Thioredoxin-1 Regulates Cellular Heme Insertion by Controlling S-Nitrosation of Glyceraldehyde-3-phosphate Dehydrogenase*

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Background: NO inhibits heme insertion into inducible NOS by causing buildup of S-nitroso-GAPDH in cells.

Results: Recovery of heme insertion from NO inhibition correlates with denitrosation of GAPDH. Thioredoxin-1 (Trx1) is largely responsible for the denitrosation.

Conclusion: Cellular Trx1 levels can determine the NO sensitivity of heme insertion.

Significance: Trx1 levels may impact the inflammatory response by affecting maturation of inducible NOS and other heme proteins.

NO generated by inducible NOS (iNOS) causes buildup of S-nitrosated GAPDH (SNO-GAPDH) in cells, which then inhibits further iNOS maturation by limiting the heme insertion step (Chakravarti, R., Aulak, K. S., Fox, P. L., and Stuehr, D. J. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 18004–18009). We investigated what regulates this process utilizing a slow-release NO donor (NOC-18) and studying changes in cellular SNO-GAPDH levels during and after NO exposure. Culturing macrophage-like cells with NOC-18 during cytokine activation caused buildup of heme-free (apo) iNOS and SNO-GAPDH. Upon NOC-18 removal, the cells quickly recovered their heme insertion capacity in association with rapid SNO-GAPDH denitrosation, implying that these processes are linked. We then altered cell expression of thioredoxin-1 (Trx1) or S-nitrosoglutathione reductase, both of which can function as a protein denitrosylase. Trx1 knockdown increased SNO-GAPDH levels in cells, made heme insertion hypersensitive to NO, and increased the recovery time, whereas Trx1 overexpression greatly diminished SNO-GAPDH levels during and after NO exposure. Culturing macrophage-like cells with NOC-18 during cytokine activation caused buildup of heme-free (apo) iNOS and SNO-GAPDH. Upon NOC-18 removal, the cells quickly recovered their heme insertion capacity in association with rapid SNO-GAPDH denitrosation, implying that these processes are linked. We then altered cell expression of thioredoxin-1 (Trx1) or S-nitrosoglutathione reductase, both of which can function as a protein denitrosylase. Trx1 knockdown increased SNO-GAPDH levels in cells, made heme insertion hypersensitive to NO, and increased the recovery time, whereas Trx1 overexpression greatly diminished SNO-GAPDH buildup and protected heme insertion from NO inhibition. In contrast, knockdown of S-nitrosogluthione reductase expression had little effect on these parameters. Experiments utilizing C152S GAPDH confirmed that the NO effects are all linked to S-nitrosation of GAPDH at Cys-152. We conclude (i) that NO inhibition of heme insertion and its recovery can be rapid and dynamic processes and are inversely linked to the S-nitrosation of GAPDH and (ii) that the NO sensitivity of heme insertion can vary depending on the Trx1 expression level due to Trx1 acting as an SNO-GAPDH denitrosylase. Together, our results identify a new way that cells regulate heme protein maturation during inflammation.

Protein S-nitrosation is thought to be an important means through which NO functions in physiology and pathology (1–3). Cellular proteins can become S-nitrosated through either chemical or enzymatic mechanisms (2, 4, 5), and the extent and pattern of protein S-nitrosylation in cells may be determined by a balance between active S-nitrosation and denitrosation pathways (1). Proteins implicated in S-nitrosation and/or denitrosation pathways include thioredoxin/thioredoxin reductase, S-nitrosoglutathione reductase (GSNOR),2 GAPDH, protein-disulfide isomerase, and xanthine oxidase (1, 6–8). These systems have been implicated in controlling the S-nitrosation level of a variety of proteins, including caspase-3 (9, 10), caspase-8 (11, 12), PtpB (13), the β-adrenergic receptor (14), and GRK2 (14, 15).

