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Ascorbic Acid Uptake and Regulation of Type I Collagen Synthesis in Cultured Vascular Smooth Muscle Cells[1]

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Abstract

Background/Aims—Vascular smooth muscle cells contribute both to the structure and function of arteries, but are also involved in pathologic changes that accompany inflammatory diseases such as atherosclerosis. Since inflammation is associated with oxidant stress, we examined the uptake and cellular effects of the antioxidant vitamin ascorbic acid in cultured A10 vascular smooth muscle cells.

Methods/Results—A10 cells concentrated ascorbate against a gradient in a sodium-dependent manner, most likely on the SVCT2 ascorbate transporter, which was present in immunoblots of cell extracts. Although ascorbate did not affect A10 cell proliferation, it stimulated radiolabeled proline incorporation and type I collagen synthesis. The latter was evident in the cells as increases in proα(I) collagen and conversion of proα1(I) and proα2(I) collagen to mature forms that were released from the cells and deposited as extracellular matrix. Intracellular type I procollagen maturation was optimal at intracellular ascorbate concentrations of 200 μM and below, which were readily achieved by culture of the cells at plasma physiologic ascorbate concentrations.

Conclusion—These results show that the SVCT2 facilitates ascorbate uptake by vascular smooth muscle cells, which in turn increases both the synthesis and maturation of type I collagen.

Keywords

SVCT; vitamin C; type I collagen; Western blotting; collagen subtypes; A10 smooth muscle cells

Introduction

In response to acute arterial injury, such as from angioplasty/stenting or to chronic injury as a result of damage from oxidized low density lipoprotein (LDL), cytokines and growth factors are released from damaged endothelial and macrophage foam cells [1,2]. These factors reach vascular smooth muscle cells (VSMCs) in the media or intima and cause them to dedifferentiate, proliferate, and migrate to form the neointima [1,3]. This uncontrolled proliferation of VSMCs is an important factor in the pathogenesis of atherosclerosis and of restenosis following angioplasty [1,4]. There is accumulating evidence that this response is mediated, at least in part, by oxidant stress in the vascular wall [1,5]. This opens the possibility that antioxidant therapies might ameliorate either its occurrence or its detrimental effects. For example, a role for ascorbic acid in preventing VSMC proliferation and dedifferentiation is suggested by the results of a clinical trial of restenosis after angioplasty, in which subjects receiving oral ascorbate supplements had 25% larger luminal diameters

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and required 50% less intervention compared to a clinically matched control population [6]. Similar results regarding maintenance of luminal diameter were observed in studies of coronary restenosis in pigs using combinations of ascorbate and α-tocopherol [7,8].

The mechanism(s) underlying the effects of ascorbate on VSMC proliferation, migration, and differentiation have not been clearly defined, but may relate to the role of the vitamin in collagen synthesis and deposition. Ascorbate enhances both the synthesis [9,10] and hydroxylation of procollagen in VSMCs [11], such that without ascorbate cultured VSMCs make little collagen [12]. Indeed, although ascorbate deficiency in atherosclerosis-prone mice also unable to make ascorbate does not affect aortic lesion size, the collagen content of atherosclerotic lesions is markedly decreased [13]. Whereas collagen synthesis is required for VSMC migration and proliferation [14,15], the overall effect of ascorbate in these cells seems to be to decrease proliferation [14,16] and enhance maturation [17–19]. The latter mechanisms could contribute to decreased neointimal smooth muscle cell accumulation seen in the above-noted *in vivo* studies.

Although several studies have documented that ascorbate stimulates collagen synthesis in VSMCs, none have compared either the ability of the cells to take up ascorbate or intracellular ascorbate concentrations with effects on collagen synthesis. Since it is intracellular ascorbate that stimulates type I collagen synthesis and hydroxylation in VSMCs, it is relevant to correlate intracellular ascorbate with collagen synthesis, especially with regard to the pattern of collagen subtype maturation and release. Accordingly, we evaluated ascorbate uptake and its mechanism, as well as the resulting effects of intracellular ascorbate on cell proliferation and collagen formation in the A10 cell line of VSMCs. This line was derived from the thoracic aorta of embryonic rats [20] and has been extensively used as a model of VSMCs [21]. Subsequent characterization of these cells with multiple smooth muscle cell markers showed them to be non-differentiated neointimal VSMCs [21], and thus a good model for assessing ascorbate effects on type I collagen. The results of this study show that A10 cells take up ascorbate on the SVCT2 and that low intracellular ascorbate concentrations stimulate synthesis and maturation of type I collagen sub-types that are deposited as extracellular matrix.

Materials and methods

Materials

Sigma/Aldrich Chemical Co. (St. Louis, MO) supplied the dehydroascorbic acid (DHA), ethyl-2,4-dihydroxybenzoic acid, N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid, *L*-buthionine sulfoxime, diethylmaleate, 1,3-bis(2-chloroethyl)-1-nitrosourea, and sulfinpyrazone. Ethanol was used to dissolve ethyl-2,4-dihydroxy benzoic acid and dimethylsulfoxide to dissolve diethylmaleate before further dilution in Krebs-Ringer-*N*-2-hydroxyethylpiperazine-NN-2-ethanesulfonic acid (KRH) buffer, such that the concentration of neither solvent was greater than 0.8% (v/v). KRH buffer consisted of 20 mM N-2-hydroxyethylpiperazine-NN-2-ethanesulfonic acid, 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. Perkin-Elmer Life and Analytical Sciences, Inc. (Boston, MA) supplied the radionuclides used.

Cell Culture

A10 cells (obtained from the American Type Culture Collection) were cultured in Dulbecco's minimal essential medium containing 10% (v/v) heat-inactivated fetal bovine serum, which was prepared by the Cell Culture Core of the Vanderbilt Diabetes Research and Training Center. Cells were cultured to near confluence at 37 °C in humidified air

containing 5% CO_2. Just before an experiment, cells were rinsed 3 times in 2 ml of KRH buffer at 37 °C.

Assay of ascorbate transport

Following treatments as noted, near confluent A10 cells in 12-well plates were incubated at 23 °C in KRH that contained 5 mM D-glucose, 0.5 mM GSH, and 0.05 μCi of L-[1-^{14}C]ascorbic acid. The total ascorbate concentration was 6–9 μM, unless otherwise stated. After 30 min of incubation, the supernatant was aspirated, and the cells were rinsed twice in 2 ml of ice-cold KRH. The cell monolayer was treated with 1 ml of 0.05 N NaOH, the cells were scraped from the plate, and the combined extract was added to 5 ml of Ecolume liquid scintillation fluid (ICN, Costa Mesa, CA) and mixed. The radioactivity of duplicate samples was measured in a Packard CA-2200 liquid scintillation counter, after allowing at least 1 h for decay of chemiluminescence.

Assay of ascorbate and GSH

Following culture and incubations as indicated in 6-well plates, the medium was aspirated, and the adherent cells were gently rinsed twice with 2 ml of ice-cold KRH. The last rinse was removed and the cell monolayer was treated with 0.1 ml of 25% metaphosphoric acid (w/v) for several minutes, followed by partial neutralization with 0.35 ml of a buffer containing 0.1M Na_2HPO_4 and 0.05 mM EDTA, pH 8.0. Adherent material was scraped from the plate, and the lysate was removed and centrifuged at 3 °C for 1 min at 13,000 × *g*. Duplicate aliquots of the supernatant were taken for assay of ascorbic acid by high performance liquid chromatography as previously described [22], except that ascorbate was detected by its ultraviolet absorption at a wavelength of 260 nm on a Waters Model 2487 spectrophotometer. In some experiments, ascorbate was also measured in 0.1 ml of the incubation medium by adding 0.1 ml of 25% metaphosphoric acid (w/v), mixing, neutralizing with 0.35 ml of the above phosphate/EDTA buffer, and centrifuging to remove and precipitated solids before assay of ascorbate. GSH was assayed in duplicate by the method of Hissin and Hilf [23]. Intracellular concentrations of ascorbate and GSH were calculated based on the intracellular distribution space of 3-*O*-methylglucose in A10 cells and normalized to measured protein in an experiment. This was measured as described previously for endothelial cells in culture [24] and was 3.7 ± 0.6 μl/mg protein (N=24, ± SD).

