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Antioxidant and Vasodilatory Effects of Heme Oxygenase on Mesenteric Vasoreactivity Following Chronic Hypoxia

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Abstract

Objective—Chronic hypoxia (CH) results in impaired vasoconstriction associated with increased expression of heme oxygenase (HO). We hypothesized that enhanced HO activity minimizes reactive oxygen species (ROS) in arteries from CH rats, thereby normalizing endothelium-dependent vasodilation and concurrently produces carbon monoxide (CO), resulting in tonic vasodilation.

Methods—ROS were quantified in mesenteric arteries from control and CH Sprague-Dawley rats. Reactivity to the endothelium-dependent vasodilator, acetylcholine (ACh), and the vasoconstrictor, phenylephrine (PE), were also assessed.

Results—Basal ROS levels did not differ between groups and were similarly increased by HO inhibition. In contrast, catalase inhibition increased ROS in CH rats only. Vasodilatory responses to ACh were not different between groups. Combined inhibition of catalase and HO impaired PE-induced vasoconstriction in both groups. CH-induced impairment of vasoconstriction was reversed by either catalase or HO inhibition supporting the protective roles of the HO and catalase pathways following CH. Increased vascular smooth muscle calcium was observed with inhibition in the CH group, suggesting that catalase and HO-derived CO elicit reduced calcium influx, leading to the impaired vasoconstriction.

Conclusions—Our data suggest that although the HO pathway is an important antioxidant influence, impaired vasoconstriction following CH appears to be due to effects of ROS and HO-derived CO.

Keywords

hypoxia; heme oxygenase; carbon monoxide; oxidative stress; mesenteric arteries

Chronic hypoxia (CH) can arise from pathological conditions, such as chronic obstructive pulmonary disease (COPD) as well as prolonged residence at high altitude. Chronic hypoxemia in patients [18] or in normal individuals exposed to 36 hours of hypobaric hypoxia [19] results in blunted reflex vasoconstriction to a challenge of lower body negative pressure. In addition, forearm vasodilation is observed in COPD patients even when oxygenation is normalized [2]. Similarly, exposure of rats to CH produces decreased systemic vasoconstrictor reactivity, as demonstrated by the impaired agonist as well as myogenic-induced vasoconstriction of systemic arteries [1,8,10,11,21,36]. Interestingly, these deranged vasoconstrictor responses persist upon restoration of normoxia [1,8,21]. Although several potential pathways have been examined to account for this CH-induced

alteration in vasoreactivity, the role of increased oxidative stress and generation of reactive oxygen species (ROS) has not been explored.

ROS, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), and hydroxyl radicals ($\bullet OH$), are implicated in the pathogenesis of many diseases, including COPD [40], hypertension [29,43], and ischemia reperfusion injury, as well as other cardiovascular diseases through the development of pathologies, such as atherosclerosis, cardiac hypertrophy, and cardiomyocyte apoptosis [4,25,29,43]. Sources of ROS include nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, as well as uncoupling of the mitochondrial electron transport chain and of nitric oxide synthase (NOS) [28]. Mechanisms to scavenge ROS are, therefore, important to minimize their impact in disease states. One well-characterized ROS-scavenging pathway present constitutively in cells occurs through the conversion of $O_2^{\bullet-}$ by superoxide dismutase (SOD) to form H_2O_2 , which is further broken down by catalase to form water and oxygen [7,32,48]. As a scavenger of the vasodilator NO, $O_2^{\bullet-}$ can have profound inhibitory effects on endothelium-dependent vasodilation [43]. As a potent oxidant, H_2O_2 can inhibit vasoconstrictor reactivity [30] but has additionally been reported to act as a vasodilator, depending on the concentration and tissue examined [15]. Endogenous antioxidant pathways, therefore, play important roles in protecting vascular function [30].

The heme oxygenase (HO) pathway is an additional mechanism that diminishes vascular oxidative stress. HO catalyzes the degradation of heme to the gaseous vasodilator carbon monoxide (CO), ferrous iron (sequestered by ferritin), and biliverdin, which is reduced to the potent antioxidant bilirubin by biliverdin reductase (BVR) [14,39]. Oxidation of bilirubin by ROS reverts it to biliverdin, providing an additional substrate for BVR [41]. This recycling of bilirubin greatly amplifies the antioxidant capacity of this pathway.

