# Low-level Nitric Oxide Blunts Oxidant Injury *via* Up-regulating Glutathione Synthesis

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The elevation of cellular glutathione (GSH) level induced by low concentrations of an nitric oxide (NO)-donor, sodium nitroprusside (SNP), and its effect on oxidant-induced cell injury were examined in RAW264.7 cells. The cellular GSH level increased 6 hr after exposure of the cells to SNP at low concentrations ranging from 0.1 to 0.5 mM, and the elevation followed the induction of mRNA coding for $\gamma$-glutamylcysteine synthetase, the rate-limiting enzyme of the de novo glutathione synthesis pathway. Pre-treatment of cells with low concentration of SNP (less than 0.25 mM) at 12 hr prior to exposure to menadione (MEND), an superoxide anion ($O_2^-$)-donor, significantly suppressed the cell injury induced by MEND alone. Simultaneous treatment with a higher concentration of SNP (1.0 mM or more) also blunted the MEND-induced cell injury. Low and high doses of NO both seem to show a preventive effect against oxidant injury: NO may protect against oxidant injury by up-regulating GSH synthesis at low concentrations, while at high concentrations it may directly react with radical oxygen species (ROS), thus acting as a free radical scavenger and blunting oxidant injury. These results suggest that modulation of the cellular glutathione metabolism through intracellular NO is a potential mechanism for enhancing the antioxidant defense of cells.

**Key words** —— nitric oxide (NO), glutathione, inhibition, oxidant injury

## INTRODUCTION

Nitric oxide (NO) is an endogenously produced free radical and has many biological functions, including role in vasorelaxation, neurotoxicity, bacteriostasis, and host defense against infection.[1] The reactive nature of NO with transition metals, oxygen, and other radical oxygen species (ROS) suggests several biological pathways through which NO might either promote or reduce oxidant-induced cell injury. It has been reported that NO can either enhance ROS-mediated vascular damage, while it reduces oxidant-mediated vascular injury in the pulmonary,[2–5] coronary,[6] splanchnic,[7,8] and renal circulation.[9] There are conflicting data in the literature to support both a protective and a cytotoxic role of NO in biological systems. With respect to the protective role of NO, the following mechanisms have been proposed. Released NO reacts with ROS, *i.e.*, superoxide anion ($O_2^-$), to form peroxynitrite ($ONOO^-$), thus acting as a free radical scavenger and blunting oxidantive injury.[10,11] In a model of myocardial ischemia/reperfusion, NO interferes with neutrophil adherence to coronary endothelium and attenuates neutrophil superoxide production, thereby blunting myocardial injury.[12] NO has also been shown to inhibit neutrophil ROS production through a direct inhibitory effect on NADPH oxidase[13] and/or to act as an antioxidant *via* the formation of NO-iron adducts, reducing the availablity of ferrous iron and thereby ROS production.[14]

In our previous study, changes in endogenous glutathione (GSH) levels in mice after low-dose $\gamma$-ray irradiation were examined, and the GSH levels in organs such as liver, brain and pancreas were found to be significantly induced by a low dose of ionizing radiation. This elevation was accompanied by elevated activity of $\gamma$-glutamylcysteine synthetase ($\gamma$-GCS), which is the rate-limiting enzyme for *de novo* GSH synthesis.[15–18] It was subsequently confirmed that this phenomenon is a post-transcriptional event; the elevation of glutathione levels in these organs follows the induction of mRNAs coding for $\gamma$-GCS.[19,20] This phenomenon is considered to be an adaptive response of living cells against ROS. Most radiation effects including toxicity and adaptive response are thought to be mediated by ROS, such as hydroxyl radicals and superoxide anion radicals.[21,22]

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Thus, similar adaptive responses to those in the case of ionizing radiation may occur with the NO radical, because NO is also one of the ROS; NO may also play a regulatory role in basal GSH biosythesis and NO treatment may increase cellular GSH levels, resulting in elevation of the defense system against the oxidative stresses.

