ANTIOXIDANTS & REDOX SIGNALING
Volume 2, Number 4, 2000
Mary Ann Liebert, Inc.

Original Research Communication

Glutathione-Dependent Regulation of Nitric Oxide Production in Isolated Rat Hepatocyte Suspensions

M.A. TIRMENSTEIN, F.A. NICHOLLS-GRZEMSKI, T.D. SCHMITTGEN, B.A. ZAKRAJSEK, and M.W. FARISS

ABSTRACT

Freshly isolated suspensions of rat parenchymal liver cells (hepatocytes) spontaneously produce large amounts of nitrite following collagenase isolation. Our previous studies indicate that nitrite production is associated with the expression of inducible nitric oxide synthase (iNOS) and reflects NO production. Depletion of glutathione (GSH) with diethylmaleate (DEM) inhibited nitrite production, and this inhibition was time-dependent. DEM was more effective in blocking nitrite production if it was added within the first 1 hr of the start of the incubation. The reducing agent dithiothreitol (DTT) and the alkylating agent ethyl methanesulfonate (EMS) also inhibited hepatocyte nitrite production, and this inhibition was also greatest if they were added within 1 hr of initiating the incubation. However, EMS added at 3 hr still reduced 6-hr nitrite production by about 70%. This reduction in nitrite production by EMS added at 3 hr may be due to the direct modification of thiol groups on the iNOS protein because we have determined that iNOS activity is inhibited by the sulfhydryl modifying reagent *N*-ethylmaleimide (NEM). Western blots also indicate that the iNOS protein is expressed when EMS is added at 3 hr. The addition of DEM, DTT, or EMS at 0 time greatly reduced the levels of cellular iNOS mRNA relative to controls as determined by quantitative RT-PCR. Based on our results with mRNA levels, both DTT and depletion of cellular GSH appear to inhibit the early signaling events leading to iNOS expression and suggest that the control of iNOS induction in hepatocytes is sensitive to the thiol redox status of the cell. Antiox. Redox Signal. 2, 767–777.

INTRODUCTION

In previous studies conducted in this laboratory, we have demonstrated that isolated rat parenchymal liver cell (hepatocyte) suspensions generate large amounts of nitrite following an initial 3- to 4-hr incubation period (Nicholls-Grzemski *et al.*, 1999). Nitrite represents a stable breakdown product of nitric oxide (NO) and is commonly used to assess NO production *in vitro*. The enzyme nitric oxide synthase (NOS) converts L-arginine to NO. At least three isoforms of NOS are known to exist in tissues. The inducible form of the nitric oxide synthase (iNOS) is expressed in several cell types, including hepatocytes, smooth muscle cells, and macrophages (Harbrecht and Billiar, 1995). In our experiments, the production of nitrite by hepatocyte suspensions was associated with a time-dependent increase in iNOS mRNA, protein, and activity levels (Tirmenstein *et al.*, 2000). The formation of nitrite by hepatocytes was inhibited by iNOS inhibitors aminoguanidine and L-NAME as well as the transcription inhibitor, actinomycin D, and the protein synthesis inhibitor, cycloheximide

Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Pullman, WA, 99164-6534.

(Nicholls-Grzemski *et al.*, 1999; Tirmenstein *et al.*, 2000). These results indicate that nitrite is formed as a result of iNOS expression in hepatocyte suspensions. Hepatocytes were identified as the major source of nitrite production because removal of Kupffer cells after isolation did not significantly lower nitrite production (Nicholls-Grzemski *et al.*, 1999). Recently, the spontaneous production of nitric oxide has also been reported with cultured rat hepatocytes (Lopez-Garcia, 1998; McMillan, 1999).

A number of different exposure treatments have been shown to influence iNOS induction. Endotoxin and cytokines are potent inducers of iNOS and act synergistically to stimulate NO production (Nussler *et al.*, 1993). Oxidative stress has also been proposed to induce iNOS expression in rat hepatocytes (Duval *et al.*, 1995). Jones and Czaja (1998) suggested that NO is generated in liver in response to injury. Hepatotoxicants, ischemia-reperfusion (Jones and Czaja, 1998), and partial hepatectomy (Hortelano *et al.*, 1995) have been shown to increase NO production by the liver. The cause for the spontaneous expression of iNOS in hepatocyte suspensions is not known but is most likely a response to the stresses generated during collagenase isolation procedures. This response appears to be triggered by a complex interaction between several different factors, including Kupffer cell activation, reactive oxygen species (ROS) generation, and endotoxin contamination of collagenase preparations (Tirmenstein *et al.*, 2000).

Vos *et al.* (1999) reported that iNOS expression in hepatocytes isolated from endotoxemic rats was dependent on cellular glutathione (GSH) levels. In this study, rats were treated with endotoxin to induce iNOS expression (Vos *et al.*, 1999). Harbrecht *et al.* (1997) also demonstrated that inhibition of GSH synthesis with buthionine sulfoximine (BSO) inhibited the induction of iNOS in hepatocytes. In contrast, Kang *et al.* (1999) reported that depletion of GSH *in vivo* with diethylmaleate (DEM) inhibited iNOS expression in the livers of endotoxemic mice, but BSO did not. In addition to regulating iNOS expression, GSH is known to react with NO under aerobic conditions to produce *S*-nitrosoglutathione, which can transnitrosylate thiol groups on proteins and modulate enzyme activity (Wink *et al.*, 1994). These data suggest that GSH and NO metabolism are not independent but instead interact with one another in the cell.

The activation of transcription factor nuclear factor-κB (NF-κB) is generally believed to be required for iNOS expression in hepatocytes (Vos *et al.*, 1999). NF-κB is present in the cytoplasm bound to the inhibitor IκB. Upon induction, IκB is phosphorylated and/or rapidly degraded. After the dissociation of IκB, NF-κB is translocated into the nucleus where it binds to DNA and activates target genes such as the iNOS gene (Sun and Oberley, 1996; Vos *et al.*, 1999). There is increasing evidence that NF-κB activation is redox sensitive and may be influenced by cellular levels of reduced GSH and oxidized glutathione (GSSG) (Sun and Oberley, 1996). In experiments conducted by Galter *et al.* (1994), GSSG was shown to inhibit the binding of NF-κB to DNA in human T-lineage cells (Molt-4).

