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Peroxynitrite stimulates vascular smooth muscle cell cyclic GMP synthesis

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Abstract Peroxynitrite stimulated the synthesis of cyclic GMP by rat aortic smooth muscle in a time- and dose-dependent manner. Peak formation of cyclic GMP occurred at 1 min with 100 μM peroxynitrite and was inhibited by oxyhemoglobin. Peroxynitrite was less potent than nitric oxide in stimulating cyclic GMP synthesis. Peroxynitrite also enhanced endothelial-dependent cyclic GMP synthesis, via generation of a long-lived substance, which was prevented by inhibition of glutathione synthesis. These data show that peroxynitrite stimulates cyclic GMP synthesis, inferring production of low yields of nitric oxide or associated derivatives. Additionally, vascular exposure to peroxynitrite potentiates endothelial-dependent activation of guanylate cyclase.

Key words: Peroxynitrite; Superoxide; Nitric oxide; Cyclic GMP; Endothelial cell; Free radical

1. Introduction

It has been recognized for several years that the vasoactive properties of nitric oxide (•NO) are diminished by superoxide anion ($O_2^{\bullet-}$) [1,2]. The rapid reaction of •NO with $O_2^{\bullet-}$ is almost diffusion-limited in rate ($k = 6.7 \times 10^9$ $M^{-1} \cdot s^{-1}$) [3], forming peroxynitrite anion. Peroxynitrite is a potent oxidant that directly oxidizes a wide spectrum of biological molecules, such as DNA constituents [4], lipids [5], and amino acids [6]. Alternatively, peroxynitrite can be protonated to peroxynitrous acid (ONOOH), yielding a product with hydroxyl radical (•OH)-like activity and nitrogen dioxide ($•NO_2$) [4,7].

It is unclear whether the inhibitory effects of $O_2^{\bullet-}$ on •NO-mediated vasodilation are due to decreased steady state concentrations of •NO available to activate vascular soluble guanylate cyclase, the direct inhibition of guanylate cyclase activity, or the generation of a product with less potent guanylate cyclase activating properties. Paradoxically, recent reports have noted that peroxynitrite causes vasodilation both in endothelium-intact coronary artery and endothelium-denuded pulmonary artery [8,9], possibly by formation of *S*-nitroso derivatives. The purpose of the present study is to examine the influence of peroxynitrite on cGMP production by cultured rat aortic smooth muscle cells (RASM) and its effects on endothelial-dependent increases in cGMP synthesis by a reporter cell, RFL-6 fibroblasts [10]. It is concluded that while peroxynitrite is a less potent activator of cGMP synthesis than •NO, peroxynitrite can enhance endothelial-dependent production of cGMP in a thiol- dependent fashion.

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Abbreviations: BAEC, bovine aortic endothelial cells; BSO, buthionine sulfoximine; cGMP, cyclic GMP; •OH, hydroxyl radical; IBMX, isobutylmethylxanthine; •NO, nitric oxide; NO_3^-, nitrate; NO_2^-, nitrite; $•NO_2$, nitrogen dioxide; HbO_2, oxyhemoglobin; ONOOH, peroxynitrous acid; PBS, phosphate-buffered saline; RASM, rat aortic smooth muscle cells; SNP, sodium nitroprusside; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase.

2. Materials and methods

2.1. Cell culture

Rat aortic smooth muscle cells were isolated and cultured as described previously [11]. Briefly, cells were grown in 21% O_2, 5% CO_2 at 37°C in Dulbecco's Modified Eagle Media supplemented with 10% newborn calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin on collagen-coated plates. Cells were subcultured at a split ratio of 1:4, fed twice weekly, and used within 24 h of confluency. Experiments were performed with RASM between passage 4 and passage 10. Smooth muscle cells were routinely identified by the presence of smooth muscle α-actin. Bovine aortic endothelial cells (BAEC) were cultured in M199 with 5% newborn calf serum, 5% iron-supplemented calf serum and antibiotics as described above. Experiments were carried out on passage 5 to passage 10 cells within 36 h of confluency. The purity of BAEC cultures was determined on the basis of their characteristic cobblestone morphology, positive immunostaining for factor VIII antigen and selective incorporation of fluorescently labeled acetylated-LDL. The rat lung fibroblast line, RFL-6 (ATCC, CCL192; Rockville, MD) was grown in Kaighn's F-12 media (Irvine Scientific, Santa Ana, CA) supplemented with 10% calf serum, penicillin and streptomycin. Cells were subcultured at a split ratio of 1:3, fed three times weekly, and used within 24 h of confluency. Cells between passage 12 and passage 20 were used.

