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Calcitonin Gene-Related Peptide-Dependent Vascular Relaxation of Rat Aorta

AN ADDITIONAL MECHANISM FOR NITROGLYCERIN

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ABSTRACT. We investigated the involvement of calcitonin gene-related peptide (CGRP) in the vasodilatory mechanism of action of nitric oxide (NO) donors. The functional role of CGRP in NO donor-induced vasodilation of isolated rat aortic rings was determined by incubating these drugs with and without $CGRP_{8-37}$, a selective CGRP receptor antagonist. $CGRP_{8-37}$ (0.63 μM) induced rightward shifts in the vasodilatory concentration–response curves for nitroglycerin (NTG), Piloty's acid (PA), and SIN-1 (linsidomine). The EC_{50} values for NTG, PA, and SIN-1 were increased by 8.3-, 5.2-, and 2.3-fold, respectively ($P < 0.05$). The release of CGRP from rat aorta in response to NTG and PA was measured specifically by radioimmunoassay. Thirty-minute incubations of NTG or PA with rat aorta induced 189.5 and 214.6% increases, respectively, in CGRP release when compared with the control ($P < 0.05$). The concentration–response curves of sodium nitroprusside (SNP), *S*-nitroso-acetylpenicillamine (SNAP), tetranitromethane (TNM), diethylamine NO complex (DEA-NO), and diethylenetriamine/nitric oxide adduct (DETA NONOate) were not inhibited significantly by $CGRP_{8-37}$ co-incubation ($P > 0.05$). NO donors also were incubated with aortic strips, and NTG and PA alone induced significant formation of hydroxylamine, a NO^- metabolite (232.4 and 364.9%, respectively, $P < 0.05$). These results indicate that only NTG and PA, and to a lesser extent SIN-1, stimulate the release of CGRP from the rat aorta, which subsequently contributes to the vasodilatory activity of these agents. The hydroxylamine formation suggests a possible link between NO^- generation and CGRP release from the vascular wall. BIOCHEM PHARMACOL **59**;12:1603–1609, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. nitroglycerin; CGRP; vasodilation; nitroxyl anion; hydroxylamine; NO donors

As a class of pharmacological agents, NO‡ donors exert their effects by generating NO. The paradox of the NO donors is that, despite the common mechanism of action, the NO donors have disparate pharmacodynamic effects [1]. For example, NTG is predominantly a venodilator, and the continuous use of this nitrovasodilator produces pharmacological tolerance [2]. On the other hand, SNP is an arteriodilator, which is not limited by tolerance [3]. Furthermore, it has been demonstrated that NTG is a useful therapy in acute myocardial infarction, whereas SNP can exacerbate the situation [4]. Why two drugs with the same mechanism of action can produce opposite outcomes is a question that has not been answered completely.

One possible explanation may be that some NO donors may also rely on a second pharmacological mechanism, in addition to NO production [5]. Recently, it has been demonstrated that the ability of NTG to inhibit platelet aggregation is partly dependent upon CGRP [5], a small neuropeptide located in peptidergic neurons, which is capable of potent vasodilation [6]. CGRP can induce its effects by stimulating cyclic AMP production [7], K^+ channel activation [8], and/or NOS III activation [9] in the vascular wall. The platelet inhibition experiments with NTG also revealed the necessity of vascular tissue for this mechanism [5]. This finding suggested that the vasodilation induced by some NO donors may also depend on the action of this peptide. However, the dependence upon CGRP for platelet inhibition is a mechanism that apparently is not shared with SNP [10]. Therefore, it appears that only certain NO donors may function by this mechanism. The purpose of these experiments was to test the hypothesis that

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‡ *Abbreviations*: CGRP, calcitonin gene-related peptide; $CGRP_{8-37}$, calcitonin gene-related peptide fragment 8–37; CGRP-LI, calcitonin gene-related peptide-like immunoreactivity; DEA-NO, diethylamine NO complex; DETA NONOate, diethylenetriamine/nitric oxide adduct; NANC, non-adrenergic, non-cholinergic; NO, nitric oxide; NOS, nitric oxide synthase; NTG, nitroglycerin; PA, Piloty's acid, *N*-hydroxybenzenesulfonamide; SIN-1, linsidomine; SNAP, *S*-nitroso-acetylpenicillamine; SNP, sodium nitroprusside; and TNM, tetranitromethane.

