Stimulation of cystine uptake by nitric oxide: regulation of endothelial cell glutathione levels

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Stimulation of cystine uptake by nitric oxide: regulation of endothelial cell glutathione levels. Am. J. Physiol. 276 (Cell Physiol. 45): C803–C811, 1999.—Nitric oxide (NO) is known to produce some of its biological activity through modification of cellular thiols. Return of cellular thiols to their basal state requires the activity of the GSH redox cycle, suggesting important interactions between NO signaling and regulation of cellular redox status. Because continuous exposure to NO may lead to adaptive responses in cellular redox systems, we investigated the effects of NO on cellular GSH levels in vascular endothelial cells. Acute exposure (1 h) of cells to >1 mM S-nitroso-N-acetyl-penicillamine (SNAP) led to depletion of GSH. On the other hand, chronic exposure to lower concentrations of SNAP (<1 mM) led to a progressive increase in cytosolic GSH, reaching fourfold above basal by 16 h. The mechanism may involve an increase in GSH biosynthesis through effects on biosynthetic enzymes or through increased supply of cysteine, the limiting substrate. In this regard, we report that chronic exposure to SNAP led to a concentration-dependent increase in cystine uptake over a time course similar to that seen for elevation of GSH. The effect of SNAP on cystine uptake was inhibitable by either cycloheximide or actinomycin D, suggesting a requirement for both RNA and protein synthesis. Furthermore, uptake was Na⁺ independent and was blocked by extracellular glutamate. Extracellular glutamate also blocked SNAP-mediated elevation of cytosolic GSH. Finally, in a coculture model, NO produced by cytokine-pretreated RAW 264.7 cells increased both GSH levels and cystine uptake in naïve endothelial cells. These findings strongly suggest that NO leads to adaptive induction of the Xc⁺ amino acid transport system, increased cystine uptake, and elevation of intracellular GSH levels.

cystine; amino acid transport; nitrosative stress; oxidative stress

GLUTATHIONE is a critical intracellular reductant that functions in protecting cells from free radicals, reactive oxygen species, and toxic substances. The intracellular concentration of GSH is in the range of 1–10 mM in many cells and is maintained by controls on its biosynthesis. Several mechanisms are important. For example, the rate-limiting enzyme γ-glutamylcysteine synthase is regulated by GSH levels (18, 26) and by oxidants (20) such that depletion of GSH or the presence of oxidants increases its activity. GSH synthesis is also dependent on the availability of the amino acid precursors glutamate, glycine, and cysteine (7, 8, 10, 18). Because glutamate and glycine occur at relatively high intracellular concentrations, cysteine availability largely determines GSH synthesis. Therefore, intracellular GSH levels are dependent on cysteine levels in the extracellular space and thus on transport of cysteine or cystine into cells.

A number of mechanisms are available for transport of cysteine or cysteine equivalents (cysteinylglycine) into cells. Cysteine can be taken up directly via the Na⁺-dependent amino acid transport system called the ASC system (4, 14, 29). In many cells, this is a major route for supplying intracellular cysteine to maintain GSH levels. However, because cystine levels are generally higher than cysteine levels in extracellular fluids (and in cell culture media), mechanisms for cystine uptake are also crucial for GSH biosynthesis. Cystine is taken up by an amino acid transport system designated Xc, which is Na⁺ independent, inducible, and exhibits a high degree of specificity for cysteine and glutamate (7, 14, 19, 27, 33). Once inside a cell, cystine is rapidly reduced to cysteine. In some cells, intracellular cysteine may result from the activity of γ-glutamyl transpeptidase utilizing extracellular GSH and cysteine as substrates (33).

Oxidant stress imposed by reactive oxygen species is known to increase GSH levels as part of an adaptive response (7, 8, 19, 27). Although regulation of γ-glutamylcysteine synthase activity is certainly involved after acute exposure (18, 20, 26), oxidants also induce cystine uptake in some cells. For example, exposure of endothelial cells (7, 19), V79 cells (21), and macrophages (27) to agents that cause oxidant stress [H₂O₂, arsenite, diethyl maleate (DEM), hyperoxia, and cadmium] leads to increased activity of the Xc⁺ but not the ASC uptake system. Induction of the Xc⁺ system occurs over 6–12 h in endothelial cells (6) or V79 cells (21) exposed to arsenite, leading to a two- to threefold increase in GSH levels by 16 h.

