Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1744 (2005) 58–67

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

http://www.elsevier.com/locate/bba

Increase of intracellular glutathione by low-level NO mediated by transcription factor NF-κB in RAW 264.7 cells

Risa Kurozumi*, Shuji Kojima

The Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba 278-8510, Japan

Received 13 July 2004; received in revised form 15 November 2004; accepted 15 November 2004
Available online 7 December 2004

Abstract

The mechanism underlying the elevation of intracellular glutathione (GSH) in RAW 264.7 cells exposed to low concentrations of sodium nitroprusside (SNP), a well-known nitric oxide (NO) donor, was investigated. The peak of intracellular GSH was reached at 6 h after exposure of the cells to SNP (0.1–0.5 mM), and this was preceded by the induction of mRNA for γ-glutamylcysteine synthetase (γ-GCS; the rate-limiting enzyme of de novo GSH synthesis), which peaked at 3 h. *N*-α-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) and caffeic acid phenethyl ester (CAPE), specific inhibitors of NF-κB, significantly suppressed the SNP-induced elevation of GSH protein and γ-GCS mRNA, while curcumin, an inhibitor of AP-1, was less effective. Electrophoretic mobility shift assay (EMSA) showed that SNP exposure markedly increased the DNA binding of NF-κB, but not that of AP-1. Deletion or mutagenesis of the NF-κB site in the γ-GCS gene promoter abolished the SNP-induced up-regulation of GSH protein and γ-GCS mRNA.

These results suggest that the elevation of intracellular GSH in RAW 264.7 cells exposed to low concentrations of SNP occurs through the operation of the de novo GSH pathway, and is mediated by transcriptional up-regulation of the γ-GCS gene, predominantly at the NF-κB binding site in its promoter.

Keywords: Sodium nitroprusside; Nitric oxide; γ-GCS mRNA; NF-κB

1. Introduction

Nitric oxide (NO), which has been identified as the endothelium-derived relaxing factor [1], has a wide range of biological activities, acting as a neurotransmitter, mediator of inflammation, regulator of immune response and inducer or inhibitor of apoptosis. NO is produced by enzymatic oxidation of the amino acid L-arginine to L-citrulline by a family of enzymes termed nitric oxide synthases (NOS). High concentrations of NO can increase oxidative stress and induce tissue injury and apoptosis, while low concentrations of NO can induce biosynthesis of intracellular antioxidants including glutathione and thereby protect cells against oxidative stress. Whether NO is cytotoxic or protective thus depends on the circumstances.

Reduced glutathione (GSH), a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) containing a thiol (sulfhydryl) group, is a critical intracellular reductant, protecting cells from electrophilic compounds, free radicals, reactive oxygen species (ROS), and toxic substances, and it has been implicated in immune modulation and inflammatory responses [2–4]. The intracellular concentration of GSH is in the range of 5–10 mM in many cells and is maintained by control of the biosynthesis. The rate-limiting enzyme, γ-glutamylcysteine synthetase (γ-GCS), is regulated by the level of GSH [5,6] and by oxidants [7], and depletion of GSH or the presence of oxidants increases its activity. GSH synthesis is also dependent on the availability of the amino acid precursors, glutamate, glycine, and cysteine. A normal reducing environment, required for cellular integrity, is provided by reduced GSH.

* Corresponding author. Tel.: +81 4 7121 3691x6443; fax: +81 4 7121 3613.
E-mail address: j3102702@ed.noda.tus.ac.jp (R. Kurozumi).

0167-4889/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2004.11.005

In previous studies, it was found that low doses of radiation elevated intracellular total glutathione, and it was suggested that this elevation is a consequence of the formation of ROS. Reactive oxygen species, which include hydroxyl radicals ($^{\cdot}OH$), superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and nitric oxide (NO), are produced during normal cellular functions.

Currently, the central issue concerning the signal transduction pathways activated by changes of intracellular redox status is the transcription factors. It is well known that nuclear factor κB (NF-κB) and activator protein-1 (AP-1) rapidly respond to a wide range of agents, factors, and chemicals that alter cellular redox status, and that both factors regulate the expression of a variety of genes [8–12]. γ-GCS is composed of two subunits, heavy (γ-GCSh) and light (γ-GCSl) chains, expressing catalytic and regulatory functions, respectively. These two subunits are encoded by different genes, and dissociate under reducing conditions [13–15]. Furthermore, the γ-GCSh gene has binding motifs for both NF-κB and AP-1 in the 5′ regulatory region [16,17]. The induction of γ-GCSh mRNA by several stimuli that cause redox disruption is regulated by stimulus-dependent transcription factors [18–21], but the factor(s) that modulate gene expression in response to low-level NO remain to be identified. In the present study, we investigated the mechanisms of induction of γ-GCSh mRNA expression by SNP, a well-known NO donor.

2. Materials and methods

2.1. Materials

N-α-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), caffeic acid phenethyl ester (CAPE) and curcumin were purchased from Sigma (St. Louis, MO, USA). Reduced glutathione (GSH), oxidized glutathione (GSSG), sodium nitroprusside (SNP), and 5.5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Wako Pure Chemicals (Osaka, Japan). β-NADPH and GSH reductase (GR, 120 U/mL) were from Oriental Yeast (Tokyo, Japan).

2.2. Cell culture

Mouse macrophage-like RAW 264.7 cells were purchased from American Type Culture Collection, USA. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2/95% air atmosphere. The cells were tested and found to be free of mycoplasma.

2.3. SNP treatment

Cells in the logarithmic phase were used throughout the experiment. Freshly harvested RAW264.7 cells were resuspended in DMEM medium containing 10% FBS at a density of 1×10^6/mL, and the suspension (2 mL) was seeded into six-well plates (Corning, NY). SNP was then added to the medium to give a final concentration of 0, 0.1, 0.25, 0.5, or 1.0 mM, and incubation was continued for an appropriate time.

2.4. Measurement of NO metabolite production

Concentrations of nitrite (NO_2^-) and nitrate (NO_3^-) in the medium were assayed. NO_2^- and NO_3^- were separated and measured by using an HPLC-Griess system, which consists of separation and reduction columns (ENO-10, EICOM, Kyoto, Japan). Briefly, the collected medium was mixed with 1 vol of methanol and centrifuged at 10,000×*g* at 4 °C for 30 min. Then, 10 μL of the supernatant was injected into the system, in which NO_2^- and NO_3^- were separated on a reverse-phase separation column packed with polystyrene polymer (NO-PAK, EICOM), and nitrate was reduced to nitrite in a reduction column packed with copper-plated cadmium filings (NO-RED, EICOM). Nitrite was mixed with a Griess reagent (1.25% HCl containing 5 g/L sulfanilamide with 0.25 g/L *N*-naphthylethylenediamine) to form a purple azo dye in the reaction coil. The separation and reduction columns and the reaction coil were kept at 35 °C in a column oven. The absorbance of the product dye at 540 nm was measured by a flow-through spectrophotometer (NOD-10, EICOM). The mobile phase, which was delivered by a pump at a rate of 0.33 mL/min, was 10% methanol containing 0.15 N NaCl/NH_4Cl and 0.5 g/L 4 Na-EDTA. The Griess reagent was delivered at a rate of 0.1 mL/min. Total NO metabolites (NO_x^-) was calculated by summing NO_2^- and NO_3^-.