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some NO donors evoke the release of CGRP, and that this peptide subsequently contributes to the vasodilatory mechanism of these NO donors.

MATERIALS AND METHODS

Drugs

NTG was obtained as an injectable solution from Schwarz Pharma. DETA NONOate, DEA-NO, and SNAP were obtained from Research Biochemicals Inc. SIN-1 was purchased from Alexis Biochemicals. PA was obtained from Fluka. Ketamine was obtained from Fort Dodge, and xylazine was obtained from Bayer. TNM, $CGRP_{8-37}$, a specific CGRP antagonist [11], phenylephrine, and SNP were obtained from the Sigma Chemical Co. All drugs, except NTG and TNM, were dissolved in Kreb's buffer, which consisted of (in mM): NaCl, 120; KCl, 5.6; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; dextrose, 10; $NaHCO_3$, 25; $CaCl_2$, 2.5. NTG was dissolved in 5% dextrose, and TNM was dissolved in ethanol.

Vasodilatory Concentration–Response Curves

All procedures were conducted in accordance with SUNY Institutional Animal Care and Use Committee guidelines. Male Sprague–Dawley rats, 325–350 g (Harlan), were anesthetized i.m. with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), the abdomen was exposed, and the rat was exsanguinated via the abdominal aorta. The thoracic cavity was opened quickly, and the thoracic aorta was excised and placed in ice-cold Kreb's buffer. The aortas were cleaned of extraneous tissue, and 3- to 4-mm vascular rings were cut. Vascular rings were then suspended by stainless steel hooks in 10-mL tissue baths containing Kreb's buffer, which were maintained at 37°, and aerated with 95% O_2:5% CO_2. One hook was fixed in place, and the other was connected to a Grass FT83 force transducer, which was connected to a Grass model 7D Physiograph. Vascular rings were washed with fresh Kreb's buffer every 15 min for an hour, and the basal tension was adjusted gradually to 4 g. The vascular rings then were contracted submaximally with 2 μM phenylephrine. Inhibitors were added and incubated for 15 min prior to the addition of the vasodilators. Then cumulative concentration–response curves ranging from 0.5 nM to 58 μM were obtained for NTG, SNP, SNAP, SIN-1, PA, TNM, DETA NONOate, and DEA-NO in the presence and absence of 0.63 μM $CGRP_{8-37}$. Drugs were added to the tissue baths as 100-μL aliquots.

CGRP Release from Rat Aorta

Male Sprague–Dawley rats, 350 g (Harlan), were anesthetized i.m. with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), and the aortas were isolated as described above, except that the vascular tissues were cut longitudinally. The aortas then were added singly to polypropylene test tubes containing Kreb's buffer, 69.7 KIU/mL of aprotinin (Sigma), and 0.73 mM NTG or PA, and pre-warmed to 37°. The tissues were aerated with 95% O_2:5% CO_2. Incubations were maintained for 30 min, and the reaction was stopped by removing the supernatant to clean test tubes, and adding 0.5 mL of 1 N HCl. Then CGRP was isolated from the supernatant by solid phase extraction with 200-mg C_{18} cartridges (Peninsula Laboratories) according to the manufacturer's protocol. CGRP samples were eluted into polypropylene test tubes with 20 mL of 60% acetonitrile in distilled water, dried down under a stream of N_2, and frozen at −70° until assayed. CGRP-LI was determined by radioimmunoassay (Peninsula Laboratories), and normalized to the wet weight of the aorta.

