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Oxidized lipoprotein induces the macrophage ascorbate transporter (SVCT2): protection by intracellular ascorbate against oxidant stress and apoptosis

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Abstract

To assess whether ascorbic acid decreases the cytotoxicity of oxidized human low density lipoprotein (oxLDL) in cells involved in atherosclerosis, its interaction with oxLDL was studied in murine RAW264.7 macrophages. Macrophages took up ascorbate to millimolar intracellular concentrations and retained it with little loss over 18 h in culture. Culture of the macrophages with oxLDL enhanced ascorbate uptake. This was associated with increased expression of the ascorbate transporter (SVCT2), which was prevented by ascorbate and by inhibiting the NF-κB pathway. Culture of RAW264.7 macrophages with oxLDL increased intracellular dihydrofluorescein oxidation and lipid peroxidation, both of which were decreased by intracellular ascorbate. Ascorbate also protected the cells against oxLDL-induced cytotoxicity and apoptosis, but it did not affect macrophage accumulation of lipid from oxLDL or oxLDL-induced increases in macrophage cytokine secretion. These results suggest that ascorbate protects macrophages against oxLDL-induced oxidant stress and subsequent apoptotic death without impairing their function.

Keywords

Ascorbate transport; oxidant stress; RAW264.7 macrophages; oxidized LDL; SVCT2; dihydrofluorescein; apoptosis; malondialdehyde; atherosclerosis; NF-κB

The macrophage is a crucial cell type in initiating the atherosclerotic process, where it has several roles in perpetuating the inflammatory nature of this disease. Macrophages take up oxidized low density lipoprotein (oxLDL)[1] to become a lipid-laden foam cells, secrete cytokines and other activators that enhance the inflammatory nature of this disease, and generate reactive oxygen species (ROS) that may damage both the macrophage and surrounding cells and tissue [1,2]. Since macrophages are relatively long-lived cells compared to neutrophils [3], they must have mechanisms for protection against the excess reactive oxygen species that they generate.

Macrophage antioxidant defenses are similar to those of other cells and include several antioxidant enzymes [4–7] and low molecular weight antioxidants such as GSH [4,5,8,9], vitamin E [5,8], and ascorbic acid [8–10]. GSH is generally considered the major low

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[1]*Abbreviations used*:
AI, 6-amino-4-(4-phenoxyphenylethylamino)quinazoline; DHA, dehydroascorbic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid); KRH, Krebs-Ringer Hepes; LDL, low density lipoprotein; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; oxLDL, oxidized low density lipoprotein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; ROS, reactive oxygen species, SVCT2: Sodium-Dependent Vitamin C Transporter, type 2

molecular weight antioxidant in most cells, based on intracellular concentrations in the low millimolar range and on the high capacity for its recycling via glutathione reductase. However, ascorbate may also be important in macrophages for several reasons. First, circulating human monocytes, the precursors of macrophages, have intracellular ascorbate concentrations 2–3-fold higher than found in circulating neutrophils [10,11], or about 3 mM [12]. Second, freshly prepared peritoneal macrophages have ascorbate contents in the low millimolar range [10,13] that are comparable to GSH concentrations measured in other studies [4,14]. In a direct comparison, we found that thioglycollate-induced peritoneal macrophages from mice retained endogenous ascorbate to concentrations of 3 mM, which were slightly higher than GSH concentrations of 2 mM [8]. Moreover, the cells could further take up ascorbate to reach intracellular concentrations of 6–8 mM [8]. Finally, when challenged with lipopolysaccharide [9] or during phagocytosis of latex beads [8], macrophage ascorbate decreased, whereas GSH did not change. The latter results show that ascorbate is consumed during oxidant stress in these cells, which supports a role for the vitamin in macrophage antioxidant defense.

The major oxidant stress encountered by macrophages that have been attracted to the sub-endothelial space is generated by oxLDL [15]. Intracellular ascorbate has been shown to decrease both macrophage uptake of oxLDL [16–18] and the oxidant stress induced in macrophages by oxLDL [19,20], although the latter has not always been observed [21]. Protection against oxidant stress by ascorbate might be expected to decrease cytotoxicity and macrophage death. However, macrophage death due to oxLDL relates to apoptosis rather than necrosis [19,20,22,23] and ascorbate has been shown either to have no effect on macrophage apoptosis [19], or to modestly enhance it [20].

To assess whether ascorbate might protect macrophages from oxLDL-induced cytotoxicity, and to determine whether oxLDL might in turn affect ascorbate homeostasis, we studied the interaction of an oxidized preparation of human LDL and ascorbate in the murine macrophage cell line RAW264.7. We had previously shown that these cells avidly take up ascorbate from the culture medium on the SVCT2 (the major transporter for ascorbate in non-epithelial cells), that they carry out GSH-dependent recycling of dehydroascorbic acid (the 2-electron oxidized form of ascorbate), and that treatment with lipopolysaccharide enhances GSH-dependent recycling of dehydroascorbate (DHA) to ascorbate. In the present work we used a novel preparation of human oxLDL to assess whether intracellular ascorbate protects macrophages against an oxidant stress or affects macrophage function and survival.

Materials and methods

Materials

Sigma/Aldrich Chemical Co. (St. Louis, MO) supplied the reagent chemicals including ascorbic acid, DHA, *N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid (Hepes), and Trizol reagent. Molecular Probes, Inc. (Eugene, OR) provided dihydrofluorescein diacetate, which was initially dissolved in dimethylsulfoxide, such that the final dimethylsulfoxide concentration in the incubation was never above 0.2% (v/v). The NF-κB Activation Inhibitor (AI, 6-amino-4-(4-phenoxyphenylethylamino)quinazoline) was obtained from EMD Chemicals Inc. (Darmstadt, Germany).

RAW264.7 macrophage preparation and culture [24]

RAW264.7 cells, which were established from a tumor induced by Abelson murine leukemia virus, were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in Dulbecco's minimal essential medium that contained 10% (v/v) fetal bovine serum, penicillin (100U/ml), and streptomycin (100μg/ml). The culture medium was

prepared by the Cell Culture Core of the Vanderbilt Diabetes Research and Training Center. Cells were cultured in a water-saturated atmosphere of 5% CO_2 and 95% air at 37°C, and the medium was replaced every 2–3 days until confluence, at which time they were either sub-cultured onto new plates or used for experiments. Just before an experiment, cells were rinsed three times in 2 ml of Krebs-Ringer Hepes buffer at 37 °C. The latter consisted of 20 mM Hepes, 128 mM NaCl, 5.2 mM KCl, 1 mM NaH_2PO_4, 1.4 mM $MgSO_4$, and 1.4 mM $CaCl_2$, pH 7.4.

Acquisition and preparation of oxLDL

Lipoproteins that were highly enriched in LDL were obtained from a subject with familial hypercholesterolemia who was undergoing biweekly plasma apheresis to remove LDL. The subject gave informed consent and the protocol was approved by the Vanderbilt University Institutional Review Board. Apolipoprotein B-containing lipoproteins were eluted from the dextran sulfate-cellulose adsorbent column (Liposorber, Kaneka Co., Osaka, Japan). Purity of the LDL preparation was assessed by electrophoresis using the Titan Gel Electrophoresis System, as described by the manufacturer (Helena Laboratories, Beaumont, TX). LDL preparations were either stored in the dark at 3 °C or oxidized in the dark at 23 °C for 24 h in the presence of 5 μM $CuSO_4$. The reaction was terminated with the addition of ethylenediaminetetraacetic acid (EDTA) to 5 mM, and the oxidized lipoproteins (termed oxLDL) were dialyzed against three 100-volumne changes of phosphate-buffered saline (PBS, 140 mM NaCl and 12.5 mM sodium phosphate, pH 8.0) before storage up to 1 week at 3 °C in the dark at a protein concentration of 1.8–2.2 mg/ml.

Assays of cell viability and mitochondrial reduction capacity

The ability of RAW264.7 cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was determined as described by Mossmann [25]. Cells plated at 5×10^4 per well were cultured in 96-well plates and treated with ascorbate or oxLDL for 18 h. The culture medium was replaced with 0.05% (w/v) MTT in fresh medium. After 1 h of incubation at 37 °C, the medium was aspirated, and 200 μl of dimethylsulfoxide was added to each well to solubilize the formazan crystals in the cells. After shaking for 10 minutes, absorbance was measured on a plate reader at 540 nm.

