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Denitrosation of 1,3-Dimethyl-2-cyano-1-nitrosoguanidine in Rat Primary Hepatocyte Cultures

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ABSTRACT. *N*-Nitrosoguanidines are potential carcinogens. However, the toxicity of these agents is attenuated significantly in laboratory rodents by processes that remove the nitroso group to generate the relatively innocuous parent guanidinium compound. The denitrosation of 1,3-dimethyl-2-cyano-1-nitrosoguanidine (CyanoDMNG) mediated by rat hepatocytes in primary culture was investigated. At concentrations ≤200 μM, applied CyanoDMNG was converted efficiently to 1,3-dimethyl-2-cyanoguanidine (CyanoDMG). In trials using 50 μM CyanoDMNG (5 mL dosing solutions), it was demonstrated that hepatocytes are capable of denitrosating a 40 μM concentration of the applied compound with little change in the total or oxidized glutathione levels. The process was inhibited by coincidently applied ethacrynic acid, a glutathione transferase inhibitor. Reduction of hepatocyte glutathione to 20% of control levels by buthionine sulfoximine pretreatment had little effect on CyanoDMG production; total depletion of cytosolic glutathione by diethyl maleate pretreatment arrested CyanoDMNG processing. Hepatocyte-mediated CyanoDMNG denitrosation did not generate nitrite; nitrate yields were 10% relative to the CyanoDMG produced. The mercuric chloride/azo dye response of cultures lysed at times during 50 μM CyanoDMNG processing indicated intact CyanoDMNG as the only dye-sensitive material present. At applied CyanoDMNG > 100 μM, *S*-nitrosoglutathione (GSNO) yields were detectable; 4 μM GSNO was generated (concentration in 5 mL lysates) and maintained during 60 min at the 200 μM CyanoDMNG treatment level; this yield decayed if CyanoDMNG was withdrawn. Based on these and previous findings, it is hypothesized that CyanoDMNG is converted to CyanoDMG and GSNO by glutathione transferases and that GSNO is catabolized to eventually regenerate reduced glutathione. The fate of most of the NO moiety released remains to be determined. BIOCHEM PHARMACOL 53;9:1297–1306, 1997. © 1997 Elsevier Science Inc.

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The potential carcinogens NC† and MNNG are efficiently detoxified, apparently by first pass catabolism, in laboratory rodents [1–4]. This processing is likely to be among the reasons why NC is non-toxic and non-tumorigenic to rats and mice after administration by any of several routes, and why the cytotoxicity and tumorigenicity of MNNG are generally limited to regions local to the site of application. The *in vivo* detoxification process involves the removal of the nitroso group to generate high yields of the parent guanidinium compound, observed to be nearly 100% in the NC case [1, 2]. It is yet to be determined whether this extensive detoxification by denitrosation is a characteristic unique to *N*-nitrosoguanidinium compounds, or if these agents represent an extreme case in a continuum of *N*-nitroso compound vulnerability to this processing.

We have discovered that members of the mu class of glutathione transferase isoenzymes, found in abundance in laboratory rodent liver, catalyze *N*-nitrosoguanidinium compound denitrosation [5]. The reaction products, generated in a 1:1 ratio, are the parent guanidinium compound and GSNO, a relatively stable S-nitrosated thiol. The specific activities of the competent isoenzymes for the *N*-nitrosoguanidinium substrates are impressively high. We have also demonstrated that the denitrosation reaction proceeds

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† *Abbreviations:* NC, nitrosocimetidine: MNNG, 1-methyl-2-nitro-1-nitrosoguanidine: GSNO, *S*-nitrosoglutathione; GSH, reduced glutathione; GSSG, oxidized glutathione; CyanoDMNG, 1,3-dimethyl-2-cyano-1-nitrosoguanidine; CyanoDMG, 1,3-dimethyl-2-cyanoguanidine; BN, *n*-butyl nitrite; DMNU, 1,3-dimethyl-1-nitrosourea; BSO, L-buthionine-[*S,R*]-sulfoximine; DEM, diethyl maleate; EA, ethacrynic acid; LDH, lactate dehydrogenase; D-PBS, Dulbecco's phosphate-buffered saline; TCA, trichloroacetic acid; PCA, perchloric acid; SAX-HPLC, strong anion exchange-high pressure liquid chromatography; RP-HPLC, reverse phase-high pressure liquid chromatography; and CDNB, 1-chloro-2,4-dinitrobenzene.

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efficiently in rodent liver cytosol fraction, that glutathione is consumed in the process, and that included glutathione transferase inhibitors effectively quench the reaction [6, 7]. The denitrosated product is generated in a 1:1 stoichiometry with the nitroso compound consumed. While the GSNO yields in cytosol-mediated reactions are observed to be high, they are not equivalent to the guanidinium product produced. We have demonstrated that GSNO is capable of passing the NO moiety to cytosolic protein thiols, generating S-nitrosated proteins, in what is perhaps a dynamic NO-exchange equilibrium. This process would reduce the apparent GSNO yield in favor of increased levels of GSH. Incubation of GSNO with cytosol fraction also promotes the formation of detectable levels of nitric oxide as well as GSSG and nitrite, further operating to deplete the GSNO yield. The key observation is that the same GSNO yields, and associated redistribution of glutathione units, are generated in cytosol incubates formulated with equimolar *N*-nitrosoguanidinium compound and GSH as are found in those prepared with the anticipated 100% yield concentration of GSNO. Thus, the stoichiometry of the glutathione transferase reaction in rat liver cytosol incubates is demonstrated [7].

Another important observation derives from our cytosol experiments. GSNO itself is actively catabolized in cytosol incubates by a process that utilizes NADPH as a cofactor and that generates GSH together with a partially characterized glutathione derivative [7]. This cytosolic processing of GSNO does not produce nitric oxide. Also, the yields of nitrite and S-nitrosated protein evident in the absence of NADPH vanish when the cofactor is present. The chemical form assumed by the NO moiety released from GSNO remains to be identified. We recently isolated the GSNO catabolizing enzyme ("GSNO terminase")* and determined that GSSG is the prevailing immediate reaction product. GSSG is evidently converted to GSH in our cytosol incubates by NADPH-dependent glutathione reductase.

We hypothesize that *N*-nitrosoguanidinium compounds are detoxified in rodents via the action of mu class glutathione transferases, that the consequent GSNO product is dispatched by GSNO terminase, and that the glutathione units involved eventually are recovered as GSH. Support for this model is provided by the observation that depletion of glutathione levels is not evident in the livers of hamsters 15 min after intravenous administration of the highest practical doses of NC [6].

