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Nitroprusside Increases Cyclic Guanylate Monophosphate Concentrations during Relaxation of Rabbit Aortic Strips and Both Effects Are Antagonized by Cyanide

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The authors have confirmed previous observations that sodium cyanide (CN^-) partially reverses the vasodilator effects of sodium nitroprusside (SNP) on vascular smooth muscle. As tested on rabbit aortic strips contracted by norepinephrine (NE), the final tension is independent of the order of addition of reagents. In the same concentration, CN^- alone had no effect on tension also as reported by others. The ED_{50} values for relaxation of aortic strips for a series of directly acting agonists ("nitric oxide vasodilators") were: sodium azide (N_3^-) 2.1×10^{-7} M; SNP 2.7×10^{-7} M; hydroxylamine (H_2NOH) hydrochloride 2.5×10^{-6} M; human nitric oxide hemoglobin (HbNO) 3.5×10^{-6} M; and sodium nitrite (NO_2^-) 1.2×10^{-4} M. In addition to SNP, CN^- antagonized the vasodilator effects of N_3^- and H_2NOH, but it failed to reverse relaxation by HbNO, NO gas, NO_2^- (as observed by us), glyceryl trinitrate, adenosine, or papaverine (as observed by others). The only change noted in cyclic-adenosine monophosphate (c-AMP) concentrations in aortic strips exposed to 1) NE, 2) NE + NO_2^- or SNP, or 3) NE + NO_2^- or SNP + CN^- was an increase due to NE. The only statistically significant change noted in cyclic-guanosine monophosphate (c-GMP) concentrations exposed to 1) NE, 2) NE + NO_2^- or 3) NE + NO_2^- + CN^- was also an increase due to NE. In contrast, SNP resulted in further increases in c-GMP after NE, and when cyanide was added, a significant decrease in c-GMP followed. These results are only partially consistent with a role for c-GMP in relaxation of vascular smooth muscle, but cyanide may become a useful tool for the study of mechanisms of action of the nitric oxide vasodilators. (Key words: Arteries: aortic strips. Muscle, smooth: vascular. Pharmacology: nitroprusside. Toxicity: cyanide; nitroprusside.)

IN MOST PATIENTS, SNP is a potent, directly acting vasodilator, but a few rare individuals appear to be resistant to its hypotensive effects. Since CN^- is released from SNP *in vivo* via a non-enzymatic redox reaction with hemoglobin,[1] patients resistant to SNP are potentially at greater risk of CN^- poisoning. It has been suggested that CN^- accumulation may be responsible for the resistance to SNP in these patients by directly antagonizing its vasodilator effects. Evidence for CN^- antagonism of SNP has in fact been obtained using both the isolated, acutely denervated, perfused canine gracilis muscle[2] and isolated rabbit aortic strips.[3]

That CN^- might have effects on vascular smooth muscle is suggested by the observation that mice given $KC^{14}N$ or $KSC^{14}N$ accumulated higher levels of radioactivity in the walls of large arteries than in the blood,[4] and by the discovery that CN^- stimulates guanylate cyclase activity in a number of tissues from rats.[5] Blood vessels, however, were not tested. A considerable body of evidence, most of which was obtained using bovine coronary arteries, indicates that c-GMP is involved in drug-induced relaxation.[6] A series of directly acting agonists, sometimes referred to as the "nitric oxide vasodilators," are thought to have a common mechanism of action in activating guanylate cyclase in bovine coronaries.[7] These organic nitrites and nitrates, SNP, N_3^-, H_2NOH, and NO may act through a common intermediate in the form of S-nitrosothiols to activate guanylate cyclase and to relax vascular smooth muscle.[8] Since a common mechanism is proposed for many of these agents, it seemed advisable to investigate whether the vasodilator effects of all are antagonized by cyanide and to examine cyclic nucleotide levels in the muscle for possible correlations with mechanical effects.