In the following study, we have used spontaneously NO-producing hepatocyte suspensions as a model system to investigate the involvement of GSH and changes in cellular redox status on the control of NO formation and iNOS expression in hepatocytes. NO production has been shown to influence many biochemical parameters (Harbrecht and Billiar, 1995). Therefore, it becomes important to characterize further the factors responsible for the regulation of iNOS expression in hepatocytes. The use of our model system allows us easily to examine how the timing and extent of GSH depletion affects the spontaneous production of NO in hepatocyte suspensions and to determine if the control of iNOS expression of GSH is similar to those reported in cultured hepatocytes or other cell types. Most other studies examining the regulation of iNOS expression in hepatocytes by GSH have been conducted in cultured cells exposed to cytokines (Duval *et al.*, 1995; Harbrecht *et al.*, 1997) or in cultured hepatocytes isolated from rats treated with endotoxin (Vos *et al.*, 1999). Our results extend these findings, and indicate that GSH is an important determinant of iNOS expression in hepatocyte suspensions.

MATERIALS AND METHODS

Chemicals

2,3-Diaminonaphthalene was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Collagenase (type L), the components of modified Waymouth's medium, DEM, *N*-ethylmaleimide (NEM), dithiothreitol (DTT), ethyl methanesulfonate (EMS) and all other chemicals were obtained from Sigma (St. Louis, MO).

Preparation of isolated hepatocyte suspensions

Adult male Sprague-Dawley rats (180–220 grams) were obtained from Simonsen Laboratories Inc, (Gilroy, CA), housed in small groups and given food and water *ab libutum* in a 12-hr light/dark cycle for at least 1 week prior to use. All procedures involving animals were approved by the Washington State University Animal Care and Use Committee. Rat hepatocytes were prepared by collagenase perfusion as previously described (Fariss *et al.*, 1985). Briefly, the liver was cannulated via the portal vein, surgically removed, and perfused in a recirculating system at 30 ml/min for 3 min with Hanks'-bicarbonate buffer containing 0.6 m*M* EGTA. The liver was then perfused in a recirculating system with Hanks'-bicarbonate buffer containing 3 m*M* calcium chloride and 60–100 mg of standard collagenase (Sigma L blend) for up to 10 min. After dispersion and washing, cells were assessed for viability by Trypan blue exclusion. A yield of 4.5–5.5 $\times$ 10^8 was routinely obtained with over 93% viability. Hepatocyte suspensions were prepared as described in Fariss *et al.* (1997). Briefly, hepatocyte suspensions (2 $\times$ 10^6 cells/ml, 12 ml total) were prepared in serum-free modified Waymouth's medium as previously described Farris *et al.* (1997) with the addition of 0.12 m*M* ornithine but without cystine and methionine. Cells were placed in 125-ml boiling flasks and rotated at 37°C under ambient air. After a 15-min equilibration time, an aliquot of cells was taken as the zero timepoint. Following collection of the zero timepoint, the chemical treatments were immediately added. At hourly intervals thereafter, cells were sampled and pelleted, and supernatants were collected for nitrite and lactate dehydrogenase (LDH) leakage analysis. Cell pellets were stored at −80°C for iNOS Western blot analysis and quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

Enzyme assays

Hepatocyte calcium-independent NOS (iNOS) activity was determined by measuring the formation of nitrite as previously described (Nicholls-Grzemski *et al.*, 1999). LDH activity was determined by monitoring the enzymatic formation of NADH from NAD^+ in the presence of L-lactic acid. Post-centrifugation supernatants were diluted 1:25 with phosphate-buffered saline (PBS), pH 7.4. A 100-μl aliquot was mixed with 100 μl of reagent to give a final concentration of 3.75 m*M* NAD^+ and 25 m*M* L-lactic acid in 125 m*M* Tris-HCl buffer, pH 8.9, in a 96-well plate. The increase in absorbance at 340 nm due to the formation of NADH was immediately monitored at room temperature using a Biorad Benchmark plate reader and analyzed Microsoft Manager III data analysis software (Hercules, CA). The percent LDH leakage was calculated by comparing values to total LDH activity. Total LDH was measured from a sample of hepatocytes collected at zero time and lysed with a final concentration of 0.2% Triton X-100.

Nitrite determinations

Nitrite levels in media were determined with 2,3-diaminonaphthalene according to the procedures of Misko *et al.* (1993) with slight modifications as previously described (Nicholls-Grzemski *et al.*, 1999). Nitrite standards were prepared fresh and standard curves were constructed in the same media as samples. Samples and standards were read at 360 nm ex and 460 nm em with a gain setting of 90 on a CytoFluor 4000 fluorescence plate reader, PerSeptive Biosystems (Framingham, MA). In some cases, nitrite levels were determined by adding 100 μl of samples to an equal volume of the Griess reagent (Green *et al.*, 1982), [0.5% sulfanilamide, 0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid] and read at 550 nm after 10 min. Stan-

dards were prepared with sodium nitrite in the same media as samples.

GSH determinations

GSH and nitrosoglutathione (GSH-NO) levels in hepatocytes were determined using a modification of the method of Saville (1958) to accommodate a 96-well colorimetric assay. At each indicated timepoint, about 4×10^5 of cells were collected, mixed with 0.2 ml of 10% trichloroacetic acid (TCA) and stored at −20°C. Samples were centrifuged at 16,000 × *g* for 5 min. In a 96-well plate, 50 μl of 1 m*M* sodium nitrite in 0.54 *M* sulfuric acid was added to 20 μl of supernatant. For GSH-NO assays, 0.54 *M* sulfuric acid was added without sodium nitrite. After a 10-min incubation at room temperature, 20 μl of 22 μ*M* ammonium sulfamate was added and gently agitated to mix. After an additional 5 min, 100 μl of 10 m*M* mercuric chloride and 0.15 *M* sulfanilamide in 0.25 *M* hydrochloric acid were added followed by 80 μl of 4 m*M* (*N*-(1-naphthyl)ethylenediamine dihydrochloride. Absorbance values were measured at 550 nm after 10 min on a Biorad Benchmark plate reader.

Real-time quantitative RT-PCR

The amount of iNOS mRNA relative to the β-actin endogenous control was determined using real-time, quantitative PCR as previously described (Tirmenstein *et al.*, 2000). Total cellular RNA was isolated from the hepatocytes using the RNeasy Mini, RNA isolation kit from Qiagen (Valencia, CA) per the manufacturer's protocol, and residual genomic DNA was removed by incubating the RNA solution with 15 units of RNase-free DNase I in 2 m*M* $MgCl_2$ for 10 min at 37°C followed by 5 min at 90°C to inactivate the DNase. DNase-treated RNA solution (25 μl) was reverse transcribed to cDNA as previously described (Horikoshi *et al.*, 1992).