Assays of cell proliferation

Cellular uptake of [^{3}H]thymidine was measured as described by Totzke, et al [25], with minor modifications. Subconfluent cultures (70–90%) of A10 cells in 24-well plates were washed and then incubated in cultured medium containing 0.2% bovine fetal bovine serum and glutamine for 24 h to growth arrest cells. Ascorbate (0–300 μM) was then added for 24 h and [^{3}H]thymidine (1 μCi/well) was added for the final 16 h of the incubation. After this, the supernatant was aspirated and cells were rinsed twice with phosphate-buffered saline (12.5 mM sodium phosphate, 140 mM NaCl, pH 7.4) before being fixed with methanol (100%)/glacial acetic acid (3:1) for at least 1 h at room temperature. Two further rinses with methanol/water (4:1) were performed before lysing cells with 1 ml of 1 M NaOH and scintillation counting.

Incorporation of 5-bromo-2-deoxyuridine into synchronized A10 cells pre-treated with 0–300 μM ascorbate was measured as described by Meyer, et al. [26]. Cells grown on eight-chamber slides were stained using mouse monoclonal antibodies to 5-bromo-2-deoxyuridine (Ab-2, 1:100; Oncogene Science, San Diego, CA), Before staining for incorporated 5-bromo-2-deoxyuridine, DNA was partially denatured with 2 N HCl for 30 min. As a control for specific staining, cells were also incubated with assay buffer but the primary antibody

was omitted. To stain for 5-bromo-2-deoxyuridine, cells were serially stained and bound biotinylated anti-5-bromo-2-deoxyuridine antibody (Ab-3; Oncogene Science) was visualized using 3,3'-diaminobenzidine (brown color) as a peroxidase substrate.

Assay of proline uptake and incorporation

A10 cells at near confluence in culture were treated with 1 μCi of [2,3-^{3}H]proline and cultured for an additional 3 h at 37 °C, following which they were rinsed 3 times in KRH and prepared for radioactive counting as described for the assay of ascorbate.

Polyacrylamide gel electrophoresis and immunoblotting of SVCTs and type I collagen

Cells cultured in T-25 flasks were rinsed once in phosphate-buffered saline, and solubilized in a lysis buffer consisting of 150 mM NaCl, 1% Nonidet P40 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% sodium lauryl sulfate (w/v). To prevent proteolysis, this buffer also contained 0.1 mg/ml phenylmethylsulfonyl fluoride, and leupeptin, pepstatin, and aprotinin, each at 0.01 mg/ml. After mixing, the lysate and stored on ice for 30 min. The lysate was combined with an equal volume of sample buffer, which consisted of 125 mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) sodium lauryl sulfate, 10% (v/v) mercaptoethanol, and 0.0025% bromphenol blue (w/v), pH 6.8. Samples were centrifuged for 10 s at 13,000 × *g*, and 20–30 μg protein of solubilized material was subjected to 5 and 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis for type I collagen and SVCT2, respectively, according to the method of Laemmli [27]. Electrophoresis and transfer to poly(vinylidine difluoride) membrane, was performed as previously described [28]. The SVCT2 was probed with a 1:200 dilution of an affinity purified rabbit polyclonal antibody that was specific for the SVCT2 transporter (#SVCT21-A, Alpha Diagnostic International, San Antonio, TX). The SVCT1 was probed with the same dilutions of two different affinity-purified goat polyclonal antibodies made to different portions of the SVCT1 sequence (sc-9924 and sc-30113, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Type I collagen was detected using 1:8000 dilutions of affinity purified polyclonal rabbit antibodies against rat type I collagen. One antibody (SC-8784-R, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was specific for full-length proα1(I) collagen and the mature α1(I) collagen lacking both terminal propeptides. A second antibody (ab34710, Abcam, Inc., Cambridge, MA) detected collagen of both the 1α and 2α subtypes. Actin served as a control for the amount of sample loaded and was detected in immunoblots using an antibody to actin at a dilution of 1:200 (SC-1616-R, Santa Cruz Biochemicals, Santa Cruz, CA). The secondary antibody for all immunoblots was anti-rabbit IgG conjugated to horseradish peroxidase (#A0545, Sigma-Aldrich, Inc., St. Louis, MO) and was used at a 1:5000 dilution. Membranes were blocked with 5% (w/v) nonfat dry milk at 23 °C for 1 h, rinsed in phosphate-buffered saline containing 1% Tween-20, and sequentially incubated with both primary and secondary antibodies overnight at 4 °C. Bands were stained using ECL Plus Western blotting reagents (RPN 2132, Amersham Biosciences, Piscataway, NJ). Band locations were determined using pre-stained molecular weight markers. Immunoblotting results were confirmed at least 3 times.

Immunofluorescence Microscopy for Type I Collagen

A10 cells were seeded onto multi-well slides and cultured for 24 h, after which the medium was replaced fresh medium containing either 0 or 50 μM sodium ascorbate. Cells were maintained under these conditions for 6 days with daily addition of ascorbate. The cells were rinsed free of medium with phosphate-buffered saline, fixed in cold acetone, blocked for 30 min at 23 °C with 1 mg/ml bovine serum albumin plus 0.3% Triton X-100 or with bovine serum albumin alone, followed by incubation at 23 °C for 2 h with rabbit antiserum to type I collagen (ab34710). The bound primary antibody was detected with Cy2-conjugated goat anti-rabbit IgG (1:400 dilution) at 23 °C for 1 h. After 3 rinses in phosphate-buffered saline,

cells were treated with glycerol/ phosphate-buffered saline (9:1, vol/vol) and evaluated by fluorescence microscopy.

Data Analysis

Results are shown as mean + standard error. Statistical comparisons were made using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). Differences between treatments were assessed by one-way analysis of variance with post-hoc testing using Dunnett's test.

Results

A10 cells contained little or no ascorbate in culture (Fig. 1A, zero time). However, cells incubated with 0.2 mM ascorbate progressively took up the vitamin over 2 h, reaching intracellular concentrations as high as 0.5 mM (Fig. 1A). Incubation of cells with increasing amounts of ascorbate for 16–18 h resulted in intracellular ascorbate concentrations that rose to 2–3 mM (Fig. 1B, circles). During such overnight loading, intracellular ascorbate was typically twice the initial loading concentration. Further, at the end of the incubations, extracellular ascorbate concentrations were less than 5% of those added initially (results not shown). Together, these results indicate that A10 cells take up and retain ascorbate against a concentration gradient.

To determine the mechanism by which A10 cells take up ascorbate, specific ascorbate transport was measured over 30 min. This time for sampling was chosen since uptakes of both unlabeled (Fig. 1A) and radiolabeled ascorbate (results not shown) were also linear for 1 h. As shown in Fig. 2A, uptake of radiolabeled ascorbate was inhibited 50% by competition with 1 mM unlabeled ascorbate, 45% by omission of sodium from the incubation medium, and 67% by the organic anion transport inhibitor sulfinpyrazone. Ascorbate transport was not inhibited by either 25 μM cytochalasin B or by 40 mM 3-*O*-methylglucose. Both are known inhibitors of glucose transport. As depicted in Fig. 2B, the SVCT2 protein was detected on immunoblots in A10 cells as bands in the 74–76 kDa range. The SVCT1 was not detected with either of two SVCT1 antibodies (results not shown). Together, these results show that ascorbate enters the cell via a transporter with features of the SVCT2 transporter, and not on the glucose transporter.

Specific ascorbate transport is known to be sensitive to the thiol-dependent redox state of the cell [29], and this was tested for A10 cells. As shown in Fig. 3, pre-incubation of A10 cells with *L*-buthionine sulfoximine modestly decreased ascorbate transport, whereas 1,3-bis(2-chloroethyl)-1-nitrosourea and diethylmaleate had no effect. In contrast, these agents all significantly depleted GSH. Ascorbate transport in A10 cells did not correlate well with GSH depletion by these reagents.

