Carbon Monoxide Produced by Heme Oxygenase-1 in Response to Nitrosative Stress Induces Expression of Glutamate-Cysteine Ligase in PC12 Cells via Activation of Phosphatidylinositol 3-Kinase and Nrf2 Signaling*

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Induction of heme oxygenase-1 (HO-1) expression has been associated with adaptive cytoprotection against a wide array of toxic insults, but the underlying molecular mechanisms remain largely unresolved. In this study, we investigated the potential role of carbon monoxide (CO), one of the by-products of the HO-1 reaction, in the adaptive survival response to peroxynitrite-induced PC12 cell death. Upon treatment of rat pheochromocytoma (PC12) cells with the peroxynitrite generator 3-morpholinosydnonimine hydrochloride (SIN-1), the cellular GSH level decreased initially, but was gradually restored to the basal level. This was accompanied by increased expression of the catalytic subunit of glutamate-cysteine ligase (GCLC), the rate-limiting enzyme in GSH biosynthesis. The SIN-1-induced GCLC up-regulation was preceded by induction of HO-1 and subsequent CO production. Inhibition of HO activity by zinc protoporphyrin IX or knockdown of HO-1 gene expression by small interfering RNA abrogated the up-regulation of GCLC expression and the subsequent GSH restoration induced by SIN-1. In contrast, additional exposure to the CO-releasing molecule (CO-RM) restored the GSH level previously reduced by inhibition of HO activity using zinc protoporphyrin IX. Furthermore, CO-RM treatment up-regulated GCLC expression through activation of Nrf2. The CO-RM-induced activation of Nrf2 was under the control of the phosphatidylinositol 3-kinase/Akt signaling pathway. In conclusion, CO produced by HO-1 rescues PC12 cells from nitrosative stress through induction of GCLC, which is mediated by activation of phosphatidylinositol 3-kinase/Akt and subsequently Nrf2 signaling.

Heme oxygenase (HO) is the rate-limiting enzyme involved in the oxidative degradation of free heme and produces biliver-
array of toxic insults (17, 18), we attempted to examine whether CO could elevate the GSH level in PC12 cells challenged with peroxynitrite, thereby providing cytoprotection against nitrosative stress. Here we report that the CO produced as a consequence of peroxynitrite-induced up-regulation of HO-1 expression induces the expression of GCLC via activation of phosphatidylinositol 3-kinase (PI3K)/Akt and subsequently Nrf2/antioxidant-responsive element (ARE) signaling.

EXPERIMENTAL PROCEDURES

Materials—Tricarbonyldichlororuthenium(II) dimer ((Ru(CO)3Cl2)2, a CO-releasing molecule (CO-RM)), buthionine (S, R)- sulfoximine (BSO), poly-d-lysine, N-acetyl-l-cysteine, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. The peroxynitrite generator 3-morpholinosydnonimine hydrochloride (SIN-1) was supplied by BIOMOL (Plymouth Meeting, PA). Dulbecco’s modified Eagle’s medium, fetal bovine serum, horse serum, nutrient mixture F-12, and N-2 supplement were obtained from Invitrogen. Zinc protoporphyrin IX (ZnPP IX) was the product of OXIS International, Inc. (Portland, OR). [γ-32P]ATP was purchased from PerkinElmer Life Sciences. Antiserum against GCLM was a generous gift from Dr. Terrance J. Kavanagh (University of Washington, Seattle, WA).

Cell Culture and Viability Measurement—PC12 cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum in a humidified atmosphere of 10% CO2 and 90% air. All cells were cultured in poly-D-lysine-coated culture dishes. Cells were plated at an appropriate density according to the scale of each experiment, and the medium was changed at 3-day intervals. After a 24-h subculture, cells were switched to a serum-free N-2 supplement defined medium for treatment with CO-RM and/or other agents. Cell viability was determined by the MTT assay as described previously (19).