There are three known HO isoforms: HO-1, HO-2, and HO-3. The expression of the highly inducible HO-1 isozyme is enhanced by stimuli that increase oxidative stress, such as hypoxia [17,31], ischemia/reperfusion [44], hyperthermia [12], and NO [20], among other factors. HO has also been reported to limit ROS production through decreasing heme availability, thereby limiting NAD(P)H oxidase activity as well as by producing bilirubin [42]. HO-1 is present in many tissues, including the vascular endothelium [14,21,34] and smooth muscle [5]. Protein expression of HO-1 is augmented in rat aortae as well as mouse cardiac tissues after exposure to hypoxia [17,21]. In contrast, HO-2 is biochemically distinct from HO-1 and its activity appears to be constitutive. Last, HO-3 is structurally similar to HO-2 but is a poor heme catalyst [14].

Although we have previously demonstrated a role for the HO pathway in reduced vasoconstrictor reactivity following CH [16,21], it is unclear if this effect is due to enhanced production of the vasodilator CO or, alternatively, to antioxidant properties of the enzyme. Therefore, the goals of the present study were to 1) evaluate the importance of HO in regulating ROS levels in arteries from control and CH rats, 2) examine the effects of CH and the HO pathway on endothelium-dependent vasodilation, and 3) determine if the antioxidant effects of HO contribute to reduced vasoconstrictor reactivity following CH. We hypothesized that the HO pathway concurrently 1) scavenges elevated ROS generated in response to CH, thereby normalizing impaired endothelium-dependent vasodilation, and 2) impairs agonist-induced vasoconstriction through CO production.

MATERIALS AND METHODS

All protocols employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center.

Experimental Groups

Male Sprague-Dawley rats (250–300 g body weight, Harlan Industries, Indianapolis, IN, USA) were divided into normoxic and CH groups for each experimental protocol. Animals used for CH studies were housed in a hypobaric chamber with barometric pressure maintained at ~380 Torr for 48 hours. On the day of the experiment, rats were removed from the hypobaric chamber and immediately transferred to a Plexiglas® chamber continuously flushed with a 12% O₂–88% N₂ gas mixture to reproduce the inspired P_{O₂} (70 mmHg) within the hypobaric chamber. CH rats were maintained in this environment until the time of euthanasia for vessel removal (see below). Control (i.e., normoxic) animals were housed in the same animal facility under normobaric (630 Torr) conditions. All animals were housed in identical cages under a 12:12-hour light-dark cycle.

Isolation of Mesenteric Resistance Arteries

Rats were anesthetized with sodium pentobarbital (32.5 mg/kg, intraperitoneally [i.p.]) under normoxic conditions, and a midline laparotomy was performed to expose the mesenteric arteries. Rats were euthanized by exsanguination secondary to removal of the mesenteric arcade. The arcade was immediately placed in ice-cold HEPES buffer (in mM: 134.4 NaCl, 6 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, 10 glucose; pH 7.4 with NaOH) pinned out in a silastic coated dissection dish, and fifth-order small mesenteric resistance arteries (~1 mm length; 175–250 µm, intradermally [i.d.]) were dissected free. Isolated arteries were transferred to a HEPES-filled vessel chamber (CH-1; Living Systems Instrumentation, Burlington, Vermont, USA), then cannulated with glass pipettes, and secured in place with silk ligatures. The vessels were stretched longitudinally to approximate *in situ* length, pressurized to 60 mmHg with either a buffer-filled column or servo-controlled peristaltic pump (Living Systems Instrumentation), and the chamber was placed on a microscope stage for observation. At this pressure, vessels from neither group developed significant tone, since mesenteric arteries are resistant to the development of spontaneous tone [13,38,47]. Vessels were superfused during each experimental protocol with warm physiological saline solution (PSS) (37 °C) containing (in mM): 129.8 NaCl, 5.4 KCl, 0.5 NaH₂PO₄, 0.83 MgSO₄, 19 NaHCO₃, 1.8 CaCl₂, and 5.5 glucose at a rate of 10 mL/min. PSS was aerated with 21% O₂, 6% CO₂, and a balanced N₂ gas mixture throughout the experiments to maintain normoxic conditions. Arterial viability was verified prior to each experiment by adding the vasoconstrictor, phenylephrine (PE; 10⁻⁶ M), followed by the endothelium-dependent vasodilator, acetylcholine (ACh; 10⁻⁶ M), to the superfusate. All vessel experiments in both groups were performed under normoxic conditions to eliminate any complicating influences of acute hypoxia and to focus on the sustained alterations in reactivity previously documented following CH [1,8,10,21,36].