Here, we show that treatment of RAW264.7 cells with low doses of NO can cause a significant increase in cellular GSH levels and thereby blunt oxidant-induced cell injury.

## MATERIAL AND METHODS

**Materials** —— GSH, oxidized glutathione (GSSG), and menadione (MEND) and were purchased from Sigma (St. Louis, MO, U.S.A.). 5,5′-Dithiobis (2-nitro-benzoic acid) (DTNB) were obtained from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). $\beta$-NADPH and GSH reductase (GR, 120 U/ml) were from Oriental Yeast Co. Ltd. (Tokyo, Japan).

**Cell Cultures** —— Mouse macrophage-like RAW 264.7 cells were purchased from Riken Cell Bank (Tsukuba, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a 5% $CO_2$/95% air atmosphere. Cells in the logarithmic phase were used throughout the experiment. Freshly harvested RAW264.7 cells were resuspended in DMEM medium containing 10% FBS at a density of $1 \times 10^6$ ml, and the suspension (2 ml) was seeded into 6-well plates (Corning Co., NY, U.S.A.). SNP was then added to the medium to final concentrations of 0, 0.1, 0.25, 0.5, 1.0 and 5.0 mM and incubation was continued for an appropriate time. In another experiment, MEND was added to the medium to final concentrations of 0, 0.025, 0.05, 0.1, 0.25 and 0.50 mM and incubation was continued for an appropriate time.

**Effect of SNP Pretreatment on MEND-Induced Cell Injury** —— RAW264.7 cells resuspended in DMEM medium containing 10% FBS at a density of $1 \times 10^6$ ml were exposed to SNP at 0.1, 0.25, 0.5, 1.0 and 5.0 mM for 12 hr at 37°C in a $CO_2$-incubator. MEND was then added to each well to a final concentration of 0.25 mM. Incubation was continued for another 6 hr and the medium of each well was subjected to lactate dehydrogenase (LDH) assay.

**Effect of Simultaneous SNP Treatment on MEND-Induced Cell Injury** —— RAW264.7 cells resuspended in DMEM medium containing 10% FBS at a density of $1 \times 10^6$ ml were incubated with SNP at 0.1, 0.25, 0.5, 1.0 and 5.0 mM in the presence of a constant MEND concentration of 0.25 mM for 12 hr at 37°C in a $CO_2$-incubator, and the medium of each well was subjected to LDH assay.

**Assay of Lactate Dehydrogenase** —— LDH released into the medium from the cells was assayed as a marker of cell damage. Aliquots of the supernatant of the cultured medium were collected at each time interval and subjected to LDH assay with LDH CII-Test Wako (Wako Chemicals Co., Ltd., Tokyo, Japan). The activity was expressed in Wroblewski unit/ml of the medium (Wroblewski unit/ml).

**Assay of Total Glutathione (GSH + GSSG)** —— For the determination of intracellular total glutathione levels, the cultured cells at each time interval were washed 3 times with ice-cold phosphate-buffered saline (PBS) and then harvested from the dishes with a silicon rubber policeman into Eppendorf tubes. The cell pellet was suspended in PBS containing 5 mM EDTA, 0.01% digitonin and 0.25% sodium cholate. The cells were disrupted by sonication and the sonicate was centrifuged at 10000 $\times$ $g$ for 20 min. An aliquot of the supernatant was removed for protein assay. An equal volume of 10% trichroloacetic acid (TCA) was added to another aliquot, and the solutions were kept for 30 min on ice. The acid-soluble fraction was obtained by centrifugation at 10000 $\times$ $g$ for 20 min. The fraction was subjected to total glutathione assay after repeated removals (5 times) of TCA with ether. Total glutathione (GSH + GSSG) content was measured by using a modified spectrophotometric technique.[23] Briefly, each sample fraction was diluted 1 : 5, and a 25 $\mu$l aliquot was mixed with 250 $\mu$l of 1 mM DTNB, 733 $\mu$l of 0.3 mM NADPH, and 10 $\mu$l of GR (2 U/ ml). The rate of change in absorbance was measured at 412 nm. Authentic GSH (0–25 $\mu$M) was analyzed in the same manner. The GSH concentration of each sample was calculated as nmol/mg protein. Protein was determined by the method of Lowry *et al.*,[24] using bovine serum albumin (BSA) as a standard.