2.2. Peroxynitrite synthesis

Peroxynitrite was synthesized as previously described [4], with concentrations determined spectrophotometrically ($\varepsilon_{302} = 1670$ $M^{-1} \cdot cm^{-1}$) [12]. Working solutions of peroxynitrite were prepared by dilution in 0.1 N NaOH and used within 15 min.

2.3. Preparation of nitric oxide

Saturated solutions of •NO were prepared by equilibrating •NO gas (Matheson) in vacuum containers containing argon-saturated phosphate-buffered saline, pH 7.4 (PBS): 140 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl; 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4. The concentration of •NO was determined by oxidation of oxyhemoglobin (HbO_2) to methemoglobin [13] and ranged from 1.6 to 1.7 mM. Oxyhemoglobin was prepared by sodium dithionite reduction of methemoglobin. Dithionite was removed by dialysis in distilled H_2O and HbO_2 concentration determined spectrophotometrically [14]. For cell treatments, aliquots of •NO-containing buffer were removed via gas-tight syringes, diluted in de-gassed PBS as necessary, and added to RASM cells within 5 s, a time period observed to not affect dissolved •NO concentration.

2.4. Cyclic GMP quantitation

Confluent RASM, grown on 35-mm dishes, were washed three times with PBS. All experiments were carried out in 1.0 ml of PBS in the presence of isobutylmethylxanthine (IBMX; 100 μM). Sodium nitroprusside (SNP; 1 μM) was included as a positive control for soluble guanylate cyclase activation. Peroxynitrite was added to cells at the concentrations and times specified. Control cells were treated with a volume of 0.1 N NaOH equivalent to that added during peroxynitrite treatment of cells. Separate studies showed no change in pH or cGMP

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levels from untreated cells. For cell cGMP analyses, the buffer was removed and 0.5 ml of 6% TCA added for 30 min. Acid extracts were transferred to microcentrifuge tubes and centrifuged at $8000 \times g \times 5$ min. Supernatants were extracted 3 times with H_2O-saturated ether; the solvent evaporated with a stream of N_2, and extracts resuspended in 50 mM sodium acetate, pH 6.2, and stored at −20°C until assayed for cGMP by ELISA (Cayman Chemical, Ann Arbor, MI). Cell pellets were resuspended in 0.1 N NaOH and analyzed for protein content [15].

2.5. Endothelial cell studies

Confluent BAEC were rinsed three times with and maintained in PBS. Bradykinin (100 nM) and/or peroxynitrite (100 μM) were added to cells for 3 min in a 1.0 ml final volume. Control cells were treated with an equal volume of 0.1 N NaOH. The supernatant was immediately transferred to 100 μM IBMX-treated RFL-6 cells and incubated for an additional 2 min. Preliminary experiments demonstrated that these were optimal concentrations and times for detection of bradykinin-enhanced cGMP synthesis in RFL-6 cells. Cells were then analyzed for cGMP and protein content as described for RASM. In some experiments, endothelial monolayers were pre-treated for 24 h with 200 μM buthionine sulfoximine (BSO) to inhibit glutathione synthesis [16]. Cell glutathione content was measured according to the method of Murphy et al. [17].

2.6. Chemicals and reagents

Fetal calf serum and iron-supplemented calf serum were from Hyclone, Logan, UT. Cell culture reagents were from Gibco, Grand Island, NY. Superoxide dismutase was obtained from Diagnostic Data Inc., Mountain View, CA. All other chemicals were purchased from Sigma, St. Louis, MO.

2.7. Statistical analysis

Data was analyzed by one-way analysis of variance, followed by post-hoc application of Bonferroni's test.