To determine if intracellular ascorbate affects A10 cell proliferation, incorporation of both [^{3}H]thymidine and 5-bromo-2-deoxyuridine were measured with increasing concentrations of ascorbate under the conditions noted in Fig. 1B, except that the time in culture was 24–48 h before assay. There was no effect of ascorbate in either of these assays (results not shown). However, ascorbate at initial concentrations as low as 30 μM did enhance cellular uptake and incorporation of L-2,3-[^{3}H]proline into A10 cells by about 40% over 72 h (Fig. 4). Since proline will be incorporated into proteins other than collagen, to further assess effects of ascorbate on collagen synthesis by A10 cells, type I collagen was measured in cell extracts by Western blotting.

Immunoblots with an antibody specific for the α1(I) collagen showed that sub-confluent A10 cells contained little of α1(I) collagen after initial plating (Fig. 5, top panel). With time in culture, however, they generated increasing amounts of both proα1(I) collagen and

mature α1(I) collagen. Initially, ascorbate had little effect, but after 3 days in culture, daily additions of 100 μM ascorbate increased conversion of proα1(I) collagen to mature α1(I) collagen. Three days of culture with ascorbate decreased the cell content of proα1(I) collagen in this experiment. Evaluation of α2(I) collagen was complicated in that the antibody to the α2(I) subtype recognized both the α1(I) and the α2(I) subtypes. As shown in the middle panel of Fig. 5, as with proα1(I) collagen, an increase in proα2(I) collagen with time in culture was evident, but there was overlap of bands for the mature α1(I) and proα2(I) collagen at about 150 kDa. The intensity of this overlapping band was decreased by ascorbate. However, a band corresponding to mature α2(I) collagen was progressively induced by culture with 100 μM ascorbate. Together, these results show that the effect of ascorbate was to convert both proα1(I) and proα2(I) collagen into the mature forms for secretion into the medium. The lower panel of Fig. 5 shows that despite different cell densities with different times in culture, the amounts of cell material loaded on to the gels were similar, based on staining for actin. The effect of ascorbate to increase proα1(I) synthesis and the maturation of both α1(I) and α2(I) collagen was reversed within 1–2 days after removal of ascorbate from the medium (Fig. 6). Decreases in the cell content of mature α1(I) and α2(I) collagen were associated with an increase in proα2(I) but not proα1(I) collagen in the cells (Fig. 6). To determine the specificity of the effect of ascorbate on increased generation of the mature subtypes of type I collagen, cells were treated with the prolyl hydroxylase inhibitor ethyl-2,4-dihydroxy benzoic acid for 2 days during ascorbate treatment. As shown in Fig. 7, generation of both collagen subtypes was inhibited by ethyl-2,4-dihydroxy benzoic acid, but cell accumulation of the mature α1(I) collagen (top panel) was not completely inhibited by ethyl-2,4-dihydroxy benzoic acid, whereas mature α2(I) collagen was completely suppressed by 250 μM ethyl-2,4-dihydroxy benzoic acid (bottom panel).

To determine the minimum concentration of ascorbate effective for maturation of type I collagen subunits, we carried out ascorbate concentration-response studies on cells cultured for 3 days, with the results shown in Fig. 8. As shown in the top panel, cell contents of both proα1(I) collagen and mature α1(I) collagen increased to nearly a maximal level at about 25 μM ascorbate. In contrast, intracellular levels of the mature α2(I) collagen were maximally increased by ascorbate concentrations as low as 5 μM.

Ascorbate increased both intracellular and extracellular type I collagen in A10 cells, as detected by immunofluorescence using the antibody that cross-reacts with both the α1(I) and α2(I) subunits (Fig. 9). In cells cultured for 6 days and permeabilized with Triton X-100, intracellular type I collagen staining was enhanced by ascorbate (panel A). Cultures not permeabilized showed a marked increase in extracellular sheets of collagen in response to ascorbate (panel B), as expected if ascorbate accelerated generation and export of mature α1(I) and α2(I) subunits, which subsequently deposited as fibrils around and under the cells.

Discussion

Ascorbate was taken up by A10 VSMC's on what is very likely the SVCT2 ascorbate transporter, since the SVCT1 was not detected by immunoblotting. Specificity for this transporter was evident in that uptake of unlabeled and radiolabeled ascorbate was concentrative and inhibited by 1) ascorbate itself, 2) removal of sodium from the medium, and 3) the organic anion transport inhibitor sulfinpyrazone. Similar results were also observed by Holmes, et al. [30] in cultured pig coronary artery smooth muscle cells, suggesting a common transport mechanism in smooth muscle cells. Another mechanism of ascorbate accumulation against a concentration gradient is uptake of DHA on the GLUT-type glucose transporter and intracellular reduction to ascorbate, which is then trapped in the cell due to its negative charge at physiologic pH. Voskoboink, et al. [31] showed in human

umbilical vein smooth muscle cells that initial rates of DHA uptake and reduction were comparable to those of ascorbate and that the glucose transport inhibitor phloretin prevented uptake of both DHA and ascorbate. This led them to conclude a vital role for DHA uptake on glucose transporters in supplying intracellular ascorbate to smooth muscle cells. However, phloretin is also known to inhibit ascorbate transport in *Xenopus* oocytes that had been injected with mRNA for the SVCT2 [32]. Additionally, in the present studies ascorbate transport was not inhibited by either cytochalasin B or 3-*O*-methylglucose. These are known inhibitors of the GLUT-type glucose transporters [33], so the results observed are not due to uptake of DHA on glucose transporters. Cytochalasin B was also shown not to inhibit ascorbate uptake by pig coronary smooth muscle cells [30]. Although A10 cell ascorbate transport was not as sensitive to reduced glutathione (GSH) depletion by thiol reagents as that of cultured endothelial cells [29], immunoblotting of the SVCT2 confirmed the presence of this transporter in A10 cells, making it most likely to account for the transport features observed.

A10 cells treated in culture with ascorbate did not show increased proliferation above that due to fetal bovine serum as measured either by thymidine uptake and 5-bromo-2-deoxyuridine labeling. In previous studies, ascorbate effects on cell proliferation were variable, and depended on the ascorbate concentration and on the composition of the extracellular matrix [12,14,34]. For example, during cell proliferation, treatment of guinea pig VSMCs with ascorbate concentrations of 0.5 mM and higher inhibited DNA synthesis and growth [14], whereas lower ascorbate concentrations either had no effect (as in the present studies), or showed a modest 25% stimulation [14]. Thus, although ascorbate enhances VSMC attachment and migration [15], at least at physiologic plasma concentrations, it has little effect on proliferation of VSMCs. This contrasts with endothelial cells, in which similar ascorbate concentrations stimulate cell proliferation [35,36]. These differential effects are in line with postulated anti-atherosclerotic roles of the vitamin to enhance endothelial cell regeneration and prevent dedifferentiation and subsequent proliferation of VSMCs [19].

As expected, ascorbate enhanced type I collagen generation and maturation in sub-confluent A10 cells [19]. More specifically, the major effect of ascorbate was to increase intracellular generation of mature α1(I) and α2(I) subunits from the respective procollagen species, which likely reflects ascorbate-stimulated hydroxylation of proline and lysine residues in existing procollagen. A10 cells are typically cultured in medium lacking ascorbate, resulting in a very low to undetectable intracellular ascorbate concentrations. When treated for 16–18 h with ascorbate at physiologic plasma concentrations (50–100 μM), intracellular ascorbate concentrations increased to about 200 μM. In contrast, generation of mature α1(I) collagen subtype in A10 cells was maximal at ascorbate loading concentrations of 25 μM and even less for the mature α2(I) subtype. Given that the apparent K_m of purified prolyl hydroxylase for ascorbate is about 300 μM [11,37], the high sensitivity of procollagen maturation induced by ascorbate in A10 cells suggests either that somewhat higher concentrations of ascorbate are present at the enzyme than measured in the whole cell, or that the affinity of the cellular enzyme for ascorbate is increased compared to that of the purified enzyme. The results for type I collagen subtypes in A10 smooth muscle cells contrast with those we previously found for type IV collagen released into the incubation medium by cultured endothelial cells. Maximal release of type IV collagen into the incubation medium of EA.hy926 endothelial cells required intracellular ascorbate concentrations of 2 mM and greater [38]. This difference could relate to the different types of cells and collagen involved, or to the fact that the endothelial cells were confluent and contact-inhibited, whereas the A10 cells in the present study were in the growth phase of culture and were likely synthesizing collagen to allow migration and spreading [14,15]. The present results

clearly suggest that ascorbate-dependent type I collagen maturation in A10 occurs well within the physiologic plasma range of ascorbate concentrations.