The present report presents a series of experiments that considered *N*-nitrosoguanidinium compound denitrosation mediated by rat hepatocytes in primary culture. Glutathione transferase activity was evidenced as being involved in the observed processing which, in turn, implies the intracellular production of GSNO in yields equivalent to the denitrosated product detected. We found only low yields of GSNO, little perturbation of cellular glutathione levels, and no nitrite production attending extensive *N*-nitrosoguanidinium compound denitrosation. The results are consistent with the notion that GSNO is subject to processing by GSNO terminase.

Intracellular GSNO is of some interest in that it has been envisioned as an important intermediate in the biological activity of nitric oxide: GSH may be a scavenger of NO moiety as it reacts with nitric oxide autoxidation products to generate GSNO [8], and may as well be a recipient of the NO moiety from various intra- and extracellular NO donors [9]. GSNO, in turn, is capable of passing the NO group to other reactive centers, perhaps with regulatory consequence [10]. Our results suggest that in rat hepatocytes the half-life of GSNO is short.

The prototype *N*-nitrosoguanidinium compound, CyanoDMNG, utilized in these experiments is particularly well suited for this investigation of intracellular denitrosation. It is polar but not charged; aqueous solutions of up to 20 mM are readily prepared yet the compound is highly lipophilic (octanol:aqueous partition coefficient > 20; [6]), evidently permitting transit across membrane barriers. In addition, CyanoDMNG is stable in neutral pH buffer and has a relatively low rate of non-enzymic decomposition when incubated in solution with GSH. The denitrosation product, CyanoDMG, also lipophilic, is metabolized minimally in hepatocyte cultures.

MATERIALS AND METHODS

Materials

CyanoDMNG, CyanoDMG, GSNO, and BN were prepared or obtained as previously described [7] as was DMNU [6]. BSO, DEM, EA, glutathione reductase (Type IV from baker's yeast; NAD[P]H:oxidized-glutathione oxidoreductase; EC 1.6.4.2), LDH (Type XXXV from porcine heart; [S]-lactate:NAD^+ oxidoreductase; EC 1.1.1.27), isocitric dehydrogenase (Type IV from porcine heart; isocitrate: $NADP^+$ oxidoreductase [decarboxylating]; EC 1.1.1.42), superoxide dismutase (from bovine liver; superoxide:superoxide oxidoreductase; EC 1.15.1.1) and catalase (from bovine liver; H_2O_2:H_2O_2 oxidoreductase: EC 1.11.1.6) were purchased from the Sigma Chemical Co., St. Louis, MO. Collagenase (from *Clostridium histolyticum;* Clostridiopeptidase A; EC 3.4.24.3) was obtained from the Worthington Biochemical Corp., Freehold, NJ. All other chemicals were of reagent grade and were purchased from commercial sources. Female Sprague-Dawley rats (150–200 g) were obtained from Charles River Laboratories, Wilmington, MA.

Preparation of Rat Primary Hepatocytes and Pretreatment/Dosing Procedures

Hepatocytes were isolated by the collagenase perfusion method described by Seglen [11] using the buffers suggested by Bonney *et al.* [12]; all preparations used experimentally

* Jensen DE and Belka GK, manuscript in preparation.

had a cell viability of greater than 90% as assessed by the trypan blue exclusion assay. The hepatocytes were plated in 25 cm^2 flasks (Nunc, Inc., Naperville, IL) at a density of 3.33×10^6 cells per flask in 5 mL of nutritional medium [Williams' Medium E (Gibco BRL, Life Technologies, Inc., Grand Island, NY) containing 25 mM HEPES buffer (Sigma), pH adjusted to 7.4 with KOH, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.01 U/mL insulin (all from Gibco BRL) and 15% (v/v) heat-inactivated fetal bovine serum (55° for 15 min; Gibco BRL)]. After incubating the cells for 3 hr, 37°, in a high humidity incubator, the medium was replaced with fresh nutritional medium and the cells were incubated for another 20 hr. This medium was replaced with warmed D-PBS (Gibco BRL) 30 min before the dosing zero time, and the cells were washed twice more with D-PBS just before dosing. Finally, the hepatocytes were placed under 5 mL of fresh D-PBS (dosing solution).

CyanoDMNG, CyanoDMG, and DMNU solubilized in DMSO or BN delivered into ethanol were prepared immediately before each experiment, subsequently diluted 20-fold with D-PBS, and then delivered as 357 μL to the dosing solution overlaying the cells; total DMSO or ethanol was 0.33%, v/v.

In some experiments, the hepatocyte cultures were treated with 0.5 mM BSO during the 20-hr incubation in nutritional medium; the subsequent D-PBS washes and D-PBS dosing solutions were free of BSO. In other experiments, the cultures were treated with 1.0 mM DEM during the 30-min D-PBS incubation period immediately before the experimental zero time; the subsequent D-PBS washes and the D-PBS dosing solutions were free of DEM. In EA experiments, the inhibitor, solubilized in D-PBS, was added to the dosing solution simultaneously with CyanoDMNG.

Recovery of Dosing and Lysing Solutions/Analytical Procedures

At times after compound application, the dosing solution was removed from the various cell culture flasks for immediate processing and replaced with lysing solution according to the experimental requirements. Unless otherwise noted, flasks containing lysing solution were shaken vigorously, refrigerated for 30 min, shaken again, and the recovered suspensions clarified by centrifugation and retained on ice for further processing.

A portion of recovered dosing solution was octanol-extracted and the isolated organic phase subjected to analytical RP-HPLC for CyanoDMG and CyanoDMNG or similarly for DMNU yield quantification as previously described [7]. In some experiments, possible hepatocyte damage due to treatment was assessed by measuring the LDH activity in the D-PBS dosing solution as well as in hepatocyte osmotic lysates. The lysing solution in these experiments was 5 mL of 10 mM K_2HPO_4, pH 7.4 (osmotic lysates). Sample LDH activity was measured by monitoring the decrease in absorbance at 340 nm, 30°, in solutions also containing 0.5 mM sodium pyruvate and 0.1 mM NADH [13]. The results are expressed in U/5 mL relative to the activity of purified LDH standards. Protein concentrations in D-PBS dosing solutions, in osmotic lysates, or in freeze-thaw lysates (see below) were determined using a modification of the Bradford dye-binding procedure [7] relative to BSA standards.

