Adaptive responses to peroxynitrite: increased glutathione levels and cystine uptake in vascular cells

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Buckley, Barbara J., and A. Richard Whorton. Adaptive responses to peroxynitrite: increased glutathione levels and cystine uptake in vascular cells. Am J Physiol Cell Physiol 279: C1168–C1176, 2000.—We and others recently demonstrated increased glutathione levels, stimulated cystine uptake, and induced γ-glutamylcysteinyl synthase (γ-GCS) in vascular cells exposed to nitric oxide donors. Here we report the effects of peroxynitrite on glutathione levels and cystine uptake. Treatment of bovine aortic endothelial and smooth muscle cells with 3-morpholinosydnonimine (SIN-1), a peroxynitrite donor, resulted in transient depletion of glutathione followed by a prolonged increase beginning at 8–9 h. Concentration-dependent increases in glutathione of up to sixfold occurred 16–18 h after 0.05–2.5 mM SIN-1. Responses to SIN-1 were inhibited by copper-zinc superoxide dismutases and manganese(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride, providing evidence for peroxynitrite involvement. Because glutathione synthesis is regulated by amino acid availability, we also studied cystine uptake. SIN-1 treatment resulted in a prolonged increase in cystine uptake beginning at 6–9 h. Increases in cystine uptake after SIN-1 were blocked by inhibitors of protein and RNA synthesis, by extracellular glutamate but not by extracellular sodium. These studies suggest induction of the $\chi_\alpha$ pathway of amino acid uptake. A close correlation over time was observed for increases in cystine uptake and glutathione levels. In summary, vascular cells respond to chronic peroxynitrite exposure with adaptive increases in cellular glutathione and cystine transport.

endothelial cells; smooth muscle cells

ACUTE EXPOSURE TO HIGH LEVELS of nitric oxide (NO) and peroxynitrite (ONOO$^-$) may lead to alterations in cellular signaling and function through the modification of lipids and protein thiols (20, 34, 35). Chronic exposures to lower levels may have different effects. For example, work from our laboratory and others demonstrated increased cellular glutathione (GSH) levels in endothelial and smooth muscle cells treated with S-nitrosothiols and other NO donors (21, 27). Elevation of GSH levels is a common response to oxidant stress and can modulate cell injury by enhancing the capacity to scavenge radical species and repair oxidized protein thiols and lipid peroxides (7, 23, 31). Recently, cytokine-stimulated NO production was found to be essential for the upregulation of GSH synthesis and the prevention of oxidant injury in hepatocytes (18, 19). These results suggest that the proposed antioxidant role of NO may involve more than the direct scavenging of radical species and may also be related to increases in cellular GSH. This adaptive response may result from induction of γ-glutamylcysteinyl synthase (γ-GCS), the rate-limiting enzyme in GSH biosynthesis, induction of cystine uptake resulting in greater substrate availability, or a combination of both (7, 23). In our previous study of endothelial cells treated with S-nitrosothiols, elevated GSH levels appeared to result from the increased uptake of cystine by the $\chi_\alpha$ pathway of amino acid transport (21). By contrast, in vascular smooth muscle cells treated with S-nitrosothiols, elevated GSH was reported to be due to induction of γ-GCS, the rate-limiting enzyme for GSH synthesis (27).

In the case of ONOO$^-$, many deleterious effects have been reported in vascular cells exposed acutely to high levels, including altered calcium signaling (11), collapse of mitochondrial membrane permeability (14), and decreased cell viability (14). Adaptive responses to ONOO$^-$ have not been reported. However, ONOO$^-$ is not always injurious. For example, ONOO$^-$ is capable of increasing cGMP levels in endothelial cells (25), stimulating relaxation of vascular smooth muscle (42), and inhibiting platelet aggregation (28). Because ONOO$^-$ is a stronger oxidant than NO, we reasoned that vascular cells exposed to this mediator would respond in a similar manner by elevating GSH levels. In this study, we investigated GSH synthesis and cystine uptake in vascular cells using 3-morpholinosydnonimine (SN-1), which generates ONOO$^-$ from the superoxide ($O_2^-$) and NO formed during decomposition.