2.5. Assay of cellular glutathione levels

For the determination of intracellular total glutathione levels, the cultured cells at each time interval were washed three times with ice-cold phosphate-buffered saline (PBS) and then harvested from the dishes with a silicon rubber policeman into Eppendorf tubes. The cell pellet was suspended in PBS containing 5 mM EDTA, 0.01% digitonin and 0.25% sodium cholate. The cells were disrupted by sonication and the sonicate was centrifuged at 10,000×*g* for 20 min. An aliquot of the supernatant was removed for protein assay. An equal volume of 10% trichloroacetic acid (TCA) was added to another aliquot, and the solutions were kept for 30 min on ice. The acid-soluble fraction was obtained by centrifugation at 10,000×*g* for 20 min. The fraction was subjected to total glutathione assay after repeated removals (5 times) of TCA with ether. Total glutathione (GSH+GSSG) content was measured by using a modified spectrophotometric technique [22]. Briefly, each sample fraction was diluted 1:5, and a 25-μL aliquot was mixed with 250 μL of 1 mM DTNB, 733 μL of 0.3 mM NADPH, and 10 μL of GR (2 U/mL). The rate of change in absorbance was measured at 412 nm. Authentic GSH (0–25 μM) was analyzed in the same manner. The GSH concentration of each sample was calculated as nmol/mg protein. Cell protein determinations were performed using the Bradford reagent (Bio-Rad, USA) after cell lysis by the method of Gogstad and Krutnes [23].

2.6. RNA isolation and Northern blot analysis

Expression of γ-GCS mRNA was analyzed by Northern blotting. Total RNA was isolated from the cells by means of the acid guanidium isothiocyanate–phenol–chloroform extraction method. The RNA was quantified spectrophotometrically at 260 nm (the ratio of A_{260} nm to A_{280} nm always exceeded 1.8), and 8.5 μg aliquots of total RNA were size-fractionated by electrophoresis on a 1.0% agarose gel (Nippon Gene, Toyama, Japan). RNA was then blotted onto a nylon membrane using 0.02 M MOPS buffer (pH 7.0), and immobilized by UV cross-linking. The relative amounts of RNA were determined by hybridization with a mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Mouse γ-GCS and GAPDH cDNAs were synthesized by RT-PCR (Titan, Boehringer Mannheim, Mannheim) from mouse liver total RNA using oligo DNA primers for γ-GCS heavy subunit (γ-GCSh) (5′-CACATCTACCACG-CAGTCA-3′ and 5′-TTCGCTTTTCTAAATCCTGA-3′) and GAPDH (5′-TGAAG-GTCGGTGTGAACG-GATTTGGC-3′ and 5′-CATGTAGGCCATGAGGCCAC-CAC-3′). cDNA was amplified (35 cycles, 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min) and the PCR products were subcloned into the pGEM-T vector (Promega, Madison, WI) for amplification. Hybridization was carried out in a solution consisting of 5× SSPE (20× SSPE=3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 10× Denhardt's reagent (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA), 50% formamide, 1.4% sodium dodecyl sulfate (SDS), and 0.1 mg/mL herring sperm DNA with ^{32}P-labeled probes at 42 °C. After hybridization, the membrane was washed with 6× SSC (20× SSC=3 M NaCl, 0.3 M trisodium citrate) and 0.1% SDS at 42 °C for 30 min, 1× SSC and 0.1% SDS at 55 °C for 30 min, and 0.1× SSC and 0.1% SDS at 60 °C for 30 min. Quantitation was done with a laser image analyzer (Fujix BAS 2500, Fuji Film, Kanagawa). The membrane was also exposed to an X-ray film (Fuji HR-HA30, Fuji Film) with an intensifying screen at −80 °C.

2.7. Treatment with transcription factor inhibitor

RAW264.7 cells were incubated with NF-κB and AP-1 inhibitors for 30 min under conventional conditions. Then, the cells were exposed to 0.25 mM SNP, as described above, and incubated for an appropriate time at 37 °C. The inhibitors used here were inhibitors of NF-κB, i.e., TPCK or CAPE, and an inhibitor of AP-1, curcumin, all of which were dissolved in ethanol. The concentration of each inhibitor and the incubation period were determined on the basis of a preliminary experiment.

2.8. Isolation of nuclear proteins

Nuclear extracts were isolated in the following manner. Namely, the cells (1.0×10^7 cells) were washed twice with ice-cold washing buffer (pH 7.5) composed of 10 mM Tris–HCl, 130 mM NaCl, 5 mM KCl, and 8 mM $MgCl_2$, and then harvested in Eppendorf tubes. Next, the cells were incubated in hypotonic lysis buffer (pH 7.9) composed of 10 mM HEPES–NaOH, 10 mM KCl, 0.15 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 15 min. Then, 10% Igepal CA-630 solution (Sigma) was added to the swollen cells in lysis buffer to give a final concentration of 0.6%, and the suspension was centrifuged at $10{,}000\times g$ for 30 s. The supernatant was removed, and the precipitated nuclear fraction was suspended in extraction buffer (pH 7.9) composed of 20 mM HEPES–NaOH, 0.2 mM EDTA–NaOH, 0.42 M NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, and 0.5 μg/mL pepstatin A (Sigma), and stirred for 30 min at 4 °C. The suspension was centrifuged at $15{,}000\times g$ for 5 min. The supernatant was divided into small volumes and stored at −80 °C as the nuclear fraction. Aliquots of the extracts were removed for protein assay using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories).

2.9. Electrophoresis mobility shift assay (EMSA)

Oligonucleotides containing the consensus sequence for NF-κB (5′-AGTTGAGG-GGACTTTCCCAGG-3′) and AP-1 (5′-CGCTTGATGATCAGCCGGAA-3′) were labeled with [γ-^{32}P]ATP (111 TBq/mmol, NEN™ Life Science Products, USA), using the T4 polynucleotide kinase (Takara Shuzo, Japan). This labeling reaction was terminated with 50 mM EDTA. The ^{32}P-labeled oligonucleotides were purified by using a Sephadex G-50 Micro Column.

The binding reaction proceeded in a 22 μL reaction mixture containing 5 μg of nuclear extract, 2 μg of poly(dI-dC) as a nonspecific competitor DNA, and ^{32}P-labeled oligonucleotides including either AP-1 or NF-κB specific binding domain (2.0×10^4 cpm), at room temperature for 30 min. For the competitive binding study, an excess (100-fold) of nonlabeled consensus oligonucleotide was added. Further confirmation of the identity of the binding proteins was done by means of antibody supershift assays. For NF-κB, anti-p50 and anti-p65 antibodies were used (Santa Cruz Biotech, Santa Cruz, CA). Samples were incubated with 2.5 μL of antibody for 30 min at 4 °C, then loaded on a 4% poly-acrylamide gel and subjected to electrophoresis in TAE buffer (6.7 mM Tris–HCl, 3.3 mM NaOAc, and 1 mM EDTA, pH 7.5), at 4 °C for 100 min. The DNA-binding activity of each nuclear extract is presented as the amount of ^{32}P-labeled DNA-transcription factor complex measured with the Bio-imaging Analyzer System, using an imaging plate.

