Peroxynitrite and nitric oxide differ in their effects on pig coronary artery smooth muscle

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Submitted 4 September 2002; accepted in final form 5 November 2002

Walia, Mandeep, Sue E. Samson, Tracey Schmidt, Kelly Best, Melinda Whittington, Chiu Yin Kwan, and Ashok K. Grover. Peroxynitrite and nitric oxide differ in their effects on pig coronary artery smooth muscle. Am J Physiol Cell Physiol 284: C649–C657, 2003. First published November 13, 2002; 10.1152/ajpcell.00405.2002.—Peroxynitrite generated in arteries from superoxide and nitric oxide (NO) may damage their function. Here, we compare the effects of peroxynitrite and peroxynitrite/NO-generating agents SIN-1 (3-morpholinosydnonimine hydrochloride), SNAP (S-nitroso-N-acetyl-penicillamine), SNP (sodium nitroprusside), and NONOate (spermine NONOate) on pig coronary artery. Deendothelialized artery rings were pretreated with these agents and then washed before examining their contractility. Pretreatment with all agents (200 μM) results in a decrease in the force of contraction in response to the sarco(endo)plasmic Ca2+ (SERCA) pump inhibitor cyclopiazonic acid (CPA): SNAP > NONOate ≥ peroxynitrite ≥ SIN-1 > SNP. Pretreatment with SNAP, NONOate, or SIN-1 also inhibits the force of contraction produced with 30 mM KCl, with SNAP being the most potent. Including catalase plus superoxide dismutase (SOD) during the preincubation has no effect. Including an NO scavenger [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] or aguanylate cyclase inhibitor (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) partially protects against SNAP. Pretreatment of cultured cells with peroxynitrite, but not with SNAP, inhibits the Ca2+ transients produced in response to CPA. Pretreating isolated membrane vesicles with peroxynitrite inhibits the Ca2+ uptake due to the SERCA pump, with all the other agents being less effective. Thus peroxynitrite and NO both inhibit the CPA-induced contractions in deendothelialized artery rings, peroxynitrite by damage to the SERCA pump and NO possibly by a step downstream from the increase in cytosolic Ca2+.

ATPase; free radicals; oxidative stress; vascular diseases; ischemia reperfusion

REACTIVE OXYGEN SPECIES (ROS) are pivotal as intermediates in mitochondrial function and as signaling molecules in many physiologically important processes (5, 14, 22, 25). Their rates of formation and dissolution are rigorously controlled. The enhanced production of ROS during ischemia reperfusion and in vascular pathologies such as atherogenesis and inflammation is implicated in cardiovascular damage (1, 9, 22). The damage caused by ROS to transport processes may disturb homeostasis in vascular smooth muscle and endothelium. Such damage is not uniform to all transport processes. Ca2+-dependent K+ channels in rat cerebral artery smooth muscle cells are more sensitive to damage by ROS than are voltage-sensitive Ca2+ channels (6). In pig coronary artery smooth muscle, the sarco(endo)plasmic reticulum Ca2+ (SERCA) pump is more sensitive to damage by peroxide and superoxide than the plasma membrane Ca2+ (PMCA) pump (16, 19). Consequently, contractions to angiotensin II and to the SERCA pump inhibitors thapsigargin and cyclopiazonic acid (CPA) are more sensitive to such damage by pretreatment of the arteries with peroxide and superoxide than are the contractions to membrane depolarization by KCl or endothelins (12, 20). The damage may also be tissue dependent, e.g., smooth muscle may be more sensitive to damage by peroxide than endothelium (18). Individual physiological processes within each tissue may also differ in their susceptibility to different species of ROS.

