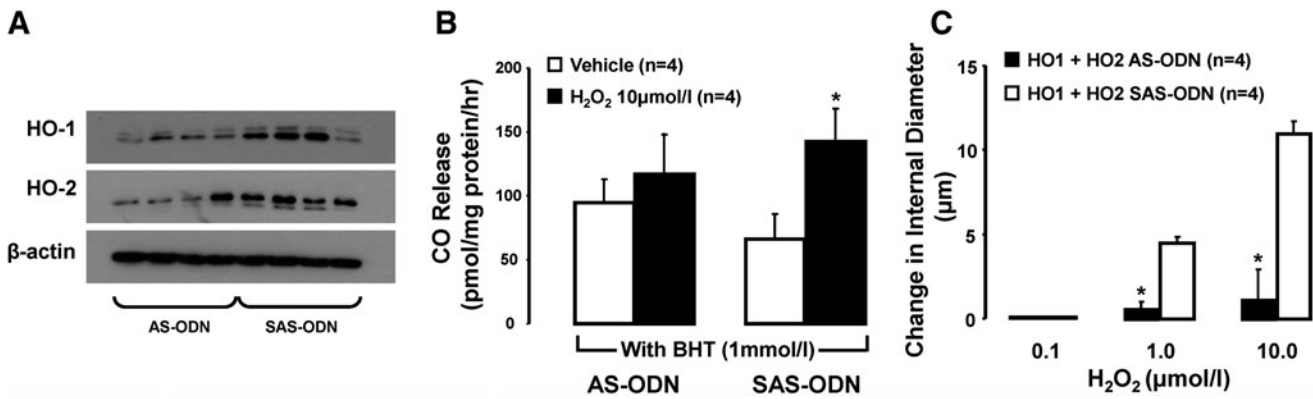


FIG. 7. Effect of H₂O₂ on CO release and change in ID in arteries from rats treated with either SAS-ODN or AS-ODN for HO1 and HO2. **(A)** Western blot analysis of HO1 and HO2 expression in renal interlobar arteries from rats treated with either AS or SAS-ODN for HO1 and HO2. **(B)** Effect of H₂O₂ on vascular CO release in mesenteric resistance arteries exposed to BHT. Results are means \pm SE; * p < 0.05 versus vehicle treated arteries from HO1+HO2 SAS-ODN. **(C)** Effect of H₂O₂ on change in ID in renal interlobular arteries exposed to BHT (1 mM). Results are means \pm SE; * p < 0.05 versus arteries from HO1+HO2 SAS-ODN. AS-ODN, antisense oligodeoxynucleotides; SAS-ODN, scrambled oligodeoxynucleotides.

combination featured, relative to control data in arteries from rats pretreated with the corresponding SAS-ODN, diminished expression of HO-1 (HO-1/ β -actin ratio: 0.25 ± 0.03 vs. 0.61 ± 0.13) and HO-2 protein (HO-2/ β -actin ratio: 0.35 ± 0.10 vs. 0.63 ± 0.06 , $n = 4$, $p < 0.05$), along with attenuation of H₂O₂-induced CO release and dilation.

Discussion

H₂O₂ is a mediator of oxidative stress *via* complex mechanisms involving promotion of lipid peroxidation, stimulation of cellular oxidases that increase O₂⁻ levels, and generation of OH \cdot *via* the Fenton reaction (1, 45). The first key finding of our study is that exogenous H₂O₂ constricts pressurized isolated rat resistance arteries, RIA and MA, a response that is prevented or in some cases converted to dilation by pretreatment with antioxidant agents. Importantly, not all antioxidants affect the same change in the vascular response to H₂O₂: pretreatment with PEG-SOD and tempol prevent H₂O₂-induced vasoconstriction without uncovering a dilatory response, whereas pretreatment with BHT, DES, BV, and DMTU prevents H₂O₂-induced vasoconstriction, but also uncovers a vasodilator response. The differential effect of these antioxidants on vascular responsiveness to H₂O₂ may be a consequence of differences in the profile of ROS targeted by the antioxidants in question. Hence, while the primary target of PEG-SOD and tempol is O₂⁻ (34), the primary targets of BHT (24), DES (2), BV (38, 39), and DMTU (22) are free radicals downstream of H₂O₂, including OH \cdot .

