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Involvement of nitroxyl (HNO) in the cyanamide-induced vasorelaxation of rabbit aorta

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Abstract—Relaxation of precontracted rabbit aortic rings *in vitro* by cyanamide, a clinically used alcohol deterrent drug, required catalase and H_2O_2, suggesting that a bioactivation mechanism was involved. Since the oxidation of cyanamide by catalase/H_2O_2 had been shown previously to lead to nitroxyl (HNO) generation via the intermediate *N*-hydroxycyanamide, and aortic ring relaxation was inhibited by the catalase inhibitor, 3-aminotriazole, HNO appears to be responsible for the vasorelaxation mediated by cyanamide. This was further supported by the observation that *N,O*-dibenzoyl-*N*-hydroxycyanamide (DBHC), a derivative of *N*-hydroxycyanamide that releases HNO in the *absence* of catalase/H_2O_2, was a potent vasorelaxant, with an EC_{50} of $4.2 \pm 1.3 \times 10^{-6}$ M.

Key words: nitroxyl; cyanamide; vasorelaxation; catalase; aldehyde dehydrogenase; cGMP

Cyanamide (H_2NCN) is currently used as an alcohol deterrent drug in Europe, Canada and Japan. Administration of cyanamide results in a drug–ethanol reaction manifested by the inhibition of the enzyme aldehyde dehydrogenase (EC 1.2.1.3; AlDH2), thereby leading to alcohol avoidance [1]. Cyanamide itself is inactive *in vitro* and must be bioactivated before inhibition of aldehyde dehydrogenase is observed [2, 3]. It has been deduced that cyanamide is oxidized by the catalase/hydrogen peroxide (H_2O_2) system to generate an *N*-hydroxycyanamide intermediate which spontaneously decomposes to give nitroxyl (HNO)* and hydrogen cyanide [4–6]. HNO has been postulated to be the species responsible for the inhibition of aldehyde dehydrogenase, since other HNO-generating substances were also found to be potent inhibitors of this enzyme [7–9].

In previous studies unrelated to the above, it was found that HNO generated from other chemical compounds of diverse structures is capable of eliciting smooth muscle relaxation by increasing cGMP levels, presumably by activating guanylate cyclase [10]. Since cyanamide has been shown to release HNO (*vide supra*), it would be expected, *a priori*, to cause vasorelaxation as well. Recent results indicated that cyanamide is indeed capable of eliciting the vasorelaxation of bovine intrapulmonary artery *in vitro*, albeit, at a somewhat high concentration [10]. However, the mechanism by which cyanamide caused vasorelaxation was not investigated thoroughly in that study. For example, the role of catalase and/or the requirement for bioactivation of cyanamide was not specifically addressed, as was demonstrated for aldehyde dehydrogenase inhibition by this compound. Herein, we report that the mechanism of cyanamide-induced vasorelaxation is likely the result of HNO generation in the oxidative metabolism of cyanamide by catalase/H_2O_2.

Materials and Methods

Chemicals and solutions. Cyanamide was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Hydrogen peroxide (30%) and catalase were purchased from the Sigma Chemical Co. (St. Louis, MO). Phenylephrine and superoxide dismutase were purchased from DDI Pharmaceuticals Inc. (Mountain View, CA). DBHC was synthesized as previously described [7]. Krebs-bicarbonate solution consisted of (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 1.5; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; glucose, 11; and disodium ethylenediaminetetraacetic acid, 0.023. All other chemicals were purchased from commercial sources and were of the highest purity available.

