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Oxidative stress impairs endocytosis of the scavenger receptor class A

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Abstract

We report the characterization of a cell system employing Chinese hamster ovary (CHO) cells and CHO cells transfected with the scavenger receptor class A (CHO-SRA) using extracellularly produced reactive oxygen species (ROS) in order to study the endocytic function of the scavenger receptor. The oxidative environment was produced using *tert*-butyl hydroperoxide (TBH) and characterized by flow cytometry and cell viability. Once an adequate oxidative environment was established, binding and internalization studies of radiolabeled acetylated LDL particles (^{125}I-labeled Ac-LDL) with CHO-SRA cells were carried out. RT-PCR analysis using total RNAs from CHO-SRA cells revealed that oxidative stress does not alter the expression of the scavenger receptor. However, internalization of ^{125}I-labeled Ac-LDL through this receptor carried out by these cells was completely abolished under extracellularly oxidative conditions. Together, these results support the idea that an oxidative stress produced extracellularly, inhibiting the endocytosis of the scavenger receptor, could help to understand and explain the mechanisms by which several physiologically important ligands are accumulated in the extracellular space with its consequent cell damage.

Keywords: Scavenger receptor class A; Free radicals; Oxidative stress; Lipoprotein internalization; Endocytosis; Acetylated low-density lipoproteins

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries [1]. Early lesions of atherosclerosis consist of subendothelial accumulations of cholesterol-engorged macrophages called foamy cells [2,3]. It has been suggested that accumulation of oxidatively modified low-density lipoproteins (LDL) in the arterial intima contributes significantly to the recruitment of monocytes toward the arterial wall and the formation of foamy cells [4]. Reactive oxygen species (ROS) produced by vascular cells, monocyte-derived macrophages, and enzymes, such as myeloperoxidase, sphingomyelinase, and secretory phospholipase have been proposed to mediate the oxidation of these lipoproteins [5–9]. These oxidatively modified LDLs are recognized and internalized by scavenger receptors present in macrophages [10]. These scavenger receptors belong to a large family of receptors composed of six classes, all related through their capacity to internalize chemically modified LDLs [11]. Scavenger receptor class A (SR-A) types I and II identified in macrophages [12,13] have been considered to play an important role in the genesis of atherosclerosis [14]. SR-A receptor types I and II are membrane-integral trimeric glycoproteins produced by editing and alternative splicing of a single gene [13,15]. SR-A type I additionally presents a C-terminal cysteine domain with no known function to date [16]. Both receptors are formed by six domains, including an α helical coiled-coil domain [13,17] and a collagenous-like domain that contains a lysine-rich cluster forming a region with positive charges essential for modified LDL binding [15,18]. Both receptors show very similar ligand-binding properties and have been reported to bind acetylated LDL (Ac-LDL), oxidized LDL (Ox-LDL), maleinated serum albumin, polysaccharides, such as dextran sulfate and fucoidin, polyribonucleotides (poly(I) and poly(G)), and phospholipids [13,15,17–19].

The expression of SR-A is largely restricted to macrophages, including alveolar, thymic, and splenic macrophages as well as Kupffer cells, but also is expressed in endothelial cells and smooth muscle cells present in atherosclerotic lesions [20–24]. Additionally, SR-A

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expression has been reported on microglial cells in the central nervous system (CNS) and, therefore related to the development of several neuronal diseases [25], such as neurodegeneration in Alzheimer's disease [26]. The expression of the SR-A has been localized in active microglia surrounding amyloid plaques [27], and its function has been associated to the endocytosis of the β-amyloid (βA) peptide [27], as well as the adhesion of microglia to amyloid fibrils with the consequent production of neurotoxins, such as ROS and reactive nitrogen species (RNS) [28].