That H₂O₂-induced vasoconstriction is impeded by a diverse group of antioxidants suggests a critical connection between the constrictor action and the generation of a pro-oxidant milieu. This conclusion is in alignment with the finding that arterial vessels challenged with H₂O₂ display increased levels of O₂⁻ and lipid peroxides. Also, all antioxidants that prevent H₂O₂-induced vasoconstriction also prevent the associated rise in lipid peroxides. That H₂O₂-induced vasodilation is uncovered by pretreatment with BHT, DES, BV, or DMTU, but not with PEG-SOD or tempol, suggests that the dilatory action is linked to elimination of a free radical other than O₂⁻, which interferes with the expression of such an

action. All in all, the emerging conclusion is that the response of resistance arteries to exogenous H₂O₂ is conditioned by the redox status: constriction *versus* blunted constriction or dilation in vascular preparations not afforded and afforded protection from oxidative stress.

The second key finding of our study is that the redox-dependent vasoconstrictor action of H₂O₂ is linked to TP receptor activation, relying on a mechanism involving enhanced sensitivity of the vessels to TP receptor stimulation. Other investigators have reported a connection between the constrictive action of H₂O₂ and constrictor prostanoids (13). We found that pretreatment of vascular preparations with agents that inhibit COX, thromboxane synthase, or TP receptors eliminates constrictor responsiveness to H₂O₂. This implies a primary role of TxA₂ as a mediator of this constrictor response. However, in our studies, H₂O₂ did not increase vascular TxB₂ production; rather, it enhanced the sensitivity of the arteries to a synthetic agonist of TP receptors, U46619. Relevant to this conclusion, previous investigators have shown that H₂O₂ can result in an immediate and sustained increase in the cell surface expression of TP receptors (3). This effect has been linked to oxidative stress-mediated increased TP recycling to the plasma membrane, thus increasing functional TP expression (42, 44). Increasing the plasma membrane TP receptor density should enhance the number of spare receptors available for activation by endogenous agonists. Consequently, TP receptor occupancy needed for the half-maximal response (EC₅₀) should be lowered without affecting the maximal response. These conclusions are indeed corroborated by our results showing the lowering of EC₅₀ for the TP agonist in the presence of exogenous H₂O₂. Even though the precise molecular events leading to TP-sensitization remain unknown, consideration should be given to the possibility that this is linked to oxidant-induced activation of PKC signaling, as previously suggested (14).

Upon pharmacological blockade of vasoconstriction, we expected H₂O₂-induced vasodilation to prevail. However, neither antioxidants targeting O₂⁻ (PEG-SOD & tempol) nor agents interfering with the synthesis or actions of constrictor prostanoids allowed expression of H₂O₂-induced vasodilation, even in the face of blunted vasoconstriction. These

observations, in addition to the findings that H₂O₂-induced vasodilation necessitates pretreatment with BHT, BV, DES, or DMTU, suggest that exclusion of radicals downstream of H₂O₂, rather than interference with the expression of vasoconstriction, enables H₂O₂ to elicit vasodilation.

The third key finding of our study is that the redox-dependent vasodilatory action of H₂O₂ is linked to a mechanism involving enhanced vascular production of HO-derived CO. Two reciprocal lines of evidence substantiate this relationship: first, the experimental conditions found to favor expression of H₂O₂-induced vasodilation; for example, pretreatment of the arterial vessels with antioxidants such as BHT, BV, and DES also enables exogenous H₂O₂ to increase the release of vascular CO. Second, experimental interventions that interfere with H₂O₂-induced release of CO; for example, pretreatment with inhibitors of HO activity or HO protein expression effectively prevents the oxidant from eliciting a vasodilatory response. Accordingly, the redox-dependent vasodilatory action of H₂O₂ may be regarded as a manifestation of the associated increase in vascular production of HO-derived CO. That only arterial vessels pretreated with BHT, BV, or DES are stimulated by H₂O₂ to release HO-derived CO implies that this response is also redox dependent, necessitating an antioxidant setting for its expression. These results suggest that free radicals, other than O₂⁻, somehow prevent H₂O₂ from increasing HO-dependent CO; however, our study falls short from addressing the mechanism underlying this redox-dependent stimulatory action of H₂O₂ on vascular CO release. According to previous reports, higher concentrations of H₂O₂ have been shown increase HO activity in purified enzyme systems and in renal homogenates, *via* acting as an electron donor (23, 29). However, in the present study, a stimulatory action of H₂O₂ on HO activity, measured in arterial homogenates *in vitro*, could not be documented, either in the absence or presence of a vasodilation promoting antioxidant. However, one cannot exclude the possibility that the ability of exogenous H₂O₂ to increase vascular release of HO-derived CO relies on stimulation of HO activity *via* engagement of second-messenger systems that are only operational when cellular integrity is preserved. In this regard, interaction between H₂O₂-activated kinases (6–8, 30) and kinase-dependent modulation of HO (5, 47) may link H₂O₂ to stimulation of HO-dependent CO release in arterial vessels.