Methods

New Zealand white rabbits, 2 to 4 kg, were killed by a sharp blow to the base of the skull. The thoracic aorta between the aortic arch and the diaphragm was removed, and helical strips were prepared.[9] Silk ligatures were attached to each end of segments approximately 2 cm long and 4 mm wide. Two such segments were mounted vertically on a glass rod and placed in a thermostatted tissue bath having a working volume of 50 ml. The upper ends were attached to Grass FT03 force-displacement transducers. The composition (mM) of the bathing medium was: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 19.3, glucose 11, and $CaNa_2EDTA$ 0.026. When aerated with a gas mixture of 95% O_2 and 5% CO_2, the *p*H was 7.4 at 37° C.

Each strip was incubated at a resting tension of 1 g until the tension became constant (at least 1 h). An isometric, cumulative dose-response recording was then traced using either NE (as the bitartrate) or methoxamine hydrochloride as the vasoconstrictor agent. This

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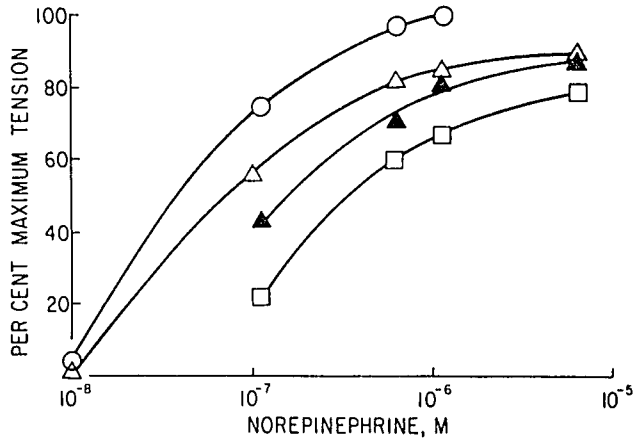

FIG. 1. Dose-response curves for NE-induced contraction of rabbit aortic drtips: O = NE alone; □ = NE in the presence of a constant 1×10^{-6} M concentration of SNP; △ = NE in the presence of a constant 1×10^{-6} M concentration of SNP plus 1.4×10^{-5} M CN^-; and ▲ = NE in the presence of a constant 1×10^{-6} M concentration of SNP plus 2×10^{-5} M sodium sulfide. Each curve is the mean of either two (△, ▲) or four (O, □) complete experiments. The standard deviations fell within 2 to 5 percentage units on the ordinate.

procedure also was repeated at the end of every series of experiments. In one type of experiment, dose-response curves with the vasoconstrictor agent were constructed in the presence of a constant concentration of a vasodilator agent with and without CN^-. In the other type of experiment, the strips were exposed to a constant, submaximal concentration of vasoconstrictor agent and then the vasodilator agent and cyanide were added sequentially. The effects of sodium sulfide ($Na_2S \cdot 9H_2O$) and of sodium thiocyanate (NaSCN) were compared with those of CN^- under the same conditions. Between experiments, the strips were washed at 10-min intervals until a constant tension was reached (at least 1 h), and the tension was then readjusted to 1 g. Responses were recorded with a Grass Model 5 polygraph.

The cyclic nucleotides were determined by radioimmunoassay using kits obtained from Collaborative Research, Inc. and the basic procedure of Brooker *et al.*[10] Rabbit aortic strips as described above were suspended by stainless steel clips in the tissue baths, but not mounted under tension. The bathing fluid was changed every 30 min during a 2-h equilibration period. The times at which strips were removed for analyses after the addition of various agents coincided with a steady-state mechanical response as determined above, and are indicated in the footnote for table 3. At those times strips were removed from the bath and rapidly frozen between blocks of dry ice.[11]

Frozen strips were put in cold 6% trichloroacetic acid and homogenized using first a Polytron and then glass Duall tissue grinders. The sample tubes were constantly kept in an ice bath. The homogenates were centrifuged at 3,000 *g* for 15 min in a refrigerated centrifuge. Supernatant fractions were extracted four times with water-saturated ether and lyophilized. The precipitates were assayed for total protein.[12]

The lyophilized residues were dissolved in 50 mM sodium acetate buffer, pH 6.2, and aliquots of this solution were taken for radioimmunoassay. The kits were used as directed, and acetylation of the samples was required for c-GMP but not c-AMP. Recoveries were checked by adding tritiated cyclic nucleotides to the original homogenates.[11] The validity of the cyclic nucleotide assays was tested by making a tissue extract dilution curve and also by spiking with known amounts of cyclic nucleotides. The specificity of the assay was verified by adding phosphodiesterase to some sample tubes.[13] Statistical comparisons were by conventional Student's unpaired *t* tests. A probability level of less than 0.05 was accepted as significant.