Nitric oxide (NO) is well known to activate guanylate cyclase. In addition to this signaling role, NO is also known to interact with cellular redox systems, especially thiol groups. Thus NO exposure or synthesis may impose a nitrosative stress similar to that imposed by oxygen-derived species. Whether or not nitrosative stress produces adaptive responses in cells is not well studied. However, exposure of cells to NO protects them from apoptosis through induction of heat shock proteins (13) or caspase 3 (9), and NO is known to react with and modify cellular responses to oxygen-derived species (35, 36). Furthermore, others have reported increases in GSH in cells exposed to NO for 2 h (17), and recently, in preliminary experiments, we have observed that exogenous NO leads to accumulation of GSH over
an extended time course. Because oxidative stress from oxygen-derived species promotes an increase in GSH biosynthesis by inducing cystine transport, we speculated that the accumulation of GSH in cells exposed to NO occurred via a similar mechanism. In the present study, we present data that show that NO leads to elevation of intracellular GSH in vascular endothelial cells through a mechanism that involves induction of cystine uptake. This adaptive response is potentially important in protecting cells from exposure to NO synthesized endogenously or NO produced in an inflammatory setting.

MATERIALS AND METHODS

Materials. Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). All other cell culture reagents were from Gibco (Grand Island, NY). Tissue culture plasticware was obtained from NUNC (Fisher Scientific, Raleigh, NC), and Costar coculture plates were from Corning (Alton, MA). L-nitroso-N-acetyl-penicillamine (SNAP) was from Research Biochemicals International (Natick, MA). L-[35S]cystine was synthesized by SNAP or cells from the coculture model were rinsed twice with warm HBSS-HEPES and incubated for 60 min in the same buffer in room air at 37°C. After two more rinses with HBSS-HEPES, the cells were incubated in 1 ml of buffer containing L-[35S]cystine (1 μCi/60 μM) in room air at 37°C for 10 min. After removal of the buffer, cells were rinsed four times with ice-cold HBSS-HEPES buffer containing 600 μM cystine. Cells were lysed in 1% Triton X-100 in HBSS-HEPES, and cystine uptake was measured by determining the amount of 35S in an aliquot of the lysate using a liquid scintillation counter. With these procedures, cystine uptake was linear for at least 20 min. In experiments in which the Na+ dependence of cystine uptake was determined, Li+ (as LiCl) was substituted for Na+.

Glutathione assay. Cells were rinsed twice with HBSS-HEPES and incubated into 1 M perchloric acid containing 2 mM EDTA and extracted for 30 min on ice. After centrifugation at 2,000 g in a microcentrifuge, the acid extract was neutralized with 4 M KOH containing 0.6 M MOPS, and samples were centrifuged to remove the potassium perchlorate precipitate. Total glutathione levels (GSH and GSSG) were determined spectrophotometrically using the glutathione reductase-linked 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) recycling assay (34). The assay conditions were 100 mM KPO4 (pH 7.0), 100 μM DTNB, 300 μM NADPH, and 0.2 U/ml glutathione reductase. Each assay was individually calibrated using known concentrations of GSH. All data were normalized per milligram of cellular protein. Protein concentrations were determined using the Bradford dye binding technique with BSA as a standard (3).

Immunoblot procedures. After incubation of RAW 264.7 cells with cytokines, cells on inserts were lysed with boiling sample buffer containing 63 mM Tris (pH 6.8), 10% glycerol, and 2% SDS. After sonication, an aliquot was removed for protein determination, and β-mercaptoethanol was added to a final concentration of 5%. Extracts were boiled for 5 min at 100°C, and equivalent amounts of extract prepared from treated and untreated cells were loaded onto 8% SDS-polyacrylamide gels and resolved by SDS-PAGE. Proteins were transferred from the gel to a nitrocellulose membrane by electrophoretic elution in a buffer containing 25 mM Tris (pH 8.3), 130 mM glycine, 0.1% SDS, and 20% methanol for 12 h. Nonspecific binding sites on the membrane were blocked with 5% nonfat dry milk in 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 at room temperature with agitation for 1 h. The blot was probed with an affinity-purified polyclonal anti-iNOS antibody raised against a 21-kDa protein fragment from the COOH-terminal region of mouse macrophage iNOS (Transduction Laboratories, Lexington, KY). The antibody was diluted 1:100,000 in blocking buffer and incubated with the membrane for 1 h at room temperature with agitation. The blot was washed five times with 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 and incubated with agitation for 1 h with rabbit anti-mouse IgG conjugated to horseradish peroxidase, diluted 1:2,000 in blocking buffer. The blot was rinsed five times, exposed to ECL reagents according to the manufacturer’s instructions, and exposed to film.