2.10. Luciferase constructs

A human γ-GCSh promoter construct (kindly provided by Dr. J. Gipp, Madison, WI) was cloned into the *Hin*dIII site of the luciferase reporter vector pGL3-Basic (Promega) and used for the transfection of RAW264.7 cells. A promoter fragment lacking the AP-1 element (−3349:

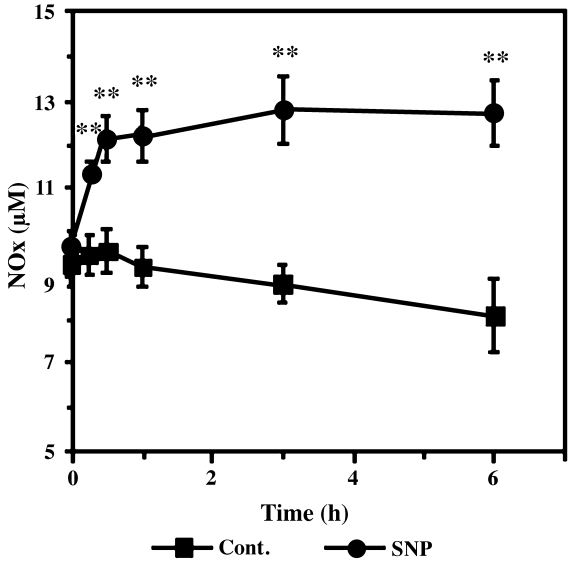

Fig. 1. Changes in NOx level after treatment of RAW 264.7 cells with SNP. Time course of SNP-induced increase of NOx in the medium. After 0.25 mM SNP treatment, the concentration of NOx (nitrite and nitrate) in the medium was measured by means of the Greiss reaction. Each point indicates the mean±S.D. of three independent assays. **: Significantly different from the respective nontreated control group at $P<0.01$.

−3343) was constructed by digestion with *Sac*I. To generate the deletion construct for the NF-κB site (−1099: −1091), the γ-GCSh promoter template was digested with *Kpn*I. To mutate the NF-κB site, site-directed mutagenesis of NF-κB was conducted using a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). The sequence of the NF-κB site was changed from 5′-GGAAATCCC-3′ to 5′-CTCAATCCC-3′.

2.11. Transient transfections and luciferase assay

RAW 264.7 mouse macrophage-like cells were seeded in 60-mm dishes at a density of 2.0×10^5 cells/mL 1 day prior to transfection. Cells were transfected with 2 μg of pGL3 luciferase reporter vector to construct various γ-GCSh promoters and 0.5 μg of pRL-TK control vector (Promega) as an internal control to normalize transfection efficiency using the TransFast™ Transfection Reagent (Promega), usually for 48 h before the experimental procedures. Cells were treated with 0.25 mM SNP for 15 h, and then harvested using passive lysis buffer (Promega). Luciferase activity in cell lysates was assayed using the Dual-Luciferase Reporter assay system (Promega). All values in transfection experiments were normalized for transfection efficiency based on the result of co-transfection with the pRL-TK vector.

2.12. Statistical analysis

The statistical significance of differences was determined by using Student's *t* test for comparison between two groups or two-way repeated measures analysis of variance and Dunnett's tests for multiple comparisons, where appropriate. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Changes in NOx production and intracellular total glutathione induced by SNP exposure

We investigated the change of NO metabolite (nitrite and nitrate) production in the medium of RAW 264.7 cells after SNP exposure, because of difficulty in measuring NO itself. Several methods are available to measure nitrite and nitrate, but the most common procedure is application of the Griess reaction after reduction of nitrate to nitrite. The NOx concentration was significantly increased at 15 min post

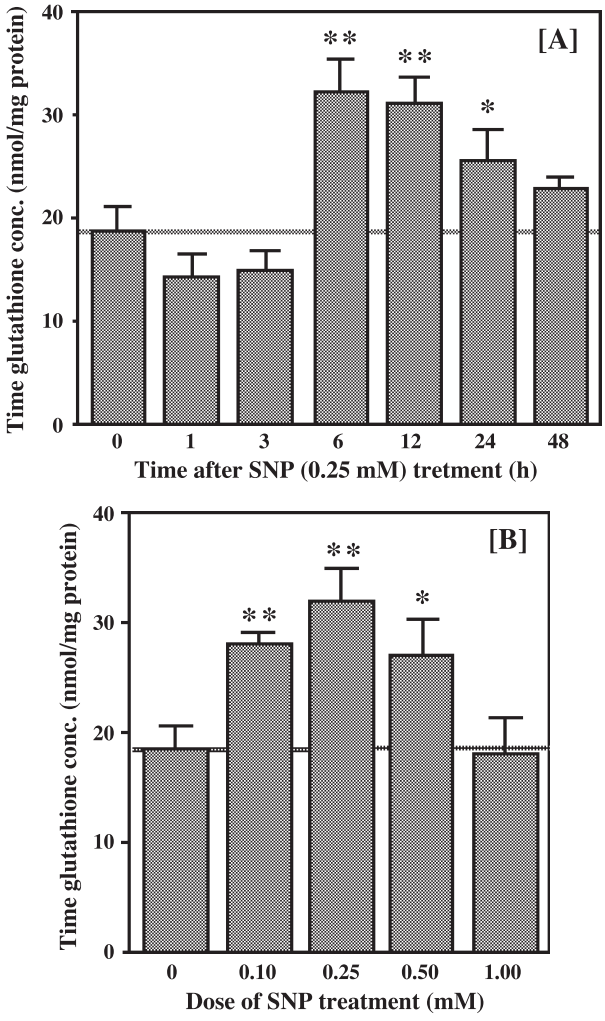

Fig. 2. Changes in total glutathione content after treatment of RAW 264.7 cells with SNP. (A) Intracellular total glutathione content after 0.25 mM SNP treatment. (B) Dose dependence of the effect of SNP on intracellular total glutathione content at 6 h post-treatment. Each point indicates the mean±S.D. of three independent assays. * and **: Significantly different from the respective nontreated control group at $P<0.05$ and $P<0.01$, respectively.