Preparation of Experimental Solutions

The cell-permeant ROS-sensitive fluorescent indicator, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCF; Molecular Probes, Carlsbad, CA, USA), was dissolved in anhydrous dimethyl sulfoxide (DMSO) at a concentration of 50 µg/mL. Immediately prior to loading, DCF was mixed with a 20% v/v solution of pluronic acid in DMSO, and this mixture was diluted with HEPES buffer to yield a final concentration of 5 µM DCF and 0.05% pluronic acid. A dose of 0.5 µM zinc protoporphyrin IX (ZnPPIX; Frontier Scientific, Inc., Logan, Utah, USA) was used for each experiment, since this dose was verified in prior studies to be effective at reducing HO activity in this preparation [36]. ZnPPIX was prepared in the dark, as previously described [46]. Specifically, 50 mg of ZnPPIX was solubilized in 0.5 mL of 10% ethanolamine and 2 mL of 0.9% NaCl was slowly added. This solution was brought to a pH of 7.6–8.0, using 1 M HCl, then brought to a final volume of 5 mL, using deionized water. The solution was then filter sterilized (0.2-µm filter), aliquoted, and frozen (–80 °C) until use. Since ZnPPIX dissociates with light

exposure, all preparation steps and experiments using ZnPPIX were performed in the dark. The final ZnPPIX concentration was verified spectrophotometrically. The ZnPPIX vehicle was prepared the same way minus the addition of ZnPPIX.

PE (Sigma, St. Louis, MO, USA) and ACh (Sigma, St. Louis, MO, USA) were dissolved in deionized water, aliquoted, and frozen (-20°C) until use. 3-amino-1,2,4-triazole (ATZ; Sigma), 4,5-dihydroxy-1,3-benzene-disulfonic acid (tiron; Sigma) and catalase (Sigma) were prepared fresh for every experiment by adding directly to PSS. Fura 2-AM (Invitrogen Corp., Carlsbad, CA, USA) stock solution was prepared by dissolving in anhydrous DMSO at a concentration of 1 mM. Aliquots of this solution were stored at -80°C until use. Immediately prior to vessel loading, stock solutions of fura 2-AM were mixed with a 20% v/v solution of pluronic acid in DMSO, and this mixture was diluted with HEPES buffer to yield a final concentration of 2 μM fura 2-AM and 0.05% pluronic acid.

Measurement of Vascular ROS

Cannulated normoxic ($n=5-9$ per protocol) and CH ($n=5-7$ per protocol) vessels were transferred to a Nikon Diaphot 300 microscope (Nikon Instruments Inc., Melville, NY, USA) equipped with a 10 $\times$ fluorescence (fluorescein isothiocyanate; FITC) objective for analysis. Following the 30-minute equilibration in aerated PSS, vessels were superfused for one hour with either PSS or PSS with the addition of either: the catalase inhibitor ATZ (5 mM), the HO inhibitor ZnPPIX (0.5 μM), ZnPPIX vehicle, ATZ+ ZnPPIX vehicle, or ATZ +ZnPPIX. Vessels were then loaded with DCF in the dark in a vessel chamber attached to a temperature controller (Living Systems). DCF is oxidized by cytoplasmic peroxynitrite (ONOO^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot\text{OH}$) to produce a fluorescent product [6,24,33]. Images were collected prior to DCF loading (for background) and 50 minutes later using a cooled, digital charge-coupled device (CCD) camera (SenSys 1400; Photometrics, Pleasanton, CA, USA). MetaFluor 4.5 software (Universal Imaging; Molecular Devices, Sunnyvale, CA, USA) was used to process the images. Sensitivity of DCF fluorescence as a measure of ROS was verified by noting the inhibitory effect on the signal resulting from a combination of 10 mM tiron (an SOD mimetic) and 1200 U/mL catalase. Previous studies from our laboratory have also demonstrated increased DCF fluorescence in isolated arteries in response to xanthine/xanthine-oxidase, which augments ROS production [22]. Since DCF may leak from cells, fluorescence from the extravascular solution was subtracted from the total to estimate intravascular ROS [24].