We recently reported that NO can inhibit heme protein maturation in cells, specifically by preventing heme insertion into the apoproteins (16). The effect appeared to be general because NO inhibited heme insertion into catalase, hemoglobin, three NOS isoforms, and two P450 cytochromes (16, 17). Our related study suggested that NO inhibits cellular heme insertion by causing S-nitrosation of GAPDH (18). GAPDH is known to undergo S-nitrosation in cells (19, 20) in a process that is reversible and sensitive to the cellular redox level (21, 22). Thioredoxins and GSNOR participate in regulating the cellular redox state (1, 22–24), and altering Trx1 or GSNOR expression impacts the S-nitrosation level of a variety of proteins, including SNO-GAPDH (10, 25–28). However, whether Trx1 or GSNOR might help mitigate the effects of NO on cellular heme insertion is still unknown. To address this question, we investigated how a cell’s capacity to drive heme insertion into apoproteins is related to the buildup and disappearance of SNO-GAPDH in cells during and after NO exposure and examined how altering the Trx1 or GSNOR expression levels would impact these processes.

EXPERIMENTAL PROCEDURES

Reagents—All the chemicals were purchased from Sigma unless stated otherwise. The sequences used to silence Trx1

2 The abbreviations used are: GSNOR, S-nitrosoglutathione reductase; Trx1, thioredoxin-1; SNO-GAPDH, S-nitrosated GAPDH; iNOS, inducible NOS; L-NAME, N\textsuperscript{\textcircled{\textminus}}-nitro-L-arginine methyl ester.
Regulation of Heme Insertion by Thioredoxin-1

(GAGACAAGCTTGTGTTGTTAGCT, CATTTCCCTCTGTGACAATGTATTCCA, and CAGGATGTGTGCTGACAGCTTGAGAG and GSNOR (CUGGAUUGGGAAAGUUG and AACAUUUCACAAUUGCA) and their respective negative controls were purchased from Invitrogen. Antibodies were purchased from the indicated companies: mouse anti-GAPDH (Fitzgerald Industries International, Acton, MA), rabbit polyclonal anti-Trx1 (Cell Signaling Technology, Inc., Danvers, MA), anti-GSNOR (Proteintech Group, Inc., Chicago, IL), and mouse anti-iNOS (BD Transduction Laboratories). NOC-18 was from Alexis Biochemicals (San Diego, CA). IFN-γ was purchased from PeproTech (Rocky Hill, NJ). HA-tagged wild-type and C152S GAPDH genes in the pRK5 expression vector were gifts from Dr. Solomon H. Snyder (John Hopkins University School of Medicine, Baltimore, MD).

Cell Culture—RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were cultured as described (29). Cells were activated by 25 μg/ml LPS and 10 units/ml IFN-γ for 17 h at 37 °C and 5% CO2 in a humidified incubator. For generation of apo-iNOS, 250 μM NOC-18 was included during the induction period. After induction, cells were quickly washed with PBS and either harvested directly or cultured for various times with fresh medium containing 5% nitro-arginine methyl ester (L-NAME; 3 mM) and cycloheximide (10 μg/ml). In the dose-response experiments, cells were induced for iNOS expression in the presence of 3 mM L-NAME and various NOC-18 concentrations as indicated. To harvest, cells were quickly washed with PBS and immediately frozen on a sheet of dry ice for 5 min. Later, cells were thawed on ice, and supernatants were prepared as described (18). Silencing of Trx1 or GSNOR in RAW 264.7 cells was done by transfecting 5 nm Trx1 or GSNOR and the respective control siRNA with siPORT transfection reagent (Ambion, Inc., Austin, TX) according to the manufacturer’s directions. After 24 h of transfection, cells were induced with LPS, IFN-γ, and L-NAME. Human Trx1 cDNA (IMAGE Consortium) was purchased from American Type Culture Collection and cloned into the pLVX-ZS-IRES vector (Clontech). Cloned Trx1 was used to generate lentivirus containing the Trx1 gene, and expression was monitored by Western blotting.