GSH Measurement—The intracellular GSH levels in PC12 cells were assessed using a commercially available colorimetric assay kit (BIOXYTECH GSH-400, OXIS International, Inc.). After exposure to SIN-1 and/or other agents, PC12 cells were harvested and homogenized in metaphosphoric working solution. After centrifugation, 50 µl of R1 solution (solution of chromogenic reagent in HCl) was added to 700 µl of supernatant, followed by gentle vortex mixing. Subsequently, following the addition of 50 µl of R2 solution (30% NaOH), the mixture was incubated at 25 °C for 10 min. After centrifugation, the absorbance of the clear supernatant was read at 400 nm. The protein concentration was determined using the BCA protein assay kit (Pierce).

Western Blot Analysis—After treatment with SIN-1 or other agents, cells (1 × 10^7/ml in a 100-mm dish) were collected, washed with phosphate-buffered saline (PBS), and centrifuged. Cell lysis was carried out at 4 °C by vigorous shaking in cell lysis buffer (Cell Signaling Technology, Beverly, MA). Following centrifugation at 10,000 × g for 15 min, the supernatant was collected and stored at −70 °C until used. The protein concentration was determined using the BCA protein assay kit. After mixing with loading buffer, protein samples were electrophoresed on a 12% SDS-polyacrylamide gel. Separated proteins were transferred to polyvinylidene difluoride membrane at 300 mA

![Image](https://example.com/image.png)

**FIGURE 1.** Time-dependent changes in the total GSH level, expression of GCLC and HO-1, and CO production in SIN-1-treated PC12 cells. A, PC12 cells were treated with SIN-1 (2.5 mM) for the indicated times and harvested for the GSH assay. The intracellular GSH level was measured as described under "Experimental Procedures." B, the expression of GCLC and HO in PC12 cells stimulated with SIN-1 (2.5 mM) was measured by Western blot analysis, and actin levels were measured to ensure equal protein loading. Quantitative data on the expression levels of GCLC, HO-1, and HO-2 are provided next to the blot data. C, shown is the effect of SIN-1 (2.5 mM) on the rate of CO production. The details are described under “Experimental Procedures.” Data are the means ± S.D. (n = 3). Asterisks indicate data that were significantly different from the control group data: *, p < 0.05; **, p < 0.01.

for 3 h. To prevent nonspecific protein binding, the polyvinylidene difluoride membrane was blocked for 1 h at room temperature using the blocking buffer (0.1% Tween 20 in Tris-buffered saline (pH 7.4) containing 5% nonfat dried milk). Dilutions of primary antibodies, including anti-HO-1 (catalog no. SPA-895), Stressgen, Victoria, Canada); anti-GCLC (catalog no. RB-1697, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), were made in PBS containing 3% nonfat dried milk. Following three washes with PBS and 0.1% Tween 20, the blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature in PBS containing 3% nonfat dried milk. After three additional washes with PBS and 0.1% Tween 20, the blots were incubated for 1 min in ECL substrate solution (Amersham Biosciences) according to the manufacturer’s instructions and visualized by exposure to x-ray film.

Measurement of CO Production—Production of CO resulting from degradation of hemin added to the lysed cell suspen-
sion was determined by gas chromatography as described by Srisook et al. (20). Briefly, after counting the number of harvested cells, they were suspended in ice-cold lysis buffer (500 μl) and lysed further by brief sonication. Hemin (20 μM) and NADPH (80 μM) were added to the lysate (0.9-ml total final volume). After capping the reaction vial (5-ml capacity), it was incubated at 37 °C for various times, generally for 30 min. The reaction was stopped at various times by injecting 100 μl of 50 mm 4-hydroxymercury benzoate through the septum. Subsequently, 1.0 ml of headspace gas was withdrawn and injected into a multigas analyzer (Taiyo Instruments Inc., Osaka, Japan) equipped with a semiconductor detector. Areas under the CO peak and the CO production rate were calculated using the software provided by the manufacturer.

