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Quiescin sulfhydryl oxidase (QSOX) is expressed in the human atheroma core: possible role in apoptosis

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Abstract Quiescin sulfhydryl oxidases (QSOXs) catalyze the formation of disulfide bonds in peptides and proteins, and in vertebrates comprise two proteins: QSOX1 and QSOX2. QSOX1, the most extensively studied type, has been implicated in protein folding, production of extracellular matrix, redox regulation, protection from apoptosis, angiogenesis, and cell differentiation. Atherosclerosis is an immunopathological condition in which redox processes, apoptosis, cell differentiation, and matrix secretion/maturation have critical roles. Considering these data, we hypothesized that QSOX1 could be involved in this disease, possibly reducing apoptosis and angiogenesis inside the plaque. QSOX1 labeling in normal human carotid vessels showed predominant expression by endothelium, subendothelium, and adventitia. In atherosclerotic plaques, however, QSOX1 was highly expressed in macrophages at the lipid core. QSOX1 expression was also studied in terms of mRNA and protein in cell types present in plaques under apoptotic or activating stimuli, emulating conditions found in the atherosclerotic process. QSOX1 mRNA increased in endothelial cells and macrophages after the induction of apoptosis. At the protein level, the correlation between apoptosis and QSOX1 expression was not evident in all cell types, possibly because of a rapid secretion of QSOX1. Our results propose for the first time possible roles for QSOX1 in atherosclerosis, being upregulated in endothelial cells and macrophages by apoptosis and cell activation, and possibly controlling these processes, as well as angiogenesis. The quantitative differences in QSOX1 induction may depend on the cell type and also on local factors.

Keywords QSOX · Atherosclerosis · Apoptosis · Microparticles · HUVEC · THP-1

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Introduction

Sulfhydryl oxidases are enzymes that catalyze the formation of disulfide bonds in peptides and proteins with the reduction of molecular oxygen to hydrogen peroxide through the reaction: $2R\text{-}SH+O_2 \rightarrow R\text{-}S\text{-}S\text{-}R+H_2O_2$ (Chang and Morton 1975; Ostrowski and Kistler 1980; Zheng et al. 2011). One subgroup of these enzymes, named flavin adenine dinucleotide (FAD)-linked sulfhydryl oxidases, includes the quiescin sulfhydryl oxidase (QSOX) family whose members share strong cDNA sequence identities and amino acid sequence features (Hoober et al. 1999; Thorpe et al. 2002; Houston et al. 2005). They display an N-terminal protein disulfide isomerase (PDI)-like domain bearing a thioredoxin family motif and a C-terminal ERV1-like domain, which contains the FAD-binding site (Coppock et al. 1998). Vertebrates have two main types of QSOX, QSOX1 and QSOX2 (or SOXN). Studies generally refer to the most abundant form of QSOX, whose gene has recently named QSOX-1 (for a review, please see Kodali and Thorpe 2010).

The first reported member and the most extensively studied type, QSOX1, is overexpressed in human lung fibroblasts (Coppock et al. 1993; Jaje et al. 2007) and abundantly expressed in adult rat seminal vesicle, from where it was first identified and sequenced (Benayoun et al. 2001). QSOX2 (or SOXN), a paralogue of QSOX1, was only reported in neuroblastoma cells (Wittke et al. 2003). In most human tissues, QSOX2 appears to be expressed at levels lower than QSOX1 (Coppock and Thorpe 2006).

Transcripts of *QSOX1* were described as *QSOX1a* and *QSOX1b* (Francavilla et al. 1994; Bach et al. 2008; Baker et al. 2008). *QSOX1a* has a single transmembrane region which is lacking in isoform *1b* due to an alternative splicing of the *QSOX1* message, which generates a shorter QSOX form (*QSOX1b*; Musard et al. 2001; Mairet-Coello et al. 2002; Thorpe et al. 2002; Wittke et al. 2003). QSOX1a (long isoform, QSOX-L) is a 74-kDa glycoprotein containing 743 amino acids, whereas QSOX1b (short isoform, QSOX-S) is a 65-kDa glycoprotein containing 604 amino acids (Antwi et al. 2009). It has been implicated in essential cellular functions such as protein folding (Thorpe and Coppock 2007), elaboration of extracellular matrix, redox regulation, and cell cycle control (Coppock et al. 2000; Musard et al. 2001; Heckler et al. 2008). Besides, it was shown by *in vitro* experiments that QSOX1 was induced after oxidative stress and that transgenic cells overexpressing QSOX were more resistant to oxidative stress-induced apoptosis (Morel et al. 2007). However, the interaction of QSOX1 with the various components of the immuno-inflammatory system and its potential role in pathophysiological processes are largely unknown.

Atherosclerosis is a chronic inflammatory disease of the vessel wall resulting from interactions between modified lipoproteins, immune cells such as monocytes/macrophages and lymphocytes, and vascular cells (Hansson 2005). Despite the large quantity of data addressing the role of redox processes in atherosclerosis, the possible involvement of QSOX1 in plaque evolution has not been explored. Such involvement can be postulated considering the role of QSOX1 in apoptosis, cell differentiation, and matrix secretion/maturation. In addition, atherosclerosis is associated with the increased expression of Nox family NADPH oxidases, which may associate with PDI (Laurindo et al. 2008; Liberman et al. 2008), a known substrate of QSOX1 (Zheng et al. 2011). Atherosclerosis progression is also associated with apoptotic cell death and accumulation of microparticles (MPs) within the lesion (Kockx and Herman 1998; Mallat et al. 2001). Interestingly, we previously showed that MPs from fetal serum, in contrast to postnatal serum, have high levels of active QSOX1 (Zanata et al. 2005), a feature similar to that of some MPs from the plasma of patients with clinical coronary atherosclerosis (unpublished observations). Besides apoptosis, QSOX1 has also been associated with anti-angiogenesis since QSOX1 knockdown increases endothelial cell proliferation and sprouting (Hellebrekers et al. 2007). Taking these considerations together, we hypothesized that QSOX1 might regulate apoptotic events associated with the atherosclerotic process and also inhibit intra-plaque angiogenesis, possibly reducing disease progression.

Material and Methods

In order to analyze whether QSOX might participate in atherosclerosis, we first evaluated the presence of QSOX in normal vessels and atherosclerotic plaques by immunohistochemistry using an antibody that recognizes both QSOX1 and QSOX2. We then assessed its expression in cell types relevant to atherosclerosis under apoptotic and activating stimuli, emulating conditions found during the natural history of plaque development. We observed QSOX1 expression in endothelial cells and macrophages in carotid normal vessels and plaques and verified its increase in endothelial cells and macrophages by apoptosis and cell activating stimuli. This study is the first to suggest that QSOX1 is induced after intra-plaque cell apoptosis and to propose that the molecule may have roles in the control of plaque progression.