Biotin Switch Assay—The biotin switch assay was performed as described (30), and the pulldowns from NeutrAvidinTM resin (Pierce) were probed by Western blotting.

iNOS Activity Measurement, and In-gel Heme Staining—Cell supernatants were processed as described (16, 17, 29). All experiments were repeated at least three times, and the average of independent experiments is shown.

GAPDH Activity Measurement—Activities in cell supernatants were determined in a spectrophotometer by monitoring the disappearance of NADH at 340 nm (31).

RESULTS

Recovery of Cellular Heme Insertion following NO Inhibition—We began by investigating how cellular heme insertion would recover from NO-mediated inhibition once we removed the NO source, which, in our case, was the slow-release NO donor NOC-18, with a half-life in solution of 20 h (32). We induced RAW 264.7 cells to express apo-iNOS by treating them with cytokines for 17 h in the presence of 250 μM NOC-18, which was shown previously to block heme insertion into iNOS (16). After this period, the NOC-18-containing medium was replaced with fresh medium that was free of NOC-18 but contained 3 mM L-NAME to prevent any NO production from iNOS, and we monitored the cells’ capacity to insert endogenous heme into the apo-iNOS versus time. As shown in Fig. 1A, the presence of NOC-18 during the induction period did not diminish iNOS protein expression but did prevent heme incorporation into iNOS (time = 0 min) (Fig. 1, A and C). Once the NO donor was removed, the cells quickly initiated heme insertion into apo-iNOS as judged by three independent measures (gain in P450 heme content, iNOS NO synthesis activity, and iNOS in-gel heme staining) (Fig. 1, A–C). Most of the heme insertion took place within the first 30 min of the recovery period. By 40–60 min, the cells had restored an amount of heme-replete iNOS that was comparable with the amount contained in cells that had been similarly induced in the absence of the NO donor. This implies that by 60 min of recovery, the NOC-18-treated cells had inserted heme into most or all of their apo-iNOS. Excluding L-NAME during the recovery phase resulted in the cells achieving a lower extent of heme insertion (supplemental Fig. S1), probably due to this condition allowing NO generation from newly formed heme-replete iNOS, which can actively inhibit subsequent heme insertion into apo-iNOS (16–18).

Correlation between Recovery of Heme Insertion and Denitrosation of SNO-GAPDH—GAPDH becomes S-nitrosated in cells that make NO or that are exposed to NO donors (10, 18, 19, 22, 33), and previously, we showed that NO inhibits cellular heme insertion through a mechanism involving SNO-GAPDH formation (18). Thus, we examined if the recovery of heme insertion after NOC-18 removal would be related to changes in the SNO-GAPDH level in the cells. We confirmed that the NOC-18 treatment increased the buildup of SNO-GAPDH (supplemental Fig. S2) (18, 33). After removing NOC-18 from the cultures, we observed that SNO-GAPDH was rapidly denitrosated such that its level decreased by 80% within 20 min (Fig. 1D and supplemental Fig. S2). Somewhat less SNO-GAPDH denitrosation occurred in recovering cultures in the absence of L-NAME (supplemental Fig. S2), consistent with some NO being made by iNOS during the recovery period. Denitrosation of SNO-GAPDH occurred without any change in the total GAPDH protein level (supplemental Fig. S2) and was inversely correlated with a 10-fold gain in the cellular GAPDH enzymatic activity (Fig. 1D). These results suggest that a majority of the cellular GAPDH had become S-nitrosated during the NOC-18 treatment, primarily at the catalytically essential Cys-152 residue (18, 21, 33). The rate of SNO-GAPDH denitrosation correlated well with the recovery of heme insertion capacity during the same time period (Fig. 1, compare D with A–C). Thus, NO inhibition of cellular heme insertion is accompanied by significant SNO-GAPDH formation (18), and recovery of cellular heme insertion correlates with SNO-GAPDH denitrosation.

