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Involvement of Nitric Oxide in Nitroprusside-Induced Hepatocyte Cytotoxicity

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ABSTRACT. Sodium nitroprusside (SNP) cytotoxicity towards rat hepatocytes was accompanied by peroxynitrite formation, lipid peroxidation, inhibition of glycolysis, cyanide (CN) release, partial inhibition of hepatocyte respiration, and ATP depletion. Antioxidants and desferoxamine prevented both cytotoxicity and lipid peroxidation induced by SNP. The CN antidote thiosulfate or the CN trapping agents dihydroxyacetone and glyceraldehyde increased SNP metabolism, SNP-induced peroxynitrite formation, cytotoxicity, and lipid peroxidation. On the other hand, addition of non-toxic concentrations of CN to hepatocytes prevented SNP metabolism and SNP-induced lipid peroxidation and cytotoxicity. SNP depleted hepatocyte GSH immediately upon addition, and GSH-depleted hepatocytes were more susceptible to SNP. The results of this study suggest that nitric oxide rather than CN mediates SNP cytotoxicity in isolated cells. BIOCHEM PHARMACOL 51;8:1031–1039, 1996.

KEY WORDS. nitroprusside; nitric oxide; cyanide; hepatocytes; cytotoxicity

SNP† is a potent hypotensive agent widely used to control hypertension during surgery (especially heart bypasses), in hypertension emergencies, and to improve heart function after myocardial infarction [1, 2]. The use of SNP resulted in deaths in some cases possibly due to CN released from SNP in the body [3–5], but this has been disputed [6, 7]. However, it is generally agreed that SNP undergoes biotransformation *in vivo* to release CN [8–13]. Infusion of the CN antidote thiosulfate has been shown to lower the blood CN level in patients treated with SNP [5, 11, 14] and to protect against CN toxicity following acute or chronic SNP administration to dogs [9, 15]. Thiosulfate and the CN trap α-ketoglutarate have also been reported to antagonize the lethality of SNP given subcutaneously to mice [16].

The vasodilating effect of SNP is believed to be due to its ability to release its NO next to the vascular wall. The mechanism of NO liberation from SNP is not precisely known. While some investigators believe that release of NO is spontaneous [17, 18], others believe that NO is released from SNP following one-electron reduction [19–22]. It has been reported that the release of NO from SNP was blocked by CN in vascular tissue [19].

We have shown previously that SNP readily undergoes one-electron reductive metabolism by isolated hepatocytes to the metal-nitrosyl radical [20], but the cytotoxic mechanism was not investigated. Recently, we have shown that butyl nitrite and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) cause cytotoxicity towards hepatocytes as a result of NO formation [23, 24]. Furthermore, focal necrotic lesions in the rat hippocampus following SNP intracerebral injection have been attributed to NO [25] rather than CN formation.

Although there have been no clinical reports of SNP-induced hepatotoxicity, SNP is metabolized by the liver [12, 20]. We have used isolated rat hepatocytes as a model cell to study the intracellular mechanisms of metabolic activation and the cytotoxicity of SNP. It was found that cytotoxicity was associated with peroxynitrite formation and lipid peroxidation. CN trapping compounds or sodium thiosulfate increased while CN prevented SNP-induced cytotoxicity, lipid peroxidation, and peroxynitrite formation. These results suggest that NO rather than CN is the cytotoxic metabolite of SNP in isolated rat hepatocytes.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (300–350 g), fed a standard chow diet and water *ad lib.*, were used in all experiments.

Chemicals

Collagenase (from *Clostridium histoliticum*) and HEPES were purchased from Boehringer-Mannheim (Montreal, Canada). Dihydroxyacetone, glyceraldehyde, trypan blue, thiobarbituric acid, quercetin, morin, fluoro-2,4-dinitrobenzene, and iodoacetic acid were obtained from the Sigma

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† *Abbreviations:* SNP, sodium nitroprusside; CN, cyanide; NO, nitric oxide; GSH, reduced glutathione; and GSSG, oxidized glutathione.

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Chemical Co. (St. Louis, MO, U.S.A.). SNP, sodium thiosulfate, and potassium cyanide were purchased from the Fisher Scientific Co. (Mississauga, ON, Canada). Dithiothreitol (DTT), 1-bromoheptane, purpurogallin, and pyrogallol were obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Desferoxamine was a gift from Ciba Geigy Ltd. (Mississauga, ON, Canada). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (carboxy-PTIO) was purchased from the Calbiochem-Novabiochem Corp. (La Jolla, CA, U.S.A.). Other chemicals were of the highest commercial grade available.

Isolation and Incubation of Hepatocytes

Hepatocytes were isolated by collagenase perfusion of the liver as described by Moldeus *et al.* [26]. Approximately 85–90% of the freshly isolated hepatocytes excluded trypan blue (0.2%, w/v). Cells were incubated at a concentration of 10^6 cells/mL in Krebs-Henseleit buffer containing 12.5 mM HEPES under an atmosphere of 95% O_2 and 5% CO_2 at 37° in continuously rotating round-bottomed 50-mL flasks. GSH-depleted hepatocytes were obtained by preincubating hepatocytes with 0.2 mM 1-bromoheptane for 20 min [27].

Cell Viability

Hepatocyte viability was assessed by plasma membrane disruption as determined by trypan blue uptake [26], as well as by lactate dehydrogenase release. Viability was determined immediately after isolation and at different time points following incubation.

Glutathione Determination

The total GSH and GSSG contents of hepatocytes were measured, on deproteinated samples (5% metaphosphoric acid), after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene, by high performance liquid chromatography, using a C18 μBondapak NH_2 column (Waters Associates, Milford, MA, U.S.A.) [28]. GSH and GSSG were used as external standards. A Waters 6000A solvent delivery system equipped with a model 600 solvent programmer, a Wisp 710 automatic injector, and a Data Module were used for analysis.