3. Results

3.1. Peroxynitrite and •NO stimulation of smooth muscle cell guanylate cyclase

The time course of peroxynitrite stimulation of cGMP production in RASM showed cGMP concentrations rose within 15 s of addition of 100 μM peroxynitrite, remained elevated for at least 2 min and returned to basal levels within 5 min following addition of peroxynitrite (Fig. 1). Dose–response analysis of peroxynitrite and •NO stimulation of cGMP in RASM revealed the minimum concentration of peroxynitrite that enhanced cGMP synthesis to be 1 μM, with a maximal effect at 100 μM. This contrasts with the dose–response curve of cGMP synthesis stimulated by •NO, where threshold stimulation of cGMP synthesis occurs at 10 nM, with a maximal effect at 1 μM. Peak cGMP levels achieved for peroxynitrite-stimulated cells was about 30% of those for •NO-stimulated cells (Fig. 2).

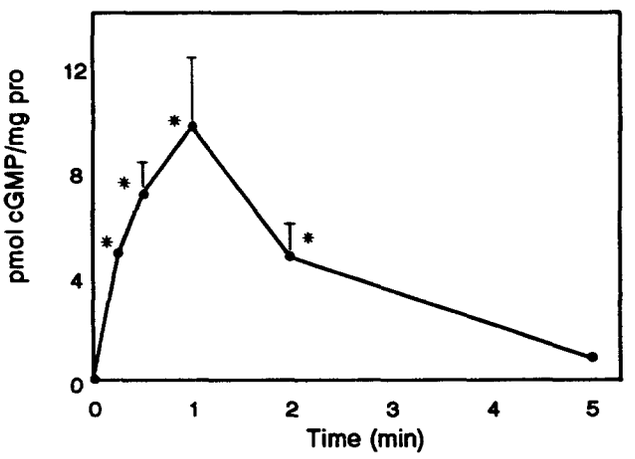

Fig. 1. Time course of peroxynitrite-induced cGMP synthesis in rat aortic smooth muscle cells. Following pretreatment with 100 μM IBMX, RASM were exposed to 100 μM peroxynitrite, the media removed and the cells analyzed for cGMP content at the times indicated. Data are the mean ± S.D. of 3 experiments, each performed in duplicate. $^*P < 0.05$ compared to 0 time.

Table 1
Effect of $ONOO^-$ on rat aortic smooth muscle cell cGMP synthesis

Condition	pmol cGMP/mg cell protein
Control	2.24 ± 0.69
$ONOO^-$	10.80 ± 2.22*
NO_2^-/NO_3^-	3.69 ± 0.64
$ONOO^- + HbO_2$	4.22 ± 0.90
SNP	13.69 ± 3.97*
SNP + $ONOO^-$	23.18 ± 3.35†

Following pretreatment with 100 μM IBMX with or without 2 μM HbO_2, cells were incubated for 1 min with 100 μM peroxynitrite or an equal volume of decomposed peroxynitrite. To study the effect of peroxynitrite on SNP-stimulated cGMP formation, cells were exposed to 1 μM SNP for 10 min; parallel cultures were treated with 1 μM SNP with 100 μM peroxynitrite added in the final min. Data are mean ± S.D. of 3 experiments carried out in triplicate.
$^*P < 0.05$; $^†P < 0.01$.

At physiologic pH, peroxynitrite rapidly decomposes in buffer to stable products, principally nitrate (NO_3^-), small amounts of nitrite (NO_2^-), with residual contaminating NaCl, NaOH and H_2O_2. To determine if the increased cell cGMP synthesis was the result of direct peroxynitrite reaction or secondary to production of breakdown products, RASM were incubated with 100 μM peroxynitrite, or to an equal volume of decomposed peroxynitrite. There was no significant enhancement of cGMP synthesis induced by peroxynitrite decomposition products while direct addition of peroxynitrite stimulated cGMP production 5-fold. Addition of 2 μM HbO_2 prevented the peroxynitrite-induced increase in cGMP (Table 1).

To ascertain if peroxynitrite inhibited guanylate cyclase activity, the effect of peroxynitrite on SNP-mediated formation of cGMP was studied. Peroxynitrite enhanced SNP-induced stimulation of cGMP production by RASM. A 10 min incubation with 1 μM SNP increased RASM production of cGMP 6-fold. The addition of 100 μM peroxynitrite to cell reaction systems doubled SNP-induced cGMP synthesis (Tables 1 and 2).