Hydroxylamine Assay

The hydroxylamine (NH_2OH) assay was a modification of the assay reported by Schmidt *et al.* [12]. Following dissection of the aorta as described, the vessel first was cut longitudinally, and then into 2–3 large strips. An aortic strip was incubated for 60 min in aerated Kreb's buffer at 37° with or without a NO donor. A volume of 540 μL of Kreb's buffer was added to 300 μL of monosodium phosphate buffer (final concentration of 5 mM, pH 6.8), 300 μL HCl (1 mM), 180 μL 8-hydroxyquinoline [0.2% (w/v); Fisher Scientific Co.] and 180 μl Na_2CO_3 (0.2 M; J. T. Baker Chemical Co.), mixed on a vortex mixer, and heated for 1 min at 95°. The samples then were cooled for 15 min on ice, and centrifuged for 10 min at 13,000 *g*. Absorbance at 680 nm was determined on a Cary spectrophotometer, and the concentration of NH_2OH was determined from a standard curve of NH_2OH (Eastman Kodak) prepared as described.

Statistical Analysis

The effects of the inhibitors on the concentration–response curves of each vasodilator were subjected to ANOVA with a Newman–Keuls post-hoc test for significance. The EC_{50} was calculated by fitting the data with the sigmoid E_{max} model using the computer program GraphPAD (Version 2.0). Significant differences between EC_{50} values were determined by Student's paired *t*-test. Statistically significant production of NH_2OH or CGRP release from the aorta was determined by ANOVA with the Newman–Kuels post-hoc test. Significant differences were deemed to exist if $P < 0.05$.

RESULTS

Vasodilatory Concentration–Response Curves

The physiograph tracings in Fig. 1 show the results of typical concentration–effect experiments with NO donors and isolated vascular rings. Experiments using NTG and SNP are shown in Fig. 1, A and B, respectively. The addition of 0.63 μM $CGRP_{8-37}$ by itself had no apparent