For the immunohistochemistry experiments, normal carotid samples were obtained from organ donors (during surgery for heart transplantation) and atherosclerotic plaques were obtained from endarterectomy specimens, both at the Heart Institute (InCor HC-FMUSP). Informed consent was obtained from families of donors or patients according to regulations, and all procedures were approved by the local human ethics

committee (CAPPESQ protocol 568/05). Both types of samples were transferred right after removal to tubes containing formalin 10% in PBS and embedded in paraffin after 24 h.

Analysis of QSOX (either QSOX1 or QSOX2; Portes et al. 2008), α-actin (HHF35) and CD68 (KP1) (Santa Cruz Biotechnology, Inc; Santa Cruz, CA) expression was carried out in paraffin-embedded sections. Sections were deparaffinized and antigen retrieval was performed by boiling in Tris buffer. Endogenous peroxidase was inactivated using excess H_2O_2. The slides were blocked for 1 h at room temperature with Protein Block (Dako, Carpinteria, CA) and incubated overnight at 4°C with the primary antibodies. Staining was performed with LSAB kit (Dako), used according to the manufacturer's instructions, with diaminobenzidine (Dako) as the substrate and hematoxylin as the counterstain.

Human umbilical vein endothelial cells (HUVEC; ATCC) were cultured in 199 medium (Gibco-Invitrogen, Carlsbad, CA), HeLa cells (cervical carcinoma cell line) in minimum essential medium (Gibco-Invitrogen), and human monocytic THP-1 in RPMI-1640 medium (Gibco-Invitrogen). All cells were grown at 37°C, 5% CO_2, and supplemented with 10% bovine calf serum and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin).

Cells were plated at a density of 2×10^6 cells/well in six-well culture plates. THP-1 cell differentiation to macrophages was induced by 40 ηM PMA for 20 h. Apoptosis was triggered with 5 •M camptothecin (Sigma-Aldrich, St. Louis, MO) and Golgi transport inhibition with 5 μg/ml Brefeldin A (BFA, Calbiochem, Merck, Darmstadt, Germany). HUVECs were in some cases stimulated by angiotensin II (Ang II, 100 μM) in the presence or absence of BFA. Different combinations of such treatments were performed. In selected experiments, after 48 h, the supernatant was harvested from each well and the microparticles isolated by centrifugation (100,000×*g* for 1.5 h; Simák et al. 2002). Protein extracts were prepared from supernatants. Cells were lysed for protein and RNA isolation. Experiments were repeated at least three times.

Cell samples were analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and CELLQUEST software was used for forward scatter (FSC) and side scatter (SSC) analyses in the linear mode setting. Samples were analyzed using FSC vs. SSC plot and propidium iodide (PI) fluorescence vs. FSC plot. Single cells were gated separately in gate G1 and apoptotic bodies and other cell fragments in gate G2. We analyzed 10,000 gated particles for each sample. The apoptotic and necrotic status of cells was assayed using Annexin V-FITC and PI cell labeling, respectively. The percentages of PI-negative cells binding Annexin V were evaluated. Flow cytometric assays for the detection/analysis of apoptotic status were carried out using the Annexin V-FITC Kit (GE Healthcare, Uppsala, Sweden) and PI according to the manufacturer's protocol. Briefly, trypsinized cells were resuspended in buffer with Annexin V-FITC and PI and incubated for 15 min in the dark. Samples were acquired on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA) and then analyzed using FlowJo software (version 8.7.1, Tree Star, San Carlo, CA). We acquired 10,000 events in the cell gate. Gating strategy was first performed in FSC vs. SSC dot plot, followed by subsequent analysis on annexin vs. PI dot plot.

RNA isolation was performed using Trizol (Invitrogen, Carlsbad, CA) and purity/integrity assessed by spectrophotometry and gel electrophoresis. cDNA was prepared from 2 μg RNA using 2.5 ηg/•l of "random primer," 12.5 ηg/•l oligodT, Superscript II enzyme, and reaction buffers (Invitrogen). Real-time RT-PCR was performed with SYBR green system and ABI Prism 7500 equipment. All reactions were performed in triplicate using cDNA diluted 1:10. GAPDH was used for normalization. QSOX primers were designed based on QSOX1 sequence and showed no similarity to the QSOX2 sequence. QSOX primers (forward: 5′-GAAGACGTCAAAGCCTGGAG-3′; reverse: 5′-CACGTGTCATGATGGGACTC-3′) and GAPDH primers (forward: 5′-GAGTCAACGGATTTGGTCGT-3′; reverse: 5′-TTGATTTTGGAGGGATCTCG-3′) were used at final concentrations of 300 and 200 ηM, respectively. PCR conditions were: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Dissociation curves were analyzed to ensure specificity of the amplification. The efficiencies of both primer pairs were calculated as described by Pfaffl (2001). The expression ratios were calculated as previously described (Livak and Schmittgen 2001; Pfaffl 2001).

Cells were lysed in buffer containing 20 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1.0 mM PMSF. Cell extracts (50 μg) or microparticles were separated through 10% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). After incubation with a blocking solution containing 5% skim milk in TBS-T (TBS, pH 7.6, Tween 0.1%), the membrane was incubated with a 1:500 dilution of a goat antibody (Bethyl Co., Montgomery, TX) against synthetic QSOX peptide EDPQFPKVQWPPRE (epitope in the N-terminal region of QSOX1 and QSOX2 isoforms), followed by 1:2,000 anti-goat IgG conjugated to horseradish peroxidase (Calbiochem). In this study, we infer that the QSOX isoform observed is the QSOX1 because of the molecular weight observed—65 kDa. Bands corresponding to QSOX1 were visualized by chemiluminescence detection with an ECL kit (GE Healthcare). Membranes of whole cell extracts were then washed and incubated with β-actin antibody (Fisher Scientific, Loughborough, UK) and developed as described above. For membranes of the microparticles,

normalization was done using Ponceau intensity due to the absence of a known internal control. In another set of experiments, membranes were incubated with a primary antibody specific for PARP (New England Biolabs, Beverly, MA) in 5% BSA in TBS-T overnight at 4°C.

Each sample set was submitted to Kolmogorov–Smirnov test of normality. All datasets followed parametric distribution; thus, we employed one-way analysis of variance (ANOVA) followed by post hoc comparisons using Bonferroni's multiple comparison test (significance level, 0.05). Results are expressed as the mean±SEM.

Results

QSOX expression was analyzed by immunohistochemistry in two normal carotid arteries and three atherosclerotic plaques, and one representative specimen of each is shown (Fig. 1*A*, *B*). Labeling of macrophages and foam cells with CD68 and smooth muscle cells with actin was performed in parallel with QSOX. Figure 1*A* shows that QSOX was expressed in normal vessels in two main patterns: (1) intracellular staining, evident at the endothelial layer and in scattered cells in the media and adventitia, and (2) extracellular staining in subendothelial and adventitial regions.

In atherosclerotic plaques, QSOX expression was somewhat heterogeneous, as expected from the heterogeneity of the plaques, but a constant feature was that the lipid-rich necrotic core showed strong staining, more evident extracellularly, while macrophage-rich regions (identified through parallel CD68 staining) were also positive, with a predominantly intracellular staining pattern (Fig. 1*B*).