Whereas ascorbate clearly enhanced maturation of type I collagen subunits, its effects on levels of proα1(I) collagen were variable. Although most experiments showed an increase in proα1(I) collagen with ascorbate treatment (Figs. 6–8), some failed to show an increase (e.g., Fig. 5). This was likely due to differences in ascorbate-dependent maturation of proα1(I) collagen, which would deplete it from the cells. A similar explanation could account for the apparent decrease in proα2(I) collagen in response to ascorbate. Nonetheless, the overall effect of ascorbate was to stimulate collagen synthesis, evidenced by the modest 30% increase in proline incorporation into cellular protein observed. These results also agree with a previous study showing that ascorbate increased collagen generation in pig aortic smooth muscle cells by a pre-translational mechanism [10]. Along with the ascorbate-induced increases in intracellular mature α1(I) and α2(I) collagen, increased procollagen synthesis contributed to the increased deposition of collagen fibrils observed outside the cells in the present study.

In conclusion, this work documents the role of the SVCT2 transporter in supplying ascorbate for type I collagen synthesis and hydroxylation in A10 VSMCs. It also shows that the predominant effects of ascorbate are to enhance synthesis of proα1(I) as well as conversion of both proα1(I) and proα2(I) collagen subtypes into mature subtypes that can then form stable fibrils required for efficient export from the cell to form the extracellular matrix. Whereas this function of ascorbate on VSMC function is crucial for vascular growth and development, it may also be important in vascular remodeling [8] and stability of collagen [13] in atherosclerotic vessels.

Abbreviations used

DHA	dehydroascorbic acid
GSH	reduced glutathione
KRH	Krebs-Ringer Hepes
SVCT1	sodium-dependent vitamin C transporter type 1
SVCT2	sodium-dependent vitamin C transporter type 2
VSMCs	vascular smooth muscle cells

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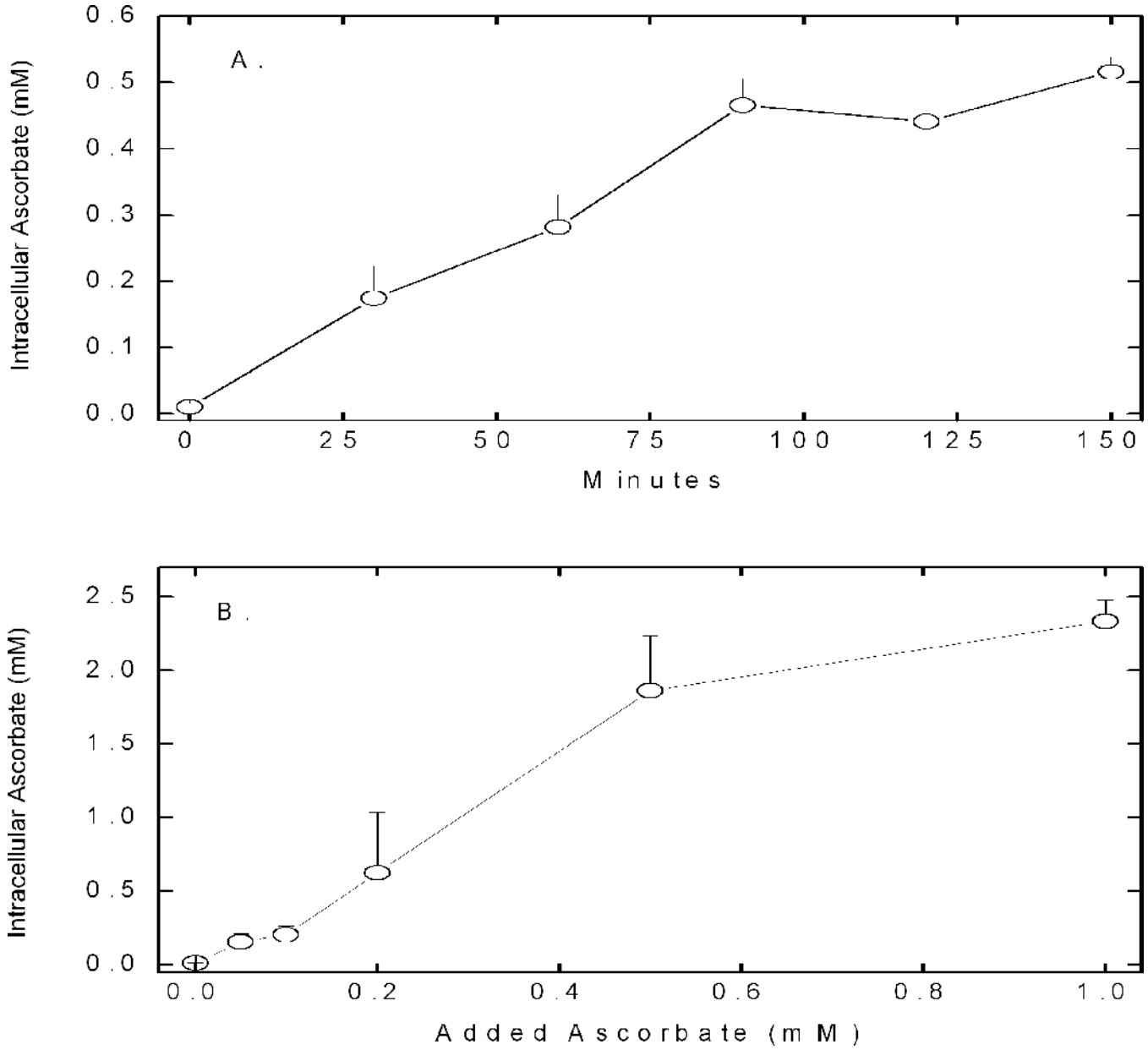

Figure 1. A10 cells take up and accumulate ascorbate in culture

Panel A: Near confluent A10 cells were treated with 0.2 mM ascorbate in culture medium for the times indicated followed by 3 rinses of the cells in KRH and assay of their ascorbate content. Panel B: Cells were treated at 37 °C with the indicated concentrations of ascorbate in culture medium. After 16–18 h in culture for ascorbate-treated cells, medium was removed and the cells were rinsed three times in KRH and taken for assay of intracellular ascorbate. Results are shown from 4 experiments.