Cell viability was assessed as exclusion of trypan blue. Cells were plated at 2×10^5 cells per ml in 24-well plates and cultured 18 h with oxLDL or ascorbate as noted. The culture medium was replaced with 200 μl of 0.4% (w/v) trypan blue in KRH and allowed to stand for 5 min at 23 °C (room temperature). Trypan blue-stained non-viable cells as well as viable cells were counted under a microscope.

Assays of apoptosis

Annexin V-conjugate assay: RAW264.7 cells plated at 5×10^3 per well were cultured in 384-well plates and treated with oxidized oxLDL and ascorbate as noted. After 16 h of treatment, 20 μl of a 1:3000-fold dilution of the annexin V conjugate (Alexa Fluor 647, A23204, Molecular Probes, Inc., Eugene, OR) was added to each well, and the cells were incubated at 37 °C for 2 h. The fluorescence of the plated cells exclusive of the medium was then analyzed using the macroconfocal FMAT 8100 system (Applied Biosystems Inc, Foster City, CA, USA) as described [24].

Assay of caspase 3/7 activity: This was measured in cells treated as described for the annexin V-conjugate assay by assessing the cleavage of N-acetyl-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin) using a kit according to the manufacturer's instructions (Apo-ONE® Homogeneous Caspase-3/7 Assay Kit, Promega, Inc., Madison, WI USA). Fluorescence was measured on a SpectraMax M5 microtiter plate reader (Molecular

Devices, Sunnyvale, CA) with SoftMax Pro software. The excitation wavelength was 485 nm, and the emission wavelength was 520 nm.

Assay of intracellular ascorbate and GSH

Following cell culture and incubation in 6-well plates, the medium was aspirated, and the cells were gently rinsed twice with 2 ml of ice-cold KRH. For assay of ascorbate and GSH, KRH was removed and the cell monolayer was treated with 0.1 ml of 25% metaphosphoric acid (w/v), the cells were scraped from the plate, and the lysate was diluted with 0.35 ml of a buffer containing 0.1M Na_2HPO_4 and 0.05 mM EDTA, pH 8.0. The lysate was centrifuged at 3 °C for 1 min at 13,000 × g. Duplicate aliquots of the supernatant were taken for assay of ascorbic acid as previously described [26] by high performance liquid chromatography with electrochemical detection, using tetrapentylammonium bromide as the ion pair reagent. GSH was assayed in duplicate by the method of Hissin and Hilf [27]. Intracellular concentrations of ascorbate were calculated based on the measured intracellular 3-*O*-methylglucose space in RAW 264.7 cells. The latter was determined to be 2.38 ± 0.63 μl/mg protein (N=12, ± SD), determined as described for endothelial cells in culture [28].

Polyacrylamide gel electrophoresis and immunoblotting of ascorbate transporters

Cells cultured in 6-well plates were rinsed in pH 7.4 PBS and then solubilized in a lysis buffer termed RIPA that consisted of 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholic acid, 0.1 % (w/v) sodium dodecyl sulfate, 150 mM NaCl, 50 mM TrisHCl, 2mM EDTA, 1mM sodium orthovanadate and a complete Mini Protease Inhibitor Cocktail (Roche, Mannheim, Germany). The lysate was mixed, stored on ice for 10 min and centrifuged at 4 °C for 10 min at 13,000 ×*g*. The supernatant was solubilized with NuPAGE sample buffer (Invitrogen, Carlsbad, CA), loaded at 40 μg of protein per lane, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the method of Laemmli [29]. Electrophoresis and transfer to Immobilon-P transfer membrane (Millipore Corporation, Billerica, Massachusetts), was carried out as previously described [30]. The bands were probed with an affinity purified goat polyclonal antibody reactive with the mouse SVCT2 transporter that was used at a 1:200 dilution (SC-9926, Santa Cruz Biotechnologies, Santa Cruz, CA). The secondary antibody was a 1:5000 dilution of horseradish peroxidase-conjugated anti-goat IgG (A8919, Sigma-Aldrich, Inc., St. Louis, MO). Bands were stained using Amersham ECL Western blotting detection reagent (RPN-2106, GE Healthcare, UK Limited, Little Chalfont Buckinghamshire, UK). Locations of the bands were determined using pre-stained molecular weight markers.

Detection of SVCT2 mRNA by polymerase chain reaction (PCR)

Cells were rinsed in PBS containing 10mM sodium phosphate and 0.9% NaCl, pH 7.4. Total RNA was isolated from plated macrophages using the Trizol reagent (Sigma Chemical Co., St. Louis, MO), reverse transcribed (RT) into cDNA, and amplified by PCR using two sets of primers specific for the mouse *SVCT2* (NM_018824: GAGAGCCGGAAAGCACTGG and ACCGCCGTGATCATTCCAAAGAGT, product size: 711), for *IL-1b* (NM_008361: GAGTGTGGATCCCAAGCAAT and CTGCCTAATGTCCCCTTGAA, product size: 478), for *TNF-a* (M11731: TCAGCCTCTTCTCATTCCTG and TGAAGAGAACCTGGGAGTAG, product size : 333), for *IL-6* (NM_031168: CCGGAGAGGAGACTTCACAG and GGAAATTGGGGTAGGAAGGA, product size: 421), and for *β-actin* (GTTTGAGACCTTCAACACCCC and GTGGCCATCTCCTGCTCGAAGTC product size: 318). The PCR product was resolved by 1.2% agarose gel electrophoresis and stained with ethidium bromide.

Measurement of intracellular oxidant stress

RAW264.7 macrophages that had been plated to near confluence in a 48-well plate were rinsed twice in KRH to remove medium, and loaded with 20 μM dihydrofluorescein diacetate for 30 min at 37 °C, rinsed 3 times with 0.2 ml of KRH, and treated with agents as indicated in 0.2 ml of KRH containing 5 mM D-glucose. The plate was loaded into a microtiter plate reader SpectraMax M5 (Molecular Devices, Sunnyvale, CA) with SoftMax Pro software to measure the fluorescence in and above the cells. The excitation wavelength was 480 nm, and the emission wavelength was 520 nm. Fluorescence readings from four separate wells in each experiment were averaged.

Measurement of cell malondialdehyde content

Lipid peroxidation was measured as the appearance of malondialdehyde in cells using HPLC separation of the cellular extract for increased specificity. Cells in 6-well plates that had been rinsed 3 times in KRH to remove medium were triturated with 0.5 ml of 5% (w/v) trichloroacetic acid. Aliquots of the extract (100 μl) and standards were added to 100 μl of 0.02 M 2-thiobarbituric acid and were treated for 35 min at 95 °C followed by 10 min at 4 °C. Malondialdehyde was assayed by HPLC using fluorescence detection, as previously described [31] and normalized to cell protein.

Analysis of Oil Red O staining of macrophages

Following aspiration of the medium from each well of a 6-well plate, the cells were fixed by incubating for 1 h at 23 °C in 10% formalin. The formalin was removed and the cells were rinsed three times over 5–10 minutes with PBS. The Oil Red O dye solution was prepared from a stock solution of 30 mg/ml Oil Red O in isopropanol by mixing 3 parts of the dye solution with 2 parts of deionized water and allowed to stand for 10 min in a fume hood before staining the cells. The fixed cells were treated with the dye solution for 10 min at 23 °C, followed by removal of excess dye by several water mixes. Retained dye was then extracted with 100% isopropanol and the optical density of the extract was measured at 500 nm on a spectrophotometer (Beckman DU 640, Beckman Instruments Inc., Fullerton, CA).

Statistical Analysis

All results were expressed as mean ± standard deviation. Statistical analyses among groups were made using SPSS16.0 software (SPSS Inc. Chicago, Illinois USA) to carry out one-way analysis of variance with post hoc multiple comparisons.

Results

RAW264.7 macrophages that were cultured in medium not supplemented with ascorbate contained very low amounts of the vitamin (80–90 μM), which was probably due to uptake of ascorbate in the fetal calf serum. To establish the extent to which ascorbate is taken up and retained by the cells, RAW264.7 macrophages were cultured for either 6 h or 18 h with increasing concentrations of ascorbate. As shown in Fig. 1, after 6 h of exposure to an initial loading concentration of 0.5 mM ascorbate, the cells achieved ascorbate concentrations as high as 17 mM ascorbate (Fig. 1, circles). Additional culture for 18 h did not appreciably decrease intracellular ascorbate concentrations (Fig. 1, squares), suggesting that ascorbate loss in culture was minimal under these conditions.