Biological assay. Rabbit aortic rings were prepared and the changes in isometric force were measured as previously described [11]. Briefly, New Zealand white male rabbits (2.5 to 3.0 kg) were killed by lethal injection (50 mg/kg pentobarbital). The thoracic aorta was then removed, cleaned of connective tissue, and cut into 3 mm rings. The rings were mounted under 1 to 1.5 g of tension in a 25-mL tissue bath in oxygenated Krebs solution. After 1–2 hr of equilibration, the tissues were precontracted submaximally with phenylephrine prior to the addition of the test substance. DBHC had only limited solubility in water and had to be dissolved in a methanol–water mixture prior to addition to the tissue bath. Control experiments with the same vehicle were therefore performed to assure that the biological responses were not due to methanol. The activity of cyanamide alone was tested over the concentration range (final concentration in the bath) of 1×10^{-7} to 1×10^{-2} M by sequential addition of concentrated stock solutions to phenylephrine-precontracted tissues. The activity of cyanamide was then retested in the presence of hydrogen peroxide. Hydrogen peroxide was added to the tissue bath after precontraction with phenylephrine to a final concentration of 1×10^{-5} M, and a concentration–response curve was generated for cyanamide as before. Cyanamide activity was also evaluated in the presence of H_2O_2 and catalase (100 U/mL). Catalase and H_2O_2 (1×10^{-5} M final concentration) were added to the baths after precontraction with phenylephrine and, again, a concentration–response curve for cyanamide in this system was generated. Inhibition of cyanamide-elicited vasorelaxation by 3-AT was tested by adding 3-AT (1×10^{-2} M final bath concentration), catalase (100 U/mL) and H_2O_2 (1×10^{-5} M) to the tissue bath. After phenylephrine precontraction *without* cyanamide, another concentration–response curve for cyanamide was determined. Without cyanamide, the addition of 3-AT, catalase and H_2O_2 had no effect on the tissues.

Results

Cyanamide alone was unable to elicit significant vasorelaxation of rabbit thoracic aorta at concentrations as high as 1×10^{-2} M. Only up to 25% relaxation was ever observed at this high concentration. In the presence of H_2O_2, cyanamide was found to be a slightly more viable

* Abbreviations: HNO, nitroxyl; DBHC, *N,O*-dibenzoyl-*N*-hydroxycyanamide; and 3-AT, 3-aminotriazole.

Table 1. Relaxation of rabbit thoracic aorta *in vitro* by cyanamide and DBHC

Compound/Conditions*	EC_{50}†	N‡
Cyanamide alone	>1 M§	12
Cyanamide + H_2O_2	5×10^{-1} M‖	9
Cyanamide + H_2O_2 + catalase	$2.6 \pm 0.5 \times 10^{-4}$ M	4
Cyanamide + H_2O_2 + catalase + 3-AT	$4.8 \pm 2.0 \times 10^{-2}$ M	4
DBHC	$4.2 \pm 1.3 \times 10^{-6}$ M	10

* See Materials and Methods for specific incubation conditions and concentrations used.

† Unless stated otherwise, values are means ± SEM.

‡ Represents number of individual tissue bath experiments.

§ A >25% relaxation of the tissues was never observed even at the highest concentration used (0.001 M).

‖ This value is very approximate since >50% relaxation was never obtained at even the highest cyanamide concentration.

vasorelaxant. Thus, by the addition of H_2O_2 at a concentration of 1×10^{-5} M, as much as 50% relaxation was observed for a 1×10^{-2} M concentration of cyanamide. Although H_2O_2 itself is a vasorelaxant at high concentrations (EC_{50} of approximately 1×10^{-3} M), the concentration of H_2O_2 used here (1×10^{-5} M) did not cause any significant vasorelaxation. When catalase was added to the tissue baths at a concentration of 100 U/mL, the potency of cyanamide was increased dramatically. The EC_{50} for cyanamide in the presence of both H_2O_2 and catalase was found to be 2.6×10^{-4} M. The catalase inhibitor, 3-AT, was found to substantially inhibit this cyanamide response to added H_2O_2 and catalase, the EC_{50} for cyanamide being raised approximately 180-fold in the presence of 3-AT ($EC_{50} = 4.8 \times 10^{-2}$ M). These data are summarized in Table 1.

The above data are consistent with the postulate that cyanamide was oxidized by a catalase/H_2O_2-dependent reaction to generate a vasorelaxing species. To further test the hypothesis that an *N*-hydroxycyanamide intermediate may be involved in the generation of the vasorelaxant, DBHC was tested as a vasorelaxant, since *N*-hydroxycyanamide itself is unstable and cannot be prepared chemically [5]. In accord with expectations, DBHC was found to be a potent vasorelaxant in the *absence* of either catalase or H_2O_2, with an EC_{50} of 4.2×10^{-6} M (Table 1).