Originally, ROS were recognized for their role in mammalian host defense during the respiratory burst of phagocytic cells [29,30]. At present, they are recognized as signaling molecules important in many biological systems under physiologic as well as pathologic conditions. There is important evidence related to the participation of ROS in the progress of various degenerative conditions, such as cardiovascular [31] and neurological [32,33] diseases. ROS can be generated intracellularly through enzymatic systems, such as NADPH oxidases, nitric oxide synthase (NOS), and lipoxygenase as a response to growth factor stimulation or by means of normal mitochondrial metabolism [34]. On the other hand, extracellular ROS production can be catalyzed by ionizing radiation, ultraviolet light (UV), environmental toxins, and cytokines produced during the inflammatory response [34]. During the activation of macrophages and microglial cells, the release of growth factors, cytokines, and cytotoxic mediators such as ROS, intracellularly as well as extracellularly, has been observed and associated with the modification of LDL particles [35]. It has also been demonstrated that interaction of these oxidized lipoproteins with the SR-A expressed on the surface of these cell type promotes activation of transcription factors that promote a further intracellular production of ROS [36,37]. Nonetheless, to date little is known concerning the role that these extracellularly produced ROS play on SR-A activity. Therefore, in this study, we investigated the effect of ROS produced exogenously on the endocytic function of the SR-A. The data presented support the notion that extracellularly produced ROS are able to inhibit the internalization of ligands through the SR-A without altering their expression.

Materials and methods

Materials. Benzamidine, *tert*-butyl hydroperoxide (TBH), collagen IV, poly-D-lysine, fucoidin, and trypan blue dye were purchased from Sigma Chemicals (St. Louis, MO). Rat anti-mouse SR-A was obtained from Serotec (Kidlington, England). Rabbit anti-human collagen CIV was purchased from Chemicon International (Temecula, CA). The ECL Kit was purchased from Pharmacia (Uppsala, Sweden). All reagents for cell culture were purchased from Life Technologies (Gaithersburg, MD), while tissue culture dishes and other plasticware were obtained from Nalgen Nunc (Rochester, NY). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR). ^{125}I-labeled sodium iodide was purchased from Amersham (Piscataway, NJ), and the protein assay and Iodogen Kits were obtained from Pierce (Rockford, IL).

Lipoproteins. Human LDL ($d = 1.019$–1.063 g/ml) were isolated from plasma of normal fasted donors by sequential density ultracentrifugation at 4 °C in a Beckman Ti-60 rotor [38]. All lipoproteins were dialyzed against 0.15 M NaCl and 0.01% EDTA at pH 7.2 as previously reported [38]. The LDL concentration is given in terms of its protein content determined by the BCA method following instructions issued by the manufacturer, using bovine serum albumin as a standard. Ac-LDL was prepared by LDL treatment with acetic anhydride [39,40]. The Iodogen method was used to iodinate Ac-LDL and LDL to reach a specific activity of 50–150 counts/min/ng protein.

Flow cytometry assays. The different cell lines employed were cultured in serum-free medium for 5 h, washed, and resuspended in a phosphate-buffered saline solution (PBS), pH 7.4. One milliliter cell aliquots were treated without or with DCFH-DA, dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 400 μM, stored in the dark at −20 °C, and used in a 1:100 dilution with PBS. Cells were preincubated for 15 min with 4 μM DCFH-DA in the absence or presence of different concentrations of TBH prior to the cytometric analysis. Fluorescence was measured on a FACScalibur instrument (Becton–Dickinson). The FL1 fluorescence distribution was displayed as a single histogram [41]. Cell viability was determined using the trypan blue dye exclusion method.

RT-PCR. Control Chinese hamster ovary (CHO) cells lacking the presence of the SR-A were cultivated in Dulbecco's modified Eagle's medium (DMEM)/F12 (50/50, v/v) containing penicillin (50 U/ml)/streptomycin (50 μg/ml) and supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Transfected CHO cells expressing the SR-A (CHO-SRA) (kindly provided by Dr. Robert E. Pitas, Gladstone Institute of Cardiovascular Disease, University of California, San Francisco) were grown in the same medium, but containing Geneticin (400 μg/ml) [42]. RNA was isolated from CHO and CHO-SRA cells that were cultured under control (absence of TBH) and oxidative stress (presence of TBH) conditions for 5 h, as mentioned above. Cells were washed and resuspended in 1 ml Trizol (Life Technologies) according to the manufacturer's instructions. Scavenger receptor from CHO and CHO-SRA cells was determined by PCR after reverse transcription of 2–3 μg of total RT-RNA using the GeneAmp RNA Kit (Applied Biosystems). cDNAs were derived using 2.5 μM random hexanucleotides and oligo(dT)$_{16}$, and 2.5 U of MuLV of reverse transcriptase at 42 °C for 45 min followed by denaturalization at 99 °C for 5 min. cDNAs were amplified by PCR using specific primer pairs (0.2 μM) and 2.5 U of AmpliTaq DNA polymerase in 100 μl during 25 cycles; denaturalization at 99 °C for 2 min, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. The primers for SR-A were selected from the cytoplasmic region (50 amino acids). The sense primer was 5′-ATGGCAC AGTGGGATGACTTT-3′ and the antisense primer 5′-TTTATAAGA CTTCATCCTCTC-3′ (PCR product, 150 bp). For the β-actin gene, the sense primer was 5′-GGGTCAGAAGGATTCC-TATG-3′ and the antisense primer 5′-GGTCTCAAACATGATCTG GG-3′ (PCR product, 233 bp).