Statistical analysis. Group means were compared by one-way ANOVA and Student’s t-test.
RESULTS

In these studies, we examined the effects of nitrosative stress imposed by incubation of vascular endothelial cells with SNAP. SNAP decomposes slowly, presumably by homolytic decomposition to release NO. Metal ions, especially copper ions, and visible light are known to catalyze decomposition and may dramatically increase the rate of NO release (28). The half-life is thus dependent on the conditions. We determined the rate of decomposition of SNAP (measured as loss of the S-NO absorbance at 335 nm) incubated with confluent endothelial cells in DMEM containing 1% FBS under fluorescent light and found the half-life to be 4 h. When vascular endothelial cells were exposed to SNAP, two responses were seen. The nature of the response depended on the concentration of SNAP and on the length of exposure. Acute exposure (1 h) to concentrations from 1 to 10 mM led to a fall in intracellular glutathione (GSH and GSSG) concentrations (Fig. 1). The decline in GSH seen in response to a 1-h incubation with 5 mM SNAP was presumably the result of ~0.8 µmol/ml NO released into solution (based on the half-life). However, it is unlikely that the intracellular compartment is exposed to all of the NO released, since NO produced in the medium may diffuse into the atmosphere or may react with either O₂ or metal ions both outside and within the cell. Furthermore, because NO itself does not react appreciably with cellular thiols, oxidation to more reactive species such as NO⁺ is likely responsible for the effects shown in Fig. 1. This acute response to NO exposure has been previously reported by us and others (5, 11, 13, 25).

In contrast to these effects resulting from acute exposure to millimolar concentrations, chronic exposure to lower concentrations of SNAP (those that do not deplete GSH) led to a time-dependent elevation of intracellular GSH (Fig. 2). The increase in GSH was first evident at 6 h, continued to increase to about four times the control levels, and remained elevated for 16–18 h. The effect of SNAP on GSH levels was concentration dependent (Fig. 3), reaching a maximum at 1 mM. At higher concentrations (5 mM), cells appeared to be damaged (some were detached). Incubation with N-acetyl-penicillamine did not produce a similar increase in GSH. In fact, at higher concentrations (~1 mM N-acetyl-penicillamine), GSH levels were lower than those in untreated cells. Long-term incubation with other nitrosothiols (GSNO) also led to a rise in cytosolic GSH levels (Fig. 3B). In contrast to the effect of SNAP, the effect of GSNO was still evident at 5 mM, perhaps due to the longer half-life of GSNO (slower NO release) and lack of overt signs of injury (no detachment).

Others have seen increases in intracellular GSH levels after exposing cells to arsenite, DEM, or H₂O₂ (6, 7, 19, 21). In each case, the rise in GSH was dependent on induction of cystine transport. Untreated confluent monolayers of bovine aortic endothelial cells transport cystine at a nearly linear rate (180 pmol·10⁶ cells⁻¹·10 min⁻¹) for 20 min (Fig. 4A). As reported by others, when cells were incubated for 24 h with the thiol-modifying agent DEM (125 µM), the rate was significantly increased (Fig. 4A). Similarly, when endothelial cells were incubated for 24 h with SNAP, we observed a concentration-dependent increase in the rate of cystine uptake (Fig. 4B). Incubation with 1 mM SNAP increased the rate of cystine uptake by approximately twofold. These data are the first demonstration that NO or its redox derivatives increase cystine uptake in endothelial cells.

