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Induction of Glutathione Peroxidase in Response to Inactivation by Nitric Oxide

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To determine effect of nitric oxide (NO) on cellular glutathione peroxidase (GPX) level in living cells, we measured the activity, protein and mRNA of GPX in rat kidney (KNRK) cells under a high NO condition. Combined treatment of lipopolysaccharide (LPS, 1 μg/ml) and tumor necrosis factor-α (TNF-α, 50 ng/ml) synergistically enhanced (23-folds) nitrite production from KNRK cells. This was suppressed by an inducible NO synthase (iNOS) inhibitor (aminoguanidine, N-nitro-L-arginine methylester hydrochloride) and arginase. iNOS expression was detected by RT-PCR in the treated cells. GPX was inactivated irreversibly when the cells had been homogenized before exposure to a NO donor, S-nitroso-N-acetylpenicillamine (SNAP). In living KNRK cells, SNAP and LPS + TNF-α exerted a transient effect on the GPX activity. The treatment with SNAP (200 μM) or sodium nitroprusside (200 μM) enhanced GPX gene expression, which was blocked by a NO scavenger, 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide. GPX mRNA was markedly increased by the treatment with LPS + TNF-α, and aminoguanidine blocked the effect. In cells metabolically labeled with ^{75}Se, LPS + TNF-α accelerated the incorporation of radioactivity into GPX molecule by 2.1-fold. These results suggest that inactivation of GPX by NO triggers a signal for inducing GPX gene expression in KNRK cells, thereby restoring the intracellular level of this indispensable enzyme.

Keywords: Glutathione peroxidase, nitric oxide, antioxidant enzymes, gene expression, cell line, free radicals

INTRODUCTION

Nitric oxide (NO) is a free radical messenger molecule with diverse roles in vascular homeostasis, immune system and neurotransmission.[1–3] It is synthesized from L-arginine by NO synthase (NOS) in mammalian cells. Basically two groups of NOS exist. One is a constitutive NOS (cNOS; neuronal NOS and endothelial NOS) whose activity is dependent on calcium. The other is an inducible NOS (iNOS) which is synthesized *de novo* in response to a variety of inflammatory stimuli. Both cNOS and iNOS have the same catalytic activity, but they differ

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from each other in the structure, the chromosomal localization and the regulation for gene expression. NO is involved in protective or regulatory functions in the cells at a low level, whereas it can be toxic at a high concentration. Once iNOS is induced, it remains active for a long time and continuously produces NO excessively. Peroxynitrite ($ONOO^-$), generated by reaction of NO with superoxide (O_2^-), is considered to play a major role in the cytotoxic process.[1–3]

Reactive oxygen species (ROS; O_2^-, H_2O_2 and $HO^•$) and reactive nitrogen species (RNS; NO and $ONOO^-$) are implicated in the pathophysiology of various human diseases.[4,5] The ROS are scavenged by both enzymatic and non-enzymatic antioxidant defense systems. If not fully detoxified, the ROS and RNS can attack cellular DNA, proteins and lipid membranes, resulting in tissue damage and cellular dysfunction. Antioxidant enzymes such as catalase, glutathione peroxidase (GPX) and superoxide dismutase (SOD) are the first line of defense for maintaining the balance of cellular redox status.[4,5]

GPX (Se-dependent cellular type), which possesses a selenocysteine in the active center, catalyzes the reduction of organic hydroperoxides and hydrogen peroxide (H_2O_2) using reduced glutathione as the substrate.[6] GPX is widely distributed in a cell; cytosol, mitochondria and peroxisomes.[7,8] However, little is known about the regulatory mechanism of the GPX gene expression except that by selenium (Se) status.[9] Recently NO is reported to directly inactivate GPX molecule by binding to its cysteine sulfhydryl (SH) residue in a cell free system.[10,11] The effect of inactivation of GPX by NO on the subsequent gene expression in living cells has not yet been elucidated. The activity of other antioxidant enzymes such as catalase, copper-zinc SOD (CuZnSOD) and manganese SOD (MnSOD) are not affected by a NO donor *in vitro*.[10] However, in living cells the levels of these enzymes are also reported to be modulated by NO or its metabolite.[12–14]

In the present study, we studied the effect of NO on GPX expression in rat kidney (KNRK) cells by exposure of both exogenously administered and endogenously produced NO, and we elucidated the initial inactivation and subsequent *de novo* synthesis of GPX by NO. Such regulatory mechanism for balancing cellular redox status plays a pivotal role in the living cells.

