Thioredoxin reductase is the major selenoprotein expressed in human umbilical-vein endothelial cells and is regulated by protein kinase C

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Damage to the endothelium by reactive oxygen species favours atherogenesis. Such damage can be prevented by selenium, which is thought to exert its actions through the expression of selenoproteins. The family of glutathione peroxidases (GPXs) may have antioxidant roles in the endothelium but other intracellular and extracellular selenoproteins with antioxidant actions may also be important. The selenoproteins expressed by cultured human umbilical-vein endothelial cells (HUVECs) were labelled with [⁷⁵Se]selenite and separated using SDS/PAGE. HUVECs secreted no extracellular selenoproteins. There were distinct differences between the intracellular selenoprotein profile of ⁷⁵Se-labelled HUVECs and those of other tissues. A single selenoprotein with a molecular mass of 58 kDa accounted for approx. 43 % of the intracellular ⁷⁵Se-labelled proteins in HUVECs. This protein was identified by Western blotting as the redox-active lipid-hydroperoxide-detoxifying selenoprotein, thioredoxin reductase (TR). TR expression in HUVECs was down-regulated by transiently exposing cells to the phorbol ester PMA for

periods as short as 1 min. However, there was a delay of 48 h after PMA exposure before maximal down-regulation of TR was observed. The protein kinase C (PKC) inhibitor bisindolylmaleimide I hydrochloride had no effect on TR expression when added alone, but the agent prevented the down-regulation of TR expression seen with PMA. The calcium ionophore A23187 increased TR expression in HUVECs after a 12-h exposure, but the maximal effect was only observed after a 35-h exposure. These findings suggest that TR may be an important factor in the known ability of Se to protect HUVECs from peroxidative damage. Furthermore, the results also suggest that TR expression can be negatively regulated through PKC. It is possible that TR expression may be positively regulated by the calcium-signalling cascade, although TR induction by A23187 may be due to toxicity.

Key words: atherogenesis, endothelium, oxidative damage, selenium.

INTRODUCTION

The endothelium of the vascular system regulates vessel tone, smooth-muscle proliferation and blood coagulability. Damage to the endothelium by reactive oxygen species, free radicals and oxidized lipids and lipoproteins favours atherogenesis [1]. Selenium is essential for the expression of several peroxidase and redox enzyme systems, which protect cells from oxidative stress and, as shown *in vitro*, selenium supplementation can protect human endothelial cells (ECs) from oxidative injury [2]. A possible mechanism for this protection is through the selenium-containing glutathione peroxidase (GPX) enzyme family [2]. Indeed, treatment of human ECs with selenium enhances GPX activity [3,4]. Furthermore, the activity of GPX in human ECs is induced by *n*–3 polyunsaturated fatty acids, which may have a role in the prevention of vascular disease [5].

Selenium exerts many of its effects through the expression of both extracellular and intracellular selenoproteins [6]. At least 30 such selenoproteins have been identified by SDS/PAGE of ⁷⁵Se-labelled tissue, but only approx. 12 have been characterized by purification and cloning. The intracellular selenoproteins identified to date that are most likely to protect against oxidative damage include cytoplasmic GPX (cyGPX), phospholipid hydroperoxide GPX (PHGPX) and thioredoxin reductase (TR).

Extracellular selenoproteins, including extracellular GPX (EGPX) and selenoprotein P, are secreted by many tissues and may also have an antioxidant action. It is not known if ECs

synthesize and secrete these extracellular selenoproteins, although selenoprotein P is associated with vascular ECs in brain, capillary ECs in the renal glomeruli and ECs of the liver [7].

The expression of some selenoenzymes is regulated through second-messenger systems. For example, in human thyrocytes and in the human fetal liver cell line, HepG2, the expression of TR is greatly increased by the addition of the calcium ionophore A23187 and PMA [8]. In contrast, EGPX secretion is inhibited by A23187, whereas PMA has little or no effect [9]. These observations have led to the suggestion that the Ca²⁺/phosphoinositol signalling cascade may be an important modulator of selenoprotein expression in certain tissues [8]. Such studies have not been performed in ECs, although phorbol ester has been reported to increase cyGPX mRNA levels in human umbilical-vein endothelial cells (HUVECs) [10].