METHODS

Materials. Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). All other cell culture reagents were from Gibco BRL (Grand Island, NY). Tissue culture plasticware was obtained from Nunc (Fisher Scientific, Raleigh, NC). S-nitroso-penicillamine (SNAP), SIN-1, and man-
ganese(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloro-
zide (MnTMPyP) were from Alexis (San Diego, CA). Cu,Zn SOD (copper-zinc superoxide dismutases) was from Roche Molecular Biochemicals (Indianapolis, IN). L-[35S]cysteine was obtained from Amersham (Piscataway, NJ). All other chemicals were from Sigma (St. Louis, MO).

**Cell isolation and culture.** Bovine aortic endothelial cells and smooth muscle cells were isolated and cultured by established methods as previously described (4, 21). Briefly, bovine aortic segments were cleaned, opened, and the endothelium was removed by collagenase treatment. Endothelial cell isolates were plated on collagen-coated tissue culture plastic ware. Smooth muscle cells were grown from explants of freshly collected aortas. Cells were incubated at 37°C in an atmosphere of 7.5% CO2 in air in Dulbecco’s modified Eagle’s medium (DMEM) that contained 10% (vol/vol) FBS and antibiotic/antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B). Cells were passaged by treatment with trypsin-EDTA.

**Experimental conditions.** Early passage primary cultures of endothelial and smooth muscle cells were used. Confluent monolayers of cells were incubated for 1–24 h in DMEM (phenol red free) that contained 1% FBS, 1% antibiotic/antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B). Cells were passaged by treatment with trypsin-EDTA.

**Materials.** Antibiotic/antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were from Sigma (St. Louis, MO).

**RESULTS**

We have previously demonstrated that treatment of endothelial cells with S-nitrosothiols resulted in increased levels of cellular GSH (21). Endothelial cells responded similarly to SIN-1, with 2.4- and 6-fold increases in cellular GSH 17 h after treatment with 0.1 and 1 mM, respectively (Fig. 1). Increased cellular GSH was also observed using 1 mM SNAP as a positive control (Fig. 1). Decomposed SIN-1 and SNAP (0.5 mM) failed to stimulate an increase in GSH levels (data not shown). Concentrations of SIN-1 >1 mM were associated with a decline in cell protein levels of >20% and were not routinely used in these studies. Elevations in cellular GSH of 1.9-fold were observed with concentrations of SIN-1 as low as 0.05 mM (Figs. 2 and 3). To determine whether this response required de novo protein and RNA synthesis, cells were treated with 0.5 mM SIN-1 for 8 h in the presence of cycloheximide (1 μg/ml) or actinomycin D (2.5 μg/ml). Both agents effectively blocked the response (Fig. 4). Control levels were increased with cycloheximide treatment, an effect attributed to a shift in cysteine utilization from protein synthesis to GSH synthesis (6).

Because SIN-1 can act as a ·NO donor rather than an ONOO− donor under some conditions (15, 37), we investigated the response to SIN-1 in the presence of Cu,Zn SOD and the SOD mimetic MnTMPyP. By scavenging O2•−, these agents would be expected to block the formation of ONOO− during SIN-1 decomposition, leaving ·NO as the major decomposition product. Incubation of cells for 17 h with 0–0.5 mM SIN-1 in the presence of 100 U/ml Cu,Zn SOD resulted in a 32–67% inhibition of the GSH response (Fig. 2). No further inhibition was observed using 250 U/ml Cu,Zn SOD (data not shown). However, boiled enzyme was much less effective in blocking the response to 0.5 mM SIN-1,
demonstrating that inhibition required active enzyme (Fig. 2, inset). With the use of 0.1 mM MnTMPyP, the increase in cellular GSH observed in response to SIN-1 was diminished by 69–92% (Fig. 3). A concentration of 0.05 mM MnTMPyP was equally effective. These results indicate that at least part of the response to SIN-1 was mediated by ONOO\(^{-}\) and that \(\cdot\)NO, which may have been generated during SIN-1 decomposition, could not fully account for the response.

We also tested the effects of Cu,Zn SOD on SNAP-mediated increases in cellular GSH levels because \(\cdot\)NO produced endogenously by endothelial cells might react with SNAP-derived \(\cdot\)NO, forming ONOO\(^{-}\). These experiments demonstrated a small and variable effect of Cu,Zn SOD that was not altered by boiling the enzyme (data not shown). Additionally, we found that Cu,Zn SOD had no effect on increases in cellular GSH levels in endothelial cells treated with another \(\cdot\)NO donor, spermine NONOate (data not shown). These results provided evidence that the effects of the \(\cdot\)NO donors were not secondary to ONOO\(^{-}\) production.