Determination of Malondialdehyde in Hepatocytes

Malondialdehyde was determined by treating 1-mL aliquots of hepatocytes with 0.25 mL of 70% trichloroacetic acid and 1 mL of 0.8% thiobarbituric acid and heating for 20 min. Butylated hydroxytoluene (10 μL of 1 M in DMSO) was added to prevent oxidation that may occur in the heating stage of the assay. Samples were monitored spectrophotometrically at 535 nm after centrifugation [29].

Hepatocyte Respiration

Hepatocyte respiration was measured at different time points following the addition of SNP to hepatocytes using a Clark-type oxygen electrode (model 5300; Yellow Spring Instrument Co. Inc., Yellow Spring, OH, U.S.A.) in a 2-mL incubation chamber maintained at 37°. Prior to oxygen consumption measurements, hepatocytes (10^6 cells/mL) were kept at 37° in Krebs-Henseleit buffer, plus HEPES (12.5 mM), pH 7.4, under 95% oxygen and 5% CO_2.

Determination of Peroxynitrite Formation

Peroxynitrite formation was determined by luminol chemiluminescence as previously described [30]. Chemilu-

TABLE 1. Effect of removing SNP from the medium or addition of CN or CN trapping agents to the incubation on SNP cytotoxicity

	Cytotoxicity (% trypan blue uptake)			
Addition	**30 min**	**60 min**	**120 min**	**180 min**
None	14 ± 2	15 ± 2	17 ± 2	19 ± 2
CN 0.4 mM	15 ± 2	17 ± 2	20 ± 2	23 ± 2
SNP 2 mM	17 ± 2	31 ± 3	48 ± 4*	72 ± 4*
+ CN 0.4 mM at 0 min	16 ± 2	19 ± 2	24 ± 3†	28 ± 3†
+ CN 0.4 mM at 15 min	17 ± 2	20 ± 2	26 ± 3†	32 ± 3†
+ CN 0.4 mM at 30 min	15 ± 2	30 ± 3	45 ± 3	69 ± 4
+ Thiosulfate 10 mM	22 ± 3	40 ± 3	91 ± 5†	100†
+ Dihydroxyacetone 10 mM	24 ± 3	45 ± 4	87 ± 5†	100†
+ Glyceraldehyde 10 mM	23 ± 2	47 ± 3	90 ± 5†	100†
+ Acetaldehyde 10 mM	20 ± 2	37 ± 3	67 ± 4	95 ± 5†
SNP 2 mM (resuspended at 5 min)	21 ± 2	24 ± 2	28 ± 3†	30 ± 3†
SNP 2 mM (resuspended at 15 min)	23 ± 2	32 ± 3	37 ± 3†	50 ± 4†
SNP 2 mM (resuspended at 30 min)	18 ± 2	34 ± 3	45 ± 3	64 ± 4

Hepatocytes (10^6 cells/mL), freshly isolated from normal rats, were incubated in Krebs-Henseleit buffer, pH 7.4. SNP and CN trapping agents were added to hepatocytes at the same time. CN was added at different time points after SNP addition. To remove SNP from the medium, hepatocytes were centrifuged at determined time points and resuspended in fresh Krebs-Henseleit buffer. Cytotoxicity was determined as the percent of cells that took up trypan blue. Values are expressed as means ± SD of 3–5 different experiments.

* Significantly different from untreated cells ($P < 0.001$).

† Significantly different from SNP alone treatment ($P < 0.005$).

TABLE 2. Effects of CN traps, antioxidants, ascorbate, and GSH depletion on lipid peroxidation induced by SNP

Addition	Malondialdehyde (μmol/10^6 cells)		
	60 min	120 min	180 min
None	0.26 ± 0.12	0.31 ± 0.14	0.42 ± 0.15
SNP 1 mM	0.99 ± 0.20	1.32 ± 0.34*	1.70 ± 0.28*
+ Cyanide 0.4 mM	0.27 ± 0.11	0.30 ± 0.08†	0.52 ± 0.14†
+ Thiosulfate 10 mM	2.05 ± 0.60	3.97 ± 0.53†	5.93 ± 0.86†
+ Dihydroxyacetone 10 mM	2.24 ± 0.53	4.65 ± 0.84†	6.85 ± 1.15†
+ Ascorbate 1 mM	1.25 ± .12	2.70 ± 0.50†	3.92 ± 0.85†
+ Desferoxamine 200 μM	0.18 ± 0.07	0.22 ± 0.10†	0.43 ± 0.21†
+ GSH depletion	2.80 ± 0.58	4.45 ± 1.15†	5.40 ± 1.08†

Hepatocytes (10^6 cells/mL), freshly isolated from normal rats, were incubated in Krebs-Henseleit buffer, pH 7.4. SNP and other compounds were added to the incubation mixture at the same time. Malondialdehyde was measured as explained under Materials and Methods. Values are expressed as means ± SD of 3–8 different experiments.

* Significantly different from untreated cells ($P < 0.001$).

† Significantly different from SNP-treated cells ($P < 0.05$).

minescence was measured by a Luminometer LB 9501-Berthold Lumat. SNP was added to 1 mL of hepatocytes (10^6/mL) at room temperature in the presence of 1 mM luminol, and the relative light unit (RLU) was recorded every 12 sec.

Determination of Thiocyanate Formation

Thiocyanate formation from SNP in hepatocytes was measured according to the method described by Bowler [31]. Briefly, aliquots of hepatocytes incubated with SNP and thiosulfate were taken at different time points, and proteins were precipitated with 5% trichloroacetic acid. After centrifugation, 1 mL of supernatant was added to 1 mL of ferric nitrate reagent (400 mM ferric nitrate in 1 N nitric acid), and the absorbance was measured at 470 nm.

Determination of SNP

SNP was measured at different time points following addition to hepatocytes according to the method described by Trablesi *et al.* [32]. Aliquots of hepatocytes were taken, the proteins were precipitated in acidic medium, and 0.02 M thiourea was added to the alkalinized supernatant. Following acidification of the medium, the absorbance was measured at 600 nm.

Statistics

Values shown are means ± SD of at least three separate experiments, and statistically significant differences between controls and experimental groups were obtained using ANOVA.