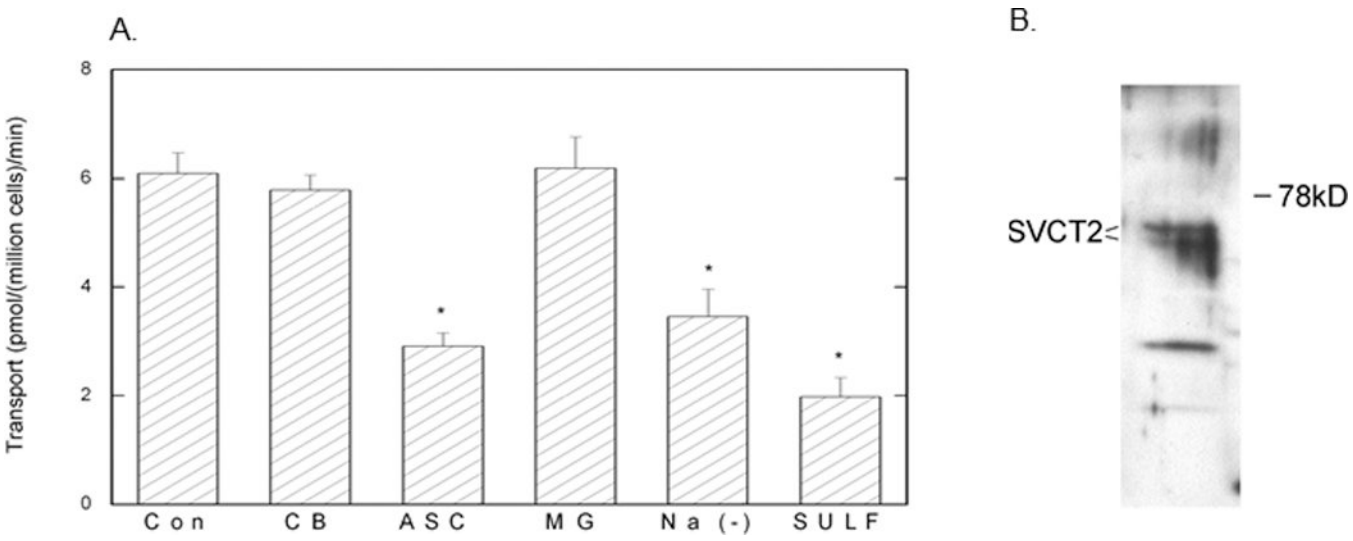

Figure 2. A10 cells transport ascorbate by both the SVCT1 and SVCT2

Panel A: Rinsed cells were incubated in KRH at 23 °C in the presence of 5 mM D-glucose, 0.5 mM GSH, and 0.05 μCi of [1-^{14}C]ascorbate in the absence of further treatments (Con), or in the presence of 25 μM cytochalasin B (CB), 1 mM ascorbate, 40 mM 3-*O*-methylglucose (MG), KRH in which sodium chloride was replaced with 140 mM choline chloride (Na (-)), or 1 mM sulfinpyrazone (SULF). After 30 min, the cells were rinsed 3 times in KRH and taken for assay of ascorbate transport. Results are shown from 4 experiments, with an asterisk (*) indicating $p < 0.05$ compared to the control sample. Panel B: Immunoblotting of the SVCT2 transporter.

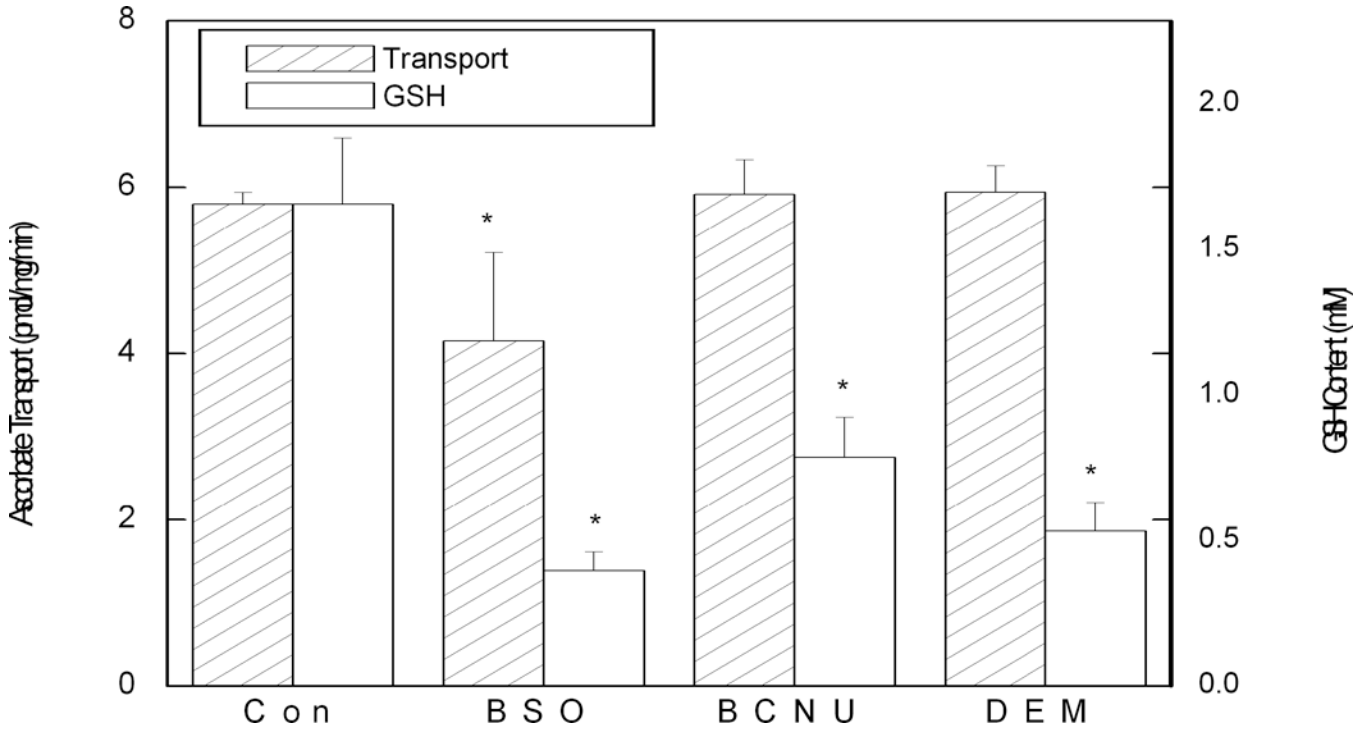

Figure 3. Ascorbate transport does not correlate with GSH content of A10 cells

Cells were cultured overnight without or with 0.5 mM *L*-buthionine sulfoximine (BSO), as indicated. Cells were then rinsed and incubated for 30 min at 23 °C in KRH that contained 5 mM D-glucose and no additions (Con and BSO), or either 0.5 mM 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or 1 mM diethylmaleate (DEM). Assays of ascorbate transport (hatched bars) and intracellular GSH (open bars) were then carried out on separate batches of cells. Results were normalized to the transport activity or GSH content of cells not treated with an agent in the same assay. Results are shown for at least 4 experiments using each agent.