ACh-Induced Vasodilation

Cannulated arteries from normoxic ($n=5-6$ per protocol) and CH ($n=4-6$ per protocol) rats were transferred to an inverted microscope (model TMS; Nikon) equipped with a 10 $\times$ objective, video camera, and monitor for analysis. Arteries remained pressurized at 60 mmHg for a 30-minute equilibration period and were then superfused for one hour with either a control PSS solution or PSS with the addition of antagonists. Increasing doses of PE were administered until vessels were constricted to 50% of resting inner diameter. Subsequent vasodilation in response to increasing concentrations of ACh (10^{-9} to 10^{-5} M, three minutes each step) was assessed by measuring intraluminal diameter (ID), using a video dimension analyzer (Living Systems). Data were digitized by using DATAQ A/D software for analysis (DATAQ Instruments, Akron, Ohio, USA).

PE-Induced Vasoconstriction

Cannulated vessels from normoxic ($n=5-6$ per protocol) and CH ($n=5$ per protocol) rats were transferred to an inverted microscope (model Eclipse TS100; Nikon) equipped with a Nikon S Fluor 20 $\times$ objective, video camera, and monitor for analysis. Vessels were equilibrated for 30 minutes by superfusion (10 mL/min) with heated PSS (37°C), followed

by loading with the cell-permeant ratio-metric Ca^{2+} -sensitive fluorescent dye fura 2-AM (2 μM) in 4 mL of HEPES buffer. Vessels were incubated in this solution for 45 minutes in the dark at room temperature. Abluminal exposure to fura 2-AM, using this protocol, has previously been shown to selectively load vascular smooth muscle (VSM) cells [26]. Prior to experimentation, fura 2-AM was washed from the superfusate for 15 minutes with warmed PSS to remove excess dye and allow for hydrolysis of AM groups by intracellular esterases. Vessels were then superfused for one hour in either a control PSS solution or PSS with the addition of antagonists. Agonist-induced vasoconstrictor and calcium responses were determined by exposing fura-loaded vessels to increasing concentrations of PE (10^{-9} to 10^{-5} M, three minutes each step) in the superfusate. The inner diameter was continuously monitored by using video microscopy and edge-detection software (IonOptix, Milton, Massachusetts, USA). Fura-loaded vessels were alternatively excited at 340 and 380 nm, and the respective 510-nm emissions were quantified by using a photomultiplier tube (IonOptix) and recorded by using IonWizard software (version 4.4; IonOptix).

Statistics

Data are expressed as the means $\pm$ SEM. DCF fluorescence was analyzed by one way analysis of variance (ANOVA). When significance was indicated, groups were compared using Tukey post-hoc analysis. Percent vasodilation and vasoconstriction to ACh and PE, respectively, were calculated as the percent difference of ID observed at each concentration vs. baseline values. This was calculated as the following equation: $((\text{ID at baseline} - \text{ID at each concentration}) \div \text{ID at baseline}) \times 100$. Prior to analysis, percentage data were arcsine transformed to approximate a normal distribution. Change in VSM calcium was calculated as the difference in F_{340}/F_{380} emissions at each concentration from baseline. Data from vasodilation and vasoconstriction studies were analyzed by two-way repeated measures ANOVA. Where significant differences were indicated, individual groups were compared by using Student-Newman-Keuls post-hoc analysis. A probability (P) of ≤ 0.05 was accepted as statistically significant for all comparisons.