Measurement of Bilirubin Production—HO activity was measured by determining the rate of bilirubin production according to the previously described method (3, 21). Harvested PC12 cells (1 × 10⁷/7 ml in a 100-mm dish) were washed twice with cold PBS, scraped in the presence of cold homogenization buffer (30 mM Tris-HCl (pH 7.5), 0.25 M sucrose, and 0.15 M NaCl) containing a mixture of protease inhibitors (10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), homogenized, and centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was collected and centrifuged again at 100,000 × g for 1 h at 4 °C. The microsomal pellets obtained were resuspended in 100 mM potassium phosphate buffer (pH 7.4) containing 2 mM MgCl₂, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The microsomal fraction (200 μl) was added to the reaction mixture (200 μl) containing 0.8 mM NADPH, 2 mM glucose 6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase, 20 μM hemin, 100 mM potassium phosphate buffer (pH 7.4), and 2 μg of rat liver cytosol as the source of biliverdin reductase. Mixtures were incubated at 37 °C for 1 h in the dark, and the samples were left in an ice bath at least for 2 min to terminate the reaction. Bilirubin formed was quantified by calculating the differences in absorbances measured at 464 and 530 nm (extinction coefficient of 40 mM⁻¹ cm⁻¹ for bilirubin). HO activity is expressed as picomoles of bilirubin formed per mg of protein/h.

Preparation of HO-1 Small Interfering RNA (siRNA) and Plasmids—An oligonucleotide sequence for HO-1 siRNA was selected to knock down HO-1 expression using siRNA Target FIGURE 2. Possible involvement of HO-1 in SIN-1-induced up-regulation of GCLC expression and GSH biosynthesis. A, effect of ZnPP IX on the SIN-1-dependent increase in HO activity as measured by bilirubin production. Cells were pretreated for 12 h in the absence and presence of 0.5 μM ZnPP IX and then incubated for an additional 12 h with SIN-1 (2.5 mM). B, effect of ZnPP IX, an inhibitor of HO activity, on SIN-1-induced GCLC expression. PC12 cells were exposed to SIN-1 (2.5 mM) for 24 h with and without ZnPP IX (0.5 μM) pretreatment for 12 h. Blockage of SIN-1-dependent activation of GCLC expression as a consequence of HO-1 inhibition was verified by immunoblotting. C, blockage of SIN-1-dependent activation of HO-1 mRNA expression by siRNA determined by RT-PCR. D, effect of HO-1 siRNA transfection on the SIN-1-induced up-regulation of GCLC protein expression. Bands were quantitated, and values are expressed as the ratio of HO-1 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PC12 cells were transfected transiently with 250 nM nonspecific siRNA or with the same amount of HO-1-specific siRNA prior to SIN-1 (2.5 mM) treatment. E, inhibitory effects of ZnPP IX on SIN-1-induced GCLC expression. Cells were pretreated for 12 h with or without ZnPP IX (0.5 μM) and then incubated with SIN-1 (2.5 mM) for 24 h in the presence or absence of CO-RM (50 μM). The intracellular GSH level was measured as described under “Experimental Procedures.” Data represent the means ± S.D. (n = 3). Significant differences between the compared groups are indicated: *, p < 0.05; **, p < 0.01.
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Find a method (Invitrogen). The rat HO-1-specific siRNA (5’-CCG UGG CAG UGG GAA UUU AUG CCA U-3’) and non-specific siRNA (5’-CCG ACG GUG AGG UUA AUU CGU GCA U-3’) were provided by Invitrogen. pEF (control vector); pEF-DN-Nrf2; and the reporter gene fusion constructs for luciferase vector (pTi-luciferase), wild-type ARE, and GC mutant ARE were kindly provided by Dr. Jeffrey A. Johnson (University of Wisconsin, Madison, WI) (22, 23). Full-length Akt and Akt with the K179M mutation (kinase-dead Akt) were kind gifts from Dr. An-Sik Chung (Korea Advanced Institute of Science and Technology) (24). The GCLC promoter-luciferase construct was generously provided Dr. Shelly C. Lu (University of Southern California) (25).