MATERIALS AND METHODS

Cell Culture and Treatments

KNRK cells, which was originated from a normal rat kidney and transformed by Kirsten sarcoma virus (American Type Culture Collection, Rockville, MD, No. 514882), were grown to confluency in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Rockville, MD.) containing 10% fetal bovine serum. They were then cultured in the same medium without serum and treated with the following peptides and chemicals. Lipopolysaccharide (LPS) from Escherichia coli (Sigma Chemical Co., St. Louis, MO.) and recombinant tumor necrosis factor-α (TNF-α), kindly provided from Dainihon Pharmaceutical Co., Osaka, Japan, were used as iNOS inducers. S-nitroso-N-acetylpenicillamine (SNAP; Calbiochem, San Diego, CA.) and sodium nitroprusside (SNP; Sigma Chemical Co.) were as NO donors. Aminoguanidine hydrochloride (AG; Wako Pure Chemicals, Osaka, Japan), N-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma Chemical Co.) and arginase (Sigma Chemical Co.) were used as NO blockers, and 2-phenyl-4,4,5,5-tetra-methylimidazoline (PTIO; Calbiochem) as a NO scavenger.

Cell Viability

The viability of the cells was estimated by the spectrophotometric measurement of rate of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium

bromide (MTT, Sigma Chemical Co.) reduction as described previously.[15]

NO Production

NO synthesis was estimated by the assay for nitrite, a stable reaction product of NO and molecular oxygen. Briefly, 600 µl of culture supernatant was incubated with 100 µl of Griess-Romijn reagent (Wako Pure Chemicals). After incubating for 15 min at 25 °C, the samples were measured spectrophotometrically at 520 nm. Fresh culture medium (DMEM) was used as the blank. Nitrite concentrations were calibrated using sodium nitrite as the standard.[15]

RNA Isolation and Reverse-Transcriptase Coupled Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from KNRK cells by the acidic guanidine isocyanate/phenol/chloroform extraction method using Ultraspec-II RNA isolation system (Biotecx Laboratories Inc., Houston, TX.). Reverse transcription was carried out on 1 µg of the treated RNA using Superscript Preamplification System (Gibco BRL), according to the manufacturer's instruction. PCR reaction was carried out by commercial kit (Takarashuzo Co., Osaka, Japan). Amplification was performed on a Thermal Cycler (Perkin Elmer Co., Norwalk, CT.) under the following conditions; 94 °C (5 min) for 1 cycle, 94 °C (30 sec), 54 °C (1 min), 72 °C (1 min) for 35 cycles. 72 °C (10 min) for 1 cycle. Oligonucleotide primers for iNOS were synthesized based on the sequences described by Geller *et al.*[16] The PCR amplified products, which corresponded to the nucleotide positions of 447 to 1176 (730 bp) for iNOS and 183 to 667 (484 bp) for GPX, were confirmed by restriction mapping. Oligonucleotide primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as control. The PCR products were visualized by electrophoresis in a 2% agarose gel containing ethidium bromide (0.5 µg/ml).

GPX Activity in KNRK Cells

For homogenization, KNRK cells were suspended in 0.01 M potassium phosphate with 0.01% digitonin (pH 7.4) and sonicated. GPX activities were measured using *tert*-butyl hydroperoxide as a substrate by the method described previously.[17] One unit of the enzyme is defined as µmol of NADPH oxidized per min per mg protein. The contribution of non-Se dependent isoenzyme, glutathione S-transferase as GPX activity in the present system is minimal.[18,19] The protein concentration was determined by using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA.).

Immunoblot Analysis

The mono-specific polyclonal antibodies against rat cellular GPX have been described previously.[7] Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli using 12.5% slab gels.[19,20] After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane, incubated overnight at 4 °C with rabbit anti-GPX antiserum at a dilution of 1:3000, followed by incubation with goat anti-rabbit IgG Fab fraction conjugated to horseradish peroxidase (MBL Co., Nagoya, Japan) for 60 min at room temperature. The peroxidase activity was visualized by an ECL western blotting analysis system kit from Amersham Life Science (Arlington Heights, IL.).[15]

Metabolic Labelling and Immunoprecipitation

One day after the 75% confluence (day 0), ^{75}Se-selenious acid (specific radioactivity 7742 mCi/mmol) were added into the culture medium of KNRK cells at the concentration of 92.5 kBq/ml (2.5 µCi/ml) for metabolic labeling.[6] On day 3,

the KNRK cells were treated with LPS and TNF-α. After 12 h of treatment, the cells were lysed in the buffer containing 0.2% SDS, 2 mM N-ethylmaleimide, 4 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 1% deoxycholate and 4 mM EDTA in 0.1 M Tris HCl (pH 8.3). Anti-GPX antibody was coupled to Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Uppsala, Sweden) by incubation for 30 min at 37 °C. Then the cell lysate was added to the gel and incubated for 2 h by gentle mixing at room temperature. The immunoprecipitate, which was coupled to the gel, was exhaustively washed with the same buffer, and then the radioactivity of the gel was counted with Auto-well-γ-system, ARC-380 (Aloka, Tokyo, Japan).

