Thiol oxidation inhibits nitric oxide-mediated pulmonary artery relaxation and guanylate cyclase stimulation

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IT IS WELL ESTABLISHED that nitric oxide (NO) stimulates the soluble form of guanylate cyclase (sGC) by binding the reduced or ferrous form of the heme of this enzyme (4, 10, 11). Stimulation of sGC by NO in a manner consistent with its function as an important regulatory process is thought to occur through oxidation of the heme of sGC to its ferric form, which has low affinity for binding NO (7, 36). Studies in isolated endothelium-denuded bovine pulmonary arteries (BPA) have provided evidence that a cytosolic, NADPH-dependent flavoprotein-containing methemoprotein reductase system appears to maintain the redox status of the heme of sGC in its ferrous NO-binding form (13). In this previous study, it appeared that a heme oxidant (ferricyanide or the ODQ inhibitor of sGC stimulation) or inhibition of methemoprotein reductase activity was needed for attenuation of BPA relaxation to NO. The role of cytosolic NADPH generation by the pentose phosphate pathway (PPP) appeared to be one of restoring relaxation to NO in the presence of the heme oxidants, presumably through reduction of the heme of sGC.

Observations from our studies on the effects of NADPH on homogenates from BPA, depleted of reducing cofactors, suggested that we were detecting a second heme redox-independent mechanism of enhancing sGC activity (13). This mechanism of enhancing sGC activity appeared to be reproduced by the thiol reductant DTT, suggesting that an additional process, potentially involving thiol redox, might also be controlling the activity of sGC. The early literature on the regulation of sGC demonstrates the mechanisms whereby thiol oxidation can enhance and inhibit basal cGMP production and sGC activation by agents thought to release NO (1, 3, 6, 14, 19, 22, 32, 35). It is known that sGC is regulated by multiple mechanisms related to the metabolism of reactive oxygen species and sources of NO that could be influenced by thiol redox (10, 33). For example, many of these effects of thiols on sGC stimulation appear to originate from improvement in the generation of NO from drugs (20), reconversion of reactive NO-derived metabolites to NO (5), or modulation of the redox status of sGC thiols influencing activation by NO (22). Thiols are thought to be essential for the generation of cGMP by sGC, and incubation of sGC with oxidized thiols has been observed to inhibit sGC by an S-thiolation (1, 30). The oxidant of glutathione and adjacent protein thiols, diamide, has been observed to stimulate sGC activity in platelets at low concentrations and to inhibit sGC activity at higher concentrations (35). Because our preliminary studies detected an inhibition of NO-mediated relaxation and activation of sGC in homogenates of arteries treated with diamide, we developed studies to define whether a thiol oxidation mechanism functions to inhibit relaxation to NO by attenuating the ability of NO to activate sGC in BPA.

The cellular control of oxidation or reduction of sites on proteins, such as cysteine thiols, and levels of oxidized and reduced forms of cofactors, such as glutathione and NAD(P)H, which are used by enzymes to regulate the redox status of thiols on proteins, can serve as important signaling mechanisms. Insomuch as cytosolic NADPH reductase is a potential regulator of multiple aspects of thiol redox signaling (9, 33), we examined whether inhibition of NADPH generation by the PPP with 6-aminonicotinamide (6-AN) (12) could function as an inhibitory regulator of relaxation responses to NO that are mediated through its stimulation of sGC. On the basis of...
evidence for GSSG-dependent enzymatic activity in BPA homogenates inhibiting sGC stimulation by NO, we focused on examining the potential role of GSSG in the actions of 6-AN and diamide on sGC regulation.

MATERIALS AND METHODS

Materials. Spermine-NONOate, cGMP enzyme immunoassay (ELISA) kits, GSH/GSSG kits, and ODQ were purchased from Cayman Chemical. Vasodilator-stimulated phosphoprotein (VASP) antibodies were purchased from Cell Signaling (Beverly, MA). S-nitroso-N-acetylpenicillamine (SNAP) was prepared in our laboratory using previously published methods (19). All gasses were purchased from Tech Air (White Plains, NY), all salts were analyzed reagent grade from Baker Chemical, and all other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Determination of changes in force in BPA. Bovine lungs were obtained from a slaughterhouse and maintained in ice-cold oxygenated PBS solution during transport to our laboratory. Isolated endothelium-denuded pulmonary artery rings were prepared by adaptation of previously utilized methods. Briefly, the intralobar pulmonary arteries were isolated from surrounding tissue, cleaned, and cut into rings (~4 mm diameter and length), and the endothelium was removed by gentle rubbing of the lumen. The rings were mounted on wire hooks attached to force displacement transducers (model FT03, Grass) for measurement of changes in isometric force. Tension was adjusted to 5 g, which is the optimal passive force for maximal contraction. Changes in force were recorded on a polygraph (model 7, Grass).