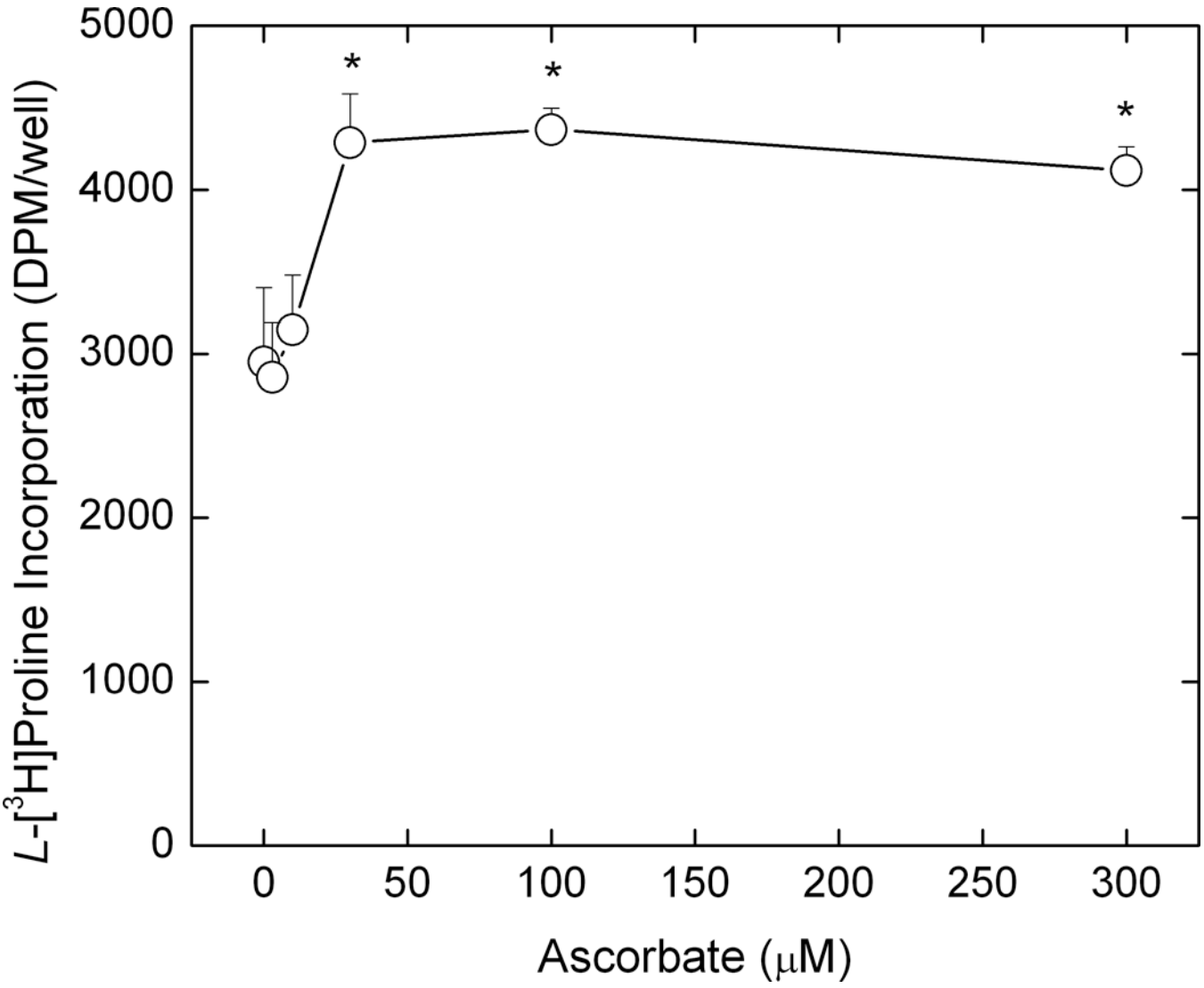

Figure 4. Ascorbate stimulates proline incorporation into A10 cells

Sub-confluent A10 cells were cultured with the indicated ascorbate concentrations added every 24 h for 72 h, followed by assay of the cell content of radioactivity. Results are shown from 3 experiments with an "*" indicating $p < 0.05$ compared to cells not treated with ascorbate.

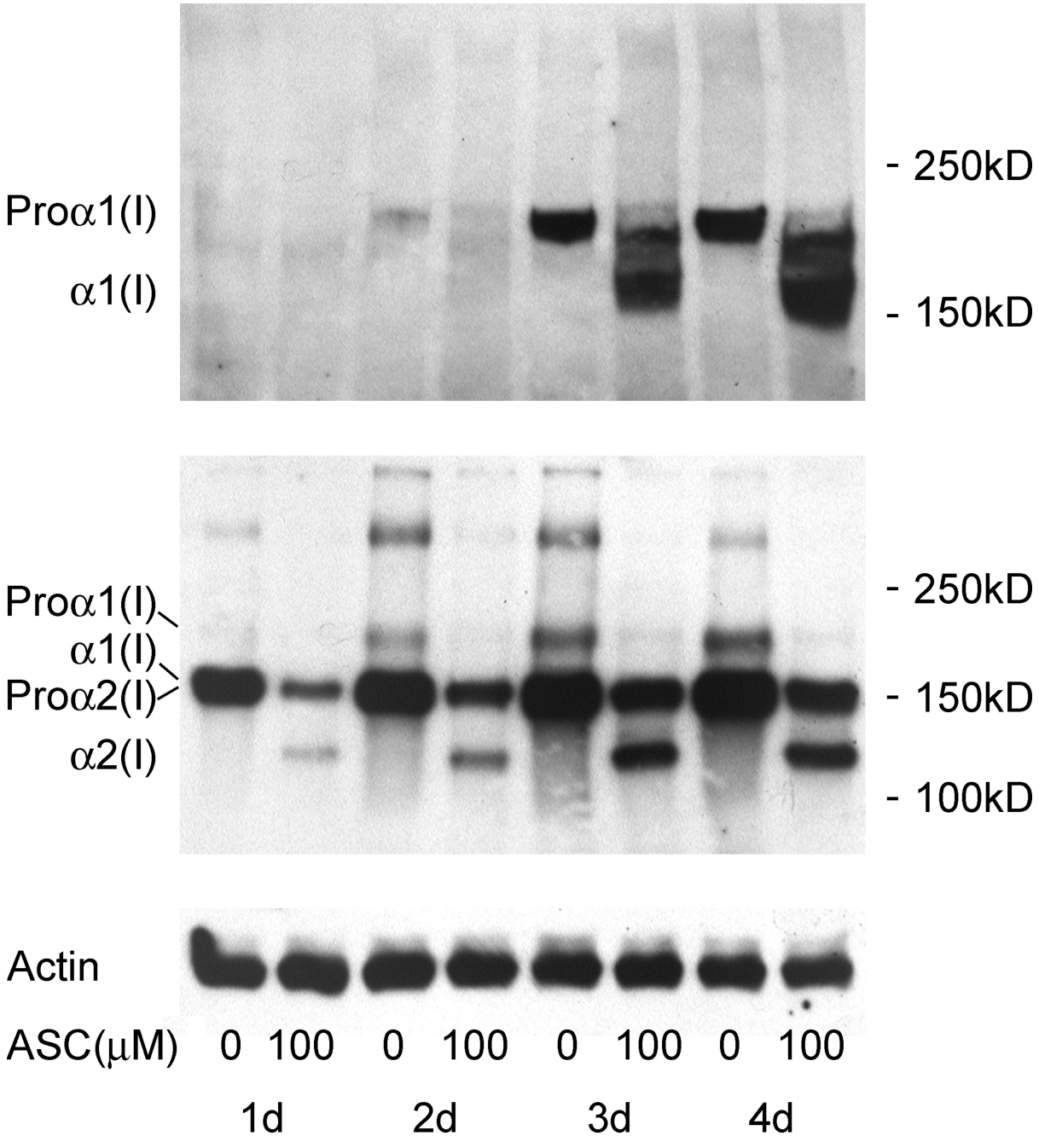

Figure 5. Ascorbate stimulates type I collagen generation in A10 cells as measured by immunoblotting

A10 cells were cultured for up to 4 days in the absence or presence of ascorbate as noted, before 3 rinses with KRH and removal for SDS-gel electrophoresis. The top panel shows results with an antibody specific for α1(I) collagen, the middle panel shows results with an antibody cross-reactive with both α1(I) and α2(I) collagen, and the bottom panel shows the corresponding immunoblots of cell actin, which was used to assess gel protein loading. Locations of molecular weight markers are shown on the right of the immunoblot.