Peroxynitrite differs from ROS such as peroxide, superoxide, and hydroxyl radicals (3, 24, 33). It can modify proteins by nitrosylation of thiols and tyrosine residues, in addition to the protein and lipid oxidation pathways used by other ROS. The effects of peroxynitrite on the pig coronary artery have not been reported. Peroxynitrite is produced in arteries from nitric oxide (NO) and superoxide generated by macrophages during injury or atherogenic inflammatory responses. It may also be produced in the arteries during ischemia reperfusion (1, 3, 24, 30). In the isolated perfused rat heart, it induces both vasodilation and impaired vascular relaxation (41). Here, we set out to determine the effects of peroxynitrite on pig coronary artery smooth muscle at three different levels of organization: contractility in artery rings, intracellular calcium concentration ([Ca2+]i) transients in cultured cells, and oxalate-stimulated-azide-insensitive Ca2+ uptake in the SERCA-enriched subcellular membrane fraction. The
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effects of peroxynitrite can only be determined by adding a bolus of this agent because it is unstable at the physiological pH. There are no agents that can slowly generate only peroxynitrite. Most peroxynitrite-generating agents produce mixtures of various ROS, including NO. NO-generating agents may also cause S- and O-nitrosylation of proteins, although it is not clear whether they are all equally effective in doing so (5, 24). Therefore, we also compared the effects of peroxynitrite with different peroxynitrite/NO-generating agents.

**EXPERIMENTAL METHODS**

**Contractility experiments.** Pig hearts were obtained from Maple Leaf Meats (Burlington, Canada) and placed immediately in an ice-cold physiological saline solution (20). The left anterior descending coronary artery was dissected, deendothelialized, and placed in a Krebs solution bubbled with 95% O2-5% CO2. The Krebs solution contained (in mM) 115 NaCl, 5 KCl, 22 NaHCO3, 1.7 CaCl2, 1.1 MgCl2, 1.1 KH2PO4, 0.03 EDTA, and 7.7 glucose. From the middle region of each artery, 3-mm long rings were cut and hung under 3 g of tension in 4-ml organ baths at 37°C for contraction as previously described (20). The maximum force of contraction was determined for each artery ring by adding 3 M KCl to a final concentration of 60 mM. The rings were then washed five times with 5 ml of Krebs solution over a total period of 20 min and then allowed to equilibrate for another 20 min. The force of contraction with 30 mM KCl (20) was examined, and the tissues were washed again. Aliquots of peroxynitrite (Calbiochem) under nitrogen were stored at ~80°C and used within 3 mo. The peroxynitrite concentration was determined by its absorbance at 302 nm (extinction coefficient = 1670 M⁻¹cm⁻¹) in 0.1 M NaOH immediately before use. SNAP (S-nitroso-N-acetyl-penicillamine) and SNP (sodium nitroprusside) were prepared in chilled Krebs solution just before use. NONOate (spermine NONOate), CPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide], and SIN-1 (3-morpholinosydnonimine hydrochloride) were prepared as concentrated stock solutions in water and stored at ~80°C. Concentrated stock solutions of CPA and ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) were prepared in dimethylsulfoxide and stored at ~80°C. Aliquots of the frozen solutions were thawed only once. In all instances, aliquots of 4 μl were added to the organ baths containing 4 ml of Krebs solution. Peroxynitrite or other agents were added, and the tissues were incubated for another 30 min. They were then washed again five times with 5 ml of Krebs solution over a total period of 45 min and allowed to equilibrate for another 10 min. Their contraction to 30 mM KCl was examined for 20 min, and the tissues were washed again over a 30 min period and then used for monitoring their contraction with 10 μM CPA.

**[Ca2⁺]** measurements. Smooth muscle cells in passage 4 were cultured on cover slips and used for the [Ca2⁺] measurements. Isolation and characteristics of these cells have been previously described (15, 20). These cells express smooth muscle actin, contract to 2-3 μM angiotensin II, or endothelin-1 but not to membrane depolarization by KCl. The cover slips coated with cultured cells were removed from the culture medium and rinsed with a solution containing (in mM) 115 NaCl, 5.8 KCl, 2 CaCl2, 0.6 MgCl2, 12 glucose, and 25 HEPES-Na, pH 7.4, at 37°C. These were then incubated in this buffer at 37°C for 30 min in the presence of 200 μM peroxynitrite or other agents, washed 3 times to remove these agents, loaded with fluo 3-AM, and then used for [Ca2⁺] measurements. The total time period between the end of the pretreatment with any agents and the [Ca2⁺] measurements was 90 min.