The sequence of the β-actin primers was 5′-ACCAACTGGGACGATATGGAGAAGA-3′ bases 1,557–1,581 forward primer and 5′-TACGACCAGAGGCATACAGGGACAA-3′ bases 2210–2234 reverse primer of the genomic β-actin sequence (Nudel *et al.*, 1983). The sequence of the iNOS primers was 5′-CACGACACCCTTCACCACAAG-3′ bases 301–321, forward primer and 5′-TTGAGGCAGAAGCTCCTCCA-3′ bases 419–438, reverse primer of the iNOS cDNA sequence (Kosuga *et al.*, 1994). Real-time quantitative PCR was performed in the PE Biosystems GeneAmp 5700 Sequence Detection System (Foster City, CA) using the SYBR green PCR kit as recommended by the manufacturer (PE Biosystems, Foster City, CA). The amount of iNOS mRNA relative to the β-actin was determined as previously described (Tirmenstein *et al.*, 2000).

Western blot analysis of iNOS

Western plots were performed as previously described (Tirmenstein *et al.*, 2000). Briefly, hepatocyte pellets were resuspended in PBS, pH 7.4, and protein levels were determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL). Expression of iNOS protein was detected with rabbit anti-mouse antibody (Transduction Laboratories, Lexington, KY). Specific immunocomplexes were detected using peroxidase-labeled anti-rabbit antibody with the ECL Western Blot Analysis System Amersham Pharmacia (Piscataway, NJ). Developed blots were immediately exposed to Kodak X-Omat scientific imaging film, and both bands were quantified together using a Hewlett-Packard ScanJet 4C/T (Palo Alto, CA) equipped with RFLPscan version 2.01 software, Scanalytics (Billerica, MA).

Statistics

Results are presented as means ± SD. Analysis of variance (ANOVA) followed by Dunnett's *post hoc* test were performed with the InStat 2.03 GraphPad Software Inc. (San Diego, CA) statistical package.

RESULTS

The effects of the GSH depletor DEM on spontaneous nitrite production in isolated hepatocyte suspensions is reported in Figure 1. In our studies, rat hepatocytes were isolated from rats by established collagenase perfusion techniques. Following isolation, nitrite levels were measured in the extracellular media as a measure of NO production. There was a clear

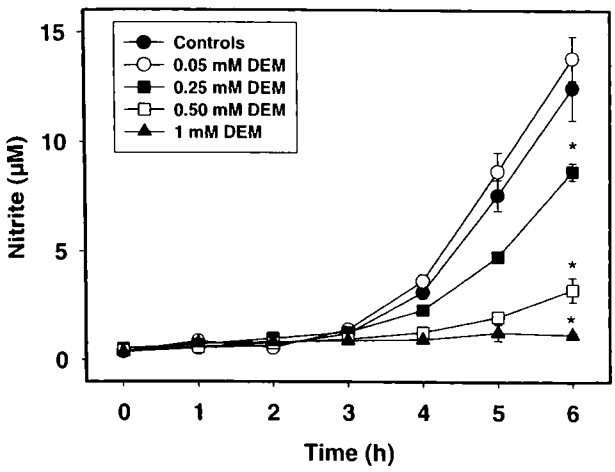

FIG. 1. Effect of DEM concentration on nitrite production by hepatocytes suspensions. The indicated concentrations of DEM were added at zero time, and extracellular nitrite levels were monitored over time. Control hepatocytes represent no additions. Values represent mean ± SD (n = 3 for all determinations). *Significantly different from 6-hr controls ($p < 0.05$) as determined by ANOVA followed by Dunnett's *post hoc* test.

dose–response relationship with DEM treatment in which the addition of 1 m*M* DEM at the start of the incubation (0 time) completely blocked nitrite production throughout the course of the incubation, whereas lower doses (0.5 and 0.25 m*M*) attenuated nitrite production and the addition of 0.05 m*M* DEM had no significant effect. The inhibitory effects of DEM on nitrite production were not due to cell death. The concentrations of DEM used in this study did not significantly increase cell death as measured by LDH release or lipid peroxidation. In all of our experiments, control hepatocytes were typically 70% viable after a 6-hr incubation, and none of the treatments used in this study (DEM, DTT, or EMS) decreased cellular viability to below 65% after 6 hr (data not shown).

Various concentrations of DEM were added to hepatocytes at the start of the incubation, and GSH and nitrite levels were measured over time. We found that GSH levels at 1 hr were a good determinant of 6-hr nitrite levels (Fig. 2). In an attempt to clarify the relationship between GSH levels and NO production, we plotted 1-hr GSH levels versus 6-hr nitrite levels (Fig. 2). Our results indicate that depletion of GSH levels to below 4 nmol/10^6 cells at 1 hr completely inhibited nitrite production. If GSH levels were above 15 nmol/10^6 cells at 1 hr, there was no effect on nitrite production. GSH values from 4–15 nmol/10^6 cells at 1 hr produced intermediate 6-hr nitrite levels (Fig. 2). In control cells, GSH levels decreased over time and were measured at 10.6 ± 1.61 nmol/10^6 cells (n = 3) at 6 hr. Levels of GSH-NO in control cells were below the limits of detection (1 nmol/10^6 cells) at 0, 2, and 4 hr. After 6 hr, GSH-NO levels were 1.66 ± 0.14 nmol/10^6 cells (n = 3) or about 16% of the total GSH present at 6 hr.

The effects of the timing of GSH depletion on nitrite production were examined by adding 1 m*M* DEM at 0, 1, 2, and 3 hr after the initiation of the hepatocyte incubations. Because increases in nitrite production were first detected at 3–4 hr, these timepoints all preceded nitrite production by hepatocytes. The effects of adding 1 m*M* DEM at these times on hepatocyte GSH levels are shown in Fig. 3. The 1 m*M* DEM concentration produced almost complete (90–95%) depletion of GSH in hepatocytes within 1 hr of addition (Fig. 3). In our experiments, hepatocytes were incubated in Waymouth's medium lacking methionine, cysteine, and cystine to prevent the resynthesis of cellular GSH. The effects of time of addition of DEM on hepatocyte nitrite production are reported in Fig. 4. The time of addition of DEM had a large effect on subsequent nitrite production.

