Effects of Selenium Deficiency on Expression of Selenoproteins in Bovine Arterial Endothelial Cells

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Selenium (Se) is an essential trace element and functions primarily in the form of selenoproteins.1–5) Se is incorporated into selenoproteins in the form of selenocysteine, and its normal function is directed by a specific UGA codon, which normally functions as a stop codon in both prokaryotes and eukaryocytes.5) In mammals, 11 selenoproteins have been found to date.2,3) These include four types of glutathione peroxidase (GPx),3) three types of thyroid hormone deiodinase,8) three types of thioredoxin reductase (TrxR),9,10) selenophosphate synthetase 2,11) selenoprotein W12) selenoprotein P (SelP),13) and the 15-kDa selenoprotein.14) In addition to these selenoproteins, a computational screening identified three other new mammalian selenoproteins.15,16)

Se deficiency causes a fall in the activities and mRNA levels of selenoproteins.2,3) In humans, severe Se deficiency is associated with cardiac disease (Keshan disease) in some provinces of China.2,17) It has also been reported that deficiency of Se is associated with an increased incidence of myocardial infarction and other atherosclerotic cardiovascular diseases.18,19) Reactive oxygen species (ROS) have been implicated in the pathogenesis of many cardiovascular diseases including atherosclerosis.20) The vascular endothelium represents a critical cell target in these disorders. ROS stimulate endothelial lipid peroxidation,21) perturb the barrier function of vascular endothelial cells (EC),22) and then interfere with endothelial functions. Selenoproteins such as GPx isozymes, TrxR isozymes and SelP have an activity to scavenge ROS and lipid peroxides.23,24) Thomas et al. found that Se supplementation confers resistance on EC to oxidative injury.25) The possible mechanism for this protection is considered to be through the expression of selenoproteins. However, the expression and the regulation of selenoproteins in vascular EC have not yet been well understood.

In the present study, we detected the expression of several selenoproteins including TrxR isozymes, GPx isozymes and SelP in cultured bovine arterial EC (BAEC) and then investigated the effect of Se depletion on the expression of these selenoproteins.

MATERIALS AND METHODS

Cell Culture BAEC were kindly provided by Dr. M. Masuda of the National Cardiovascular Research Institute (Osaka, Japan). BAEC were cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS). In order to prepare Se-sufficient and Se-deficient cells, the cells were grown in 0.5% FCS-containing DMEM supplemented either with insulin (5 μg/ml), transferrin (0.5 μg/ml) and sodium selenite (100 nm) (Se-sufficient medium) or with insulin and transferrin (Se-deficient medium) alone.

Measurement of Sensitivity of BAEC to tert-Butylhydroperoxide (t-BuOOH) The sensitivity of BAEC to t-BuOOH was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as described previously.26) BAEC cultured in Se-deficient or Se-sufficient medium for 6 d were resuspended in each medium. One hundred microliter aliquots (3×10⁴ cells) were dispensed to each well of 96-well plates and cultured for 24 h at 37 °C. These cells were incubated for 24 h in the presence of various concentrations of t-BuOOH. After incubation, 10 μl of MTT (5 mg/ml) was added and incubation was continued for an additional 2 h. The resultant formazan product was solubilized in 100 μl of 20% SDS solution containing 50% N,N-dimethylformamide (pH 4.7) and the concentration was measured spectrophotometrically at 550 nm.

Measurements of t-BuOOH-Induced 8-Isoprostane Formation in BAEC BAEC cultured in Se-deficient or Se-sufficient medium for 6 d (2.5×10⁵ cells/well) were dispensed to each well of 24-well plates and cultured for 24 h at...
37 °C. For measurement of t-BuOOH-induced 8-isoprostane formation, these cells were incubated for 24 h in the presence of various concentrations of t-BuOOH. After incubation, the culture medium was collected, and then the amount of 8-isoprostane was measured by 8-isoprostane EIA kit (Cayman, Ann Arbor, MI, U.S.A.).