In order to obtain adequate amounts of LDL for study and to ensure that the source and conditions of preparation of the LDL were consistent, we used apolipoprotein B-containing particles obtained from a single subject with familial hypercholesterolemia who was undergoing biweekly plasmapheresis to remove excess LDL. As shown in lane 2 of Fig. 2A, the lipoprotein content of this preparation was almost exclusively LDL, in comparison to

normal human plasma (lane 1), which demonstrates chylomicrons at the origin, and more rapidly migrating pre-beta and alpha lipoproteins. Given the relative purity of this preparation, we have termed it “LDL” in this work. Oxidation of this LDL for 18 h with 5 μM $CuCl_2$ to yield oxLDL did not affect the migration of this preparation in the electrophoretic gel (Fig. 2, lane 3), suggesting that this is minimally oxidized LDL.

As shown in Fig. 2B, 18 h of culture of RAW264.7 macrophages with 0.1 mM ascorbate and increasing concentrations of oxLDL resulted in a significant increase in intracellular ascorbate at oxLDL concentrations between 0.05 and 0.2 mg/ml. No effect of non-oxidized LDL was observed over the same concentration range (Fig. 2B, squares). OxLDL had no effect over the same concentration range on intracellular GSH concentrations (results not shown).

To determine the mechanism for the increase in intracellular ascorbate induced by oxLDL, expression of the SVCT2 in RAW264.7 macrophages was assessed. As shown in the representative gel in Fig. 3A, SVCT2 mRNA measured by PCR increased during 18 h of culture of the cells with increasing concentrations of oxLDL and then tended to fall off with higher LDL concentrations. This is shown in Fig. 3B as averages from 3 experiments when the results were normalized to β-actin expression. LDL that had not been oxidized had little or no effect on SVCT2 mRNA, as shown in Fig. 3C.

To confirm that the oxLDL effect extends to SVCT2 protein, cells were treated for 18 h with oxLDL and extracted protein was subjected to electrophoresis and immunoblotting of the SVCT2. As shown in the first lane of Fig. 4A, RAW264.7 macrophages expressed the SVCT2 protein as a band of about 70 kDa. Cells treated with increasing concentrations of ascorbate showed progressive and significant decreases in SVCT2 expression, both in the representative gel (Fig. 4A, Fig. 2nd group of lanes), and in when the results from 3 experiments were normalized to actin (Fig. 4B, 2nd group of bars). In contrast, cells treated with increasing amounts of oxLDL showed increased SVCT2 expression compared to control cells in the 3rd set of lanes and bars in panels A and B of Fig. 4. The increase in SVCT2 expression induced by oxLDL was prevented when the cells were cultured with ascorbate (last 2 lanes and bars in panels A and B of Fig. 4).

To examine the mechanism of how oxLDL might increase SVCT2 message and protein, we used an inhibitor of the NF-κB pathway, AI. At the concentration used (1 μM), this inhibitor was not toxic to the cells over 18 h of culture as judged by cell counting and morphology. As shown in Fig. 5A, AI treatment during the 18 h exposure to oxLDL markedly decreased the effect of LDL to induce SVCT2 expression (compare lanes 2 and 3). Further, treatment of the cells with AI alone decreased SVCT2 expression relative to that in control cells maintained in culture without any additions (compare lanes 1 and 4). These results were also evident when SVCT2 immunoblotting results of several experiments were expressed relative to β-actin staining (Fig. 5B).

To assess whether oxLDL induces an oxidant stress in RAW264.7 macrophages, oxidation of intracellular dihydrofluorescein and lipid peroxidation were assessed. As shown by the second set of bars in Fig. 6, culture of cells for 18 h with oxLDL markedly increased oxidation of dihydrofluorescein to fluorescein compared to cells alone (1st bar in Fig. 6). When cells were cultured with increasing concentrations of ascorbate at either 0.1 or 0.2 mg/ml oxLDL, dihydrofluorescein oxidation was progressively decreased.

Lipid peroxidation was assessed by measuring the cell content of malondialdehyde. As shown in Fig. 7A, there was little malondialdehyde in the cells at baseline, but malondialdehyde was markedly increased by culture of the cells with increasing concentrations of oxLDL. However, as shown by lipid staining using Oil Red O in the first

two bars of panel B of Fig. 7, at least part of this increase could be due to the uptake of oxLDL by the cells, since an 18 h culture with oxLDL modestly but significantly increased staining of intracellular lipid by Oil Red O. Inclusion of increasing amounts of ascorbate was without effect on the amount of oxLDL taken up by the cells over 18 h in culture (Fig. 7B). On the other hand, a different pattern emerged when the effects of ascorbate on the cell content of malondialdehyde was measured. As shown in Fig. 7C, the increase in cellular content of malondialdehyde induced by culture for 18 h with 0.2 mg/ml oxLDL was partially reversed when the cells were cultured with increasing concentrations of ascorbate as well as oxLDL (squares). The effect was more pronounced if the cells were allowed to take up ascorbate for 2 h before addition of LDL (circles). Thus, the results of studies of both ROS generation and lipid peroxidation support a role for ascorbate to decrease oxidant stress in RAW264.7 macrophages treated with oxLDL.

Since oxLDL caused an oxidant stress in RAW264.7 cells, it was important to determine whether this affected cellular function and survival. Cellular mitochondrial function was assessed by following the reduction of MTT. As shown in Fig. 8A, the ability of the cells to reduce MTT was decreased after culture for 18 h with both 0.2 and 0.3 mg/ml oxLDL (first group of bars). Ascorbate had no effect on MTT reduction alone (middle group of bars), but partially reversed that due to culture with 0.2 mg/ml oxLDL. Cell viability as assessed by exclusion of trypan blue showed that culture for 18 h with 0.3 mg oxLDL modestly but significantly increased cell death (Fig. 8B). Ascorbate had no effect on cell viability and prevented death due to 0.3 mg/ml oxLDL. As expected from previous studies in other macrophage types, culture of RAW264.7 macrophages for 18 h with increasing amounts of oxLDL increased apoptosis, which was measured both as annexin-V binding to the surface of the cells (Fig. 8C) and as caspase 3/7 activity (Fig. 8D). Although ascorbate treatment alone had no effect on macrophage apoptosis, ascorbate added initially at 0.25 and 0.5 mM did significantly decrease the extent of apoptosis induced by 0.2 mg/ml oxLDL in both assays.

Macrophages secrete various cytokines when activated, so effects of oxLDL and ascorbate on IL-1, IL-6, and TNF-α were studied by measuring their mRNA by RT-PCR (Fig. 9). In RAW264.7 macrophages cultured for 18 h with the indicated concentrations of oxLDL, generation of both IL-1 (Panel A) and IL-6 (Panel B) were markedly increased, although TNF-α was not affected (Panel C). Ascorbate had no consistent effect on cytokine expression, either alone or when present with oxLDL.

Discussion

The marked ability of RAW264.7 macrophages to take up and retain ascorbate reflects similar increases seen in other types of macrophages [19,20]. The steep concentration gradient for ascorbate that persisted after 18 h of culture of RAW264.7 macrophages after a single treatment with ascorbate is likely due both to function of the high affinity SVCT2 protein to scavenge any ascorbate that is present around the cells and to robust intracellular recycling of the oxidized forms of ascorbate by RAW264.7 macrophages [9]. Indeed, the SVCT2 is the major determinant of intracellular ascorbate concentrations in cells that express it. Support for this statement derives from the observation that human erythrocytes, which also have a high capacity to recycle ascorbate [32], but which lack the SVCT2 [33], have intracellular ascorbate concentrations similar to those in plasma [32,34].

Despite its key role determining intracellular ascorbate levels, little is known about regulation of the SVCT2 at either the pre- or post-translational level. Regarding the latter, the SVCT2 has several putative phosphorylation sites for both protein kinases A and C on its intracellular cytoplasmic tail [35]. The functional relevance of these sites is supported by

results showing that ascorbate transport is decreased by both dibutyryl cyclic AMP [36,37] and by protein kinase C agonists [38,39]. Additionally, a novel mechanism has been proposed wherein a truncated non-functional form of the SVCT2 interacts in a dominant-negative fashion with functional full length transporters, resulting in inhibition of ascorbate uptake [40]. Human platelets have also been shown to regulate the SVCT2 at the translational level [41].