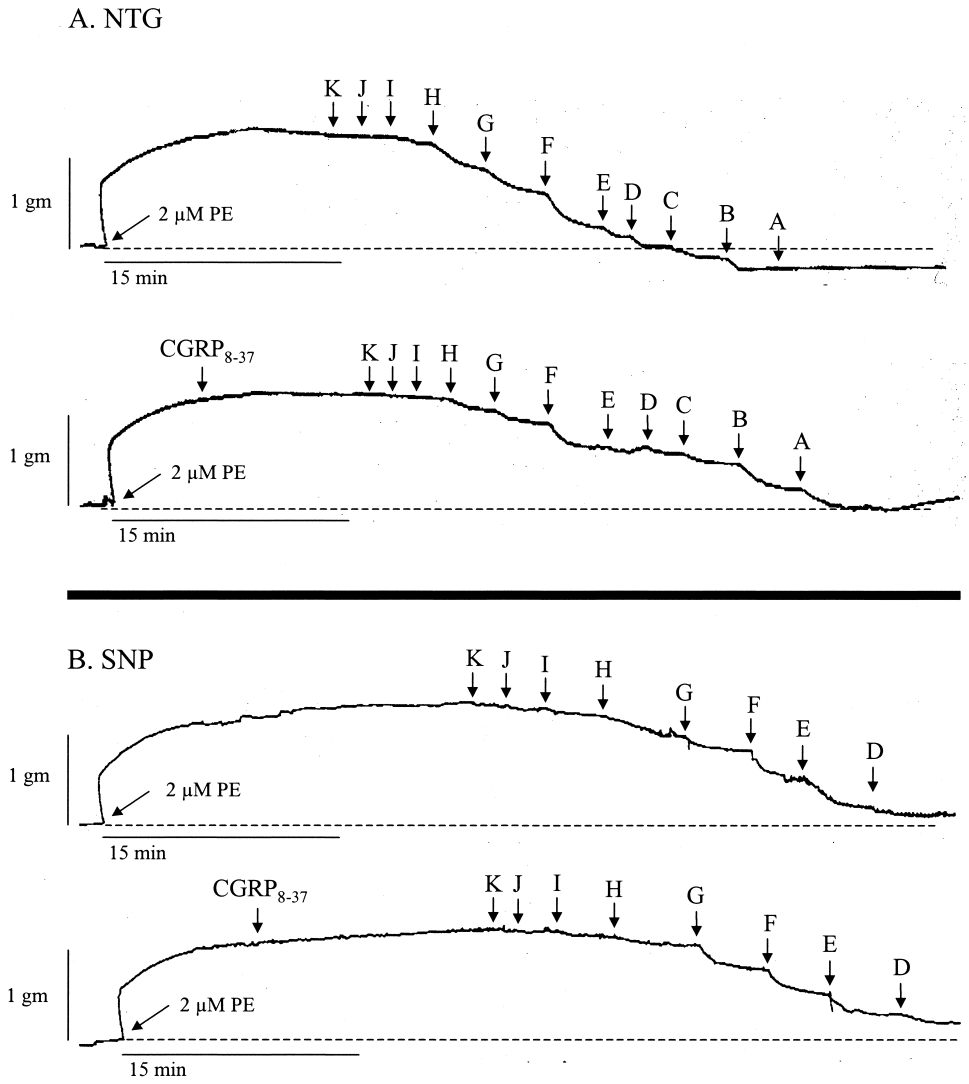

FIG. 1. Typical physiograph recordings of vascular relaxations of phenylephrine (PE)-contracted rat aortic rings in response to cumulative additions of (A) NTG and (B) SNP, with and without $CGRP_{8-37}$ preincubation (0.63 μM). NO donors were added from the lowest concentration (K) to the highest (A). For NTG, K was 0.22 nM and A was 58.7 mM. For SNP, K was 0.5 nM and A was 66.7 mM.

effect on smooth muscle tension (Fig. 1). However, vascular relaxation in response to NTG clearly was inhibited by $CGRP_{8-37}$. In contrast, the vasorelaxation induced by SNP appeared unaffected by the incubation with $CGRP_{8-37}$.

The effect of $CGRP_{8-37}$ on the NTG concentration–response curve is shown graphically in Fig. 2. $CGRP_{8-37}$ caused a significant rightward shift in the concentration–response curve over the range of 15 nM to 4 μM (Fig. 2; Table 1; $P < 0.05$). This resulted in an 8.3-fold increase in the EC_{50} of NTG. Conversely, the concentration–response curve and the EC_{50} of SNP were unaffected by the addition of $CGRP_{8-37}$ (Fig. 3; Table 1; $P > 0.05$). Similar experiments with other NO donors revealed that PA and SIN-1 also were affected by $CGRP_{8-37}$. The EC_{50} of PA was increased by greater than 5-fold (Table 1; $P < 0.01$), whereas that of SIN-1 was affected only modestly (a 2.3-fold increase in EC_{50}; Table 1; $P < 0.05$).

SNAP, TNM, and DETA NONOate, like SNP, were unaffected by $CGRP_{8-37}$ (Table 1; $P > 0.05$). The EC_{50} of DEA-NO was enhanced slightly by $CGRP_{8-37}$ (0.6-fold, Table 1, $P < 0.05$), which may have resulted from a physicochemical interaction between the two agents that yielded a mild enhancement of NO production.

CGRP Release from Aorta

In vitro incubations of NTG and PA with rat aorta induced a significant release of CGRP-LI from rat aorta within 30 min ($P < 0.05$). NTG induced the release of 217.3 ± 81.1 fmol CGRP-LI/g aorta (wet weight; mean ± SD; N = 4), which was an 189.5% increase over control tissue ($P < 0.05$). PA induced the release of 246.2 ± 30.4 fmol CGRP-LI/g aorta (mean ± SD; N = 4), which was 214.6% greater than the control ($P < 0.05$).