Results

Our confirmation and extension of the findings of Grayling *et al.*[3] is shown in figure 1 where the vasodilator effects of SNP on rabbit aortic strips contracted by various concentrations of NE are at least partially antagonized by cyanide. We have extended their findings to show that the same is true for a similar concentration of sodium sulfide. Other observations of Grayling *et al.*[3] which we have confirmed are: 1) cyanide alone, even in

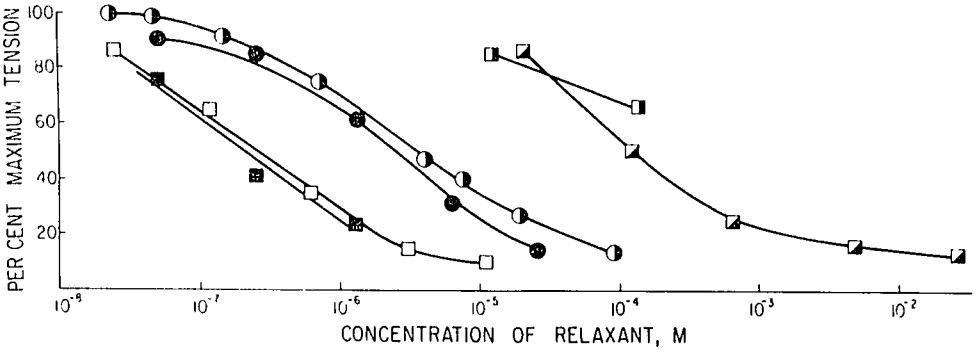

FIG. 2. Dose-response curves for vasodilator agonists on rabbit aortic strips contracted in the constant presence of 1×10^{-6} M NE: □ = N_3^-; □ = SNP; ● = H_2NOH; ◑ = HbNO; ◨ = NO (concentrations are only approximate) and ◪ = NO_2^-. Each curve is the mean of two to 10 complete experiments. The standard deviations fell within 2 to 5 percentage units on the ordinate.

much higher concentrations, had no effect on a NE-contracted strip except at concentrations above 1×10^{-4} M, where a very slow loss in tension occurred, which we interpreted as a nonspecific poisoning of the preparation secondary to inhibition of cytochrome oxidase; 2) the order of the addition of the reagents had no significant effect on the final tension; 3) the effect is completely reversible with washing; 4) cyanide did not reverse the vasodilator effects of papaverine; and 5) if lower concentrations of SNP were used, *e.g.*, 1×10^{-7} M, the vasodilator effect could be antagonized by a lower concentration of CN^-, *e.g.*, 1×10^{-6}, but the antagonism was never complete.

In our hands, thiocyanate, the chief metabolic product of CN^- *in vivo*, had no effect on rabbit aortic strips in concentrations up to 1×10^{-3} M. At concentrations of 5×10^{-3} and higher, thiocyanate appeared to potentiate slightly the vasoconstrictor effect of NE (data not shown).

Dose-response curves for a variety of so-called nitric oxide vasodilators on rabbit aortic strips contracted in the constant presence of 1×10^{-6} M NE are shown in figure 2. Clearly, SNP and N_3^- are the most potent of the agents tested. Human HbNO and H_2NOH were about equipotent, and NO_2^- was a very weak agonist. The concentrations shown for NO gas are only approximations based on solubility. The actual numerical values for the ED_{50} values for those agents in which we can have confidence about their concentrations in the bath are listed in table 1.

Each of these vasodilator agents was then tested as shown in table 2 to see if their effects could be reversed by CN^-. In addition to SNP, the vasodilator effects of N_3^- and of H_2NOH were partially antagonized by CN^-, whereas the vasodilator effects of HbNO, NO gas, and NO_2^- were not antagonized by CN^-.