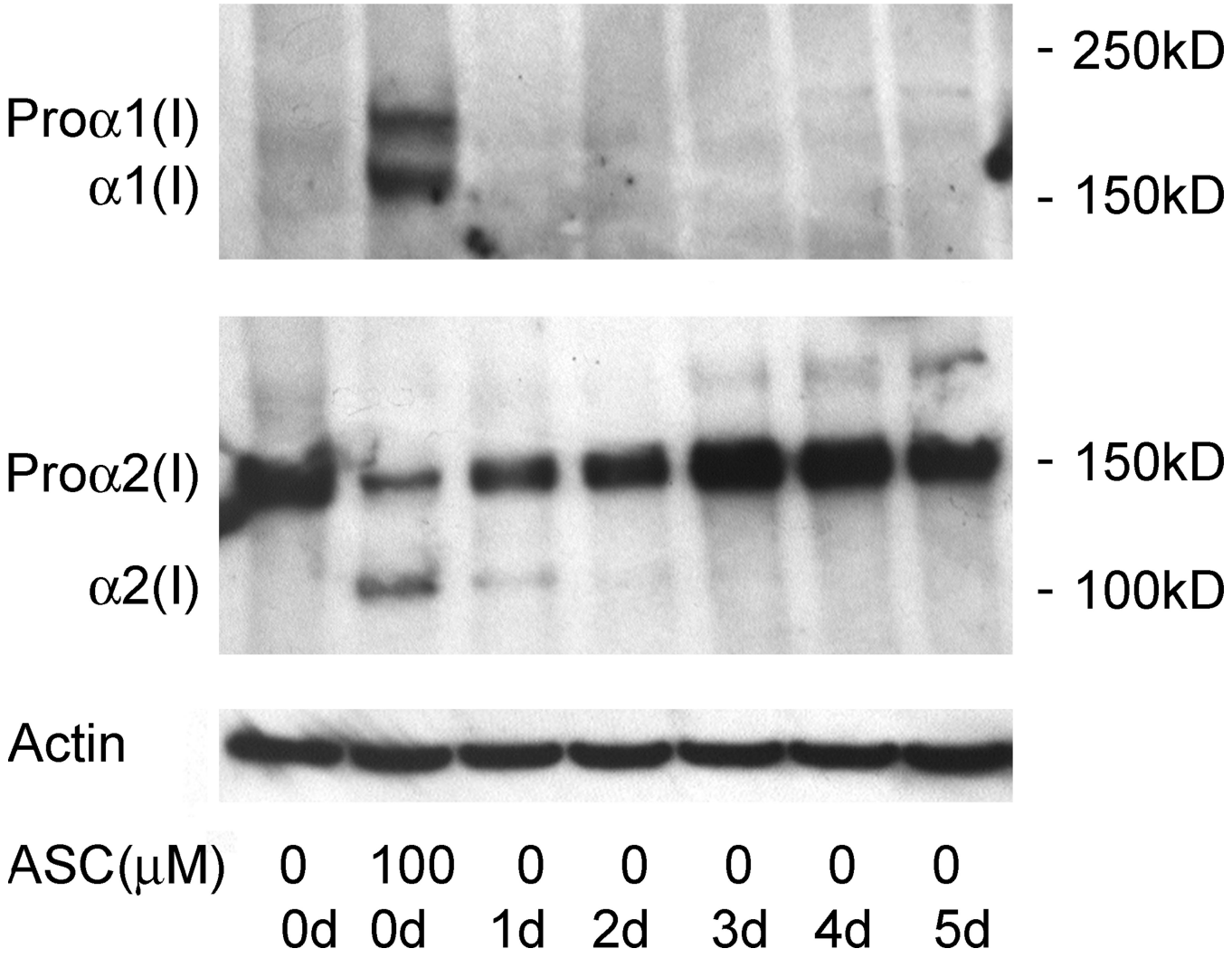

Figure 6. Removal of ascorbate decreases type I collagen generation in A10 cells

A10 cells were cultured for 3 days without or with daily additions of 100 μM ascorbate ("0d"), then rinsed in culture medium and ascorbate-treated cells were cultured for the number of days indicated before rinsing, cell lysis, and immunoblotting for the content of α1(I) collagen (top panel), for both α1(I) and α2(I) collagen (middle panel), and for actin (bottom panel). Actin staining was used to assess gel protein loading.

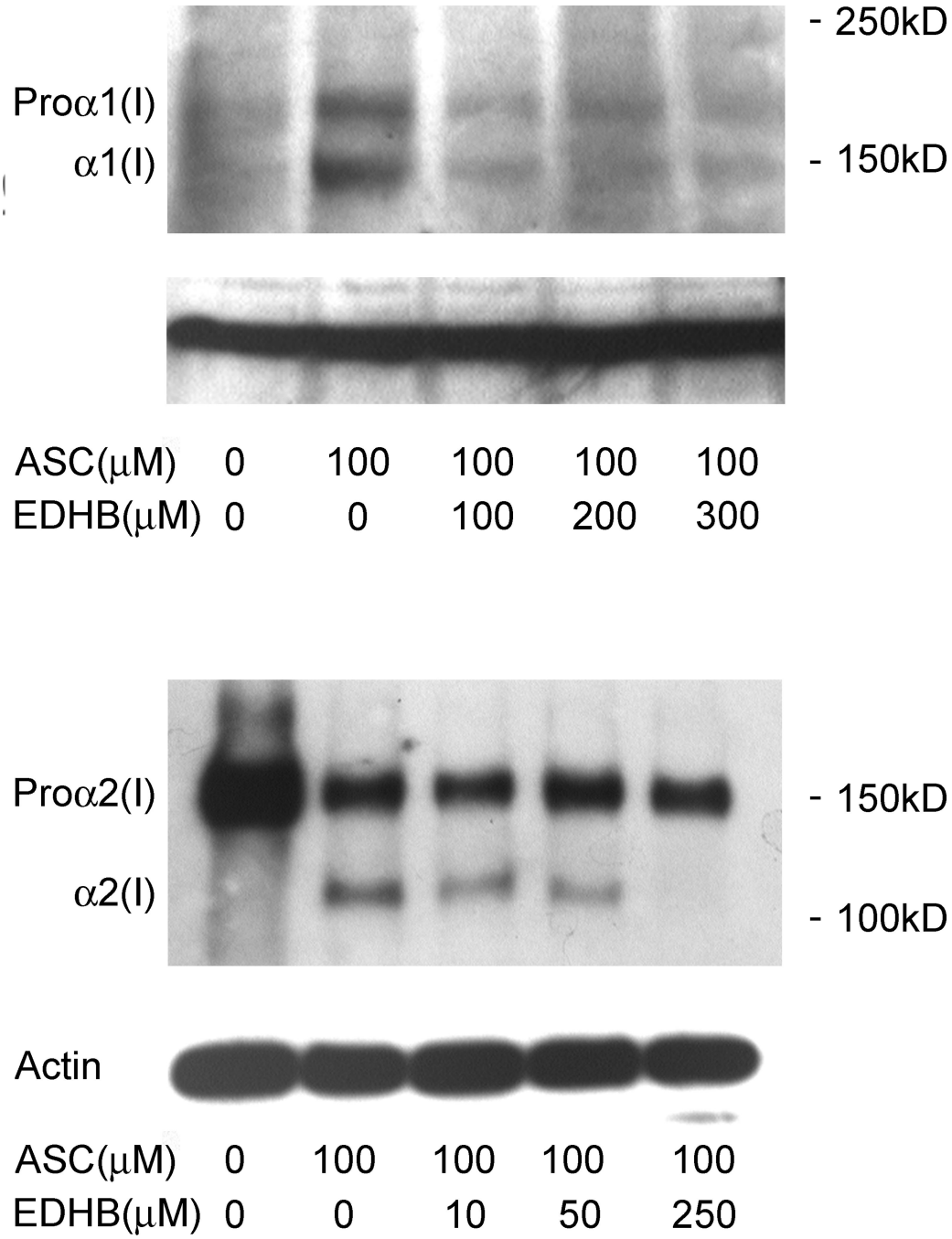

Figure 7. Ethyl-2,4-dihydroxy benzoic acid inhibits ascorbate-stimulated type I collagen synthesis in A10 cells

A10 cells that had been cultured for 2 days in the absence or presence of daily additions of 100 μM ascorbate were also treated with the indicated concentrations of ethyl-2,4-dihydroxy benzoic acid before cell removal for immunoblotting for the content of α1(I) collagen (top panel), both α1(I) and α2(I) collagen (middle panel), and for actin (bottom panel). Actin staining was used to assess gel protein loading.