Level of Trx1 Expression Controls NO Sensitivity of Heme Insertion—If SNO-GAPDH denitrosation is required for cells to recover their heme insertion, then cellular denitrosylases might be able to modulate the NO sensitivity. Trx1 functions as...
a protein denitrosylase enzyme in several cell models (8, 10, 26) and can denitrosate SNO-GAPDH (10, 26). We therefore examined how modulating the Trx1 expression level would impact heme insertion into apo-iNOS, hypothesizing that lower Trx1 expression would sensitize the process to NO inhibition, whereas higher Trx1 expression would be protective and increase resistance to NO. In our experiments, we manipulated cellular Trx1 expression levels and then induced iNOS expression in the cells during exposure to different concentrations of NOC-18 for 17 h. We measured iNOS protein expression and heme content immediately following NOC-18 exposure or at various times during a recovery period that started after removing NOC-18.

As shown in Fig. 2A, Trx1 knockdown using siRNA reduced the level of Trx1 expression without affecting the expression of iNOS or GAPDH proteins. However, it increased SNO-GAPDH levels in both cytokine-induced and non-induced cells cultured in the absence of NOC-18 (supplemental Fig. S3A). The Trx1 knockdown sensitized cellular heme insertion to NO inhibition, as judged by its shifting the NOC-18 dose-response curve toward the left compared with control cells that were treated with scrambled siRNA (Fig. 2B). It also greatly diminished the cells’ ability to recover their heme insertion capacity after NOC-18 removal (Fig. 2C), and the poor recovery was associated with a defect in SNO-GAPDH denitrosation during the recovery period (Fig. 2D).
We next examined the effect of Trx1 overexpression using lentiviral transduction. The transfected cells had increased Trx1 protein expression and maintained normal iNOS protein expression (Fig. 3A). The sensitivity of their heme insertion reaction to NO inhibition was shifted to the right compared with the vector-transfected control cells (Fig. 3, B and C). This is consistent with the higher Trx1 expression protecting heme insertion from NO inhibition. To understand the mechanism, we checked if Trx1 overexpression affected the cellular SNO-GAPDH levels. Fig. 3D shows that the transfected control cells increased their SNO-GAPDH level in response to NOC-18 in a dose-dependent manner, as expected. In comparison, the Trx1-overexpressing cells had much lower levels of SNO-GAPDH buildup across the entire range of NOC-18 concentrations. Together, our results show that altering Trx1 expression is a powerful means to control the NO sensitivity of heme insertion and suggest that it is linked to an SNO-GAPDH denitrosylase activity of Trx1.

Impact of GSNOR Expression—It is conceivable that GSNOR could similarly function like Trx1 to limit SNO-GAPDH buildup and protect heme insertion against NO inhibition. To investigate this possibility, we examined if knockdown of GSNOR expression would impact these parameters. Knockdown of GSNOR lowered its protein expression level in the cells without altering iNOS protein expression (Fig. 4A). However, the lower GSNOR expression did not dampen recovery of iNOS heme insertion from NO inhibition and only slightly retarded the SNO-GAPDH denitrosylation that occurred during the
recovery period (Fig. 4, A and B). It also did not increase the sensitivity of iNOS heme insertion to NOC-18, as judged by its not shifting the NOC-18 dose curve to the left relative to the control (Fig. 4C). These results imply that GSNOR operates distinctly from Trx1 and plays, at most, a minor role in controlling SNO-GAPDH levels in our system.