Increased cellular GSH in smooth muscle cells after treatment with \(\text{S-nitrosothiols}\) has also been recently reported (27). To determine whether SIN-1 has similar effects, we incubated smooth muscle cells with 0–2.5 mM SIN-1 or SNAP for 18 h. Increases in cellular GSH of 2.6- to 2.7-fold were observed with these agents (Fig. 5). However, a decline in cellular protein levels of 40% was observed in smooth muscle cells incubated with 1 and 2.5 mM SNAP (data not shown). Cellular protein levels decreased by 5–10% with the same doses of SIN-1 (data not shown). It should be noted that smooth muscle cell GSH levels were variable between experiments, as has been previously reported (27).

The availability of precursor amino acids, particularly cysteine, is an important regulator of GSH synthesis (2, 7), and cells in culture rely on extracellular cystine for their intracellular cysteine pool (2). We have previously reported that \(\cdot\)NO donors increase cystine uptake in endothelial cells, but the effects of ONOO\(^{-}\) were not determined. Furthermore, the role of increased cystine uptake in elevating GSH levels in vascular smooth muscle treated with \(\cdot\)NO donors or ONOO\(^{-}\) has not been investigated. Thus we next studied the effects of SIN-1 on cystine uptake. Endothelial and smooth muscle cells were incubated with 1 mM SNAP and SIN-1 for 12 h, and the uptake of radiolabeled cystine during 10 min was determined. No decline in cellular protein levels was observed under these experimental conditions. The response to SIN-1 was similar in both cell types (2.3- to 2.4-fold), whereas the response to SNAP was much greater in endothelial cells (3.7-fold) than in smooth muscle cells (52%; Fig. 6). Interestingly, basal cystine uptake was fourfold higher in smooth muscle cells than in endothelial cells.
To determine whether the increase in cystine uptake was due to induction of amino acid transport, endothelial and smooth muscle cells were incubated with cycloheximide (5 mg/ml) or actinomycin D (1 mg/ml) to inhibit de novo protein and mRNA synthesis, respectively, during treatment with SIN-1. Endothelial cells were incubated with 0.5 mM SIN-1 for 8 h and smooth muscle cells were incubated with 1 mM SIN-1 for 12 h. Different protocols were employed because the two cell types exhibited different sensitivities to cycloheximide and actinomycin D and different lag periods before the increase in cystine uptake occurred. Cycloheximide and actinomycin D fully blocked the increase in cystine uptake in both cell types in response to SIN-1 (Fig. 7).

A common pathway for cystine uptake, which is upregulated by oxidants, thiol reagents, and heavy metals is the \( \text{x}_c \) pathway (2, 7). This pathway is characterized by a sodium-independent counterexchange of cystine and glutamate. To determine whether the increase in cystine uptake observed in SIN-1-treated cells might be occurring by this mechanism, cystine uptake was measured in the presence or absence of extracellular glutamate after SIN-1 treatment. Glutamate (5 and 10 mM) completely blocked the SIN-1-mediated increase in cystine uptake observed in both cell types (Fig. 8). In addition, 10 mM glutamate blocked basal uptake by 82% in smooth muscle cells and by 38% in endothelial cells. These results indicate that the exchange of cystine for glutamate accounts for the majority of basal cystine uptake in smooth muscle cells, a minority of basal cystine uptake in endothelial cells, and all of the increase in cystine uptake observed in both cell types.

To provide further evidence for the involvement of the \( \text{x}_c \) pathway in the SIN-1-mediated increase in cystine uptake, we investigated the sodium dependence of the response. Endothelial and smooth muscle cells were incubated with 1 mM SIN-1 for 19 h, and cystine uptake was measured in the presence and absence of extracellular sodium. We found that the increase in cystine uptake after SIN-1 was independent of sodium status in both cell types (Fig. 9).