3.2. Peroxynitrite stimulation of endothelial cell-dependent cGMP synthesis by RFL-6 cells

Direct exposure of RFL-6 cells to 100 μM peroxynitrite for 2 min caused a doubling of cGMP levels over control (control, 2.01 ± 0.18 pmol cGMP/mg protein; 2 min, 4.14 ± 0.28 pmol cGMP/mg protein; $P < 0.05$); when the incubation period was increased to 5 min (the total time period of the BAEC-RFL-6 incubations), cGMP levels were no longer increased (5 min, 2.32 ± 0.22 pmol cGMP/mg protein). Cyclic GMP production by the lung fibroblast cell line, RFL-6, increased 100% over controls when incubated for 2 min with media transferred from BAEC, presumably due to the constitutive release of •NO by endothelial cells. Incubation of BAEC with 100 nM bradykinin for 3 min resulted in a 3-fold increase in RFL-6 cGMP content, while BAEC exposure to 100 μM peroxynitrite also induced a 3-fold increase in RFL-6 generation of cGMP. Co-incubation

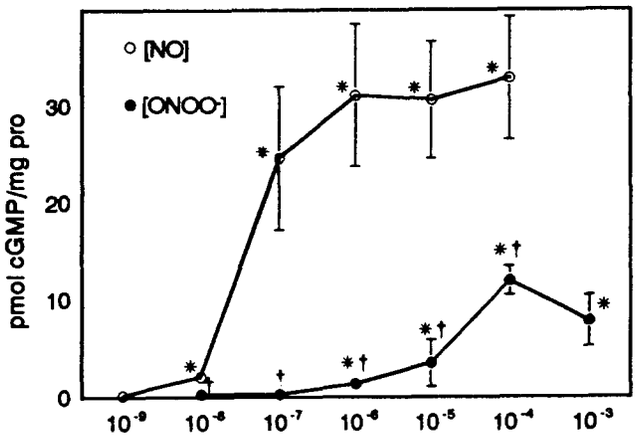

Fig. 2. Dose–response of nitric oxide- and peroxynitrite-induced cGMP synthesis in rat aortic smooth muscle cells. Cells, pre-treated with 100 μM IBMX, were incubated with ·NO or peroxynitrite. At 1 min, the media was removed, and the cells were analyzed for cGMP content. Data are the mean ± S.D. of 3 experiments, each performed in triplicate. $^{*}P < 0.05$ compared to control; $^{\dagger}P < 0.05$ compared to equimolar dose of •NO.

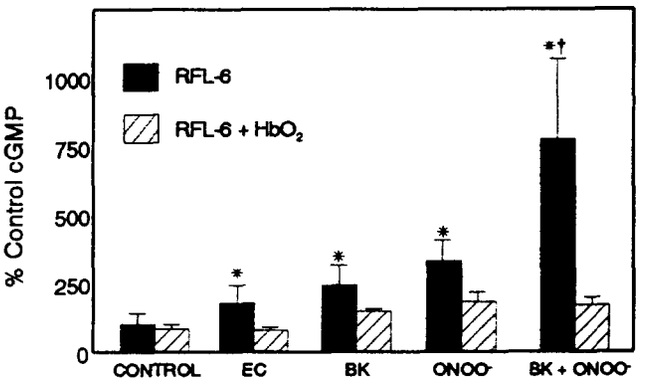

Fig. 3. Effect of peroxynitrite on endothelial cell-dependent cGMP synthesis in RFL-6 cells. Confluent BAEC in 35 mm dishes were treated for 3 min with 100 nM bradykinin and/or 100 μM peroxynitrite. The media was then transferred to IBMX treated RFL-6 cells and incubated for 2 min before analyzing for cGMP. Filled bars represent control RFL-6 cells. Hatched bars represent RFL-6 cells treated with 10 μM HbO_2 in addition to IBMX. Data are the mean ± S.D. of 4 experiments, each performed in triplicate. $^{*}P < 0.05$ compared to control; $^{\dagger}P < 0.05$ compared to bradykinin.

of BAEC with bradykinin and peroxynitrite led to an 8-fold increase in RFL-6 cGMP content (Fig. 3). Addition of 10 μM HbO_2 to the medium of RFL-6 cells prevented the rise in BAEC-stimulated RFL-6 cGMP levels in all treatments. When the incubation period was increased from 3 to 10 min, there was no significant change in the effects of either bradykinin or peroxynitrite on RFL-6 cGMP synthesis. However, there was a significant decrease in cGMP formation following co-incubation of BAEC with peroxynitrite and bradykinin for 10 min before transfer of medium to RFL-6 cells (Table 1). When BAEC were treated for 24 h with 200 μM BSO to inhibit glutathione synthesis, cGMP formation following co-incubation with peroxynitrite and bradykinin was also diminished (Fig. 4). Control cell glutathione content was 20 ± 2.1 μmol/mg protein. The glutathione content in BSO-treated cells was below the sensitivity of assay detection. There was no effect of 100 μM NO_2^- or decomposed 100 μM peroxynitrite on RFL-6 cGMP content, either when first added to BAEC and the media then transferred to RFL-6 cells or when added directly to RFL-6 cells (data not shown).