Labelling with [⁷⁵Se]selenite demonstrates clear tissue differences in the pattern of selenoprotein expression [11] but little information of this nature is available for ECs. Although studies *in vitro* have found that selenite supplementation confers resistance on ECs to oxidative injury, the mechanisms by which selenium acts are not clear [2]. Resistance to oxidative damage may arise from increased expression of cyGPX and PHGPX [2], but the expression of other selenoproteins with antioxidant properties, such as TR, EGPX and selenoprotein P, was not investigated. Here we describe experiments performed to examine the range of intracellular and extracellular selenoproteins labelled

Abbreviations used: HUVECs, human umbilical-vein endothelial cells; TR, thioredoxin reductase; PKC, protein kinase C; EC, endothelial cell; GPX, glutathione peroxidase; cyGPX, cytoplasmic GPX; PHGPX, phospholipid hydroperoxide GPX; EGPX, extracellular GPX; EBS, Earle's balanced salt solution; GF109203X, bisindolylmaleimide I hydrochloride; (15S)-HPETE, (15S)-hydroperoxy-(5Z), (8Z), 11(Z), 13(E)-eicosatetraenoic acid; EGM-2, endothelial-cell growth medium 2; DMEM, Dulbecco's modified Eagle's medium.

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by [^{75}Se]selenite and report on the effects that PMA and A23187 exert on the expression of TR.

EXPERIMENTAL

Materials

Endothelial-cell growth medium 2 (EGM-2) bullet-kit and endothelial-cell basal medium (EBM-2) were both supplied by BioWhittaker UK (Wokingham, Berks., U.K.). Earle's balanced salt solution (EBS), M199, Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 nutrient mix, fetal bovine serum, glutamine, penicillin, streptomycin, amphotericin B and Ca^{2+} - and Mg^{2+} -free Hanks balanced salt solution were obtained from Life Technologies (Paisley, Scotland, U.K.). Collagenase was purchased from Worthington Biochemicals via Lorne Laboratories (Twyford, Berks., U.K.). [^{75}Se]Selenite (specific radioactivity, 16 MBq/nmol) was obtained from the Reactor Center, University of Missouri (Columbia, MO, U.S.A.). Low-range SDS/PAGE molecular-mass standards were purchased from Bio-Rad (Hemel Hempstead, Herts., U.K.). EDTA was supplied by Boehringer Mannheim UK (Lewes, East Sussex, U.K.). Glycerol and poly(ethylene glycol) were both purchased from Merck (Lutterworth, Leics., U.K.). Antisera to human and rat TR were raised in rabbits to TR purified from rat liver and human placenta as described previously [8,12]. Bisindolylmaleimide I hydrochloride (GF109203X) was supplied by Calbiochem Novabiochem UK (Nottingham, U.K.). Antisera to rat PHGPX were raised in rabbits to PHGPX purified from rat liver. Antiserum to human cyGPX was obtained from The Binding Site (Birmingham, U.K.). Sigma (Poole, Dorset, U.K.) supplied all other reagents.

Isolation and culture of HUVECs and human thyrocytes

Human umbilical cords (> 100 mm in length) were obtained at normal deliveries or Caesarean sections from non-smoking women. Immediately after delivery, the cords were placed into sterile EBS containing penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and amphotericin B (2.5 $\mu\text{g}/\text{ml}$) at 4 °C. ECs were isolated within 20 h of delivery using a method adapted from that described previously by Jaffe et al. [13]. Briefly, the umbilical vein was cannulated with a Venflon (17 gauge, 45 mm) and the vein was washed with 100 ml of EBS (pre-warmed to 37 °C). One end of the vein was clamped shut and the opposite end infused with 0.07% (w/v) collagenase in EBS (5–15 ml). The cord was then incubated at 37 °C in an atmosphere of 5% $\text{CO}_2/95\%$ air. After 10 min the cord was removed and massaged gently, and the contents of the umbilical vein were flushed out with 30 ml of Ca^{2+} - and Mg^{2+} -free Hanks balanced salt solution. The resulting cell suspension was collected, centrifuged at 450 g for 10 min and the pellet washed once with EGM-2 containing penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and amphotericin B (2.5 $\mu\text{g}/\text{ml}$). The cells were resuspended in 15 ml of EGM-2, plated out into one 75-cm² flask and cultured at 37 °C in an atmosphere of 5% $\text{CO}_2/95\%$ air.

After 24 h, and then on alternate days, the culture medium was changed and replaced with a further 15 ml of EGM-2. Cells reached confluence in 3–7 days, and were then subcultured as required, seeding at a density of approx. 3000 cells/cm². HUVECs were identified by their cobblestone appearance under phase-contrast microscopy and by the presence of factor-VIII-related antigen detected by a specific antibody using immunofluorescent microscopy.

Human thyrocytes were isolated as described previously using thyroid tissue (surplus to routine histopathological examination) obtained from a patient with Graves' disease [14,15].

Maintenance of HepG2 cell line

HepG2 cells, a human fetal liver-derived cell line, were obtained from the European Collection of Cell Cultures (Salisbury, Wilts., U.K.) and maintained in DMEM/Ham's F-12 nutrient mix plus L-glutamine and 15 mM Hepes containing 5% (v/v) fetal bovine serum. The cells were cultured in 75-cm² culture flasks and incubated at 37 °C in an atmosphere of 5% $\text{CO}_2/95\%$ air.

