Antioxidant Function of the Mitochondrial Protein SP-22 in the Cardiovascular System*

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The mitochondrial protein SP-22 has recently been reported to be a member of the thioredoxin-dependent peroxide reductase family, suggesting that it may be one of the antioxidant systems in mitochondria, which are the major site of reactive oxygen intermediate generation. The aim of this study was to examine whether SP-22 is involved in mitochondrial antioxidant mechanisms and whether its expression is induced by oxidative stresses, particularly those in mitochondria. The expression of SP-22 protein was enhanced by about 1.5–4.6-fold when bovine aortic endothelial cells (BAEC) were exposed to various oxidative stresses, including mitochondrial respiratory inhibitors which increased the superoxide generation in BAEC mitochondria. The expression of SP-22 mRNA increased 2.0–3.5-fold with a peak at 3–6 h after exposure to Fe**/dithiothreitol or a respiratory inhibitor, antimycin A. BAEC with an increased level of SP-22 protein caused by pretreatment with mild oxidative stress became tolerant to subsequent intense oxidative stress. On the other hand, BAEC that had been depleted of SP-22 with an antisense oligodeoxynucleotide against SP-22 mRNA became more labile to oxidative stress than control BAEC. The inducement of SP-22 protein by oxidative stress in vivo was demonstrated in an experimental model of myocardial infarction in rat heart. These findings indicate that SP-22 functions as an antioxidant in mitochondria of the cardiovascular system.

Mitochondria play an important role in aerobic energy metabolism of living cells. The mitochondrial electron transport system consumes approximately 85% of the oxygen utilized by the cell, and about 5% of the oxygen is converted to reactive oxygen intermediates (ROIs)1 (1, 2). The generation of ROIs in the cell, and about 5% of the oxygen is converted to reactive oxygen intermediates such as protein, DNA, and lipid (3–6). Mitochondrial permeability transition, which may be an initial event in the process of cell death induced by Ca** and inorganic phosphate, has recently been reported to be mediated by ROIs and prevented by antioxidants in vitro (7).

The mitochondrial protein SP-22 was originally isolated from bovine adrenal cortex as a substrate protein for mitochondrial ATP-dependent protease (8, 9). An analysis of its amino acid sequence revealed that SP-22 is a member of the thioredoxin-dependent peroxide reductase family like the C22 component of alkyl hydroperoxidase in Salmonella typhimurium (10), thiol-specific antioxidant enzyme (11), 23-kDa macrophage stress protein (MSP23) (12), natural killer cell enhancing factor (NKEF) (13), and MER5 (14) in mammalian cells. MER5 is 92% similar to SP-22 protein and considered to be a mouse homolog of SP-22 (15). Members of this family have a highly conserved active site sequence among a wide range of species and are believed to act as antioxidant systems together with the NADPH-thioredoxin-thioredoxin reductase system (11, 16). Among the members of the thioredoxin-dependent peroxide reductase family, SP-22 is the only protein located in mitochondria. To test the hypothesis that SP-22 functions as an antioxidant system in mitochondria, which are the major site of cellular ROI generation, we investigated the oxidant-induced expression of SP-22 using cultured bovine aortic endothelial cells (BAEC) and an in vivo model of experimental myocardial infarction. Furthermore, we also examined the antioxidant function of SP-22 using BAEC with decreased or increased levels of SP-22 protein produced by treatment with antisense oligodeoxynucleotide or mild oxidant preconditioning. The present results indicate that SP-22 plays a crucial role in the antioxidant defense mechanism of mitochondria in the cardiovascular system.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—BAEC were harvested from bovine thoracic aorta obtained from a local slaughterhouse and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) as described by Kaku et al. (17). The identity of endothelial cells was verified by their characteristic morphology and the presence of factor VIII-associated antigen. Endothelial cells at up to passage 10 were used for these experiments. Mouse anti-manganese superoxide dismutase (MnSOD) monoclonal antibody was purchased from Chemicon International (Temecula, CA), anti-4-hydroxy-2-nonal (HNE) monoclonal antibody was from NOF Co. (Tokyo, Japan), 1-methyl-4-phenylpyridinium was from Aldrich, paracetamol from Nakalai Tesque (Kyoto, Japan), antimycin A and superoxide dismutase were from Sigma, and glucose oxidase and xanthine oxidase were from Boehringer Mannheim (Mannheim, Germany). Other chemicals were standard commercial products.