4. Discussion

Nitric oxide is constitutively synthesized by the oxidation of L-arginine in endothelial cells and neural tissue. Nitric oxide is also generated in large amounts by numerous cell types upon exposure to inflammatory stimuli such as lipopolysaccharide and cytokines, following the expression of inducible •NO synthase [18]. The effects of •NO on vessel tone are related to heme iron binding and subsequent activation of soluble guanylate cyclase, formation of cGMP and relaxation of vascular smooth muscle. The vasoactive properties of •NO are diminished in the presence of $O_2^{\bullet-}$ [1,2,19] with the radical-radical reaction of •NO with $O_2^{\bullet-}$ occurring at near diffusion-limited rates: 6.7×10^9 $M^{-1} \cdot s^{-1}$ [3]. Since the rate constant for $O_2^{\bullet-}$ scavenging by SOD is 2×10^9 $M^{-1} \cdot s^{-1}$, the facile reaction of •NO reaction with $O_2^{\bullet-}$ effectively competes with the dismutation of $O_2^{\bullet-}$ by SOD. It was not clear if this interaction of •NO with $O_2^{\bullet-}$ leads to the formation of a compound that directly inhibits the production of cGMP or whether peroxynitrite formation merely decreases the concentration of •NO available to activate guanylate cyclase. Thus, the aims of this study were to evaluate the direct effect of peroxynitrite on vascular smooth muscle cGMP formation and the effect of peroxynitrite on endothelial-dependent guanylate cyclase activation.

Direct exposure of vascular smooth muscle, maintained in a protein- and thiol-free buffer, to peroxynitrite results in a limited stimulation of cGMP synthesis. The increase in cGMP following exposure to peroxynitrite was much less than that achieved with equimolar amounts of •NO, with the precise proximal chemical species causing stimulation of vascular smooth muscle cell guanylate cyclase unclear. The increase in cGMP was not due to the stable decomposition products of peroxynitrite, NO_2^- and NO_3^-, as these species did not result in stimulation of RASM guanylate cyclase activity at the concentrations employed. The ability of HbO_2 to attenuate peroxynitrite stimulation of cGMP synthesis suggests that peroxynitrite enhancement of guanylate cyclase activity was •NO mediated [20]. This data concurs with the recent report of peroxynitrite-induced relaxation in isolated endothelium-

Table 2
Endothelial-dependent cGMP synthesis

Condition		% Control cGMP
RFL-6		100 ± 10
$ONOO^-$	3 min	280 ± 18
	10 min	214 ± 39
BK	3 min	280 ± 80
	10 min	215 ± 48
$ONOO^-$ + BK	3 min	771 ± 100
	10 min	310 ± 104*

Confluent BAEC in 35 mm dishes were treated for 3 or 10 min with 100 nM bradykinin and/or 100 μM peroxynitrite. The media was then transferred to IBMX-treated RFL-6 cells and incubated for 2 min before analyzing for cGMP. Data are mean ± S.D. of 3 experiments performed in triplicate. $^{*}P < 0.05$ compared to 3 min.