Internalization assays and cell toxicity. Lipoprotein internalization assays were performed for 5 h at 37 °C and 5% CO_2 in serum-free medium [42]. Lipoprotein binding assays were performed at 4 °C following the same procedure employed for internalization assays. Nonlabeled Ac-LDL, fucoidin, or LDL were used to determine nonspecific internalization. Cells were grown for 2–3 days prior to the experiment, controlling density up to 10,000 cells/cm^2 for CHO and CHO-SRA cells. Oxidative stress assays were performed by adding 30 μM TBH each hour into the incubation medium during the internalization and binding assays. For competition experiments, cells were washed with serum-free medium and incubated for 5 h at 37 °C in serum-free medium containing either ^{125}I-labeled Ac-LDL, ^{125}I-labeled LDL, or the indicated concentrations of competitors.

Immunoblots. After 5 h had elapsed for each internalization assay under control or oxidative stress conditions, cytosolic extracts from CHO and CHO-SRA cells were obtained for protein analysis, as previously described [43]. Protein samples of extracts were analyzed by SDS–PAGE on 10% gels and transferred to nitrocellulose paper. Membranes were blocked for 1 h at room temperature with a solution containing 5% fat-free milk and 0.05% Tween 20 in Tris-buffered saline solution (TBS), pH 7.6. Incubation with the anti-scavenger receptor antibody and the anti-collagen antibody was carried out in the same solution, but by using 0.1% fat-free milk and incubating the membrane for 1 h at room temperature. Immunoblots were visualized with the chemiluminiscent reagent ECL from Amersham according to the supplier's instructions.

Results and discussion

Production of free radicals and oxidative stress conditions

Several sources have been described to contribute to a free radical production and an oxidative environment during the natural course of several degenerative processes. One consequence of the establishment of this oxidative environment is the oxidation of ligands highly susceptible to being recognized by the scavenger receptor, such as Ox-LDL. Although the SR-A has been involved in the pathogenesis of several degenerative diseases, no one has examined the influence of free radicals upon the function of the scavenger receptor itself. For this propose, first, we established the experimental conditions needed to maintain an oxidative environment during the culture of the two cell lines employed in this study. Therefore, TBH, a lipophilic alkylhydroperoxide, was added in sublethal doses to the culture medium of CHO and CHO-SRA cells. We then examined the oxidative response of each cell line studied in response to different concentrations of TBH using FACS analysis. After addition of TBH (30–500 μM) to the culture medium of CHO-SRA cells, the fluorescence emitted by conversion of DCFH-DA to the fluorescent compound DCF was stimulated by the presence of free radicals released into the medium, whereas control CHO cells showed a lower level of fluorescence intensity (Fig. 1). Basal fluorescence was obtained with cells stimulated in the presence of DCFH-DA (4 μM) for 15 min. After stimulation with TBH at concentrations varying from 30, 50, 100 to 500 μM, fluorescence emitted by CHO-SRA cells increased ~5-, 13-, 14-, and 17-fold, respectively (Fig. 1). This response was directly related to the production of ROS by the cells. Table 1 shows the relative fluorescence intensity values of CHO and CHO-SRA cell lines, showing that fluorescence increases proportionally with increasing amounts of TBH. In our hands, free radical production by addition of TBH to the culture media provided an adequate method in order to maintain optimal conditions for a controlled oxidative cell environment.