The lysing solution for total glutathione ([GSH + GSSG]) analysis was 5 mL of 5% TCA; total glutathione was determined using the glutathione recycling assay developed by Tietze [14]. In some experiments, the intracellular GSH and GSSG concentrations, and the concentrations of these same compounds released into the overlaying dosing solutions, were determined separately using the CM-DNP Procedure/SAX-HPLC methodology previously described (free sulfhydryls first carboxymethylated, then all primary amines dinitrophenylated; [7]). In these experiments, the isolated dosing solution was adjusted to 10% PCA and the cell lysing solution was 10% PCA.

The lysing solution for GSNO analysis was 5 mL of 5 mM acetic acid, pH 3.5. A portion of the lysing solution was octanol-extracted, and the isolated aqueous phase was subjected to the RP-HPLC method for quantifying the GSNO yield as previously described [7].

Azo dye-sensitive material was determined using the mercuric chloride/azo dye assay previously described [7]. In these experiments, the flasks containing the cells and the dosing solution were frozen by placement on CO_2 pellets at the requisite times and subsequently thawed (freeze-thaw lysates); the recovered suspensions were clarified by centrifugation and aliquots of the supernatants were utilized in the assay. Aliquots of these same supernatants were analyzed for nitrite and nitrate anion yields using the SAX-HPLC methodology previously described [7] and for protein content as noted above.

The lysing solution for NADPH analysis was 5 mL of 0.33 M KOH in 30% ethanol solution [15]. NADPH was recovered in the aqueous phase of octanol extracts (500 μL of 0.33 M KOH in 30% ethanol lysing solution sample into 300 μL of 0.5 M KH_2PO_4, pH 4.25, plus 1.0 mL octanol) and quantified on a μBondapac C-18/Radial-Pak HPLC column (8 × 100 mm; Waters Chromatography Division, Millipore Corp., Milford, MA) equilibrated with 10 mM K_2HPO_4, pH 7.0, and eluted at a flow rate of 3.0 mL/min; sample injection volume was 50 μL. The elution profile was monitored at 334 nm. The system was programmed to effect a 0–60% linear methanol gradient in 10 mM K_2HPO_4, pH 7.0, buffer over min 4.5 to 6.5 of the elution process to clear the column of other sample solutes, followed by 4 min of equilibration buffer. The NADPH retention time was 5.3 min.

Preparation of Rat Liver Microsomes and Glutathione Transferase Isoenzymes/Analytical Procedures

Phenobarbital-induced female Sprague–Dawley rat liver microsomes were prepared and microsomal inhibition stud-

ies were carried out essentially as previously described [16] but in the present experiments with the inclusion of an NADPH-recycling system (1.0 mM NADPH, 10 mM trisodium isocitrate, 10 mM $MgCl_2$, and 0.5 U of isocitric dehydrogenase/mL), and also 100 U/mL catalase and 64 U/mL superoxide dismutase. The isolation of rat glutathione transferase isoenzymes 3-4 and 4-4 from liver cytosol as highly enriched fractions and the analysis of isoenzyme activity as a function of included inhibitor concentration were carried out as described in an earlier report [5].

RESULTS

The volume of the various dosing and lysing solutions in all of the rat hepatocyte experiments was 5.0 mL overlaying plated cells seeded at 3.33×10^6 live cells/flask. Solute concentrations delivered to, and detected in, these solutions are expressed in micromolar units. The [GSH + GSSG] concentration assessed in 5% TCA lysate solutions from non-pretreated, control hepatocyte preparations was in the range of 27 to 35 μM or 40 to 52 nmol/10^6 cells seeded.

CyanoDMNG Processing by Rat Hepatocytes in Primary Culture and the Effect of GSH Depletion

CyanoDMNG is stable in the D-PBS dosing solution (2% loss over 60 min, 37°). Applying 50 μM CyanoDMNG to hepatocyte cultures resulted in a steady loss of the nitroso compound from the dosing solution volume with the concurrent, and nearly quantitative, production of the denitrosated product CyanoDMG (Fig. 1A). In the illustrated experiment, a 40 μM concentration of the nitroso compound had been processed by the hepatocytes during the 60 min of observation. The total glutathione ([GSH + GSSG]) in these cultures (assessed as 32 μM in zero time cell lysates) decreased approximately 12% (4 μM) during this period. It should be noted that the D-PBS dosing solution does not contain the nutrients required for *de novo* glutathione synthesis.

The glutathione levels were reduced in the hepatocytes by treatment with 1.0 mM DEM during the 30 min immediately prior to nitroso compound application. DEM depletes glutathione via the formation of glutathione conjugates, a process that is rate enhanced by cytosolic glutathione transferases [17, 18]. It has been shown that DEM will reduce substantially the levels of cytosolic GSH in rat hepatocytes with minimal concurrent effect on mitochondrial glutathione levels (generally representing 10–15% of the total cellular glutathione) [19]. In the experiment illustrated in Fig. 1B, DEM treatment depleted hepatocyte GSH to about 10% of non-treated control levels. These cultures were found to be incapable of processing the applied 50 μM CyanoDMNG during the 60-min observation period. An assessment of hepatocyte damage based on the release of the intracellular enzyme LDH into the dosing solution overlaying control and DEM-treated cultures indicated that the pretreatment procedure had no effect on this measure of cell viability during the subsequent 60 min (see Fig. 5C, below).

Overnight incubation in nutritional medium containing 0.5 mM BSO, a compound that inhibits the *de novo* synthesis of glutathione by interacting with γ-glutamylcysteine synthetase [20], depleted the GSH levels in hepatocyte cul-