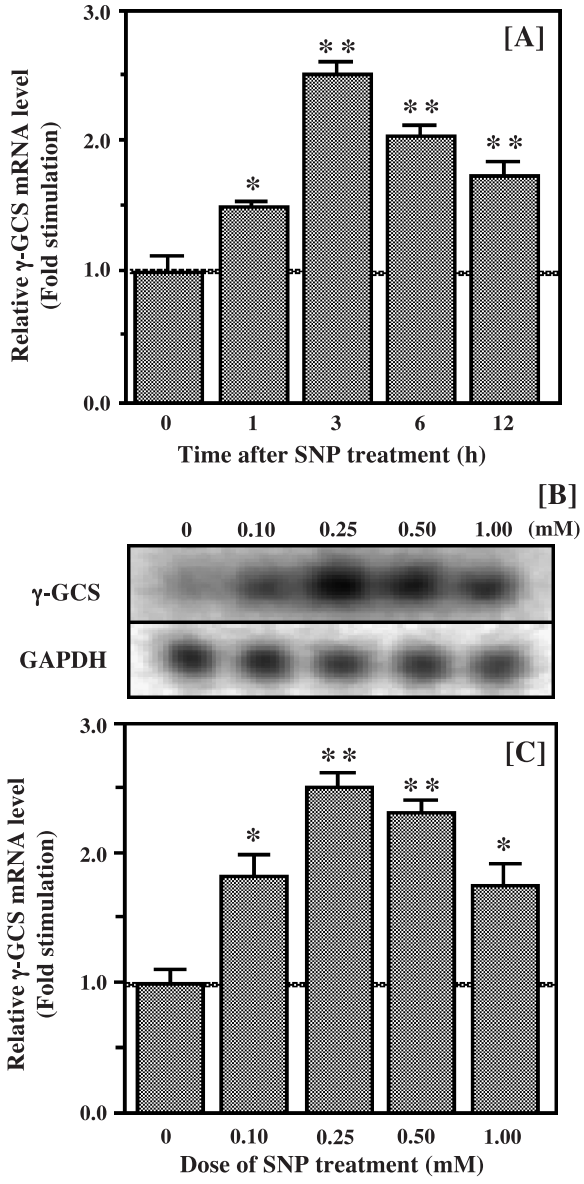

Fig. 3. Time and dose dependence of the effects of SNP on γ-GCS mRNA expression in RAW 264.7 cells. (A) Total RNA from RAW 264.7 cells was extracted at various times post-treatment with 0.25 mM SNP, hybridized with cDNA for γ-GCS and analyzed by Northern blotting as described in Materials and methods. Relative mRNA levels are indicated as the ratio of the γ-GCS mRNA level to the mRNA level of the housekeeping gene GAPDH. Each point indicates the mean±S.D. of three independent assays. * and **: Significantly different from the nontreated control group at $P<0.05$ and $P<0.01$, respectively. (B,C) Dose dependence of the effect of SNP on the expression of γ-GCS mRNA in RAW 264.7 cells after 3 h. Total cellular RNA was extracted and Northern analysis for γ-GCS (B, upper bands) and GAPDH mRNA (B, lower bands) was performed as described in Materials and methods. (C) Results of quantification of mRNA levels by densitometric analysis of autoradiogram of RAW 264.7 cells treated with SNP. Relative mRNA levels are indicated as the ratio of the γ-GCS mRNA level to the mRNA level of the housekeeping gene GAPDH. Each point indicates the mean±S.D. of three independent assays. * and **: Significantly different from the nontreated control group at $P<0.05$ and $P<0.01$, respectively.

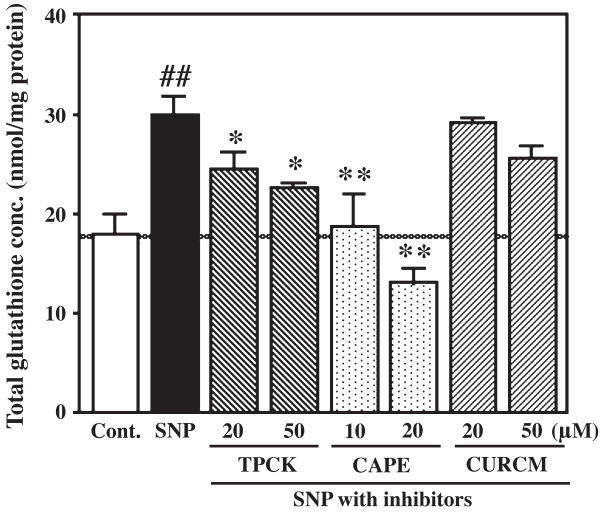

Fig. 4. Effect of transcription factor inhibitors for NF-κB and AP-1 on the increase of total glutathione induced by SNP. Cellular total glutathione was assayed at 6 h after 0.25 mM SNP treatment. Cells were exposed to SNP in the presence or absence of inhibitors. Values are the mean±S.D. of three independent assays. ##: Significantly different from the nontreated control group at $P<0.01$. * and **: Significantly different from the SNP-treated group at $P<0.05$ and $P<0.01$, respectively. SNP, sodium nitroprusside; TPCK, *N*-α-tosyl-L-phenylalanine chloromethyl ketone; CAPE, caffeic acid phenethyl ester; CURCM, curcumin.

SNP-treatment, and the increase was maintained at 6 h post-treatment (Fig. 1). Next, changes in the intracellular total glutathione (GSH+GSSG) level induced by SNP treatment were examined in RAW 264.7 cells after exposure to 0.25

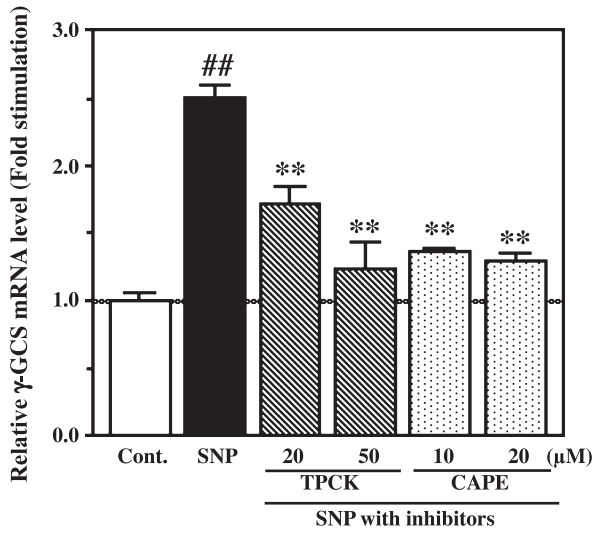

Fig. 5. Effect of NF-κB transcription factor inhibitors on the elevated γ-GCS mRNA expression induced by SNP. Results of quantification of mRNA level by densitometric analysis. Relative mRNA level is indicated as the ratio of the γ-GCS mRNA level to that of the housekeeping gene, GAPDH. Values are the mean±S.D. of three independent assays. ##: Significantly different from the nontreated control group at $P<0.01$. **: Significantly different from the SNP-treated group at $P<0.01$. SNP, sodium nitroprusside; TPCK, *N*-α-tosyl-L-phenylalanine chloromethyl ketone; CAPE, caffeic acid phenethyl ester.

mM SNP. As shown in Fig. 2(A), the glutathione level in RAW 264.7 cells was significantly increased at 6 h post-treatment, and returned to almost the nontreated control level at 48 h post SNP treatment. Next, the dose dependence of the effect of SNP on the total glutathione level at 6 h post-treatment was investigated. As shown in Fig. 2(B), total glutathione increased dose-dependently up to 0.25 mM SNP, while at 0.5 mM SNP, the increase was smaller than that at 0.25 mM SNP, though it was still significant.