Discussion

The results presented here support the hypothesis that cyanamide is metabolized by a catalase/H_2O_2-mediated oxidation process to generate a vasoactive entity. A previous study using ^{15}N- and ^{13}C-labeled cyanamide provided evidence indicating that the metabolism of cyanamide by catalase/H_2O_2 results in HNO generation [6]. Also, since previous reports indicated that other, structurally unrelated HNO precursors were potent vasorelaxants [10], it is likely that HNO is the entity generated from cyanamide oxidation which is responsible for the observed vasorelaxation. The participation of catalase in the bioactivation of cyanamide was substantiated by the observations that (a) the potency of cyanamide was increased in the presence of added catalase, and (b) cyanamide potency was attenuated markedly by addition of the catalase inhibitor 3-AT. It should be recognized, however, that inhibition of cyanamide activation does not, *a priori*, indicate that HNO was the vasorelaxant species, as other potential mediators may be generated.

The mechanism of HNO generation from cyanamide has been proposed to occur through initial formation of an *N*-hydroxycyanamide intermediate by a catalase-mediated oxidation [5, 6]. Spontaneous chemical decomposition of the N-hydroxylated intermediate releases HNO. The intermediacy of *N*-hydroxycyanamide would be persuasive if this compound could be tested directly for its ability both to release HNO and to elicit vasorelaxation. Unfortunately, the inherent instability of this compound precluded its chemical synthesis. However, a derivative of *N*-hydroxycyanamide was able to be synthesized [7] and this *N,O*-dibenzoylated derivative of *N*-hydroxycyanamide, viz. DBHC, was found to be a potent vasorelaxant (Table 1). The release of HNO from this species has previously been demonstrated indirectly via the measurement and detection

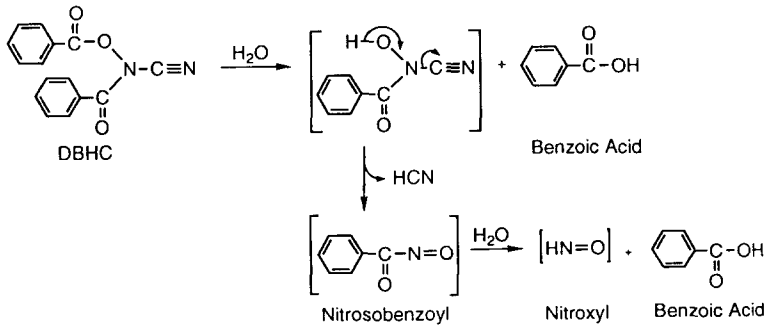

Fig. 1. Proposed mechanism of HNO release from DBHC [7].

of its stable decomposition product, N_2O [7]. Briefly, DBHC can be hydrolyzed non-enzymatically at the labile benzoate ester to give an *N*-hydroxy-*N*-cyano intermediate, followed by spontaneous cyanide release to generate a nitrosobenzoyl species. Further hydrolysis of this latter intermediate results in the generation of HNO and benzoic acid [7] (Fig. 1). This mechanism is related to the proposed mechanism of cyanamide bioactivation such that both have, as a crucial step, the expulsion of cyanide from an *N*-hydroxy intermediate. This step has some precedent in that other *N*-hydroxy compounds with an appropriately situated leaving group other than cyanide were also able to expel the leaving group with concomitant formation of an acylnitroso intermediate that hydrolyzed to HNO [8, 9].

These results support our postulate that cyanamide is metabolized to a vasoactive species—very likely HNO—by a catalase/H_2O_2-dependent process, and provide further evidence that HNO has vasoactive properties. Also, based on the relative lack of potency of cyanamide *in vitro* without added catalase to the aortic rings in the tissue bath, it does not appear likely that cyanamide will cause significant vasodilatation in *in vivo* systems, especially if the levels of catalase present in vascular tissues are low. In addition, the metabolism of cyanamide by the liver by an alternative major pathway, viz. by *N*-acetylation, is expected to be substantial *in vivo*, [12], thus lowering the circulating levels of free cyanamide. Accordingly, derivatives of *preformed* *N*-hydroxycyanamide are likely to be substantially more potent *in vivo*, although concomitant release of cyanide ion is undesirable. In this regard, other compounds that release HNO (i.e. prodrugs of HNO) that do not give rise to cyanide may have significantly greatly greater potential as pharmacologically useful vasorelaxants [13].

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