Pre-translational regulation of SVCT2 expression has been documented in response to diverse stimuli in a variety of cultured cell types, including brain microvascular endothelial cells (increased SVCT2 protein expression when placed in primary culture [42]), trophoblasts (increased SVCT2 mRNA due to treatment with fetal bovine serum or epidermal growth factor in culture [43]), osteoblasts (increased SVCT2 mRNA when exposed to calcium/phosphate [44], glucocorticoids [45], and zinc [46]), skeletal muscle cells (decreased by differentiation [47]). Of relevance to the antioxidant effects of ascorbate are additional results by Savini, et al. [48], who found that treatment of C2C12 myotubes with the oxidant H_2O_2 enhanced SVCT2 function and expression. In the present work, we found that oxLDL, but not native LDL, increased intracellular ascorbate and SVCT2 message and protein expression in RAW264.7 macrophages during 18 h in culture. These findings, in a cell type where oxidant stress may well have physiologic relevance, support the conclusion of Savini, et al. [48] that SVCT2 synthesis is subject to redox regulation. Perhaps the strongest evidence that oxidant stress-induced SVCT2 up-regulation is relevant *in vivo* derives from the study of Berger, et al. [49] involving middle cerebral artery ischemia and reperfusion in rats, a procedure known to induce oxidant stress in surviving cells in the area surrounding the infarct. These investigators found by *in situ* hybridization that SVCT2 mRNA markedly increased in both neurons and astrocytes in these areas.

We found that specific inhibition of the NF-κB pathway completely prevented SVCT2 induction by oxLDL. Activation of the NF-κB pathway due to oxidative stress and oxLDL in particular is well documented [50]. The NF-κB pathway may indeed be fundamental to SVCT2 expression, since blockade of the NF-κB cascade significantly decreased basal SVCT2 expression in both C2C12 myotubes [48] and in RAW264.7 macrophages. Additional support for a role of the NF-κB pathway in SVCT2 transcription is the observation that although the two known promoters for the human SVCT2 appear to lack NF-κB recognition sites [51], two such sites have been identified on the mouse SVCT2 gene upstream of the transcription start site [48]. Notwithstanding this caveat for the relevance to human disease, the NF-κB pathway provides a plausible route for oxLDL-induced activation of SVCT2 transcription as a counterpoint to increased cellular oxidant stress, as discussed next.

An oxLDL-induced increase in expression of the high affinity SVCT2 (apparent K_m = 40 μM in RAW264.7 cells [9]), will increase ascorbate uptake of even low extracellular concentrations of ascorbate by the cells, which will help to alleviate intracellular oxidant stress. However, increased intracellular ascorbate will also affect SVCT2 synthesis. Thus, previous studies in osteoblasts [52], astrocytes [53], and lung epithelial cells [54] have shown that SVCT2 function or expression varies inversely with intracellular ascorbate in culture. We found in primary cultures of brain microvascular endothelial cells that whereas low intracellular concentrations of ascorbate up-regulated SVCT2 protein expression, higher concentrations, such as those used in the present study, down-regulated SVCT2 expression [42]. Our current finding that ascorbate down-regulated SVCT2 protein expression both in the basal state and that induced by oxLDL in RAW264.7 macrophages could indicate that self-regulation of its transporter by ascorbate takes precedence over changes in SVCT2 expression induced by oxidant stress.

If increased oxidant stress does enhance SVCT2 expression, then decreased oxidant stress might be expected to decrease it. Savini, et al. [48], have shown, for example, that lipoic acid, which is converted to a potent antioxidant dithiol in cells, inhibited both ascorbate uptake and expression of the SVCT2 in C2C12 myotubes [48].

The effect of ascorbate to blunt oxidant stress and toxicity induced by overnight culture of oxLDL with RAW264.7 macrophages was evident as decreased oxidation of intracellular dihydrofluorescein (reflecting generation of ROS) and as decreased malondialdehyde formation (evidence of ROS damage to cell membrane lipids). These results are in agreement with the previous finding that ascorbate decreased expression of heme oxygenase-1, a marker of intracellular oxidant stress [20]. In previous studies using macrophages, ascorbate did not protect against oxLDL-induced apoptosis [19,20], which was interpreted as protection of the apoptotic cell machinery by ascorbate [20]. In the present work, we found a modest protection against both early and late apoptosis of RAW264.7 macrophages by ascorbate after 18 h in culture. This also correlated with the ability of intracellular ascorbate to enhance oxLDL-induced decreases in mitochondrial function in the MTT assay. Reasons for these discrepant findings regarding apoptosis likely relate to one or more differences in cell culture conditions, such as macrophage type, degree of LDL oxidation (ours was very minimally oxidized), and duration of culture with oxLDL. Resolution of these differences awaits the results of *in vivo* studies of ascorbate effects on macrophage apoptosis.

Whereas ascorbate decreased oxidant stress and cytotoxicity in RAW264.7 macrophages, it did not affect the ability of the cells to take up oxLDL or the marked increases in IL-1 and IL-6 mRNA induced by oxLDL. Insofar as these activities reflect macrophage function, the effects of ascorbate appear to decrease the toxic effects of oxLDL and thus to protect the cells. Indeed, oxLDL up-regulated the SVCT2, which would serve to increase intracellular ascorbate and enhance its protective effects.

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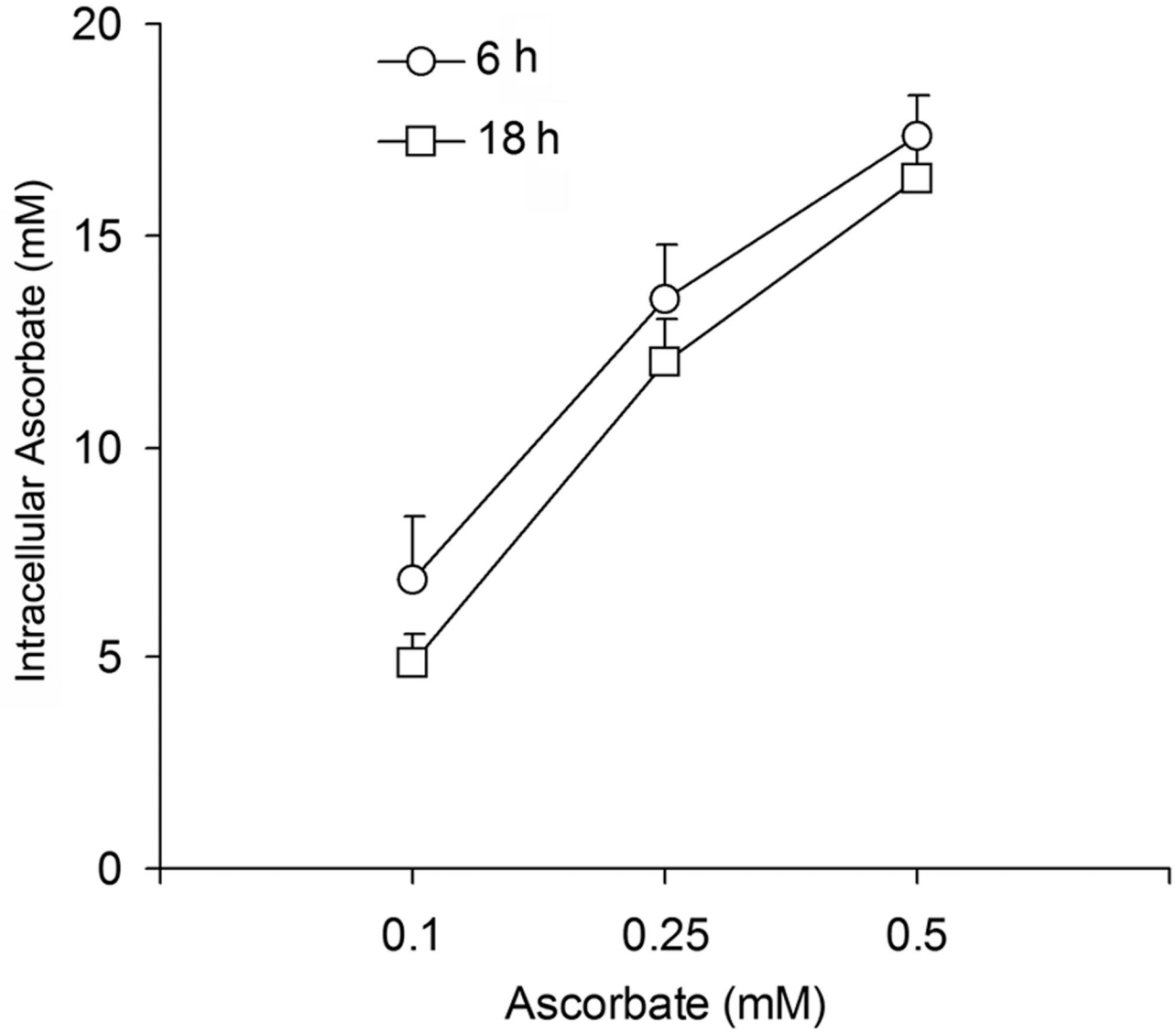

Fig. 1. Uptake of ascorbate by RAW264.7 macrophages

Cells were plated in 6-well plates to a density of 2×10^6 cells per well. Ascorbate was added at the indicated concentrations for 6h (circles) or 18h (squares) followed by assay of intracellular ascorbate. Results were shown from 6 experiments.