**Ca2⁺** uptake in isolated membranes. Subcellular membrane fraction F3 enriched in sarcoplasmic reticulum (SR) was isolated from smooth muscle. Isolation of these membranes and their characteristics have been previously reported (16, 19, 21). The membranes were incubated with peroxynitrite or other agents at 37°C, and then 1 mM DTT was added to quench any remaining peroxynitrite or peroxide produced from the decomposition. The samples were placed on ice until use. Ca2⁺ uptake was carried out as described earlier (16, 19). The final reaction mixture consisted of creatine kinase (70 units/ml) and the following concentrations (in mM) of the other components: 100 KCl, 5 Na-azide, 5 MgCl2, 5 ATP, 10 creatine phosphate, 1 EGTA, 0.85 CaCl2 (plus trace amounts of 45Ca), 30 imidazole-HCl (pH 6.8 at 37°C), 0.33 DTT, 0 or 5 K-phosphate (with F3 fraction), and 0 or 5 K-phosphate (with F2 fraction, pH 6.8, at 37°C). This resulted in 5 μM free Ca2⁺ as described earlier (16, 19).

**Nitroblue tetrazolium reduction assay.** Reduction of nitroblue tetrazolium was used to monitor superoxide generation as described earlier (23). Briefly, 75-μl aliquots of each agent were prepared and pipetted into a 96-well plate on ice and 175 μl of ice-cold nitroblue tetrazolium solution was added with or without catalase and SOD. The final reaction mixtures contained 200 μM peroxynitrite or other specified agent, 1.5 mM nitroblue tetrazolium, and 0 or 200 units each of catalase and SOD in the HEPES-Krebs solution described above for the contractility experiments. Absorbance of the samples at 595 nm was monitored immediately, and then the foil-wrapped samples were placed in a 37°C oven and absorbance monitored again after 30, 60, and 90 min. The difference in the absorbance changes with and without catalase plus SOD after 30 min was used to express the activity.

**Data analysis.** Curve fitting was carried out by nonlinear regression using the FigP software (BioSoft; Ancaster, Canada). Statistical significance was determined using the Student’s t-test. All experiments were replicated three to five times.

**RESULTS**

Effects of pretreatment with peroxynitrite and NO-generating agents on contraction. Deendothelialized arteries in Krebs solution contract when challenged with the SR Ca2⁺ pump inhibitor CPA (31) or when membranes are depolarized with high concentrations of KCl. The mean force of contraction produced by 30 mM KCl is 23.9 ± 1.2 mN (mean ± SE, 100 tissues), whereas with 10 μM CPA it is 4.9 ± 0.4 mN (100 tissues). To compare the damage to the artery rings by various agents with that of peroxide and superoxide reported previously, we used the same protocols as described earlier and given in EXPERIMENTAL METHODS (20). This protocol includes extensive washing to remove from the organ baths any agents and their decomposition products used during the pretreatment. This was done to ensure that these substances did not directly interfere in the subsequent measurements. Figure 1 shows the concentration dependence of the effects of pretreatment with peroxynitrite, SNAP, or NONOate. Pretreatment with peroxynitrite does not
alter the force of contraction produced with 30 mM KCl, but SNAP or NONOate inhibits the contraction with 30 mM KCl, with SNAP ($r^2 = 0.9454$, IC$_{50} = 5.7 \pm 0.9$ μM) being more potent than NONOate ($r^2 = 0.9366$, IC$_{50} = 47 \pm 15$ μM) (Fig. 1A). Pretreatment with these agents decreases the force of contraction produced with CPA with the following order of potency: SNAP ($r^2 = 0.9145$, IC$_{50} = 1.6 \pm 0.5$ μM) > NONOate ($r^2 = 0.9719$, IC$_{50} = 32 \pm 8$ μM) > peroxynitrite ($r^2 = 0.9935$, IC$_{50} = 87 \pm 6$ μM) (Fig. 1B). We also determined the effects of single concentrations of SNP and SIN-1. Pretreatment with 200 μM SNP decreased the force of contraction with KCl and CPA to $76 \pm 9$ and $61 \pm 16\%$ of the control, respectively (data not shown). The corresponding values with SIN-1 were $62 \pm 11$ and $32 \pm 10\%$. The rank order of effectiveness of these agents (at 200 μM) for the CPA contraction is SNAP > NONOate ≥ peroxynitrite ≥ SIN-1 > SNP. This order is similar but not identical to that obtained for the contractions with KCl (SNAP ≥ NONOate > SIN-1 > SNP = peroxynitrite). Because peroxynitrite was added as a bolus in the presence of fivefold molar excess of NaOH, we also examined the effects of pretreatment with NaOH up to 1,000 μM. Pretreatment with NaOH does not significantly affect the force of contraction ($P > 0.05$). For example, in the tissues pretreated with 1,000 μM NaOH, the force of contraction with KCl is $96 \pm 8\%$ of the control and with CPA is $86 \pm 11\%$.