RESULTS

Figure 1 summarizes the change in GPX activity in KNRK cell homogenates during exposure to SNAP. The GPX activity declined in a dose- and time-dependent manner. The effect of SNAP was not significant over 90 min of incubation time at a concentration of 1 μM. When the SNAP concentration was increased to 10 and 100 μM, the activity declined to 88.6% (ns) and 13.8% ($p < .05$), respectively, of that of the untreated control homogenate at 30 min, and to 33.0% ($p < .05$) and 12.4% ($p < .05$), respectively, at 60 min. At 90 min, GPX activities declined to around 10% of the control at the SNAP concentration of both 10 and 100 μM. The GPX activity was not restored toward control level thereafter (data not shown), indicating that inactivation of the GPX molecules by SNAP was irreversible process when the cells had been homogenized.

Figure 2 summarizes the NO production (nitrite) from KNRK cells into the culture medium by the treatment with LPS and TNF-α. The nitrite production was increased 4.6-fold by the treatment with LPS alone, but not with TNF-α alone. The combination of LPS with TNF-α exhibited a synergistic effect on the nitrite production. The nitrite level was increased 23-folds by the combined treatment from the control level. The nitrite production induced by LPS and TNF-α was suppressed dose-dependently by the co-treatment with iNOS inhibitors, AG and L-NAME. Arginase is an enzyme that depletes arginine, the substrate of iNOS. This NO blocker also suppressed the nitrite production induced by LPS and TNF-α.

When the KNRK cells were incubated for 24 h as intact cells with LPS + TNF-α, LPS + TNF-α

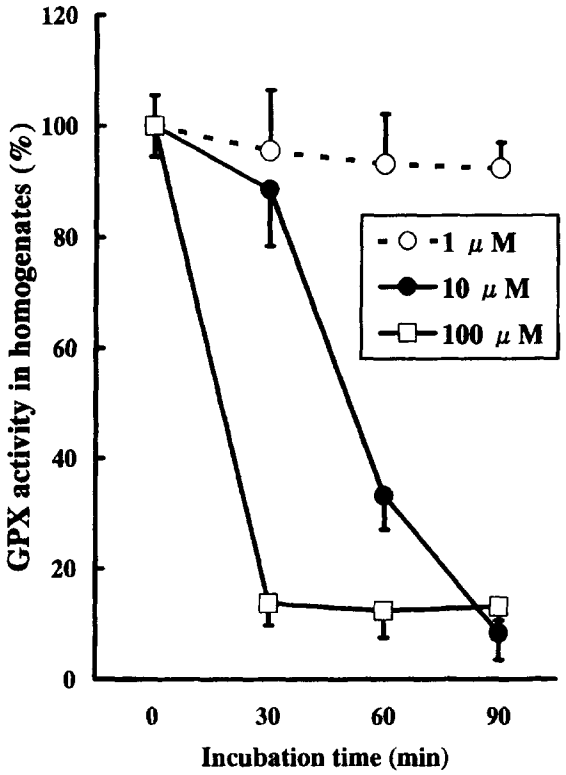

FIGURE 1 GPX activities in KNRK cell homogenates incubated with S-nitroso-N-acetylpenicillamine (SNAP). KNRK cell homogenate was incubated at 37 °C in the presence of 1, 10 or 100 μM of SNAP. GPX activities were measured at 30, 60 and 90 min. Data are expressed as the percent of the activity in untreated control. The brackets indicate SD ($n = 4$). The GPX activities in the homogenate treated with 100 μM of SNAP declined rapidly and were significantly ($p < .05$) decreased by 30 min compared with controls. Those treated with 10 μM of SNAP were significantly ($p < .05$) decreased by 60 min. GPX activities did not decrease significantly in the homogenate treated with 1 μM of SNAP (Mann-Whitney test).