Transient Transfection and Luciferase Reporter Assay—PC12 cells were seeded at a density of 1 × 10⁵/well in 6-well plates. For each sample, cells were transfected with 2.5 μg of pGL3 vector (control) or GCLC-luciferase construct using DOTAP liposomal transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s protocol. After 12 h, the medium was changed, and the cells were treated with CO-RM. The treated cells were washed twice with PBS and lysed in 1× reporter lysis buffer (Promega Corp., Madison, WI). Luciferase activity was measured using a luciferase assay system (Promega Corp.), and the protein concentration was determined using the BCA protein assay kit.

Reverse Transcription (RT)-PCR—Total RNA was isolated from PC12 cells using TRizol® (Invitrogen) following the manufacturer’s instructions. RT-PCR was performed according to standard techniques. The PCR primers used in this study were as follows: GCLC, 5’-AGA ACA GAC GCC ATC TCT CAG TT-3’ (sense) and 5’-CTG ACA GTG AGC CTC GGT AA-3’ (antisense); GCLM, 5’-AGA CCG GGA ACC TGC TCA AC-3’ (sense) and 5’-CAT CAC CCT GAT GCC TAA GC-3’ (antisense) (26); and glyceraldehyde-3-phosphate dehydrogenase, 5’-AGT GTA GCC GAG CAG GCT GCC CTT-3’ (sense) and 5’-GCC AAG GTC ATC CAT GAC AAC CAC-3’ (antisense). Amplification products were resolved by 1.0% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light. All primers were purchased from Bionics (Seoul, South Korea).

Preparation of Nuclear Extracts—After treatment with CO-RM, the PC12 cells (1 × 10⁷/7 ml in a 100-mm dish) were washed with cold PBS, collected by centrifugation, and resuspended in ice-cold isotonic cell membrane lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride). Following incubation in an ice bath for 10 min, the broken cells were centrifuged again, the supernatant was removed, and the pellet was resuspended in ice-cold lysis buffer (20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride). After incubation at 0 °C for 20 min, the suspension was centrifuged, and the collected supernatant of the nuclear extract was stored at −70 °C for the Nrf2-ARE binding assay. The protein concentration of nuclear extracts was determined by the Bradford method using the Bio-Rad protein assay kit.

Electrophoretic Mobility Shift Assay for the Measurement of Nrf2-ARE Binding—A synthetic double-stranded oligonucleotide containing the Nrf2-binding domain (ARE) was labeled with [γ-32P]ATP using T4 polynucleotide kinase and separated from the free [γ-32P]ATP by gel filtration using a NICK spin column (Amersham Biosciences). The sequences of oligonucleotides in the double strands used in this study were 5’-TTT TCT GCT GAG TCA AGG TCC G-3’ and 3’-AAA AGA CGA CTC AGT TCC AGG C-5’). The oligonucleotide was synthesized by Bionics. Prior to the addition of the radiolabeled oligonucleotide (100,000 cpm), 10 μg of the nuclear extract was kept on ice for 15 min in gel shift binding buffer (4% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 10 mM Tris-Cl (pH 7.5), and 0.1 mg/ml sonicated salmon sperm DNA). The DNA-protein complexes were resolved on a 6% non-denaturing polyacrylamide gel at 145 V for 2 h and subjected to autoradiography.

Measurement of Intracellular ROS Accumulation—The fluorescent probe 2’,7’-dichlorofluorescein diacetate (DCF-DA) was used to monitor net intracellular accumulation of ROS. After treatment with CO-RM for 2 h in the presence or absence of N-acetyl-L-cysteine, cells (1 × 10⁶/ml on a 4-well chamber slide) were rinsed with Krebs-Ringer solution and loaded with 10 μM DCF-DA. After a 15-min incubation at 37 °C, cells were
examined under a confocal microscope equipped with an argon laser (488 nm, 200 milliwatt).

**Statistical Analysis**—Where necessary, data were expressed as the means ± S.D., and Student’s t test was used to perform statistical analysis for a single comparison. The criterion for statistical significance was $p < 0.05$.