RESULTS

Effect of CN on SNP Cytotoxicity

SNP was toxic towards isolated rat hepatocytes in a concentration-dependent manner with an EC_{50} for 2 h of about 2 mM, as indicated by trypan blue uptake. SNP cytotoxicity was prevented if SNP was removed within 15–20 min of

TABLE 3. Effect of antioxidants on SNP cytotoxicity

Addition	Cytotoxicity (% trypan blue uptake)			
	30 min	60 min	120 min	180 min
None	14 ± 2	15 ± 2	17 ± 2	19 ± 2
SNP 2 mM	17 ± 2	31 ± 3	48 ± 4*	72 ± 4*
+ Pyrogallol 100 μM	16 ± 2	21 ± 2	27 ± 3†	34 ± 3†
+ Purpurogallin 100 μM	16 ± 2	17 ± 2	20 ± 2†	25 ± 2†
+ Quercetin 200 μM	14 ± 2	18 ± 2	21 ± 2†	23 ± 2†
+ Morin 100 μM	15 ± 2	19 ± 2	24 ± 2†	32 ± 3†
+ BHA 200 μM	18 ± 2	23 ± 2	31 ± 2†	41 ± 3†
+ Desferoxamine 1 mM	17 ± 2	18 ± 2	23 ± 2†	26 ± 2†

Hepatocytes (10^6 cells/mL), freshly isolated from normal rats, were incubated in Krebs-Henseleit buffer, pH 7.4. SNP and antioxidants or desferoxamine were added to the incubation mixture at the same time. Cytotoxicity was determined as the percent of cells that took up trypan blue. Values are expressed as means ± SD of 3–5 different experiments. BHA = β-hydroxyanisole.

* Significantly different from untreated cells ($P < 0.001$).

† Significantly different from SNP-treated cells ($P < 0.001$).

TABLE 4. Effects of ascorbate, NO oxidant, or hydrogen peroxide on SNP cytotoxicity

Addition	Cytotoxicity (% trypan blue uptake) 30 min	60 min	120 min	180 min
None	14 ± 2	15 ± 2	17 ± 2	19 ± 2
SNP 300 μM	14 ± 2	17 ± 2	22 ± 3	25 ± 3
+ Ascorbate 5 mM	27 ± 3	45 ± 3	95 ± 5*	100*
+ Ascorbate 5 mM + Desferoxamine 200 μM	18 ± 2	20 ± 2	26 ± 3†	34 ± 3†
+ Carboxy-PTIO 300 μM	24 ± 3	38 ± 3	76 ± 5*	93 ± 6*
SNP 1 mM	15 ± 2	18 ± 3	27 ± 3	46 ± 4‡
Glucose 10 mM/GO 0.1 U	16 ± 2	20 ± 2	22 ± 2	26 ± 3
+ SNP 1 mM	18 ± 2	29 ± 2	93 ± 4‖	100‖

Hepatocytes (10^6 cells/mL), freshly isolated from normal rats, were incubated in Krebs-Henseleit buffer, pH 7.4. SNP and other compounds were added to the incubation mixture at the same time. Cytotoxicity was determined as the percent of cells that took up trypan blue. Values are expressed as means ± SD of at least 3 different experiments. GO = glucose oxidase.

* Significantly different from SNP-treated cells ($P < 0.001$).

† Significantly different from ascorbate + SNP-treated cells ($P < 0.001$).

‡ Significantly different from untreated cells ($P < 0.05$).

‖ Significantly different from SNP (1 mM)-treated cells ($P < 0.001$).

addition (Table 1), suggesting that SNP metabolic activation was not rapid. However, cytotoxicity was not prevented if SNP was removed from the medium at 30 min after addition (Table 1). CN at a non-toxic concentration (0.4 mM) protected hepatocytes from SNP cytotoxicity if added before or up to 15 min following SNP addition (Table 1). Addition of CN 30 min after SNP addition did not affect SNP cytotoxicity. SNP cytotoxicity was accompanied by lipid peroxidation, and addition of CN also decreased the amount of lipid peroxidation (Table 2).

Effects of Thiosulfate or CN Trapping Agents on SNP Cytotoxicity

As shown in Table 1, the CN antidote, sodium thiosulfate, increased SNP cytotoxicity towards isolated rat hepatocytes. The CN trapping carbohydrate metabolites dihydroxyacetone and glyceraldehyde [33] also had effects similar to the effect of thiosulfate on SNP cytotoxicity (Table 1). Acetaldehyde, which binds CN, also potentiated SNP toxicity, but its effect was less than dihydroxyacetone or glyceraldehyde (Table 1). However, α-ketoglutarate or pyruvate, which also trap CN [34–36], did not affect SNP cytotoxicity (data not shown). Dihydroxyacetone and thiosulfate increased lipid peroxidation induced by SNP more than 4-fold (Table 2).

Effects of Antioxidants on SNP Cytotoxicity

The antioxidants pyrogallol, purpurogallin, quercetin, morin, or β-hydroxyanisole (BHA) prevented both cytotoxicity (Table 3) and lipid peroxidation induced by SNP (data not shown). The ferric iron chelator desferoxamine also prevented cytotoxicity and lipid peroxidation induced by SNP (Tables 2, 3).

Effects of Ascorbate or NO Oxidant on SNP Cytotoxicity

Addition of ascorbate to hepatocytes increased SNP cytotoxicity by a factor of about 10-fold (Table 4). Ascorbate also greatly increased SNP-induced lipid peroxidation (Table 2). Desferoxamine prevented both cytotoxicity and lipid peroxidation induced by SNP plus ascorbate. The NO oxidizing agent carboxy-PTIO, which converts NO to the NO_2 radical [37], also increased SNP cytotoxicity (Table 4). Inhibition of NADPH–P450 reductase by diphenyliodonium chloride prevented SNP cytotoxicity (Table 5), suggesting that a one-electron reduction of SNP by P450 reductase is necessary for SNP cytotoxicity. Hydrogen peroxide, produced by a glucose/glucose oxidase system, also markedly increased SNP cytotoxicity (Table 4).