3.2. Changes in expression level of γ-GCS mRNA induced by SNP exposure

Changes in mRNA for γ-GCS, the rate-limiting enzyme of de novo GSH synthesis, after treatment with SNP were examined. As shown in Fig. 3(A), the γ-GCS mRNA level peaked at 3 h post-treatment with 0.25 mM SNP, and then declined slowly. It remained significantly increased at 12 h post-treatment.

Next, the SNP dose dependence of the increase in the γ-GCS mRNA level was investigated at 3 h post-treatment with SNP in the concentration range of 0 to 1 mM. As shown in Fig. 3(B,C), γ-GCS mRNA expression in RAW 264.7 cells was significantly elevated at SNP concentrations ranging from 0.1 to 1.0 mM, and the maximal effect was seen at 0.25 mM SNP.

3.3. Effect of NF-κB and AP-1 inhibitors on the elevation of total glutathione and γ-GCS mRNA expression induced by SNP

It has recently been reported that the promoter region of the γ-GCS gene contains binding sites for nuclear factor-κB

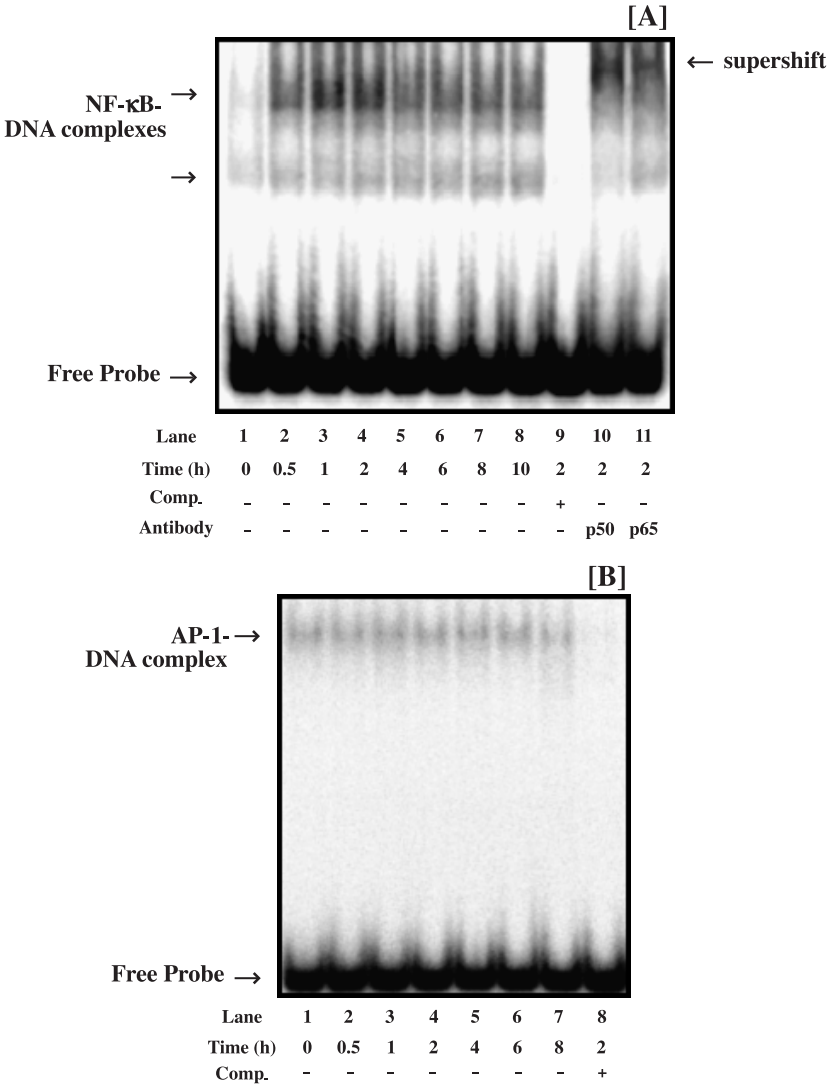

Fig. 6. Changes of NF-κB and AP-1 DNA-binding activities in RAW 264.7 cells treated with 0.25 mM SNP. Nuclear extracts obtained at the indicated times were incubated with an NF-κB-or AP-1-specific ^{32}P-oligonucleotide for 30 min and loaded on a 4% nondenaturing polyacrylamide gel. The DNA-binding activities of the extracts were estimated by electrophoretic mobility shift assay. (A) NF-κB activity; lanes 1–8, extracts from RAW 264.7 cells; lane 9, +competitor for NF-κB; lanes 10 and 11, supershift analysis with p50 and p65 antibodies, respectively. (B) AP-1 activity; lanes 1–7, cell extracts from RAW 264.7 cells; lane 8, +competitor for AP-1. Arrows indicate positions of specific DNA-binding complexes and the free probe.

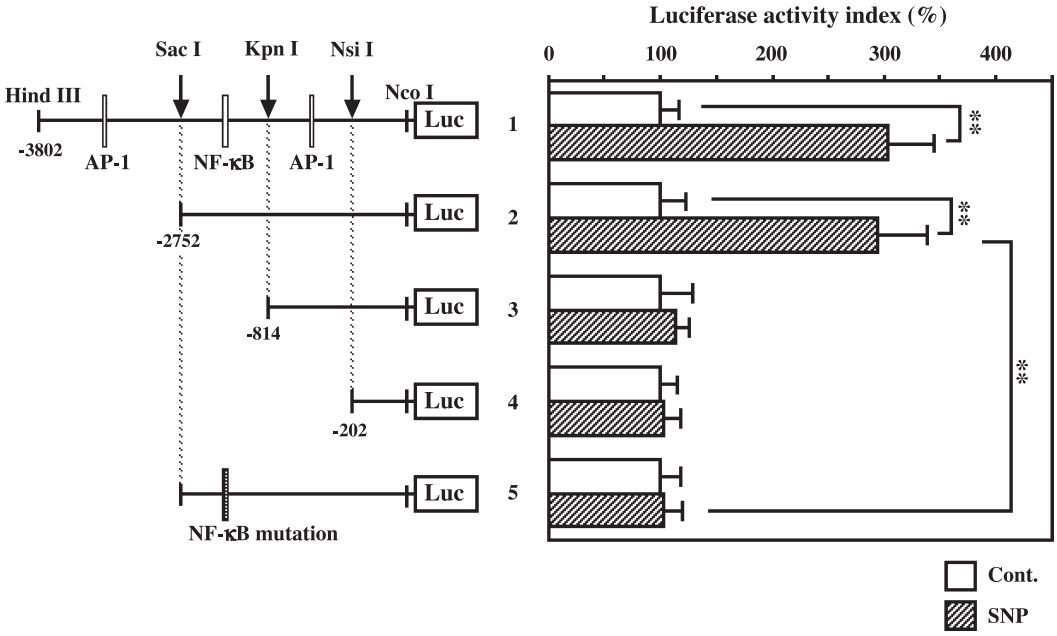

Fig. 7. Luciferase activity of γ-GCS promoter. RAW 264.7 cells were treated with 0.25 mM SNP and incubated for 15 h. Putative sequences in the 5′-upstream region of the human γ-GCSh gene and restriction enzymes used are shown (upper left). Numbers indicate the distance in base pairs from the transcription start site. 1, a *Hin*dIII fragment; 2, a *Sac*I fragment lacking the AP-1 site; 3, a *Kpn*I fragment lacking the AP-1 and NF-κB sites; 4, a *Nsi*I fragment lacking the AP-1, NF-κB, and AP-1 like sites; 5, 2 with a mutation at the NF-κB site. Each point indicates the mean±S.D. of three independent assays. **P*<0.05, ***P*<0.01.