Because both the increase in cystine uptake and the increase in cellular GSH appeared to be induced in SIN-1-treated cells, it seemed likely that increases would occur over similar time frames. The next experiments examined the time course of the two responses in both cell types. When endothelial cells were incubated with 0.5 mM SIN-1 for 1–24 h, GSH levels were decreased by 41% at 2 h and increased 6.9-fold at 16 h (Fig. 10). Cystine uptake was increased 4.4-fold by 9 h and remained elevated at 23 h. The increase in cystine uptake was first observed at 6 h, whereas the increase in GSH was first observed at 9 h. When smooth muscle cells were similarly treated, GSH levels were decreased by 22% at 6 h and increased 2-fold at 9 h and 2.2-fold at 24 h (Fig. 11). Cystine uptake was increased by 49% at 9 h and remained elevated at 23 h. No decrease in cellular viability was observed in endothelial or smooth muscle cells treated with 0.5 mM SIN-1 for 24 h (data not shown). In fact, at the completion of the study, cell protein levels were similar in control...
and SIN-1-treated endothelial cells and higher in SIN-1-treated smooth muscle cells compared with controls.

It should be noted that the increase in cystine uptake occurred several hours later in smooth muscle cells than in endothelial cells. However, in both cell types, increased cystine uptake preceded by a few hours or occurred concomitantly with increased cellular GSH levels. These results demonstrate a close correlation in time for the two responses and suggest a causal relationship.

**DISCUSSION**

Our results demonstrate for the first time that vascular cells respond to ONOO$^-$ by increasing cellular GSH levels. Treatment of endothelial and smooth muscle cells with 0.05–2.5 mM SIN-1 for 16–18 h resulted in 1.9- to 6-fold increases in cellular GSH. In endothelial cells, the magnitude of the response was similar to that seen with SNAP. In smooth muscle cells, the elevation in GSH was greater in response to SIN-1 than to SNAP. Reasons for the smaller response to SNAP than to SIN-1 in smooth muscle cells are unknown but are possibly related to differences in metabolism of S-nitrosothiols and ONOO$^-$. Elevations in GSH have been reported in endothelial cells 8–24 h after treatment with diethyl maleate, hyperoxia, cadmium, arsenite, $N,N'$-bis(2-chloroethyl)-$N$-nitrosourea, disulfiram, and pyrrolidine dithiocarbamate (6, 8, 9, 26, 39).

In smooth muscle cells, elevations in GSH were found 24 h after treatment with SNAP, sodium nitroprusside, isosorbide dinitrate, and diethyl maleate (27, 33, 41).

In the present investigation, GSH levels began to increase 6–12 h after treatment. This response was blocked by inhibitors of protein and RNA synthesis.

Because S-nitrosothiols and SIN-1 both increased cellular GSH levels, we considered the possibility that SIN-1 was acting as a $\dot{\text{NO}}$ donor rather than a ONOO$^-$ donor under the conditions of our study. It is known that SIN-1 generates ONOO$^-$ in aqueous solutions from the $\dot{\text{NO}}$ and $O_2^-$ produced during decomposition (16) and that in MEM, $\dot{\text{NO}}$ is produced from 1 mM SIN-1 in the presence of SOD (15). Recent reports have shown that SIN-1 can also act as a $\dot{\text{NO}}$ donor in the presence of one-electron oxidizing agents that can be found in plasma (37). Our incubations with SIN-1 were, in fact, carried out in the presence of 1% FBS. If SIN-1 was acting as a $\dot{\text{NO}}$ donor by this mechanism in our studies, one would expect that the addition of $O_2^-$ scavengers would have a minimal or enhancing effect on the response. We found that Cu,Zn SOD inhibited a large part of the SIN-1 response. These results indicate that SIN-1 was not primarily acting as a $\dot{\text{NO}}$ donor in our studies and that ONOO$^-$ mediated at least part of the response to SIN-1. Incomplete inhibition of the response by Cu,Zn SOD was not surprising, given the greater efficiency of $\dot{\text{NO}}$ compared with SOD for scavenging $O_2^-$.