**RNA Isolation and Northern Blot Analysis** —— Expression of $\gamma$-GCS mRNA was analyzed by Northern blotting. Total RNA was isolated from the cells by means of the acid guanidium isothio-cyanate-phenol-chloroform extraction method. The RNA was quantified spectrophotometrically at 260 nm (the ratio of $A_{260}$ nm to $A_{280}$ nm always exceeded 1.8), and 15 $\mu$g aliquots of total RNA were sized-frac-

tionated by electrophoresis on a 1.0% agarose gel (Nippon Gene, Toyama, Japan). RNA was then blotted onto nylon membrane using 0.02 M 2-morpholinoethanesulfonic acid buffer (pH 7.0), and immobilized by UV cross-linking. The relative amounts of RNA were judged by hybridization with a mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Specific cDNA probes were obtained as follows. Mouse GR cDNA was a kind gift from Prof. Dieter Werner, German Cancer Research Center.[25] Mouse $\gamma$-GCS and GAPDH cDNAs were synthesized by RT-PCR (Titan, Boehringer Mannheim, Mannheim) from mouse liver total RNA using oligo DNA primers for $\gamma$-GCS (5'-CACAT-CTACCAC-GCAGTCA-3' and 5'-TTCGCTTTT-CTA-AATCCTGA-3') and GAPDH (5'-TGAAG-GTCGGTGTGAACGGA-TTTGGC-3' and 5'-CA-TGTAGGCCATGAGGCCACCAC-3'). cDNA was amplified (35 cycles, 94°C, 1 min; 55°C, 1 min; 72°C, 1 min) and PCR products were sub-cloned into the pGEM-T vector (Promega, Madison, WI, U.S.A.) for amplification. Hybridization was carried out in a solution consisting of $5 \times$ SSPE ($20 \times$ SSPE = 3.6 M NaCl, 200 mM $NaH_2PO_4$, 20 mM EDTA, pH 7.4), $10 \times$ Denhardt's reagent (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA), 50% formamide, 1.4% sodium dodecyl sulfate (SDS), and 0.1 mg/ml herring sperm DNA with $^{32}$P-labeled probes at 42°C. After hybridization, the membrane was washed with $6 \times$ SSC ($20 \times$ SSC = 3 M NaCl, 0.3 M trisodium citrate) and 0.1% SDS at 42°C for 30 min, $1 \times$ SSC and 0.1% SDS at 55°C for 30 min, and $0.1 \times$ SSC and 0.1% SDS at 60°C for 30 min. Quantitation was done with a laser image analyzer (Fujix BAS 2500, Fuji Film, Kanagawa, Japan). The membrane was also exposed to an X-ray film (Fuji HR-HA30, Fuji Film) with an intensifying screen at –80°C.

**Statistical Analysis** —— The statistical significance of differences was determined by using Student's $t$ test for comparison between two groups or two-way repeated measures analysis of variance and Dunnett's tests for multiple comparison where appropriate. $p$ Values of less than 0.05 were considered significant.