### Effects of pretreatment with various agents with catalase plus SOD on contraction

To ascertain which of these agents produced measurable amounts of superoxide, we used an assay based on change in the absorbance of nitro blue tetrazolium (NBT) upon reduction. We incubated these agents with NBT with or without excess catalase plus SOD. SIN-1 shows the largest amount of SOD-sensitive reduction of NBT, and peroxynitrite is next (Table 1). The change in the absorbance of NBT with SNAP, NONOate, and SNP was no different from the water used as a control.

Pretreatment with peroxide and superoxide is known to inhibit the contractions to CPA (20). Therefore, we considered the possibility that the hydrogen peroxide or superoxide produced by decomposition of these agents may cause the inhibition of the contractions observed in Fig. 1. We have shown previously that catalase plus SOD can overcome the effect of superoxide and that catalase alone can abolish the effect of hydrogen peroxide in this system (11, 16). Hence, we examined the effects of preincubation with varying agents with catalase plus SOD on contraction. To ascertain which of these agents produced measurable amounts of superoxide, we used an assay based on change in the absorbance of nitro blue tetrazolium (NBT) upon reduction. We incubated these agents with NBT with or without excess catalase plus SOD. SIN-1 shows the largest amount of SOD-sensitive reduction of NBT, and peroxynitrite is next (Table 1). The change in the absorbance of NBT with SNAP, NONOate, and SNP was no different from the water used as a control.

### Table 1. SOD-sensitive nitro blue tetrazolium reduction

<table>
<thead>
<tr>
<th>Additive, in μM</th>
<th>Change in Absorbancy</th>
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<tbody>
<tr>
<td>Water</td>
<td>0.029 ± 0.009(11)</td>
</tr>
<tr>
<td>Peroxynitrite, 200</td>
<td>0.127 ± 0.015(6)</td>
</tr>
<tr>
<td>SIN-1, 200</td>
<td>0.400 ± 0.063(3)</td>
</tr>
<tr>
<td>SNP, 200</td>
<td>0.015 ± 0.003(6)</td>
</tr>
<tr>
<td>NONOate, 200</td>
<td>−0.007 ± 0.007(6)</td>
</tr>
<tr>
<td>NaOH, 200</td>
<td>−0.018 ± 0.007(6)</td>
</tr>
</tbody>
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Values are means ± SE of the number of replicates shown in parentheses. SNAP, S-nitroso-N-acetyl-penicillamine; SIN-1, 3-morpholinosyndnonimine hydrochloride; SNP, sodium nitroprusside; NONOate, spermine NONOate.
SIN-1, peroxynitrite, and SNAP with or without excess catalase plus SOD. Figure 2 shows that peroxynitrite inhibited only the CPA-induced contractions (P < 0.05), and all the other agents significantly decreased the force of contractions with KCl and CPA (P < 0.05). Catalase plus SOD had no effect (P > 0.05), indicating that the observed effect is primarily due to peroxynitrite and not due to its decomposition products.