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‡ The abbreviations used are: ROI, reactive oxygen intermediate; SP-22, 22-kDa substrate protein; MSP23, 23-kDa macrophage stress protein; BAEC, bovine aortic endothelial cells; MnSOD, manganese superoxide dismutase; HNE, 4-hydroxy-2-nonal; SMP, submitochondrial particles; RT, reverse transcription; PCR, polymerase chain reaction; DIG, digoxigenin; DTT, dithiothreitol; ODN, oligodeoxynucleotide; CuZnSOD, copper and zinc superoxide dismutase; SOD, superoxide dismutase; TNF, tumor necrosis factor; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum.
of analytical grade.

Oxidative Stresses—Monolayers of confluent BAEC were incubated for the indicated periods with or without oxidative stress agents in Eagle’s minimal essential medium (Nissui, Japan) containing 0.5% FCS at 37 °C. After washing the monolayers, cells were disrupted in 1 mL of lysis buffer (10 mM Tris-HCl, pH 7.4, containing 0.2% (w/v) Triton X-100, 1 mM EDTA, 5 μg/mL chymostatin, 10 μg/mL each leupeptin, antipain, and pepstatin, and 20 μg (p-aminophenyl)mercaptanesulfon fluoride hydrochloride) and homogenized with a Teflon homogenizer. After centrifugation at 7000 × g for 10 min at 4 °C, lactate dehydrogenase activity in the supernatant was assayed as described by Bergmeyer et al. (17).

Polyclonal Rabbit Antibodies against SP-22—The peptide SPTASTREYFFKVR, corresponding to residues 192–203 of the SP-22 protein, was synthesized and conjugated with hemocyanin. Female Japanese white rabbits were subcutaneously immunized with the conjugated peptide (500 μg) emulsified with adjuvant (Titer Max, Sigma). The first booster injection (250 μg) was given 4 weeks later, and this was followed by three booster injections (250 μg each) at 2-week intervals. Sera were obtained 2 weeks after the last booster injection.

Immunocytochemistry—For immunofluorescence microscopy, KB cells grown on coverslips in minimal essential medium/10% FCS were fixed with 4% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.1% saponin. The permeabilized cells were reacted with anti-SP-22 antibody and then stained with rhodamine-conjugated second antibody. Electron immunocytochemistry of human mitochondria was carried out as described previously (18). The crude mitochondrial fraction of KB cells was pelleted, fixed with 8% paraformaldehyde in 0.1 mM phosphate buffer, pH 7.4, and embedded in LR white resin at 4 °C. Thin sections of the mitochondria were incubated with the antibody against the SP-22, and then incubated with anti-rabbit IgG-gold. Immunolabeled sections were then stained with uranyl acetate and lead citrate and examined under a Hitachi HU 12 electron microscope at 100 kV. Control experiments done with preimmune serum gave no immunoreactive signals.

Superoxide Production in BAEC Submitochondrial Particles—BAEC submitochondrial particles (SMP) were prepared essentially as described by Kang et al. (18). Briefly, BAEC were washed in an isotonic sucrose buffer composed of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 15 μg/mL leupeptin, 5 μg/mL (p-aminophenyl)mercaptanesulfon fluoride hydrochloride, and 50 ng/mL pepstatin, and suspended in the same buffer (1 × 10^6 cells/mL). The cells were homogenized in a Potter-Elvehjem homogenizer and centrifuged twice at 600 × g for 10 min to obtain post-nuclear supernatant. The post-nuclear supernatant was centrifuged at 7000 × g for 10 min. The pellet (crude mitochondrial fraction) was sonicated and centrifuged at 320,000 g for 1 h. The resultant pellet was homogenized in an isotonic sucrose buffer and served as the SMP fraction. Superoxide production by SMP was determined by the oxidation of adrenaline to adrenochrome, and was corrected by subtracting the rate in the presence of 10 μM tetramethyl-p-phenylenediamine as described by Takeshige et al. (19).