![Fig. 1. Effect of S-nitroso-N-acetyl-penicillamine (SNAP) on intracellular GSH levels. Confluent monolayers of vascular endothelial cells were incubated at 37°C with 0, 1, 5, or 10 mM SNAP for 1 h in DMEM (1 ml total volume) containing 1% serum and 1% antibiotics. Cells were rinsed and treated with perchloric acid to precipitate protein, and acid extracts were used to measure intracellular GSH + GSSG concentrations. Data are expressed as means ± SE in nmol GSH/mg protein and represent 3 similar independent experiments with triplicate observations in each experiment. *Significantly different from control, P < 0.05.](image-url)

![Fig. 2. Time course for SNAP-induced elevation of GSH levels. Confluent monolayers of vascular endothelial cells were treated with 1 mM SNAP in DMEM (2 ml) containing 10% serum and 1% antibiotics at 37°C. Incubations were stopped at times indicated, cells were rinsed and treated with perchloric acid to precipitate protein, and acid extracts were used to measure intracellular GSH + GSSG. Data are expressed as means ± SE in nmol GSH/mg protein and represent 3 similar independent experiments with triplicate observations in each experiment.](image-url)
In subsequent experiments, we examined the mechanisms for increased uptake and characterized the transport system. The increase in cystine transport activity was observed as early as 4 h after exposure to 1 mM SNAP and continued to be elevated for at least 24 h (Fig. 5). The mechanism for increased cystine uptake appeared to require both RNA and protein synthesis, since either actinomycin D or cycloheximide blocked the response to SNAP (Fig. 6). As such, the effect of SNAP appears to be the induction of expression of a cystine transport system. The time course for NO-mediated effects on cystine uptake is similar to that seen for NO-stimulated increases in cellular GSH reported in Fig. 2 and is consistent with a role for induced cystine uptake in the elevation in GSH.

We further characterized SNAP-induced cystine uptake by examining the Na\(^+\) dependence and effect of glutamate. As shown in Fig. 7, substituting Li\(^+\) for Na\(^+\) in the medium had little effect on the extent of cystine uptake (compared with control without Na\(^+\)) induced following a 24-h exposure to SNAP, suggesting that stimulated uptake is primarily Na\(^+\) independent. Na\(^+\)-independent cystine uptake is characteristic of the \(x_c\) system. The cystine transport system is also known to have a high affinity for glutamate and exchanges intracellular glutamate for extracellular cystine. Another glutamate transporter, \(\gamma\)-glutamyl transpeptidase, which is also inhibited by glutamate and is Na\(^+\) independent, participates in cystine uptake in some cells (33). We have examined the potential role of this enzyme by investigating the effects of acivicin, a specific \(\gamma\)-glutamyl transpeptidase inhibitor, on SNAP-induced cystine uptake. Acivicin (5 mM) did not significantly alter cystine uptake following treatment of cells with SNAP (Fig. 9), suggesting that \(\gamma\)-glutamyl transpeptidase is not involved in the NO-mediated stimulation of cystine uptake.

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Fig. 3. Effect of S-nitrosothiols on GSH levels in endothelial cells. Confluent monolayers of vascular endothelial cells were incubated in DMEM containing 1% fetal bovine serum (FBS) and 1% antibiotics with various concentrations of either SNAP or S-nitrosogluthathione (GSNO) for 16 h at 37°C. Cells were then rinsed with ice-cold Hanks’ balanced salt solution-10 mM HEPES (HBSS-HEPES; pH 7.4) and treated with perchloric acid, and acid extracts were used to measure intracellular GSH + GSSG. A: incubation with SNAP or N-acetyl-penicillamine (NAP). B: incubation with GSNO or GSSG. Data are expressed as means ± SE in nmol GSH/mg protein; n = 4 measurements.

Fig. 4. Stimulation of cystine uptake by SNAP. Confluent monolayers of endothelial cells were treated with SNAP for 24 h in DMEM (2 ml) containing 10% serum and 1% antibiotics at 37°C. To measure cystine uptake, cells were rinsed and incubated in HBSS-HEPES containing 1 µCi \[^{35}S\]\(\text{cysteine} \) (60 µM) for 10 min. After times indicated, cells were rinsed 4 times with ice-cold HBSS-HEPES buffer containing 600 µM cystine and lysed in 1% Triton X-100 in HBSS-HEPES, and cystine uptake was measured by determining amount of \[^{35}S\]\ in an aliquot of lysate using a liquid scintillation counter. A: time course of cystine uptake in control and with diethyl maleate (DEM; 125 µM). Data are expressed as counts/min (cpm) of \[^{35}S\]\cysteine taken up into cells per microgram of protein (means ± SE, n = 6). B: cystine uptake after 24-h incubation of endothelial cells with SNAP at indicated concentrations. Data are expressed as multiples of control value and represent 3 similar independent experiments with triplicate observations in each experiment. Basal uptake was 71.17 ± 2.31 cpm·µg protein\(^{-1}\)·10 min\(^{-1}\) (mean ± SE), which is equivalent to 370 pmol·10\(^6\) cells\(^{-1}\)·10 min\(^{-1}\). *Significantly different from control, P < 0.05.
not responsible for the glutamate-sensitive cystine uptake we have measured. Taken together, these data strongly suggest that NO increases cystine uptake by inducing some critical component of the $\text{xc}_2$ transport system.