Vessels were incubated in 10-ml baths (Metro Scientific, Farmingdale, NY), which were individually thermostatized (37°C) in Krebs buffer gassed with 21% O2-5% CO2-balance N2. Krebs buffer contained (in mM) 118 NaCl, 4.7 KCl, 1.5 CaCl2, 25 NaHCO3, 1.1 MgSO4, 1.2 KH2PO4, and 5.6 glucose. Arteries were incubated for 2-12 h, during which passive tension was adjusted to maintain 5 g. The vessels were then depolarized with Krebs solution containing 125 mM KCl (high K+), 5 min before measurement of cGMP by ELISA, as described in the manual supplied by manufacturer of the ELISA kit.

Detection of changes of force in BPA homogenate sGC activity. Guanylate cyclase activity of BPA was measured by enzyme immunoassay in homogenates obtained from control BPA and arteries treated with redox-modulating agents under the incubation conditions used for studies of vascular responses, as previously described (13). The activity of sGC was measured in a reaction mixture (0.2 ml final volume) containing 20 mM MOPS-KOH (pH 7.4), 0.1 mM GTP, 2 mM MgCl2, 0.3 mM IBMX (a phosphodiesterase inhibitor), a GTP-regenerating system consisting of 10 mM phosphocreatine and 150 U/ml creatine phosphokinase, 0.1 mM of concentrated (13) or diluted (17) homogenate, and test agents. Diamide did not appear to impair the maintenance of GTP needed to support sGC activity by the GTP-regenerating system, because it altered NO-stimulated sGC activity without changing the basal levels of cGMP generation in the absence of NO. Assays of sGC activity were initiated by the addition of arterial protein. Incubations were conducted for 10 min at 37°C and terminated by the addition of 0.1 ml of preheated 12 mM EDTA. Then the assay mixtures were boiled for 10 min, and 5% trichloroacetic acid solution was added. The precipitate was removed by centrifugation, and the supernatant was washed three times with water-saturated diethyl ether. Residual ether was removed by incubation at 70°C for 5 min before measurement of cGMP by ELISA, as described in the manual supplied by manufacturer of the ELISA kit.

Statistical analysis. Values are means ± SE of the number of arterial segments (n) from different animals. Statistical analyses were performed with a paired Student’s t-test, and a one-way ANOVA with Bonferroni’s correction was used for comparison between multiple groups.

RESULTS

Thiol oxidant diamide inhibits BPA relaxation to NO donors. The dose-dependent relaxation to the NO donor spermine-NONOate of BPA precontracted with 30 mM K+ is shown in Fig. 1A. NONOate elicited an almost complete relaxation of BPA. The thiol oxidant diamide (1 mM) significantly inhibited relaxation of BPA to various doses of NONOate, and relaxation to the largest dose of NONOate, i.e., 10 μM (91 ± 6%; n = 8), was also inhibited by 1 mM diamide (43 ± 6%, n = 10, P < 0.05). Data in Fig. 1A demonstrate that the inhibition of relaxation to NO by diamide can be reversed when this thiol oxidant is washed out, suggesting that reversible redox-regulated mechanisms were controlling the response to NO. Furthermore, lower doses of diamide (100 μM, not shown) did not do so.
inhibit relaxation to 1 mM NONOate (81 ± 6% and 81 ± 13% relaxation in control and diamide-treated vessels, respectively, n = 5). Figure 1B shows that diamide also inhibits relaxation to the NO donor SNAP, a nitrosothiol that spontaneously releases NO in physiological buffers and promotes relaxation of BPA primarily by stimulating sGC (8, 16, 25). Relaxation of 30 mM K+ precontracted BPA to the NO donor SNAP at 10 μM (81 ± 7%, n = 7) was substantially inhibited by 1 mM diamide (36 ± 8%, n = 7, P < 0.05) in a manner similar to that of the NONOate donor of NO.