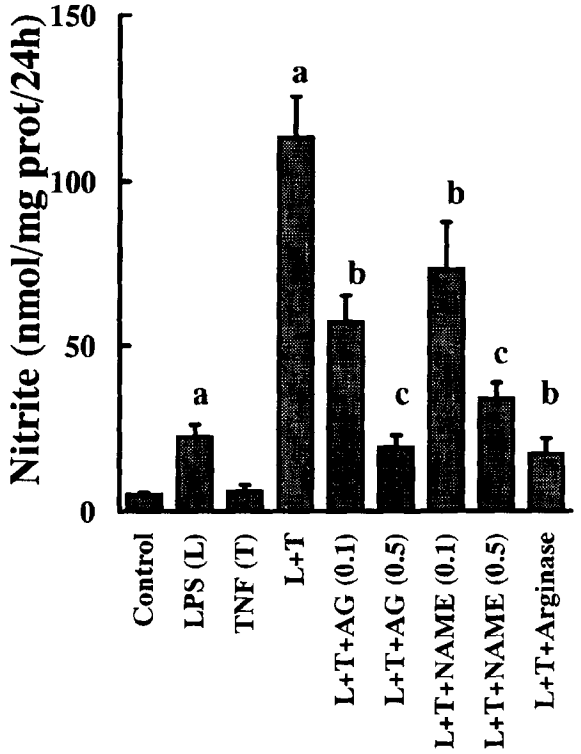

FIGURE 2 Effect of LPS and TNF-α (TNF) on NO production from KNRK cells. KNRK cells were treated with LPS (1 μg/ml) and/or TNF (50 ng/ml) in the presence or absence of a NO blocker. The nitrite concentration in culture medium was measured 24 h after the treatments as per mg cell proteins. The brackets indicate SD ($n = 5$). Aminoguanidine (AG; 0.1 or 0.5 mM), N-nitro-L-arginine methylester hydrochloride (L-NAME; 0.1 or 0.5 mM) or arginase (100 U/ml) was administered 1 h before the combined treatment of LPS and TNF (L + T). a: $p < .01$ (vs control), b: $p < .01$ (vs L + T), c: $p < .01$ (vs the group with a lower concentration of NO blocker: Mann-Whitney test).

+AG or SNAP alone, GPX activities were similar between the treated groups and controls (Figure 3A). The immunoblot analysis also depicted the single protein bands in a similar intensity in all lanes (Figure 3B). Figure 4 summarizes the time course of the changes in GPX activities in living KNRK cells after the treatment with LPS + TNF-α or SNAP. GPX activity initially decreased and reached the nadir in 2 h (SNAP, 84.6% of the initial activity) and 4 h (LPS + TNF-α, 85.8%), and then was gradually restored toward the baseline level by 4 h in SNAP treatment and 6 h in the combination treatment.

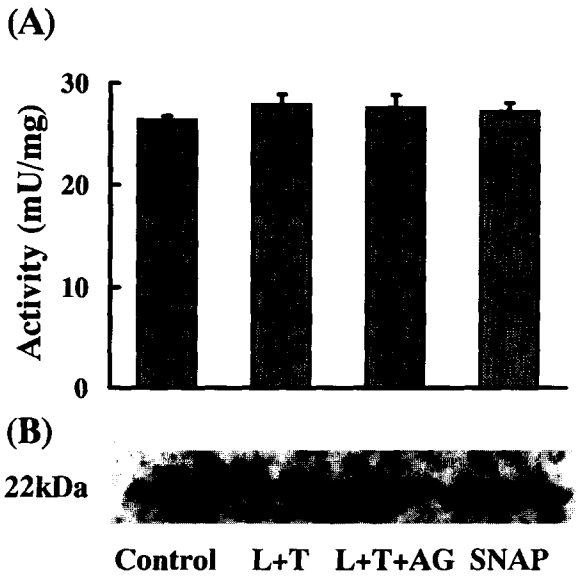

FIGURE 3 The activity and protein level of GPX in KNRK cells treated with 1 μg/ml of LPS plus 50 ng/ml of TNF-α (L + T), L + T + AG (0.5 mM) and SNAP alone (200 μM). The cells were incubated for 24 h and then the GPX activity was measured (A). The same samples were subjected to immunoblot analysis (B). A specific band was detected at the 22 kDa level in each lane. GPX activities and the protein level were similar in the four groups. Data are the results of a representative experiment from 4 observations.

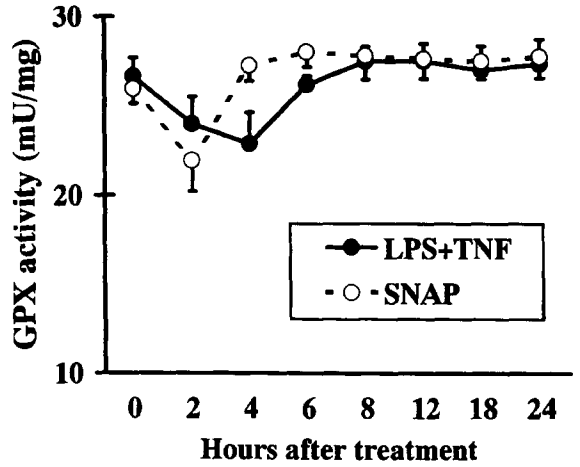

FIGURE 4 The time course of the change in GPX activity in KNRK cells after the treatment with the combination of LPS (1 μg/ml) and TNF-α (TNF, 50 ng/ml) or SNAP (200 μM). In both cases the GPX activity declined initially and then gradually restored to the baseline level. LPS + TNF ($n = 4$): 0 h vs 2 h; $p < .05$, 2 h vs 4 h; ns, 4 h vs 6 h; $p < .05$. SNAP ($n = 4$): 0 h vs 2 h; $p < .01$, 2 h vs 4 h; $p < .005$.