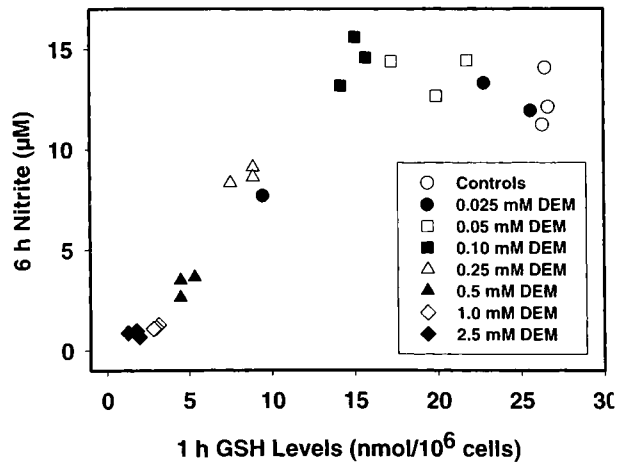

FIG. 2. Relationship between 1-hr GSH levels and 6-hr nitrite production in hepatocyte suspensions. Hepatocytes were treated with the indicated concentrations of DEM at zero time and the 1-hr GSH levels and 6-hr extracellular nitrite levels were subsequently determined. Each point represents a separate incubation (n = 24).

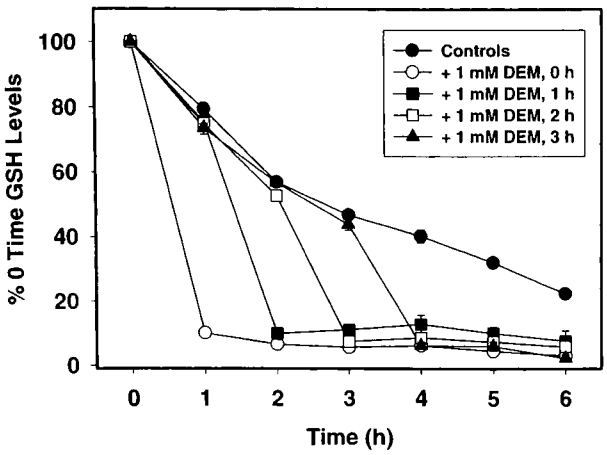

FIG. 3. DEM-dependent depletion of GSH in hepatocyte suspensions. Hepatocytes were treated with 1 m*M* DEM at 0, 1, 2, and 3 hr after incubations were initiated. Control hepatocytes represent no additions. At the indicate timepoints GSH levels were determined. Values represent mean ± SD ($n = 3$ for all determinations).

The addition of DEM at 0 or 1 hr had a much greater influence on nitrite production than the addition of DEM at 2 or 3 hr (Fig. 4). When 1 m*M* DEM was added at zero time, 6-hr nitrite levels were reduced by about 90% of controls. The addition of 1 m*M* DEM at 3 hr only reduced 6-hr nitrite levels by about 25% of controls.

We also examined the influence of the reducing agent DTT on nitrite production in hepatocytes (Fig. 5). In these experiments, nitrite production was measured by the Griess reagent because the 1 m*M* DTT treatment interfered with the fluorometric determination of nitrite by 2,3-diaminonaphthalene as previously reported by Miles *et al.* (1996). As with DEM, the addition of 1 m*M* DTT had a greater influence on hepatocyte nitrite production when it was added at zero time than at 3 hr.

The effects of the alkylating agent EMS on nitrite production were also examined (Fig. 6). EMS has been shown to alkylate thiol groups and deplete GSH and protein thiol levels in hepatocyte suspensions (Fariss *et al.*, 1997). The addition of 10 m*M* EMS at zero time completely blocked nitrite production by hepatocytes. This concentration of EMS depletes cellular GSH to less than 3% of 0 time values within 1 hr of addition but did not significantly increase cell death or lipid peroxidation at 6 hr regardless of when it was added (data not shown). The effects of EMS on nitrite production are similar to those of DEM. However, the time in which EMS was added had less of an influence on nitrite production than DEM (Fig. 4). The addition of 10 m*M* EMS at 3 hr still produced an almost 70% decrease in nitrite production by hepatocytes. We used Western blot analysis to determine if this loss of nitrite production was due to a decrease in iNOS protein expression. The iNOS protein levels collected following a 6-hr incubation were easily measured in control hepatocytes and hepatocytes treated with EMS or DEM at 3 hr. The addition of DEM or EMS at 3 hr reduced the intensity of the 6-hr iNOS band by about 30% and 50%, respectively, from control bands, but this decrease in intensity was not significantly different from controls (data not shown).

Hofmann and Schmidt (1995) reported that protein thiol reagents inactivate the neuronal form of NOS. To test whether iNOS could also be inactivated by protein thiol modification, postmitochondrial fractions were prepared as previously described (Nicholls-Grzemski *et al.*, 1999) from hepatocytes suspensions producing nitrite (5 hr incubation). Postmitochondrial fractions were used because the iNOS protein is located in the cytoplasm. Following isolation,

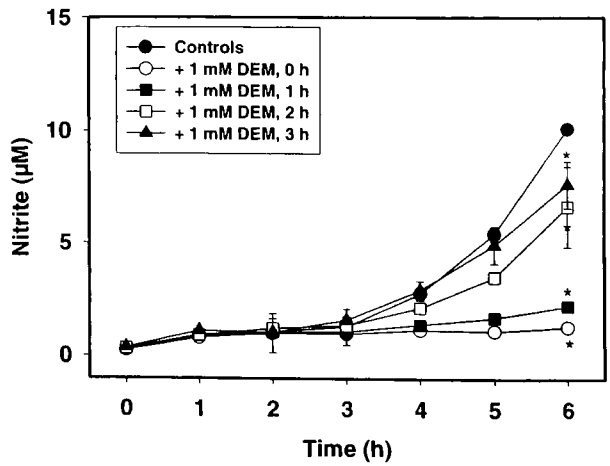

FIG. 4. Time-dependent effect of DEM on nitrite levels in hepatocyte suspensions. At the indicated times, 1 m*M* DEM was added to the incubations, and extracellular nitrite levels were measured. Controls contained no additions. Values represent mean ± SD ($n = 3$ for all determinations). *Significantly different from 6-hr controls ($p < 0.05$) as determined by ANOVA followed by Dunnett's *post hoc* test.