C152S GAPDH Makes Heme Insertion Resistant to NOC-18 Inhibition—To confirm that reducing the SNO-GAPDH level is the mechanism that allowed Trx1 to protect heme insertion from NO, we performed similar experiments using cells that stably express C152S GAPDH, which is a variant that cannot become nitrosated at Cys-152 (33). In our previous study, heme insertion in these cells was resistant to NO inhibition in a 3-h NO exposure assay (18). Here, we found that insertion of endogenous heme into apo-iNOS by these cells was also resistant to the NOC-18 inhibition, particularly in the range from 0 to 225 μM (Fig. 5, A and B). This is consistent with SNO-GAPDH inhibiting heme insertion in our study and with Trx1 controlling the process by determining the buildup of SNO-GAPDH in the cells.

DISCUSSION

Heme insertion is a key step in heme protein maturation. We know that NO generated by cells (or provided to cells by an NO donor) can inhibit maturation of iNOS through its causing SNO-GAPDH formation in cells, which in turn inhibits the insertion of heme into the apoenzyme (16, 18). Here, we studied how heme insertion recovers from NO inhibition and what might regulate the process, specifically examining what controls SNO-GAPDH buildup in cells and how the SNO-GAPDH level may relate to a cell’s capacity to drive heme insertion both during and after NO exposure.

Cells quickly recovered their heme insertion capacity upon removal of the NO donor, with measurable heme incorporation...
Remarkably, this took place after the cells had been exposed continuously to the NO donor for 17 h. This implies that the NO-treated cells still had enough of their endogenous heme available to allow for heme insertion to begin almost immediately. NO can also inhibit cellular heme insertion fairly quickly (16). Thus, the inhibition of heme insertion by NO and the reversal of this inhibition can be a rapid and dynamic process in cells.

SNO-GAPDH builds up rapidly in cells exposed to NO (18), and here, we found that the cells could rapidly denitrosate their SNO-GAPDH once the NO source was removed. A similar time course for SNO-GAPDH denitrosation was observed in endothelial cells after removing the NO source (22). Importantly, both the buildup and denitrosation of SNO-GAPDH occurred fast enough in our system to be kinetically linked to the inhibition and recovery of the cells’ capacity for heme insertion. Such similarity in the kinetic profiles for the two processes is compelling evidence that NO inhibits cellular heme insertion by causing buildup of SNO-GAPDH and that SNO-GAPDH denitrosation allows heme insertion to recover.

Our experiments using a cell line that stably expresses C152S GAPDH lend further support to the above concepts and confirm that Cys-152 is the critical site of NO-based modification in GAPDH (6, 18, 33). Our results with this cell line (Fig. 5) also suggest that there is a range of NO exposure (between 0 to 200 μM NOC-18 in our case) at which inhibition of heme insertion is predominantly linked to S-nitrosation of GAPDH at Cys-152. However, at higher NO exposures (beyond 200 μM NOC-18 in our case), NO appeared to inhibit heme insertion by additional mechanisms that are independent of SNO-GAPDH. Although potentially interesting, we did not pursue these mechanisms because at the lower and more physiologic NO range, it is clear that heme insertion is predominantly inhibited by the SNO-GAPDH mechanism.

FIGURE 4. GSNOR knockdown has minor impacts. RAW 264.7 cells were transfected with control (Control-si) or GSNOR (GSNOR-si) siRNA and cytokine-induced for 17 h to express iNOS in the presence of L-NAME and the indicated concentrations of NOC-18. The cells were then harvested and analyzed, or NOC-18 (250 μM) was removed, followed by further culture for various times with L-NAME before harvest. A, upper panel, Western analysis of iNOS and GSNOR expression in control versus knockdown cell supernatants created at the indicated times during the recovery period. Lower panel, heme stain band intensities from the cell supernatants. Relative intensities were determined using Image J (mean ± S.D. of three independent experiments). B, levels of SNO-GAPDH and total GAPDH in the supernatants at the indicated recovery times. C, upper panel, in-gel heme staining of iNOS protein bands and total iNOS (Western blotting) in the supernatants at 17 h from cells receiving the indicated NOC-18 concentrations. Lower panel, heme stain band densities as quantified using ImageJ (mean ± S.D. of at least three independent experiments).