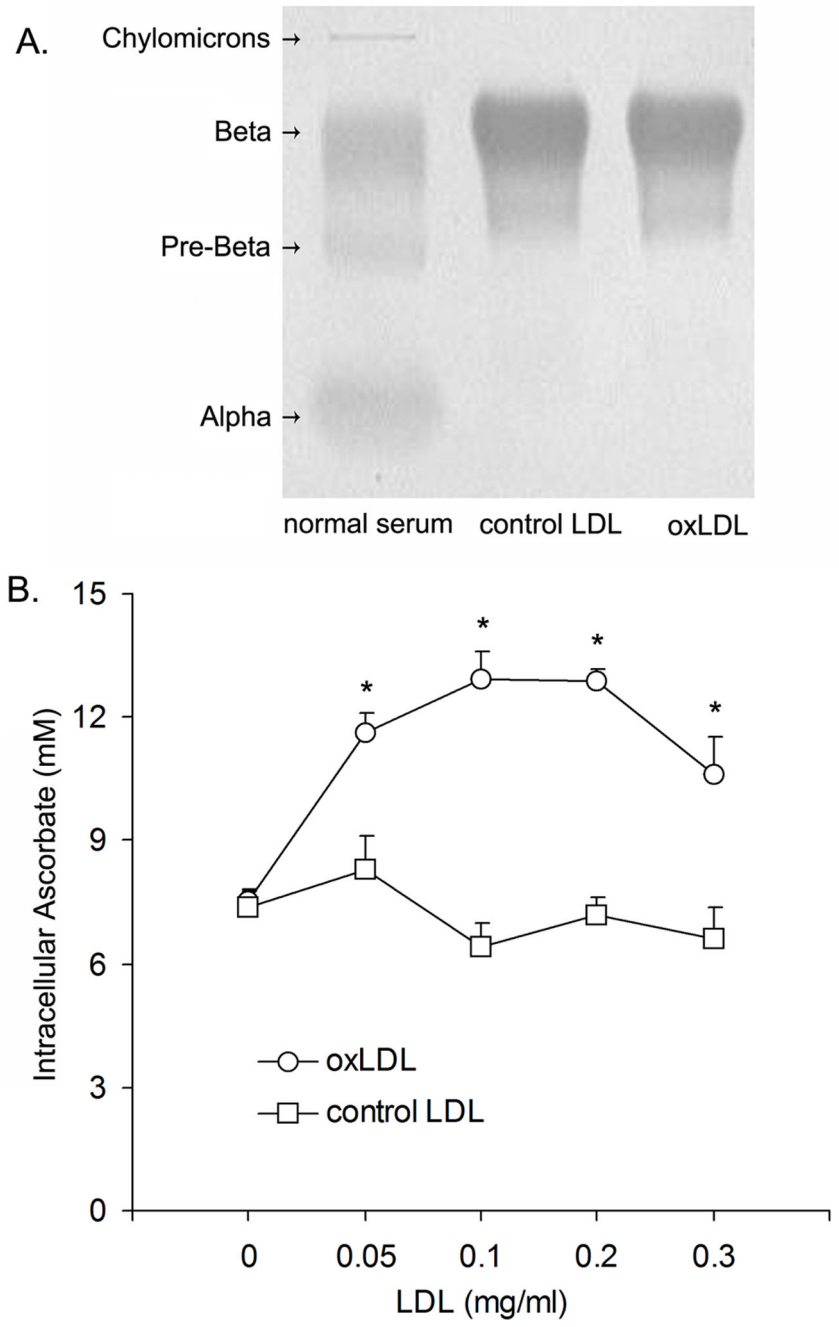

Fig. 2. LDL quality and effect on ascorbate uptake

Panel A: Titan Gel electrophoresis of normal serum (Lane 1), non-oxidized apolipoprotein B-rich preparation (Lane 2), and oxidized apolipoprotein B-rich preparation. Panel B: cells were plated to a density of 2×10^6 cells per well and treated for 18 h in culture with a single addition of ascorbate to 0.1 mM and the indicated concentration of oxidized (circles) or non-oxidized LDL (squares). The cells were rinsed 3 times in KRH and taken for assay of intracellular ascorbate as described in Materials and methods. Results are shown from 6 experiments, with an asterisk (*) indicating $P<0.05$ compared to the sample not treated with either type of LDL.

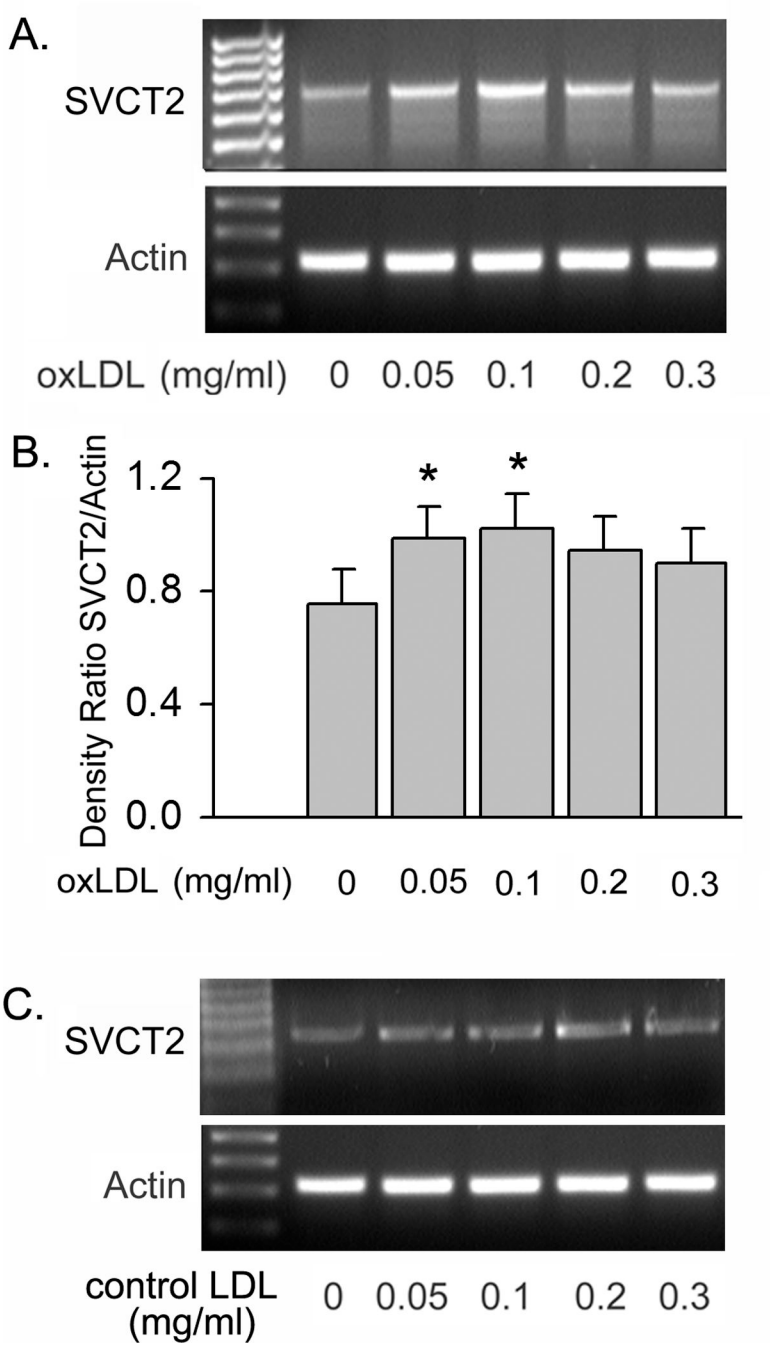

Fig. 3. RT-PCR amplification of SVCT2 in RAW264.7 macrophages

Cells were cultured in 6-well plates with the indicated treatment for 18 h, rinsed free of culture medium, and taken for RT-PCR amplification using 2 μg of total mRNA. Panel A shows a representative gel when cells were treated with oxLDL, with β-actin staining to indicate gel loading amounts. Panel B shows results from three experiments, normalized to β-actin. An asterisk (*) indicates $P < 0.05$ compared to cells not treated with oxLDL. Panel C shows results from a typical experiment using non-oxidized LDL, again with β-actin staining.