Monitoring selenoprotein expression

Intracellular and extracellular selenoproteins expressed by HUVECs were detected by labelling cells with [^{75}Se]selenite as described previously for thyrocytes [9]. Labelling with [^{75}Se]selenite provides a reliable, precise and sensitive method for assessing the expression of selenoproteins, where the incorporation of ^{75}Se is directed to specific selenocysteine residues by a UGA codon. However, equilibration of exogenous [^{75}Se]selenite with the endogenous pool of selenium and selenoproteins can take in excess of 27 h [16] and therefore cells are usually labelled for between 32 and 48 h to ensure that a steady state of labelling has been achieved. If it is required to examine changes in selenoprotein expression over short time periods it is essential to use cells pre-labelled with [^{75}Se]selenite. In the time-course experiments described in this article, cells pre-labelled for 48 h with [^{75}Se]selenite were used. The mass of exogenous selenium present in the culture medium was maintained at adequate levels over the labelling period by using media containing selenite at a concentration of 30 nM.

Therefore, to investigate intracellular selenoprotein expression in the steady state, confluent cultures of HUVECs or other cell types were incubated for 48 h with EGM-2 (which contains 30 nM selenite) in the presence of 0.02 MBq/ml [^{75}Se]selenite. After labelling, the culture medium was removed and the cells were washed three times with EBS and harvested into 20 ml of EBS by scraping followed by centrifugation at 2000 g for 10 min. The cells were resuspended in 1 ml of 60 mM Tris buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol and lysed by sonication at 4 °C.

After dilution to a common final protein concentration, the HUVECs were diluted 2:1 with 'boiling mix' (35 mM SDS/1.4 mM glycerol/0.3 mM 2-mercaptoethanol/15 mM Bromophenol Blue) and heat-treated at 90 °C for 10 min.

To monitor the extracellular selenoprotein expression, HUVEC cultures were incubated for 48 h in the presence of 0.02 MBq/ml [^{75}Se]selenite. The culture medium was changed from EGM-2 to protein-free EBM-2 for the last 18 h of the incubation period, but the [^{75}Se]selenite concentration was maintained throughout.

After the final 18-h labelling period, the culture medium was removed, centrifuged at 2000 g for 20 min and dialysed over 2 days to remove the unbound [^{75}Se]selenite. Dialysis was against 2 litres of 60 mM Tris buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol at 4 °C (which was changed four times over the 2-day period). The dialysed solution was then concentrated by placing the dialysis sac into a saturated solution of poly(ethylene glycol) prepared in the dialysis buffer. The protein content of the dialysed medium was then measured and the medium treated with boiling mix as described above.

The [^{75}Se]selenoproteins present in 25 μg of protein in the HUVECs and in 12.5 μg of protein in the culture medium were separated by SDS/PAGE on a 12% gel, the resulting gel was dried and the ^{75}Se -labelled selenoproteins were visualized by autoradiography using Kodak X-OMAT XAR-5 film.

The gels were scanned using a Bio-Rad model GS-525 Molecular Imager System to create a digitized image. The radio-

activity in each band was quantified using the Bio-Rad Molecular Analyst/PC image-analysis software. The data were presented as a percentage of the total intracellular ^{75}Se -labelled proteins expressed. Quantification of TR in the Western blots was carried out on an Epson_{GT}-9500 scanner using Phoretix software.

The intracellular selenoproteins expressed by human thyrocytes and HepG2 cells were determined in an identical fashion to the HUVECs.

To investigate the changes in selenoprotein expression that occurred over time in response to various agents, HUVECs pre-labelled to a steady state with [^{75}Se]selenite (0.02 MBq/ml) for 48 h were used. Monitoring of selenoprotein expression in these cells was then carried out as described above. These experiments are described in detail below.

Western-blot analysis

Proteins (20 μg per sample) resolved by SDS/PAGE were transferred to Immobilon P membranes that were then blocked using 10 % (v/v) horse serum in 0.025 M Tris buffer/0.5M NaCl (pH 7.5) containing 0.05 % (v/v) Tween before being probed with affinity-purified anti-rat TR antibody at a final dilution of 1:500. Chemiluminescence was used to visualize the immunoreactive proteins [8].

Previous studies that we have performed with this antiserum and the purified 58-kDa selenoprotein isolated from ^{75}Se -labelled HepG2 cells confirmed that the antiserum reacted with TR and not another protein of similar molecular mass [8]. Western blotting for PHGPX was performed as for TR, using an antibody raised to rat testes PHGPX.

Changes in expression of the 58-kDa selenoprotein in HUVECs following exposure to PMA and A23187 for different times

Selenoprotein expression in cells can be modified by PMA and A23187 [8,9]. Whereas these agents may produce a rapid effect on signalling systems in the cell, the changes in selenoprotein expression that they elicit may only be observable after several hours. The following experiments were performed to determine (i) the time at which maximal changes in the expression of the 58-kDa selenoprotein occur when HUVECs are exposed continually to PMA and A23187 and (ii) the influence of short 'pulsed' exposure times to PMA and A23187 on the subsequent maximal changes observed in the expression of the 58-kDa selenoprotein.