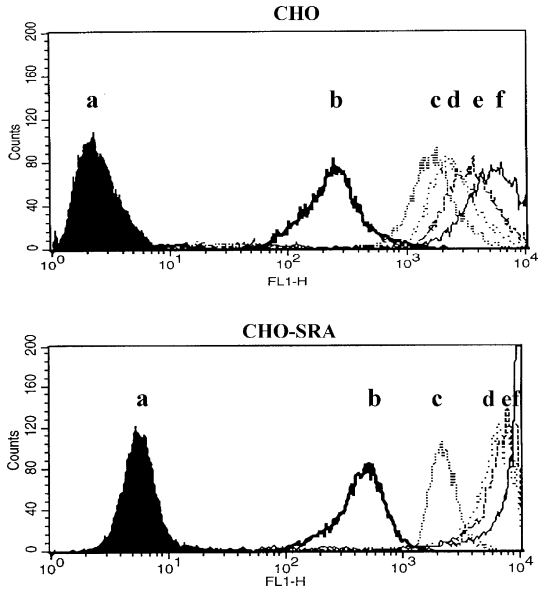

Fig. 1. Flow cytometry measurements of control and stimulated CHO and CHO-SRA cells by TBH. The histogram shows the DCF fluorescent shift emitted by the CHO and CHO-SRA cell lines without or with treatment using different concentrations of TBH. CHO and CHO-SRA monolayers were washed, scraped, and resuspended in PBS buffer (pH 7.4). The suspension was divided in aliquots to determinate oxidative conditions. (a) Intrinsic cell fluorescence without the presence of TBH or DCFH-DA. (b) Basal fluorescence of unstimulated cells emitted by the addition of 4 μM DCFH-DA (15 min). (c) Fluorescence emitted by CHO and CHO-SRA cells stimulated with (c) 30, (d) 50, (e) 100, and (f) 500 μM TBH.

Table 1
Relative fluorescence intensity values obtained after stimulation with TBH

	Cell lines	
	CHO	CHO-SRA
No dye and unstimulated	3	6
DCFH-DA (basal)	268	512
DCFH-DA + 30 μM TBH	1716	2458
DCFH-DA + 50 μM TBH	2738	6464
DCFH-DA + 100 μM TBH	3868	7033
DCFH-DA + 500 μM TBH	5601	8720

Cell viability during the internalization assays

Once we established the oxidative response of each cell line studied using TBH as an adequate source for ROS production, we examined cell viability at the different cell incubation times used during the internalization assays. First, we examined cell viability by adding TBH at zero time and following the experiment for 5 h. Since under these conditions we found that cells recovered with time, it is most probable that their intrinsic antioxidant capabilities allowed them to eliminate ROS along the experiment (data not shown). Therefore, we tested TBH (30, 50, and 75 μM) added every hour

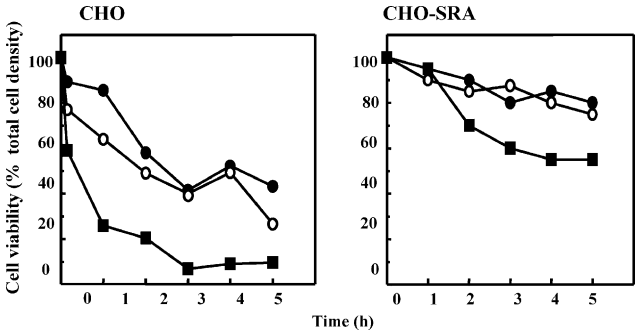

Fig. 2. Time course of cell viability after treatment with different concentrations of TBH. CHO and CHO-SRA cells were incubated at 37 °C with 30 μM (●), 50 μM (○), and 70 μM (■) TBH added each hour for 5 h. After this time, cells were recovered and viability was measured by the trypan blue dye exclusion method. Cell viability is expressed as a percentage of the total number of cells found at zero time.

throughout the time employed for the internalization assays. A set of experiments with the cell lines employed in the form of cell monolayers was performed in order to monitor the process of cell viability by trypan blue dye exclusion during the experiments when TBH was added every hour to the culture medium. As shown in Fig. 2, cell viability was maintained up to 50% in the CHO cell line throughout the 5-h internalization assay, depending on the amount of TBH added to the incubation medium. In contrast, when the CHO-SRA cell line was tested, cell viability improved up to 80% using the same amounts of TBH in the incubation medium. These results indicated the higher susceptibility to an oxidative stress as shown by the control CHO cells in comparison