Results using the other $O_2^-$ scavenger, MnTMPyP, are less clear-cut but also provide evidence that ONOO$^-$ plays a major role in mediating the response to SIN-1. Recent reports demonstrate that MnTMPyP scavenges ONOO$^-$ in addition to $O_2^-$ (12). Further-
more, in the presence of a low-molecular-weight reductant, MnTMPyP also scavenges \( \cdot\text{NO} \) (12, 32). In our experiment, most of the SIN-1 response was inhibited by MnTMPyP, suggesting that either MnTMPyP efficiently scavenged \( \cdot\text{O}_2 \), resulting in little or no \( \text{ONOO}^- \) production, or that MnTMPyP efficiently scavenged the \( \text{ONOO}^- \) generated by SIN-1. A role for MnTMPyP in scavenging \( \cdot\text{NO} \) generated in the culture media seemed unlikely, given the low concentrations of low-molecular-weight reductants present. Furthermore, a role for MnTMPyP in scavenging intracellular \( \cdot\text{NO} \) in the presence of high concentrations of low-molecular-weight reductants seemed unlikely because it is not clear that MnTMPyP enters cells (32). Recent studies have also demonstrated that \( \text{ONOO}^- \) reacts rapidly with \( \text{CO}_2 \) in bicarbonate-\( \text{CO}_2 \)-buffered media (10, 13), as was used in our experiments. This results in the production of nitrosoperoxycarbonate anion (ONOOCO\(_2\)), which decomposes to form the highly reactive carbonate radical (CO\(_3^\cdot\)) (3). MnTMPyP effectively competes with \( \text{CO}_2 \) for reaction with \( \text{ONOO}^- \) and also scavenges ONOO\(_2\) (12). In our studies, the nearly complete inhibition of the SIN-1 response by MnTMPyP indicates that \( \text{ONOO}^- \) or products of its reaction with \( \text{CO}_2 \) played a major role in the GSH response.

Although \( \text{ONOO}^- \) can oxidize proteins, carbohydrates, lipids, and DNA (10, 34, 35) and can be scav-
engaged by GSH, ascorbate, and uric acid (12), the biological effects of ONOO\(^-\) more likely depend on its reaction with CO\(_2\) to form ONOOCO\(_2\) and CO\(_3\)\(^-\) (3). These reactants mediate one-electron oxidations and nitrations (3, 10, 13) and can cause the formation of thyl radicals, albumin disulfide dimers, and 3-nitrotyrosine-containing proteins in plasma (30, 36). The cellular targets of ONOO\(^-\) in the present study have not been identified. However, SIN-1 decreased cellular GSH levels in both endothelial and smooth muscle cells 1–4 h after treatment.

Our studies show that ONOO\(^-\) stimulated an increase in cystine uptake, apparently by inducing the x\(_{-}\) transport pathway. Treatment of endothelial and smooth muscle cells with 1 mM SIN-1 led to a 2.5-fold increase in cystine uptake after 12 h. SNAP treatment also increased cystine uptake in both cell types, although the response was much smaller in smooth muscle cells. The increased uptake in SIN-1-treated endothelial and smooth muscle cells exhibited characteristics of the x\(_{-}\) transport pathway, including sodium independence, inhibition by extracellular glutamate, and a requirement for new protein and RNA synthesis (2, 7). This is the first evidence for an inducible x\(_{-}\) transport pathway in smooth muscle cells. However, increased GSH and glutamate uptake in smooth muscle cells treated with diethyl maleate (33) and increased GSH and \(\gamma\)-GCS in smooth muscle cells treated with SNAP (27) have previously been reported.

Interestingly, basal cystine uptake in smooth muscle cells was fourfold higher than in endothelial cells. In addition, the majority of basal cystine uptake was inhibited by high extracellular glutamate in smooth muscle cells but not in endothelial cells. These results suggest that the x\(_{-}\) pathway is much more active in smooth muscle cells in culture than in endothelial cells. A recent report demonstrated upregulation of the x\(_{-}\) pathway in hepatocytes during isolation and culture (23). Whether or not culture conditions have a similar effect on smooth muscle cells used in the current study is not known. Others have demonstrated cell density-dependent alterations in sodium-independent glutamate uptake, presumably by the x\(_{-}\) pathway, in endothelial cells (38).

Neither the molecular components of the x\(_{-}\) transport pathway nor the mechanisms by which it is regulated have been defined. However, agents that induce the x\(_{-}\) pathway, such as pyrrolidine dithiocarbamate, disulfiram, arsenite, cadmium, hyperoxia, \(N,N'\)-bis(2-chloroethyl)-\(N\)-nitrosourea, and SNAP, share common features as oxidants and/or thiol reagents (6, 8, 9, 26, 29, 39). These agents stimulate induction of the x\(_{-}\) pathway in endothelial cells, fibroblasts, and Chinese hamster ovary cells (6, 8, 21, 26) but not in alveolar epithelial cells (17). The significance of the x\(_{-}\) pathway of cystine uptake is that it provides a mechanism whereby cells can increase their supply of intracellular cysteine. Although the ASC pathway of cysteine uptake in cells is generally more active than the x\(_{-}\) pathway, it is not inducible (2, 7). Furthermore, cystine is more readily available in cell culture medium than cysteine, which is rapidly oxidized (2). After uptake of cystine, it is reduced to cysteine and made available for protein or GSH synthesis (2). Cellular GSH synthesis is regulated by negative feedback by GSH, the activity of the rate-limiting enzyme \(\gamma\)-GCS, and the availability of precursor amino acids (7, 23). Because cysteine concentrations are often limiting, the x\(_{-}\) pathway can play an important role in enhancing cellular GSH levels (7, 23).