Effects of GSH Depletion on SNP Cytotoxicity

Hepatocyte GSH was depleted to less than 50% of control immediately upon addition of SNP, which then stayed constant during the incubation (Fig. 1A). GSSG levels were

TABLE 5. Effects of inhibition of NADPH:TP450 reductase or GSH depletion on SNP cytotoxicity

Addition	Cytotoxicity (% trypan blue uptake) 30 min	60 min	120 min	180 min
None	14 ± 2	15 ± 2	17 ± 2	19 ± 2
SNP 2 mM	17 ± 2	31 ± 3	48 ± 4*	72 ± 4*
+ DPIC 50 μM	14 ± 2	17 ± 2	24 ± 2†	31 ± 2†
+ GSH depletion‡	52 ± 4§	100§		
+ DTT 5 mM	32 ± 3	69 ± 3†	100†	

Conditions were similar to those described for Table 1. Values are expressed as means ± SD of at least three separate experiments. DPIC = diphenyliodonium chloride.

* Significantly different from untreated cells ($P < 0.001$).

† Significantly different from SNP-treated cells ($P < 0.005$).

‡ GSH depletion was achieved as explained in Materials and Methods. Viability of control GSH-depleted hepatocytes was the same as normal controls.

§ Significantly different from normal SNP-treated cells ($P < 0.001$).

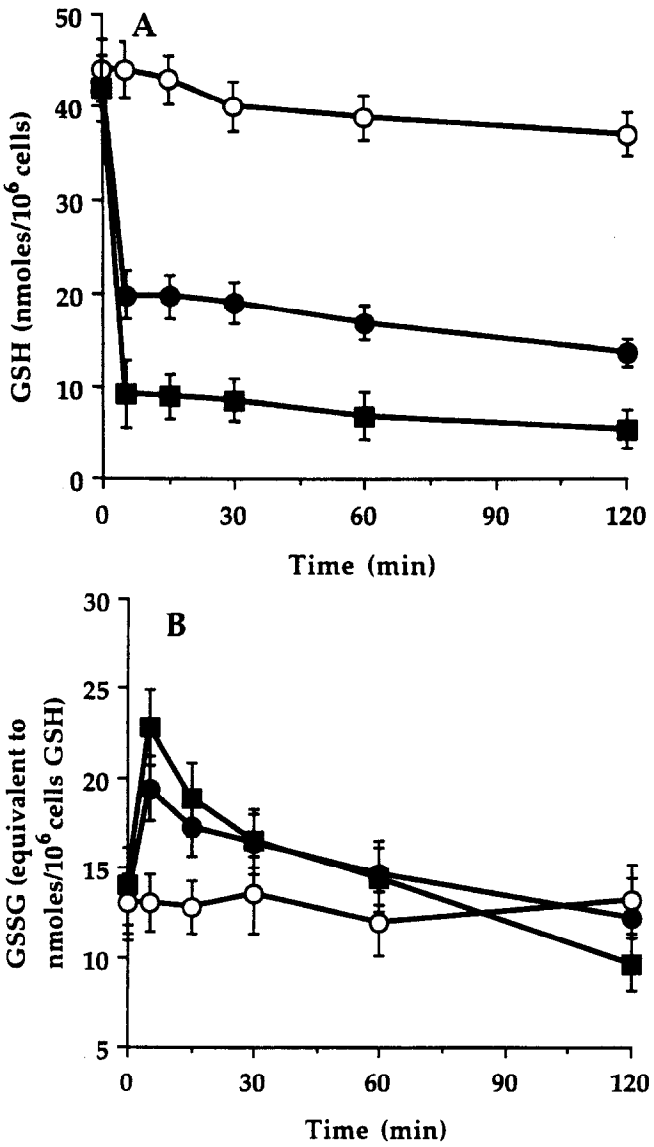

FIG. 1. Effect of SNP on hepatocyte GSH (A) and GSSG (B) content. GSH and GSSG levels were measured as explained under Materials and Methods. Values are means ± SD of three separate experiments. Key: (○) control untreated cells; (●) 2 mM SNP; and (■) 3 mM SNP.

increased at the beginning but returned to control levels after 30 min of incubation (Fig. 1B). Depleting hepatocyte GSH before addition of SNP markedly increased SNP cytotoxicity towards isolated rat hepatocytes (Table 5). Three times more lipid peroxidation was also induced by SNP in GSH-depleted hepatocytes than in normal hepatocytes (Table 2).

Peroxynitrite Formation from SNP

Addition of SNP to hepatocytes resulted in the formation of peroxynitrite, as detected by luminol chemiluminescence, which lasted for only a short period of time (about 2 min) (Fig. 2). However, trapping CN released from SNP by

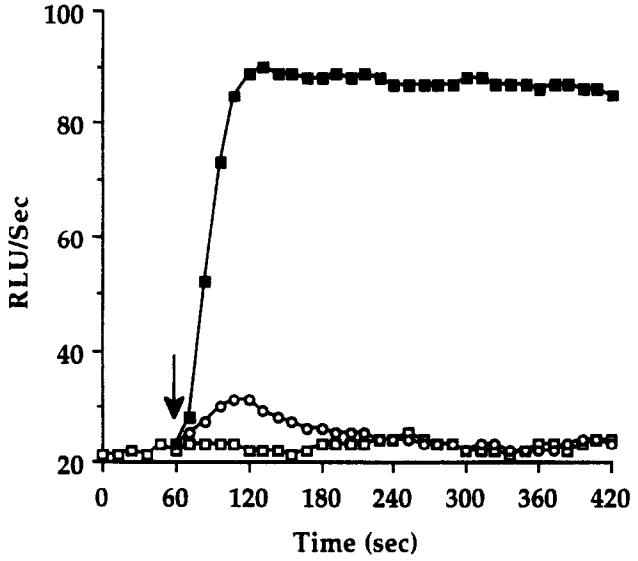

FIG. 2. Formation of peroxynitrite from SNP. SNP was added to 1 mL of hepatocytes (10^6 cells/mL) incubated with 1 mM luminol, and the relative light unit (RLU) was measured every 12 sec as explained under Materials and Methods. The arrow shows the time of addition of SNP. Key: (□) no addition; (○) 3 mM SNP; and (■) 10 mM dihydroxyacetone + 3 mM SNP. Dihydroxyacetone treatment was not significantly different from no addition.

dihydroxyacetone (Fig. 2) or thiosulfate (data not shown) enhanced peroxynitrite formation from SNP about 7- and 5-fold, respectively, and was much longer lasting.