We then selected SNP as an example of an agent where the vasodilator effect is antagonized by CN^- and NO_2^- as an example of an agent where the vasodilator effect is not antagonized by CN^-. Results of cyclic nucleotide assays in aortic strips exposed sequentially to NE, to vasodilator, and to CN^- are summarized in tables 3 and 4. In table 3, it can be seen that NE consistently produced an increase in the c-AMP content of rabbit aorta. There were no further changes with either NO_2^- or SNP with or without CN^-.

In table 4 it can be seen that NE also consistently produced an increase in the c-GMP content of rabbit aortic strips. When NO_2^- was added, there was a further increase in c-GMP which was very close to statistical significance ($P = 0.06$). The subsequent addition of CN^- produced no further change in c-GMP. In contrast, SNP produced a statistically significant increase in c-GMP over and above the NE effect. When CN^- was then added, the c-GMP content fell to a value not significantly different from that produced by NE alone. The decrease in c-GMP produced by cyanide was statistically significant. Thus, the mechanical effects and the c-GMP content are correlated in the case of SNP and CN^- in such a way as to suggest that c-GMP has a role in vasodilation.

TABLE 1. The ED_{50} Values for Nitric Oxide Vasodilators on Rabbit Aortic Strips Contracted by NE

Agonist	ED_{50} (M)
N_3^-	2.1×10^{-7}
SNP	2.7×10^{-7}
H_2NOH	2.5×10^{-6}
HbNO	3.5×10^{-6}
NO_2^-	1.2×10^{-4}

Discussion

Results summarized here at least partially support the hypothesis of a role for c-GMP in the relaxation of vascular smooth muscle.[7] Both SNP and NO_2^- increased c-GMP in rabbit aorta at concentrations in which they had clear-cut vasodilator effects. The increase after NO_2^-, however, just escaped the usually accepted level of statistical significance. It has been suggested that the much less potent vasodilator effects of NO_2^- *vs.* SNP (*e.g.*, fig. 2) might be due to a greater lipophilicity of SNP.[8] If so, such a difference in lipophilicity might also account for the weaker effect of NO_2^- on the c-GMP content of the aorta as well. When CN^- reversed the vasodilator effect of SNP, there was a corresponding fall in the c-GMP content of the aortic strip, whereas CN^- failed to alter the mechanical response to NO_2^- and to alter the c-GMP levels. At least to this extent there was a certain concordance between the mechanical responses and the c-GMP concentrations.

TABLE 2. Concentrations at Which Agonists Were Tested for Cyanide Reversal of Their Vasodilator Effects

	Agonists Reversed by Cyanide		
Agonist	Concentration (M)	Cyanide (M)	Vasoconstrictor (M)*
N_3^-	1.0×10^{-6} 1.3×10^{-6}	2.0×10^{-4} $1\text{-}6 \times 10^{-4}$	1.0×10^{-7} 1.0×10^{-6}
SNP	1.0×10^{-6} 1.0×10^{-6} 3.0×10^{-6}	4.0×10^{-5} 4.0×10^{-5} 1.0×10^{-4}	1.0×10^{-7} 1.7×10^{-6}* 1.0×10^{-6}
H_2NOH	2.6×10^{-5}	$1\text{-}6 \times 10^{-4}$	1.0×10^{-6}
	Agonists Not Reversed by Cyanide		
HbNO	1.2×10^{-5}	2.4×10^{-4}	1.0×10^{-6}
NO	1.2×10^{-4}†	2.0×10^{-4}	1.0×10^{-6}
NO_2^-	1.0×10^{-4}	2.0×10^{-4}	1.0×10^{-7}

* Methoxamine; NE in all other cases.
† Approximate concentration only.