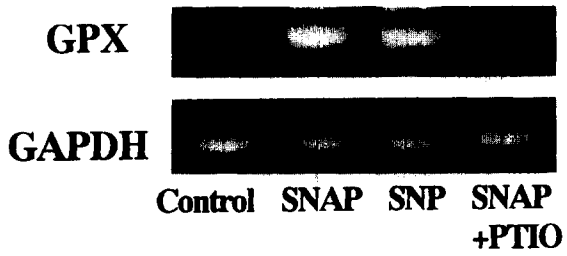

FIGURE 5 RT-PCR analyses of mRNA for GPX in KNRK cells treated with NO donors. The GPX mRNA expression in KNRK cells was increased by the 24 h treatment with SNAP (200 μM) or SNP (200 μM). PTIO (0.5 mM), a NO scavenger, appears to block the effect of SNAP. GAPDH: glyceraldehyde 3-phosphate dehydrogenase was used as the control. Data are the results of a representative experiment from 5 observations.

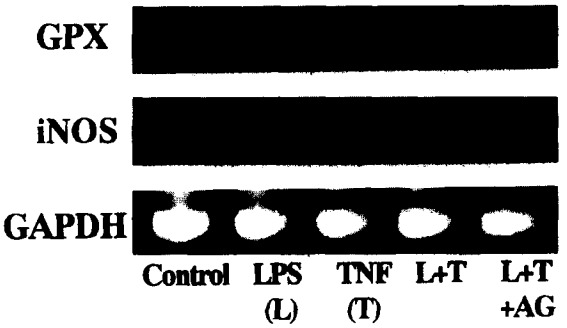

FIGURE 6 RT-PCR analyses of mRNA for GPX and iNOS in KNRK cells treated with the LPS (1 μg/ml) or/and TNF-α (TNF, 50 ng/ml). GPX mRNA expression was enhanced by the 24 h treatment with LPS or TNF alone. The combination treatment (L + T) synergistically enhanced the expression. AG (0.5 mM) completely blocked the effect of combination treatment. The mRNA for iNOS was clearly detected in KNRK cells treated with combination of LPS and TNF. AG, a competitive inhibitor for iNOS, did not affect the expression of iNOS. GAPDH was used as the control. Data are the results of a representative experiment from 5 observations.

In RT-PCR analysis, GPX mRNA signal was more intense in the cells treated with NO donors, SNAP and SNP, than in the controls (Figure 5). Pretreatment with PTIO, a NO scavenger, completely abolished the effect of SNAP on the GPX expression. Figure 6 summarizes the RT-PCR analysis of the mRNA for GPX and iNOS in KNRK cells treated with the combination of LPS and TNF-α. GPX expression was more intensely depicted in the lanes for the cells treated with LPS or TNF-α alone compared with that for the untreated control cells. The combined treatment further enhanced the expression of GPX. AG, a competitive inhibitor for iNOS, completely inhibited the GPX mRNA induction by the combined treatment in KNRK cells. The iNOS mRNA was clearly depicted in the lane of the cells with the combined treatment. AG did not affect the expression of iNOS mRNA.

The result of the metabolic labeling by ^{75}Se-selenious acid is shown in Figure 7. The radioactivity in the immunoprecipitate of the anti-GPX antibody was 2.1-fold higher in the cells treated with combination of LPS and TNF-α than in untreated control, indicating that the combined treatment accelerated the *de novo* incorporation of selenium into GPX molecule.

The viability of the KNRK cells was not affected by the 24 h treatment with LPS, TNF-α,