## RESULTS

### Kinetics of LDH Release from Raw246.7 Cells and Cellular Total Glutathione Levels after Treatment with SNP

LDH activity released from RAW264.7 cells after treatment with SNP, an NO-donor, was assayed
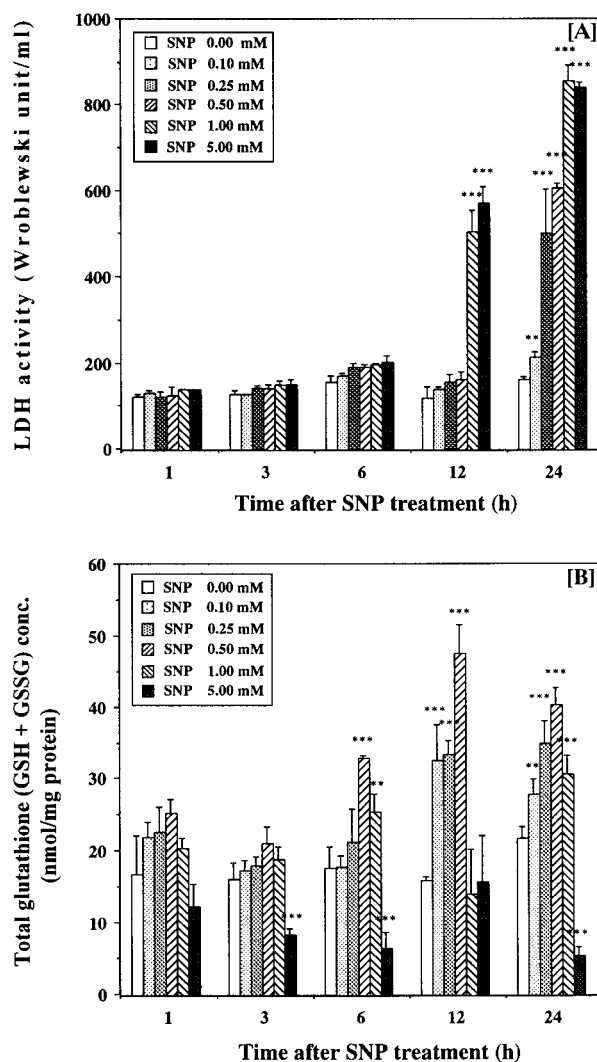


**Fig. 1.** Changes in LDH Activity and Total Cellular Glutathione (GSH + GSSG) after Treatments of RAW 264.7 Cells with Various Concentrations of Sodium Nitroprusside (SNP)

[A] LDH activity in the medium. The activity was expressed in Wroblewski unit/ml of the medium (Wroblewski unit/ml). [B] Total cellular glutathione (GSH + GSSG). Each point indicates the mean ± S.D. of 4 wells. ** and *** Significantly different from the respective non-treated control group at $p < 0.01$ and $p < 0.005$, respectively.

in the medium as a measure of cell injury. As can be seen in Fig. 1 [A], the activity did not show any significant elevation at SNP concentrations ranging from 0.1 to 0.5 mM in comparison with the control during 12 hr after treatment. However, SNP at more than 1.0 mM significantly increased the activity, indicating serious cell injury. Exposure to SNP at all doses except 0.1 mM caused drastic elevations of the LDH activity at 24 hr.

Changes in the intracellular glutathione levels after treatment with SNP were next examined in RAW 264.7 cells. As shown in Fig. 1 [B], signifi-

cant increases in cellular glutathione (GSH + GSSG) levels were observed from 0.1 mM to 0.5 mM SNP at 12 hr post-treatment. In contrast, the highest dose of SNP (5 mM) tested in this experiment markedly decreased the cellular glutathione levels during 24 hr post-treatment.

## Induction of mRNAs for GSH Synthesis-Related Enzymes

Expression of mRNA for $\gamma$-GCS, a rate-limiting enzyme of the *de novo* GSH synthesis pathway was examined after SNP treatment at a concentration of 0.25 mM. As shown in Fig. 2 [A], the $\gamma$-GCS mRNA level increased soon after treatment, peaked at 3 hr post-treatment, and then declined slowly. Next, dose–dependent effect of SNP on the expression of the $\gamma$-GCS mRNA levels was investigated at 3 hr post-treatment. As shown in Fig. 2 [B], significant elevations of the mRNA expression were observed at concentrations ranging from 0.1 mM to 1.0 mM, and the effect on $\gamma$-GCS mRNA expression in RAW264.7 cells was maximal at 0.25 mM.