75Se Metabolic Labeling and Separation of Radiolabeled Proteins Sodium [75Se]selenite (2.0 Ci/mg) purchased from the Research Reactor Facility, University of Missouri-Columbia, U.S.A., was used to label the selenoproteins of BAEC. BAEC (1 × 10^6 cells) were labeled with 100 nCi/ml sodium [75Se]selenite for 96 h. Labeled cells were collected after washing with ice-cold phosphate-buffered saline and lysed by three subsequent freeze/thaw cycles. Cell lysates (2000 cpm) were subjected to SDS-PAGE analysis on a 12.5% separating gel. 75Se-labeled proteins in culture medium were precipitated by the addition of trichloroacetic acid (final 15%) and also loaded onto SDS-PAGE. Bands of dried gels were visualized with a Fujix Bio-Imaging Analyzer (BAS2000; Fuji Photo Film Co., Tokyo, Japan).

RNA Isolation Total RNA from the harvested cells was isolated according to the acid guanidinium thiocyanate procedure. Poly(A)^+ RNA was purified by oligo (dT)~30~ latex (Nippon Roche, Tokyo, Japan).

RT-PCR Analysis of GPx Isozymes in BAEC Six degenerated oligonucleotide primers were designed and synthesized based on two highly conserved sequences in known mammalian GPx isozymes (NVAS(L/Y/Q)UG(T/L/K)T (U denotes selenocysteine) and WNF(E/T)KFL(V/I)(G/D)). Sense primers GFw1, GFw2 and EcoGFw2 are 5'-CTCTTCTTCA-3', GRv2 and XhoGRv2 are 5'-GG(CT)TT(ATGC)CC(ATGC)GG-3', and 5'-TG-3'. Antisense primers TRv1 and TRv2 are 5'-GC(AG)CA(CT)TC(CT)A(AG)(AG)GC(A-TGC)(AC)(AG)TA-3' and 5'-AC(CA)(CT)AC(AG)(AG)GC(A-TGC)(AC)(AG)TA-3', respectively. Antisense primers TRv1 and TRv2 are 5'-GC(AG)CA(CT)TC(CT)A(AG)(AG)GC(A-TGC)(AC)(AG)TA-3' and 5'-AC(CA)(CT)AC(AG)(AG)GC(A-TGC)(AC)(AG)TA-3', respectively. We designed and synthesized SelP-specific primers, SP1 (5'-A-3') and TRv2 using the first PCR products as templates. The second stage amplification was carried out with the primers TRv1 and TRv2 and the first PCR products as templates. The PCR was run for 30 cycles in the following cycle profile: 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 1 min. The major PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI, U.S.A.) and then subjected to sequence analysis.

RT-PCR Analysis of TrxR Isozymes in BAEC Four degenerated oligonucleotide primers were designed and synthesized based on two highly conserved sequences in known mammalian TrxR isozymes (GLGGTCVNVGCIPK and PGKTLVGVASYVALECA). Sense primers TFw1 and TFw2 are 5'-GG(CT)CT(CT)G(GGC)(GCA)(AC)(ATGC)(TGC-GT-3' and 5'-AA(CT)GT(AG)(ATGC)G(GC)(ATGC)TGC-GT-3', respectively. Antisense primers TRv1 and TRv2 are 5'-GC(AG)CA(CT)TC(CT)A(AG)(AG)GC(A-TGC)(AC)(AG)TA-3' and 5'-AC(CA)(CT)AC(AG)(AG)GC(A-TGC)(AC)(AG)TA-3', respectively. We first performed RT-PCR using the primers TFw1 and TRv1 and 1 µg BAEC poly(A)^+ RNA as templates, and then the second stage amplification was carried out with the primers TFw2 and TRv2 and the first PCR products as templates. The PCR was run for 30 cycles in the following cycle profile: 94 °C for 45 s, 48 °C for 1 min, and 72 °C for 1 min. Approximately 400 bp PCR products were extracted, ligated into pGEM-T Easy Vector, and then subjected to sequence analysis.