(NF-κB), activator protein (AP-1), and other factors. We examined the involvement of the transcription factors NF-κB and AP-1 in the SNP-induced elevations of glutathione level and γ-GCS mRNA expression by using the NF-κB inhibitors TPCK and CAPE, and the AP-1 inhibitor curcumin. As shown in Fig. 4, the elevation of total glutathione level by 0.25 mM SNP was dose-dependently suppressed by TPCK or CAPE, while curcumin was less effective. The elevation of γ-GCS mRNA expression was also significantly suppressed by both inhibitors of NF-κB (Fig. 5), suggesting that SNP-induced enhancement of γ-GCS gene expression was mainly mediated through the NF-κB response element in the promoter.

3.4. Effects of SNP on DNA-binding activity of NF-κB and AP-1

To test whether activation of NF-κB and AP-1 mediates the induction of γ-GCS mRNA in RAW 264.7 cells exposed to SNP, changes in the DNA-binding activities of the transcription factors were assessed by EMSA. As shown in Fig. 6(A), the DNA-binding activity of NF-κB increased within a short period after treatment with 0.25 mM SNP, and peaked at 1–2 h. The nature of the activated NF-κB–oligonucleotide complex in RAW 254.7 cells was further examined by supershift analysis (Fig. 6(A)). The binding activity of the activated NF-κB with antisera to the p50 and p65 subunits of NF-κB was assayed. The complex was shifted to a higher molecular weight by addition of both subunits (Fig. 6(A), lanes 10 and 11). In contrast, there was no detectable change in AP-1 binding activity in nuclear extracts from RAW 264.7 cells treated with 0.25 mM SNP (Fig. 6(B)).

3.5. Effect of SNP on transcription of the γ-GCS gene

RAW 264.7 cells were transiently transfected with pGL3 basic vector containing the γ-GCS promoter constructs, and then exposed to 0.25 mM SNP for 15 h. As shown in Fig. 7, SNP-stimulated activation of luciferase was seen in the cells transfected with γ-GCS promoter containing the NF-κB binding site. Deletion or mutagenesis of the NF-κB site absolutely blocked the SNP-induced elevation of luciferase activity. These results suggest that SNP induction of the transcription of this gene is mediated mainly by NF-κB.

4. Discussion

The ability to induce cellular defense mechanisms in response to environmental changes is a fundamental characteristic of eukaryotic and prokaryotic cells. Cellular levels of glutathione are influenced by multiple factors, including the activities of enzymes in the γ-glutamyl cycle, the availability of precursors such as cysteine, and the rate of consumption or efflux of GSH. These pathways are physiologically regulated so that the cellular level of total glutathione (GSH+GSSG) is properly maintained. Glutathione synthesis is signaled by many kinds of physiological and nonphysiological stimuli under various conditions, including oxidative stress and liver regeneration, and NO

is one stimulus that induces elevation of glutathione level through the de novo pathway [24]. Besides, the signaling function of NO for vasodilation through activation of guanylate cyclase, both injurious and protective physiological effects of NO have been recognized. Interest has recently been focused on induction of GSH synthesis as one mechanism by which NO exerts its cytoprotective effects [24,25]. In the present study, we investigated the SNP-induced GSH synthesis in order to elucidate the mechanism of the enhanced GSH synthesis induced by NO.

NO-induced glutathione synthesis in macrophage-like cells, RAW264.7 cells, appeared to occur through enhanced induction of γ-GCSh mRNA. This is the same mode of induction as that observed in rat aortic smooth muscle cells [24] and cultured rat hepatocytes [26], whereas in endothelial cells the induction involves enhanced cysteine uptake through the Xc-pathway of amino acid transport, not enhanced γ-GCS expression [27]. Thus, the mechanism seems to depend on the cell type and the function to be modified. Besides, NO, many other stimuli, including cytokines [19,26,28], hormones [29,30], GSH conjugation [31], heat shock [21], ionizing radiation [32,33] and oxidative stress [34,35], have been reported to induce elevated expression of γ-GCS. A common consequence of these stimuli is oxidative stress. Since the functional activities of glutathione are closely related to its antioxidant or SH-reductant capacity owing to the ability to release the thiol hydrogen atom, it is reasonable that oxidizing conditions affecting the cell induce γ-GCS expression to enhance the antioxidant capacity, as an adaptive response.

The promoter (5′-flanking) region of the γ-GCS gene contains consensus NF-κB and AP-1 binding sites [16,17]. Though several oxidative stimuli induce γ-GCS gene expression through the regulation of both transcription factors [17–19,29], there has been no information available on the characteristics of the transcriptional regulation in response to low-level NO exposure. To elucidate the involvement of transcription factors in the signaling pathways of intracellular glutathione induction by NO, the effects of various inhibitors on the NO-induced glutathione elevation and γ-GCS gene expression were examined in RAW 264.7 cells. TPCK and CAPE are inhibitors of NF-κB, while curcumin is an inhibitor of AP-1 [36–38]. Induction of glutathione and γ-GCS mRNA expression was significantly inhibited by TPCK or CAPE. In EMSA, low-level NO activated NF-κB DNA binding, but not AP-1 DNA binding. Therefore, the increase of γ-GCS mRNA expression involves transcriptional regulation at the NF-κB site in the promoter. Finally, in order to confirm that NF-κB-DNA-binding activity induced by NO is required for the increased expression of γ-GCS mRNA, the effect of NO on the transcription of γ-GCS was examined by using γ-GCS promoter luciferase constructs. It was found that NF-κB is directly involved in the induction of γ-GCS mRNA by SNP, leading to an increase in the intracellular glutathione level. On the other hand, curcumin is also considered as an inhibitor of NF-κB [39,40] as well as AP-1. However, the AP-1 DNA-binding activity showed no change in EMSA following SNP treatment, and the AP-1 DNA binding site on the γ-GCS promoter was not associated with the increase of γ-GCS promoter luciferase activity. Taken together, these results suggest that curcumin mainly suppressed AP-1 in this case.