TABLE 3. Effects of Various Agonist and Antagonist Drugs on the c-AMP Content of Rabbit Aortic Strips* (Means ± SD)

	c-AMP (picomol/mg protein)		
	NE	NE + NO_2^-	NE + NO_2^- + CN^-
Control 3.9 ± 1.8 (N = 6)	6.2 ± 2.6†	6.9 ± 3.6†	7.0 ± 5.6†
	NE	NE + SNP	NE + SNP + CN^-
Control 2.6 ± 1.2 (N = 5)	5.1 ± 2.9†	4.8 ± 2.0†	4.1 ± 1.6†

* Concentrations of reagents were: NE 1×10^{-7} M (about 2/3 maximal contraction), NO_2^-; 1×10^{-4} M (about 1/2 relaxation of a maximally contracted strip), CN^- 4×10^{-5} M, SNP 1×10^{-6} M (about 1/2 relaxation of a maximally contracted strip). The times selected for cyclic nucleotide analyses were times at which the mechanical effects appeared to be approaching a steady state, namely, 5 min after NE, 6 min after NO_2^-, 3 min after SNP, and 2 min after CN^-. Thus, the total duration of a NO_2^- experiment was 13 min and for an SNP experiment it was 10 min.

† Significantly different from control ($P < 0.05$) as evaluated by unpaired samples Student's t test.

It is much more difficult to fit the observation that NE increased the concentrations of both c-AMP and c-GMP while contracting aortic strips into this hypothesis. Perhaps certain basal concentrations of both cyclic nucleotides are essential before further changes can occur. Although it is said to be sparse, it may be significant that the innervation of the aorta, like that of most vascular smooth muscle, is exclusively sympathetic.[14,15] Since NE is the only neurotransmitter involved in the maintenance of vascular smooth muscle tone, it may be able to regulate the concentrations of both cyclic nucleotides. With respect to circulating hormones or neurotransmitters, we have confirmed the observation[16] that rabbit aortic strips are relaxed by low concentrations of acetylcholine, but they are contracted by higher concentrations. Furthermore, we have confirmed the finding that relaxation by acetylcholine required the presence of endothelial cells.[16] Thus, with respect to some agents, rabbit aortic strips exhibit responses which are unusual if not unique. In our experiments, endothelial cells were not required for the nitric oxide vasodilators to be active.

TABLE 4. Effects of Various Agonist and Antagonist Drugs on the c-GMP Content of Rabbit Aortic Strips.* Values are means ± SD

	c-GMP, (femtomol/mg protein)		
	NE	NE + NO_2^-	NE + NO_2^- + CN^-
Control 112 ± 64 (N = 5)	218 ± 56†	346 ± 151†	356 ± 239†
	NE	NE + SNP	NE + SNP + CN^-
Control 152 ± 33 (N = 10)	242 ± 29†	719 ± 115‡	261 ± 38‡

* See table 3 for concentrations of reagents and times of analyses.

† Significantly different from control ($P < 0.05$).

‡ Significantly different from control and from the value to the immediate left ($P < 0.05$).

It seems unlikely that the effect of CN^- reported here is due to a poisoning of the aortic strip secondary to an inhibition of cytochrome oxidase. Sulfide, CN^- and N_3^- are all recognized inhibitors of cytochrome oxidase, although N_3^- appears to act by a different mechanism and is the least potent by a significant margin.[17] Under certain conditions, NO_2^-, and perhaps H_2NOH and NO, are also said to inhibit cytochrome oxidase.[18] Sulfide, CN^-, N_3^-, and NO_2^- all bind to methemoglobin heme,[19,20] whereas NO is bound by both hemoglobin and methemoglobin.[8] Among the agents tested here, SNP, H_2NOH, and NO_2^- have in common the ability to participate in certain redox reactions such as the oxidation of hemoglobin to methemoglobin.[1,21] Finally, there is evidence for the participation of a heme complex in the activation of guanylate cyclase.[22] Despite these tantalyzing similarities in biochemical effects and chemical properties, no single hypothesis occurs to us which fits all the observations reported here.