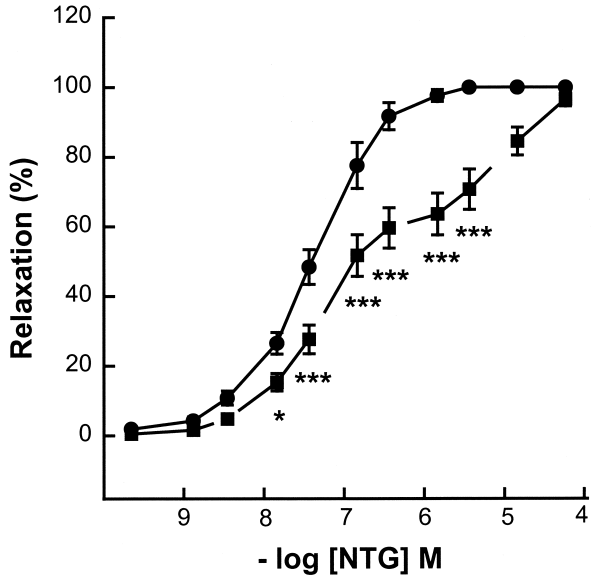

FIG. 2. Concentration–response curve of the vasodilatory effect of NTG in the presence (■) and absence (●) of 0.63 μM $CGRP_{8-37}$ in isolated rat aortic rings. Data are means ± SEM (N = 8–10). Key: (*) $P < 0.05$; and (***) $P < 0.001$.

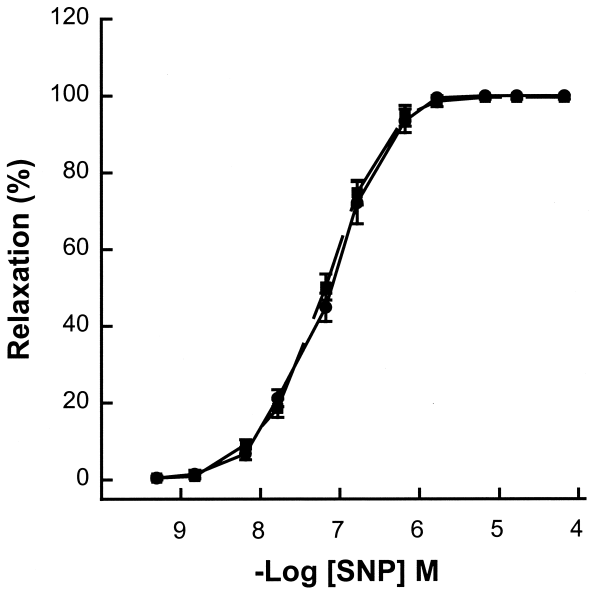

FIG. 3. Concentration–response curve of the vasodilatory effect of SNP in the presence (■) and absence (●) of 0.63 μM $CGRP_{8-37}$ in isolated rat aortic rings. Data are means ± SEM (N = 9–12).

Hydroxylamine Production

The vascular production of hydroxylamine, a putative metabolite of NO^-, by the different NO donors is shown in Fig. 4. The absorbance spectra of these samples (300–800 nm) indicated that no other absorbance peak interfered with the detection of NH_2OH at 680 nm. Incubation of NTG with aorta for 1 hr yielded a 2.3-fold increase in NH_2OH compared with the control ($P < 0.01$). PA induced a 3.6-fold increase in hydroxylamine concentration ($P < 0.001$), whereas none of the remaining NO donors induced any significant change in this nitroxyl anion metabolite ($P > 0.05$). There was no significant difference in mean weights of the aorta among the groups in this experiment ($P > 0.05$). None of the NO donors tested produced hydroxylamine in the absence of vascular tissue at the concentrations tested ($P > 0.05$).