Further experiments on the effects of SNAP on force of contraction. Because SNAP has the highest potency, we considered the possibility that a stable decomposition product of SNAP was responsible for the inhibition. To test this hypothesis, we incubated a stock solution of SNAP in Krebs solution overnight at 37°C and then pretreated the artery rings with it. Whereas a preincubation with 10 μM freshly prepared SNAP decreases the force of contraction with KCl to 35% of the control and with CPA to 7% of the control, in 3 tissues pretreated with the decomposed SNAP, the force of contraction is 95% of the control with KCl and 103% with CPA. Thus the decomposed SNAP is ineffective. This loss of ability of SNAP to inhibit the force of contraction indicates that a metastable product of SNAP (such as NO) is responsible for the inhibition. Because SNAP is known to generate NO, we next conducted an experiment in which CPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] was used to quench the NO generated by SNAP (32). In preliminary experiments, we established that CPTIO was active in that it inhibited the endothelium-dependent relaxation, thereby showing that CPTIO can effectively quench the NO produced by the endothelium. We next examined whether CPTIO could quench the NO produced by SNAP and thus overcome its inhibition of the CPA-induced contraction in deendothelialized artery rings. We preincubated the deendothelialized artery rings with 10 μM SNAP with and without 50 μM CPTIO (data not shown). Including this concentration of CPTIO in the preincubation mixture does not overcome the effect of 10 μM SNAP on the force of contraction produced by 30 mM KCl or by CPA. We then preincubated the deendothelialized artery rings with 10 μM SNAP with and without 200 μM CPTIO. It alleviates the inhibition by 10 μM SNAP significantly (P < 0.05) but only partially on the force of contraction produced with KCl or CPA (Fig. 3).

The established mode of action of NO on smooth muscle is stimulation of guanylate cyclase (24), and, hence, we next examined the effects of the guanylate cyclase inhibitor 1 ODQ (38). In a preliminary experiment, we determined that ODQ at 10 μM completely inhibits the endothelium-dependent relaxation in this tissue, but preincubating deendothelialized artery rings with ODQ does not affect the force of contraction with KCl or CPA (data not shown). We next preincubated the deendothelialized artery rings with 10 μM SNAP with and without 10 μM ODQ (Fig. 4A). Including ODQ in the preincubation mixture completely overcomes the effect of 10 μM SNAP on the force of contraction produced by 30 mM KCl. However, its effect is only partial when the CPA-induced contraction is examined (Fig. 4B).

Effects of peroxynitrite pretreatment on [Ca^{2+}]_{i} transients. Smooth muscle cells cultured from the coronary artery show an increase in [Ca^{2+}]_{i} transients when challenged with 10 μM CPA. When cells are pretreated with 200 μM peroxynitrite and then washed as de-
scribed in EXPERIMENTAL METHODS, a challenge with CPA gives a smaller increase in \([Ca^{2+}]_i\) transients \((P < 0.05\), Fig. 5). Including catalase plus SOD with peroxynitrite does not alter its effects (data not shown). However, a similar pretreatment with 200 \(\mu\)M SNAP does not significantly diminish the CPA-induced \([Ca^{2+}]_i\) transients \((P > 0.05\), Fig. 5), suggesting that peroxynitrite and SNAP act via different mechanisms. This result is in contrast to the contractility experiments in which pretreatment with this concentration of SNAP decreases the force of contraction with CPA by >90\% (Fig. 1).