## Kinetics of LDH Release from Raw246.7 Cells and Cellular Total Glutathione Levels after MEND Treatment

LDH activity released from RAW264.7 cells after treatment with MEND, a chemical $O_2^-$-donor, was assayed. As shown in Fig. 3 [A], the activity did not show any significant for 24 hr post-treatment at MEND concentrations less than 0.05 mM, while 0.05 mM produced small but significant increase at 12 and 24 hr, and concentrations of 0.1 mM or more markedly elevated the activity from 6 hr to 24 hr post-treatment in a time- and dose–dependent manner.

Changes in the intracellular total glutathione levels after treatment of RAW 264.7 cells with MEND are shown in Fig. 3 [B]. Significant increases of the cellular glutathione levels were induced by MEND at doses of 0.025 mM and 0.05 mM after 12 hr. In contrast, large decreases were observed in the cells exposed to MEND at doses of more than 0.1 mM during 24 hr post-treatment.

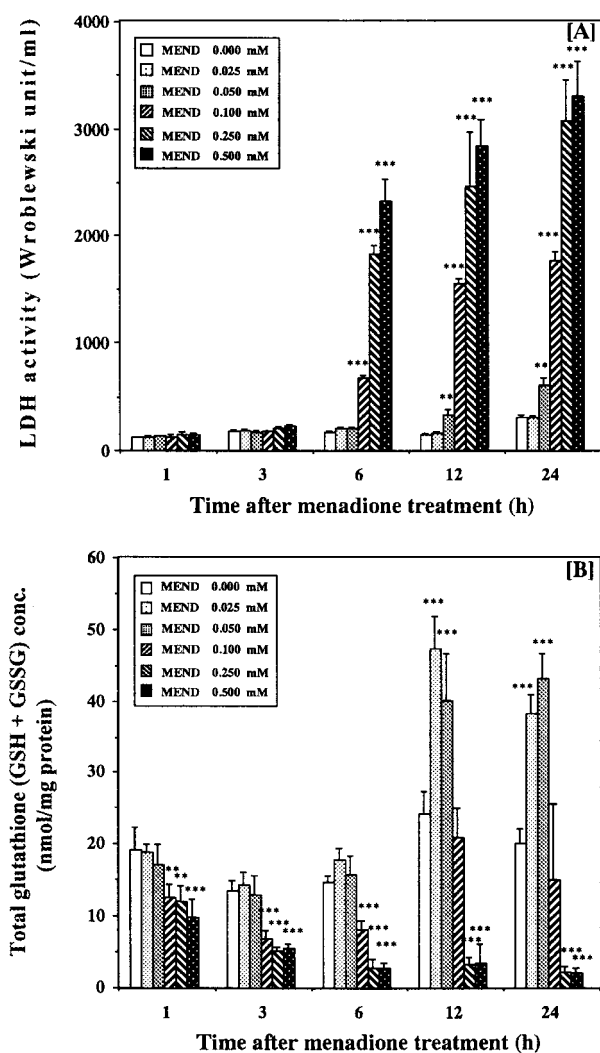## Effect of Pre-treatment with SNP on 0.25 mM MENAD-Induced Cell Injury