CN Release from SNP

SNP was slowly metabolized by hepatocytes. Dihydroxyacetone or thiosulfate markedly increased the metabolism of SNP whereas addition of CN inhibited the metabolism of SNP by hepatocytes (Table 6). As shown in Table 7, thiocyanate was readily formed from SNP, hepatocytes, and thiosulfate. Thiocyanate formation slowly increased with time, and the CN trap dihydroxyacetone or the reductant ascorbate markedly increased the rate of thiocyanate formation. Prior hepatocyte GSH depletion also increased thiocyanate formation from SNP (Table 7), suggesting that CN originates from nitroreduction rather than GSH conjugate formation.

Effects of SNP on ATP Levels

Hepatocyte ATP levels were decreased by SNP before cytotoxicity occurred, and this effect was potentiated by the addition of dihydroxyacetone (Fig. 3). Lactate formation from fructose by hepatocytes was inhibited by preincubating hepatocytes with SNP (Table 8). CN, on the other hand, increased lactate formation from fructose (data not shown).

Effects of SNP on Mitochondrial Respiration

SNP did not affect hepatocyte respiration immediately, but at 60 min hepatocyte respiration was inhibited 15% (con-

TABLE 6. Metabolism of SNP by hepatocytes

Addition	SNP (nmol/10^6 cells)				
	1 min	10 min	30 min	60 min	120 min
SNP 1 mM	992 ± 32	978 ± 27	952 ± 40	880 ± 32	781 ± 35
+ TS 2 mM	965 ± 38	829 ± 30*	753 ± 26*	650 ± 37*	533 ± 49*
+ DHA 5 mM	936 ± 53	781 ± 42*	610 ± 122*	492 ± 84*	350 ± 58*
+ CN 0.4 mM	994 ± 40	985 ± 32	988 ± 62	971 ± 61	906 ± 43

Conditions were similar to those described for Table 1. SNP and other compounds were added to the hepatocytes at the same time. SNP was measured as explained under Materials and Methods. Values are expressed as means ± SD of three separate experiments. Abbreviations: TS, thiosulfate; and DHA, dihydroxyacetone.

* Significantly different from SNP alone ($P < 0.05$).

trol cells: 11.40 nmol O_2/min/10^6 cells; SNP-treated cells: 9.76 nmol O_2/min/10^6 cells). Respiration was restored immediately upon addition of the CN trap dihydroxyacetone (5 mM), or thiosulfate (5 mM). SNP did not cause significant CN-resistant respiration.

DISCUSSION

SNP toxicity *in vivo* has been attributed to CN liberation [10, 12], and sodium thiosulfate, the regular antidote of CN, is sometimes administered concomitantly with the infusion of SNP [14, 38]. We also found that incubation of SNP with hepatocytes resulted in a slight inhibition of mitochondrial respiration, which could be attributed to CN as it was prevented by thiosulfate and thiocyanate was formed. However, the addition of sodium thiosulfate or CN trapping agents such as dihydroxyacetone or glyceraldehyde, which prevents CN-induced hepatocyte cytotoxicity [33], increased SNP-induced cytotoxicity and lipid peroxidation. On the other hand, the addition of non-toxic concentrations of CN prevented SNP toxicity and lipid peroxidation. These results suggest that the toxicity of SNP in isolated rat hepatocytes is not due to CN liberation from SNP but to another metabolite(s) of SNP.

Our results suggest that the metabolism of SNP is controlled by the equilibrium between free CN and SNP (Fig. 4) so that thiosulfate or CN trapping agents pull the equilibrium towards the release of CN and NO, as thiosulfate or CN trapping agents markedly increased SNP metabolism to CN and peroxynitrite. SNP disappearance and thiocyanate formation from SNP were also increased markedly by thiosulfate and dihydroxyacetone. CN, on the other hand, inhibited the disappearance of SNP. It was reported previously that CN also decreases the release of NO from SNP in the presence of vascular tissue [19].

The work of several laboratories is consistent with the theory that NO is released from SNP following one-electron reduction of SNP by cytochrome P450 reductase and the release of CN ion [19–22]. Ascorbate increased the toxicity of SNP about 10-fold, whereas inhibition of cytochrome P450 reductase with diphenyliodonium chloride prevented SNP cytotoxicity and lipid peroxidation. This further suggests that the one-electron reduction of SNP leads to the release of NO, which in the presence of oxygen forms cytotoxic peroxynitrite (Fig. 4).

SNP decreased hepatocyte GSH levels, and GSH-depleted hepatocytes were much more susceptible to SNP, which suggests that intracellular GSH detoxifies SNP and the GSH conjugate $[Fe(CN)_5N(O)SG]^{3-}$ does not contribute to SNP cytotoxicity. The initial GSH oxidation probably results from peroxynitrite formation [39] or breakdown of the GSH conjugate [19] (Fig. 4) as SNP did not induce significant CN-resistant respiration. This also suggests that redox cycling mediated oxygen activation was minimal.