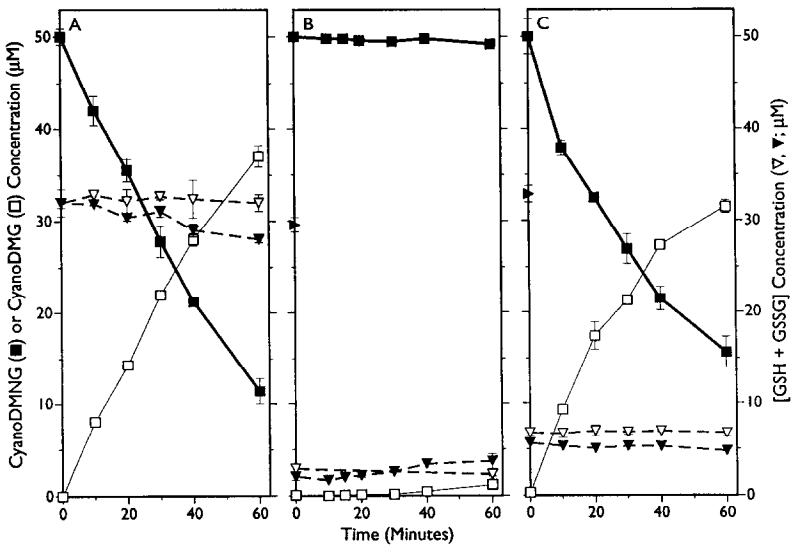

FIG. 1. Intact CyanoDMNG concentration (■; ——) and the concentration of the denitrosation product, CyanoDMG (□; ——) detected in the dosing solution overlaying primary rat hepatocyte cultures at times after applying 50 μM CyanoDMNG. (A) no pretreatment; (B) cultures pretreated with 1.0 mM DEM for the 30 min prior to dosing; (C) cultures pretreated overnight with 0.5 mM BSO. Indicated are [GSH + GSSG] concentrations (– – –; ▽, control cultures; ▼, CyanoDMNG-treated cultures) detected in the several 5% TCA cell lysates. Also indicated in panels B and C are the [GSH + GSSG] concentrations in parallel, non-pretreated, control cultures (▶). All data points are means ± SD, N = 3. Error bars not evident are smaller than the data symbol.

tures to about 20% of control levels (Fig. 1C). Application of 50 μMCyanoDMNG to the BSO-treated cultures resulted in a slight decrease in the rate of CyanoDMNG processing relative to non-pretreated controls (Fig. 1A), but the essentially quantitative denitrosation of the compound was sustained.

Evidence for Glutathione Transferase Involvement in Hepatocyte-Mediated CyanoDMNG Denitrosation

The diuretic drug EA is a competitive inhibitor of glutathione transferases [21, 22]. Figure 2A illustrates the results from *in vitro* experiments that demonstrate the capacity of EA to inhibit the CyanoDMNG denitrosation reaction catalyzed by mu class glutathione transferase isoenzymes 3-4 and 4-4 isolated from rat liver. The analogous experiments using the benchmark glutathione transferase substrate CDNB are summarized in Fig. 2B. At high input concentrations, EA can also serve as a mu class glutathione transferase substrate [5, 23]. EA–glutathione conjugation results in an optical density increase at 295 nm, the wavelength monitored in the kinetics assay for glutathione transferase-mediated CyanoDMNG denitrosation and characterized as a decrease in optical density [5]. At the applied concentrations at which the EA conjugation reaction proceeds at a significant rate, the CyanoDMNG kinetics assay is thus corrupted; the upturn in the inhibitor titration curve shown in Fig. 2A reflects this condition. Important in the evaluation of the hepatocyte experiments described below is the fact that EA is an inhibitor of the relevant mu class glutathione transferase isoenzymes at much lower concentrations than required for it to serve as a substrate for these same isoenzymes.

A series of hepatocyte cultures were treated with a range of EA concentrations concurrently with 50 μM CyanoDMNG (Fig. 2C); a 50% diminution of the CyanoDMG yield at 10 min, relative to EA-free controls, was estimated to occur at an EA concentration of 65 μM. At this level of EA treatment, the interpolated cellular glutathione level represented 85% of that detected in EA-free cultures. Noting that CyanoDMG production at this same applied 50 μM concentration of CyanoDMNG was little perturbed even when the glutathione levels in hepatocytes were reduced to 20% of control levels (BSO experiment; Fig. 1C), the present result suggests that the effect of EA on CyanoDMG production was due to the inhibition of glutathione transferase isoenzymes. We submit that the decrease in cellular glutathione levels detected in this series of cultures at the higher concentrations of applied EA (Fig. 2C) was probably due to EA conjugation reactions.

The capacity of rat liver microsomes to utilize CyanoDMNG as substrate in an NADPH-dependent process has been noted [16]. In this case, the CyanoDMG yield represents 50–70% of the CyanoDMNG decomposed and nitrite is a major product. In the present experiments, we found that EA is a weak inhibitor of the microsomally mediated conversion of CyanoDMNG to CyanoDMG;