Diamide does not alter relaxation to stimuli of adenylate cyclase. BPA are also relaxed by cAMP-mediated signaling pathways, and we employed the β-adrenergic receptor agonist isoproterenol and the adenylate cyclase activator forskolin to determine whether the thiol oxidant diamide influenced vascular relaxation through this alternative mechanism. Relaxation to isoproterenol and forskolin was not significantly inhibited in the presence of 1 mM diamide, suggesting that this cAMP-mediated response is not affected by thiol oxidation elicited by 1 mM diamide in BPA (Fig. 2).

Diamide decreases NO-elicited VASP phosphorylation. The effects of diamide on changes in the phosphorylation of VASP on Ser239 in BPA were measured by Western blot analysis to provide evidence that this thiol oxidant was altering vascular function through changes in cGMP-dependent protein kinase (PKG) activity. Stimulation of sGC with 10 μM SNAP significantly increased VASP phosphorylation by 360 ± 120% (P < 0.05), and diamide inhibited this phosphorylation (190 ± 70%, P < 0.05, n = 4) as normalized to total VASP (Fig. 3). Although diamide appeared to lower basal levels of VASP

Fig. 1. A: effects of thiol oxidation by diamide on relaxation of endothelium-denuded bovine pulmonary arteries (BPA) precontracted with 30 mM K+ to spermine-NONOate (n = 8–10). Inhibition of relaxation to NONOate was restored after washout of diamide (post), as demonstrated by relaxation similar to control in vessels treated after diamide washout. B: effects of diamide on BPA relaxation to S-nitroso-N-acetylpenicillamine (SNAP). Soluble guanylate cyclase (sGC)-dependent relaxation by the nitric oxide (NO) donor SNAP was significantly inhibited by diamide (n = 8).

Fig. 2. Relaxation of BPA to adenylate cyclase activators isoproterenol (A, n = 7) and forskolin (B, n = 7) was unaffected by 1 mM diamide.
Because sGC has been reported to be stimulated by NO, a reduction in phosphorylation of VASP is decreased because of the actions of diamide on the basis of mediated stimulation of sGC and a subsequent increase in PKG. This suggests that protein phosphorylation regulated by NO- phosphorylation, this decrease was not statistically significant. However, the addition of 2 mM GSH or GSH containing the higher concentration of 5% GSSG (100 μM) to dilute homogenates did not influence the stimulation of sGC by NO (Fig. 5B), suggesting that the inhibition is due to enzymatic reactions that require interactions in the concentrated BPA homogenate preparation. Thus GSH redox is potentially regulating an enzymatic process that is inhibiting sGC stimulation by NO.

**Attenuation of BPA relaxation to NO by inhibition of NADPH generation by the PPP with 6-AN is reversed by the thiol reductant DTT.** Because the maintenance of GSH redox in bovine arteries seems to be controlled by NADPH generation by the PPP (12), the effects of inhibition of NADPH generation by the glucose-6-phosphate dehydrogenase inhibitor 6-AN on NO-mediated regulation of sGC and force in BPA were examined. 6-AN (1 mM) decreased relaxation to the NO donor spermine-NONOate (Fig. 6A), whereas 100 μM 6-AN (not shown) did not have an effect (1 μM NONOate, 81 ± 6% and 89 ± 4% relaxation in control and 6-AN-treated vessels, respectively, n = 5). Treatment with 1 mM 6-AN also decreased NADPH levels in control vessels from 6.5 ± 1.4 to 2.2 ± 0.7 nmol/g. This effect of 6-AN on the attenuation of vascular relaxation was not observed in the presence of the thiol reductant 1 mM DTT, suggesting that a thiol redox process was mediating the effects of the PPP inhibitor 6-AN. The effects of DTT on reversing the inhibition of BPA relaxation to NO by the oxidant of the sGC heme with 0.01 mM ODQ were examined to provide evidence that this thiol reductant does not function through heme reduction. The data in Fig. 6B show that DTT does not reverse the inhibitory effects of ODQ. The presence of DTT did not accelerate the restoration of relaxation to NO after washout of ODQ (post-ODQ, n = 4; Fig. 6B). Relaxation to forskolin and the β-adrenergic