The effect of pre-irradiation with various concentrations of SNP on the cell injury induced by MEND at a constant concentration of 0.25 mM was investigated. As shown in Fig. 4, no synergistic effects were seen. Significant inhibitions were caused
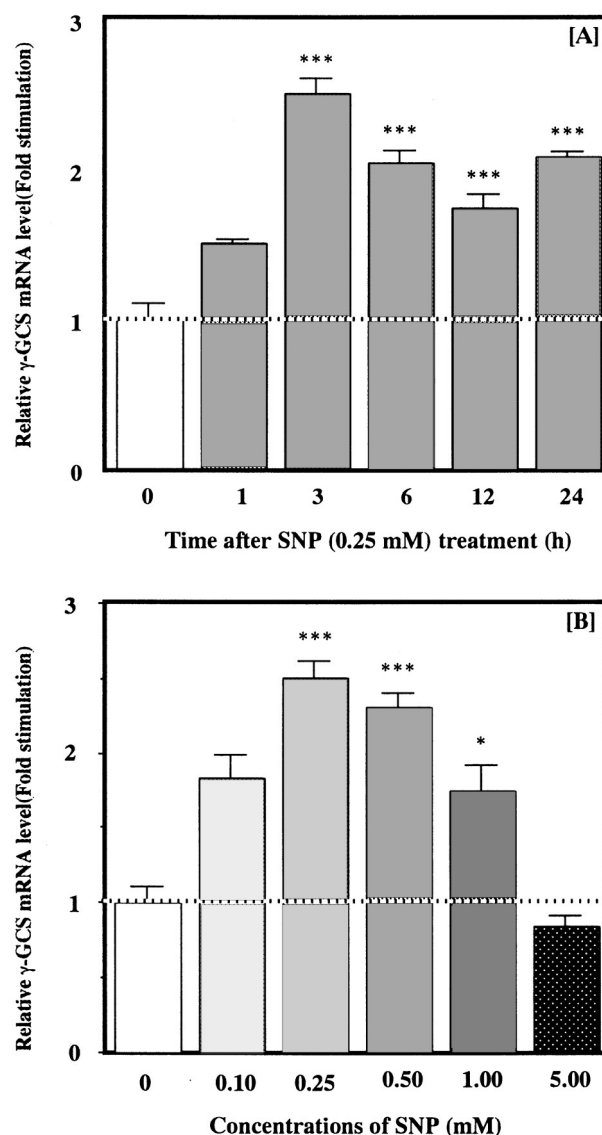


**Fig. 2.** Time- and Dose–dependent Effects of Sodium Nitroprusside (SNP) on $\gamma$-Glutamylcysteine Synthetase ($\gamma$-GCS) mRNA Expression in RAW 264.7 Cells.

[A] Changes in $\gamma$-GCS mRNA level after treatment with SNP. Expression of mRNA for $\gamma$-GCS was examined after SNP treatment at a concentration of 0.25 mM. [B] Dose–dependent effects of SNP on $\gamma$-GCS mRNA level. Dose–dependent effects of SNP on $\gamma$-GCS mRNA level were examined at 3 hr after treatment with SNP at concentrations ranging from 0.1 mM to 5.0 mM. Results of mRNA quantification by densitometric analysis of the autoradiogram for RAW 264.7 cells treated with SNP are shown. Relative mRNA levels are indicated as the ratio of the $\gamma$-GCS mRNA level to the mRNA level of the housekeeping gene *GAPDH*. Each point indicates the mean ± S.D. of 3 independent assays. * and *** Significantly different from the time 0 or the non-treated control group at $p < 0.05$ and $p < 0.005$, respectively.

by pre-treatment with SNP at low and high concentrations. The lowest dose of SNP (0.1 mM) tested in this experiment markedly inhibited the MEND-induced cell injury. Inhibitory effects were also obtained at high doses of SNP (1.0 mM or more).

**Fig. 3.** Changes in LDH Activity and Total Cellular Glutathione (GSH + GSSG) after Treatments of RAW 246.7 Cells with Various Concentrations of Menadione (MEND)

[A] LDH activity in the medium. The activity was expressed in Wroblewski unit/ml of the medium (Wroblewski unit/ml). [B] Total cellular glutathione (GSH + GSSG). Each point indicates the mean ± S.D. of 4 wells. ** and *** Significantly different from the respective non-treated control group at $p < 0.01$ and $p < 0.005$, respectively.
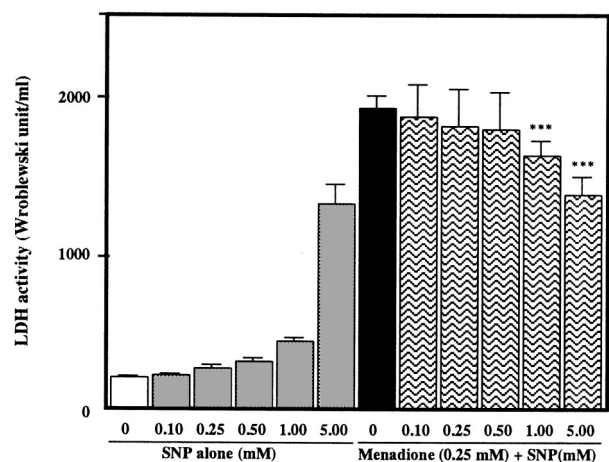
## Effect of Simultaneous Treatment with SNP on 0.25 mM MENAD-Induced Cell Injury