FIGURE 5. C152S GAPDH decreases NO sensitivity of heme insertion. Cells overexpressing either wild-type or C152S GAPDH were induced for 17 h to express iNOS in the presence of the indicated NOC-18 concentrations, and cell supernatants were prepared and analyzed. A, spectrophotometrically determined INOS heme content (normalized to 0 [NOC-18] = 100%) in samples of equal total protein from each supernatant. Data are the mean ± S.D. of three independent experiments. B, relative heme content of iNOS (upper panel; in-gel heme staining) and Western analysis of the total iNOS protein (lower panel) in the supernatant samples (equal total protein loaded). Data are representative of three similar experiments.
Manipulating the Trx1 expression level in the cells had remarkably strong and reciprocal influences on the SNO-GAPDH buildup and on the sensitivity of heme insertion to NO. Trx1 overexpression almost eliminated SNO-GAPDH buildup and made heme insertion immune to NO within the probable physiologic range (0–200 μM NOC-18). In contrast, Trx1 knockdown caused cellular heme insertion to be NO-hypersensitive such that >80% inhibition was seen even at the lowest NOC-18 dose we tested (50 μM). This effect was also manifested in the Trx1 knockdown cells not being able to recover their heme insertion following NO exposure or to denitrosate their SNO-GAPDH. The apparent strong role for Trx1 as we have seen adds to the body of evidence that Trx1-mediated denitrosation or transnitrosation reactions can impact diverse cellular functions, including apoptosis, host immune response, and gene expression (5, 7, 8, 12, 13, 34). On the other hand, GSNOR silencing did not affect the NO sensitivity or recovery of heme insertion into apo-iNOS. This is in accordance with a previous published study showing that GSNOR knock-out mice have similar iNOS activity compared with wild-type control mice (35). In our cell culture system, Trx1 appeared to act through its denitrosylase activity, as seen before in several cell types (10, 26). The molecular mechanisms by which Trx1 may alter SNO-GAPDH content or cause protein denitrosation in general are still unclear (1, 8, 36). Whether Trx1 acts through a direct protein-protein interaction with SNO-GAPDH or, alternatively, acts through an intermediary protein or through a more global effect are interesting questions for further study.

Most physiologic effects of NO are concentration-dependent (4), and this is also true for how NO affects heme insertion (16). We previously calculated that the concentrations of NOC-18 required to inhibit heme insertion (50–100 μM) result in NO release that approximates the NO flux (50–100 nM/min) measured in cultures of immunonactivated cells that express iNOS to generate NO (16). Within this context, it is now clear that a cell’s Trx1 expression level can greatly influence the NO sensitivity of its heme insertion. This is important because it implies that heme protein maturation in cells or tissues could vary broadly in their NO sensitivity, depending on the level of Trx1 expression and its inherent capacity to control buildup of SNO-GAPDH. In this way, Trx1 may help to regulate a cell’s behavior during and after NO exposure. For example, a high Trx1 expression level could allow cells to maintain high levels of active heme-replete iNOS for sustained constitutive NO release, a situation that apparently exists in the lung epithelium (37). During an inflammatory immune response, a cell might manipulate its Trx1 expression level to allow enough iNOS to mature and produce an NO flux sufficient to cause stasis or killing of pathogens while subsequently allowing enough SNO-GAPDH buildup to down-regulate continued high NO production that could damage host cells and tissues (38). In this way, Trx1 control of SNO-GAPDH level would be advantageous because it gives cells an additional, sensitive means to tune their iNOS activity at appropriate times during the inflammatory response. On a broader scope, we suspect that the level of Trx1 activity may also influence maturation of several other heme proteins whose heme insertion is known to be NO-sensitive, including neuronal NOS, endothelial NOS, hemoglobin, P450 cytochromes, and catalase (16). Our present work lays a foundation to investigate these concepts.

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