**RESULTS**

Increased GCLC Expression and GSH Synthesis by Peroxynitrite Are Mediated by the Carbon Monoxide Produced from Up-regulated HO-1—PC12 cells were treated with SIN-1 (2.5 mM), a commonly used peroxynitrite generator, and the GSH levels were measured at different intervals. With 2.5 mM SIN-1, the intracellular GSH level decreased initially for up to 7 h (Fig. 1A). After 7 h, however, the GSH level increased gradually, and by 24 h, the intracellular GSH was completely restored to the basal level (Fig. 1A). To determine whether such restoration of GSH following initial depletion in peroxynitrite-treated cells is mediated via induction of GSH biosynthesis, we examined the expression of GCLC, the rate-limiting enzyme in GSH synthesis. No appreciable changes in GCLC expression were noted at 7 h after treatment with 2.5 mM SIN-1. However, GCLC expression was increased markedly at 24 h of exposure to SIN-1 (Fig. 1B). HO-1 is an important stress-responsive antioxidant enzyme known to be induced by oxidative or electrophilic stimuli that can cause depletion of GSH (27, 28). In agreement with this, the expression of HO-1 protein was increased significantly in PC12 cells as early as 4 h after exposure to SIN-1 (Fig. 1B). However, the expression of the non-inducible HO-2 isoform remained unchanged (Fig. 1B). In parallel with HO-1 up-regulation, the CO production resulting from increased HO activity was elevated in a time-dependent manner, with a maximal increase observed at 12 h following the SIN-1 treatment (Fig. 1C). To clarify whether the peroxynitrite-derived increase in GCLC expression is causally associated with the up-regulation of HO-1, we utilized the HO inhibitor ZnPP IX. ZnPP IX pretreatment completely inhibited not only HO-1 activity (Fig. 2A), but also GCLC expression induced by SIN-1 in PC12 cells (Fig. 2B). To further verify the association of HO-1 induction with the SIN-1-induced up-regulation of GCLC expression, we examined HO-1 expression after siRNA knockdown of the gene (Fig. 2C). The SIN-1-induced up-regulation of GCLC expression was abolished by transfection with HO-1-specific siRNA, whereas transfection of the PC12 cells with the same amount of nonspecific siRNA was not effective (Fig. 2D). Consistent with the suppression of GCLC expression by ZnPP IX treatment (Fig. 2A), the GSH level was reduced (Fig. 2E). It is interesting that the suppression of GSH synthesis as a result of ZnPP IX-mediated inhibition of GCLC up-regulation was negated by the addition of exogenous CO released from CO-RM (Fig. 2E). Cells treated with the same amount of
CO-RM alone exhibited elevated GSH levels. These results suggest that CO derived from the elevated HO-1 activity can mediate the induction of GCLC expression and subsequent GSH synthesis in PC12 cells challenged with SIN-1.

Preincubation with CO-RM Increases the GSH Level and Protects against Cell Death Caused by Peroxynitrite—In a previous study, we reported that CO can protect PC12 cells from SIN-1-induced apoptotic death (6). To elucidate the plausible mechanism by which CO could protect against apoptosis caused by SIN-1, we determined the level of GSH present in cells pre-treated with CO-RM. Preincubation of PC12 cells with increasing doses of CO-RM for 1 h prevented the loss of GSH caused by exposure to SIN-1 for 7 h in a concentration-dependent manner (Fig. 3A). It is interesting that the GSH level in cells pretreated with the highest nontoxic dose of CO-RM (50 μM) was even higher than that in control cells not exposed to SIN-1. To examine whether the increased GSH biosynthesis induced by pre-exposure to CO-RM is responsible for the cytoprotective effect against the cytotoxicity caused by SIN-1, we compared the cytoprotective effects of CO-RM in the presence and absence of BSO, an irreversible and specific inhibitor of GCL. As shown in Fig. 3B, the CO-RM-induced protection of PC12 cells against SIN-1-induced cytotoxicity was negated when GCL-catalyzed GSH synthesis was blocked with BSO. Moreover, the intracellular GSH level in PC12 cells treated with CO-RM ([Ru(CO)3Cl2]2) alone was increased, but the cells treated with the metal carrier alone (RuCl3) failed to enhance the GSH biosynthesis (Fig. 3C). Combined, these results suggest that the anti-apoptotic effect of CO in peroxynitrite-treated PC12 cells is mediated, at least in part, by induced GSH biosynthesis, possibly resulting from up-regulation of GCLC expression.