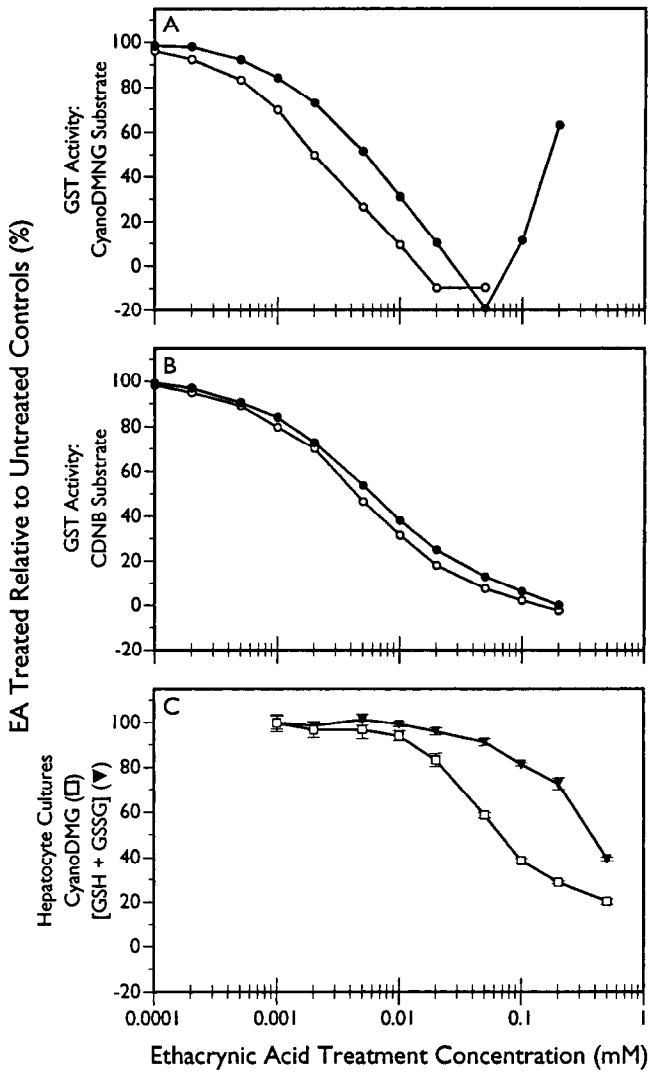

FIG. 2. (A and B) Inhibition of glutathione transferase mu isoenzyme activity (rat GST 3-4, ○; rat GST 4-4, ●) as a function of included EA concentration, represented as a percentage relative to EA-free controls, utilizing 1.0 mM CyanoDMNG as substrate (panel A; measured as a decrease in absorbance at 295 nm), or 1.0 mM CDNB as substrate (panel B; measured as an increase in absorbance at 340 nm) and 1.0 mM GSH cosubstrate. Each data point in panels A and B represents one quantitative assessment. The specific activities of the GST 3-4 and GST 4-4 preparations (in the absence of EA) at 30° in pH 7.5 phosphate buffer [5] were 58.7 ± 0.3 and 66.4 ± 0.3 μmol CyanoDMNG utilized/min/mg protein, respectively, and 82.9 ± 1.4 and 54.0 ± 0.1 μM CDNB utilized/min/mg protein, respectively (mean ± SD, N = 3). (C) CyanoDMNG concentrations (□) detected in the dosing solutions of primary rat hepatocyte cultures 10 min after applying 50 μM CyanoDMNG coincidentally with EA treatment and the corresponding [GSH + GSSG] concentrations (▼; expressed in glutathione units) detected in cell lysates. The data are represented as percentages relative to untreated culture control values and plotted as a function of the EA treatment concentration; each point represents the mean ± SD, N = 3. In the untreated control cultures, 11.1 ± 0.2 μM CyanoDMG was generated in 10 min and the [GSH + GSSG] at 10 min was 34.0 ± 0.7 μM (mean ± SD, N = 3).

50% inhibition was accomplished at an EA concentration of 10 mM (data not shown). This EA concentration was 150-fold greater than that required to inhibit CyanoDMNG denitrosation mediated by hepatocytes.

Effect of CyanoDMNG Application on Hepatocyte GSH/GSSG Levels

Utilizing the CM-DNP Procedure/SAX HPLC methodology, the concentrations of GSH and GSSG in hepatocyte lysates were assessed at times after treating the cultures with 50 μM CyanoDMNG (Fig. 3). Relative to zero time, GSH decreased 8, 13, and 21% at the 10-, 60-, and 90-min time points, respectively. Experiments presented below suggest that some of this diminution may have been due to the export of glutathione out of the hepatocytes. Over this same time interval, very little change in the intracellular GSSG levels was apparent. Thus, the hepatocytes were capable of converting 40 μM CyanoDMNG to CyanoDMG (Fig. 3) while maintaining an intracellular redox potential similar to that characteristic of the zero time condition.

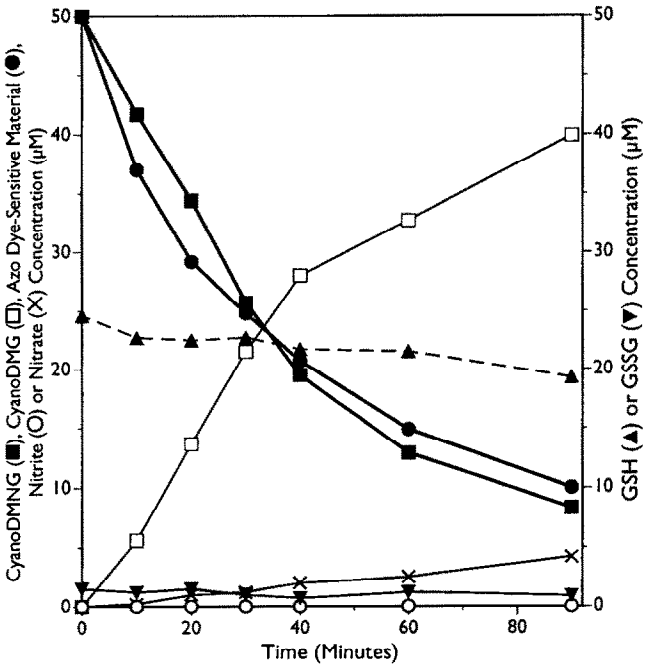

FIG. 3. CyanoDMNG (■) and CyanoDMG (□) concentrations detected in the D-PBS dosing solutions overlaying primary rat hepatocytes at times after applying 50 μM CyanoDMNG as well as the coincident GSH (▲) and GSSG (▼; expressed in glutathione units) concentrations detected in 10% PCA cell lysates using the CM-DNP Procedure/SAX-HPLC methodology. Also indicated are the mercuric chloride/azo dye assay responses of aliquots of the supernatants derived from cultures treated in parallel with CyanoDMNG and then freeze–thaw lysed at the appropriate times (azo dye-sensitive material; ●). Shown as well are the nitrite (○) and nitrate (X) concentrations (SAX-HPLC) detected in other aliquots drawn from these same freeze–thaw lysates. Each point in this figure represents one quantitative assessment.

The CyanoDMG yield generated by hepatocyte cultures appeared to be proportional, and in a 1:1 ratio, to the CyanoDMNG applied up to the 200 μM concentration (Fig. 4A and 4A *Inset*). Upon application of 100 μM and greater concentrations of CyanoDMNG, the total intracellular glutathione levels ([GSH + GSSG]) were observed to be depleted significantly within 10 min, with continued diminution detectable at the 60-min time points. More detailed analysis after application of 200 or 500 μM CyanoDMNG (CM-DNP Procedure/SAX HPLC) indicated increased GSSG in the dosing solution overlaying the hepatocytes (Table 1). Increased GSSG concentrations were also detected in the cell lysates. Cellular redox imbalance at these high concentrations was apparent. (Note that the cells in D-PBS were without a glucose source beginning at 30 min prior to nitroso compound administration and throughout the observation period.) The sum of the GSH and GSSG recovered from these cultures decreased as the concentration of CyanoDMNG applied increased.