The rate of peroxynitrite formation that accompanied SNP reductive metabolism was increased by thiosulfate or

TABLE 7. Thiocyanate formation from SNP

Addition	Thiocyanate (nmol/10^6 cells)				
	1 min	10 min	30 min	60 min	120 min
None	5 ± 1	6 ± 1	7 ± 2	9 ± 2	10 ± 3
SNP 1 mM + TS 0.2 mM	14 ± 2	15 ± 2	39 ± 4	80 ± 6	142 ± 10
SNP 1 mM + TS 0.5 mM	14 ± 2	80 ± 14	158 ± 10	263 ± 18	394 ± 25
SNP 1 mM + TS 1 mM	13 ± 4	93 ± 16	160 ± 15	267 ± 22	414 ± 36
SNP 1 mM + TS 2 mM	22 ± 6	135 ± 12	218 ± 21	336 ± 38	557 ± 40
+ TS 2 mM + DHA 5 mM	30 ± 5	224 ± 23	313 ± 18	518 ± 35	920 ± 47*
+ TS 2 mM + Ascorbate 1 mM	57 ± 11	239 ± 29	654 ± 53	853 ± 67	1245 ± 89*
+ TS 2 mM + GSH depletion	29 ± 5	159 ± 20	342 ± 28	422 ± 36	735 ± 62*

Hepatocytes (10^6 cells/mL), freshly isolated from normal rats, were incubated in Krebs-Henseleit buffer, pH 7.4. Thiocyanate was measured as explained under Materials and Methods. Values are expressed as means ± SD of at least three separate experiments. Abbreviations: TS = thiosulfate; and DHA, dihydroxyacetone.

* Significantly different from SNP + TS ($P < 0.01$).

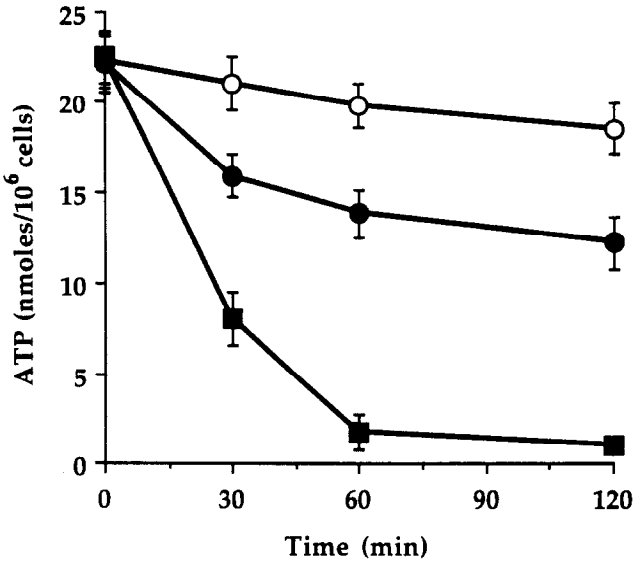

FIG. 3. Effect of SNP on ATP levels of hepatocytes. ATP was measured as explained under Materials and Methods. Values are expressed as means ± SD of three separate experiments. (○) control; (●) 1 mM SNP; and (■) 5 mM dihydroxyacetone + 1 mM SNP.

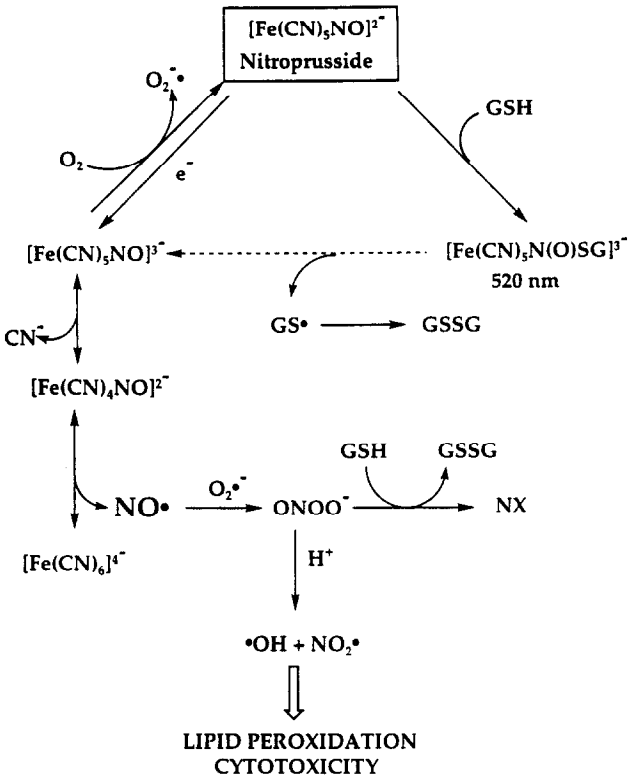

FIG. 4. Proposed mechanism of metabolism and cytotoxicity of SNP. NX = unidentified nitrogen containing metabolite(s).

dihydroxyacetone but was inhibited by CN. Peroxynitrite formation may result from the reaction of superoxide and nitric oxide (Eq. 1), which could readily initiate lipid peroxidation as a result of the decomposition of peroxynitrite to NO_2 and hydroxyl radicals (Eq. 2) [40].

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^- \quad (1)$$

$$ONOO^- + H^+ \rightarrow {}^{\bullet}OH + NO_2^{\bullet} \quad (2)$$

The NO oxidizing agent carboxy-PTIO, which converts NO to NO_2 radical [37], also increased SNP cytotoxicity and lipid peroxidation and further supports the involvement of NO in SNP cytotoxicity. Furthermore, the production of hydrogen peroxide by the glucose/glucose oxidase system greatly increased SNP cytotoxicity towards hepatocytes, which is consistent with our theory that NO may be the cause of cytotoxicity. H_2O_2 was shown previously, *in vitro*, to react with NO and produce cytotoxic oxygen species, especially singlet oxygen [41] which could contribute to NO cytotoxicity [42]. We have shown previously that the NO generating agents butyl nitrite [23] and MNNG [24] were also toxic to hepatocytes as a result of metabolic activation by hepatocytes to form NO.