RESULTS

Measurement of Vascular ROS

Levels of DCF fluorescence, indicative of ROS concentrations, were not significantly different between untreated isolated mesenteric arteries from normoxic and CH rats (Figure 1). In addition, Figure 1 shows that the ROS scavengers tiron (10 mM) and catalase (1200 U/mL) significantly reduced DCF fluorescence in both groups. To account for DCF leakage into the extravascular solution, fluorescence from the solution was subtracted from total fluorescence to obtain intravascular ROS levels [24]. For this reason, experiments with the antioxidants tiron and catalase produced negative DCF fluorescence values.

Effects of HO and Catalase Inhibition on Vascular ROS

Between-group comparisons (i.e., normoxic control vs. CH) revealed no significant differences in DCF fluorescence for similar treatments (Figure 2). Catalase inhibition with 5 mM of ATZ significantly increased DCF fluorescence in isolated vessels from CH rats but not normoxic controls (Figure 2). The vehicle for ZnPPIX slightly enhanced DCF fluorescence in vessels from the CH group but not in normoxic controls (Figure 2). In contrast, super-fusion with the HO inhibitor, ZnPPIX (0.5 μM), resulted in augmented DCF fluorescence above that of controls and ZnPPIX vehicle-treated vessels from both groups of rats (Figure 2). The combined exposure of vessels to ATZ and ZnPPIX resulted in significantly increased DCF fluorescence above that of controls in both groups but did not differ from HO inhibition alone in the CH rats. Thus, contrary to our hypothesis, these data demonstrate that vascular ROS are not elevated in response to CH exposure. In addition,

these results suggest that the antioxidant catalase and HO pathways are important in the regulation of ROS within the vasculature of both normoxic and CH rats.

ACh-Induced Vasodilation

There were no significant differences in ACh-induced vasodilation in vessels from CH vs. control rats (Figure 3). Varying concentrations of PE were required to achieve 50% vasoconstriction from baseline inner diameter measurements in vessels from each group. In general, slightly larger doses of PE were necessary to achieve 50% vasoconstriction in the untreated CH rats, compared to the untreated normoxic group.

Despite our observation that HO inhibition results in enhanced ROS levels (Figure 2), incubation of vessels with ZnPPiX was without effect on ACh-mediated vasodilation in either group. These findings suggest that CH does not result in ROS-dependent impairment of endothelium-mediated vasodilation. Further, the acute vasodilatory responses to ACh do not appear to involve CO or other products of HO in either group. Similarly, combined administration of ATZ and ZnPPiX produced no significant effect on ACh-induced vasodilation in vessels from either normoxic or CH rats (data not shown).

PE-Induced Vasoconstriction

Normoxic Control Arteries—Inhibition of HO with ZnPPiX decreased PE-mediated vasoconstriction in vessels from normoxic rats at only one point in the concentration-response curve (Figure 4A). Inhibition of catalase had no significant effect on vasoconstriction, compared to untreated arteries. Combined inhibition of HO and catalase did not result in further impairment of vasoconstriction in arteries from this control group, compared to HO inhibition alone (Figure 4A). This minor impairment of vasoconstriction to PE following the administration of the inhibitors is possibly due to diminished VSM calcium sensitivity. Of note, at lower concentrations of PE, these treatments exerted minor increases in the VSM calcium response in control arteries without affecting vasoconstriction (Figure 4A).

CH Arteries—Vasoconstriction of untreated vessels from CH rats was significantly impaired, compared to normoxic (N) controls (Figure 4B). Similarly, slightly larger doses of PE were required to reach 50% vasoconstriction in the untreated CH vessels, compared to the normoxic controls in the ACh-induced vasodilator studies. VSM calcium, however, was similar to controls, suggesting decreased calcium sensitivity in vessels from CH rats (Figure 4B). Inhibition of either HO or catalase restored vasoconstrictor reactivity to levels of normoxic controls. Similar to control arteries, VSM calcium was increased moderately by HO inhibition but was augmented dramatically by catalase inhibition (Figure 4B). Interestingly, combined HO and catalase inhibition greatly blunted PE-induced vasoconstriction in arteries from CH rats, again without major effects on VSM calcium (Figure 4B). These results suggest that the catalase and HO pathways work in concert to reduce ROS levels and vasoconstrictor reactivity in vessels from CH rats.