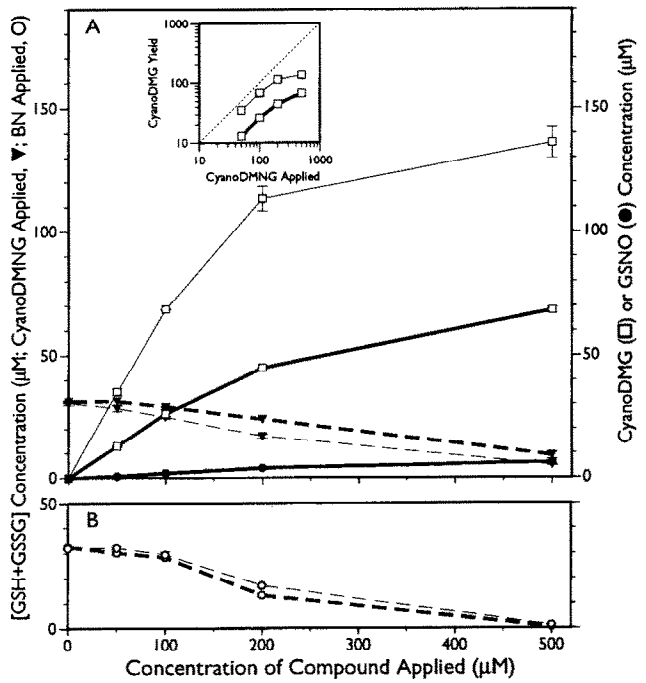

FIG. 4. (A) CyanoDMG (□; ——— / ——) concentrations detected in the D-PBS dosing solutions overlaying primary rat hepatocyte cultures and the coincident [GSH + GSSG] concentrations detected in 5% TCA cell lysates (▼; — — / — —) 10 min (heavy lines) and 60 min (light lines) after treating with a range of CyanoDMNG concentrations. The GSNO yields detected in parallel lysates 10 min after dosing are also indicated (●). All data points represent the mean ± SD, N = 3. Error bars not evident are smaller than the symbol. *Inset:* CyanoDMG yields generated by primary rat hepatocyte cultures 10 min (———) or 60 min (——) after applying various CyanoDMNG concentrations, both represented in μM units. (B) [GSH + GSSG] concentrations detected in 5% TCA cell lysates 10 min (heavy lines) and 60 min (light lines) after treating rat hepatocyte cultures with various concentrations of BN (○). Each point in this figure represents one quantitative assessment.

TABLE 1. GSH and GSSG recovered (μM in 5 mL volume) in the D-PBS dosing solution and in cell lysates at times after treating rat primary hepatocyte cultures with various concentrations of CyanoDMNG*

	D-PBS dosing solution			Cell lysate			
	GSH	GSSG (expressed in glutathione units)	Calculated GSH + GSSG	GSH	GSSG (expressed in glutathione units)	Calculated GSH + GSSG	Calculated total glutathione recovered
Control							
0 min	2.2 ± 0.2†	2.0 ± 0.4	4.2	27.8 ± 1.4	1.9 ± 0	29.7	33.9
10 min	2.5 ± 0.3	1.9 ± 0.2	4.4	26.9 ± 1.2	2.8 ± 0.2	29.7	34.1
60 min	3.6 ± 0	3.0 ± 0.2	6.6	21.2 ± 1.2	2.2 ± 0.4	23.4	30.0
200 μM CyanoDMNG							
10 min	1.1 ± 0.3	3.7 ± 0.4	4.8	15.9 ± 0.8	8.0 ± 0.3	23.9	28.7
60 min	1.8 ± 0.1	7.9 ± 0.5	9.7	5.4 ± 1.5	11.4 ± 1.1	16.8	26.5
500 μM CyanoDMNG							
10 min	0.8 ± 0	4.8 ± 0.3	5.6	2.9 ± 0.6	12.0 ± 1.2	14.9	20.5
60 min	1.1 ± 0	8.0 ± 0.8	9.1	2.5 ± 0.1	8.5 ± 1.5	11.0	20.1

* GSH and GSSG yields were determined using the CM-DNP Procedure/SAX-HPLC method after making the D-PBS solution 10% PCA (the data are corrected for volume change) and after lysing the cells in 10% PCA. The GSH and GSSG yields were calculated relative to appropriately derivatized GSH and GSSG solutions of known solute concentration.

† Mean ± SD, N = 3.

Possible cellular damage due to the applied nitroso compound was evaluated in hepatocyte cultures by monitoring the release of the intracellular enzyme LDH into the dosing solution overlaying the cells during treatment (as well as the LDH present in osmotic lysates). The results, summarized in Fig. 5A, indicate that by this measure and over the 60-min period of observation the applied CyanoDMNG had no detectable effect on hepatocyte integrity.

Control experiments demonstrated that the CyanoDMNG denitrosation product, CyanoDMG, was not catabolized significantly by rat hepatocyte cultures and had little effect on cellular glutathione levels during the 60 min consequent to application of concentrations up to 500 μM (data not shown). Similarly, a related *N*-nitroso compound, DMNU, which is not a substrate for rat liver cytosolic glutathione transferase activities [5], had minimal effect on rat hepatocyte glutathione levels at applied concentrations of up to 500 μM. Over the 60-min observation period, approximately 5% of the applied DMNU had decomposed (data not shown).

Corroborating an earlier report [24], marked depletion of total glutathione occurred in primary hepatocyte cultures treated with the O-nitroso compound BN (Fig. 4B). This loss appeared to be complete within 10 min of compound application and evidently reflects the short half-life of BN noted previously [7]. As observed in the CyanoDMNG case, BN application did not result in the release of LDH into the dosing solution during the time course of this experiment (Fig. 5B).

Production of GSNO and the Loss of Detectable NO Moiety in CyanoDMNG-Treated Hepatocyte Cultures

GSNO was detected in the lysates recovered from hepatocyte cultures that had been treated with CyanoDMNG. As indicated in Fig. 4A, the GSNO detected after 10 min of treatment was seen to increase with the concentration of CyanoDMNG applied; generally, the yield was in the range of 10% of the CyanoDMG generated in the same cultures.

The GSNO concentrations recovered in hepatocyte lysates as a function of time after application of 200 μM CyanoDMNG are indicated in Fig. 6B. GSNO had reached a concentration of 4 μM in these lysates within 5 min of CyanoDMNG application and remained at this level for the remaining 55 min of observation. During this experiment, 90 μM of the applied CyanoDMNG had decomposed and 90 μM of CyanoDMG was generated at an overall rate of 1.5 μM/min (Fig. 6A). In a parallel set of hepatocyte cultures, the CyanoDMNG dosing solution was replaced with D-PBS after the 20-min time point. The GSNO subsequently recovered in these cultures steadily decreased with time, approaching zero at the 60-min time point. The assessed total glutathione and NADPH concentrations in the hepatocyte cultures decreased by about 40% within the first 10 min of CyanoDMNG treatment and stayed at these levels throughout the remaining treatment period in spite of continuing CyanoDMNG denitrosation (Fig. 6).