Thus, as summarized in Figure 8, pleiotropism observed to exogenous H₂O₂ with regard to the ID of resistance arteries is redox dependent. In the absence of an exogenous antioxidant, H₂O₂-induced redox imbalance leads to vasoconstriction that in turn is dependent upon vascular TP sensitization. All antioxidants, in spite of their diverse specificity, attenuate oxidative stress and prevent this redox-dependent vasoconstriction. Importantly, vasodilation to H₂O₂ is only uncovered when oxidants downstream of H₂O₂ are quenched and involves increased HO-dependent CO generation. These observations lead us to conclude that a free radical, other than O₂⁻, interferes with the mechanisms linking H₂O₂ to stimulation of HO-dependent CO generation.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee of the New York Medical College approved all animal protocols.

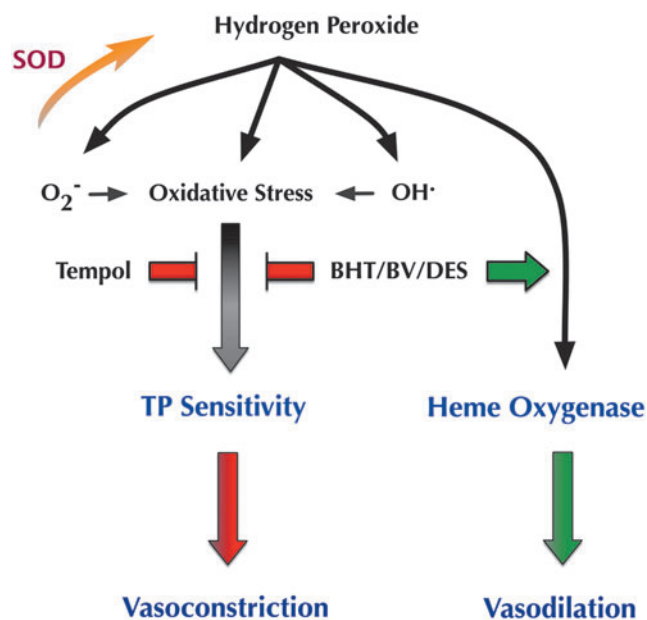


FIG. 8. A schematic representation of pleiotropic vascular effects of H₂O₂, as derived from our hypothesis and results. In the absence of exogenous antioxidants, H₂O₂ promotes a pro-oxidant setting and induces TP-dependent vasoconstriction. This effect is abrogated by a wide spectrum of antioxidants, targeting radicals both up and downstream of H₂O₂. Conversely, vasodilation to H₂O₂ is reliant upon HO-derived products and is only uncovered in the presence of antioxidants targeting radicals downstream of H₂O₂. These results imply that a radical other than O₂⁻ somehow interferes with the ability of H₂O₂ to stimulate HO product generation and to bring about vasodilation.