To investigate (i) above, confluent cultures of HUVECs were pre-labelled to a steady state for 48 h in the presence of EGM-2 containing 0.02 MBq/ml [^{75}Se]selenite. The culture medium was then removed and replaced with a further 15 ml of medium containing 0.02 MBq/ml [^{75}Se]selenite to ensure that nutrient and selenium supply were not exhausted. The effect of PMA ($0.5 \times 10^{-6}\text{M}$) or A23187 ($0.5 \times 10^{-6}\text{M}$) on the expression of the 58-kDa selenoprotein was studied by including the compounds in the culture medium for 1 min, 12 h, 24 h, 48 h, 72 h or 96 h in the continuing presence of 0.02 MBq/ml [^{75}Se]selenite. The timing of the additions were made to ensure that the cells for each data point were harvested immediately at the end of the drug-incubation period and at the same overall time point (i.e. after a total culture time of 146 h, which includes the 48-h pre-incubation interval). After harvesting the HUVECs, the intracellular [^{75}Se]selenoproteins were monitored as described above.

The concentrations of PMA and A23187 were selected on the basis of previous studies, which had shown optimal effects of these agents on the expression of the 58-kDa selenoprotein (results not shown).

To address (ii) above, confluent cultures of HUVECs were used that had been pre-labelled to a steady state with [^{75}Se]selenite (0.02 MBq/ml) for 48 h. The addition of PMA ($0.5 \times 10^{-6}\text{M}$) or A23187 ($0.5 \times 10^{-6}\text{M}$) was then made to these pre-labelled cells for 1 min, 10 min, 1 h or 12 h in the continued presence of [^{75}Se]selenite (0.02 MBq/ml). Additions were made at the same time and after each time point the culture medium was removed and the cells were washed. Fresh culture medium containing [^{75}Se]selenite (0.02 MBq/ml) but without PMA or A23187 was then added and the cells were incubated for a total incubation time of either 48 h in the case of PMA or 35 h in the case of A23187. These total incubation times were selected because the experiments performed in (i) above showed that they gave maximal changes in the expression of the 58-kDa selenoprotein. Cells were then harvested and the expression of the 58-kDa selenoprotein was monitored by autoradiography.

To control for any possible effects of DMSO, in which the PMA and A23187 were dissolved, HUVECs were also incubated with 0.05 % DMSO at each time point.

Effects of PMA and A23187 added alone and in combination on the expression of the 58-kDa selenoprotein

The effects of PMA ($0.5 \times 10^{-6}\text{M}$) and A23187 ($0.5 \times 10^{-6}\text{M}$), added individually or in combination, on the expression of the 58-kDa selenoprotein in HUVECs were investigated by the inclusion of the compounds in the culture medium while the cells were being labelled with [^{75}Se]selenite for 35 h, as described above. These conditions were chosen because they produced the maximum changes in expression of the 58-kDa selenoprotein in the presence of A23187. It was not possible to use time points in excess of 35 h for these experiments as A23187 occasionally caused some cell detachment if time periods were longer than this. Pre-labelled cells were not used for this experiment, as the incubation time (35 h) was sufficient to ensure steady-state labelling of the selenoproteins.

Effects of GF109203X and PMA on expression of the 58-kDa selenoprotein

Using cells pre-labelled to a steady state for 48 h with [^{75}Se]selenite (0.02 MBq/ml), the effect of the specific protein kinase C (PKC) inhibitor GF109203X ($4.5 \times 10^{-7}\text{M}$) on the 58-kDa selenoprotein expressed by HUVECs was studied in the presence and absence of PMA ($0.5 \times 10^{-6}\text{M}$). After pre-labelling for 48 h, the culture medium was removed from HUVECs and replaced with fresh medium containing 0.02 MBq/ml [^{75}Se]selenite. The GF109203X ($4.5 \times 10^{-7}\text{M}$) was added to the culture medium 1 h prior to any PMA additions. Previous studies have shown that PKC activity is inhibited using these conditions [17]. After the PMA ($0.5 \times 10^{-6}\text{M}$) addition, the HUVECs were incubated for a further 48 h before the cells were harvested for detection of selenoprotein expression by autoradiography.

Determination of protein

The protein content of the sonicated cells and extracellular proteins was determined using the Bradford dye-binding method using BSA standards [18].

RESULTS

Selenoproteins expressed by cell types

There were distinct differences in the ratios of the various intracellular selenoproteins expressed between the cell types

Figure 1 Autoradiograph of an SDS/PAGE gel showing various human cell types grown under basal conditions labelled with [^{75}Se]selenite (0.02 MBq/ml) for 48 h

Lane A, HUVECs; lane B, human thyrocytes; lane C, HepG2 cells. Each lane was loaded with 25 μg of protein.