The observation that CN^- reverses the vasodilator effects of N_3^-, SNP, and H_2NOH, but not that of NO_2^-, NO, or HbNO suggests to us that there are at least two different mechanisms of action for the nitric oxide group of vasodilators. This specificity of CN^- for only certain vasodilators, the promptness of the response when it is effective, and the absence of any response when it is used alone in the same concentrations, all further support the concept that some mechanism is at work other than inhibition of cytochrome oxidase and a nonspecific histotoxic poisoning of the tissue. Indeed, when the CN^- concentration was increased, a slow loss of tension occurred over several hours in a NE-contracted strip, and this effect was interpreted as being secondary to the well-known disruption by CN^- of aerobic metabolism.

In addition to CN^-, methylene blue and methemoglobin have effects on c-GMP accumulation or mechanical relaxation or both after exposure of bovine coronary arterial strips to various nitric oxide vasodilators.[7] Methylene blue blocked both effects after NO, SNP, NO_2^-, and glyceryl trinitrate. In contrast, methemoglobin blocked both effects after NO, but did not alter the responses to

SNP, NO_2^-, or glyceryl trinitrate. Obviously, cyanide must be acting by a mechanism that is different from the other two agents, or differences in the two vascular smooth muscles or in the two species studied play important roles.

The original studies[2,3] on CN^- antagonism of the vasodilator effects of SNP were undertaken with the hypothesis that CN^- accumulation secondary to SNP decomposition *in vivo* might account for the resistance of some patients to SNP. In patients where the desired hypotensive level was not readily achieved, the tendency was to increase the dose-rate of SNP leading to further CN^- accumulation in a dangerous cycle. In attempting to compare the concentrations of CN^- used in these studies with those reported in patients receiving SNP, two considerations are critical. First, the comparisons should be based on human plasma concentrations of CN^- and not whole blood. More than 90% of the whole blood CN^- is found within red cells, presumably in an inactive form. Certainly this intracellular CN^- is not available to interact with SNP on vascular smooth muscle. Vesey *et al.*[23] estimate the lethal plasma level of CN^- in an otherwise healthy human adult as 10 to 20 $\times$ 10^{-6} M. Thus, many of the experiments reported here (tables 2, 3, and 4, and fig. 1) were conducted at potentially lethal plasma concentrations of CN^-. The second consideration, however, is that the ratio of the CN^- concentration to that of SNP is as important as the absolute CN^- concentration. As noted above, clear-cut vasodilator effects of SNP *in vitro* at 1×10^{-7} M are antagonized by 1×10^{-6} M CN^-, which is an order of magnitude under the lethal plasma level. We are not aware of data on plasma concentrations of SNP in effective doses in humans which would address this question. In baboons given brief infusions of SNP, the peak SNP concentration was 15×10^{-6} M and the peak CN^- was about 6×10^{-6} M. When the infusion was stopped, however, the plasma CN^- concentration fell more slowly than the SNP concentration, and eventually the CN^- concentration became greater than that of SNP.[24]

The relative concentrations of CN^- and SNP in patients are determined by a complex series of pharmacokinetic processes including the rate of administration of SNP, the rate of SNP decomposition to yield CN^-, and the rate of conversion of CN^- to thiocyanate by rhodanese.[25] It is certainly relevant that each equivalent of SNP breaks down to yield at least four equivalents of CN^-[1]. The latter circumstance suggests that under some conditions the plasma CN^- concentration might exceed the plasma SNP concentration and lead to apparent resistance, particularly if the patient is in some way compromised with respect to cyanide detoxication. Oliguria or anuria, however, does not increase the risk of CN^- toxicity,[26] so that any hypothetical compromise must relate to the rhodanese reaction.

As noted above, CN^- antagonism of SNP vasodilation is never complete, so that another explanation must be sought for the frank hypertensive responses observed in some resistant patients. A possible explanation is that CN^- itself evokes a generalized sympathetic discharge as part of the reflex response secondary to activation of the carotid body chemoreceptors.[27]

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Erratum

There was an error in reference 14 of the article, "Precipitation of Local Anesthetic Drugs in Cerebrospinal Fluid," which was published in the August 1982 issue of ANESTHESIOLOGY.

The manuscript, "Morphological Effects of Etidocaine HCl on the Spinal Cord of Sheep," was published in *Pharmacological Research Communications* 14:533–540, 1982.