The presence of QSOX in the subendothelial region and in the necrotic core suggests its secretion by endothelial cells and macrophages/foam cells and/or a possible leakage correlating with cell death, likely of macrophages and foam cells (Fig. 1*A*, *B*). *Ex vivo* specimens (organ donor and endarterectomy specimens), however, may carry some postmortem-induced changes, making it difficult to precisely assess the concomitance of apoptosis and QSOX expression. Thus, further studies were carried out in cultured cells relevant to atheroma pathogenesis.

We analyzed the mRNA and protein expression of QSOX1 in endothelial cells and monocytes/macrophages at baseline and under stimuli promoting, according to each cell type, cell death (camptothecin), proliferation/activation (Ang II), differentiation/apoptosis (PMA), or inhibition of cell secretion (BFA). To compare such results with those from a cell type known to be absent from atherosclerotic plaques, we performed similar experiments with the epithelial cell line HeLa. In all cases, QSOX1 expression was assessed after incubation with different stimuli for 48 h. The primers for real-time RT-PCR were designed to abridge cDNA sequences corresponding to *QSOX1 variants a* and *b*, but not to *QSOX2*. Western blots for QSOX were performed using an antibody that could recognize both QSOX1 and QSOX2. However, differences in size between QSOX1 and QSOX2 would allow us to distinguish them. In fact, we observed only bands corresponding to QSOX1 size in the blots of cell lysates and microparticles, suggesting that QSOX2 was not expressed in the cell types analyzed.

In a first series of experiments, the extent as well as the mode of cell death after each stimulus were documented by both flow cytometry (fluorescence-activated cell sorting, FACS) and PARP analysis. PARP is a DNA repair-initiating enzyme that is cleaved after caspase 3 and 7 activation and is considered a good indicator of apoptosis (Hertzberg et al. 1989). PARP cleavage was enhanced by camptothecin in a dose-dependent manner in HUVEC, THP-1, and HeLa cells (data not shown). Using FACS analysis, we determined the percentages of cells in gates defined as Annexin V-positive (apoptosis) or Annexin V/PI double-positive (apoptosis and secondary necrosis). Background fluorescence of control (unstimulated) cells was subtracted from each time point in order to express the percentage of apoptotic cells above basal levels. Total apoptosis was determined as the sum of Annexin V-positive and Annexin V/PI double-positive cells. The results are shown in Figs. 2, 3, and 4.

HUVECs were particularly sensitive to apoptosis induced by camptothecin (70% apoptosis); less apoptosis was induced by Ang II (16%) and BFA (9%; Fig. 2). In THP-1 cells, the baseline levels of apoptosis were already elevated to some extent (13% apoptosis), while PMA synergized with camptothecin to induce an average 37% apoptosis (Fig. 3). In HeLa cells, either camptothecin or BFA promoted moderate increases of 7–8% in apoptosis rates (Fig. 4)

The results of real-time PCR experiments shown in Fig. 5 indicated that the induction of cell death with camptothecin was a relevant stimulus for a significant QSOX1 mRNA increase in HUVECs, while not in THP-1 monocytes/macrophages and HeLa cells. Moreover, triggering endothelial cell activation with Ang II or cell stress due to Golgi disruption (with BFA) had no independent or additional effect on QSOX1 mRNA expression. In contrast, differentiation/apoptosis by PMA in monocytes was a strong QSOX inducer, promoting at a mean of 3.4-fold increase in the three experiments performed, and not further enhanced by camptothecin or BFA. Neither camptothecin nor BFA had a significant effect on QSOX1 mRNA expression in HeLa cells. As can be observed, the conditions that promoted the highest apoptosis index by FACs (HUVECs with camptothecin and THP-1 with PMA) were the same conditions that induced the highest QSOX1 expression at the mRNA level, suggesting that an aug-