Male Sprague-Dawley rats (250–300 g; Charles River) were anesthetized (pentobarbital sodium, 60 mg/kg, intraperitoneally), and the kidneys and the intestines were removed and placed on a dish filled with oxygenated ice-cold KB (composition in mM: 118.5 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25.0 NaHCO₃, and 11.1 mM dextrose). RIA and mesenteric arteries (3rd order) were isolated for various experiments as described in the Results section. To investigate the effects of HO inhibition on vascular effects of H₂O₂, antisense oligonucleotides, HO2-AS-ODN and HO1-AS-ODN (complementary to rat HO2 and HO1 mRNA, respectively), and their scrambled controls were synthesized by Sigma-Genosys; each oligodeoxynucleotide was phosphorothioated on the first three bases of the 3' end and was purified by high-pressure liquid chromatography. The sequence of HO-1 AS-ODN is 5'-GGCGCTCCATCGCGGGACTG-3' and targets bases +10 to -9 of HO-1 mRNA, and the sequence of HO-2 AS-ODN is 5'-TCTGAAGACATTGTTGCTGA-3' and targets bases +11 to -9 of HO-2 mRNA. The sequence of HO-1 S-ODN is 5'-TCCAGCGGCGTCAGCGGTGC-3', and the sequence of HO-2 S-ODN is 5'-GATCTGACTTCAAGTGATTG-3'. The effectiveness of HO-1 AS-ODN and HO-2 AS-ODN to reduce tissue expression of HO-1 and HO-2, respectively, was documented previously (19). Oligonucleotides encapsulated in liposomes (1:1 molar ratio) prepared using DOTAP liposomal transfection reagent (Roche Diagnostics) were followed

by injection into the tail vein of rats, as described before (19). Animals were sacrificed 36 h postinjection, and renal and mesenteric arteries were collected for further studies.

Vascular function studies

RIAs or MAs, were dissected into segments 1 to 2 mm in length and mounted on a myograph (model CH/200/Q; Living System Instrumentation). All residual blood was gently flushed from vessel lumen before experiments. One end of the vessel was mounted on a glass micropipette and connected to a pressure servo-controller (model CH/200/Q; Living System Instrumentation). The opposite end of the vessel was tied to a micropipette connected to a stopcock. A video camera (Javelin), leading to a video caliper (Texas A & M), monitor (Javelin) and recorder, was used to record the vessel chamber. Vessels were superfused throughout the experiment with gassed (95% O₂-5% CO₂) KB at 1 ml/min, at 37°C. Intraluminal pressure was gradually increased to 80 mmHg and allowed to equilibrate for 1 h. Drugs were added, at indicated concentrations, to the superfusion buffer and allowed to equilibrate for 15 min; changes in ID were then recorded in response to increasing concentrations of H₂O₂, over a period of 20–25 min. Data are reported as ID (μ m) or change in ID (μ m) over baseline.

For experiments involving endothelium free arteries, the vascular endothelium of RIA was removed *via* passage of an air bubble through the lumen of the artery. Endothelium removal was confirmed by failure of these arteries to dilate in response to increasing concentrations of acetylcholine (Ach). Arteries demonstrating Ach-dependent dilation were excluded from the study.

Assessment of vascular TxB₂

Freshly dissected renal interlobar arteries were washed and pretreated with and without indicated antioxidants. Following a 15-min preincubation, arteries were removed and placed in fresh vials containing or not containing various antioxidants in the absence or presence of H₂O₂ (10 μ M). Incubations were done in closed vials at 37°C in oxygenated KB for 60 min, so as to allow detection of low concentrations of eicosanoids. After incubation, the reaction was stopped by acidification with acetic acid (~pH 4.0), and internal standard mix (IS for TxB₂- d4PGE2) was added to the buffer, followed by extraction with ethyl acetate. TxB₂ was identified and quantified with a Q-trap 3200 linear ion trap quadrupole liquid chromatography/tandem mass spectrometry equipped with a Turbo V ion source operated in a negative electrospray mode (Applied Biosystems), as previously described (36). Data are normalized to total protein and are presented as ng/mg protein/h.

Assessment of vascular CO release

Freshly dissected small mesenteric arteries (2nd and 3rd order) were pretreated with indicated antioxidants, with untreated arteries kept as controls. Following a 15-min preincubation, 10 μ M of H₂O₂ was added to the vials, and the vials were then sealed and incubated at 37°C for 90 min in oxygenated KB. Experiments were performed in arteries concurrently exposed and not exposed to CrMP (30 μ M), to differentiate between HO-dependent and independent sources

of CO. After incubation, gas-sealed vials were placed on ice to stop the reaction, and headspace gas was analyzed for CO with C¹³O¹⁶ added as an internal standard. CO measurements were performed using an Agilent 5890 GC-MS, as previously described (19, 31). Data are normalized to total protein and are presented as pmoles/mg protein/h.