DISCUSSION

The major findings of this study were: 1) exposure of rats to 48-hour CH does not result in increased vascular ROS, compared to normoxic controls (Figure 1 and 2); 2) the SOD/catalase and HO pathways effectively limit ROS levels in both control and CH arteries (Figure 2); 3) agonist-induced vasodilation is normal in arteries from CH rats and is unaffected by HO inhibition (Figure 3); 4) impaired PE-induced vasoconstriction following CH was normalized by the inhibition of either HO or catalase (Figure 4); and 5) increased ROS following combined inhibition of catalase and HO was associated with significantly

diminished PE-induced vasoconstriction in both groups (Figure 4). Our findings support the hypothesized role of HO to diminish vascular ROS; however, reduced vasoconstrictor reactivity following CH is likely due to CO produced by the enzyme rather than its antioxidant properties.

The present study found no difference in ROS levels between arteries from the control and CH groups (Figure 1). Exogenous activation of the SOD/catalase pathway, using tiron and catalase, significantly decreased DCF fluorescence, demonstrating that DCF is an effective indicator of ROS in this preparation (Figure 1). Although prior studies have shown DCF is not oxidized by $O_2^{\bullet-}$ [6,24,33], the specific ROS detected in these experiments may include H_2O_2 , $OONO^-$, and $^{\bullet}OH$. Our results support the protective role of catalase to scavenge H_2O_2 in CH rats, since inhibition of the enzyme with ATZ produced a significant increase in ROS (Figure 2). In contrast, catalase inhibition had no effect on ROS levels in normoxic rats (Figure 2). As hypothesized, HO also appears to play an important role in the regulation of ROS, as inhibition of this pathway led to significantly increased ROS levels in both groups (Figure 2). However, ROS were not elevated in CH vessels beyond that of normoxic controls with HO inhibition (Figure 2). This finding suggests that 48-hour hypoxic exposure may not elicit elevated generation of ROS in this vascular bed. This conclusion is also supported by the lack of an apparent effect of ROS on endothelium-dependent vasodilation in arteries from either group (Figure 3). Thus, although ROS generation has been shown to be elevated in other beds following CH, this does not appear to occur in the mesenteric circulation following 48 hours of the stimulus. It is also notable that inhibition of catalase and HO did not result in an additive effect on ROS levels (Figure 2), although the specific ROS generated by the inhibition of both pathways could differ. However, recent studies have demonstrated that upregulation of the HO pathway in streptozotocin-induced diabetic rats results in increased endothelial SOD protein expression and catalase activity [45]. Thus, the protective effects of HO to diminish H_2O_2 may be secondary to catalase activation, rather than the antioxidant properties of HO products themselves.

ROS can have profound effects in the vasculature, resulting in impaired endothelium-mediated vasodilation through NO scavenging by $O_2^{\bullet-}$ [43]. However, in the present study, vascular ROS were not elevated after exposure of rats to CH (Figure 1 and 2). In addition, responses to the endothelium-dependent vasodilator, ACh, were not impaired in these vessels (Figure 3). Moreover, HO inhibition had no effect on this response, suggesting that HO-derived CO does not contribute to ACh-induced dilation, even in CH arteries with documented elevation of enzyme expression [21] (Figure 3).