Figure 2 Western blot of HUVECs using antiserum to TR

Cells were incubated for 35 h in the presence and absence of various agents, as specified, prior to the Western blotting of cell lysates. Lanes 1–3, purified human TR standard (4, 2 and 1 ng respectively); lane 4, no additions; lane 5, calcium ionophore A23187 (0.5×10^{-6} M); lane 6, PMA (0.5×10^{-6} M); lane 7, A23187 (0.5×10^{-6} M) with PMA (0.5×10^{-6} M).

studied (Figure 1). In HUVEC preparations a single selenoprotein with a molecular mass of 58.1 ± 1.0 kDa (mean $\pm$ S.E.M., $n = 5$) dominated the selenoprotein profile accounting for $42.9 \pm 1.4\%$ ($n = 3$) of the total intracellular ^{75}Se -labelled proteins. Two other selenoproteins showed quite pronounced labelling with [^{75}Se]selenite, with calculated molecular masses of 21.7 ± 0.5 ($n = 5$) and 24.4 ± 0.5 kDa ($n = 5$). These two selenoproteins accounted for $14.6 \pm 1.0\%$ ($n = 3$) and $15.1 \pm 2.8\%$ ($n = 3$) of the intracellular ^{75}Se -labelled selenoproteins, respectively. Western blotting identified the 21.7-kDa selenoprotein as PHGPX (results not shown). Western blotting was also attempted using antiserum to cyGPX but no immunoreactive band was observed.

Figure 3 Autoradiograph of an SDS/PAGE gel showing the expression of the 58-kDa selenoprotein in HUVECs incubated in the continued presence of PMA (0.5×10^{-6} M) for various times

Confluent cultures of HUVECs pre-labelled for 48 h with 0.02 MBq/ml [^{75}Se]selenite were used. The effect of PMA on the expression of the 58-kDa selenoprotein was studied by including either PMA (0.5×10^{-6} M) or DMSO (0.05%) in the culture medium for 1 min, 12 h, 48 h, 72 h or 96 h in the continued presence of 0.02 MBq/ml [^{75}Se]selenite. Lane 1, PMA, 1 min; lane 2, DMSO, 1 min; lane 3, PMA, 12 h; lane 4, DMSO, 12 h; lane 5, PMA, 48 h; lane 6, DMSO, 48 h; lane 7, PMA, 72 h; lane 8, DMSO, 72 h; lane 9, PMA, 96 h; lane 10, DMSO, 96 h. Each lane was loaded with 25 μg of protein.

Figure 4 Autoradiograph of an SDS/PAGE gel showing the expression of the 58-kDa selenoprotein in HUVECs incubated with PMA (0.5×10^{-6} M) for various times

Confluent cultures of HUVECs pre-labelled for 48 h with 0.02 MBq/ml [^{75}Se]selenite were used. The effect of PMA on the expression of the 58-kDa selenoprotein was studied by including either PMA (0.5×10^{-6} M) or DMSO (0.05%) in the culture medium for 1 min, 10 min, 1 h or 12 h in the continued presence of 0.02 MBq/ml [^{75}Se]selenite. After each time point the medium was removed, the cells were washed and fresh culture medium containing 0.02 MBq/ml [^{75}Se]selenite without PMA or DMSO was added. The cells were incubated for a total incubation time of 48 h. Lane 1, PMA, 1 min; lane 2, DMSO, 1 min; lane 3, PMA, 10 min; lane 4, DMSO, 10 min; lane 5, PMA, 1 h; lane 6, DMSO, 1 h; lane 7, PMA, 12 h; lane 8, DMSO, 12 h. Each lane was loaded with 25 μg of protein.

In the human thyrocytes and HepG2 cells the ^{75}Se -labelled 58-kDa selenoprotein was not as prominent as that observed in the ^{75}Se -labelled HUVECs (Figure 1). No significant ^{75}Se -labelling of any extracellular proteins was detected (results not shown).

Identification of the 58-kDa selenoprotein by Western blotting

Using antiserum to rat TR, the 58-kDa selenoprotein expressed by HUVECs was identified by Western blotting as TR. HUVECs expressed high concentrations of TR under basal conditions (4.36 ± 0.63 $\mu\text{g}/\text{mg}$, $n = 3$; Figure 2). TR concentrations in HepG2 cells were approximately one-tenth of those found in HUVECs. The Western blot was insufficiently sensitive to detect TR in the thyrocytes grown in the basal state.