DISCUSSION

Several investigators have demonstrated that CGRP release is stimulated by NO, in various tissues [13] including the vasculature [14]. Therefore, we hypothesized that some NO donors may mediate their actions by stimulating the release of this neuropeptide, in addition to generating NO. In the present studies, the hypothesis that NO donors mediate vasodilation partly by stimulating the release of CGRP was tested by incubating isolated aortic rings with $CGRP_{8-37}$, an inactive CGRP analogue that competes for the CGRP receptor [11]. The rationale of this approach was to block the action of CGRP released in response to the exposure of the aorta to a NO donor. In these experiments, a high concentration of 0.63 μM was used to block the CGRP receptors. $CGRP_{8-37}$ reportedly binds to the CGRP receptor with a high affinity, but possesses no intrinsic activity

TABLE 1. Effect of $CGRP_{8-37}$ on the vasodilatory EC_{50} of NO donors

NO donor	Primary NO species produced	EC_{50} (−log M) control	EC_{50} (−log M) + 0.63 μM $CGRP_{8-37}$	*P*	Effect on EC_{50}
SNAP	$NO^•$, NO^+	6.63 ± 0.58	6.57 ± 0.35	NS*	NS
SNP	$NO^•$, NO^+	7.11 ± 0.12	7.20 ± 0.14	NS	NS
TNM	$NO^•$, NO_2^+	5.41 ± 0.83	5.02 ± 0.69	NS	NS
DETA-NONOate	$NO^•$	5.03 ± 0.11	5.25 ± 0.17	NS	NS
DEA-NO	$NO^•$	7.00 ± 0.18	7.19 ± 0.08	†	0.6-fold ↓
SIN-1	$NO^•$, $ONOO^-$	5.07 ± 0.32	4.71 ± 0.32	†	2.3-fold ↑
PA	$NO^•$, NO^-	6.62 ± 0.65	5.90 ± 0.64	‡	5.2-fold ↑
NTG	$NO^•$, ?	7.34 ± 0.28	6.42 ± 0.68	‡	8.3-fold ↑

Data are means ± SEM N = 6–12.
*NS = not significantly different, $P > 0.05$.
†$P < 0.05$, compared with control.
‡$P < 0.01$, compared with control.

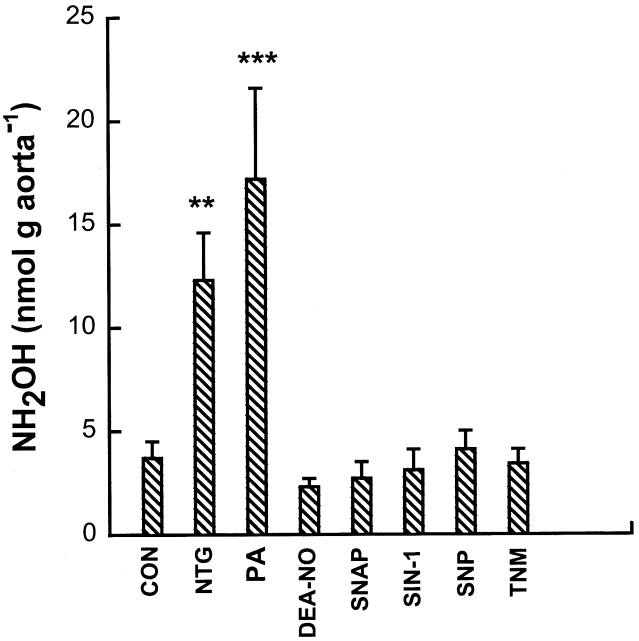

FIG. 4. Hydroxylamine production following a 60-min incubation of NO donors with rat aortic strips in Kreb's buffer at 37°. Data are means ± SEM (N = 6–9). Key: (**) $P < 0.01$; and (***) $P < 0.001$.

[11]. The experimental tracings in Fig. 1 confirmed these reports, as $CGRP_{8-37}$ alone had no effect on phenylephrine-induced contractions. Similar results have been observed in other test systems [5, 10].