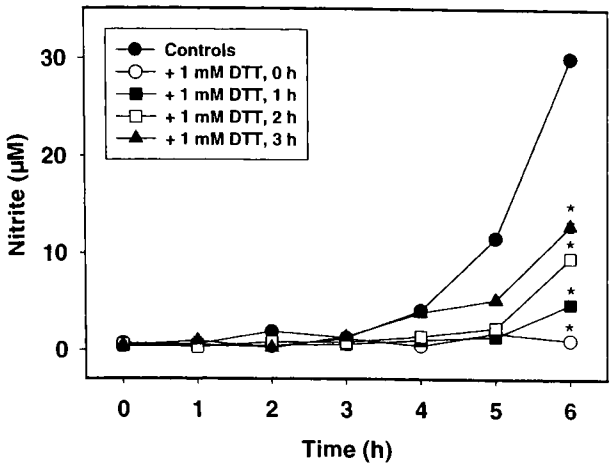

FIG. 5. Time-dependent effect of DTT on nitrite levels in hepatocyte suspensions. At the indicated times, 1 m*M* DTT was added to the incubations and extracellular nitrite levels were measured. Controls contained no additions. Values represent mean ± SD ($n = 3$ for all determinations). *Significantly different from 6-hr controls ($p < 0.05$) as determined by ANOVA followed by Dunnett's *post hoc* test.

fractions were preincubated with the thiol reactive compound NEM at a concentration of 0.5 m*M* for 15 min at 37°C and then assayed for iNOS activity (nitrite production). Preincubation with 0.5 m*M* NEM for 15 min inhibited iNOS activity by about 50%.

Previous studies have shown that iNOS mRNA peaks in hepatocyte suspensions 4 hr after the initiation of the incubations (Tirmenstein *et al.*, 2000). As Fig. 7 indicates, the addition of 1 m*M* DEM, 1 m*M* DTT, or 10 m*M* EMS at zero time dramatically reduced iNOS mRNA production measured after a 4-hr incubation. At the concentrations used in this study, EMS and DEM reduced iNOS mRNA about 20-fold over controls whereas DTT reduced iNOS mRNA about 800-fold.

DISCUSSION

Our results suggest that cellular levels of GSH are an important determinant of iNOS expression in rat hepatocytes. DEM depletes GSH by conjugating GSH through a glutathione transferase-catalyzed reaction. EMS, in contrast, is an alkylating agent that depletes GSH by alkylating GSH and forming *S*-ethyl glutathione (Fariss *et al.*, 1997). Both DEM and EMS inhibited nitrite production by hepatocyte suspensions when they were added at zero time, and our results indicate that this inhibition occurs prior to the transcription of iNOS mRNA (Fig. 7). Similar results were reported by Duval *et al.* (1995) and Harbrecht *et al.* (1997), with both groups reporting that GSH depletion inhibited iNOS mRNA formation in cultured rat hepatocytes. It seems unlikely that GSH-NO is involved in regulating the expression of iNOS because GSH-NO levels are very low and were only detectable several hours following NO production. Although we were able to measure GSH-NO levels at 6 hr of incubation (1.7 nmol/10^6 cells), this time point is after iNOS mRNA is expressed (4 hr). Our results with hepatocyte suspensions spontaneously producing NO agree well with those obtained with cultured hepatocytes and suggest that this model system may be useful for examining the control of iNOS expression in hepatocytes.

The addition of DEM at 2 or 3 hr after the initiation of hepatocyte incubations was not as effective as adding DEM at 0 or 1 hr in inhibiting nitrite production (Fig. 4). In previous studies, we have determined that iNOS mRNA formation is first detectable 2 hr after the start of hepatocyte incubations and peaks at 4 hr. Also, iNOS protein levels are detectable 3 hr after the start of the incubation and peak at 7 hr (Tirmenstein *et al.*, 2000). The time dependency

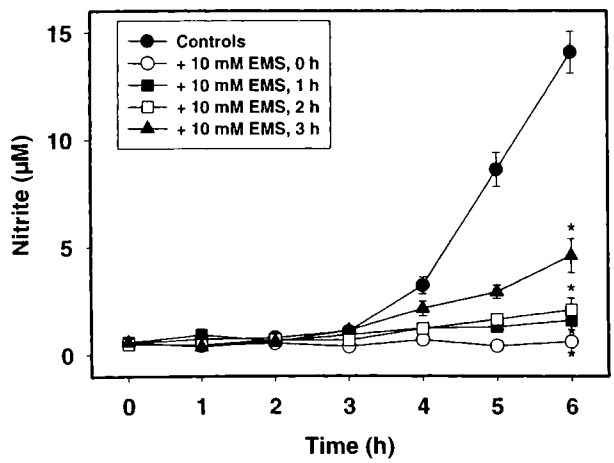

FIG. 6. Time-dependent effect of EMS on nitrite levels in hepatocyte suspensions. At the indicated times, 10 m*M* EMS was added to the incubations, and extracellular nitrite levels were measured. Controls contained no additions. Values represent mean ± SD ($n = 3$ for all determinations). *Significantly different from 6-hr controls ($p < 0.05$) as determined by ANOVA followed by Dunnett's *post hoc* test.

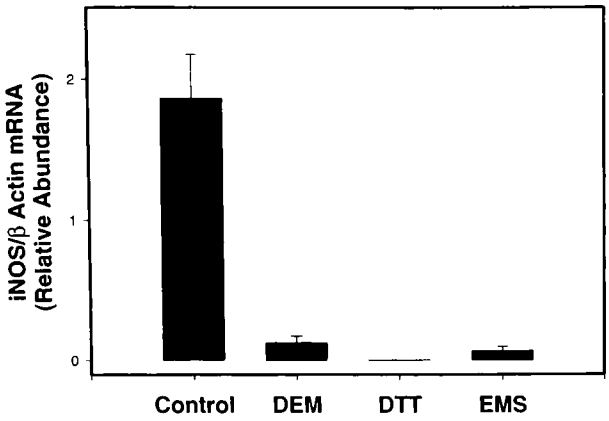

FIG. 7. Effects of thiol-depleting and -reducing agents on iNOS mRNA levels. At zero time, 1 m*M* DEM, 1 m*M* DTT, or 10 m*M* EMS were added to hepatocytes incubations, and 4 hr iNOS mRNA levels were measured by real-time quantitative PCR analysis. iNOS mRNA levels were normalized relative to β-actin. Controls contained no additions. Values represent mean ± SD ($n = 3$ for all determinations).

of DEM-inhibition of hepatocyte nitrite production suggests that DEM inhibits events that occur within 1 hr of the start of the incubation but has little effect on events occurring after 2 hr. Therefore, our results are consistent with the hypothesis that DEM inhibits early events, such as NF-κB activation, leading to iNOS induction. Our results are also for the most part in agreement with Hothersall *et al.* (1997), who demonstrated that GSH levels did not significantly affect NO production in a murine macrophage cell line (J774) after the induction of the iNOS protein.