Time-dependent changes in expression of the 58-kDa selenoprotein in HUVECs following exposure to PMA and A23187 for different times

The effects of both PMA and A23187 on the expression of the 58-kDa selenoprotein were time-dependent. The continued presence of PMA (0.5×10^{-6} M) significantly decreased the expression of the 58-kDa selenoprotein. The down-regulation of this protein was first apparent at 12 h, with the lowest level of expression seen at 48 h (Figure 3). After 72 and 96 h of exposure, the expression

Figure 5 Autoradiograph of an SDS/PAGE gel showing the expression of the 58-kDa selenoprotein in HUVECs incubated with A23187 (0.5×10^{-6} M) for various times

Confluent cultures of HUVECs were pre-labelled for 48 h with 0.02 MBq/ml [^{75}Se]selenite. The effect of A23187 on the expression of the 58-kDa selenoprotein was studied by including either A23187 (0.5×10^{-6} M) or DMSO (0.05%) in the culture medium for 1 min, 10 min, 1 h, 12 h or 35 h in the continued presence of 0.02 MBq/ml [^{75}Se]selenite. After each time point the medium was removed, the cells were washed and fresh culture medium containing 0.02 MBq/ml [^{75}Se]selenite without A23187 or DMSO was added. The cells were incubated for a total incubation time of 35 h. Lane 1, A23187, 1 min; lane 2, DMSO, 1 min; lane 3, A23187, 10 min; lane 4, DMSO, 10 min; lane 5, A23187, 1 h; lane 6, DMSO, 1 h; lane 7, A23187, 12 h; lane 8, DMSO, 12 h; lane 9, A23187, 35 h; lane 10, DMSO, 35 h. Into each lane, 25 μg of protein was loaded.

of the 58-kDa selenoprotein started to rise but the level did not return to the basal levels observed in the control DMSO-treated HUVECs.

The continued presence of A23187 (0.5×10^{-6} M) in the culture medium resulted in a significant induction of the 58-kDa ^{75}Se -labelled selenoprotein at 24 and 38 h (results not shown). However, in some experiments, where HUVECs were exposed to A23187 for 38 h and longer, cells started to detach from the monolayer, suggesting possible toxic effects of the ionophore. For all further experiments, a total incubation period of 35 h was therefore chosen, as this allowed maximal induction of the 58-kDa selenoprotein without any observable cell detachment. Controls containing DMSO showed no significant change in expression of the 58-kDa selenoprotein over the duration of the experiments.

The effects of the shorter pulse times of exposure to PMA and A23187 are shown in Figures 4 and 5. In HUVECs transiently exposed to PMA (0.5×10^{-6} M) for 1 min–12 h, a decrease in the expression of the 58-kDa selenoprotein was measured 48 h after the initial exposure to PMA (Figure 4).

HUVECs exposed to A23187 (0.5×10^{-6} M) for 1 h showed a slightly increased expression of the 58-kDa selenoprotein 35 h later (Figure 5). The observed induction of this 58-kDa selenoprotein by A23187 was increased with exposure time such that clear increases were observed after 12 h, with maximal induction after 35 h of exposure.

Effects of PMA and A23187, added alone and in combination, on the expression of the 58-kDa selenoprotein

The addition of PMA down-regulated the expression of the 58-kDa [^{75}Se]selenoprotein in HUVECs, whereas the addition of the calcium ionophore A23187 alone significantly induced its expression, as visualized by autoradiography (Figure 6). PMA and A23187 added in combination produced an overall net increase in the expression of the 58-kDa protein but the expression was lower than that seen when A23187 was added alone.

Quantification of the ^{75}Se -labelled 58-kDa selenoprotein using the Molecular Imager System showed that treatment with PMA decreased the expression of the 58-kDa band by between 43.5 and 50.7% of basal levels ($n = 2$), whereas treatment with A23187 increased the expression of the same band by 2.39–2.60-fold over basal levels ($n = 2$). PMA and A23187 added in

Figure 6 Autoradiograph of an SDS/PAGE gel of HUVECs labelled with [^{75}Se]selenite (0.02 MBq/ml) for 35 h in the presence and absence of various agents

Lane A, no additions; lane B, calcium ionophore A23187 (0.5×10^{-6} M); lane C, PMA (0.5×10^{-6} M); lane D, A23187 (0.5×10^{-6} M) with PMA (0.5×10^{-6} M). Each lane was loaded with 25 μg of protein.

combination produced an overall increase in expression of between 1.44- and 1.64-fold over basal levels ($n = 2$).