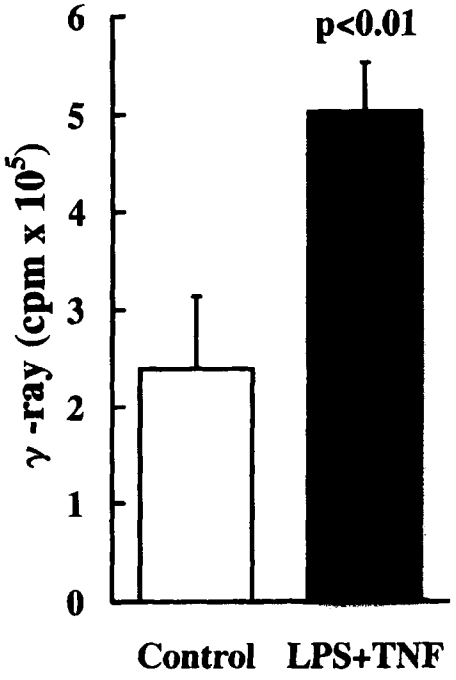

FIGURE 7 The incorporation of radioactive selenium (Se) into GPX. KNRK cells were metabolically labeled by the addition of ^{75}Se-selenious acid in the culture medium three days before the treatment with LPS (1 μg/ml) and TNF-α (TNF, 50 ng/ml). The radioactivity for the immunoprecipitate by specific GPX antibodies was counted 24 h after the combination treatment. $p < .01$ (control vs LPS + TNF; Mann-Whitney test; $n = 5$).

SNAP and SNP either in the presence or absence of NO blockers and scavengers. The mean viability ($n = 5$) was greater than 95% in each group of the KNRK cells (data not shown).

DISCUSSION

In the present study, cellular GPX was inactivated irreversibly when the KNRK cells had been broken before exposure to a NO donor, SNAP. On the other hand, the NO donor and iNOS inducers exerted a transient effect on the GPX in living KNRK cells. The restoration of GPX level in the living KNRK cells was associated with an enhanced GPX gene expression and *de novo* protein synthesis. Such induction was completely blocked by the co-incubation with either a NO scavenger or an iNOS inhibitor, suggesting that the inactivation and subsequent induction of GPX, observed here, was due to a high intracellular concentration of NO.

Asahi *et al.* reported that SNAP inactivated purified bovine GPX in a cell free system in a dose- and time-dependent manner[10] and that peroxynitrite also exerted similar effect on the GPX.[11] Excessive NO production can inactivate cellular enzyme systems by several different mechanisms; inactivation of the iron-sulfur cluster,[21] auto-ADP-ribosylation,[22] nitrosylation of free SH groups[23] and release of Zn^{2+} from zinc-sulfur clusters.[24] It has been shown by mass spectrometry that NO mainly oxidizes selenocysteine residue of the catalytic center for GPX to form a selenenyl sulfide (Se-S) with a free thiol, leading to the inactivation of biological activity.[11] The inactivation of the GPX observed in this study suggested that such oxidative reaction on the intracellular GPX by RNS also took place in the KNRK cells. Similar to our present results, Asahi *et al.* also observed the transient and a little effect of SNAP on GPX activity in intact U937 cells that GPX was maximally reduced to 75.9% of the control after 1 h treatment then gradually recovered.[10] However, this restoration mechanism of intracellular GPX has not yet been understood.

The induction of iNOS by LPS treatment in cultured cells is variable. LPS alone can enhance iNOS expression in phagocytes, however, most of other cell types require additional action of inflammatory cytokines such as TNF-α, interferon-γ or interleukin-1β for the induction of iNOS. LPS, when administered to animals *in vivo*, induces iNOS expression in various rat tissues including adipocytes,[15,25] and increases NO secretion into circulation.[15,25] The LPS administration elicits inflammatory cells to produce cytokines, resulting in iNOS induction in various other cell types as their combined effect. In the present study, combined treatment of LPS and TNF-α exhibited a synergistic effect on expression of iNOS in KNRK cells. To our knowledge, this is the first report of the induction of iNOS in KNRK cells by the combined treatment of LPS and a cytokine. RNS is known to attack nuclear DNA, inducing apoptosis of cells and conversely exhibiting carcinogenesis on different occasions.[5,26] Whether the RNS directly induces gene expression has not been reported.

The effect of *in vivo* administration of LPS to rat on the tissue GPX levels has been variously reported to be increased or unchanged depending on cell types. Dhaunsi *et al.* reported that LPS treatment increased the activity and protein level of the peroxisomal but not mitochondrial and cytosolic GPX in rat liver.[29] Thus, LPS-induced alterations in GPX enzyme level may vary depending on the cell types and also on subcellular sites. MnSOD gene is well known to be induced by inflammatory cytokines such as TNF-α, interleukin-1β and interleukin-6 in a various cell types.[27,28] Whether such direct induction by cytokines or LPS occurs in GPX gene has not been elucidated.

The results of the present study in KNRK cells suggest that the initial inactivation of GPX molecule by excessive NO itself compensatory triggers a signal for inducing GPX gene expression, thereby restoring the intracellular level

of this enzyme that is indispensable for cell survival.