**Effects of pretreatment with various agents on \([Ca^{2+}]_i\) uptake in isolated membrane vesicles.** We next examined the possibility that the pretreatment with these agents may damage the SERCA pump in the SR-enriched fraction F3 isolated from pig coronary artery smooth muscle. The subcellular fractionation and \([Ca^{2+}]_i\) uptake properties of F3 have been characterized previously (17, 21). With these membranes, one can use an ATP-regenerating system to obtain \([Ca^{2+}]_i\) uptake that is linear with time for up to 2 h. The uptake due to the SR in this fraction is ATP dependent, azide insensitive, stimulated by oxalate that prevents back flux of \(Ca^{2+}\), and inhibited by CPA. In a preliminary experiment, we observed that pretreating the membranes with 100 \(\mu\)M peroxynitrite gave similar values for inhibition of the \([Ca^{2+}]_i\) uptake as those observed for the CPA-induced contractions with 200 \(\mu\)M peroxynitrite. Therefore, we compared the effects of pretreating the membranes with 100 \(\mu\)M of each of these agents on the \([Ca^{2+}]_i\) uptake. Figure 6 shows that 1) pretreating the F3 membrane vesicles with 100 \(\mu\)M peroxynitrite results in an 80\% inhibition of the \([Ca^{2+}]_i\) uptake, 2) a similar pretreatment with 100 \(\mu\)M SNAP or SNP leads to marginal inhibition of the uptake and 100 \(\mu\)M NONOate or SIN-1 do not inhibit the uptake significantly (Fig. 6), and 3) the inhibition of the uptake by the pretreatment with any of the agents is not overcome by including catalase plus SOD during the pretreatment. Thus the rank order for inhibition appears to be peroxynitrite >> SNP = SNAP > NONOate > SIN-1.

**DISCUSSION**

Our results show that 1) pretreating the artery rings with peroxynitrite and NO-generating agents de-

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Fig. 3. Contractility experiments (A, 30 mM KCl; B, 10 \(\mu\)M CPA) with 200 \(\mu\)M 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) included during pretreatment. Artery rings were preincubated with 10 \(\mu\)M SNAP and tested as in Fig. 1. CPTIO (200 \(\mu\)M) was included during preincubation where shown. The number of tissues used was 22 (control), 44 (SNAP), and 21 (SNAP + CPTIO). All values are % of control carried out in parallel without any agents added during the preincubation. Values are means ± SE of %control.

Fig. 4. Contractility experiments (A, 30 mM KCl; B, 10 \(\mu\)M CPA) with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) included during pretreatment. Artery rings were preincubated with 10 \(\mu\)M SNAP and tested as in Fig. 1. ODQ (10 \(\mu\)M) was included during preincubation where shown. The number of tissues used was 25 (control), 19 (SNAP), and 17 (SNAP + ODQ). All values are % of control carried out in parallel without any agents added during preincubation. Values are means ± SE of %control.
creases the force of contraction in the following decreasing order of effectiveness: SNAP > NONOate ≥ peroxynitrite = SIN-1 > SNP with CPA and SNP > NONOate > SIN-1 with KCl (no inhibition with SNP and peroxynitrite), 2) the force of contraction with none of the agents is significantly altered when catalase + SOD are included during the pretreatment, 3) the effectiveness of SNAP is decreased with CPTIO or ODQ, 4) pretreatment of cultured cells with peroxynitrite, but not with SNAP, decreases the CPA-induced $[\text{Ca}^{2+}]_i$ transients, and 5) in isolated membranes, the $\text{Ca}^{2+}$ uptake due to the SERCA pump is inhibited predominantly by pretreatment with peroxynitrite with the order of effectiveness of the different agents being peroxynitrite $\gg$ SNP $\geq$ SNAP $\geq$ NONOate $=$ SIN-1. The DISCUSSION focuses on the methods used in this study, comparison of these results with the literature, a proposed model to explain the results, and possible biological implications of these findings.

The agents used during the preincubation or their decomposition products may react with the reagents used subsequently in the assays. To avoid this possibility, we washed the tissues after the treatment with the agents used for the pretreatment and before adding any other reagents. We have shown earlier that including catalase plus SOD prevents the effects of superoxide and peroxide on artery rings, cultured cells, and isolated membranes (11, 23). Thus protocols used in

Fig. 5. Effects of pretreatment with 200 μM peroxynitrite or 200 μM SNAP on CPA-induced increase in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$). The values are means ± SE from 34 replicates for control, 13 for peroxynitrite, and 9 for SNAP. *Peroxynitrite treatment inhibits the increase in $[\text{Ca}^{2+}]_i$, significantly ($P < 0.05$). SNAP does not produce a significant inhibition ($P > 0.05$).