Unlike the vasodilatory responses to ACh, inhibition of HO and catalase resulted in altered vasoconstrictor responses to PE in both normoxic control and CH groups (Figure 4). In control arteries, catalase inhibition had no effect, whereas HO inhibition caused a slight impairment of PE-induced vasoconstriction (Figure 4A). These findings conflict with previous studies of gracilis muscle arteries from normoxic rats that suggest that HO-derived CO acts tonically to blunt pressure-induced vasoconstriction [27,49]. The present results also differ from our previous findings that failed to detect an effect of HO inhibition on vasoconstrictor responses in control arteries [16]. However, these earlier experiments were performed in the presence of NOS inhibition, whereas the current study was conducted with NOS functionally intact. Thus, the reduced vasoconstrictor responsiveness observed following HO blockade in control arteries could be due to removal of an inhibitory effect of endogenous CO on endothelial nitric oxide synthase (eNOS) activity, as suggested by others [23]. The finding that combined HO and catalase inhibition did not further affect constrictor reactivity, compared to HO inhibition alone (Figure 4A), might also be interpreted as evidence for a vasodilatory role of H_2O_2 following the blockade of either of these pathways. The increased VSM calcium response suggests that the combined treatment may also

decrease calcium sensitivity, since no matching increase in vasoconstriction was observed (Figure 4A). However, the differential effects of HO inhibition vs. combined HO/catalase blockade in CH arteries (Figure 4B) discussed below suggests that HO inhibition may not act similar to catalase to effectively increase H_2O_2 .

In the present study, we observed impaired PE-induced vasoconstriction in resistance arteries from rats exposed to CH, compared to controls, that was restored by HO inhibition (Figure 4B). The moderately increased VSM calcium levels following HO inhibition alone may account for the normalization of the vasoconstrictor response (Figure 4B). This likely occurs due to inhibition of the VSM cell hyperpolarizing effects of HO-derived CO [35], which elicits vasodilation through the activation of large conductance calcium-activated potassium channels resulting in hyperpolarization of the VSM cell membrane [35]. Restoration of reactivity following HO inhibition in CH arteries (Figure 4B) suggests that enhanced production of HO-derived CO contributes to tonic vasodilation following CH that is responsible for the generalized impairment of vasoconstriction in this setting. These findings are similar to previous studies by our laboratory demonstrating increased PE-induced vasoconstriction in mesenteric arteries from CH rats following HO inhibition [16]. As discussed above, one important difference between the present experiments and earlier studies is that the present studies were performed without NOS inhibition. The similarity of the results between these studies suggests that the ability of HO inhibition to restore constrictor reactivity following CH is not due to a modulatory role of CO on NOS activity [23]. The likely role of CO in diminished constrictor reactivity following CH is suggested by other studies demonstrating that biliverdin, another product of the HO pathway, does not induce vasorelaxation in aortic rings [3]. Moreover, catalase appears to play an important role in the attenuation of vasoconstriction in CH arteries, as demonstrated by the normalization of this response with catalase inhibition (Figure 4B). This normalization is accompanied by greatly augmented VSM calcium levels following catalase inhibition with ATZ, suggesting that catalase inhibition enhances calcium signaling in the CH group (Figure 4B). Although the differences in responsiveness to PE in isolated arteries are not large, the attenuated constrictor response in CH vessels translates into significant effects on vascular resistance *in vivo*, as predicted by Poiseuille's relationship. This has been demonstrated in prior studies from our laboratory, wherein PE elicited greatly reduced pressor, total peripheral resistance, and renal vascular resistance responses following exposure to CH in conscious, chronically instrumented rats [8,21,37]. Endothelium disruption has been shown to normalize vasoconstrictor and Ca^{2+} responses between groups, suggesting that the vasoconstrictor mechanism is not altered by CH, but rather endothelial modulation of this response likely differs between groups [9]. Considering these prior results, the present findings suggest that both the catalase and HO components of altered vasoreactivity following CH likely reside within the endothelium.

Since the present study demonstrated impaired vasoconstriction in arteries from rats exposed to CH (Figure 4B), we focused on H_2O_2 as a potential mediator of this response, as it has purported vasoconstrictor as well as vasodilator activities [15]. Interestingly, combined HO and catalase inhibition resulted in significantly impaired PE-induced vasoconstriction in the CH group (Figure 4B). This finding suggests that the catalase and HO pathways work similarly to reduce ROS levels and vasoconstrictor reactivity in vessels from CH rats. The specific ROS generated following the inhibition of each pathway were not discriminated in the present study. Thus, the differential effects on vasoconstrictor reactivity of blocking HO alone or in combination with catalase inhibition could be attributed to dissimilar profiles of ROS produced by each antagonist.