Purified antioxidant enzymes such as catalase, CuZnSOD and MnSOD are not inactivated *in vitro* by NO molecules generated from SNAP.[10] On the other hand, NO donors or iNOS inducers, when administered to intact cells, modulate antioxidant enzymes including GPX in certain cell types.[12–14] Such treatment increases intracellular ROS concentration and eventually leads to cell death mediated by RNS. Hydrogen peroxide synergistically enhances the cytotoxicity of NO donors.[30,31] A variant human leukemia cell line, HL-NR6, which has higher levels of catalase and CuZnSOD compared with original HL-60 cells, is resistant to NO-induced apoptosis.[32] Cells that are transformed for over-expressing antioxidant enzymes are protected against cytotoxicity of RNS derived from ROS and NO.[30] Over-expression of GPX is reported to be more protective against the toxicity of SNAP and SNP than that of SOD.[30] Thus, the balance and the synergism of antioxidant enzymes also play a key role for protection against RNS toxicity in cells.

In the present study, GPX molecule appeared to detoxify the NO by the direct binding, and that was followed by a compensatory induction of the enzyme protein. Thus, cellular GPX acts as an intracellular NO scavenger (or as a chain-breaking antioxidant) in cells, along with its intrinsic action as a preventive antioxidant to reduce the concentration of ROS. GPX is suggested to inhibit I κ Bα phosphorylation/degradation and subsequent NF-κ B activation, thereby preventing the induction of iNOS gene.[33] Thus, cell has protective mechanisms to keep the balance in production and detoxification of ROS and RNS at different levels.

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References

[1] S. Moncada, R.M. Palmer and E.A. Higgs (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacolgical Reviews*, **43**, 109–142.
[2] C. Nathan and Q. Xie (1994) Nitric oxide synthesis: roles, tolls, and controls. *Cell*, **78**, 915–918.
[3] S.R. Jaffrey and S.H. Snyder (1995) Nitric oxide: A neural messenger. *Annual Review of Cell and Developmental Biology*, **11**, 417–440.
[4] J.M. Mates, C. Perez-Gomez and I. Nunez de Castro (1999) Antioxidant enzymes and human diseases. *Clinical Biochemistry*, **32**, 595–603.
[5] B. Halliwell, K. Zhao and M. Whiteman (1999) Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. *Free Radical Research*, **31**, 651–669.
[6] N. Avissar, J.C. Whitin, P.Z. Allen, D.D. Wagner, P. Liegey and H.J. Cohen (1989) Plasma selenium-dependent glutathione peroxidase. Cell of origin and secretion. *Journal of Biological Chemistry*, **264**, 15850–15855.
[7] K. Asayama, S. Yokota, K. Dobashi, H. Hayashibe, A. Kawaoi and S. Nakazawa (1994) Purification and immunoelectron microscopic localization of cellular glutathione peroxidase in rat hepatocytes: quantitative analysis by post-embedding methods. *Histochemistry*, **102**, 213–219.
[8] A.K. Singh, G.S. Dhaunsi, K. Asayama, J.K. Orak and I. Singh (1994) Demonstration of glutathione peroxidase in peroxisomes: Biochemical and immunochemical evidence. *Archives of Biochemistry and Biophysics*, **315**, 331–338.
[9] T. Nakane, K. Asayama, K. Kodera, H. Hayashibe, N. Uchida and S. Nakazawa (1998) Effect of selenium deficiency on cellular and extracellular glutathione peroxidase: Immunochemical detection and mRNA analysis in rat kidney and serum. *Free Radical Biology and Medicine*, **25**, 504–511.
[10] M. Asahi, J. Fujii, K. Suzuki, H.G. Seo, T. Kuzuya, M. Hori, M. Tada, S. Fujii and N. Taniguchi (1995) Inactivation of glutathione peroxidase by nitric oxide. *Journal of Biological Chemistry*, **270**, 21035–21039.
[11] M. Asahi, J. Fujii, T. Takao, T. Kuzuya, M. Hori, Y. Shimonishi and N. Taniguchi (1997) The oxidation of selenocysteine is involved in the inactivation of glutathione peroxidase by nitric oxide donor. *Journal of Biological Chemistry*, **272**, 19152–19157.
[12] G.C. Brown (1995) Reversible binding and inhibition of catalase by nitric oxide. *European Journal of Biochemistry*, **232**, 188–191.
[13] H. Ischiropoulos, L. Zhu, J. Chen, M. Tsai, J.C. Martin, C.D. Smith and J.S. Beckman (1992) Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Archives of Biochemistry and Biophysics*, **298**, 431–437.
[14] K. Dobashi, K. Pahan, A. Chahal and I. Singh (1997) Modulation of endogenous antioxidant enzymes by nitric oxide in rat C_6 glial cells. *Journal of Neurochemistry*, **68**, 1896–1903.

[15] K. Dobashi, K. Asayama, T. Nakane, K. Kodera, H. Hayashibe and S. Nakazawa (2000) Troglitazone inhibits the expression of inducible nitric oxide synthase in adipocytes *in vitro* and *in vivo*: Study in 3T3-L1 cells and Otsuka Long-Evans Tokushima fatty rats. *Life Sciences*, **67**, 2093–2101.