The effect of the simultaneous presence of SNP on MEND (0.25 mM)-induced cell injury was examined. As shown in Fig. 5, no synergistic effects were seen. The presence of SNP inhibited MEND-induced LDH elevation in a dose–dependent manner, showing significant differences ($p < 0.005$) at 1.0 mM and 5.0 mM.
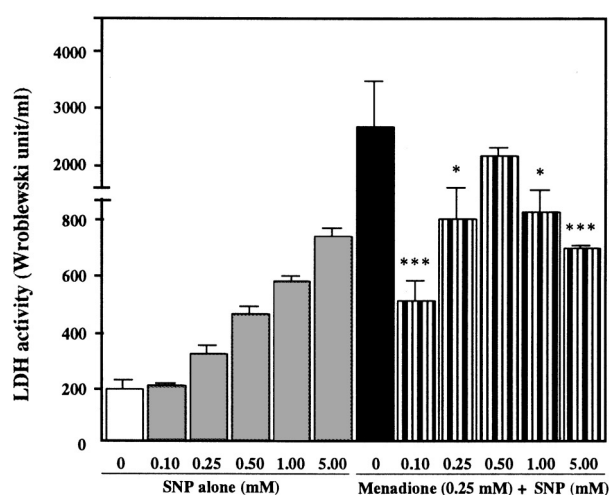


**Fig. 4.** Effect of Pre-Treatment with SNP on Menadione (MEND)-Induced RAW 264.7 Cell Injury

Cells were exposed to SNP at 0.1, 0.25, 0.5, 1.0 and 5.0 mM for 12 hr. MEND was then added to each well to a final concentration of 0.25 mM and incubation was continued for another 6 hr. The medium of each well was subjected to LDH assay. The activity was expressed in Wroblewski unit per ml of the medium (Wroblewski unit/ml). Each column indicates the mean ± S.D. of 4 wells. * and *** Significantly different from the MEND alone-treated group at $p < 0.05$ and $p < 0.005$, respectively.



**Fig. 5.** Effect of Simultaneous SNP Treatment on MEND-Induced Cell Injury

Cells were exposed to various concentrations of SNP (0.1, 0.25, 0.5, 1.0 and 5.0 mM ) and a constant concentration (0.25 mM) of MEND for 12 hr. The medium of each well was subjected to LDH assay. The activity was expressed in Wroblewski unit/ml of the medium (Wroblewski unit/ml). Each column indicates the mean ± S.D. of 4 wells. *** Significantly different from the MEND alone-treated group at $p < 0.005$.

## DISCUSSION

Bacteria and mammalian cells show an adaptive response to oxidative stress.[26–28] Pre-treatment with small doses of oxidant induces resistance to subse-

quent, and otherwise lethal, doses of oxidant. This adaptive response includes induction of superoxide dismutase (SOD),[29] GPX,[30] metallothionein,[31] heat shock proteins,[32] and other factors.[33] Though the precise mechanisms of these phenomena are still unclear, ROS are generally believed to be involved.