It has been demonstrated that GSNO yields a response in the colorimetric, mercuric chloride/azo dye assay that is equivalent to that of an equimolar preparation of sodium nitrite [7]. Also, we have determined that CyanoDMNG produces a colorimetric response equivalent to equimolar sodium nitrite. (Relative to 50.0 μM CyanoDMNG, a 50.0 μM preparation of sodium nitrite yielded values of 51.4 ± 0.5.) In addition, the mercuric chloride in the assay mixture releases NO moiety bound to protein thiols, which, in turn, yields a colorimetric response [25], as does any nitrite in the sample. In the experiment summarized in Fig. 3, a subset of flasks containing hepatocyte cultures was subjected to freeze

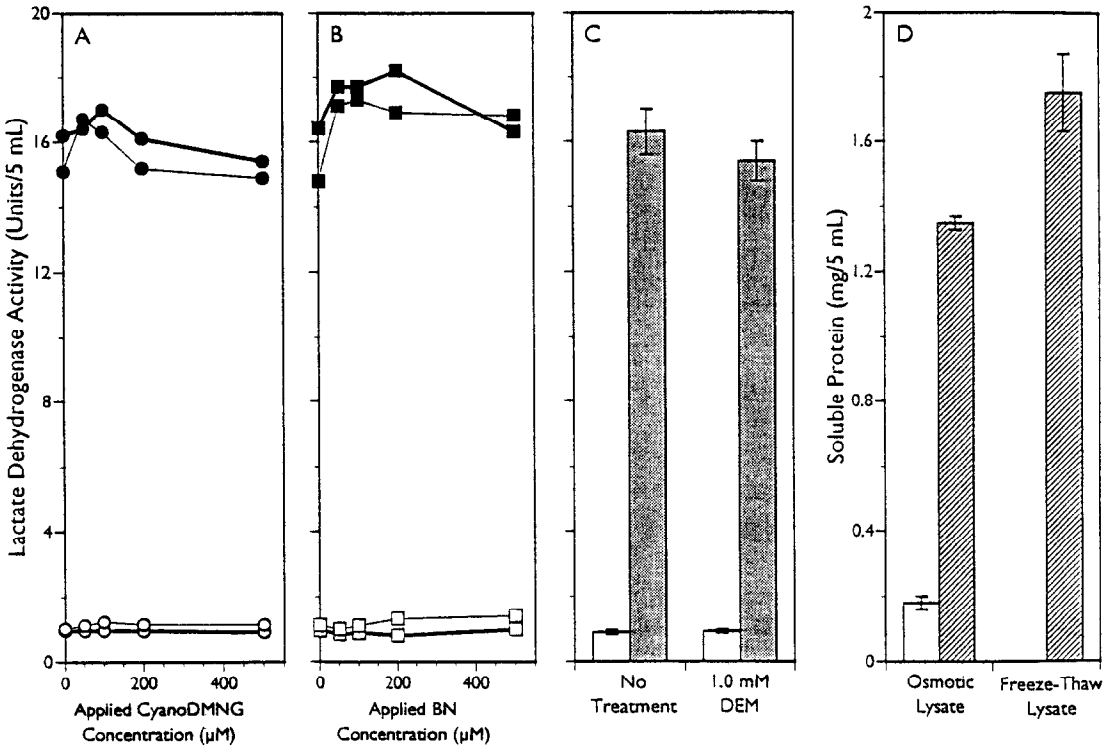

FIG. 5. (A, B, and C) LDH activity detected in primary rat hepatocyte culture D-PBS dosing solutions (open symbols or unfilled bars) and in cell osmotic lysates (closed symbols or filled bars): panels A and B, 10 min (▬) and 60 min (——) after treating with various concentrations of CyanoDMNG (○, ●) or BN (□, ■), respectively (each point in these panels represents one quantitative assessment); panel C, at zero time after treating the hepatocyte cultures with 1.0 mM DEM for the 30 min prior to zero time. (D) Soluble protein detected in untreated hepatocyte culture D-PBS dosing solutions (unfilled bars) and in cell osmotic lysates or freeze–thaw lysates (filled bars); bar heights in panels C and D represent the mean, error bars represent ± SD, N = 4.

–thaw lysis at times after applying 50 μM CyanoDMNG (the cells were thus lysed into the overlaying dosing solution) and the isolated supernatants were submitted to the mercuric chloride/azo dye assay. [Verification of lysis using the freeze–thaw method (the diagnostic enzyme LDH becomes non-functional upon freezing) derives from the control experiments illustrated in Fig. 5D; the soluble protein released into hepatocyte freeze–thaw lysates was observed to approximate the sum of the soluble protein detected in the D-PBS dosing solutions overlaying parallel cultures and the soluble protein detected in the osmotic lysates of these cultures.] It is apparent from the data summarized in Fig. 3 that most of the azo dye-sensitive material detected in the treated cultures represented that due to intact CyanoDMNG. The independently determined nitrite yields (SAX-HPLC) were at all times below detectable levels; the nitrate yields in the cultures accounted for about 10% of the CyanoDMG generated. Thus, most of the NO moiety released from CyanoDMNG disappeared from our assays.

Utilizing the analytical procedures for quantifying the yield of the as yet unidentified coproduct of NADPH-promoted GSNO degradation evident in isolated rat liver cytosol experiments (HPLC of dinitrophenyl derivatives; [7]), we were unable to detect any of this unknown compound in hepatocyte cultures at any concentration of applied CyanoDMNG (data not shown).

DISCUSSION

The present experiments utilizing rat hepatocytes in culture were undertaken to evaluate the contention that glutathione transferases are the primary mediators of the documented *N*-nitrosoguanidinium compound detoxication by denitrosation in rodents. Our results can be interpreted as indicating that this is likely to be the case. The stoichiometric conversion of applied CyanoDMNG to CyanoDMG mediated by hepatocytes indeed characterizes the glutathione transferase-catalyzed CyanoDMNG denitrosation process. Although hepatocytes were capable of sustaining this conversion even when intracellular glutathione levels were reduced substantially, the capacity to catabolize CyanoDMNG was lost in hepatocytes that had been completely depleted of cytosolic glutathione. Also, EA, which proved to be an effective inhibitor of glutathione transferase-mediated CyanoDMNG denitrosation *in vitro*, inhibited the production of CyanoDMG by hepatocytes when applied coincidently with CyanoDMNG.