The effects of PMA and A23187 on the expression of the 58-kDa selenoprotein were confirmed by Western blotting using the anti-TR antibody (Figure 2). Western-blot analysis showed that PMA significantly ($P < 0.05$, analysis of variance and test of least significant difference) decreased TR expression in HUVECs from $4.36 \pm 0.63 \mu\text{g}/\text{mg}$ of protein (mean $\pm$ S.E.M., $n = 3$), expressed under basal conditions, to $2.97 \pm 0.27 \mu\text{g}/\text{mg}$ of protein ($n = 3$), a decrease of $29.6 \pm 9.7\%$. In contrast, A23187 significantly ($P < 0.01$) increased TR expression to $8.38 \pm 0.69 \mu\text{g}/\text{mg}$ of protein ($n = 3$), a 1.96 ± 0.13 -fold increase over basal levels. When HUVECs were treated with PMA and A23187 in combination there was a net increase in TR expression to $6.58 \pm 0.42 \mu\text{g}/\text{mg}$ of protein ($n = 3$), a 1.57 ± 0.24 -fold increase over basal levels.

The addition of PMA and A23187 also had effects on some of the other ^{75}Se -labelled selenoproteins in HUVECs. For example, treatment with PMA decreased the labelling of the 64- and 72-kDa [^{75}Se]selenoproteins. However, in the presence of A23187, ^{75}Se -labelling of the 64-kDa selenoprotein increased, whereas that of the 72-kDa selenoprotein was significantly attenuated. Both PMA and A23187 down-regulated the expression of the 22-kDa selenoprotein. The expression of the 24-kDa selenoprotein was increased by PMA, whereas A23187 had no effect on the expression of this selenoprotein (Figure 6).

Figure 7 Autoradiograph of an SDS/PAGE gel showing the expression of the 58-kDa selenoprotein in HUVECs incubated with the PKC inhibitor GF109203X (7.5×10^{-7} M) and/or PMA (0.5×10^{-6} M)

Confluent cultures of HUVECs pre-labelled for 48 h with 0.02 MBq/ml [^{75}Se]selenite were used. The effect of GF109203X and PMA on the expression of the 58-kDa selenoprotein was studied by including GF109203X and PMA in the culture medium alone or in combination. GF109203X was added 1 h prior to the 48-h PMA incubation in the continued presence of 0.02 MBq/ml [^{75}Se]selenite. Lanes 1 and 2, DMSO; lanes 3 and 4, GF109203X alone; lanes 5 and 6, GF109203X and PMA; lanes 7 and 8, PMA alone. Each lane was loaded with 25 μg of protein.

Effects of GF109203X and PMA on the expression of the 58-kDa selenoprotein

Treatment of HUVECs with PMA (0.5×10^{-6} M) for 48 h decreased the expression of the 58-kDa selenoprotein, as shown in the previous experiments. Pre-incubation of HUVECs with the PKC inhibitor GF109203X (4.5×10^{-7} M) for 1 h prior to the PMA addition attenuated the down-regulation of the 58-kDa [^{75}Se]selenite-labelled selenoprotein produced by PMA (Figure 7). No change in the expression of the 58-kDa selenoprotein was observed when HUVECs were incubated with GF109203X alone.

DISCUSSION

HUVECs exhibit an intracellular selenoprotein profile that is quite distinct from the other cells studied. We have found that HUVECs show dominant expression of a [^{75}Se]selenoprotein with a molecular mass of 58 kDa (Figure 1). Using Western blotting with antiserum characterized previously [8], we have demonstrated that this 58-kDa selenoprotein is TR (Figure 2). The expression of TR in HepG2 cells was found to be only approx. one-tenth of that found in HUVECs. In human thyrocytes, its expression was so low that it could not be detected by Western blotting, but ^{75}Se -labelling experiments suggested that TR expression in thyrocytes was approx. half of that seen in HepG2 cells (Figure 1). These different patterns of expression presumably underlie the requirement for different selenoproteins to contribute to the specific functions of the different cell types.

TR is an FAD-containing homodimeric selenoenzyme, which, together with thioredoxin as a substrate and NADPH as a cofactor, forms a powerful dithiol-disulphide oxidoreductase system that has multiple roles. The TR/thioredoxin system has been associated with a number of cellular processes, including regulation of cell growth and the modification of the activity of transcription factors and receptors [19–21]. TR expression is regulated in cancer cells through selenium supply, which stabilizes TR mRNA [21].

In addition, TR can reduce and detoxify lipid hydroperoxides, hydrogen peroxides and organic hydroperoxides [22]. The accumulation of these compounds in tissues exerts deleterious effects. For example the hydroperoxide (15*S*)-hydroperoxy-(5*Z*), (8*Z*), 11*Z*), 13(*E*)-eicosatetraenoic acid [(15*S*)-HPETE] oxidizes low-density lipoprotein to a form that under some conditions renders the particle more toxic to the endothelium, with implications in the pathogenesis of atherosclerosis [22]. TR also appears to be more potent than the GPX system at detoxifying (15*S*)-HPETE and other lipid hydroperoxides [22].