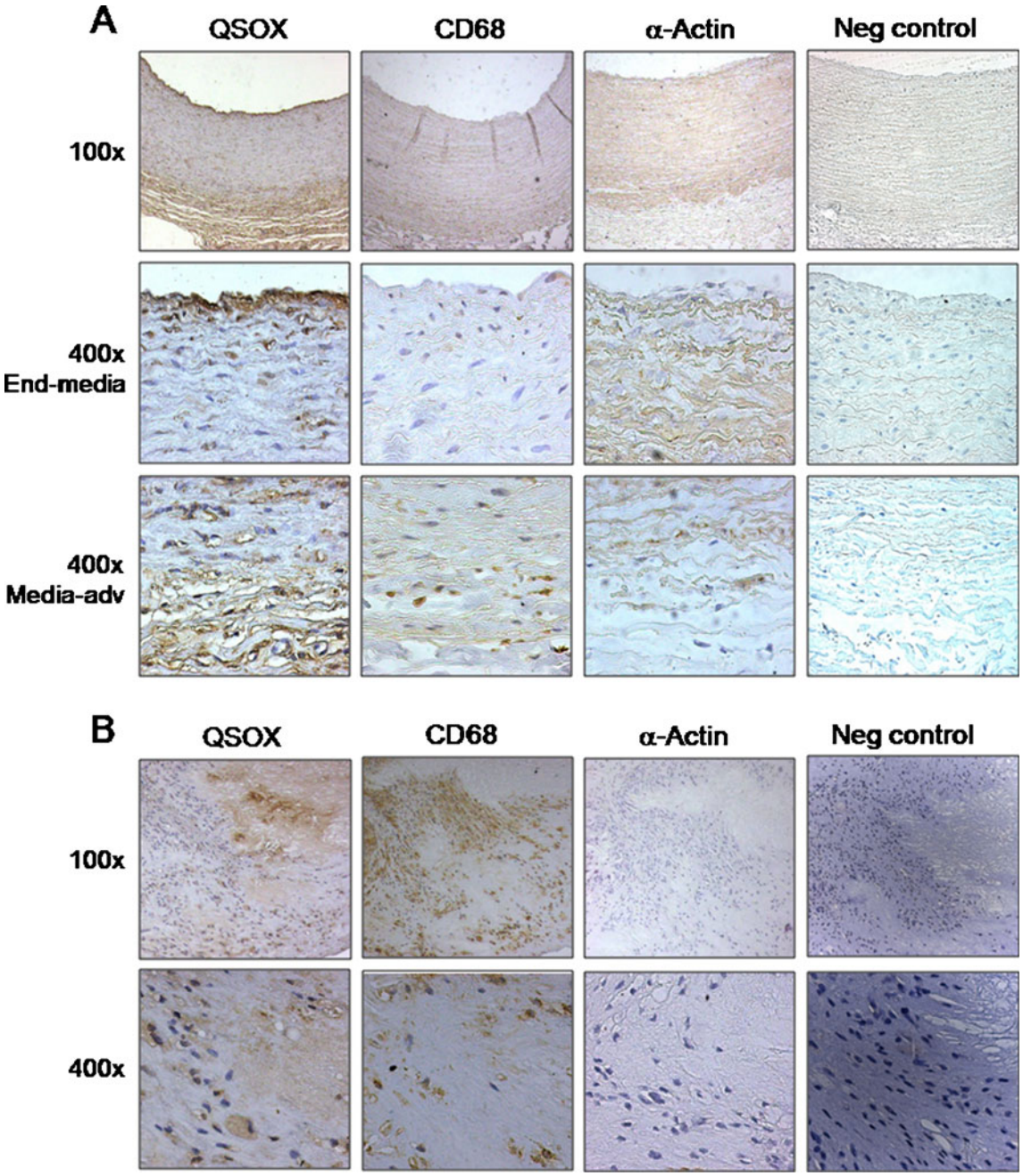

Figure 1. *A*, Immunohistochemistry staining showing QSOX, CD68, and α-actin labeling in a normal carotid artery. *Slides* represent contiguous sections of each segment labeled using specific primary antibodies (anti-QSOX, CD68, or α-actin) and LSAB secondary antibody system. Negative control was submitted to all labeling procedures, excluding the primary antibody from the first incubation. Magnification (shown on the *left side*): ×100 and ×400. *B*, Immunohistochemistry staining showing QSOX, CD68, and α-actin labeling in the core of an atherosclerotic plaque from carotid artery. *Slides* represent contiguous sections of each segment labeled using specific primary antibodies (anti-QSOX, CD68, or α-actin) and LSAB secondary antibody system. Negative control was submitted to all labeling procedures, excluding the primary antibody from the first incubation. Magnification (shown on the *left side*): ×100 and ×400.

mented transcription of QSOX1 might be a consequence of apoptosis, as similarly observed after redox stress-induced apoptosis (Morel et al. 2007).

To verify whether alterations on QSOX mRNA were reflected in translational levels, and thus in protein expression, we analyzed cellular and secreted QSOX1 protein in response to the above interventions. Because we had prior evidence of a strong QSOX1 expression in microparticles (Zanata et al. 2005), we analyzed QSOX1 protein expression in secreted microparticles from cultured HUVECs, THP-1, and HeLa cells. Specific QSOX1 identification in total supernatants was not

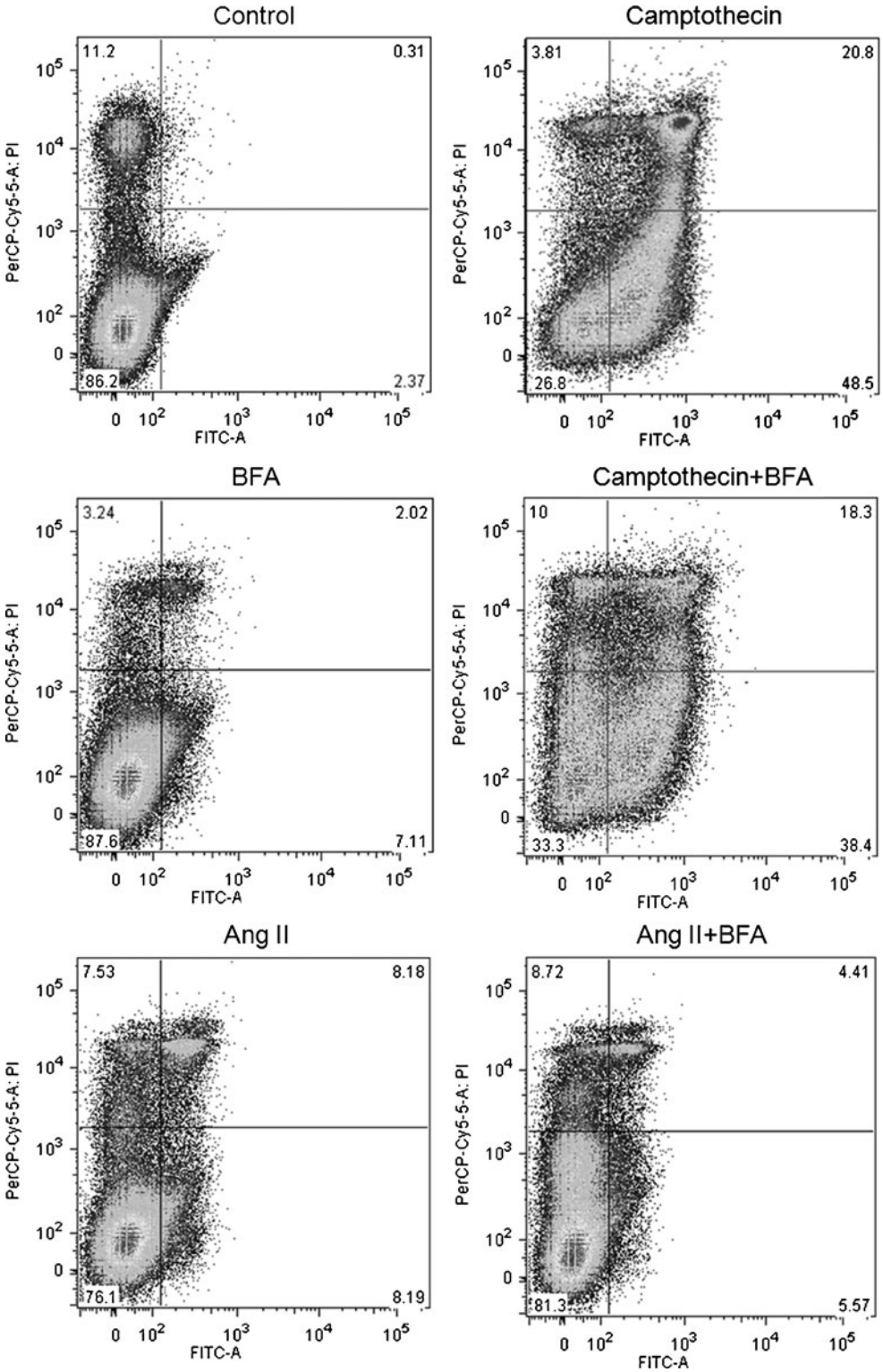

Figure 2. Quantification of apoptosis rates in HUVEC cells treated with camptothecin, BFA, and AngII. HUVEC cells were treated with camptothecin (5 μM), BFA (5 μg/ml), Ang II (100 μM), or both for 48 h. Cells were stained with Annexin V and PI and subjected to FACS analysis to determine the relative percentages of apoptotic and necrotic cells. *Graphs* are representative FACS scatter dot plots of HUVEC cells treated with the stimuli.

possible due to complete superimposition between bands of QSOX1 and serum albumin. In addition, we analyzed QSOX1 protein expression in whole cell lysates.