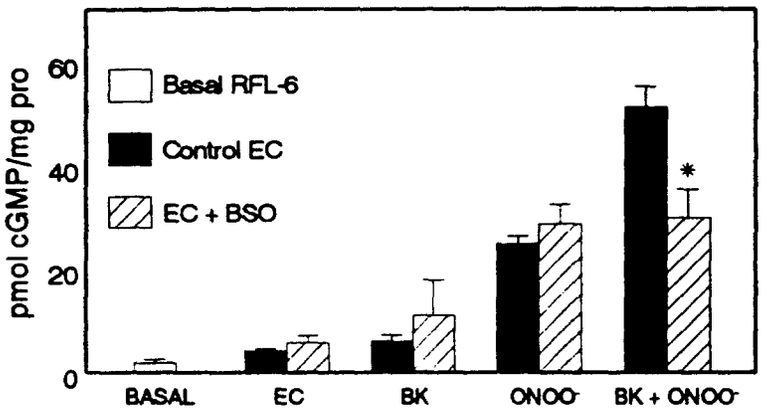

Fig. 4. Effect of buthionine sulfoximine on endothelial cell-dependent cGMP synthesis in RFL-6 cells. Confluent BAEC in 35 mm dishes were treated for 3 min with 100 nM bradykinin and/or 100 μM peroxynitrite. The media was then transferred to IBMX treated RFL-6 cells and incubated for 2 min before analyzing for cGMP. Open bar represents basal RFL-6 cGMP content. Filled bars represent control BAEC. Hatched bars represent BAEC treated with 200 μM BSO for 24 h. Data are the mean ± S.D. of 3 experiments, each performed in triplicate. $^*P < 0.05$ compared to non-BSO-treated BAECs.

denuded bovine pulmonary arterial rings [8]. Auto-oxidation of peroxynitrite can result in the formation of approximately 0.25% •NO [20]; thus the bolus addition of 100 μM peroxynitrite could generate as much as 40 nM •NO. While peroxynitrite will directly, albeit minimally, react with HbO_2 [21], the reaction of •NO with HbO_2 can be almost quantitative. The addition of 2 μM HbO_2 completely prevented the rise in cGMP following treatment with 100 μM peroxynitrite, suggesting that •NO was responsible for the stimulation of guanylate cyclase. Moreover, the importance of this direct reaction of peroxynitrite with HbO_2 at physiologic pH is unclear as the reaction rate is markedly diminished under non-alkaline conditions [21]. In addition to its direct effect on cGMP production in RASM, peroxynitrite enhanced cGMP synthesis that was stimulated by a •NO donor, SNP. Such an effect on cGMP synthesis does not suggest that peroxynitrite inhibits guanylate cyclase activity.

While peroxynitrite was a weak stimulant of smooth muscle cell cGMP formation, exposure of endothelial cells to peroxynitrite generated a long-lived substance that, when transferred to reporter cells, enhanced cGMP production in an oxyhemoglobin-inhibitable manner. It is improbable that peroxynitrite directly stimulated RFL-6 generation of cGMP, because the 3 min exposure of endothelial cells to peroxynitrite also results in complete decomposition of peroxynitrite before transfer of the supernatant to the reporter cells. While 2 min incubation of RFL-6 with peroxynitrite increased cGMP 2-fold, extending the incubation period to 5 min resulted in a return to control levels. Also, neither 100 μM NO_2^- nor decomposed peroxynitrite was capable of increasing RFL-6 synthesis of cGMP. Potential candidates for more long-lived substances that could stimulate endothelial-dependent RFL-6 cGMP synthesis include *S*-nitrosoglutathione or other reversibly *S*-nitrosated intermediates capable of activation of guanylate cyclase [8,22,23]. The concept of cellular thiols serving as intermediates in •NO transport is additionally supported by the 40% decrease in peroxynitrite-treated endothelial-dependent cGMP formation, following depletion of endothelial glutathione by treatment with BSO.

Numerous pathophysiologic processes such as sepsis, ischemia-reperfusion injury, or acute inflammation are associated with enhanced production of both $O_2^{\bullet-}$ and •NO, with these species derived from either the same or different cell types to yield peroxynitrite intracellularly, intravascularly or in extracellular compartments. It has been estimated that local production of peroxynitrite could reach 1 $mM \cdot min^{-1}$ following macrophage activation [24]. We have demonstrated that peroxynitrite stimulates cGMP production by vascular smooth muscle cells, although to a lesser extent than authentic •NO. In addition, peroxynitrite also enhances endothelial-dependent cGMP production. Since peroxynitrite induces vasorelaxation of both coronary and pulmonary artery rings [8,9] and inhibits platelet function [25], it is concluded from the observations reported herein that these effects are at least partially mediated by activation of guanylate cyclase. However, these studies do not address the target molecule reactivities of peroxynitrite towards vascular structural and functional components that are not cGMP-mediated. Thus, peroxynitrite or its secondary reactive and potentially cytotoxic products are likely to contribute as yet undefined roles in the alteration of vascular reactivity associated with enhanced production of •NO and $O_2^{\bullet-}$.

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