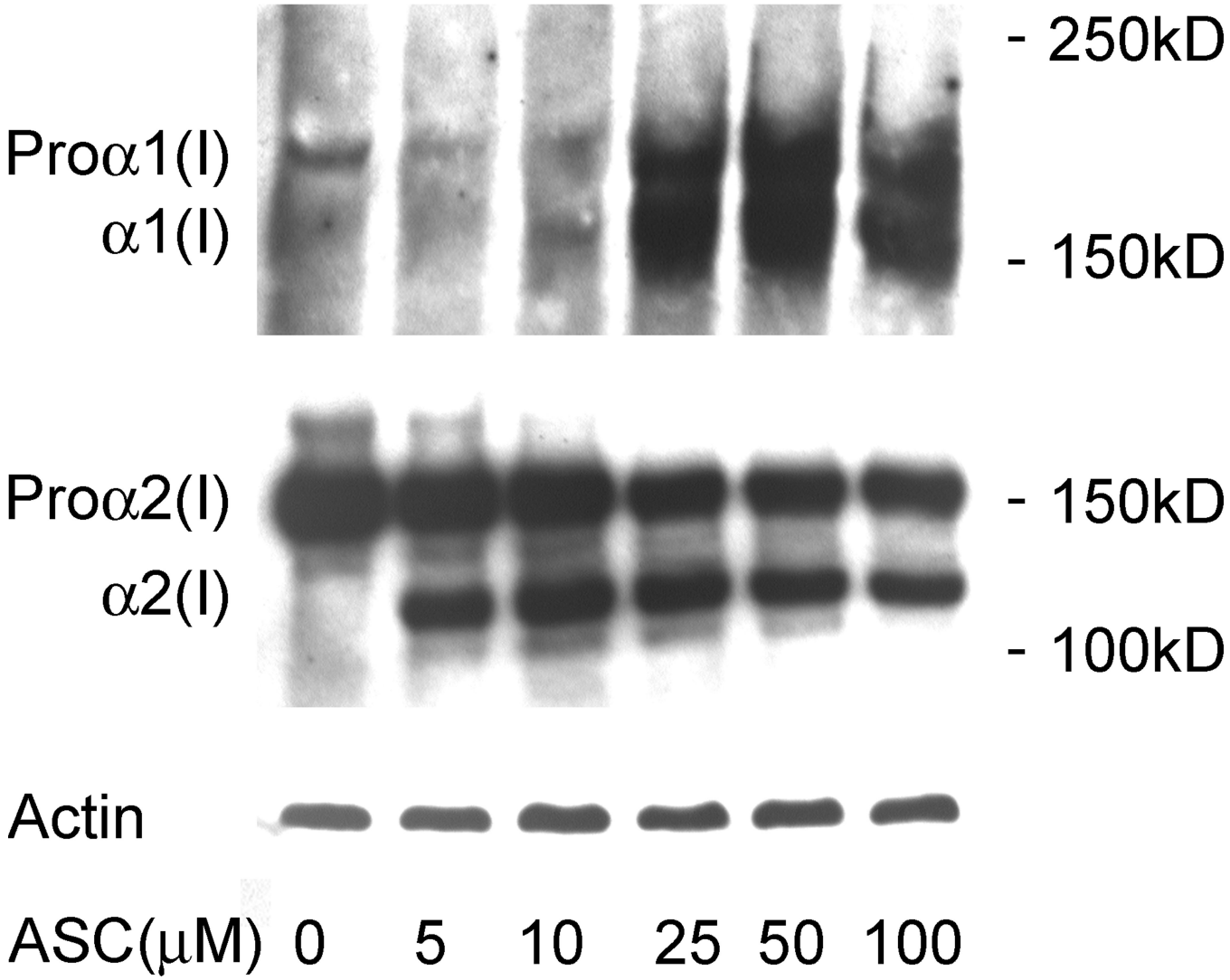

Figure 8. Stimulation of A10 cell type I collagen generation by ascorbate is concentration-dependent

A10 cells were cultured for 3 d with daily additions of the indicated ascorbate concentration before rinsing and removal for immunoblotting for the content of α1(I) collagen (top panel), both α1(I) and α2(I) collagen (middle panel), and for actin (bottom panel). Actin staining was used to assess gel protein loading.

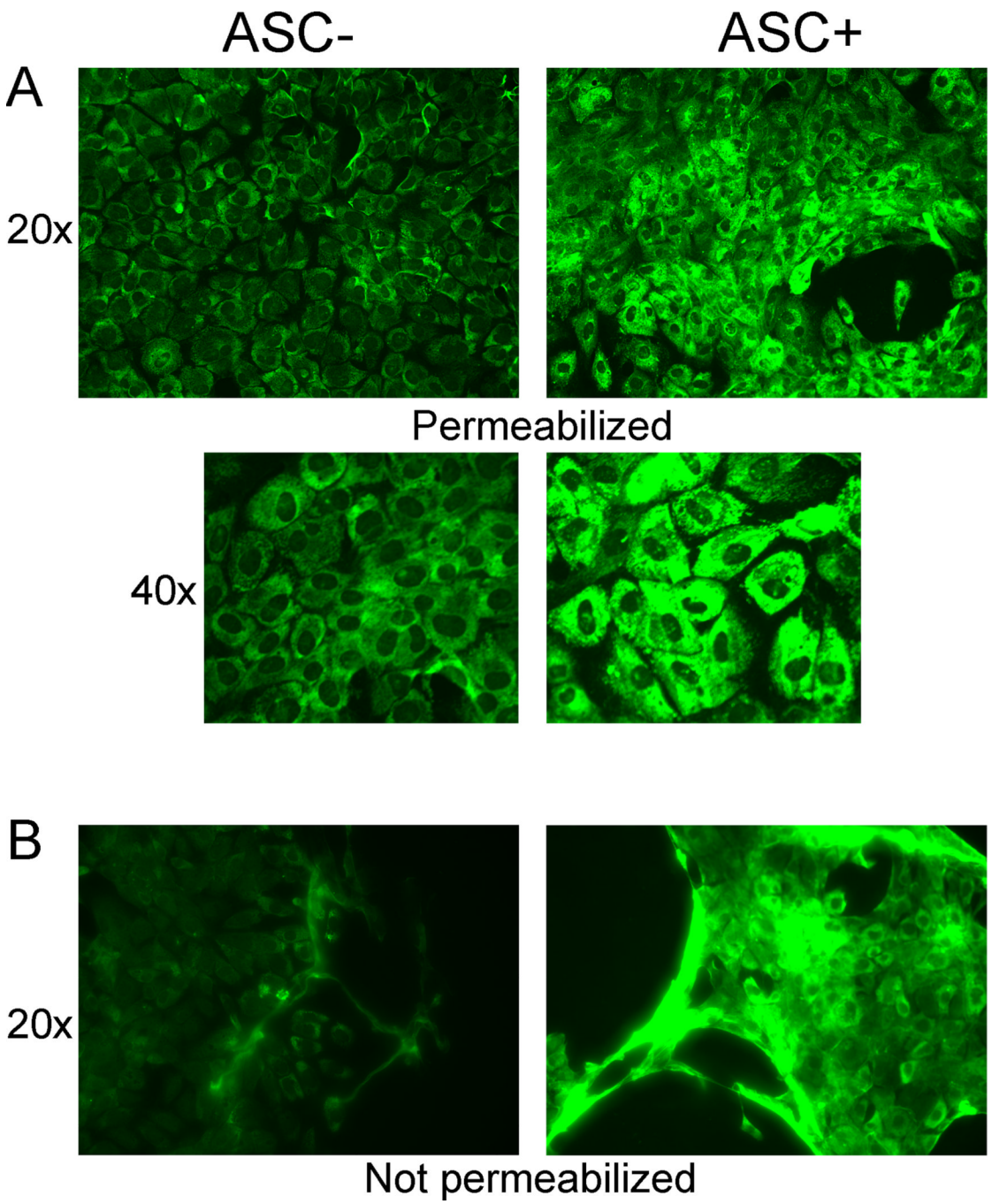

Figure 9. Ascorbate stimulates type I collagen deposition in immunostains of A10 cells

A10 cells were cultured for 6 days in the absence (column labeled ASC-) or presence of daily additions of 50 μM ascorbate (column labeled ASC+) before they were rinsed in KRH and either permeabilized with 0.3% Triton X-100 (two top panel rows, 20× or 40× magnification) or not permeabilized (bottom panel row, 20× magnification) before immunostaining with an antibody that recognizes both α1(I) and α2(I) collagen.