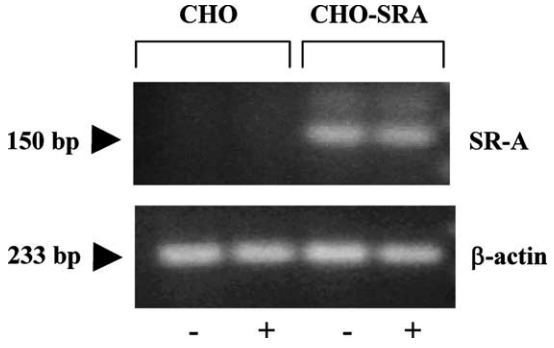

Fig. 3. Effect of oxidative stress upon the expression of RNA-SRA and RNA-β-actin. RNA was isolated from CHO and CHO-SRA cells under control (−) and oxidative conditions (+) employing 30 μM TBH added each hour for 5 h of assay. After reverse transcription of total RNA (2.3 μM) with random primers, equal amounts of cDNA template were amplified by PCR using specific oligonucleotides as shown under Materials and methods. The upper panel shows a 150-bp band corresponding to the cytoplasmic region of the scavenger receptor class A under both control and oxidative conditions in CHO-SRA cells. The lower panel shows a 230-bp band corresponding to the β-actin gene used as a control protein as well as a loading control in CHO and CHO-SRA cells under both control (−) and oxidative conditions (+).

to the results obtained with the CHO-SRA cell line. Transfection of the SR-A seems to have given this cell line the ability to better manage oxidative stress.

The mRNA levels for the CHO-SRA cell line studied after the treatment with TBH (30 μM) were similar to those of control cells without treatment (Fig. 3). Moreover, in order to test whether synthesis of other proteins might be affected with the same TBH treatment, we tested β-actin, showing no differences when either CHO or CHO-SRA cells were cultured with or without TBH (Fig. 3). These results indicate that experimental conditions employed in these series of experiments maintained an oxidative environment without altering gene expression of SR-A and β-actin as an example of other protein type. From these sets of experiments, we decided to carry out our internalization and binding experiments using the lowest concentration of TBH tested (30 μM) in order to maintain an adequate ratio between oxidative stress and cell viability.

Exogenous free radicals affect the internalization process of Ac-LDL

In order to investigate the contribution of free radicals upon scavenger receptor function, we evaluated the internalization of ^{125}I-labeled Ac-LDL in CHO-SRA cells and CHO cells under control and oxidative conditions. This last condition was maintained by adding TBH (30 μM) every hour for 5 h in order to maintain a constant oxidative stress throughout the internalization assay. As shown in Fig. 4, internalization of ^{125}I-labeled Ac-LDL by CHO-SRA cells was completely abolished in the presence of TBH, whereas CHO control cells, which do not express the SR-A, did not internalize acetylated LDL particles. These results demonstrate that internalization of modified LDL through the SR-A present in CHO-SRA cells is importantly affected by the presence of an oxidative environment. In contrast, control experiments carried out by studying the internalization of ^{125}I-labeled LDL through the LDL receptor in both cell lines showed that this lipoprotein receptor was not as dramatically affected by oxidative stress as the effect observed upon the scavenger receptor (Fig. 4).

Effect of oxidative stress upon ^{125}I-labeled Ac-LDL binding

Binding assays under control conditions as well as under oxidative stress conditions showed no significant changes in ^{125}I-labeled Ac-LDL binding, indicating that stress produced under our experimental conditions did not significantly affect the ligand binding capacity of the scavenger receptor (Fig. 5).

In order to study the possibility that oxidative stress might have affected the radioactively labeled acetylated