In contrast to mRNA levels, QSOX1 protein expression was unaffected by camptothecin in HUVEC whole cell lysates, but enhanced after BFA, BFA plus camptothecin, Ang II, and Ang II plus BFA treatments (Fig. 6). Micro-

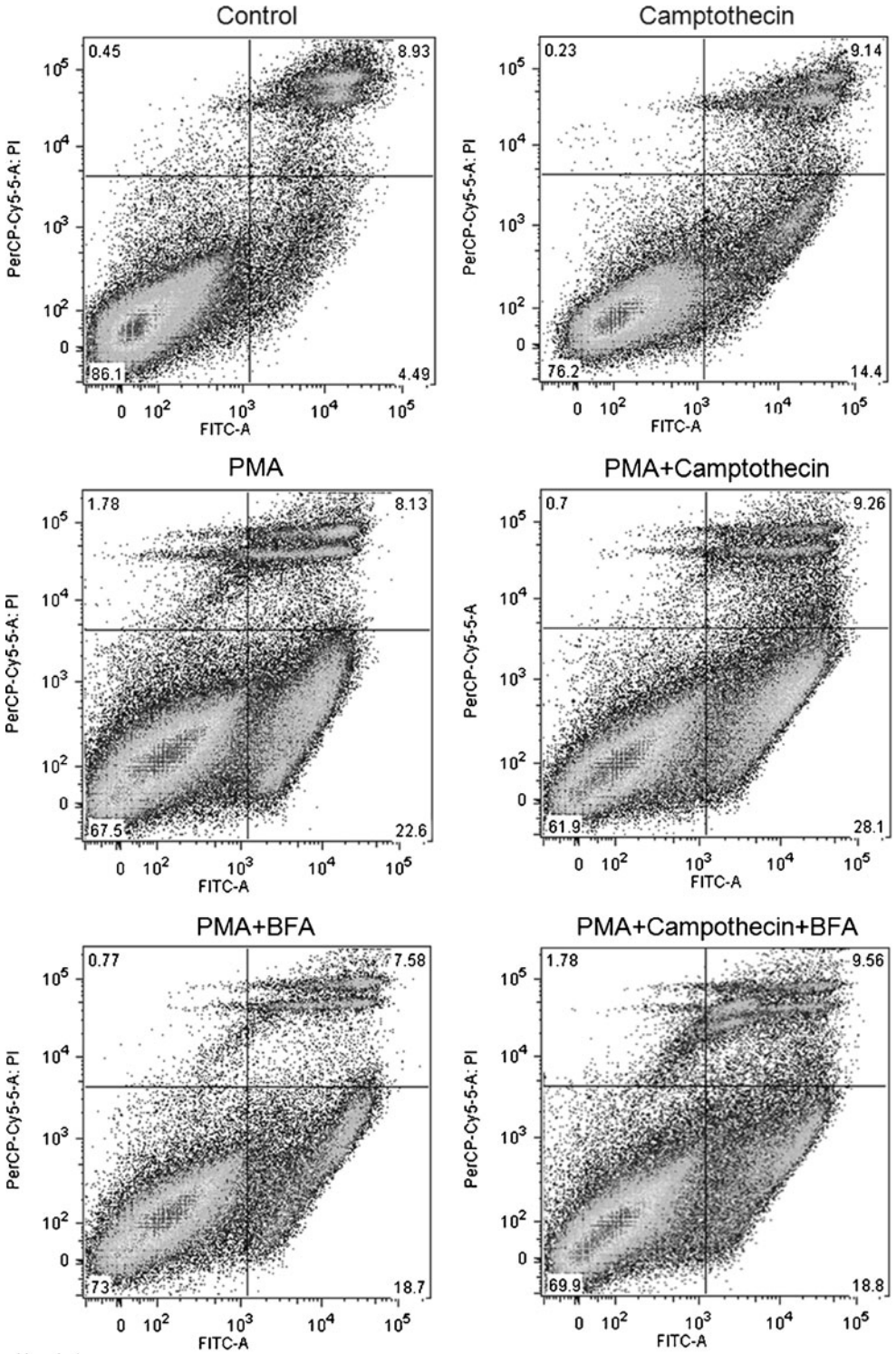

Figure 3. Quantification of apoptosis rates in undifferentiated THP-1 cells treated with PMA, camptothecin, BFA, and AngII. THP-1 cells were treated with PMA (40 nM), camptothecin (5 μM), BFA (5 μg/ml), Ang II (100 μM), or both for 48 h. Cells were stained with Annexin V and PI and subjected to FACS analysis to determine the relative percentages of apoptotic and necrotic cells. *Graphs* are representative FACS scatter dot plots of THP1 cells treated with the stimuli.

particles from HUVECs contained QSOX1 after incubation with camptothecin plus BFA, Ang II, and Ang II plus BFA (Fig. 6). Similar to HUVECs, THP-1 (monocytes) cell lysates did not show alterations in QSOX1 abundance after camptothecin treatment. In contrast, differentiation from monocytes to macrophages (PMA), as well as the treatment of these macrophages with camptothecin, enhanced QSOX1 expression (Fig. 7), which parallels mRNA data.