Antioxidants and the iron chelator desferoxamine prevented both SNP-induced cytotoxicity and lipid peroxidation, which further suggests that free radicals are involved in the mechanism of cytotoxicity. The prevention of lipid peroxidation by desferoxamine has been attributed to its reaction with peroxynitrite [40]; however, it is possible that Fe^{2+} activates peroxynitrite by a Fenton reaction (Eq. 3).

$$ONOO^- + Fe^{2+} + H^+ \rightarrow {}^{\bullet}OH + NO_2^{\bullet -} + Fe^{3+} \quad (3)$$

SNP depleted hepatocyte ATP levels, and dihydroxyacetone accelerated that effect. The ATP depletion induced by SNP is unlikely to be due to CN release as dihydroxyacetone was shown previously to restore CN-induced ATP depletion in hepatocytes [33]. Furthermore, the amount of CN formed was not sufficient to cause significant hepatocyte ATP depletion. The ATP depletion induced by SNP may be due to the inhibition of glycolysis by peroxynitrite as NO was shown previously to inhibit glyceraldehyde-3-phosphate dehydrogenase in the glycolytic pathway by activating a cytosolic ADP ribosyl transferase [43]. Recently,

TABLE 8. Effects of SNP on glycolysis of isolated rat hepatocytes

Addition	Lactate (nmol/10^6 cells)		
	30 min	60 min	120 min
None	58 ± 9	59 ± 9	62 ± 10
Fructose 10 mM	107 ± 8*	181 ± 15*	250 ± 22*
+ SNP 1 mM	85 ± 5	101 ± 5†	106 ± 9†
+ Cyanide 1 mM	174 ± 12	225 ± 23	293 ± 28

Hepatocytes (10^6 cells/mL), freshly isolated from normal rats, were incubated in Krebs-Henseleit buffer, pH 7.4. Hepatocytes were incubated with SNP for 20 min before addition of fructose. Lactate formation from fructose was measured as an index of glycolysis. Lactate was measured as explained under Materials and Methods. Values are expressed as means ± SD of three separate experiments.

* Significantly different from untreated cells ($P < 0.01$).

† Significantly different from fructose alone ($P < 0.01$).

NO and peroxynitrite were shown to reversibly inhibit mitochondrial respiration [44–46], which may be partially responsible for inhibition of mitochondrial respiration and ATP depletion in SNP-induced cytotoxicity.

The results of the present study suggest that although CN is liberated from SNP, the cytotoxicity of SNP is due to NO and derived free radicals that can inhibit glycolysis and initiate lipid peroxidation. Removal of CN liberated from SNP with thiosulfate or CN trapping agents results in a faster reductive metabolism of SNP to NO, which potentiates SNP cytotoxicity. The cytotoxic concentrations of SNP used in this investigation were much higher than SNP doses infused clinically (3–8 μg/kg/min or up to 230 μM [47]); however, it is interesting to speculate whether the administration of thiosulfate or CN trapping agent concurrently with SNP would increase the hypotensive effect of SNP, which may need the adjustment of SNP dose to prevent severe hypotension in patients receiving SNP.

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References

1. Siegel P, Moraca PP and Green JR, Sodium nitroprusside in the surgical treatment of cerebral aneurysms and arteriovenous malformations. *Br J Anaesth* **43:** 790–795, 1971.
2. Franciosa JA, Limas CJ, Guiha NH, Rodriguera E and Cohn JN, Improved left ventricular function during nitroprusside infusion in acute myocardial infarction. *Lancet* **1:** 650–654, 1972.
3. Vesey CJ, Cole PV, Linnell JC and Wilson J, Some metabolic effect of sodium nitroprusside in man. *Br Med J* **2:** 140–142, 1974.
4. Vesey CJ, Cole PV and Simpson PJ, Cyanide and thiocyanate concentrations following sodium nitroprusside infusion in man. *Br J Anaesth* **48:** 651–660, 1976.
5. Pasch T, Schulz V and Hoppelshauser G, Nitroprusside-induced formation of cyanide and its detoxication with thiosulfate during deliberate hypotension. *J Cardiovasc Pharmacol* **5:** 77–85, 1983.
6. Butler AR and Glidewell C, Recent chemical studies of sodium nitroprusside relevant to its hypotensive action. *Chem Soc Rev* **16:** 361–380, 1987.
7. Bisset WI, Butler AR, Glidewell C and Reglinski JR, Sodium nitroprusside and cyanide release: Reasons for re-appraisal. *Br J Anaesth* **53:** 1015–1018, 1981.
8. Smith RP and Kruszyna H, Nitroprusside produces cyanide poisoning via a reaction with hemoglobin. *J Pharmacol Exp Ther* **191:** 557–563, 1974.
9. Michenfelder JD and Tinker JH, Cyanide toxicity and thiosulfate protection during chronic administration of sodium nitroprusside in the dog: Correlation with a human case. *Anesthesiology* **47:** 441–448, 1977.
10. Schulz V, Clinical pharmacokinetics of nitroprusside, cyanide, thiosulfate and thiocyanate. *Clin Pharmacokinet* **9:** 239–251, 1984.
11. Vesey CJ and Cole PV, Blood cyanide and thiocyanate concentrations produced by long-term therapy with sodium nitroprusside. *Br J Anaesth* **57:** 148–155, 1985.
12. Devlin DJ, Smith RP and Thron CD, Cyanide release from nitroprusside in the isolated, perfused, bloodless liver and hindlimbs of the rat. *Toxicol Appl Pharmacol* **99:** 354–356, 1989.
13. Campbell JM, McCrae F, Reglinski J, Wilson R, Smith WE and Sturrock RD, The interaction of sodium nitroprusside with peripheral white blood cells *in vitro*: A rationale for cyanide release *in vivo*. *Biochim Biophys Acta* **1156:** 327–333, 1993.
14. Schulz V, Gross R, Pasch T, Busse J and Loeschcke G, Cyanide toxicity of sodium nitroprusside in therapeutic use with and without sodium thiosulfate. *Klin Wochenschr* **60:** 1393–1400, 1982.
15. Vesey CJ, Krapez JR, Varley JG and Cole PV, The antidotal action of thiosulfate following acute nitroprusside infusion in dogs. *Anesthesiology* **62:** 415–421, 1985.
16. Yamamoto HA, Nitroprusside intoxication: Protection of α-ketoglutarate and thiosulfate. *Food Chem Toxicol* **30:** 887–890, 1992.
17. Feelisch M, The biochemical pathways of nitric oxide formation from nitrovasodilators: Appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J Cardiovasc Pharmacol* **17 (Suppl 3):** S25–S33, 1991.
18. Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ and Gruetter CA, Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: Evidence for the involvement of *S*-nitrosothiols as active intermediates. *J Pharmacol Exp Ther* **218:** 739–749, 1981.
19. Bates JN, Baker MT, Guerra R and Harrison DG, Nitric oxide generation from nitroprusside by vascular tissue: Evidence that reduction of the nitroprusside anion and cyanide loss are required. *Biochem Pharmacol* **42** (Suppl): S157–S165, 1991.
20. Rao DNR, Elguindi S and O'Brien PJ, Reductive metabolism of nitroprusside in rat hepatocytes and human erythrocytes. *Arch Biochem Biophys* **286:** 30–37, 1991.
21. Kruszyna H, Kruszyna R, Rochelle LG, Smith RP and Wilcox DE, Effects of temperature, oxygen, heme ligand and sulfhydryl alkylation on the reaction of nitroprusside and nitroglycerin with hemoglobin. *Biochem Pharmacol* **46:** 95–102, 1993.
22. Rochelle LG, Kruszyna H, Kruszyna R, Barchowsky A, Wilcox D and Smith RP, Bioactivation of nitroprusside by porcine endothelial cells. *Toxicol Appl Pharmacol* **128:** 123–128, 1994.
23. Meloche BA and O'Brien PJ, *S*-Nitrosyl glutathione-mediated hepatocyte cytotoxicity. *Xenobiotica* **23:** 863–871, 1993.
24. Niknahad H and O'Brien PJ, Cytotoxicity induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine may involve *S*-nitrosyl glutathione and nitric oxide. *Xenobiotica* **25:** 91–101, 1995.
25. Loiacono RE and Beart PM, Hippocampal lesions induced by microinjection of nitric oxide donor nitroprusside. *Eur J Pharmacol* **216:** 331–333, 1992.
26. Moldeus P, Hogberg J and Orrenius S, Isolation and use of liver cells. *Methods Enzymol* **52:** 60–71, 1978.
27. Khan S and O'Brien PJ, 1-Bromoalkanes as new potent nontoxic glutathione depletors in isolated rat hepatocytes. *Biochem Biophys Res Commun* **179:** 436–441, 1991.
28. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW and Potter DW, High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal Biochem* **106:** 55–62, 1980.
29. Smith MT, Thor H, Hartzell P and Orrenius S, The measurement of lipid peroxidation in isolated hepatocytes. *Biochem Pharmacol* **31:** 19–26, 1982.
30. Radi R, Cosgrove TP, Beckman JS and Freeman BA, Peroxynitrite-induced luminol chemiluminescence. *Biochem J* **290:** 51–57, 1993.
31. Bowler RG, The determination of thiocyanate in blood serum. *Biochemistry* **38:** 118–124, 1944.