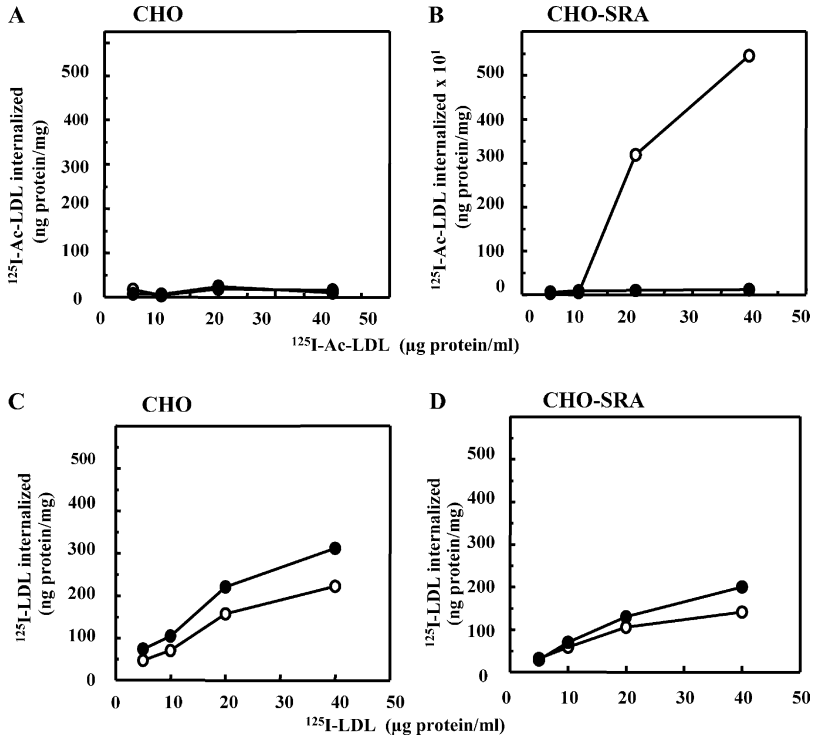

Fig. 4. Internalization of ^{125}I-labeled Ac-LDL and of ^{125}I-labeled LDL under control or oxidative conditions. CHO (A,C) and CHO-SRA (B,D) cells were incubated at 37 °C with ^{125}I-labeled Ac-LDL or ^{125}I-labeled LDL under control conditions without TBH (○) or under oxidative stress conditions adding 30 μM TBH to the culture media each hour for 5 h (●). Specific lipoprotein internalization was determined as the difference between total internalization and internalization in the presence of a molar excess of unlabeled ligand. Each point represents the mean ± SD of two experiments carried out in triplicate. Experimental error bars are contained within data points.

lipoproteins directly, making them incapable of being recognized by the scavenger receptor, we incubated 40 μg ^{125}I-labeled Ac-LDL/ml in a serum-free medium for 5 h at 37 °C in the absence or the presence of TBH (30 μM) added each hour in order to maintain ^{125}I-labeled Ac-LDL in a continuous state of oxidative stress. After this procedure, the medium with lipoproteins was removed and added to previously cultured cell monolayers. The cells were left to interact with TBH-unexposed and TBH-exposed lipoproteins for 5 h at 37 °C without additional use of hydroperoxide. The results obtained showed no significant internalization changes in the values obtained with TBH-exposed ^{125}I-labeled Ac-LDL compared with the unexposed ^{125}I-labeled Ac-LDL (data not shown).

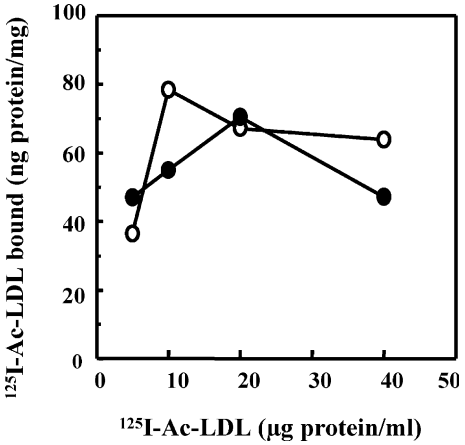

Fig. 5. Binding of ^{125}I-labeled Ac-LDL to SR-A under control and oxidative stress conditions. CHO-SRA cells were incubated at 4 °C with ^{125}I-labeled Ac-LDL under control conditions without TBH (○) or oxidative stress conditions adding 30 μM TBH, each hour for 5 h (●). Specific binding is determined as the difference between total binding and binding in the presence of a molar excess of unlabeled ligand. Binding of ^{125}I-labeled Ac-LDL to CHO cells was negligible (data not shown). Each point represents the mean of two experiments carried out in triplicate. Experimental error bars are contained within data points.