The alkylating agent EMS yielded similar results to DEM. The addition of 10 m*M* EMS at the start of the incubation depleted GSH and inhibited the transcription of iNOS (Fig. 7). This concentration has been shown to deplete cellular GSH completely but has little effect of cell viability, However, unlike DEM, EMS still reduced nitrite formation in hepatocyte suspensions by about 70% when it was added after 3 hr (Fig. 6). We speculate that EMS may be acting through two distinct mechanisms to inhibit nitrite formation. At early timepoints, EMS depletes cellular GSH and inhibits the transcription of iNOS. At later timepoints (2–3 hr), EMS may inhibit the enzyme through thiol modification of protein thiols.

We have determined with Western blot analysis that the iNOS protein is expressed when EMS is added after 3 hr, although at a reduced level (data not shown). The 6-hr iNOS protein levels in hepatocytes treated with EMS or DEM at 3 hr is not significantly different from controls. We are unable to determine with Western blot analysis whether iNOS is present in the active form. The protein may be expressed and detected by the iNOS antibody, but the iNOS enzyme activity may be inhibited by EMS alkylation. EMS can alkylate thiol groups and deplete protein thiols (Fariss *et al.*, 1997), whereas the capacity of DEM to alkylate thiol groups is more limited. This difference in the capacity of DEM and EMS to alkylate protein thiols may explain why the addition of EMS at 3 hr has more of an effect on iNOS activity than DEM, despite similar levels of the iNOS protein being present. In addition, the alkylation of iNOS by EMS may affect the affinity of the iNOS antibody toward the protein. If this is the case, then the levels of iNOS detected following the addition of EMS at 3 hr may underestimate the true amount of iNOS protein present at 6 hr.

Our work with NEM also supports the hypothesis that the iNOS protein may be sensitive to thiol modification. Preincubation with the thiol modifying agent NEM inhibited hepatocyte iNOS activity. Cubberley *et al.* (1997) have recently shown that human iNOS contains an essential cysteine residue that is required for activity. Most iNOS activity assays contain reduced thiols to maximize activity. Stuehr *et al.* (1990) reported that the omission of thiols in an iNOS activity assay led to a decrease in activity, and this decrease in activity was 70% reversible upon subsequent thiol addition. These researchers speculated that the thiols may protect against oxidative inactivation of the enzyme or keep important enzyme components reduced. Further research is required to confirm that EMS is inhibiting iNOS activity by alkylating thiol groups on the enzyme.

We also examined the effects of adding the thiol reducing agent DTT on nitrite production in hepatocytes. The addition of 1 mM DTT completely blocked hepatocyte nitrite formation (Fig. 6) and dramatically reduced iNOS mRNA formation (Fig. 7). The inhibitory effects of DTT

were greatest when added at the start of the incubation. Collectively, this work suggests that DTT is similar to DEM and is affecting the early signaling events involved in iNOS expression. However, based on relative iNOS mRNA levels, it appears that DTT is more effective at blocking these early signaling events than either DEM or EMS (Fig. 7).

The mechanism linking GSH depletion with inhibition of iNOS mRNA formation is not known, but the binding of activated NF-κB to DNA is reported to be redox sensitive with oxidants inhibiting and reducing agents, such as GSH, promoting DNA binding (Sun and Oberley, 1996). Attenuation of NF-κB binding to DNA would be expected to lower cellular iNOS mRNA levels (Vos *et al.*, 1999). Antioxidants such as *N*-acetyl cysteine are known to inhibit the activation of NF-κB in response to most known inducers (Muller *et al.*, 1997). Galter *et al.* (1994) demonstrated that 1 m*M* DTT inhibited NF-κB activation in response to phorbol esters in human T lineage cells (Molt-4). The inhibition of NF-κB activation by DTT only occurred if DTT was added at the same time as the phorbol ester. If DTT was added 1 hr later, NF-κB activation was potentiated. These researchers concluded from their data that GSSG may be required for activation of NF-κB and that the addition of DTT decreased GSSG levels (Galter *et al.*, 1994). However, it is important to note that they also determined that if cellular levels of GSSG were too high the binding of NF-κB binding to DNA was suppressed. Based on their work, they concluded that NF-κB activation may require a narrow range of GSSG. This two-step redox regulation of NF-κB may explain our data with the GSH depletors (DEM and EMS) and the thiol reducing agent DTT on iNOS expression. Further work is required to address whether these agents are acting at the level of NF-κB and to examine the importance of cellular GSSG as a signal for iNOS induction in hepatocytes.

Our results suggest that the GSH status of the cell is an important regulator of iNOS expression. In this regard, it is interesting to speculate why the GSH status of cell is linked to NO formation. NO is known to have a dual nature with respect to oxidative stress and cell injury. If NO reacts with superoxide, the powerful oxidant peroxynitrite is formed. Peroxynitrite can nitrosylate tyrosine residues on proteins and induce lipid peroxidation and DNA strand breaks. However, several studies also suggest that NO in itself can function as an antioxidant and protect cells against lipid peroxidation (Muriel, 1998; Kelley *et al.*, 1999) and oxidant-induced cell injury (Wink *et al.*, 1995; Sergent *et al.*, 1997). In fact, there is increasing evidence that cells induce iNOS and produce NO in response to oxidative stress (Muller *et al.*, 1997). It would seem logical to assume that iNOS induction in response to oxidative stress would only be beneficial if peroxynitrite is not produced. Sies *et al.* (1997) have suggested that GSH and the enzyme glutathione peroxidase are important cellular lines of defense against peroxynitrite damage. Peroxynitrite is metabolized by glutathione peroxidase in the presence of GSH to form nitrite and GSSG. Sies *et al.* (1997) have shown that glutathione peroxidase-GSH protected against peroxynitrite catalyzed formation of 3-nitrotyrosine in human fibroblast lysates. This protection required both glutathione peroxidase and GSH. Also, it has been proposed that GSH acts as scavenger of NO and may prevent the build up of toxic peroxynitrite levels (Wink *et al.*, 1994). These results suggest that glutathione peroxidase and GSH are an important protective system against peroxynitrite. However, this protective system would only be functional if there were adequate levels of GSH. Low levels of GSH would be expected to favor peroxynitrite formation by inhibiting glutathione peroxidase activity and increasing steady state levels of free NO and ROS present in the cell. Cellular GSH levels may determine whether NO reacts with superoxide and forms the potent oxidant peroxynitrite or acts as an antioxidant. In this context, the GSH regulation of iNOS expression may be an important defense limiting peroxynitrite formation.