NO is a readily diffusible pleuripotent free radical which is associated with multiple organ-specific regulatory functions as noted in the introduction.[1] However, NO is also considered to contribute to tissue and cell injury due to its reactive nature of free radical nature. An unanswered question has been whether the co-production of other ROS and NO would reduce or enhance the toxic effects of the individual free radicals. NO appears to be capable of both increasing[34–36] and decreasing oxidative stress *in vitro* and *in vivo* experiment.[10–14,37–39] Though the mechanisms by which NO protects cells from oxidative stress are likely to include direct radical scavenging by NO, it has recently been speculated that NO may also protect against oxidant injury by increasing cellular glutathione levels through the induction of gene expression.[3,40,41]

It has already been well established that sublethal oxidative stress causes an elevation of cellular glutathione levels through a transcriptional event. Our previous studies also have shown that low doses of ionizing radiation, a generator of ROS, effectively induce GSH in various tissues and in cultured cells.[15–20] Thus, a similar effect could be anticipated for NO.

To examine whether NO has an ameliorating effect against oxidative stress through the induction of increased glutathione levels, possibly *via* increased expression of $\gamma$-GCS, SNP and MEND were selected in this study as chemical donors of NO and $O_2^-$, respectively. As shown in Fig. 1, low doses of SNP (less than 0.5 mM) significantly increased the cellular glutathione levels after 6 hr post-treatment under conditions where no cytotoxicity was detected. This is consistent with a stimulation of GSH synthesis, possibly at the level of $\gamma$-GCS. $\gamma$-GCS controls the rate-limiting step and forms the dipeptide $\gamma$-glutamyl cysteine from glutamic acid and cysteine (Fig. 2). The enzyme is usually subject to negative feed-back inhibition by GSH, but oxidative stress has been shown to elevate $\gamma$-GCS enzymatic activity and mRNA expression. NO may interfere with the feed-back step by binding to thiol sites on the $\gamma$-GCS molecule and blocking negative feed-back. Alternatively, NO may up-regulate $\gamma$-GCS activity by producing a conformational change in the enzyme

or by increasing message transcription. It has been well documented that oxidative stress elevates $\gamma$-GCS enzyme activity through increasing mRNA expression. Indeed, we have previously shown that low doses of ionizing radiation, a generator of oxidative stress, effectively induces cellular glutathione by way of increasing $\gamma$-GCS mRNA gene expression.

Since elevation of cellular glutathione by NO was ascertained, we next examined the effect of NO on oxidative cell injury in RAW264.7 cells exposed to MEND. MEND, which is lipid-soluble, can enter the cells very rapidly and generate $O_2^-$ *via* a one-electron-transfer reaction.[42] It is generally considered that independent sources of NO and $O_2^-$ can act in a synergistic fashion to enhance rather than ameliorate toxicity; NO reacts with $O_2^-$ to form ONOO$^-$, which is metabolized to the hydroxyl radical (HO • ) and NO$_2$ . Furthermore, ONOO$^-$ has been shown to oxidize sulfhydryls, which may deplete the glutathione store, causing cells to become more sensitive to oxidative stress.

Low doses of SNP definitely inhibited the MEND-induced cell injury, as we had expected (Fig. 4). These low doses of SNP appear to increase cellular glutathione with little concomitant cytotoxicity, judging from the low levels of LDH activity. Inhibitory effects were also recognized at high doses of SNP. NO released at high doses of SNP may directly react with superoxide anion to form ONOO$^-$, thus acting as a free radical scavenger and reducing the toxicity of $O_2^-$ itself. In another experiment, we examined the effect of simultaneous presence of SNP on $O_2^-$-induced cell injury (Fig. 5). SNP at 1.0 mM or more significantly prevented the toxicity induced by MEND alone. It is noteworthy that synergistic effects were never seen, either on pretreatment or simultaneous treatment with NO, even at high doses. These facts are consistent with a direct reaction of NO and $O_2^-$, generating less toxic molecules. NO appears to be capable of both producing and preventing oxidant injury *in vitro*. The net outcome in any system may depend on the relative concentrations of NO and $O_2^-$. The increase in cellular glutathione induced by low doses of NO supports a role for NO in glutahtione metabolism.

In summary, we have established that modulation of cellular glutathione metabolism through intracellular NO is a potential mechanism for enhancing the antioxidant defense of cells. Our results may also be helpful in identifying redox-sensitive cell signaling pathways that can be activated by NO.

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