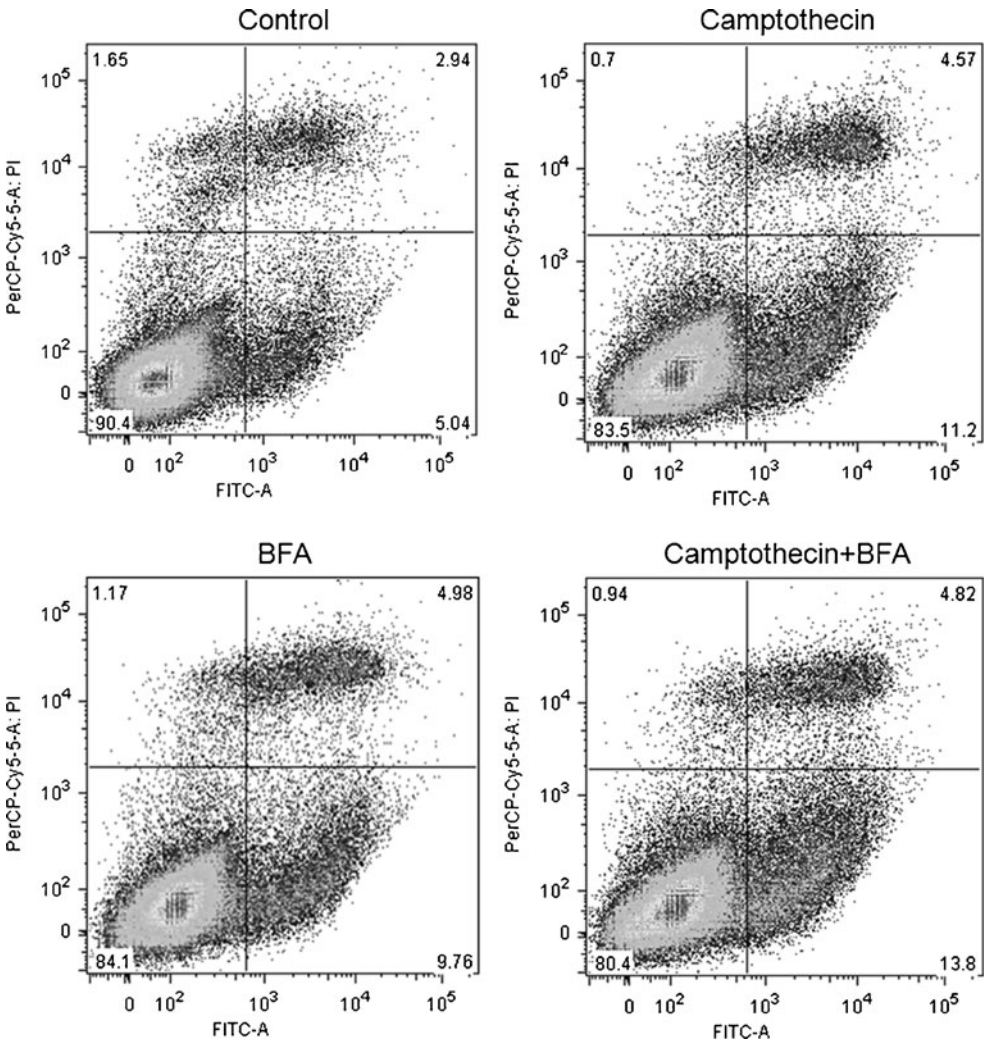

Figure 4. Quantification of apoptosis rates in HeLa cells treated with camptothecin and BFA. HeLa cells were treated with camptothecin (5 μM), BFA (5 μg/ml), or both for 48 h. Cells were stained with Annexin V and PI and subjected to FACS analysis to determine the relative percentages of apoptotic and necrotic cells. *Graphs* are representative FACS scatter dot plots of HeLa cells treated with the stimuli.

Unexpectedly, microparticles derived from THP-1 cells showed a decrease in QSOX1 expression in the presence of camptothecin and no changes with other stimuli (Fig. 7). HeLa cell lysates, contrary to the other cells analyzed, showed a mild increase in QSOX1 protein after camptothecin treatment. On the other hand, no other stimulus affected QSOX1 abundance in the lysates of these cells and no treatment modulated QSOX1 release into microparticles (Fig. 8).

Discussion

The link between apoptosis and QSOX1 was first pointed out by studies in rat pheochromocytoma cells in which QSOX1 expression, induced by oxidant exposure, was protective against apoptosis (Morel et al. 2007). Although the precise mechanisms by which QSOX1 or QSOX2 affects apoptosis have not yet been defined, it is known that QSOX1 protects from apoptosis by maintaining the polarization of mitochondria and allowing the cells to continue ATP synthesis (Ang and Lu 2009). In this direction, it seems relevant to analyze how QSOX1 influences the assembly or the biological activity of permeability transition pore and protein transport in the process of apoptosis, considering that analogous sulfhydryl oxidases from the Erv family importantly regulate mitochondrial permeability (Morel et al. 2007; Ang and Lu 2009). In addition, QSOX1 may potentially influence cell stress response by regulating redox protein folding in specific intracellular compartments (Jordan and Gibbins 2006; Ang and Lu 2009), as well as in the extracellular milieu (Wang et al. 2007; Alon et al. 2010). Our results are the first to address the possible association of QSOX1 expression and apoptosis in the context of atherosclerosis. Immunolabeling of QSOX in normal carotids and atherosclerotic plaques showed that QSOX is expressed by endothelial cells and macrophages and that it is probably secreted by these cells to the subendothelium and adventitia. In the atheroma, QSOX1 present in the lipid core may

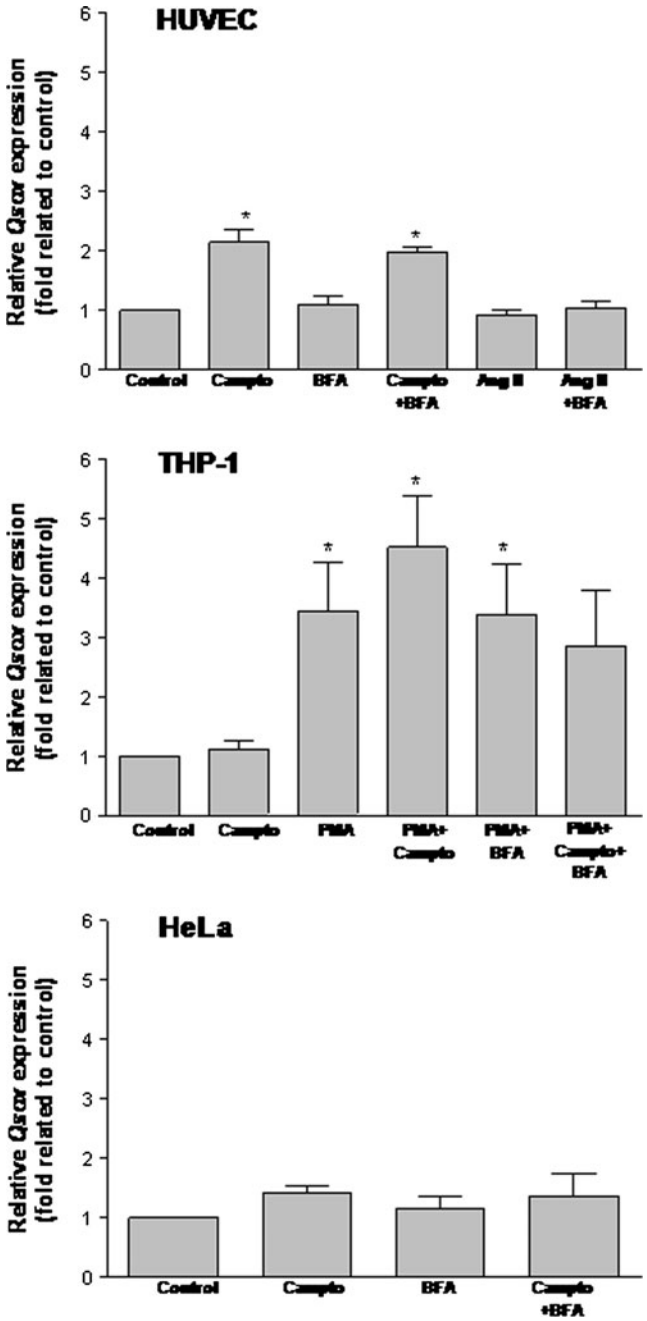

Figure 5. Relative expression of QSOX1 in different cell types evaluated by real-time RT-PCR. Values were normalized by GAPDH expression and are presented as ratios between treated relative to untreated cells. Values are the mean ± SEM of n=3–6 experiments. *p<0.05 vs control in ANOVA followed by Bonferroni's multiple comparison test.