CONCLUSIONS

In conclusion, our hypothesis that enhanced HO activity minimizes vascular ROS in CH rats and concurrently produces CO resulting in impaired vasoconstriction was only partially supported. Contrary to our hypothesis, we found that ROS were not elevated in vessels from CH rats (Figure 1 and 2). However, the catalase pathway appears to effectively limit ROS production in CH rats, since inhibition results in significantly increased ROS and enhanced vasoconstriction in CH rats but not normoxic controls (Figure 2 and 4). As scavengers of the endothelium-dependent vasodilator NO, we hypothesized an increase in vascular ROS would lead to impaired endothelium-mediated vasodilation. However, our data demonstrate no impairment of vasodilation, supporting the observed lack of increased ROS in CH rats (Figure 3). Further, HO does not appear to be involved in ACh-mediated vasodilation (Figure 3), although it has been previously localized to the endothelium of mesenteric arteries [34]. In support of our hypothesis, it is likely that ROS and HO production of CO contributes to impaired vasoconstrictor responses observed following CH.

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Figure 1. 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCF) fluorescence (average grayscale values above background and the extravascular solution) in isolated mesenteric resistance arteries from control ($n=5-9$) and 48-hour chronic hypoxia-exposed rats ($n=5-7$). 4,5-dihydroxy-1,3-benzene-disulfonic acid (tiron; 10 mM) and catalase (1200 U/mL) were used to scavenge $O_2^{\bullet-}$ and H_2O_2 , respectively, to measure the specificity of the fluorescent indicator for reactive oxygen species (ROS). Representative images are shown for control and tiron/catalase-treated arteries from normoxic and chronic hypoxia rats to demonstrate the sensitivity of DCF fluorescence to detect ROS. Data are expressed as the means $\pm$ SEM. $P=0.05$ vs. respective controls. Between-group comparisons revealed no significant differences for similar treatments.

Figure 2.

5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCF) fluorescence (average grayscale values above background and the extravascular solution) in isolated mesenteric resistance arteries from control ($n=5-9$) and 48-hour chronic hypoxia-exposed rats ($n=5-7$). Catalase was inhibited by using 5 mM of 3-amino-1,2,4-triazole (ATZ). Heme oxygenase was inhibited by using zinc protoporphyrin IX (ZnPPiX; 0.5 μ M). Data are expressed as the means $\pm$ SEM. * $P \leq 0.05$ vs. respective controls; # $P \leq 0.05$ vs. respective ZnPPiX vehicles (ZnPPiX Veh). Between-group comparisons revealed no significant differences for similar treatments.

Figure 3.

Vasodilatory responses to incremental concentrations of acetylcholine (10^{-9} to 10^{-5} M) in mesenteric resistance arteries from control and 48-hour chronic hypoxia rats preconstricted with phenylephrine to a 50% resting inner diameter. Heme oxygenase was inhibited by using zinc protoporphyrin IX (ZnPPiX; 0.5μ M). There were no significant differences in acetylcholine-induced vasodilation. Data are expressed as the means $\pm$ SEM.

Figure 4.

Vasoconstrictor and vascular smooth muscle calcium responses in mesenteric resistance arteries from control and 48-hour chronic hypoxia (CH) rats to increasing concentrations of phenylephrine (10^{-9} to 10^{-5} M). Heme oxygenase was inhibited by using zinc protoporphyrin IX (ZnPPiX; $0.5 \mu\text{M}$), and catalase was inhibited by using 3-amino-1,2,4-triazole (ATZ; 5 mM). Data are expressed as the means $\pm$ SEM. * $P \leq 0.05$ vs. normoxic controls; # $P \leq 0.05$ vs. CH controls; * $P \leq 0.05$ ATZ+ZnPPiX vs. ATZ; § $P \leq 0.05$ ATZ+ZnPPiX vs. ZnPPiX treated.