**Phosphorylation**

**VASP Phosphorylation (%) Control**

<table>
<thead>
<tr>
<th>Control</th>
<th>Diamide</th>
<th>SNAP (10 μM)</th>
<th>SNAP+Diamide</th>
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<tr>
<td>100</td>
<td>50</td>
<td>200</td>
<td>250</td>
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* p<0.05 vs. Control

**Fig. 3. Effect of diamide on vasodilator-stimulated phosphoprotein (VASP) phosphorylation by cGMP-mediated PKG activation.** SNAP increased VASP phosphorylation in a manner that was decreased in the presence of diamide (P < 0.05, n = 5). Data were normalized to total VASP.

**GSH oxidation inhibits sGC stimulation by NO in concentrated BPA homogenates.** The effects of increasing the concentration of oxidized GSH on the activation of sGC by NO in dilute and concentrated BPA homogenates were examined to detect whether GSSG influenced sGC directly or through an enzymatic reaction. To further determine whether changes in glutathione redox influence sGC activity through enzymatic reactions, homogenates were reconstituted to protein levels approximating the environment in BPA in a manner that markedly dilutes endogenous soluble redox cofactors such as GSH and NADPH (13). These homogenates were exposed to increasing GSSG/GSH levels in the presence of a total glutathione concentration of 2 mM under the conditions used to assay sGC activity. Increasing the levels of GSSG inhibited sGC activation by the NO donor SNAP (Fig. 5A). SNAP (10 μM) increased cGMP production nearly sixfold in the presence of 2 mM GSH, and the shifting of redox status of GSH by the addition of 0.5% GSSG (10 μM) significantly inhibited cGMP production in the presence of SNAP, suggesting that sGC activation is sensitive to this small alteration in the redox state of glutathione (Fig. 5A). The presence of 5% GSSG caused only a slightly greater inhibition of NO-stimulated sGC activity than 0.5% GSSG, which was not statistically significant. However, the addition of 2 mM GSH or GSH containing the higher concentration of 5% GSSG (100 μM) to dilute homogenates did not influence the stimulation of sGC by NO (Fig. 5B), suggesting that the inhibition is due to enzymatic reactions that require interactions in the concentrated BPA homogenate preparation. Thus GSH redox is potentially regulating an enzymatic process that is inhibiting sGC stimulation by NO.

**Fig. 4. Effect of diamide on sGC activity in the absence and presence of the NO donor SNAP in diluted BPA homogenates.** Activation of sGC by SNAP was attenuated by diamide (P < 0.05, n = 7) in the absence of glutathione.
receptor agonist isoproterenol, thought to be mediated through cAMP, was not altered by 6-AN treatment (Fig. 7).

Homogenate sGC activity from 6-AN-treated BPA shows a thiol-dependent attenuation of stimulation by NO. Because vascular relaxation to the NO donor NONOate was attenuated by the PPP inhibitor 6-AN, we assayed sGC activity in arterial homogenates from BPA treated with 6-AN. Stimulation of sGC activity by NO derived from spermine-NONOate was attenuated in BPA treated with 6-AN (Fig. 8). Arteries treated with a combination of 6-AN and the thiol reductant 1 mM DTT did not show an altered stimulation of homogenate sGC activity by NO, suggesting that the actions of 6-AN were mediated through a thiol redox process that regulates sGC. The presence of DTT had no effect on the generation of cGMP by sGC activity in the absence and presence of NONOate.

BPA treated with 6-AN and diamide show increased levels of oxidized GSH. To determine whether the PPP inhibitor 6-AN and the glutathione and protein thiol oxidant diamide could increase GSSG, GSH and GSSG were measured in BPA treated with or without these agents. The mechanisms regulating cellular redox (i.e., GSH/GSSG) can be modulated through the PPP or direct oxidation of GSH, leading to significant increases in oxidized GSH ($n = 7, P < 0.05$; Fig. 9).