γ-GCS consists of a heavy subunit (γ-GCSh) and a light subunit (γ-GCSl). γ-GCSh exhibits a catalytic activity, while γ-GCSl is enzymatically inactive, but plays an important regulatory function [14,15]. Both subunits have several cysteine residues, two disulfide bonds and a thiol cysteine residue at the active site, and are required for the enzyme activity [41]. Nitric oxide and NO donors, such as *S*-nitrosopenicillamine or DetaNONOate, cause transient depletion of glutathione followed by induction of glutathione synthesis via enhanced expression of the γ-GCSh and γ-GCSl genes in rat aortic vascular smooth muscle cells [24], pulmonary fibroblasts [42], and bovine aortic endothelial cells [43]. The expression of the γ-GCS subunit genes is modulated by different regulatory signals in response to diverse stimuli in specific cells. In the present study, the increased γ-GCSh mRNA expression induced by SNP was a consequence of NF-κB activation, though the mechanism of signal transduction with respect to γ-GCSl remains to be established.

NF-κB exists as a heterodimeric complex consisting usually of p50 and p65/RelA subunits. In unstimulated cells, NF-κB is found in the cytoplasm as an inactive non-DNA-binding form, associated with an inhibitor protein named IκB that masks the nuclear translocation signal and so prevents NF-κB from entering the nucleus. Oxidative stress causes rapid ubiquitination and phosphorylation of the IκB complex, leading to the activation of NF-κB [44]. Previously, we reported that the elevation of intracellular glutathione in RAW 264.7 cells exposed to low-level SNP occurs via the de novo GSH pathway through transcriptional up-regulation of the γ-GCS gene induced by peroxynitrite, and the induction was suppressed by antioxidant such as *N*-acetyl-L-cysteine. [45]. Thus, redox alteration was probably caused by peroxinitrite and resulted in the activation of NF-κB. Furthermore, 4-hydroxyhexenal induced NF-κB activation through the IKK/NIK pathway and/or p38 MAPK and ERK activation associated with peroxynitrite in endothelial cells [46]. This is in good agreement with our finding that the NF-κB DNA-binding activity was elevated post-treatment with SNP.

We previously reported that AP-1 mediates the elevation of γ-GCS gene expression by low-dose radiation [47]. It is noteworthy that induction of γ-GCS gene expression by NO is mechanistically distinct from that by hydroxyl radicals (generated by γ-rays). A detailed comparison is under way.

In conclusion, low-level NO exposure increases the intracellular glutathione level through transcriptional regu-

lation of the γ-GCSh gene, principally at the NF-κB binding site in the promoter.

References

[1] R.M.J. Palmer, A.G. Ferrige, S. Moncada, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor, Nature 327 (1987) 524–526.

[2] S.M. Deneks, B.L. Fanburg, Regulation of cellular glutathione, Am. J. Physiol. 257 (1989) L163–L173.

[3] L.A.S. Brown, Glutathione protects signal transduction in type II cells under oxidant stress, Am. J. Physiol. 266 (1994) L172–L177.

[4] W. Droge, K. Schulze-Osthoff, S. Mihm, D. Galter, H. Schenk, H. Eck, S. Roth, H. Gmunde, Functions of glutathione and glutathione disulfide in immunology and immunopathology, FASEB J. 8 (1994) 1131–1138.

[5] A. Meister, Glutathione metabolism, Methods Enzymol. 251 (1995) 3–7.

[6] H. Richman, Y. Takenaka, K. Fujisawa, M. Yamaguchi, K. Abe, S. Bannai, Increase in cystine transport activity and glutathione level in mouse peritoneal macrophages exposed to oxidized low-density lipoprotein, Biochem. Biophys. Res. Commun. 215 (1995) 154–159.

[7] T. Ochi, Arsenic compound-induced increases in glutathione levels in cultured hamster V79 cells, Arch. Toxicol. 70 (1997) 96–103.

[8] C. Viedt, G.M. Hansch, R.P. Brandes, W. Kubler, J. Kreuzer, The terminal complement complex C5b-9 stimulates interleukin-6 production in human smooth muscle cells through activation of transcription factors NF-kappaB and AP-1, FASEB J. 14 (2000) 2370–2372.

[9] M. Meyer, H.L. Pahl, P.A. Baeuerle, Regulation of the transcription factors NF-kappaB and AP-1 by redox changes, Chem.-Biol. Interact. 91 (1994) 91–100.

[10] U. Raju, G.J. Gumin, P.J. Tofilon, Radiation-induced transcription factor activation in the rat cerebral cortex, Int. J. Radiat. Biol. 76 (2000) 1045–1053.

[11] L.Z. Zhou, A.P. Johnson, T.A. Rando, NF kappa B and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells, Free Radic. Biol. Med. 31 (2001) 1405–1416.

[12] P.I. Oteiza, M.S. Clegg, M.P. Zago, C.L. Keen, Zinc deficiency induces oxidative stress and AP-1 activation in 3T3 cells, Free Radic. Biol. Med. 28 (2000) 1091–1099.

[13] N. Yan, A. Meister, Amino acid sequence of rat kidney γ-glutamylcysteine synthetase, J. Biol. Chem. 265 (1990) 1588–1593.

[14] C. Huang, M.E. Anderson, A. Meister, Amino acid sequence and function of the light subunit of rat kidney γ-glutamylcysteine synthetase, J. Biol. Chem. 268 (1993) 20578–20583.

[15] C. Huang, L. Chang, M.E. Anderson, A. Meister, Catalytic and regulatory properties of the heavy subunit of rat kidney γ-glutamylcysteine synthetase, J. Biol. Chem. 268 (1993) 19675–19680.

[16] I. Rahman, C.A.D. Smith, F. Antonicelli, W. MacNee, Characterisation of gamma-glutamylcysteine synthetase-heavy subunit promoter: a critical role for AP-1, FEBS Lett. 427 (1998) 129–133.

[17] K.S. Yao, A.K. Godwin, S.W. Johnson, R.F. Ozols, P.J. O'Dwyer, T.C. Hamilton, Evidence for altered regulation of gamma-glutamylcysteine synthetase gene expression among cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines, Cancer Res. 55 (1995) 4367–4374.

[18] I. Rahman, A. Bel, B. Mulier, M.F. Lawson, D.J. Harrison, W. MacNee, C.A. Smith, Transcriptional regulation of gamma-glutamylcysteine synthetase-heavy subunit by oxidants in human alveolar epithelial cells, Biochem. Biophys. Res. Commun. 229 (1996) 832–837.

[19] A. Morales, C. Garca-Ruiz, M. Miranda, M. Marí, A. Colell, E. Ardite, J.C. Fernandez-Checa, Tumor necrosis factor increases hepatocellular glutathione by transcriptional regulation of the heavy subunit chain of gamma-glutamylcysteine synthetase, J. Biol. Chem. 272 (1997) 30371–30379.

[20] R.T. Mulcahy, M.A. Wartman, H.H. Bailey, J.J. Gipp, Constitutive and β-naphthoflavone-induced expression of the human γ-glutamylcysteine synthetase heavy subunit gene is regulated by distal antioxidant response element/TRE sequence, J. Biol. Chem. 272 (1997) 7445–7454.

[21] T. Kondo, K. Yoshida, Y. Urata, S. Goto, S. Gasa, N. Taniguchi, γ-Glutamylcysteine synthetase and active transport of glutathione S-conjugate are responsive to heat shock in K562 erythroid cells, J. Biol. Chem. 268 (1993) 20366–20372.