CO-RM Alone Induces GCLC Expression—To further verify the increased GSH biosynthesis following CO-RM exposure, the effect of CO-RM alone on GCL expression was determined. When PC12 cells were treated with CO-RM (50 μM), up-regulation of GCLC and GCLM proteins was evident at 7 h (Fig. 4A). The induction of GCLC and GCLM gene transcription by CO-RM was confirmed by RT-PCR analysis (Fig. 4B). Furthermore, the GCLC promoter activity was transiently elevated upon CO-RM treatment as determined by the luciferase reporter gene assay (Fig. 4C).

CO-RM Up-regulates GCLC Expression through Activation of Nrf2—The promoters of both human GCL subunit genes are known to contain consensus ARE sequences that play essential roles in regulating the cellular response to oxidative stress (29, 30). Nrf2 is a redox-sensitive basic leucine zipper transcriptional activator protein that translocates into the nucleus upon oxidative stress and is involved in regulating the ARE-mediated expression of several detoxifying and antioxidant enzymes (31–33). As illustrated in Fig. 5A, CO-RM (50 μM) treatment led to a transient increase in the levels of Nrf2 protein localized in the nuclear fraction. CO-RM also induced a time-dependent increase in the ARE binding of Nrf2 (Fig. 5B) and the ARE-driven transcriptional activity (Fig. 5C). When a reporter construct containing the ARE sequence was mutated in the GC box, the CO-RM-mediated increase in ARE-luciferase activity was abolished (Fig. 5C). To confirm that the up-regulation of GCLC expression in PC12 cells is mediated by functional Nrf2, we employed a dominant-negative (DN) mutant form of Nrf2 with a truncated N terminus. In PC12 cells transiently transfected with the plasmid harboring DN-Nrf2, there was a significant decrease in the CO-RM-derived induction of Nrf2-ARE DNA binding activity (Fig. 6A). The transcriptional activity of ARE was also suppressed markedly in cells transfected with DN-Nrf2 (Fig. 6B). Western blot analyses further revealed that PC12 cells transfected with DN-Nrf2 exhibited significantly lower levels of GCLC expression upon CO-RM treatment compared with control cells transfected with the empty vector (pEF) only (Fig. 6C). Moreover, PC12 cells expressing DN-Nrf2 failed to increase the GSH level following exposure to CO-RM (Fig. 6D).

CO-RM-induced Nrf2 Activation, Up-regulation of GCLC Expression, and Cytoprotection against SIN-1 in PC12 Cells Are Mediated via Activation of the PI3K/Akt Pathway—It has been reported that PI3K and MAPKs play an important role in the activation of Nrf2 and the up-regulation of several antioxidant enzyme gene expression in various types of cultured cells (34–36). A transient increase in the phosphorylation of Akt was
observed in the CO-RM-treated PC12 cells, whereas the level of unphosphorylated Akt remained unchanged (Fig. 7A). Treatment of PC12 cells with CO-RM also increased the phosphorylation of p38 within 2 h, which declined to the basal level at 7 h (Fig. 7B). To properly assess the contribution of Akt and p38 to CO-mediated cytoprotection against SIN-1-induced apoptosis, we utilized pharmacological inhibitors of these kinases. As illustrated in Fig. 8A, the PI3K inhibitor LY294002 abrogated the cytoprotection exerted by CO-RM against cell death caused by SIN-1, whereas SB203580 (an inhibitor of p38 MAPK) did not influence the SIN-1 cytotoxicity. In line with this observation, the CO-RM-mediated up-regulation of GCLC expression was attenuated by LY294002, but not by SB203580 (Fig. 8B). To further verify the specific involvement of PI3K/Akt signaling in Nrf2 activation and induction of GCLC expression in PC12 cells exposed to CO-RM, cells were transfected with full-length functional Akt or non-functional Akt with the K179M mutation (kinase-dead Akt) and then stimulated with CO-RM. Ectopic expression of functionally inactive Akt (kinase-dead) ablated the CO-RM-mediated enhancement of Nrf2-ARE binding (Fig. 8C) and the up-regulation of GCLC expression (Fig. 8D). These findings suggest that the Nrf2-driven up-regulation of GCLC expression induced by CO-RM is mediated mainly through activation of the PI3K/Akt signal transduction pathway.