The concentration–vasodilation curve induced by NTG was shifted significantly to the right by $CGRP_{8-37}$ (by almost 10-fold), suggesting that NTG stimulates the release of CGRP, which then contributes to the overall activity of this organic nitrate. The inhibition was observed at NTG concentrations as low 15 nM (3.4 ng/mL), which are concentrations observed following therapeutic doses of NTG *in vivo* [15]. In addition to NTG, the vascular relaxation induced by PA, and to a lesser extent by SIN-1, was also inhibited by $CGRP_{8-37}$, suggesting a role for CGRP in the vasodilatory mechanism of these NO donors as well. The hypothesis was also supported by the incubation experiments of NTG or PA with aortic tissue. In these experiments, CGRP release was quantified with a specific CGRP antibody that did not cross-react with other peptides such as vasoactive intestinal peptide, substance P, or adrenomedullin. These experiments confirmed that NTG and PA could stimulate the release of significant quantities of CGRP (189.5 and 214.6%, respectively). Therefore, it seems that in addition to NO, NTG also stimulates CGRP release. CGRP may generate more NO by activating NOS III, which would further enhance cyclic GMP production and vascular smooth muscle relaxation. This pathway seems likely, because previous studies on the antiplatelet actions of NTG demonstrated a dependence of NTG on the presence of vascular tissue, CGRP, and NOS activity to mediate its actions [5]. The parallel path of CGRP-generated NO is very similar to the generally accepted direct production of NO by NTG, which may explain why this pathway has not been identified previously. Interestingly, Ahlner *et al.* [16] have suggested previously that the NTG mechanism of action consisted of two components. One of these pathways was sensitive to pertussis toxin inhibition [17], a G-protein inhibitor that is also known to inhibit CGRP [18]. Furthermore, both Wei *et al.* [19] and Fanciullacci *et al.* [20] have each independently demonstrated the phenomenon of NTG-mediated CGRP release in the cerebral circulation. The present results, in conjunction with these other findings, suggest that NTG-stimulated release of CGRP, and subsequent NO production, is likely to occur systemically in any vascular bed containing CGRP.

The release of CGRP from the vasculature is not elicited by all NO donors. Clearly, the vascular relaxations induced by SNP were unaffected by $CGRP_{8-37}$ (Fig. 3 and Table 1). This finding is consistent with the previous studies of the antiplatelet actions of SNP [10], but disagrees with those of Wei *et al.* [19] and Garry *et al.* [21]. The reason(s) for this discrepancy is unclear. Additionally, the vascular relaxations induced by the experimental NO donors SNAP, DEA-NO, DETA NONOate, and TNM were also unaffected by $CGRP_{8-37}$. The mechanisms of action of these drugs appear to be strictly mediated by NO, as previously suggested [10].

The results of these experiments provoke a number of questions. What is the source of the CGRP? What is the mechanism of CGRP release? Why do some but not other NO donors stimulate CGRP release? What is the physiological/pharmacological significance of CGRP release? With regard to the source of CGRP, these experiments did not answer that question. In the literature, there is only one report of CGRP identification in endothelial cells from human umbilical vein [22], so the endothelial cells appear to be an unlikely source. However, there are a number of reports that localize CGRP in perivascular NANC nerves of various vascular beds [23, 24]. Many of these nerves contain NOS, or are in close proximity to nerves that contain this enzyme. Therefore, it seems likely that the source of CGRP in these experiments was also the perivascular nerves.

An intriguing question is why can some NO donors stimulate CGRP release, but others apparently cannot? This issue was not resolved completely by these experiments. Among the possibilities, physicochemico differences between the NO donors may account for the difference. NTG is very lipophilic, and penetrates tissues easily, where metabolism generates NO. It is possible that NTG may generate much higher local concentrations of NO at the site of CGRP storage than SNP, which apparently generates NO extracellularly [25]. This possibility is supported by Garry *et al.* [21], who demonstrated that SNP at much higher concentrations (500 μM) could stimulate CGRP release (although so did the SNP metabolite sodium ferricyanide). However, as the *in vitro* vasodilatory EC_{50} values of NTG and SNP are similar, it can be strongly argued that

similar quantities of NO are available intracellularly, because all NO donors must provide the same of amount of NO to guanylate cyclase to produce the same amount of cyclic GMP [26].