Assessment of HO activity in vascular homogenates

Freshly dissected small mesenteric and renal interlobar arteries were homogenized in sucrose-Tris buffer (sucrose, 255 mM, Tris-HCl, 20 mM) with NP-40 (1%) and mammalian protease inhibitor cocktail (Sigma Aldrich). After protein estimation, 0.15 mg protein of the arterial homogenate was incubated with various antioxidants for 15 min. After this, homogenates were incubated with 10 μ M of H₂O₂, 30 μ M heme, and 2 mM NADPH, in the absence or presence of an HO inhibitor, CrMP (50 μ M). Vials were then sealed and incubated at 37°C for 90 min. After incubation, gas-sealed vials were placed on ice to stop the reaction, and headspace gas was analyzed for CO with C¹³O¹⁶ added as an internal standard. CO measurements were performed using an Agilent 5890 GC-MS, as previously described (19, 31). HO activity is calculated by subtracting CO levels obtained in the presence of CrMP from those obtained without. Data are normalized to total protein and are presented as pmoles/mg protein/h.

Assessment of HO protein expression

Freshly dissected renal interlobar and small mesenteric arteries were paced in oxygenated KB containing 1 mM of BHT. After a 15-min preincubation, arteries were incubated with and without H₂O₂ (10 μ M) at 37°C for 90 min. HO-1 and HO-2 protein expression was assessed in these arteries by immunoblotting, as previously described (9). Assessment of HO protein expression in SD rats treated with either AS-ODN or S-ODN for HO-1 & 2 was performed in freshly dissected renal interlobar arteries from these animals.

Assessment of the redox state

Renal interlobar and small mesenteric arteries were preincubated in the absence or presence of described antioxidants for a period of 15 min, followed by an incubation with and without H₂O₂ (10 μ M) at 37°C for 20 min in oxygenated KB. Subsequently, arteries were snap-frozen in liquid nitrogen before being analyzed for TBARS (using a Cayman Chemicals, Inc. kit # 10009055), as per manufacturers' protocol. Data are normalized to total protein and are presented as nmoles/mg protein.

For assessment of effects of H₂O₂ on arterial SOD activity, mesenteric and renal arteries were incubated with and without H₂O₂ (10 μ M) at 37°C for 20 min in oxygenated KB. Arteries were snap-frozen in liquid nitrogen and analyzed for SOD activity (Cayman Chemicals, Inc. kit # 706002), as per the manufacturers' protocol. Data are normalized to total protein and are presented as U/mg protein.

Lucigenin chemiluminescence was employed for superoxide detection, as previously described (25). Briefly, renal interlobar arteries were incubated in oxygenated KB at 37°C for 20 min with and without H₂O₂ (10 μ M). Arteries were then transferred to preblanked lucigenin vials (5 μ M) and analyzed

in a scintillation counter. Data are normalized to total protein and are presented as CPM/ μ g protein.

Data analysis

Data are expressed as means \pm SEM for the given number (*n*) of experiments. Results were analyzed by ANOVA with Tukey–Kramer *post hoc* analysis, or by Student *t*-test. The null hypothesis was rejected at *p* < 0.05.

Acknowledgments

This work was supported by the National Institutes of Health grants 2PO1HL034300 (AN) and DK068134, HL55601, and HL34300 (NGA). All authors had full access to the data and take responsibility for its integrity. All authors have read and agree with the manuscript as written. We thank Jennifer Brown for her outstanding editorial assistance in the preparation of the manuscript.

Author Disclosure Statement

The authors declare no competing financial interests.

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Date of first submission to ARS Central, February 27, 2012; date of final revised submission, August 6, 2012; date of acceptance, August 7, 2012.

Abbreviations Used

BHT	= butylated hydroxytoluene
BV	= biliverdin
CO	= carbon monoxide
COX	= cyclooxygenase
CrMP	= chromium mesoporphyrin
DES	= desferroxamine
DMTU	= dimethyl thiourea
HO	= heme oxygenase
H ₂ O ₂	= hydrogen peroxide
ID	= internal diameter
KB	= Krebs' buffer
PEG-SOD	= PEGylated superoxide dismutase
RIA	= renal interlobular
ROS	= reactive oxygen species
SOD	= superoxide dismutase
SnMP	= stannous mesoporphyrin
TP	= thromboxane
TxA ₂	= thromboxane A ₂
TxB ₂	= thromboxane B ₂