**CO-RM-induced GCLC Expression Is Triggered by ROS Generation**—Like NO, CO has been reported to inhibit mitochondrial electron transport and to cause leakage of superoxide anion, leading to accumulation of H$_2$O$_2$ in the cytoplasm (37). To examine whether cells exposed to CO-RM undergo oxidative stress and thus provide the signal for up-regulation of GCLC expression and enhanced synthesis of GSH, intracellular ROS accumulation was measured using DCF-DA, a fluorescent probe that is freely permeable to the cell membrane and that produces fluorescence upon reaction with peroxides such as H$_2$O$_2$. Cells treated with CO-RM displayed a transient increase in fluorescence (Fig. 9A, panel b), indicative of the intracellular accumulation of ROS. Treatment of the cells with the thiol antioxidant N-acetyl-l-cysteine markedly reduced the CO-RM-derived increase in DCF-DA (Fig. 9A, panel c). Likewise, the up-regulation of GCLC expression induced by CO-RM was suppressed by N-acetyl-l-cysteine (Fig. 9B). These results suggest that the CO-RM-derived up-regulation of GCLC expres-
sion is mediated by production of ROS, which may trigger redox-sensitive signaling.

**DISCUSSION**

Peroxynitrite (ONOO⁻) is a highly reactive and strong oxidant generated by rapid interaction between superoxide anion and nitric oxide radicals that are produced under normal physiological conditions. Overproduction of peroxynitrite derived from excess superoxide anion and nitric oxide has been implicated in the pathophysiology of acute endotoxemia, ischemia/reperfusion, inflammation, and other disorders (38, 39). However, the peroxynitrite produced in moderate amounts has been reported to serve as an intracellular signal molecule that induces the adaptive survival response under various stressful cellular conditions (17, 40, 41).

In support of the adaptive survival response achieved in PC12 cells challenged with peroxynitrite, we demonstrated that exposure to a moderate concentration of SIN-1 caused a transient but substantial reduction of total GSH, which was followed by a delayed rebound elevation to the control level. It is interesting that such restoration of the intracellular GSH pool was causally linked to up-regulated GCLC expression, which was preceded by pronounced induction of HO-1 expression with concomitant production of CO. Thus, upon inhibition of HO activity with ZnPP IX, the peroxynitrite-induced up-regulation of GCLC expression was abolished, but was restored by further addition of CO, an end product of HO-1 activity, in cells exposed to SIN-1. These results suggest that CO derived from elevated HO activity is responsible for the up-regulation of GCLC expression and restoration of GSH, the synthesis of which was initially diminished upon peroxynitrite exposure.

Therefore, increased expression of HO-1 with subsequent generation of CO is likely to be a part of the physiological mechanism by which PC12 cells protect themselves from oxidative/nitrosative injuries. We demonstrated recently that CO derived from elevated HO activity as a consequence of prior exposure to SIN-1 can rescue PC12 cells from apoptotic death by preventing the perturbation of the mitochondrial transmembrane potential and the release of cytochrome c caused by subsequent exposure to larger amounts of SIN-1 (5, 6). This finding prompted us to examine whether CO that can be generated normally from up-regulated HO-1 expression could enhance the biosynthesis of GSH, a ubiquitous cellular antioxidant, thereby conferring adaptive cytoprotection against peroxynitrite-induced apoptosis in PC12 cells. The results obtained in this study indicate...
that CO alone does increase GCL expression and subsequently elevates the intracellular GSH level to elicit the adaptive survival response in peroxynitrite-treated PC12 cells. The cytoprotective effect of CO was abolished by treating the CO-RM-exposed cells with BSO, a specific inhibitor of GCL, lending further support to the notion that CO targets the cellular signaling responsible for GSH biosynthesis.