Fig. 6. $\text{Ca}^{2+}$ uptake by sarcoplasmic reticulum-enriched membranes pretreated with different agents. Membranes treated without any additives showed an uptake of 804 ± 18 nmol/mg protein. Values for the $\text{Ca}^{2+}$ uptake for the samples treated with the various additives are expressed as % of this control value and are means ± SE of 6 replicates. Treatment with peroxynitrite significantly ($P < 0.05$) inhibited the uptake. SNP, sodium nitroprusside.
the study would monitor the damage produced by the agents during pretreatment on contractility or other properties rather than their effects when present during the course of the assays. This distinction is important in comparing this work with the literature. Because one cannot obtain pure plasma membranes or SR membranes from the coronary artery smooth muscle, we used the effect of oxalate on the inhibition of back flux of Ca$^{2+}$ to identify the activity of the SERCA pump (16, 17, 21). The membrane fractions used in this study and the characteristics of the oxalate-stimulated Ca$^{2+}$ uptake have been established previously (21). Thus the methods for monitoring contractility, [Ca$^{2+}$], and SR Ca$^{2+}$ pump activity in isolated membranes have been previously validated using pig coronary artery (15, 20).

We first conducted the work on artery rings with peroxynitrite. However, the damage produced in this study required high micromolar concentrations of this agent. Because in these experiments the peroxynitrite is extremely short-lived but in vivo peroxynitrite may be generated constantly during ischemia reperfusion or inflammation-like responses during atherosclerosis, we then used the peroxynitrite-generating agent SIN-1 (23, 24). Because all peroxynitrite-generating agents also produce NO, we also used several NO-generating agents. None of the NO-generating agents produce only one species of NO, and even NO gas is converted to other species and/or oxidized to nitrites and nitrates under aerobic conditions (34). The various agents not only produce NO at different rates but may also produce different species of NO (2, 23, 24, 35). Predominant species generated by some of the agents are NONOate and SNP (NO$^{-}$), SNAP (NO$^{+}$), and Angeli’s salt (NO$^{-}$) (24). We tested SNAP and NONOate and found that they were both able to produce relaxation in the deendothelialized arteries that had been precontracted with KCl. However, in inhibiting the contractility, SNAP was the most potent, even more potent than SIN-1 and peroxynitrite. In the NBT reduction assay, only SIN-1 and peroxynitrite showed any production of superoxide, indicating that SNAP, NONOate, and SNAP generated predominantly various species of NO.

Thus we verified under our experimental conditions that SIN-1 produces significantly more superoxide than SNAP. SNAP is in fact a very potent NO release agent. It produces NO at a rate several hundred times faster than SIN-1 (23, 24). SNAP activates guanylate cyclase at lower concentrations than spermine-NONOate, and the rate of NO release from SNAP is much faster than from the NONOate (2, 24). The various agents not only produce NO at different rates but may also produce different species of NO (2, 23, 24, 35). Even when NO is generated as a gas, it may form different species, depending on the aqueous environment and in a tissue-dependent manner (10, 34). In addition, S-nitrosothiols like SNAP decompose spontaneously in solution to their disulfide forms while generating NO. However, decomposed SNAP by itself was not effective, although one cannot rule out the ability of SNAP to generate simultaneously NO and disulfide groups that would allow it to act differently from other agents.

We have shown earlier that pretreatment with peroxide or superoxide causes a decrease in 1) the SR Ca$^{2+}$ pump activity in the coronary artery, 2) Ca$^{2+}$ transients produced by CPA in cultured smooth muscle cells, and 3) tissue contractility to CPA and to agents such as angiotensin II that depend on the mobilization of the SR Ca$^{2+}$ (18, 20). Peroxide and superoxide act simply in oxidation reactions, presumably involving thiol groups (3, 24). However, the action of peroxynitrite or SIN-1 did not occur via decomposition into superoxide and peroxide, because their effects were insensitive to the inclusion of catalase plus SOD during pretreatment. Peroxynitrite has been shown to cause nitrosylation of thiol groups and tyrosine residues (27, 36, 40). Such nitrosylation of SERCA2a in slow-twitch skeletal muscle decreases its enzymatic activity (42). Therefore, the most likely mechanism appears to be inactivation of the SR Ca$^{2+}$ pump by peroxynitrite directly or through SIN-1. A direct effect of peroxynitrite on the contractile machinery or on voltage-dependent Ca$^{2+}$ channels is thus ruled out, because contraction with 30 mM KCl was not affected. However, one cannot rule out the possibility that pathways such as store depletion-dependent Ca$^{2+}$ channels are also affected by the treatment.