[16] D.A. Geller, C.J. Lowenstein, R.A. Shapiro, A.K. Nussler, M. Di Silvio, S.C. Wang, D.K. Nakayama, R.L. Shimmons, S.H. Snyder and T.R. Billiar (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proceedings of the National Academy of Sciences of the USA*, **90**, 3491–3495.

[17] K. Asayama, W.D. Dettbarn and I.M. Burr (1986) Differential effect of denervation on free radical scavenging enzymes in slow and fast muscle of rat. *Journal of Neurochemistry*, **46**, 604–609.

[18] O.A. Levander, D.P. Deloach, V.C. Morris and P.B. Moser (1983) Platelet glutathione peroxidase activity as an index of selenium status in rats. *Journal of Nutrition*, **113**, 55–63.

[19] K. Dobashi, K. Asayama, T. Nakane, H. Hayashibe, K. Kodera, N. Uchida and S. Nakazawa (1999) Effect of peroxisome proliferator on extracellular glutathione peroxidase in rat. *Free Radical Research*, **31**, 181–190.

[20] U.K. Laemmli (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

[21] Y. Henry, M. Lepoivre, J.C. Drapier, C. Ducrocq, J.L. Boucher and A. Gussani (1993) EPR characterization of molecular targets for NO in mammalian cells and organelles. *FASEB Journal*, **7**, 1124–1134.

[22] B. Brüne, S. Dimmeler, L. Molina y Vedia and E.G. Lapetina (1994) Nitric oxide: a signal for ADP-ribosylation of proteins. *Life Sciences*, **54**, 61–70.

[23] J.S. Stamler (1994) Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell*, **78**, 913–936.

[24] K.D. Kröncke, K. Fehsel, T. Schmidt, F.T. Zenke, I. Dasting, J.R. Wesener, H. Bettermann, K.D. Breunig and V. Kolb-bachofen (1994) Nitric oxide destroys zinc-sulfur clusters inducing zinc release from metallothionein and inhibition of the zinc finger-type yeast transcription activator LAC9. *Biochemical and Biophysical Research Communications*, **200**, 1105–1110.

[25] S. Kapur, B. Marcotte and A. Marette (1999) Mechanism of adipose tissue iNOS induction in endotoxemia. *American Journal of Physiology*, **276**, E635–E641.

[26] H. Ohshima and H. Bartsch (1994) Chronic infections and inflammatory processes as cancer risk factor: possible role of nitric oxide in carcinogenesis. *Mutation Research*, **305**, 253–264.

[27] C.W. White and P. Ghezzi (1989) Protection against pulmonary oxygen toxicity by interleukin-1 and tumor necrosis factor: role of antioxidant enzymes and effect of cyclooxygenase inhibitors. *Biotherapy*, **1**, 361–367.

[28] K. Pahan, K. Dobashi, B. Ghosh and I. Singh (1999) Induction of the manganese superoxide dismutase gene by sphingomyelinase and ceramide. *Journal of Neurochemistry*, **73**, 513–520.

[29] G.S. Dhaunsi, I. Singh and C.D. Hanevold (1993) Peroxisomal participation in the cellular response to the oxidative stress of endotoxin. *Molecular and Cellular Biochemistry*, **126**, 25–35.

[30] M. Tiedge, S. Lortz, R. Munday and S. Lenzen (1999) Protection against the co-operative toxicity of nitric oxide and oxygen free radicals by overexpression of antioxidant enzymes in bioengineered insulin-producing RINm5F cells. *Diabetologia*, **42**, 849–855.

[31] J. Igarashi, M. Nishida, S. Hoshida, N. Yamashita, H. Kosaka, M. Hori, T. Kuzuya and M. Tada (1998) Inducible nitric oxide synthase augments injury elicited by oxidative stress in rat cardiac myocytes. *American Journal of Physiology*, **274**, C245–C252.

[32] M. Yabuki, S. Kariya, R. Ishisaka, T. Yasuda, T. Yoshioka, A.A. Horton and K. Utsumi (1999) Resistance to nitric oxide-mediated apoptosis in HL-60 variant cells is associated with increased activities of Cu, Zn-superoxide dismutase and catalase. *Free Radical Biology and Medicine*, **26**, 325–332.

[33] C. Kretz-Remy, P. Mehlen, M.E. Mirault and A.P. Arrigo (1996) Inhibition of I kappa B-alpha phosphorylation and degradation and subsequent NF-kappa B activation by glutathione peroxidase overexpression. *Journal of Cell Biology*, **133**, 1083–1093.