Immunoblotting Analysis—SP-22 protein of BAEC was determined by immunoblotting. Proteins (20 μg) from each sample of BAEC were separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred onto Immobilon P (Millipore), and probed with anti-SP-22 serum. Immunoreactive proteins were detected using horseradish peroxidase-conjugated goat anti-rabbit antibody and POD Immunostain (Wako, Osaka, Japan).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Semi-quantitative RT-PCR was performed to quantitate SP-22 mRNAs in oxidant-treated BAEC, as described previously (17). Briefly, total RNA was isolated from confluent BAEC using TRIzol reagent (Life Technologies, Inc.) based on the method reported by Chomczynski and Sacchi (20). RT reactions were carried out with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The reverse-transcribed cDNA products were amplified with Taq DNA polymerase using a 5′-catccagggcatcttgcag-gt-3′ (antisense) and 9′-catcttcgttatgcag-gt-3′ (sense) primers, which amplified a 366-bp base pair product. Each primer set yielded a single PCR product of the predicted size. The identity of the PCR products was confirmed by direct cycle sequencing. RT-PCR was also performed for the housekeeping gene β-actin as a control for the amount of RNA used in the RT reaction. A negative control, in which reverse transcriptase was omitted, was also performed to exclude the possibility of the amplification of contaminating genomic DNA. Linear relationships were observed between the quantity of RNA subjected to the RT reaction and the amount of amplified PCR product under the PCR conditions used for SP-22 (31 cycles) and β-actin (24 cycles) (Fig. 1).

Northern Blotting Analysis—SP-22 PCR products (366 base pairs) were subchoned into the pGEM-T Easy Vector (Promega) by means of the TA cloning technique. After confirming the direction of the PCR insert by sequencing, the plasmids with inserts were linearized with Bsp 120I (Fermentas Ltd., Vilnius, Lithuania) and transcribed in vitro into digoxigenin (DIG)-labeled cRNA with SP6 RNA polymerase using a DIG RNA labeling kit (Boehringer Mannheim). Total RNA (5 μg) was dissolved in 12 μL of sample buffer containing 10 mM sodium phosphate, pH 7.0, 50% (w/v) dimethyl sulfoxide, and 1 μg/μL of RNAse-free DNAse I. After denaturation (1 h at 50 °C), the samples were electrophoresed in 1% agarose gel with a 10 mM sodium phosphate buffer, and then transferred to a nylon membrane (Boehringer Mannheim) and immobilized by incubation for 30 min at 121 °C. Hybridization with the DIG-labeled cRNA probe was carried out overnight at 68 °C in 500 mM sodium phosphate, 7% SDS, 1 mM EDTA, and 1 mg/mL yeast tRNA (21). Blots were washed twice at room temperature with 200 mM sodium phosphate, 5% SDS, and 1 mM EDTA, and for 15 min at 65 °C with 0.2× SSC plus 0.2% SDS before color reaction using a DIG nucleic acid detection kit (Boehringer Mannheim) according to the instructions provided by the supplier. The amount of SP-22 mRNA was normalized by 18 S ribosomal RNA.

Preconditioning of BAEC with a Mild Oxidant—BAEC were preincubated with 3.3 μM FeSO₄, 330 μM dithiothreitol (DTT) for 24 h, which maximally increased the expression of SP-22 protein. These treated BAEC were then exposed to more intense oxidative stress consisting of 3.3 μM FeSO₄, 1 mM DTT for 24 h, and cell viabilities were evaluated by measuring the lactate dehydrogenase activities of the cell lysate.

Antisense Oligodeoxynucleotide—A 20-mer antisense phosphorothioate oligodeoxynucleotide (ODN) was synthesized by Toagosei Inc. Japan. The first antisense ODN sequence (antisense 1; 5′-5′-catccagggcatcttgcag-gt-3′) is directed against the translation initiation region of the SP-22 mRNA. The second antisense ODN (antisense 2; 5′-catcttcgttatgcag-gt-3′) is directed against the internal region of SP-22 mRNA. We also prepared a sense ODN of antisense 1 and 2, and several random ODNs for a control experiment. All of these ODNs were designed to not possess sequences homologous to other genomic sequences or strong secondary structures (22). For efficient transfection of BAEC, we used the cationic lipid TiX-50 reagent (Promega). ODNs that had been diluted in 30 mM HEPES buffer, pH 7.4 (final concentration of 3.3 μM, which was performed according to the instructions provided by the supplier, and mixed and incubated for 15 min at room temperature. The ODN/TiX-50 reagent mixture was added to each plate with 1 mL of RPMI 1640 medium without FCS, and the cells were incubated for 1 h at 37 °C in a 5% CO₂ atmosphere. At the end of the incubation period, the cells were gently overlaid with 2 mL of RPMI 1640 medium containing 10% FCS and incubated for 24 h. Subsequently, BAEC were treated with SP-22 antisense ODNs for 24 h.