The data we have presented here show that the expression of TR is far greater in HUVECs than the expression of the selenoproteins with molecular masses of 22 and 24 kDa (Figure 1). The 22-kDa band was identified as PHGPX using Western-blot analysis (results not shown). It was not possible to visualize an immunoreactive band in HUVECs using antisera to cyGPX. However, other groups have shown that HUVECs express cyGPX [10] and exhibit GPX activity [2,3,5]. The molecular mass of cyGPX has been reported to be in the range of 21–26 kDa [23,24], which corresponds to the 24-kDa selenoprotein band we have found in HUVECs. These observations suggest that the 24-kDa selenoprotein expressed by HUVECs is cyGPX.

Thomas et al. [2] have suggested that selenite confers resistance on ECs to oxidative injury by increasing expression of cyGPX and PHGPX [2]. Whereas it is likely that these selenoperoxidases do have an important role in preventing oxidative injury to the ECs, our observations concerning TR expression raise the possibility that this reductase may be more important than the peroxidases in the protection of the endothelium against oxidative damage. The molecular mass of TR appears greater in HUVECs than that of PHGPX (22-kDa selenoprotein) and cyGPX (24-kDa selenoprotein) (Figure 1) and TR can detoxify many lipid hydroperoxides more efficiently than the GPXs [22]. TR may also have effects on other antioxidant systems in ECs; it can induce manganese superoxide dismutase [25] and regenerate ascorbate from dehydroascorbate [26].

We have shown that the addition of the phorbol ester PMA and the calcium ionophore A23187 have significant effects on the expression of TR. Exposure of HUVECs to PMA for times as short as 1 min decreased TR expression by approx. 30%. However, changes in TR expression were only seen 48 h after the short exposure to PMA. A similar degree of down-regulation of TR was seen when the exposure time of HUVECs to PMA was as long as 48 h. However, if cells were exposed to PMA for times in excess of 48 h the effect of the PMA on TR expression was diminished. These observations suggest that the down-regulation of TR by PMA arises by activation of PKC rather than resulting from desensitization of PKC. However, if PMA was present for times in excess of 48 h it seems likely that PKC desensitization does occur, as reported previously in studies using long-term exposures to PMA [27,28].

Our experiments using the PKC inhibitor GF109203X produced further evidence that increased TR expression in response to PMA is not the result of desensitization of PKC. GF109203X added alone had no effect on TR expression but when added with PMA the PKC inhibitor attenuated the effect of PMA on down-regulating TR. Overall, our results suggest that TR expression in HUVECs can be down-regulated by activation of PKC by PMA. This contrasts with human thyrocytes and HepG2 cells where PMA produces an increase in TR expression [8].

The addition of A23187 increased the expression of TR 1.96 ± 0.13 -fold over basal levels in HUVECs (Figure 2). Similar effects of the ionophore on TR expression have also been reported in human thyrocytes and HepG2 cells [8]. A23187 increases intracellular calcium levels but it may also act as an uncoupler of oxidative phosphorylation and as an inhibitor of mitochondrial ATPase activity. The A23187-induced cGMP response in ECs occurs rapidly following treatment with A23187 and reaches a plateau within 3 min [29]. However, our studies have shown that clear increases in the expression of TR only occur when A23187 is present in the culture medium for periods in excess of 12 h. This suggests that regulation of TR in the presence of A23187 in HUVECs may not be the result of calcium signalling but rather the result of a stress effect produced by A23187 on the HUVECs.

If A23187 was acting through a calcium response it would appear that, in HUVECs, the two branches of the Ca^{2+} /phosphoinositol signalling pathway are acting antagonistically. There are other examples in ECs where this is also the case. For example, constitutive nitric oxide synthase activity in ECs is stimulated by A23187, but the subsequent A23187-induced nitrite release is inhibited by PKC activation by the phorbol ester PMA [30].

The activation of PKC through oxidized low-density lipoprotein has been implicated in vascular disease [31] and we have shown that PKC activation may also give rise to a decrease in TR expression. Since TR may have an antioxidant function within the EC, down-regulation of TR by this mechanism may diminish the antioxidant capacity of ECs and render the cell prone to oxidative damage and to the development of atheroma.

In our culture system, HUVECs do not secrete extracellular selenoproteins. This contrasts with human thyrocytes, which secrete a 24-kDa selenoprotein identified as EGPX [9]. The vascular endothelium thus appears to make no contribution to the circulating pool of EGPX or selenoprotein P, another extracellular selenoprotein that may have antioxidant properties [32]. Thus it would seem that the selenoprotein P found bound to ECs is unlikely to have originated from synthesis within these cells, but rather that it is synthesized in other tissues such as kidney and released into plasma [7].

In conclusion, HUVECs, unlike other human cell types examined, express high levels of TR, a selenoprotein that has the potential to detoxify hydrogen peroxide and lipid hydroperoxides [22], regenerate ascorbate [26], promote superoxide dismutase activity, maintain cell redox state and influence the activity of the transcription factors of a number of important genes [19–21]. TR may thus be an important factor in the known ability of selenium to protect HUVECs from oxidative damage.

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