Several effects of NO have been reported on vascular smooth muscle. An NO-mediated increase in store depletion-dependent Ca$^{2+}$ entry has been reported in some studies, and decrease reported in others (8, 13). NO binding to heme group of guanylate cyclase activates it, thus producing an increase in cell cGMP levels (2, 24). NO-mediated decrease in vascular reactivity has been proposed to occur by various mechanisms (7, 26, 28, 37). Our results show that pretreatment with SNAP inhibits the contractions produced by KCl and CPA. The inhibition is not affected by CPTIO at 50 μM, but it can be overcome partially with 200 μM CPTIO, indicating the involvement of NO. However, even the high concentration of CPTIO used here does not completely overcome the effects of SNAP, possibly because SNAP produces NO very rapidly (2). SNAP produces NO·. NO· and NO are both quenched with CPTIO, although NO· produced by Angeli’s salt is not quenched effectively with this agent (10, 29). Because these experiments required pretreatment at high concentrations of NO for a prolonged period under aerobic conditions, one cannot rule out the conversion of the initially generated NO into other products, including the nitroxyl ion. Gaseous NO under aerobic conditions can be converted to products that no longer react with CPTIO (34). The effect of SNAP is also overcome in part by the guanylate cyclase inhibitor ODQ, suggesting that cGMP plays a key role in this pathway. However, 10 μM ODQ does not completely overcome the effect of SNAP, even though IC$_{50}$ for guanylate inhibition of ODQ is 84 nM (38). This may be because ODQ may not as readily cross the cell membranes as NO or because NO has been converted into other products or another pathway is involved in the observed effects of

AJP-Cell Physiol • VOL 284 • MARCH 2003 • www.ajpcell.org
SNAP. Finally, the pretreatment of cultured cells with SNAP does not produce a decrease in Ca\(^{2+}\) transients in response to CPA. Together, these results suggest that SNAP acts by a step downstream of Ca\(^{2+}\) mobilization.

**Physiological implications.** The effects of peroxynitrite and NO may be important in aging, ischemia reperfusion, inflammation, atherosclerosis, and sepsis (5, 14, 22, 25). SERCA2a in rat slow twitch skeletal muscle shows that nitrosylated tyrosine residues increase with aging (42). Such data are not currently available for the coronary artery. Damage to the SERCA pump in this tissue would mean a loss of reactivity to agents such as angiotensin and \(\alpha\)-adrenergic agents that depend on mobilization of the SR Ca\(^{2+}\) pools (4, 20). NO signaling pathways are of central importance in both the maintenance of vascular homeostasis and the progression of vascular disease. Multiple interactions of NO with other pathways can lead to either vascular protection or potentiation of inflammatory vascular injury (33). During sepsis, an increase in [Ca\(^{2+}\)] may be an early event responsible for cellular injury. This would normally lead to an increased contractile force in the affected areas (39). A decrease in the Ca\(^{2+}\) sensitivity of the contractile apparatus would thus provide a useful protection.

We conclude that peroxynitrite and NO both inhibit the CPA-induced contractions in deendothelialized artery rings, peroxynitrite via damage to the SERCA pump and NO at a step downstream from a change in [Ca\(^{2+}\)], as one of the possibilities. Both types of interactions have important pathophysiological ramifications.

We thank Dr. N. Narayanan of University of Western Ontario for critical comments on the manuscript. This work was funded by Heart and Stroke Foundation of Ontario Grant-in-Aid T-4815. A. K. Grover received a Career Investigator Award from the Heart and Stroke Foundation of Ontario.

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