![Figure 1](http://www.jbc.org/content/199/5/2731/F1.large.jpg)
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RESULTS

Immunological Detection of SP-22—We prepared an antibody against SP-22 protein and used it for immunological analysis. In immunoblotting, this antibody detected a single band with a molecular mass corresponding to SP-22 (22 kDa) in total homogenate and the mitochondrial fraction of BAEC, but not in the cytosol fraction (Fig. 2A). Intracellular distribution of SP-22 protein was further examined by immunofluorescence microscopy. When cultured KB cells were stained with the anti-SP-22 antibody, the signals of immunoreactive SP-22 exhibited the mitochondrial staining pattern characterized by a reticular staining appearance (Fig. 2B). Immunostaining signals were hardly visible when preimmune sera for SP-22 were used. The localization of the SP-22 protein in the mitochondria was further confirmed by electron microscopic immunocytochemistry. The mitochondrial fraction isolated from KB cells was stained with the anti-SP-22 antibody, followed by gold-labeled second antibody. Proteins reactive to the antibody were located in the mitochondria (Fig. 2C). These results indicate that SP-22 protein is located in mitochondria of culture cells, which is consistent with the results of the biochemical analysis by Watabe et al. (8).

Induction of the SP-22 Protein by Oxidative Stress—Exposing BAEC to Fe²⁺/DTT, which produces the hydroxyl radical through the Fenton reaction (24, 25), increased the expression of SP-22 protein in a time-dependent manner, with the maximal elevation occurring after 24 h of exposure. On the other hand, the expression of MnSOD was induced only slightly in this condition (Fig. 3). SP-22 expression was completely suppressed with Fe chelators, such as defereroxamine and diethylthiocarbamide, which interact with sulfhydryl groups (28), also en-

Fig. 2. Immunological analysis of subcellular localization of SP-22. A, proteins in the total homogenate and subcellular fractions of BAEC were separated on 15% SDS-PAGE and immunoblotted with the anti-SP-22 antibody (lanes 2–4). Lane 1, total BAEC homogenate stained by Coomassie Brilliant Blue; lane 2, total homogenate; lane 3, cytosol; lane 4, crude mitochondria. Twenty μg of protein was applied to lanes 1 and 2, and 60 μg was applied to lanes 3 and 4. Molecular size standards are indicated on the left (in kDa). B, cultured KB cells were stained with the anti-SP-22 antibody and examined by immunofluorescence microscopy as described under “Experimental Procedures.” C, isolated mitochondria from KB cells were reacted with the anti-SP-22 antibody and then stained with colloidal gold-conjugated second antibody for immunoelectron microscopy as described under “Experimental Procedures.” The bars in B and C indicate 10 and 0.2 μm, respectively.

Fig. 3. Induction of SP-22 and MnSOD proteins by Fe²⁺/DTT in BAEC. BAEC were incubated with 3.3 μM Fe²⁺, 500 μM DTT for 0, 3, 6, 12, 24, or 48 h. Proteins of BAEC were separated on 5% SDS-PAGE and immunoblotted with the anti-SP-22 antibody (upper) and anti-MnSOD antibody (lower). SP-22 and MnSOD in each sample were quantified and are expressed as intensity of the stained band/mg of protein (arbitrary units). Twenty μg (upper) and 40 μg (lower) of protein were applied to each lane, respectively.
noblotted with the anti-SP-22 antibody. Twenty
ide roughly at a rate of 0.13 nmol/min/mg protein (assuming
which is about 800-fold lower than the rate of superoxide gen-
ric analysis of the stained bands. The abundance of SP-22 protein is
applied to each lane. Quantitative data were obtained by a densitomet-
the stained bands. The abundance of SP-22 protein is expressed as a
ratio relative to that of the control BAEC (defined as 1.0). DTT, dithi-
ofreitol; DETAPAC, diethylenetriamineacetic acid.