Our results also demonstrate that increases in GSH and cystine uptake follow a similar time course, suggesting that the availability of cysteine is rate limiting for GSH synthesis after SIN-1 treatment. Results of the current study do not rule out the possibility that \(\gamma\)-GCS was induced in response to SIN-1 treatment and contributed to the elevations in GSH observed in endothelial and smooth muscle cells. Induction of \(\gamma\)-GCS has been demonstrated in cytokine and hormone-treated hepatocytes (18, 23), tert-butyldihydroquinone-treated lung epithelial cells (22), oxidized low-density lipoprotein-treated endothelial cells (5), and SNAP-treated smooth muscle cells (27). However, different patterns of induction of the heavy (catalytic) and light (regulatory) subunits of \(\gamma\)-GCS were observed in both tert-butyldihydroquinone-treated epithelial cells and SNAP-treated smooth muscle cells (22, 27). In addition, the increase in heavy subunit mRNA and immunoreactive protein preceded by several hours the increase in cellular GSH levels in the smooth muscle cell study (27). These results suggest that other factors regulating \(\gamma\)-GCS activity, such as substrate availability, may play a role in elevating GSH levels. Furthermore, induction of \(\gamma\)-GCS is not always required for increases in GSH levels to occur, as was demonstrated in arsenite-treated fibroblasts (29).

Results of the present study demonstrate a close correlation over time between the increases in cystine uptake and the increases in cellular GSH in endothelial and smooth muscle cells treated with SIN-1. We found a similar correlation in SNAP-treated endothelial cells (21). These results suggest that intracellular cysteine levels may be rate limiting for GSH synthesis and that increased cystine uptake by the x\(_{-}\) pathway may play a key role in elevating cellular GSH levels.

Our studies demonstrate that ONOO\(^-\) stimulates adaptive responses in vascular cells. These results suggest that ONOO\(^-\), like NO\(^-\), may protect cells against oxidative/nitrosative stress by increasing cellular GSH levels. This may be considered an adaptive response due to the many cytoprotective functions of this low-molecular-weight antioxidant. In fact, a GSH-dependent detoxification mechanism has recently been reported in vascular tissue exposed to ONOO\(^-\) (24).

In the present study, it should be noted that GSH levels were decreased over the first few hours of exposure to ONOO\(^-\) in both endothelial and smooth muscle cells. This suggests that cellular GSH may have reacted directly with ONOO\(^-\) or ONOO\(^-\)-derived species such as ONOO\(_2\)\(^-\) and CO\(_3\)\(^-\) and may have been consumed in the process (36). The possibility that significant amounts of \(S\)-nitrosoglutathione were formed by
the direct reaction of ONOO− with GSH seems unlikely, given the extremely low yield of this product in other models (1). Because the concentration of low-molecular-weight thiols in the media was very low, extracellular reactions of ONOO− or its byproducts with GSH was most likely to be negligible. However, one-electron oxidation reactions of ONOOCO2− or CO2 with protein thiols in the media or cells may have occurred and resulted in the formation of thiol radicals, protein disulfides/mixed disulfides, and nitrothiols, which can release ·NO under certain circumstances (36). In addition, cellular GSH may have been consumed by glutaredoxin-catalyzed reduction of protein mixed disulfides formed subsequent to the reaction of protein thiols with ONOO− or its byproducts (31). Alternatively, GSH may have participated as a cofactor in GSH peroxidase-catalyzed repair of lipid peroxidation that resulted from ONOO− exposure (7, 23). In these ways, cellular GSH may have played a key role in maintaining cellular integrity and function after exposure to ONOO−. Consequently, the elevation in cellular GSH observed may represent an important adaptive response to chronic oxidative and/or nitrosative stress.

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