Northern Blot Analysis Poly(A)^+ RNAs (10 µg) or total RNAs (30 g) from BAEC were denatured with formamide, electrophoresed on a 1.0% agarose gel, and transferred to a PhotoGene nylon membrane (Life Technologies Inc., Gaithersburg, MD, U.S.A.). The probes were labeled with [α-32P]dCTP by Random Primer DNA Labeling Kit (Takara, Otsu, Japan). Hybridization was carried out as described previously. The mRNA levels were calculated on the basis of hybridization signals measured by a Fujix Bio-imaging analyzer BAS2000 (Fuji Photo Film Co., Tokyo, Japan).

Measurements of Cytosolic GPx (cGPx) and Phospholipid Hydroperoxide GPx (PHGPx) Activities Activities of cGPx and PHGPx were measured by recycling assay with NADPH and glutathione reductase as described previously. Substrates for cGPx and PHGPx were t-BuOOH and phosphatidylcholine hydroperoxide, respectively.

Measurements of TrxR Activity 13000 × g supernatant was prepared from the cell lysates and dialyzed against phosphate-buffered saline containing 1 mM EDTA. The TrxR activity was measured by the method of Hill et al. Briefly, the reaction was started by the addition of 250 µM supernatant to 650 µl of a cocktail containing 5',5'-dithiobis(2-nitrobenzoic acid) (DTNB) (final 5 mM) in buffer A (0.1 M potassium phosphate, pH 7.0, with 10 mM EDTA and 0.2 mg/ml BSA) with or without gold thioglucose (final 20 μM). Measurement of absorbance at 412 nm was started immediately after addition of the enzyme source. Six and a half minutes later, 100 µl of 2 mM NADPH was added to the reaction mixture to give a final concentration of 0.2 mM NADPH, and then DTNB reduction per minute was measured. TrxR activity was estimated as the difference between the reducing activity of the sample in the absence of gold thioglucose and its activity in the presence of gold thioglucose.
RESULTS

Effects of Se Deficiency on t-BuOOH Cytotoxicity and 8-Isoprostane Formation in BAEC. In order to elucidate the role of Se and selenoproteins in endothelial defense against oxidative injury, we first examined the effects of Se deficiency on t-BuOOH cytotoxicity and lipid peroxidation in BAEC. As shown in Fig. 1A, BAEC cultured in Se-deficient medium became more sensitive to t-BuOOH than those cultured in Se-sufficient medium.

We then measured the amounts of 8-isoprostane in the culture medium as an index of lipid peroxidation. 8-Isoprostane is a prostaglandin-like compound produced by non-enzymatic peroxidation of arachidonic acid.33,34) Treatment of BAEC with t-BuOOH increased the concentration of 8-isoprostane in a dose-dependent manner. As shown in Fig. 1B, Se deficiency enhanced t-BuOOH-induced 8-isoprostane formation in BAEC.

Expression of Various Selenoproteins in BAEC. We next investigated the expression of selenoproteins in BAEC by 75Se metabolic labeling analysis, and identified several selenoproteins which migrated at 14 to 66 kDa in the cell lysates on SDS-PAGE analysis (Fig. 2A, lane 1). Four bands were detected in the range between 19 to 26 kDa which correspond to the molecular weight of GPx isozymes.35) To examine which GPx isozymes BAEC expressed, we next performed RT-PCR analysis using degenerated primers as described in Materials and Methods. Amplified cDNA fragments were cloned and then subjected to sequence analysis. The sequence analysis revealed that two kinds of cDNAs encoding GPx isozymes were amplified. The first one encoded the nucleotide sequences identical to that of bovine cGPx cDNA,36) and the second one encoded the sequence which was highly homologous to those of known mammalian PHGPx cDNAs.37—39) However, neither cDNAs encoding other GPx-like proteins including extracellular GPx (eGPx) or gastrointestinal GPx (GI-GPx) were amplified using degenerated primers as described in Materials and Methods.

In addition to these four bands, 57 kDa proteins were strongly labeled. The molecular weight of SelP and TrxR isozymes is approximately 57 kDa. Among known mammalian selenoproteins, SelP and eGPx are secreted by many tissues. We next precipitated proteins in culture medium of 75Se-labeling BAEC with trichloroacetic acid and investigated extracellular 75Se-labeled proteins. As shown in Fig. 2A, 75Se-containing 57 kDa protein was observed in the culture medium as well as in the cell lysates. As it was reported that SelP had an affinity for heparin,40) whether the 75Se-containing 57 kDa protein could bind to heparin-Sepharose or not was next examined. After incubation of the culture medium with heparin-Sepharose, the bound proteins were eluted with 2 M NaCl and subjected to SDS-PAGE analysis.