### TABLE I

**Induction of SP-22 protein in BAEC exposed to the Fe²⁺ / DTT system**

| Stress agents | Relative SP-22 protein levels |
|---------------|-----------------------------|
| Fe²⁺ (3.3 μM/DTT (330 μM) + DETAPAC (1 mM)) | 1.92 ± 0.19 |
| Fe²⁺ (3.3 μM/DTT (500 μM) + DETAPAC (1 mM)) | 2.65 ± 0.53 |
| Fe²⁺ (3.3 μM/DTT (1 mM) + DETAPAC (1 mM)) | 4.61 ± 0.53 |
| Fe²⁺ (3.3 μM/DTT (330 μM) + DETAPAC (1 mM)) | 0.92 ± 0.08 |

### TABLE II

**Induction of SP-22 protein in BAEC exposed to various oxidative stresses**

| Stress agents | Relative SP-22 protein levels |
|---------------|-----------------------------|
| H₂O₂ (500 μM) | 1.47 ± 0.19 |
| tert-Butylhydroperoxide (250 μM) | 1.82 ± 0.32 |
| Glucose oxidase (10 milliunits/ml) | 1.44 ± 0.10 |
| Xanthine (50 μM)/xanthine oxidase (50 milliunits/ml) | 1.39 ± 0.05 |
| Na₃AsO₃ (100 μM) | 1.89 ± 0.36 |
| CdCl₂ (100 μM) | 1.69 ± 0.23 |
| Buthionine sulfoximine (100 μM) | 1.09 ± 0.09 |
| Diethylmaleate (50 μM) | 1.26 ± 0.16 |
| Rotenone (100 μM) | 1.75 ± 0.26 |
| Paraquat (330 μM) | 1.95 ± 0.22 |
| MPP⁺ (500 μM) | 2.49 ± 0.54 |
| Antimycin A (10 μM) | 3.23 ± 0.67 |
| KCN (1 mM) | 1.60 ± 0.16 |
| 2-Deoxyglucose (20 mM) | 1.03 ± 0.04 |

### TABLE III

**Generation of superoxide by SMP treated with respiratory chain inhibitors**

| Conditions | Adrenochrome formation (nmol/min/mg) |
|------------|-------------------------------------|
| Control system | 1.97 ± 0.02 |
| + Rotenone (100 μM) | 2.76 ± 0.07 |
| + Antimycin A (10 μM) | 4.01 ± 0.84 |

SMP were prepared from BAEC as described under "Experimental Procedures." Superoxide production by SMP was determined by the
xanthine/xanthine oxidase treatment, suggesting that su-
the xanthine/xanthine oxidase treatment, suggesting that su-
SP-22 expression in BAEC exposed to oxidative stress was also examined in
the media level. Total RNA was obtained from BAEC exposed to
for 0.5, 1, 3, 6, and 24 h, and the amount of
SP-22 mRNA was analyzed by Northern blotting and semi-
quantitative RT-PCR. In Northern blotting, SP-22 mRNA be-
gained to increase after 0.5 h of exposure to Fe²⁺/DTT or antimy-
achieved a maximal level at 3 h, and decreased to the
initial level after 24 h (Fig. 4A). In semiquantitative RT-PCR,
almost the same results were obtained (Fig. 4B). To investigate the
mechanism of the apparent induction of SP-22 in antimycin A-treated BAEC, we examined the effect of antimycin D on
increase in SP-22 mRNA expression after antimycin A treatment. As shown in Fig. 4C, antimycin D suppressed the
antimycin A-mediated increase in SP-22 mRNA expression to
the control level. This result indicates that the observed increase
in SP-22 mRNA was due at least in part to the transcrip-
tional activation of SP-22 mRNA.