As shown above, exposure to low levels of SNAP ($\leq 1$ mM) increased GSH levels over a time course similar to that seen for induction of the cystine transport system. This suggests that increased cystine uptake participates in increased intracellular GSH levels. In the following experiments, we explored this possibility using glutamate in the medium bathing cells to block the newly expressed $\text{xc}_2$ uptake system. As shown in Fig. 10, addition of 5 mM glutamate to the medium completely prevented the SNAP-induced increase in intracellular GSH, providing strong evidence that cystine uptake played at least a partial role in elevation of GSH levels.

Finally, we determined whether NO produced by one cell type could affect GSH levels and cystine uptake in a neighboring cell. To examine this, we used RAW 264.7 rat monocytes grown on the membrane insert of a Transwell apparatus. These cells were pretreated with a cytokine mixture to induce iNOS. We have shown in other studies that the combination of endotoxin and interferon-$\gamma$ used in these studies leads to expression of iNOS within 4 h. Levels of protein expression reached maximal levels by 24 h and remained elevated for at least 48 h (Fig. 11A). RAW 264.7 cells treated in this manner synthesized NO at a linear rate ($5.3\,\text{nmol} \cdot \text{ml}^{-1} \cdot \text{h}^{-1} \cdot \text{insert}^{-1}$). The inserts were rinsed and then placed in plates in which endothelial cells had been grown to confluence on the bottom of the well. The

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**Fig. 5.** Time course for SNAP-stimulated cystine uptake. Confluent monolayers of endothelial cells were treated with 1 mM SNAP in DMEM (2 ml) containing 10% serum and 1% antibiotics at 37°C. After times indicated, cells were rinsed twice with HBSS-HEPES and cystine uptake was determined as described in Fig. 4. Data are expressed as cpm [35S]cystine/µg protein (means ± SE, n = 6).

**Fig. 6.** Effect of cycloheximide or actinomycin D on SNAP-stimulated cystine uptake. Confluent endothelial cells were incubated in buffer containing 1 mM SNAP and either cycloheximide (CHX; 1 µg/ml) or actinomycin D (AD; 2.5 µg/ml) for 8 h in DMEM containing 10% serum and 1% antibiotics at 37°C. Cells were then rinsed as described in Fig. 4, and cystine uptake was determined. Data are expressed as multiples of control value (means ± SE, n = 6). Control value for this experiment was (mean ± SE) 29.3 ± 0.5 cpm·µg protein·10 min–1, which is equivalent to −173 pmol·106 cells·10 min–1. *Significantly different from control, P < 0.001. † Significantly different from SNAP-treated group, P < 0.001.

**Fig. 7.** Effect of Na$^{+}$ on SNAP-induced cystine uptake. Confluent endothelial cells were treated with 1 mM SNAP in DMEM containing 10% serum and 1% antibiotics at 37°C as described in Fig. 4. After 24 h, cells were rinsed and cystine uptake was measured in Na$^{+}$-complete buffer or buffer in which Li$^{+}$ replaced Na$^{+}$. Data are expressed as cpm [35S]cystine/µg protein (means ± SE, n = 6). *Significantly different from paired control (Cont), P < 0.05. † Significantly different from Na$^{+}$-free control, P < 0.05.