Effect of an oxidative environment upon the expression of the SR-A

It has been previously described that oxidative stress caused by PMA or reactive oxygen species up-regulates the expression of the SR-A gene as well as protein expression and activation of redox-sensitive transcription factors in human smooth muscle cell (SMC) and THP-1 cells [43,44]. This phenomenon is apparently caused by oxidized LDL and not by growth factors or cytokines secreted by macrophages. Under the present experimental conditions, we also studied whether the extracellular oxidative stress produced by TBH affected the expression of the SR-A. Since scavenger receptors class

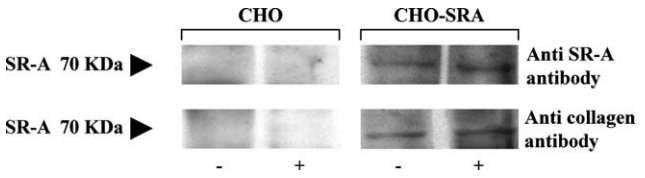

Fig. 6. Effect of TBH upon the expression of the scavenger receptor in CHO-SRA cells. Cellular extracts were obtained after incubation of CHO and CHO-SRA cells in the absence (−) or the presence (+) of TBH (30 μM) added every hour during an internalization assay carried out at 37 °C. The homogenates were fractionated according to Mietus-Snyder et al. [51]. Cytosolic extracts were isolated and 30 μg of protein was used for SDS–PAGE (10%) and transferred to nylon membranes overnight. The upper panel shows the signal detected using an anti-scavenger receptor class A antibody and the lower panel shows the signal detected using an anti-collagen antibody.

A present a collagenous-like domain recognized as the binding site for several polyanionic ligands and the support for its distinctive broad specificity [15], we compared receptor protein levels using an anti-SR-A antibody in parallel with an anti-collagen antibody using both control and oxidative stress conditions. As shown in Fig. 6, SR-A expression in the CHO-SRA cell line was shown to be similar under both control and oxidative conditions, suggesting that exogenously produced free radicals apparently only modify the internalization function of the SR-A, leaving untouched the mechanisms involved in receptor synthesis and receptor inclusion into the membrane. Several studies have shown that different cytokines and growth factors inhibit or enhance the activity and expression of SR-A in macrophages where changes in receptor activity have been associated with the establishment of an intracellular oxidative stress condition [44–50]. Previous studies have demonstrated that an intracellular oxidative stress caused by phorbol esters with the combination of H_2O_2 and vanadate up-regulates SR-A in SMC [51]. This same effect occurred when SMC were incubated in the presence of oxidized LDL, suggesting that Ox-LDL might be a source of intracellular oxidative stress [51]. In contrast, our study demonstrates that extracellularly produced reactive oxygen species exclusively alter the endocytic capacity of the scavenger receptor class A.

Interestingly, ROS did not damage the ligand binding capacity, nor did they alter the ligands directly exposed to the oxidant effect of TBH. Since we did not observe important changes in LDL internalization through the LDL receptor when normal as well as oxidative stress conditions were studied, therefore, we can infer that the structural differences present in the LDL receptor in comparison to the SR-A most probably play an important role in the susceptibility to oxidative stress showed by the two receptors.

In comparison to previous reports showing that intracellular stress contributes to an increase in mRNA expression and protein levels in different cell systems [44,51], we did not find changes in receptor levels under the extracellular oxidative stress conditions used in this study. It seems that ROS produced by exogenous agents do not have the same effect upon the receptor as intracellularly produced ROS, therefore, supporting the notion that different microdomains might present a different susceptibility to oxidative stress. Mazhul et al. [52] reported that oxidative stress induced by TBH increases the intramolecular dynamics of membrane proteins, a phenomenon that has been associated to cell death.

Changes in Ac-LDL internalization by the SR-A under extracellularly created oxidative stress conditions could help to explain how it is that several ligands, such as the β-amyloid peptide cannot be internalized and eliminated through internalization of the scavenger receptor when an oxidative environment is well established, therefore stimulating their extracellular accumulation [53]. Since the changes caused upon the scavenger receptor seem not to be related to ligand binding, it is possible to propose that changes in the organization of the receptor embed the membrane [54], which in turn might alter its association to the cytoskeleton, could be related to the alteration of the normal mechanisms for receptor internalization. These changes caused by ROS might affect the way the extracellular signal is coupled to the cytoplasm through protein–protein recognition events carried out between the intracellular segment of the receptor and the cytoskeleton. Currently in our laboratory, we are studying these mechanisms as a feasible explanation for this phenomenon.

Acknowledgments

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