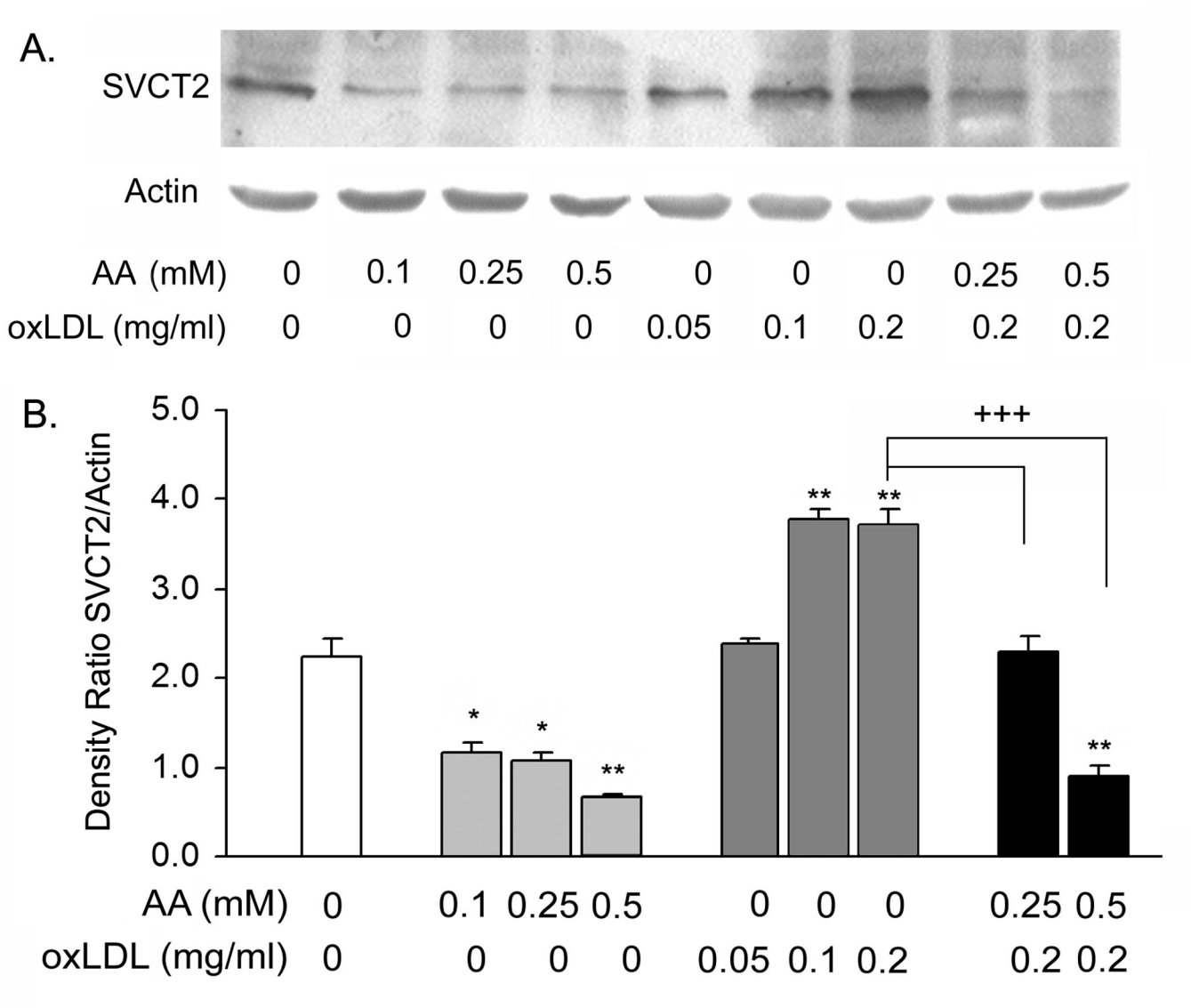

Fig. 4. Immunoblotting of the SVCT2 in RAW264.7 macrophages

Cells plated to a density of 2×10^6 cells per well were treated for 18 h in culture with agents at the concentrations indicated, rinsed in KRH to remove medium, and scraped from the plate in electrophoresis sample buffer for electrophoresis and immunoblotting. Panel A: A representative immunoblot, showing locations of the SVCT2 as a ~70 kDa band based on locations of pre-stained molecular weight markers. β-Actin was again stained in the same gel as a control for gel lane loading. Panel B: Results from 3 immunoblots were normalized to β-actin. An asterisk (*) indicates $P < 0.05$ compared to control, whereas two asterisks (**) indicates $P < 0.01$ compared to control, which did not receive oxLDL or ascorbate. Three pluses (+++) indicates $P < 0.001$ compared to cells treated with the same concentration of oxLDL. “AA” indicates ascorbic acid.

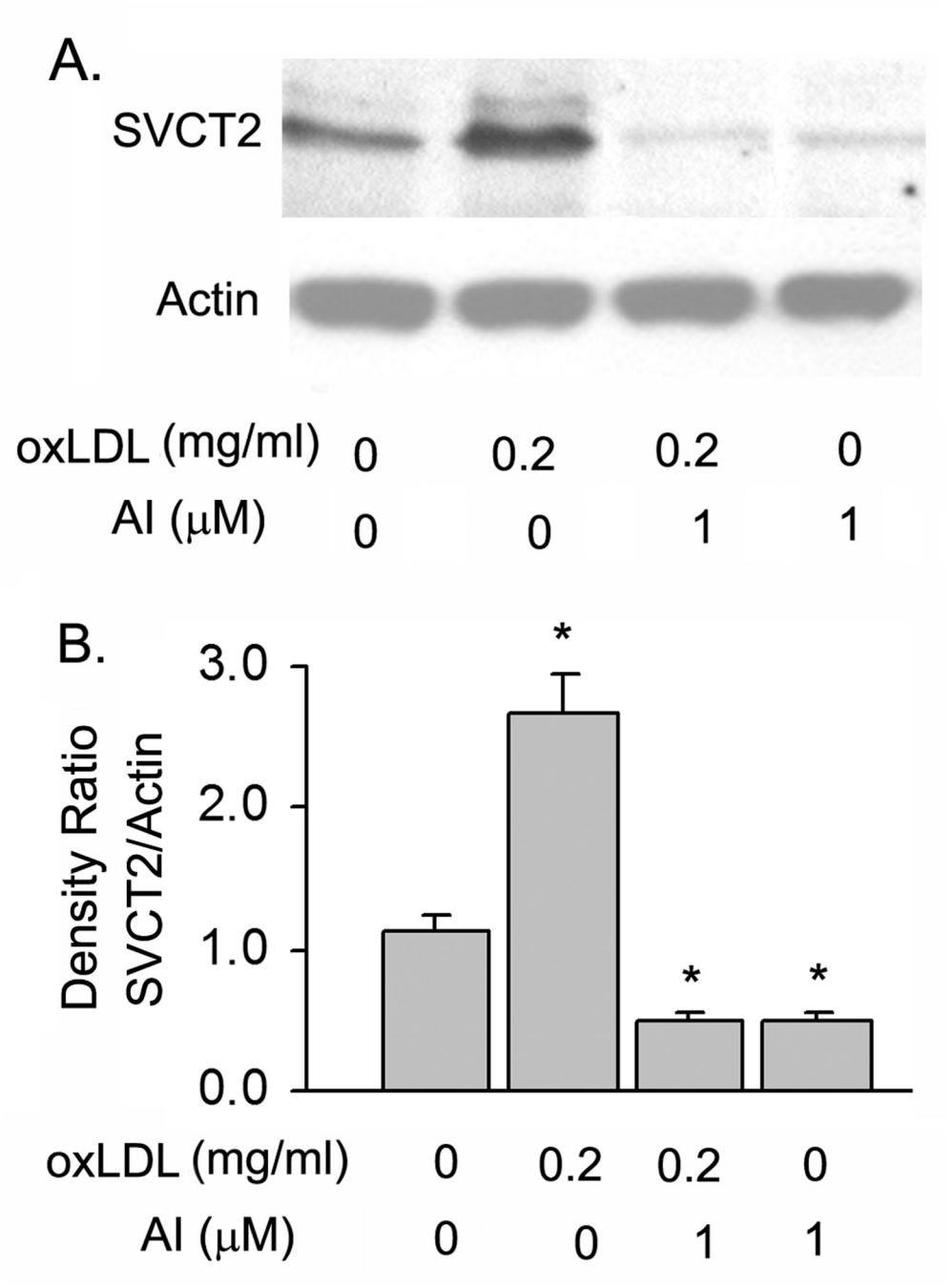

Fig. 5. Involvement of the NF-κB pathway in stimulation of SVCT2 expression by oxLDL