However, the yield of the coproduct of glutathione trans-

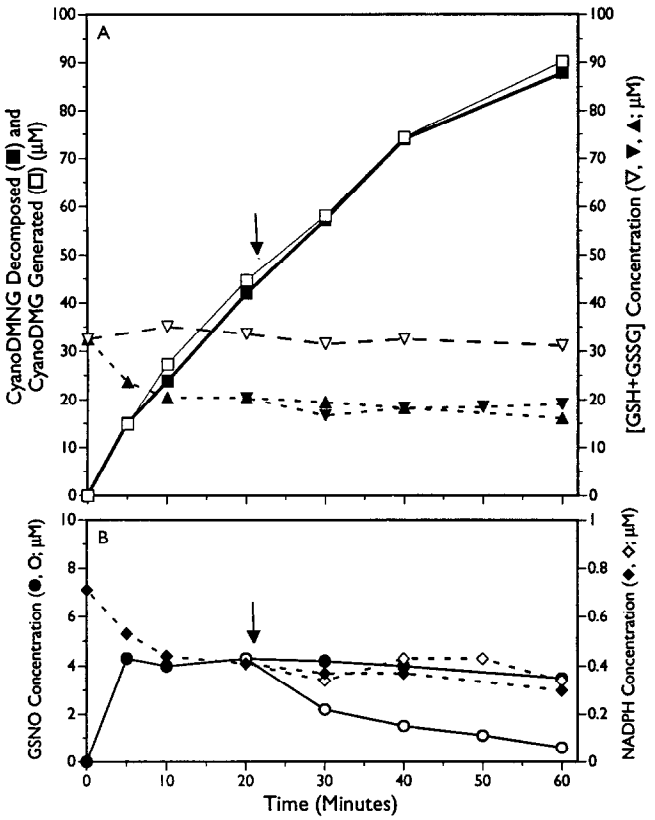

FIG. 6. (A) Concentrations of CyanoDMNG decomposed (■) and CyanoDMG generated (□) as evaluated from the analysis of the D-PBS dosing solutions, and the coincident [GSH + GSSG] concentrations (▲) detected in 5% TCA cell lysates of primary rat hepatocytes at times after treating with 200 μM CyanoDMNG; also indicated are the [GSH + GSSG] concentrations (▼) detected in the cell lysates of parallel cultures from which the dosing solution was withdrawn and replaced with D-PBS just after the 20-min data point cultures were processed (arrow). The [GSH + GSSG] concentrations detected in the lysates of control, non-treated cultures are also indicated (▽). (B) Corresponding GSNO (●) and NADPH concentrations (◆) detected in cell lysates of CyanoDMNG-treated cultures, and in the lysates of parallel cultures (○ and ◇, respectively) from which the dosing solution was withdrawn and replaced with D-PBS just after the 20-min processing period. Each point in these panels represents one quantitative assessment.

ferase-catalyzed CyanoDMNG denitrosation, GSNO, was vanishingly small in hepatocyte cultures treated with low concentrations of CyanoDMNG (≤100 μM) even though a considerable fraction of the applied nitroso compound was denitrosated. In addition, intracellular glutathione levels were little perturbed. It is our hypothesis that the GSNO generated consequent to denitrosation is catabolized by GSNO terminase, an activity first identified in rat liver cytosol [7] and presently being characterized as the purified enzyme.* This activity will utilize either NADH or NADPH as requisite cofactor and generates GSSG and an as yet unidentified glutathione-based coproduct. In cytosol fraction experiments, added NADPH converted the GSSG product to GSH via the action of glutathione reductase. It is envisioned that this conversion replenishes the GSH levels in CyanoDMNG-treated hepatocytes and allows continuing CyanoDMNG denitrosation even at low cytosolic glutathione levels. The unidentified coproduct of GSNO terminase activity was not detected in hepatocyte cultures at any CyanoDMNG treatment concentration. It is possible that this product is subject to processing in hepatocytes that is absent or nonfunctional in isolated cytosol (which represents only the soluble fraction of liver cell lysates and which, in our experiments, is cleared of solutes < 6 kDa).

Another suggestion of GSNO terminase involvement in the hepatocyte-mediated catabolism of applied CyanoDMNG was the observed progressive loss of the NO moiety from our several assays. In our cytosol experiments [7], the NADPH-dependent degradation of GSNO was found not to generate nitric oxide, nitrite, or nitrate and resulted in the concomitant loss of material responsive to the mercuric chloride/azo dye assay (the sensitivity of this assay is known to include GSNO, nitrite, and the NO moiety derived from S-nitrosated proteins). CyanoDMNG denitrosation mediated by hepatocytes also did not generate nitrite, nitrate was a minor product (corresponding to about 10% of the CyanoDMG generated), and essentially all of the treated culture response to the mercuric chloride/azo dye assay reflected the concentration of CyanoDMNG remaining intact.

Verification of GSNO production in CyanoDMNG-treated hepatocytes in yields commensurate with the CyanoDMG concentration detected awaits the development of an appropriate intracellular inhibitor of GSNO terminase activity; this objective is being pursued in our laboratory.

GSNO was readily and reliably detected in hepatocyte cultures treated with high concentrations of CyanoDMNG (≤200 μM). At the 200 μM CyanoDMNG treatment level, the 4 μM GSNO concentration detected at the first 5-min time point was maintained throughout the 60-min observation period in spite of the fact that CyanoDMG was being generated at an overall rate of 1.5 μM/min. (All concentrations were those established in 5 mL dosing or hepatocyte lysate volumes.) We conjecture that the GSNO concentration observed represents the residual concentration coincident to several processes including: GSNO production via glutathione transferase-mediated CyanoDMNG denitrosation; the presumed dynamic NO-exchange involving GSH, other small molecule thiols, and protein thiols [7]; GSNO degradation ultimately producing nitrate; and GSNO degradation via GSNO terminase.

The existence of intracellular GSNO suggests the likelihood that some fraction of the introduced NO moiety will be associated with protein as a consequence of the dynamic NO-exchange process involving GSH and protein thiols

* Jensen DE and Belka GK, manuscript in preparation.

[7]. The nature of this equilibrium remains unknown, and at present our assay for nitrosated protein thiols lacks the requisite sensitivity for the cell culture system. We have determined, however, based on the soluble protein yields recovered in hepatocyte osmotic lysates (1.35 mg/5 mL; Fig. 5) and our measure of 120 nmol of exposed thiols/mg protein (*p*-chloromercuribenzoate titrations [7]), that the concentration of exposed thiols on soluble protein is in the range of 160 mmol/5 mL of lysate or 32 μM. This number is in approximate parity with the assessed GSH concentration in cell lysates. If it is assumed as a first approximation that all thiols are equal in the NO-exchange equilibrium, the yield of nitrosated protein thiols is expected to be significant, perhaps greater than 10% of the total available when GSNO is detected as 4 μM.

Finally, it is clear that hepatocytes treated with high concentrations of CyanoDMNG suffer severe redox imbalance; GSSG appears in the medium and intracellular GSSG levels increase at the expense of GSH. It is possible that this is due, in part, to the fact that in the present protocol the treated hepatocytes were deprived of a nutrient source to compensate for the catabolic effect. We are presently extending this study to consider CyanoDMNG denitrosation mediated by hepatocytes in nutrient medium.

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