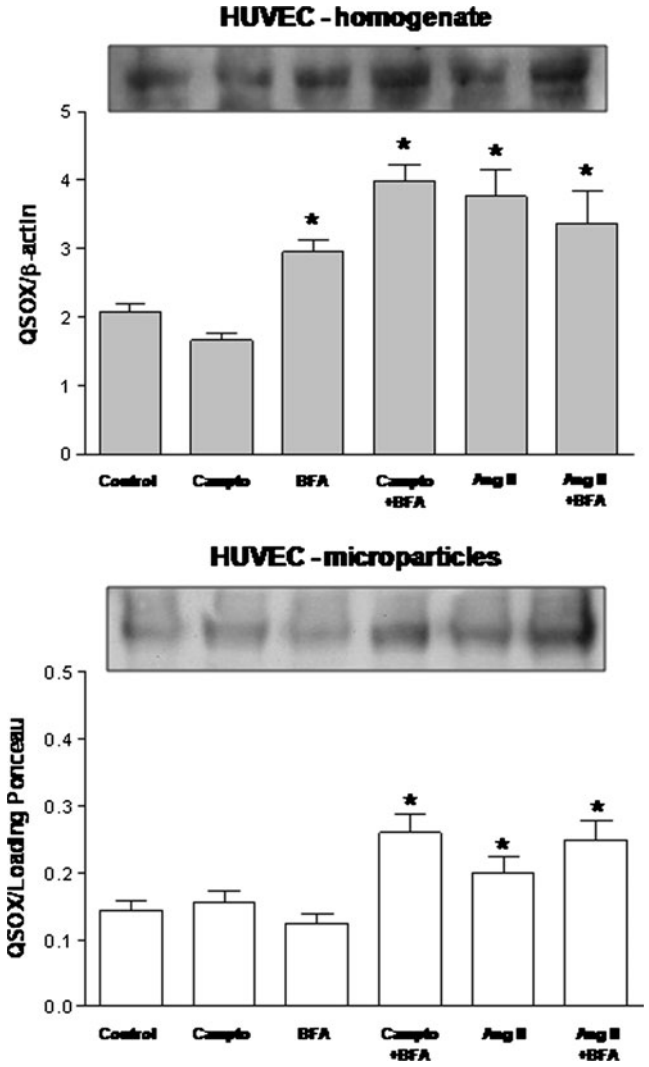

Figure 6. Western blotting depicting QSOX1 protein levels in HUVEC cell homogenates and microparticles after different stimuli for 48 h. The *figure* shows a representative Western blot membrane, and the *histogram* the densitometric data from three independent experiments, normalized by β-actin (for homogenate) or loading Ponceau (for microparticles). *p<0.05 vs. control in ANOVA followed by Bonferroni's multiple comparison test.

have either been secreted by resident macrophages and smooth muscle cells or been leaked as a consequence of their irreversible damage.

The abundant expression and secretion of QSOX1 by tissues which requires immunological tolerance has been reported (Tury et al. 2006), suggesting a possible role in vascular pathologies. Two *Qsox1* mRNA splice variants have been described in human and rodents: *Qsox1 variant a* (*Qsox-L*), encoding the full-length protein containing 747 amino acids (74 kDa), and *Qsox1 variant b* (*Qsox1-S*), which encodes a truncated protein of 604 amino acids (65 kDa; Antwi et al. 2009). Both *QSOX1* variants have important functions on protein folding, redox homeostasis, and cell cycle control (Morel et al. 2007; Portes et al. 2008). The *QSOX2* gene, mapped in chromosome 9, encodes a 78-kDa protein and was only described in human neuroblastoma cells under apoptosis (Wittke et al. 2003).

To address whether QSOX1 expression may be regulated by stimuli relevant to the atherosclerotic disease process, we assessed QSOX1 mRNA and protein expression in

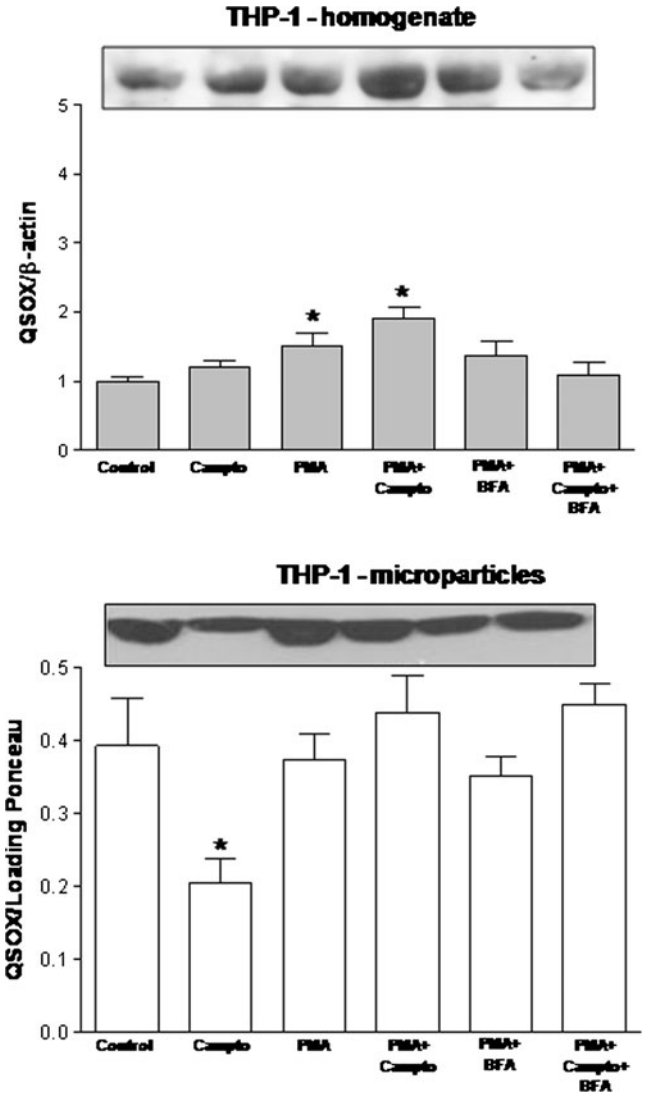

Figure 7 Western blotting depicting QSOX1 protein levels in THP-1 cell homogenates and microparticles after different stimuli for 48 h. The *figure* shows a representative Western blot membrane and the *histogram* the densitometric data from three independent experiments normalized by β-actin (for homogenate) or loading Ponceau (for microparticles). *$p<0.05$ vs. control in ANOVA followed by Bonferroni's multiple comparison test.