**Fig. 6.** A: inhibition of the pentose phosphate pathway (PPP) with 6-amino nicotinamide (6-AN) attenuated BPA relaxation to NONOate in a manner that was reversed by the thiol reductant DTT (1 mM, $n = 8–16$). B: sGC heme oxidant inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinolizin-1-one (ODQ) significantly attenuated NONOate-mediated relaxation ($P < 0.05, n = 9$) in a manner that was not reversed by DTT ($n = 5$). Partial recovery of NO-mediated relaxation after washout of ODQ (post-ODQ) was not enhanced by DTT (post-ODQ + DTT, $n = 4$).
DISCUSSION

In this study, the thiol oxidant diamide reversibly inhibited relaxation of BPA to NO in a manner that appeared to be mediated through a redox-controlled modification of sGC that inhibited stimulation of cGMP production by NO. While investigating whether oxidizing GSH and adjacent protein thiols, which are potential mechanisms whereby the activity of sGC is controlled (1, 3, 22, 30), we and others previously showed that NO appears to induce vascular relaxation in BPA under aerobic conditions, primarily through activation of sGC and cGMP production (8, 16, 25). Under the conditions examined in this study, diamide inhibited relaxation of BPA to NO donors, which spontaneously release NO without altering relaxation originating from the stimulation of adenylate cyclase by forskolin or isoproterenol. Thus, although diamide may have effects on other mechanisms that control vascular force (18, 26, 27), these alternative actions of diamide do not appear to have nonspecific effects on relaxation through cAMP. Diamide also attenuated the stimulation of sGC by NO in dilute BPA homogenates in the presence of inhibition of cGMP removal by phosphodiesterase activity, suggesting that diamide influences the function of sGC and does not stimulate phosphodiesterases, which remove cGMP. In a previous study, diamide prevented sGC stimulation by nitroprusside through a mechanism that appeared to involve the oxidation of sGC thiols (22). The stimulation of VASP phosphorylation by NO at a site known to be a target for PKG was also inhibited by diamide. Overall, these observations suggest that the thiol oxidant diamide inhibits the stimulation of sGC by NO without detectably lowering basal sGC activity, and this action of diamide appears to contribute to its attenuation of BPA relaxation to NO.

Diamide could be hypothesized to inhibit sGC in BPA by oxidation of GSH or directly by oxidation of thiols on sGC. The inhibitory actions of diamide on NO stimulation of sGC in dilute homogenates and the minimal effects of increased levels of GSSG on the actions of NO under these conditions suggest

Fig. 7. Relaxation of BPA to adenylate cyclase activators isoproterenol (A, n = 4) and forskolin (B, n = 4) was not altered by the PPP inhibitor 6-AN.

Fig. 8. Pretreatment of BPA with the PPP inhibitor 6-AN attenuates sGC activation by the NO-donor NONOate in dilute homogenates in a manner that was reversed by pretreatment of BPA with the thiol reductant 1 mM DTT (n = 9). DTT did not influence basal or NO-stimulated sGC activity (n = 5).
that diamide directly alters the activity of sGC without modifying GSH redox under the conditions of these experiments. These observations on GSSG are consistent with findings from previous studies that sGC activity was resistant to inhibition by millimolar levels of GSSG under conditions where it reacted with other oxidized thiols (1). Because NO stimulation of sGC was very sensitive to inhibition by GSH oxidation in concentrated homogenates in the absence of significant amounts of cofactors such as NAD(P)H, there appears to be an enzymatic process controlled by the availability of GSSG that inhibits NO stimulation of cGMP production. Thus these observations suggest that the control of GSH redox in BPA may regulate the ability of NO to stimulate sGC in a manner controlled by the redox status of thiols on sGC through protein-catalyzed redox interactions that remain to be identified. Because diamide increased GSSG, these experiments support the possibility that diamide could be inhibiting the stimulation of sGC by NO through GSH oxidation and direct oxidation of sGC thiols. In addition, the restoration of a normal relaxation response to NO after washout of diamide suggests that the thiol oxidation reactions caused by this agent associated with inhibition of relaxation to NO are reversible through processes that normally control thiol redox in BPA.