In experiments carried out as described in the legend to Fig. 4, cells were treated for 18 h with the indicated agents and prepared for immunoblotting. AI = NF-κB Activation Inhibitor. Panel A shows a representative immunoblot of the SVCT2 with corresponding staining of β-actin below each lane. Panel B shows the results of 4 separate experiments in which SVCT2 expression was normalized to that of β-actin, with an asterisk (*) indicating $p < 0.05$ compared to untreated cells.

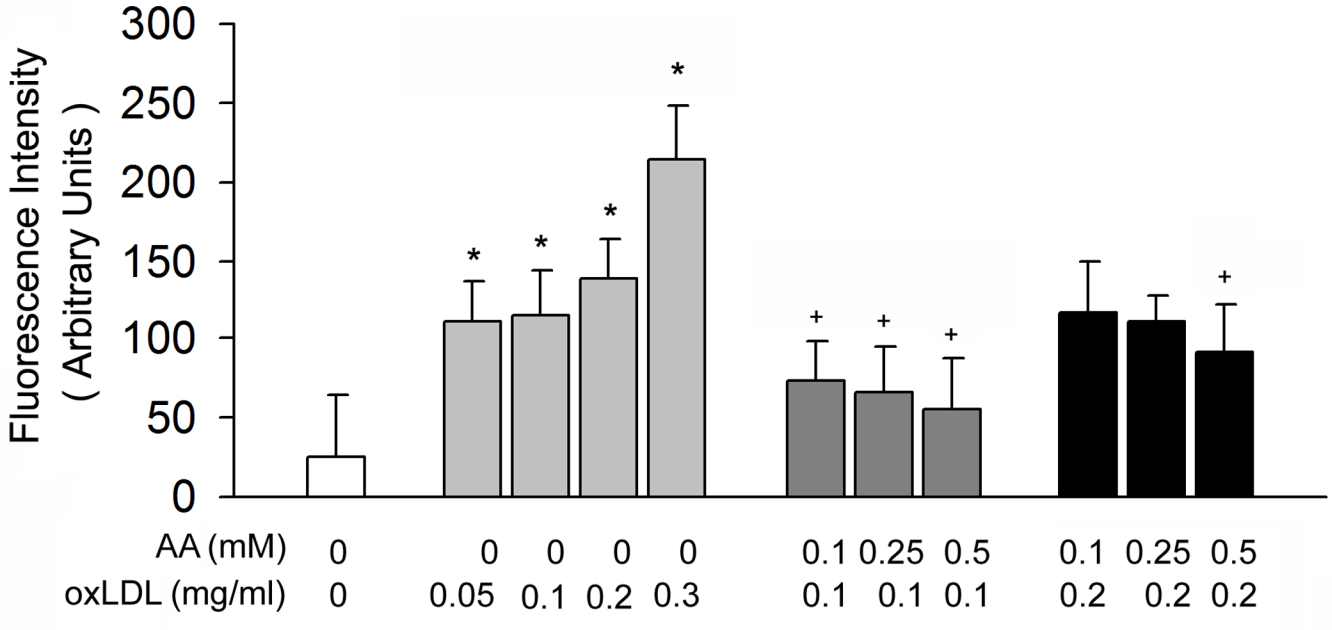

Fig. 6. Oxidant stress induction by oxLDL

Cells plated to a density of 2×10^6 cells per well that had been treated for 18 h in culture with the indicated concentrations of oxLDL and ascorbate were allowed to take up 20 μM dihydrofluorescein diacetate for 30 min at 37 °C, then rinsed 3 times in KRH and taken for measurement of fluorescence as described in Materials and methods. Results are shown from 4 experiments, with an asterisk (*) indicating $P < 0.05$ compared to control and a plus (+) indicating $P < 0.05$ compared to the corresponding bar with 0.2 mg/ml oxLDL treatment alone. “AA” indicates ascorbic acid.

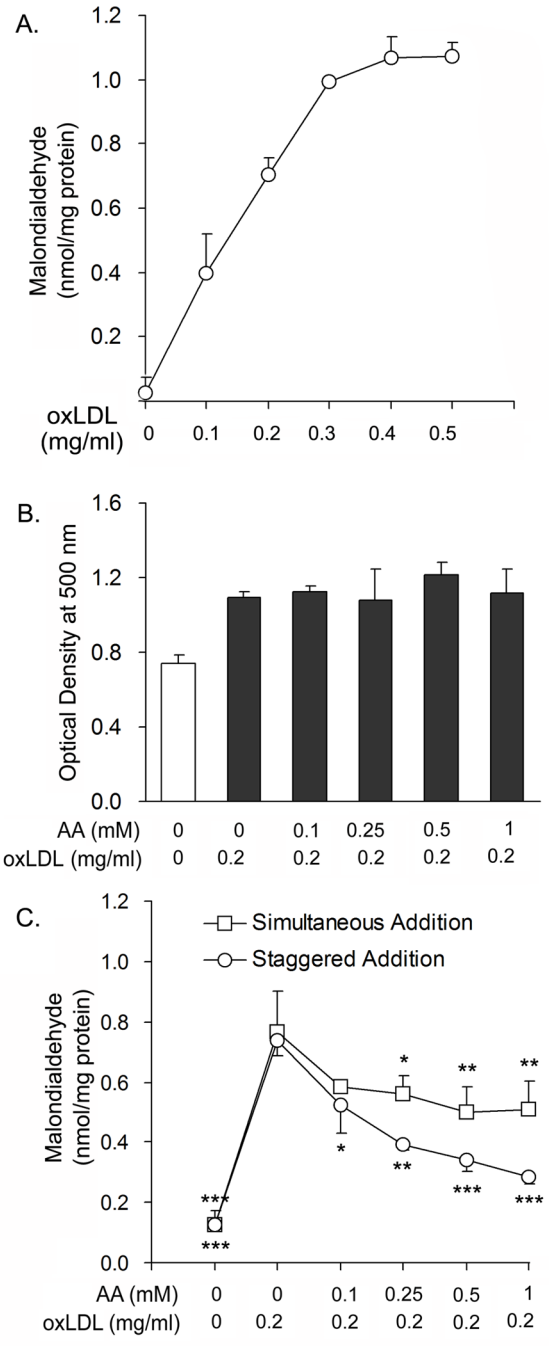

Fig. 7. Malondialdehyde appearance in RAW264.7 cells

Cells plated to a density of 2×10^6 per well of 6-well plates were treated for 18 h in culture with the indicated concentrations of oxLDL and ascorbate. Panel A: Malondialdehyde accumulation with increasing oxLDL concentrations. Panel B: Oil Red O staining of cells treated with oxLDL and ascorbate. Panel C: Malondialdehyde responses to treatment of the cells with both oxLDL and ascorbate at the indicated concentrations. The circles show results of experiments in which ascorbate was added 2 h before oxLDL, and the squares show results when both were added simultaneously. Results are from 4 or 6 experiments, with an asterisk (*) indicating $P < 0.05$, two asterisks indicating (**) $P < 0.01$, and three

asterisks (***) indicating P< 0.001 compared to cells treated with oxLDL alone of the respective treatment. “AA” indicates ascorbic acid.