To examine whether BAEC expressed TrxR isozymes or not, we next performed RT-PCR analysis using degenerated primers as described in Materials and Methods. Amplified cDNA fragments were cloned into pGEM-T Easy Vector and
then subjected to sequence analysis. The sequence analysis revealed that three kinds of cDNAs encoding TrxR isoymes were amplified. The first and the second ones encoded the nucleotide sequences identical to those of bovine cytosolic TrxR (TrxR1) and mitochondrial TrxR (TrxR2) cDNA, respectively. The third one, in contrast, encoded the sequence (accession number AB046704) which was highly homologous to that of the recently identified human third type of TrxR (termed TR2 by Sun et al.) cDNA. These results indicated that BAEC expressed three TrxR isozymes: TrxR1, TrxR2 and TrxR3.

Effects of Se Deficiency on Expression of Selenoproteins in BAEC

We next examined the effects of Se deficiency on the expression of selenoproteins in BAEC. As shown in Fig. 3A, both TrxR and cGPx activities were reduced in Se-deficient cells but Se deficiency did not affect the PHGPx activity. The activities of TrxR and cGPx in BAEC grown in Se-deficient medium for 3 d were reduced to about 70% and 60%, respectively, of those in Se-sufficient cells. The abundance of cGPx mRNA was also reduced to about 35% by Se deficiency (Fig. 3B). As well as cGPx mRNA, the SelP mRNA level was reduced by Se deficiency. The abundance of SelP mRNA in Se-deficient cells was about 70% of that in Se-sufficient cells. However, none of TrxR2, TrxR3 or PHGPx mRNA levels was reduced (Fig. 3C). TrxR1 mRNA level was conversely increased to about 1.6-fold when BAEC were grown in Se-deficient medium for 3 d. These results indicated that the effects of Se deficiency on the expressions of cGPx, PHGPx, SelP and TrxR isozymes were different from each other.

DISCUSSION

It has been reported that Se deficiency is associated with an increased incidence of myocardial infarction and other atherosclerotic cardiovascular diseases. We here showed that Se deficiency increased the susceptibility to t-BuOOH and enhanced lipid peroxidation in BAEC. Se deficiency might decrease the resistance of EC to ROS generated in vasculature and, as a result, cause cardiovascular disease.

The role of Se as an antioxidant in EC may be primarily mediated by the expression of selenoproteins. However, which kinds of selenoproteins were expressed and contributed to the resistance to ROS in EC had not been clarified. Se metabolic labeling analysis and RT-PCR analysis revealed that BAEC expressed the GPx isozymes, cGPx and PHGPx, three TrxR isozymes and SelP among selenoproteins. We further showed that Se deficiency reduced the expression of several selenoproteins in BAEC and that the effects of Se deficiency on the expression of these selenoproteins were different from each other.
teins were different from each other. The different effects of Se deficiency on cGPx, PHGPx and SelP expressions in rat tissues have also been reported. It was suggested that the different effects on cGPx and PHGPx were due to differences in stability of the mRNAs of these GPxs under conditions of low Se supply. Imai et al. reported that overexpression of PHGPx suppressed cell death due to oxidative damage in rat basophilic RBL-2H3 cells. However, we here showed that PHGPx levels were not affected by Se deficiency in BAEC. PHGPx might not make a large contribution to cellular defense against t-BuOOH-induced injury in BAEC. Se deficiency reduced cGPx levels in BAEC and none of the cDNAs encoding GPx isozymes other than cGPx and PHGPx was amplified by RT-PCR. Among GPx isozymes, cGPx might play the major role in the antioxidative defense of BAEC.