The redox status of cytosolic NADPH is thought to be the primary system that controls GSH and thiol redox in vascular tissue (33), and the function of this system could be an important factor in controlling sGC activity. The present study investigated the influence of NADPH redox on responses of BPA to NO by inhibiting the reduction of NADP to NADPH by the glucose-6-phosphate dehydrogenase reaction with 6-AN (12, 13). Relaxation to NO was attenuated by 6-AN, whereas this inhibitor of the PPP did not alter relaxation to forskolin or isoproterenol, which is thought (8) to be mediated through cAMP in BPA. When arterial homogenates were obtained from BPA treated with 6-AN, sGC stimulation by NO was attenuated, suggesting that 6-AN was causing a modification of sGC. Treatment of BPA with 6-AN was also observed to increase arterial GSSG levels. Because treatment of BPA with DTT reversed the effects of 6-AN, it appears that this modification of sGC was mediated through an alteration in thiol redox caused by inhibition of the PPP. To confirm that DTT does not reverse inhibition of sGC by changes in heme redox, the effects of DTT on the actions of the sGC heme oxidant inhibitor ODQ on relaxation to NO were evaluated. This agent attenuated relaxation to NO in a manner that was not altered by DTT, suggesting that DTT does not function through reduction of the heme of sGC. Interestingly, the actions of diamide and 6-AN on sGC suggest that these agents are attenuating the stimulation of sGC by NO without detectably altering the basal cGMP-generating activity of sGC, suggesting that thiol redox changes are controlling a site that is designed to regulate the function of sGC. It has been reported that sGC has cysteine residues at a binding site for activators such as BAY 41-2272 (28) and YC-1 (23), which function to enhance the stimulating effects of heme-binding agents such as NO. Perhaps one of the actual biological roles for this site is inhibition of NO activation through thiol redox changes. Although this site has been described as analogous to the forskolin site on adenylyl cyclase (2), the thiol-oxidizing conditions that altered sGC-mediated relaxation did not influence relaxants thought to function by stimulating cAMP generation, suggesting the absence of a thiol redox mechanism controlling adenylyl cyclase under the conditions that appear to influence the regulation of sGC. Although the ratio of GSSG to GSH measured in untreated BPA suggests that sGC stimulation by NO might be regulated through the thiol redox status, the effect of NO on this ratio remains to be determined.

**Fig. 9.** Treatment of BPA with 6-AN or diamide significantly increased GSSG. BPA levels of GSH were not significantly decreased by these treatments ($n = 7$, $P < 0.05$).

**Fig. 10.** Model showing how the thiol oxidant diamide and inhibition of the PPP by 6-AN could inhibit stimulation of sGC in BPA by NO through changes in NADPH and GSH redox via a hypothesized change in sGC thiol redox.
normally be inhibited by the thiol redox mechanism examined in this study, the thiol reductant DTT did not alter the relaxation of BPA to NO (Fig. 6A) or sGC stimulation by NO (Fig. 8). Although these high levels of GSSG could be an artifact of the extraction of tissue GSH, the assay is thought to trap tissue GSH before it has a chance to significantly oxidize. Because NADPH appears to control the activities of enzymes that reduce oxidized protein thiols (9, 33), it is possible that this process may be actively restoring the thiol oxidation process regulating sGC that is controlled by GSSG. Thus the actions of PPP inhibition on NO-mediated BPA relaxation and stimulation of sGC suggest that cytosolic NADPH redox appears to have an important role in controlling the regulation of sGC through its influence on thiol redox.

The data reported in this study develop evidence for the existence of a potentially important physiological mechanism (Fig. 10) through which the PPP and NADPH, GSH, and the thiol redox-linked system that was detected in the present study control the stimulation of sGC by NO and perhaps other activators. There is much evidence for oxidant processes promoting thiol and GSH redox, which could be functioning to inhibit the regulation of sGC under pathophysiological conditions associated with impaired vascular relaxation to NO such as pulmonary hypertension (29). However, redox-controlled changes in the expression of sGC could also be a factor in alterations in NO-mediated vascular regulation of sGC (15). The data in this study provide evidence for an additional potentially important physiological mechanism of controlling sGC through the maintenance of cytosolic NADPH by the PPP and the influence of these systems on maintaining low levels of GSSG. It appears that sGC stimulation by NO is designed to be inhibited by a thiol oxidation-controlled signaling mechanism when there is a small increase in the levels of GSSG. Although the influence of essentially all aspects of cardiovascular regulation on the function of the PPP in the vascular tissue remains to be studied, there is significant human genetic variability in the expression of glucose-6-phosphate dehydrogenase (21), and this could have a major role in disease processes associated with altered NO regulation of sGC through the thiol redox systems characterized in this study.

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