In summary, our results suggest that iNOS expression in hepatocytes is regulated by cellular GSH levels. Depletion of GSH below a critical level blocks hepatocyte NO formation. In addition, the thiol reducing agent, DTT, also effectively inhibited NO formation as judged by extracellular nitrite formation. On the basis of our results with mRNA levels, both DTT and

depletion of cellular GSH appear to inhibit the early signaling events leading to iNOS expression, and suggest that the control of iNOS induction in hepatocytes is sensitive to the thiol redox status of the cell. Hepatocyte suspensions spontaneously producing NO may provide a useful model for further studies examining the control of iNOS expression in hepatocytes.

ACKNOWLEDGMENTS

We would like to acknowledge the excellent technical assistance of Nicole Naslund in performing this study. This work was supported by NIEHS/NIH grant #R01ES05452.

ABBREVIATIONS

DEM, diethylmaleate; DTT, dithiothreitol; EMS, ethyl methanesulfonate; GSH, reduced glutathione; GSH-NO, nitrosoglutathione; GSSG, oxidized glutathione; iNOS, inducible nitric oxide synthase; NEM, *N*-ethylmaleimide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase.

REFERENCES

CUBBERLEY, R.R., ALDERTON, W.K., BOYHAN, A., CHARLES, I.G., LOWE, P.N., and OLD, R.W. (1997). Cysteine-200 of human inducible nitric oxide synthase is essential for dimerization of haem domains and for binding of haem, nitroarginine and tetrahydrobiopterin. Biochem. J. **323,** 141–146.

DUVAL, D.L., SIEG, D.J., and BILLINGS, R.E. (1995). Regulation of hepatic nitric oxide synthase by reactive oxygen intermediates and glutathione. Arch. Biochem. Biophys. **316,** 699–706.

FARISS, M.W., BROWN, M.K., SCHMITZ, J.A., and REED, D.J. (1985). Mechanisms of chemical-induced toxicity: I. Use of a rapid centrifugation technique for the separation of viable and nonviable hepatocytes. Toxicol. Appl. Pharmacol. **79,** 283–295.

FARISS, M.W., BRYSON, K.F., and TIRMENSTEIN, M.A. (1997). Role of cellular thiol status in tocopheryl hemisuccinate cytoprotection against ethyl methanesulfonate-induced toxicity. Biochem. Pharmacol. **53,** 651–661.

GALTER, D., MIHM, S. and DROGE, W. (1994). Distinct effects of glutathione disulphide on the nuclear transcription factor κB and the activator protein-1. Eur. J. Biochem. **221,** 639–648.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S., and TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Anal. Biochem. **126,** 131–138.

HARBRECHT, B.G., and BILLIAR, T.R. (1995). The role of nitric oxide in Kupffer cell-hepatocyte interactions. Shock **3,** 79–87.

HARBRECHT, B.G., DI SILVIO, M., CHOUGH, V., KIM, Y.M., SIMMONS, R.L., and BILLIAR, T.R. (1997). Glutathione regulates nitric oxide synthase in cultured hepatocytes. Ann. Surg. **225,** 76–87.

HOFMANN, H., and SCHMIDT, H.H. (1995). Thiol dependence of nitric oxide synthase. Biochemistry **34,** 13443–13452.

HORTELANO, S., DEWEZ, B., GENARO, A.M., DIAZ-GUERRA, M.J., and BOSCA, L. (1995). Nitric oxide is released in regenerating liver after partial hepatectomy. Hepatology **21,** 776–786.

HOTHERSALL, J.S., CUNHA, F.Q., NEILD, G.H., and NOROHNA-DUTRA, A.A. (1997). Induction of nitric oxide synthesis in J774 cells lowers intracellular glutathione: effect of modulated glutathione redox status on nitric oxide synthease induction. Biochem. J. **322,** 477–481.

HORIKOSHI, T., DANENBERG, K.D., STADLBAUER, T.H., VOLKENANDT, M., SHEA, L.C., AIGNER, K., GUSTAVSSON, B., LEICHMAN, L., FROSING, R., RAY, M., GIBSON, N.W., SPEARS, P., and DANENBERG, P.V. (1992). Quantitative of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. Cancer Res. **52,** 108–116.

JONES, B.E., and CZAJA, M.J. (1998). Mechanisms of hepatic injury III. Intracellular signaling in response to toxic liver injury. Am. J. Physiol. **275,** G874–G878.

KANG, K.W., PAK, Y.M., and KIM, N.D. (1999). Diethylmaleate and buthionine sulfoximine, glutathione-depleting agents, differentially inhibit expression of inducible nitric oxide synthase in endotoxemic mice. Nitric Oxide **3,** 265–271.

KELLEY, E.E., WAGNER, B.A., BUETTNER, G.R., and BURNS, C.P. (1999). Nitric oxide inhibits iron-induced lipid peroxidation in HL-60 cells. Arch. Biochem. Biophys. **370,** 97–104.

KOSUGA, K., YUI, Y., HATTORI, R., SASE, K., EIZAWA, H., AOYAMA, T., INOUE, R., and SASAYAMA, S. (1994). Cloning of an inducible nitric oxide synthase from rat polymorphonuclear neutrophils. Endothelium **2,** 217–221.

LOPEZ-GARCIA, M.P. (1998). Endogenous nitric oxide is responsible for the early loss of P450 in cultured rat hepatocytes. FEBS Lett. **438,** 145–149.

MCMILLAN, J.M. (1999). Galactosamine decreases nitric oxide formation in cultured rat hepatocytes: mechanism of suppression. J. Biochem. Mol. Toxicol. **13,** 143–148.

MILES, A.M., WINK, D.A., COOK, J.C., and GRISHAM, M.B. (1996). Determination of nitric oxide using fluorescence spectroscopy. Methods Enzymol. **268,** 105–120.

MISKO, T.P., SCHILLING, R.J., SALVEMINI, D., MOORE,

W.M., and CURRIE, M.G. (1993). A fluorometric assay for the measurement of nitrite in biological samples. Anal. Biochem. **214,** 11–16.