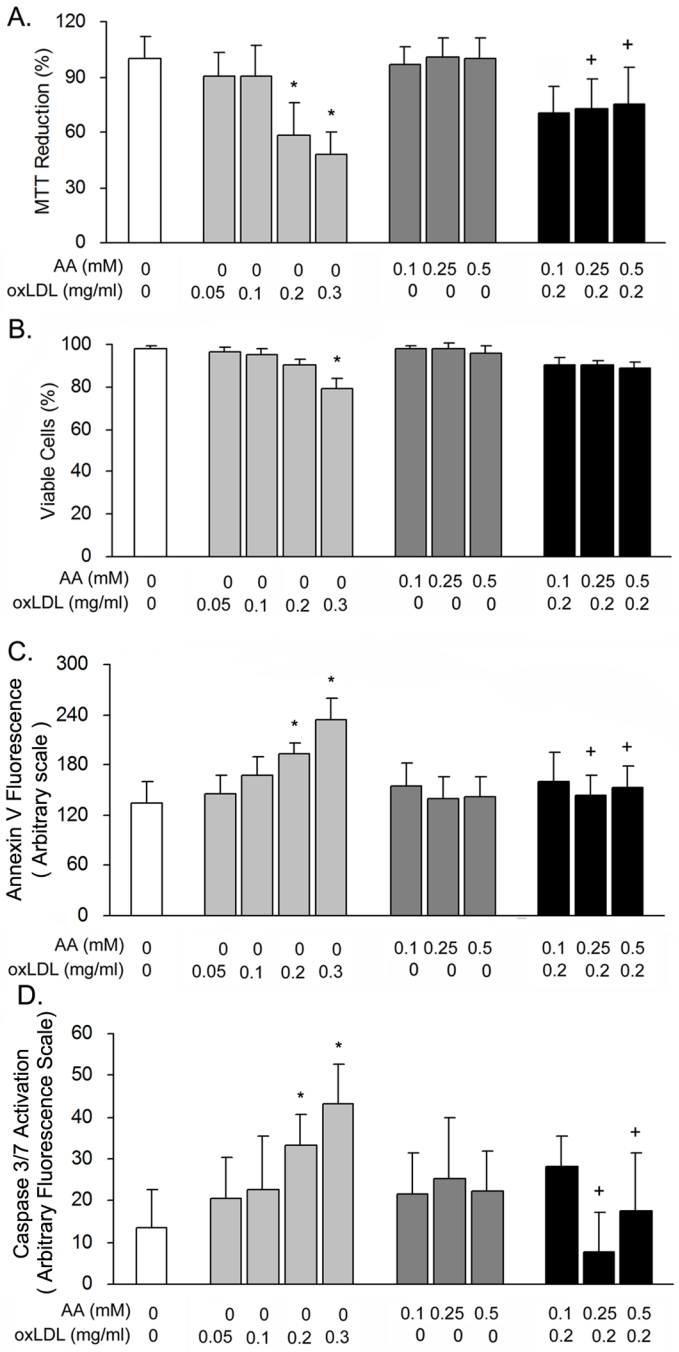

Fig. 8. Cell toxicity and viability after oxLDL treatment

Panel A: Cells plated to a density of 5×10^4 cells per well in 96-well plates were treated for 18 h with the indicated concentrations of oxLDL and ascorbate, followed by assay of MTT reduction as described in Materials and methods. Results are shown as a percent of untreated control from 16 wells on different days. An asterisk (*) indicates $P < 0.05$ compared to control, and a plus (+) indicates $P < 0.05$ compared to both the control and the result with oxLDL at 0.2 mg/ml. Panel B: Cells plated to a density of 2×10^6 cells per well in 24-well plates were treated for 18 h in culture with the indicated concentrations of oxLDL and ascorbate, followed by the trypan blue exclusion assay as described in Materials and methods. Results are shown from 6 experiments, with an asterisk (*) indicating $P < 0.05$

compared to control. Panel C: Assay of apoptosis using annexin-V: Cells were plated to a density of 5×10^3 cells per well in 384-well plates and treatments were as in Panel B. Results are shown from 2 experiments, each with 18 replicates. An asterisk (*) indicates $P < 0.05$ compared to control, and a plus (+) indicates $P < 0.05$ compared to cells treated with 0.2 mg/ml oxLDL. Panel D: Assay of apoptosis by caspase 3/7 activation was carried out after cell treatments as described under Panel C. Fluorescence readings from four separate wells in each of 3 experiments were averaged, with notation of statistical significance as in Panel C. “AA” indicates ascorbic acid.

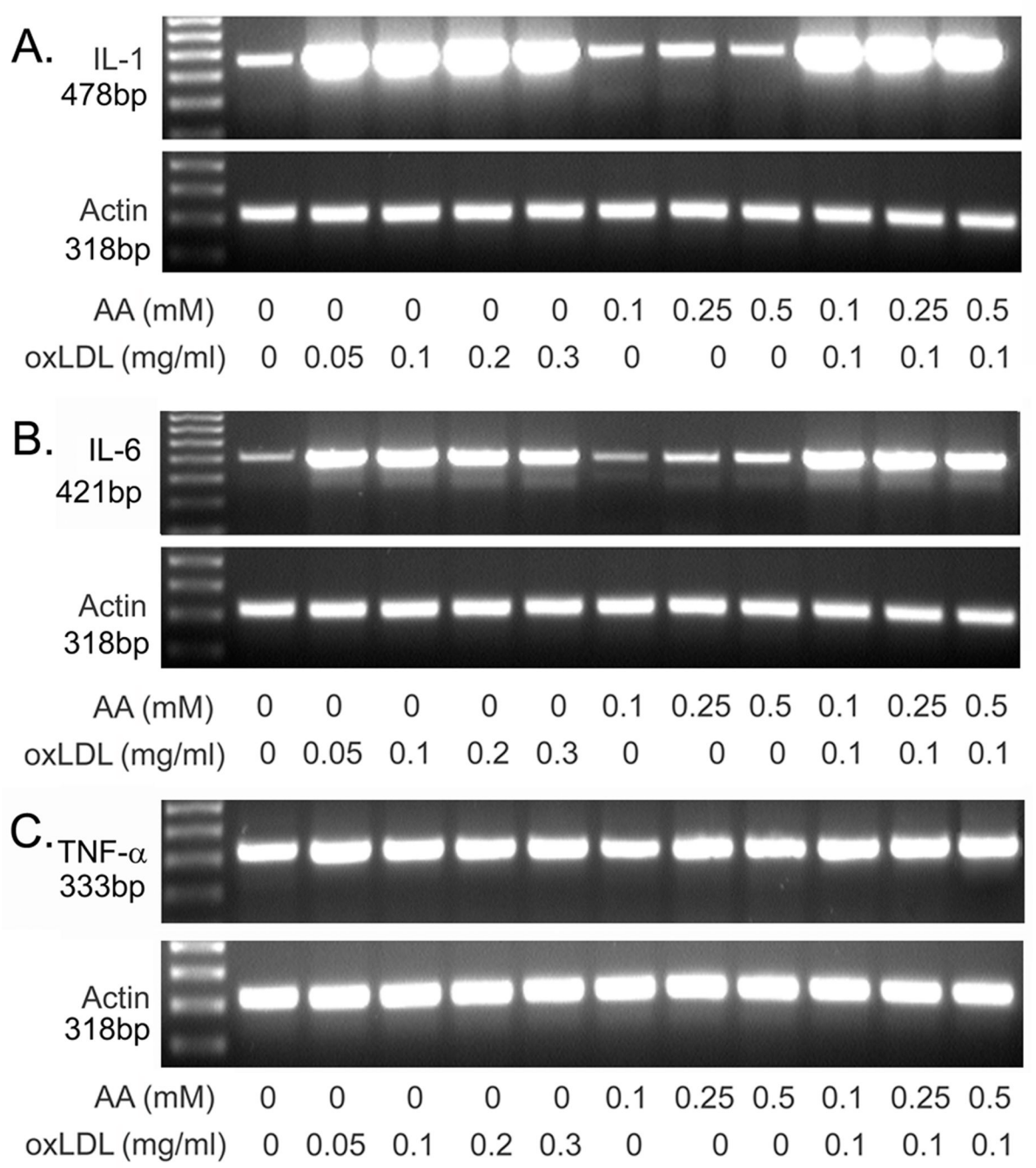

Fig. 9. RT-PCR amplification of mRNA for IL-1, IL-6 and TNF-α in RAW264.7 macrophages

Cells were plated at a density of 2×10^6 cells per well in 6-well plates and were treated as indicated for 18 h in culture. RNA was extracted, reverse-transcribed into cDNA and used as a template for RT-PCR for each message as described in Materials and methods. Actin is shown as a control for loading of the gel lanes. “AA” indicates ascorbic acid.