The MAPK pathways, which transduce the pro-oxidative stress and pro-inflammatory signals, have been reported to play important roles in mediating CO action (42, 43). The MAPKs can activate a distinct set of transcription factors capable of binding to ARE sequences for up-regulation of phase II detoxifying or antioxidant enzymes, including GCLC (44, 45). In endothelial cells, the anti-apoptotic effect of CO was found to be dependent specifically on the activation of p38 MAPK (10). However, in lung epithelial cells, CO protects against anoxia/reoxygenation-induced apoptosis through activation of the PI3K/Akt pathway, which is upstream of p38 MAPK signaling (43). In this work, we found that CO induced activation of the p38 and PI3K/Akt pathways via phosphorylation. However, pharmacological inhibition of p38 MAPK affected neither the CO-RM-induced increase in GCLC expression nor CO-dependent cytoprotection in PC12 cells treated with SIN-1. Furthermore, pretreatment of cells with LY294002, a pharmacological inhibitor of PI3K activity, negated the CO-derived up-regulation of GCLC expression and thereby abolished the cytoprotective effect of CO-RM. In addition, in PC12 cells transfected with mutant Akt retaining no kinase activity, the CO-induced increase in Nrf2-ARE binding and up-regulation of GCLC expression were substantially suppressed. On the basis of these findings, PI3K is likely to be the key regulatory signal-transducing enzyme responsible for the nuclear translocation and enhanced ARE binding of Nrf2, leading to up-regulation of GCLC expression and biosynthesis of GSH in the CO-RM-treated PC12 cells.

The molecular mechanism by which the CO activates Nrf2 and induces GCLC expression is not clear. In this study, we observed that CO-RM rapidly enhanced intracellular ROS production in PC12 cells and that pretreatment with the membrane-permeable thiol antioxidant N-acetyl-L-cysteine inhibited both the CO-RM-derived increase in ROS production and the subsequent up-regulation of GCLC expression. In this context, it is interesting that CO modulates redox signaling in airway smooth muscle cells by interacting with the heme moiety of NADPH oxidase and the mitochondrial respiratory chain (37). Several key features have emerged from an extensive study of the molecular mechanisms of Nrf2 activation by oxidative stress. Nrf2 signaling is generally considered to be regulated by a cysteine-rich protein called Keap1 (Kelch-like ECH-associated protein-1). Phase II enzyme inducers or pro-oxidants can cause oxidation or covalent modification of these cysteine residues in Keap1 (46 – 48). As a consequence, ROS could negate Keap1 suppression (49) and trigger Nrf2 nuclear translocation (50). In addition, phosphorylation of Nrf2 at serine and threonine residues by kinases such as PI3K (51), protein kinase C (52), and MAPKs (53) is considered to facilitate the dissociation of Nrf2 from Keap1. Kang et al. (54) reported that PI3K regulates Nrf2 through actin rearrangement in response to oxidative stress. Whether Nrf2 is activated through direct attack by CO-RM-induced oxidative stress or indirect actions such as phosphorylation pathways discussed here requires further investigation. Additional studies are needed to elucidate the specific mechanisms by which CO-RM induces production of ROS and activates the Nrf2 signaling pathway in PC12 cells.

In summary, we have demonstrated for the first time that CO overproduced by up-regulation of HO-1 expression mediates the increased expression of GCL and elevation of the intracellular GSH level in PC12 cells challenged with peroxynitrite. The peroxynitrite-induced nitrosative stress appears to cause initial oxidation and partial depletion of the cellular GSH level. In turn, this may trigger the up-regulation of HO-1 expression, leading to enhanced degradation of free heme. The resulting CO may serve as an endogenous oxidative stress-signaling molecule to activate the PI3K/Akt pathway and Nrf2-driven up-regulation of GCLC expression. This would replenish GSH, which had been depleted initially by exposure to peroxynitrite (Fig. 10). This underscores a potential application of CO, previously considered as a noxious gas, for the management of a variety of ailments caused by excessive oxidative or nitrosative stress.

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