MULLER, J.M., RUPEC, R.A., and BAEUERLE, P.A. (1997). Study of gene regulation by NF-κ B and AP-1 in response to reactive oxygen intermediates. Methods **11,** 301–312.

MURIEL, P. (1998). Nitric oxide protection of rat liver from lipid peroxidation, collagen accumulation, and liver damage induced by carbon tetrachloride. Biochem. Pharmacol. **56,** 773–779.

NICHOLLS-GRZEMSKI, F.A., TIRMENSTEIN, M.A., and FARISS, M.W. (1999). Time-dependent production of nitric oxide by rat hepatocyte suspensions. Biochem. Pharmacol. **57,** 1223–1226.

NUDEL, U., ZAKUT, R., SHANI, M., NEUMAN, S., LEVY, Z., and YAFFE, D. (1983). The nucleotide sequence of the rat cytoplasmic beta-actin gene. Nucleic Acids Res. **11,** 1759–1771.

NUSSLER, A.K., GELLER, D.A., SWEETLAND, M.A., DI SILVIO, M., BILLIAR, T.R., MADARIAGA, J.B., SIMMONS, R.L., and LANCASTER, J.R., JR. (1993). Induction of nitric oxide synthesis and its reactions in cultured human and rat hepatocytes stimulated with cytokines plus LPS. Biochem. Biophys. Res. Commun. **194,** 826–835.

SAVILLE, B. (1958). A scheme for the colorimetric determination of microgram amounts of thiols. Analyst **83,** 670–672.

SERGENT, O., GRIFFON, B., MOREL, I., CHEVANNE, M., DUBOS, M.P., CILLARD, P., and CILLARD, J. (1997). Effect of nitric oxide on iron-mediated oxidative stress in primary rat hepatocyte culture. Hepatology **25,** 122–127.

SIES, H., SHAROV, V.S., KLOTZ, L.O., and BRIVIBA, K. (1997). Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. J. Biol. Chem. **272,** 27812–27817.

STUEHR, D.J., KWON, N.S., and NATHAN, C.F. (1990). FAD and GSH participate in macrophage synthesis of nitric oxide. Biochem. Biophys. Res. Commun. **168,** 558–565.

SUN, Y., anD OBERLEY, L.W. (1996). Redox regulation of transcriptional activators. Free Radic. Biol. Med. **21,** 335–348.

TIRMENSTEIN, M.A., NICHOLLS-GRZEMSKI, F.A., SCHMITTGEN, T.D., ZAKRAJSEK, B.A., and FARISS, M.W. (2000). Characterization of nitric oxide production following isolation of rat hepatocytes. Toxicol. Sci. **53,** 56–62.

VOS, T.A., GOOR, H., TUYT, L., JAGER-KRIKKEN, A., LEUVENINK, R., KUIPERS, F., JANSEN, P.L., and MOSHAGE, H. (1999). Expression of inducible nitric oxide synthase in endotoxemic rat hepatocytes is dependent on the cellular glutathione status. Hepatology **29,** 421–426.

WINK, D.A., NIMS, R.W., DARBYSHIRE, J.F., CHRISTODOULOU, D., HANBAUER, I., COX, G.W., LAVAL, F., LAVAL, J., COOK, J.A., KRISHNA, M.C., DEGRAFF, W.G., and MITCHELL, J.B. (1994). Reaction kinetics for nitrosation of cysteine and glutathione in aerobic nitric oxide solutions at neutral pH. Insights into the fate and physiological effects of intermediates generated in the NO/O2 reaction. Chem. Res. Toxicol. **7,** 519–525.

WINK, D.A., COOK, J.A., PACELLI, R., LIEBMANN, J., KRISHNA, M.C., and MITCHELL, J.B. (1995). Nitric oxide (NO) protects against cellular damage by reactive oxygen species. Toxicol. Lett. **82/83,** 221–226.

Address reprint requests to:
Dr. Marc Fariss
College of Pharmacy
Washington State University
Pullman, WA 99164-6534

E-mail: fariss@mail.wsu.edu

Received for publication March 27, 2000; accepted June 11, 2000.

This article has been cited by:

1. David R. Geter, Fagen Zhang, Melissa R. Schisler, Amanda J. Wood, H. Lynn Kan, Yo-Chan Jeong, Michael J. Bartels, Lisa McFadden, B. Bhaskar Gollapudi. 2012. Genetic damage, but limited evidence of oxidative stress markers in diethyl maleate-induced glutathione depleted mouse lymphoma L5178Y (TK +/-) cell cultures. *Toxicology Mechanisms and Methods* **22**:7, 547-554. [CrossRef]
2. Brian C. Smith, Nathaniel B. Fernhoff, Michael A. Marletta. 2012. Mechanism and Kinetics of Inducible Nitric Oxide Synthase Auto- S -nitrosation and Inactivation. *Biochemistry* 120124083435001. [CrossRef]
3. Qi Lu, Frances L. Jourd'heuil, David Jourd'heuil. 2007. Redox control of G1/S cell cycle regulators during nitric oxide-mediated cell cycle arrest. *Journal of Cellular Physiology* **212**:3, 827-839. [CrossRef]
4. D BOUDKO. 2007. Bioanalytical profile of the l-arginine/nitric oxide pathway and its evaluation by capillary electrophoresis#. *Journal of Chromatography B* **851**:1-2, 186-210. [CrossRef]
5. Hosur N. Achuth, Shabbir M. Moochhala, Ratha Mahendran, Walter Tiang Lee Tan. 2005. Nitrosoglutathione triggers collagen deposition in cutaneous wound repair. *Wound Repair and Regeneration* **13**:4, 383-389. [CrossRef]
6. Ram Kannan, Hovhannes J. Gukasyan, Wenzheng Zhang, Melvin D. Trousdale, Kwang-Jin Kim, Vincent H.L. Lee. 2004. Impairment of conjunctival glutathione secretion and ion transport by oxidative stress in an adenovirus type 5 ocular infection model of pigmented rabbits. *Free Radical Biology and Medicine* **37**:2, 229-238. [CrossRef]
7. Mobeen Iqbal, Rubin I. Cohen, Kamel Marzouk, Shu Fang Liu. 2002. Time course of nitric oxide, peroxynitrite, and antioxidants in the endotoxemic heart. *Critical Care Medicine* **30**:6, 1291-1296. [CrossRef]