**Fig. 8.** Effect of glutamate (GLU) on SNAP-induced cystine uptake. Confluent endothelial cells were treated with SNAP (1 mM) in DMEM containing 10% serum and 1% antibiotics at 37°C as described in Fig. 4. Cystine uptake was determined in presence or absence of glutamate (5 mM) in uptake assay buffer. Data are expressed as multiples of control (means ± SE, n = 6). *Significantly different from control, P < 0.001. † Significantly different from SNAP-treated group, P < 0.001.
**DISCUSSION**

NO is thought to regulate cellular function by interacting with critical thiols, leading to formation of both protein and small-molecular-weight nitrosothiols (1, 12, 30, 31). This mechanism has been proposed to be involved in NO-mediated activation of p21<sup>ras</sup> (15) and Ca<sup>2+</sup>-dependent K<sup>+</sup>-dependent K<sup>+</sup> channels (2), inactivation of caspases (9, 22), and regulation of ryanodine receptor activity (32, 37). In addition, acute exposure of cells to NO is associated with a transient decline in cellular GSH levels (5, 11, 25), presumably due to conversion of GSH to GSNO (5). It should be pointed out that these effects are not mediated by NO directly, since NO is a weak oxidant (1, 31). Rather, other redox forms, especially NO<sup>-</sup> or species with NO<sup>-</sup>-like activity, produced through reaction of NO with transition metal ions, heme iron, or molecular oxygen are proposed to be intermediates (1, 31). These reactive NO-derived species are potent oxidants and pose a nitrosative stress to cells by compromising thiol redox status. In this regard, the GSH redox cycle has been shown to be an important protective mechanism that rapidly reverses NO-mediated protein modification and GSH depletion. For example, we have shown that the GSH redox cycle is required for reversal of NO-mediated glyceraldehyde-3-phosphate dehydrogenase inhibition in intact cells (23, 24) and in the recovery of GSH levels in cells exposed to exogenous sources of NO (25). In fact, both glutathione reductase and GSH levels are critical and necessary for restoring the native reduced protein thiol (24, 25). Through this mechanism, the GSH redox cycle is likely important in regulating cellular signaling mechanisms used by NO and provides a mechanism for restoring the basal state after proteins are modified (25). Similarly, the GSH redox cycle protects cells from oxidative stress imposed by oxygen-derived species. Chronic exposure of cells to oxidants leads to an array of adaptive responses. Given the importance of the GSH redox cycle, it is not surprising that one important component of the adaptive response is elevation of intracellular GSH levels. In many cells, this has been shown to occur through a mechanism that involves induction of cystine transport (7, 14, 27, 33). Because it is possible that cells respond to nitrosative and oxidative stress in similar ways, we have examined the effects of NO on cellular GSH levels and shown for the first time that NO, like reactive oxygen species, leads to an adaptive increase in GSH though a mechanism that, at least in part, requires induction of cystine transport.

Although acute exposure to NO leads to GSH depletion, chronic exposure leads to elevation of cellular GSH. In the present study, we show that 1 h of incubation with 5 mM SNAP leads to a 40% fall in GSH. Similar reductions in GSH levels have been reported by us and others in cells exposed to a variety of NO donors, including GSNO and spermine NO (25). In one study, it was suggested that the rapid fall in GSH in cells exposed to NO resulted from conversion of GSH to GSNO intracellularly (5). In this report, GSNO was identified as the NaBH<sub>4</sub>-reducible component of cellu-
lar extracts. In recent work by us (25), we report that GSNO and spermine deplete GSH in endothelial cells by promoting rapid formation of glutathionyl protein mixed disulfides. Because protein mixed disulfides are rapidly reduced by NaBH₄ to release free GSH, it seems likely that the presence of these mixed disulfides can account for GSNO identified previously by others (5) and is the likely explanation for the fall in GSH reported in the present study.