[22] J. Sedlack, R.H. Lindsay, Estimation of total protein-bound and non-protein sulfhydryl group in tissue with Ellman's reagent, Anal. Biochem. 25 (1968) 192–205.

[23] G.O. Gogstad, M.-B. Krutnes, Measurement of protein in cell suspensions using the Coomassie brilliant blue dye-binding assay, Anal. Biochem. 126 (1982) 355–359.

[24] D. Moellerring, J. McAndrew, R.P. Patel, T. Cornwell, T. Lincoln, X. Cao, J.L. Messina, H.J. Forman, H. Jo, V.M. Darley-Usmar, Nitric oxide-dependent induction of glutathione synthesis through increased expression of γ-glutamylcysteine synthetase, Arch. Biochem. Biophys. 358 (1998) 74–82.

[25] B. Buckley, A.R. Whorton, Adaptive response to peroxynitrite: increased glutathione levels and cysteine uptake in vascular cells, Am. J. Physiol., Cell Physiol. 279 (2000) C1168–C1176.

[26] P.C. Kuo, K.Y. Abe, R.A. Schroeder, Interleukin-1-induced nitric oxide production modulates glutathione synthesis in cultured rat hepatocytes, Am. J. Physiol. 40 (1996) C851–C862.

[27] H. Li, Z.M. Marshall, A.R. Worton, Stimulation of cysteine uptake by nitric oxide: regulation of endothelial cell glutathione levels, Am. J. Physiol., Cell Physiol. 276 (1999) C803–C811.

[28] Y. Urata, H. Yamamoto, S. Goto, H. Tsushima, S. Akazawa, S. Yamashita, S. Nagataki, T. Kondo, Long exposure to high glucose concentration impairs the responsive expression of γ-glutamylcysteine synthetase by interleukin-1α and tumor necrosis factor-α in mouse endothelial cells, J. Biol. Chem. 271 (1996) 15146–15152.

[29] J. Cai, Z. Huang, S.C. Lu, Differential regulation of γ-glutamylcysteine synthetase heavy and light subunit gene expression, Biochem. J. 326 (1997) 167–172.

[30] J. Cai, W. Sun, S.C. Lu, Hormonal and cell density regulation of hepatic γ-glutamylcysteine synthetase gene expression, Mol. Pharmacol. 48 (1995) 212–218.

[31] K.R. Sekhar, M. Long, J. Long, Z.Q. Xu, M.L. Summar, M.L. Freeman, Alteration of transcriptional and post-transcriptional expression of gamma-glutamylcysteine synthetase by diethyl maleate, Radiat. Res. 147 (1997) 592–597.

[32] K. Teshima, A. Yamamoto, K. Yamaoka, Y. Honda, S. Honda, S. Kojima, Involvement of calcium ion in elevation of mRNA for γ-glutamylcysteine synthetase (γ-GCS) induced by low-dose γ-rays, Int. J. Radiat. Biol. 76 (2000) 1631–1639.

[33] A. Morales, M. Miranda, A. Sanchez-Reyes, A. Colell, A. Biete, J.C. Fernandez-Checa, Transcriptional regulation of the heavy subunit chain of γ-glutamylcysteine synthetase by ionizing radiation, FEBS Lett. 427 (1998) 15–20.

[34] M.M. Shi, A. Kugelman, T. Iwamoto, L. Tian, H.J. Forman, Quinone-induced oxidative stress elevates glutathione and induces γ-glutamylcysteine synthetase activity in rat lung epithelial L2 cells, J. Biol. Chem. 269 (1994) 26512–26517.

[35] R.M. Liu, H. Hu, T.W. Robison, H.J. Forman, Differential enhancement of γ-glutamyl transpeptidase and γ-glutamylcysteine synthetase by *tert*-butylhydroquinone in rat lung epithelial L2 cells, Am. J. Respir., Cell Mol. Biol. 4 (1996) 186–191.

[36] M. Wu, H. Lee, R.E. Bella, S.L. Schauer, M. Arsura, D. Katz, M.J. FitzGerald, T.L. Rothstein, D.H. Sherr, G.E. Sonenshein, Inhibition of NF-κB/Rel induces apoptosis of murine B cells, EMBO J. 15 (1996) 4682–4690.

[37] M. Watabe, K. Hishikawa, A. Takayanagi, N. Shimizu, T. Nakaki, Caffeic acid phenethyl ester induces apoptosis by inhibition of NF-κB and activation of Fas in human breast cancer MCF-7 cells, J. Biol. Chem. 279 (2004) 6017–6026.

[38] Y.-R. Chen, T.-H. Tan, Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin, Oncogene 17 (1998) 173–178.
[39] A. Foryst-Ludwig, M. Neumann, W. Schneider-Brachert, M. Naumann, Curcumin blocks NF-κB and the motogenic response in *Helicobacter pylori*-infected epithelial cells, Biochem. Biophys. Res. Commun. 312 (2004) 1065–1072.
[40] I. Rahman, J. Marwick, P. Kirkham, Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-κB and pro-inflammatory gene expression, Biochem. Pharmacol. (2004) 1255–1267.
[41] Z. Tu, M.W. Anders, Identification of an important cysteine in human glutamate–cysteine ligase catalytic subunit by sited directed mutagenesis, Biochem. J. 336 (1998) 675–780.
[42] A.C. White, E.K. Maloney, M.R. Boustani, P.M. Hassoun, B.L. Fanburg, Nitric oxide increases cellular glutathione levels in rat lung fibroblasts, Am. J. Respir. Cell Mol. Biol. 13 (1995) 442–448.
[43] D. Moellering, Mc.J. Andrew, R.P. Patel, H.J. Forman, R.T. Mulcahy, H. Jo, V.M. Darley-Usmar, The induction of GSH synthesis by nanomolar concentrations of NO in endothelial cells: a role of γ-glutamylcysteine synthetase and γ-glutamyltranspeptidase, FEBS Lett. 448 (1999) 292–296.
[44] I. Rahman, Regulation of nuclear factor-κB, activator protein-1, and glutathione levels by tumor necrosis factor-α and dexamethasone in alveolar epithelial cells, Biochem. Pharmacol. 60 (2000) 1041–1049.
[45] R. Kurozumi, S. Tokuzumi, S. Kojima, Does peroxynitrite involve in the elevation of cellular glutathione induced by sodium nitroprusside (SNP) in RAW 264.7 cells? Biol. Pharm. Bull. 26 (2003) 1070–1075.
[46] J.H. Je, J.Y. Lee, K.J. Jung, B. Sung, E.K. Go, B.P. Yu, H.Y. Chung, NF-κB activation mechanism of 4-hydroxyhexenal via NIK/IKK and p38 MAPK pathway, FEBS Lett. 566 (2004) 183–189.
[47] Y. Kawakita, M. Ikekita, R. Kurozumi, S. Kojima, Increase of intracellular glutathione by low-dose γ-ray irradiation is mediated by transcription factor AP-1 in RAW 264.7 cells, Biol. Pharm. Bull. 26 (2003) 19–23.