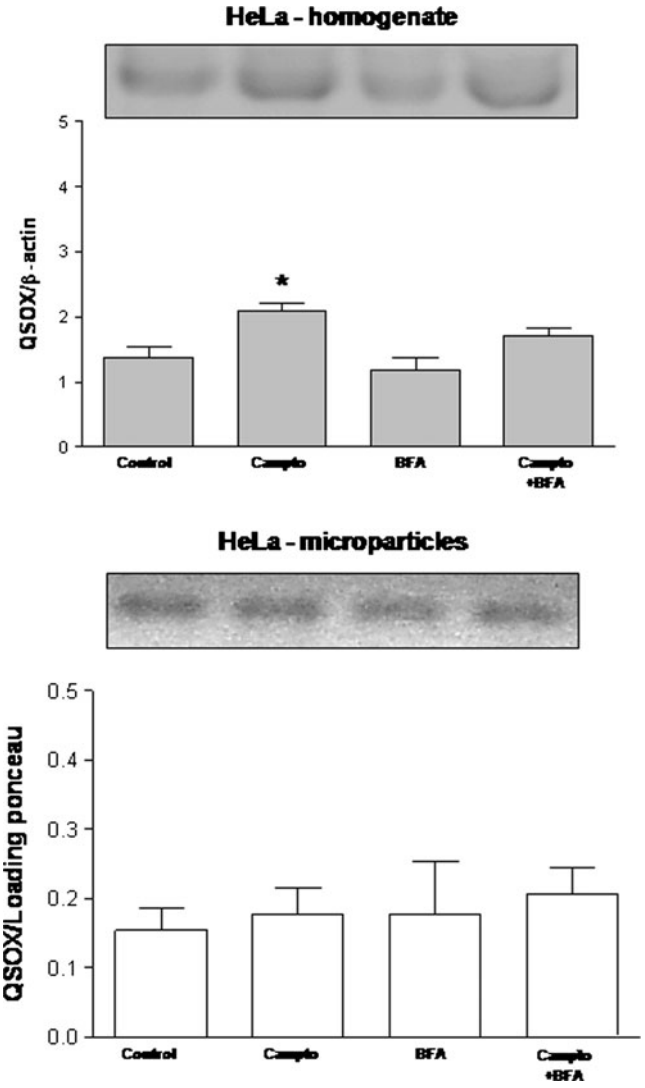

Figure 8 Western blotting depicting QSOX1 protein levels in HeLa cell homogenates and microparticles after different stimuli for 48 h. The *figure* shows a representative Western blot membrane and the *histogram* the densitometric data from three independent experiments normalized by β-actin (for homogenate) or loading Ponceau (for microparticles). *$p<0.05$ vs. control in ANOVA followed by Bonferroni's multiple comparison test.

HUVEC, THP-1, and HeLa cells exposed to camptothecin (apoptotic stimulus), BFA (Golgi apparatus inhibitor), and activators such as PMA and Ang II. Camptothecin is a commonly used topoisomerase I inhibitor which induces DNA damage and, consequently, apoptosis (Hertzberg et al. 1989). We confirmed the induction of apoptosis in our experimental conditions by Annexin V labeling and by PARP analysis. Apoptosis was induced not only by camptothecin but also to variable extents by stimuli inducing differentiation, such as PMA in THP-1 cells, and activation, such as Ang II in HUVECs. Exposure to PMA during the differentiation of monocytes into macrophages (THP-1 vs. THP-1+PMA) led to a particularly high degree of apoptosis. QSOX1 mRNA increased in HUVEC and differentiated THP-1 cells after camptothecin, suggesting that apoptosis enhances QSOX1 transcription or inhibits its degradation in these cells. However, at the protein level, a direct agreement between apoptosis and QSOX expression in cell extracts or microparticles was not always evident. Considering cell lysates, concordance was observed for THP1 and HeLa cells, in which PMA and camptothecin, respectively, induced higher apoptosis and protein expression. In HUVECs, however, camptothecin did not lead to an increase in QSOX1 protein in lysates, contrary to mRNA

and apoptosis. The differences between the levels of QSOX protein and mRNA may be due to several factors, particularly a rapid secretion of QSOX1 in supernatant and MPs and the absence of kinetics of QSOX protein in lysates, so that the data may not reflect the peak of QSOX after treatment.

Microparticles are usually released in circulation during inflammatory processes by leukocytes, vascular cells, and endothelial and smooth muscle cells under activation or apoptosis (Wang et al. 2007; Distler et al. 2011). They are found in blood in normal physiologic conditions and are increased in a variety of diseases, including atherosclerosis (Porto et al. 2011; Rautou et al. 2011). Since our focus was to study cells that participate in atherosclerosis under apoptotic and activating stimuli, we analyzed whether QSOX1 was secreted into microparticles under these circumstances. We also employed BFA, an inhibitor of protein secretion that disrupts the Golgi apparatus, causing its redistribution into the endoplasmic reticulum, as a tool to investigate whether such a forced intracellular retention would affect QSOX1 expression and secretion into microparticles. Our results show that microparticles, particularly from THP1 cells, do carry high amounts of QSOX1. Surprisingly, in THP-1 cells, camptothecin led to a decrease in QSOX in microparticles, contrary to the increase observed in QSOX mRNA and in apoptosis. Interestingly, in HUVECS, Ang II promoted an increased QSOX1 secretion into microparticles and in cell lysates. Camptothecin+BFA also promoted an increase in QSOX1 levels both in cells and microparticles, indicating that interruption of the secretory pathway does not prevent QSOX migration to microparticles. Although the pattern of QSOX1 expression and secretion into microparticles was not predictive of any specific function in our cells, these changes do not appear to be nonspecific since in HeLa cells, which are not relevant to the pathogenesis of atherosclerosis, changes in QSOX1 expression and secretion into microparticles were much less evident.

We observed better correlation between apoptosis and QSOX1 mRNA than with QSOX1 protein. Real-time primers are specific for QSOX1, while the QSOX antibody probably recognizes the two isoforms. Despite this observation, since we only detected bands of 65 kDa in Western blots, we can conclude that the isoform detected in our study was the QSOX1. The lack of a clear correlation between QSOX1 protein expression and apoptosis rates may be due to various reasons. One of them is that our estimation of total QSOX1 protein is not accurate enough because it is a sum of cell extract, supernatants, and microparticles, and unfortunately, we could not access QSOX1 abundance in the supernatant. Another explanation is that QSOX1 translation and/or degradation may be regulated by several different factors, as expected by the complex array of QSOX functions. For instance, an activating stimulus such as Ang II leads also (and perhaps predominantly) to proliferation (Boulanger et al. 2006) rather than apoptosis. In addition, the temporal pattern of QSOX1 expression may be complex and QSOX1 expression during apoptosis may be a transient process. Another important point is that the time we chose for the analysis of cells after stimuli may correspond to different moments concerning RNA and protein synthesis and stability, which could also explain the divergences observed between the abundances of the two molecules.

Taken together, our results indicate a link between QSOX1 and atherosclerosis, with this oxidase being induced in endothelial cells and macrophages by apoptosis and differentiation/activation. It is possible that each cell type responds in a particular way to these stimuli, with quantitative differences in QSOX1 induction due to sensitivity to local factors (as shown for THP-1 and THP-1 differentiated macrophages). Other cell types present in the plaque but not included in the present study, such as vascular smooth muscle cells, may also contribute by producing and secreting QSOX1. Although our study did not include functional analysis, our data indicate that QSOX is expressed in the atheroma core and raise the perspective for its possible functional pathophysiological roles. Considering previous roles of QSOX in the reduction of apoptosis and angiogenesis, we could speculate that intra-plaque expression of QSOX would ameliorate disease progression.

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