In early experiments, we had observed that, after the initial decline in GSH, levels recover and begin to exceed the concentrations present in control cells. The extent of the initial decline is concentration dependent such that low concentrations of NO donor (≤1 mM SNAP in the present study) lead to a rise in GSH without evidence of depletion. The increase in GSH was significant by 6 h. Several mechanisms may be involved in elevation of cellular GSH levels. These include increased biosynthesis of GSH or inhibition of GSH export. We have not observed any measurable GSH export from our endothelial cells and thus can rule out this mechanism, leaving us with the possibility that GSH biosynthesis is increased. GSH biosynthesis is dependent on the activity of the rate-limiting enzyme, γ-glutamylcysteine synthetase, and the availability of cysteine (18, 26). The activity of γ-glutamylcysteine synthetase is regulated by GSH, which competes with glutamate at the glutamate binding site in the catalytic domain and serves as a potent inhibitor of the enzyme. As levels of intracellular GSH fall due to mixed disulfide formation, conjugation, or export, γ-glutamylcysteine synthetase is activated. GSH also appears to maintain inhibition of the enzyme by keeping an allosteric thiol reduced (20). However, because GSH depletion is not necessarily seen in cells in which GSH levels rise and because GSH levels are above control levels for an extended period, GSH depletion and subsequent activation of γ-glutamylcysteine synthetase seems to be an unlikely explanation for increased biosynthesis. Direct effects of NO redox species on γ-glutamylcysteine synthetase have not been documented, although NO appears to induce the expression of this enzyme in lung epithelial cells (16). Possibly this ability to increase levels of enzyme protein may explain the increase in GSH. However, data showing that the NO-induced increase in GSH is inhibitable by extracellular glutamate, a competitive inhibitor of cystine uptake, suggest that induction of the xₑ transport system is quantitatively more important.

Supply of intracellular cysteine is known to limit GSH synthesis in many cases (7, 8, 10, 18). Cysteine itself is transported by the ASC amino acid transport system (4, 14, 29). This uptake mechanism is Na⁺ dependent and has a high capacity for cysteine transport (4, 14, 29). However, cysteine is oxidized to cystine outside cells, and cystine levels are generally higher than cysteine levels in the extracellular environment. Thus mechanisms for cystine uptake are important in maintaining intracellular GSH levels. A specific transport system designated κₓₑ, which is Na⁺ independent and exchanges extracellular cysteine for intracellular...
glutamate, has been described in a number of cells (7, 14, 19, 27, 33). Although the individual components have not been identified, this uptake system has been demonstrated to be activated by oxidant stress, sulfhydryl modifying reagents, arsenite, and cadmium in vascular endothelial cells, macrophages, and smooth muscle cells (7, 14, 19, 27, 33). The increase in cystine uptake stimulated by NO requires RNA and protein synthesis, becomes evident by ~6 h, and remains elevated for at least 24 h. This time course is consistent with the increase in GSH levels seen in cells exposed to SNAP. Although it had been suggested that depletion of GSH is involved in stimulation of cystine uptake (19), the effect we report with NO occurs in the absence of a detectable fall in cellular GSH levels. NO induction of cystine uptake appears to be mediated by the xϕ transport system, since it is Na+ independent and inhibitable by extracellular glutamate.

In some cells, γ-glutamyl transpeptidase may also participate in cysteine uptake. This protein is a cell surface enzyme that forms γ-glutamyl amino acids (γ-glutamylcysteine for example) and transports them into the cell, where cysteine may be released by peptidases. In studies with pancreatic duct cells (33), cystine uptake was found to be mediated by both the xϕ transport system and γ-glutamyl transpeptidase (which accounted for 40–50% of cystine uptake). Uptake of this γ-glutamyl transpeptidase is also Na+ independent and inhibitable by glutamate. However, because γ-glutamyl transpeptidase is specifically blocked by acivicin, this compound can be used to distinguish between the two possibilities. In our studies, acivicin had no effect on NO-induced cystine uptake, suggesting that in endothelial cells induction of the xϕ system is the primary mechanism. Furthermore, as mentioned above, induction of cystine uptake by NO fully explains the increase in cellular GSH levels stimulated by NO, since extracellular glutamate completely prevented this effect, and suggests the requirement of the xϕ system.

Induction of cystine uptake and elevation of intracellular GSH by NO donors suggest that NO produced by one cell might alter GSH metabolism in a neighboring cell. In our studies, NO produced by rat monocytes increased GSH levels and cystine uptake in endothelial cells after 20 h of coculture. This is important for several reasons. First, it suggests that endogenous NO production can induce cystine uptake and elevate GSH levels in cells. It also suggests that NO production by one cell can mediate adaptive responses in neighboring cells. Thus, in a setting where iNOS is induced and produces NO over an extended period, cells adapt by increasing their GSH levels and ability to reverse NO-mediated changes in cellular thiols (25). This is likely to be an important mechanism whereby tissue cells resist the potentially damaging effects of NO produced during inflammation.

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REFERENCES


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