Another alternative explanation is that a specific redox form of NO evokes CGRP release, and only certain NO donors can produce this molecule. Recent research has demonstrated that NO can also exist as different redox forms including $NO^{\cdot}$ (nitric oxide radical), NO^{-} (nitroxyl anion), and NO^{+} (nitrosonium), and it is believed that in addition to $NO^{\cdot}$, the NO donors can also generate some amount of charged NO [27]. Several groups have demonstrated that $NO^{\cdot}$ and NO^{+} can have the opposite, antagonistic effect to each other in a variety of tissue types [28]. It is possible that in the present case, a specific form of NO may be required to elicit the release of CGRP. Fukuto *et al.* [29, 30] have demonstrated that PA generates NO^{-} in addition to $NO^{\cdot}$. As both NTG and PA elicit the release of CGRP (Fig. 2, Table 1), it was hypothesized that NTG may generate NO^{-} as well, in order to release CGRP. Unfortunately, there is no pure NO^{-} donor currently available, and this hypothesis could not be tested directly. However, NO^{-} can generate a stable product, hydroxylamine, in the presence of thiols [31], which can be measured experimentally. Therefore, the hypothesis that NTG can produce NO^{-} was tested by measuring the production of hydroxylamine following incubations with aorta. In these experiments, NTG and PA alone made significant quantities of hydroxylamine, whereas none of the remaining NO donors tested differed from the control (Fig. 4). These results are encouraging, and future studies to elucidate the veracity of the hypothesis of NO^{-}-mediated CGRP release and the molecular mechanisms involved are warranted.

Finally, what is the significance of these findings? It could be argued that a 10-fold shift in the EC_{50} of NTG is not pharmacologically very important for vascular relaxation. In fact, administration of 500 pM $CGRP_{8-37}$ to anesthetized rats resulted in significant platelet inhibition (~35–40%) within minutes, without any apparent effect on mean arterial blood pressure [32]. This finding would suggest that CGRP release constitutively mediates an important effect on platelet aggregation without much effect on vasodilation. However, in the present experiments only one concentration of $CGRP_{8-37}$ (0.63 μM) was used to inhibit CGRP activity. This concentration was chosen based on the assumption that the quantity of the peptide antagonist would be much higher than the concentration of CGRP released. However, recently Edvinsson *et al.* [23] demonstrated that the CGRP concentration–response curve in human middle meningeal arteries could be shifted significantly further rightward by co-incubation with increasing concentrations of $CGRP_{8-37}$ (up to 3 μM). Therefore, it is possible that in the present experiments, the CGRP release elicited by NTG was not inhibited completely by the amount of $CGRP_{8-37}$ used in these studies.

Another consideration is the frequently made criticism that rat aorta is not an appropriate vascular site for experiments with NO donors, as NTG is considered a peripheral vasodilator. With regard to CGRP release, this may also be a valid criticism, as the aorta is not well-innervated with NANC nerves. In fact, several studies have reported that peripheral arteries and veins, including the gastrointestinal circulation, are highly innervated with CGRP-containing NANC nerves [24, 33]. Many of these arteries (mesenteric, etc.) were the tissues used by the Ahlner group and others to describe the biphasic, two-component vasodilation by NTG [16, 34]. Therefore, it is possible that the extent and action of CGRP release are much greater in peripheral vasculature, where NTG mediates its greatest systemic effects. Studies to further elucidate the nature of the NTG–CGRP interaction and the role that CGRP mediates in the cardiovascular activity of NTG and PA are needed.

In summary, NTG and PA were the only two NO donors tested that elicited the release of CGRP significantly, which subsequently contributed to the vascular relaxations induced by these NO donors. Furthermore, these two NO donors were the only NO donors of the agents tested, which produced significant quantities of hydroxylamine, a metabolite of NO^{-}. These latter results suggest that the NO^{-} redox form of NO may be responsible for CGRP release. The overall role of CGRP in NO donor-mediated vasodilation and platelet inhibition remains to be clarified.

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