32. Trablesi H, Bouzouita KA, Malbosc R and Bouguerra ML, Rapid colorimetric determination of sodium nitroprusside. *J Pharm Belg* **48:** 389–392, 1993.
33. Niknahad H, Khan S, Sood C and O'Brien PJ, Prevention of cyanide-induced cytotoxicity by nutrients in isolated rat hepatocytes. *Toxicol Appl Pharmacol* **128:** 271–279, 1994.
34. Schwartz C, Morgan RL, Way LM and Way JL, Antagonism of cyanide intoxication with sodium pyruvate. *Toxicol Appl Pharmacol* **50:** 437–441, 1979.
35. Moore SJ, Norris JC, Ho IK and Hume AS, The efficacy of α-ketoglutaric acid in the antagonism of cyanide intoxication. *Toxicol Appl Pharmacol* **82:** 40–44, 1986.
36. Norris JC, Utley WA and Hume AS, Mechanism of antagonizing cyanide-induced lethality by α-ketoglutaric acid. *Toxicology* **62:** 275–283, 1990.
37. Yoshida K, Akaike T, Doi T, Sato K, Ijiri S, Suga M, Ando M and Maeda H, Pronounced enhancement of NO-dependent antimicrobial action by an NO-oxidizing agent, imidazolineoxyl *N*-oxide. *Infect Immun* **61:** 3552–3555, 1993.
38. Rindone JP and Sloane EP, Cyanide toxicity from sodium nitroprusside: Risk and management. *Ann Pharmacother* **26:** 515–519, 1992.
39. Radi R, Beckman JS, Bush KM and Freeman BA, Peroxynitrite oxidation of sulfhydryls: The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* **266:** 4244–4250, 1991.
40. Radi R, Beckman JS, Bush KM and Freeman BA, Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* **288:** 481–487, 1991.
41. Noronha-Dutra AA, Epperlein MM and Woolf N, Reaction of nitric oxide with hydrogen peroxide to produce potentially cytotoxic singlet oxygen as a model for nitric oxide-mediated killing. *FEBS Lett* **321:** 59–62, 1993.
42. Ioannidis I and de Groot H, Cytotoxicity of nitric oxide in Fu5 rat hepatoma cells: Evidence for co-operative action with hydrogen peroxide. *Biochem J* **296:** 341–345, 1993.
43. Dimmeler S, Lottspeich F and Brune B, Nitric oxide causes ADP-ribosylation and inhibition of glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* **267:** 16771–16774, 1992.
44. Cleeter MWJ, Cooper JM, Darley-Usmar VM, Moncada S and Schapira AHV, Reversible inhibition of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide: Implications for neurodegenerative disease. *FEBS Lett* **345:** 50–54, 1994.
45. Schweizer M and Richter C, Nitric oxide potently and reversibly deenergizes mitochondria at low oxygen tension. *Biochem Biophys Res Commun* **204:** 169–175, 1994.
46. Bolaños JP, Peuchen S, Heales SJR, Land JM and Clark JB, Nitric oxide-mediated inhibition of mitochondrial respiratory chain in cultured astrocytes. *J Neurochem* **63:** 910–916, 1994.
47. Bogusz M, Moroz J, Karski J, Gierz J, Regieli A, Witkowska R and Golabek A, Blood cyanide and thiocyanate concentrations after administration of sodium nitroprusside as hypotensive agent in neurosurgery. *Clin Chem* **25:** 60–63, 1979.