### Effect of Preconditioning with a Low Dose of Oxidant—
The pretreatment of BAEC with a low dose of oxidant (3.3 μM Fe²⁺, 330 μM DTT), which did not affect cell viability, increased the
level of SP-22 protein about 3.3-fold compared with that in
control cells (Fig. 5A). In contrast to the untreated control, the
cell viability of pretreated BAEC did not markedly decrease with
subsequent treatment with a high dose of oxidant (Fig. 5B). These results indicate that BAEC in which SP-22 protein
had been induced by preconditioning with a mild oxidant be-
came tolerant to subsequent intense oxidative attack.
Antisense ODN for SP-22—We examined the cell viability of BAEC with a decreased level of SP-22 due to the treatment with antisense ODNs against SP-22 mRNA. After BAEC had been exposed to SP-22 antisense ODNs three times, the protein levels of SP-22 in BAEC were examined by immunoblotting. The amount of SP-22 in BAEC treated with antisense 1 and 2 decreased to 43.9% and 72.1%, respectively, of that in control cells, whereas that of the sense ODN-treated BAEC remained close to the level in the control (Fig. 6A). As shown in Fig. 6B, oxidative stress (3.3 μM Fe²⁺, 750 μM DTT) markedly decreased the cell viability of the antisense 1 ODN-treated BAEC (about 77% decrease), whereas the same oxidative stress produced only a slight decrease (~20%) in cell viability for the control and sense ODN-treated cells. Treatment of BAEC with antisense or sense ODNs by itself did not appreciably affect cell viability (Fig. 6B). These results indicate that BAEC with a lower level of SP-22 protein were more susceptible to oxidative stress.

Induction of SP-22 in Infarcted Rat Heart—To examine the in vivo induction of SP-22 protein, an immunohistochemical study was carried out using an experimental model of myocardial infarction in rat heart. Immunohistochemistry with anti-SP-22 and anti-HNE-protein adducts (32) showed that immunoreactive signals of both antibodies were markedly increased in the infarcted zone of the rat heart (Fig. 7, B and C), indicating that lipid peroxidation occurred in the infarct zone and SP-22 in rat heart was specifically induced corresponding to the area exposed to oxidative stress. In sham-operated animals, immunopositive signals against both antibodies were less evident, and there were no differences between the infarct and non-infarct zones (data not shown).

DISCUSSION
In cardiovascular systems, including the heart and vascular endothelial cells, mitochondria are particularly important for generating ATP. At the same time, the mitochondrial electron transport system consumes a great deal of oxygen and produces ROIs (1, 2). The generation of ROIs in cardiovascular systems...
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has harmful effects on tissues and leads to atherosclerosis (33), ischemia reperfusion injury (34), and hypertension (35). ROIs are removed by various scavenging systems, such as CuZnSOD or MnSOD (36), glutathione peroxidase (37), and catalase (38). SOD dismutates superoxide radical and produces hydrogen peroxide. Although catalase removes hydrogen peroxide in the cytosolic compartment, there has been no report on mitochondrial catalase, except for that in rat heart mitochondria (39).

SP-22 is a member of the thioredoxin-dependent peroxide reductase family (11), and by accepting electrons from the NADPH-thioredoxin-thioredoxin reductase system, it removes peroxides in vitro (15). In this study, the induction of SP-22 mRNA and protein were enhanced by various oxidative stresses, including mitochondrial respiratory chain inhibitors, in vascular endothelial cells. It is well known that respiratory chain inhibitors increase ROIs in mitochondria by blocking electron transport (29, 30), and we confirmed that antimycin A and rotenone actually increased superoxide production in sub-mitochondrial particles of BAEC. The result that intramitochondrial ROI generation may be more effective for enhancing the expression of SP-22 protein than the extracellular administration of an oxidative stressor can be explained by supposing that a redox sensor, which exists in or near mitochondria, specifically responds to intramitochondrial ROIs. Aconitase, an iron-sulfur protein located in both cytosol and mitochondria, has been reported to serve as the redox sensor and play a regulatory role in gene expression (40).