TrxR activity was also reduced by Se deficiency as well as cGPx in BAEC. The TrxR/thioredoxin system has been associated with a number of cellular processes, including regulation of cell growth and modification of the activity of transcription factors and receptors. In addition, TrxR can reduce and detoxify lipid hydroperoxides, hydrogen peroxides and organic hydroperoxides. TrxR isozymes may provide one of the main enzymatic defense systems against ROS in EC. There are currently three known forms of TrxR, TrxR1, TrxR2 and TrxR3. Northern blot analysis in mouse tissues indicated that TrxR1 and TrxR2 are expressed in a variety of tissues, whereas TrxR3 is preferentially expressed in testis.

Interestingly, BAEC expressed all three of the TrxR isozymes. Although it has been reported that TrxR1 is localized in the nucleus and cytoplasm and TrxR2 in the mitochondria, subcellular localization of TrxR3 has not been identified. We are now trying to clone TrxR3 cDNA covering an open reading frame to determine the subcellular localization and functions of TrxR3 in BAEC.

Among selenoproteins in BAEC, only TrxR1 isozyme level was up-regulated by Se deficiency. It was recently reported that the generation of ROS in human epidermoid carcinoma A431 cells increased the TrxR1 protein level and that hyperoxia increased the TrxR1 mRNA level in lungs of baboons. These observations indicated that TrxR1 expression may be regulated by redox status and ROS may induce TrxR1 expression. In BAEC, Se deficiency might affect intracellular redox status through the reduction of cGPx and TrxR activities and subsequently up-regulate TrxR1 expression.

Although none of the mRNAs of TrxR isozymes was reduced by Se deficiency, TrxR activity was reduced. These results suggested that TrxR activity might not be regulated at mRNA level when Se levels were low. Fujiwara et al. recently reported that the protein content of TrxR was not altered by Se deficiency in human lung adenocarcinoma A549 cells, although the TrxR activity was reduced. Selenocysteine residue is essential for TrxR activity, but Zhong et al. reported that TrxR purified from human placenta contained Se at a molar ratio of between 0.6 and 0.9 Se unit per TrxR. Our findings, together with these observations, suggested that Se deficiency might increase the ratio of inactive TrxR which did not contain selenocysteine residue and consequently reduce the TrxR activity in BAEC.

In addition to intracellular selenoproteins such as TrxR isozymes, we found that BAEC expressed and secreted SelP. Recently, Anema et al. reported that human umbilical-vein EC did not secrete SelP. At present, we think this discrepancy may be dependent on the difference of stems of EC. Although the precise function of SelP is unknown, it has been hypothesized to have a role in antioxidative defense. The proposed antioxidative defense function has been supported by studies in which diquat-induced liver necrosis and plasma 8-isoprostane formation in Se-deficient rats was reduced by low dose administration of selenite which increased plasma SelP levels but did not affect liver or plasma GPx activities. Recently, it was also reported that SelP had PHGPx activity. Burk et al. found that SelP associated with EC in rat tissues. SelP secreted from EC might bind to heparan proteoglycans on the cell surface, and then scavenge lipid peroxides to protect the membranes of EC in an autocrine/paracrine manner.

In the present study, as an index of lipid peroxidation, we measured the amounts of 8-isoprostane in the culture medium. 8-Isoprostanes and other F₂-isoprostanes were reported to be present in human atherosclerotic lesions and to exhibit thromboxane A₂-like activities, leading to vasoconstriction and abnormalities in platelet function. It was reported that Se deficiency inhibited prostacyclin release by human endothelial cells. SelP deficiency might cause an imbalance between the production of thromboxane A₂-like lipids including 8-isoprostane and prostacyclin. The imbalance might contribute both to the progression of atherosclerosis and to increased vascular occlusion in patients with coronary artery disease, resulting in the increased incidence of myocardial infarction and other atherosclerotic cardiovascular diseases associated with low serum Se levels.

In summary, we found that BAEC expressed two GPx isozymes, cGPx and PHGPx, and three TrxR isozymes, TrxR1, TrxR2 and TrxR3 as intracellular selenoproteins and SelP as an extracellular selenoprotein, and that Se status affected the expression of these selenoproteins. These selenoproteins might regulate ROS generation and arachidonic metabolism in vasculature and suppress the pathogenesis of many cardiovascular diseases.

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