The cytosolic members of the thioredoxin-dependent peroxide reductase family have been reported to be induced by oxidative stresses. MSP23 is inducibly expressed when mouse peritoneal macrophages are exposed to diethylmaleate (12). This expression has also been reported to be enhanced by exposure to oxidized low density lipoprotein, hydrogen peroxide, and heavy metals (12, 41, 42). Thiol-specific antioxidant from yeast is also induced by high concentrations of mercaptoethanol, iron and oxygen (43). Yeast thiol-specific antioxidant exerts a protective effect in vitro on mitochondrial oxidative damage (7). Regarding protein induction by oxidative stresses, SP-22 seems to be induced in a manner similar to other cytosolic members of the thioredoxin-dependent peroxide reductase family, except for its response to sulfhydryl-reactive agents. Diethyldimaleate and buthionine sulfoximine induce the expres-

Fig. 5. Effect of preconditioning with a low dose of oxidant. A, BAEC were either pretreated with 3.3 μM Fe⁴⁺, 330 μM DTT for 24 h or left untreated. Proteins of BAEC total homogenate were separated on 15% SDS-PAGE and immunblotted with the anti-SP-22 antibody. SP-22 in each sample was quantified and is expressed as the intensity of the stained band/mg of protein (arbitrary units). Twenty μg of protein was applied to each lane. B, before BAEC were exposed to a high dose of oxidant (3.3 μM Fe⁴⁺, 1 mM DTT) for 24 h, the cells were pretreated with a low dose of oxidant (3.3 μM Fe⁴⁺, 330 μM DTT) for 24 h. Cell viabilities are expressed by a ratio relative to the level at 0 h (defined as 1.0) in terms of the lactate dehydrogenase activities of BAEC total homogenates. Data are shown as the means ± S.E. of three separate experiments.

Fig. 6. Depletion of SP-22 by treatment with antisense ODN. A, BAEC were treated three times with an antisense ODN/Tfx-50 reagent mixture for 24 h as described under “Experimental Procedures.” Proteins of BAEC were separated on 15% SDS-PAGE and immunblotted with the anti-SP-22 antibody. SP-22 in each fraction was quantified and is expressed as the intensity of the stained band/mg of protein (arbitrary units). Thirty-five μg of protein was applied to each lane. B, after BAEC were treated with ODNs, they were exposed to oxidative stress (3.3 μM Fe⁴⁺, 750 μM DTT) for 18 h. Cell viabilities were expressed by a ratio relative to the level at 0 h (defined as 1.0) in terms of the lactate dehydrogenase activities of BAEC total homogenates. Data are shown as the means ± S.E. of three separate experiments.
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These results suggest that SP-22 plays an integral role in antioxidant systems of BAEC. We found that thioredoxin and thioredoxin reductase, which participate in a cellular antioxidant defense (46) and are essential for SP-22 activity, are also located in mitochondria of BAEC.2

Previous reports have mentioned that some cytokines such as tumor necrosis factor-α and β (TNF-α and β) and interleukin-1α and β regulated the expression of MnSOD (47). Shull et al. reported xanthine/xanthine oxidase increased MnSOD mRNA in epithelial cells without induction of catalase, CuZnSOD, and glutathione peroxidase mRNAs (48). Yamamoto et al. reported that the promoter region of murine MER5 protein (homolog of bovine SP-22) had sites for several DNA-binding proteins (AP1, AP2, SP1) and MER5 protein was enhanced after induction of differentiation by dimethyl sulfoxide (14). Kang et al. recently reported overexpression of peroxiredoxin II (thioredoxin-dependent peroxide reductase) prevented the TNF-α-induced NFκB activation by removing intracellular hydrogen peroxide (49). These results suggested that expression of cellular antioxidant enzymes, such as the thioredoxin-dependent peroxide reductase and MnSOD, can be regulated by a cytokine/ROI-mediated signal transduction. We also observed SP-22 mRNA was induced by TNF-α.3

In vivo, the expression of SP-22 was enhanced in an experimental model of rat myocardial infarction. The induction of SP-22 expression peaked at 24 h after ligation, and rapidly decreased at 48 h when necrosis reached a peak. At 24 h after coronary occlusion, the process of infarction is still incomplete in the rat heart (23). With the progression of ischemia, ROIs such as superoxide, hydrogen peroxide, and hydroxyl radical are generated through several pathways, including the leakage of electrons from mitochondria, which are especially abundant in the heart (29, 50), the metabolism of arachidonic acid (51), and the increase in xanthine oxidase activity, which produces superoxide (52). It has been reported that exogenous SOD and/or catalase reduce the extent of myocardial infarction (53, 54), suggesting that they may protect against the progression of myocardial infarction. The present results obtained in experimental myocardial infarction also suggest that SP-22 may participate in protecting against myocardial infarction.

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