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Chemico-Biological Interactions 130–132 (2001) 627–636

Chemico-Biological  
Interactions

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## Regulation of vascular smooth muscle cell growth by aldose reductase

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### Abstract

Aldose reductase (AR) is a broad-specificity aldo-keto reductase with wide species and tissue distribution. The enzyme has been implicated in the development of pleiotropic complications of long-term diabetes. However, the euglycemic function of the enzyme remains unclear. To examine its potential role in cell growth, changes in AR mRNA and protein were measured in human aortic smooth muscle cells exposed in culture to serum or thrombin. Stimulation by these mitogens led to an increase in the abundance of AR mRNA and protein. Furthermore, inhibition of the AR by tolrestat and sorbinil diminished DNA synthesis and cell proliferation in response to serum. Immunohistochemical staining with anti-AR antibodies revealed no significant expression of AR in the smooth muscle cells of rat carotid arteries. However, 10 and 21 days after balloon injury, intense staining was associated with the proliferating cells of the neointima. Treatment of these animals with 40 mg/kg/day sorbinil diminished the ratio of neointima to the media. Together, these observations suggest that, in vascular smooth muscle cells (VSMC), AR is a growth-responsive gene product and that inhibition of AR prevents VSMC growth and decreases intimal hyperplasia and restenosis. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

*Keywords:* Aldose reductase; Rat carotid artery; Restenosis; Vascular smooth muscle cells

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PII: S0009-2797(00)00290-8

## 1. Introduction

Aldose reductase (AR) is a broad-specificity aldo-keto reductase. The enzyme is widely distributed in human and animal tissues. The active form of the protein is a monomer that catalyzes the reduction of aldo-keto sugars and aliphatic and aromatic aldehydes (for a review see [1]). The physiological function of the enzyme is currently unknown. In the kidney, AR-mediated generation of sorbitol from glucose represents an intrinsic mechanism of osmoregulation [2]. However, high levels of AR are constitutively expressed in heart, skeletal muscle and brain; tissues that do not usually experience high osmotic stress, indicating involvement of the enzyme in processes other than osmoregulation. Indeed, glucose is a poor substrate of AR and that the active site of the enzyme is not optimized for carbohydrate binding [3]. Our recent data show that AR is particularly efficient at reducing medium to long-chain hydrophobic aldehydes [4]. Such aldehydes are common constituents of several food substances and drugs. However, the most abundant source of these aldehydes is lipid peroxidation [5]. Due to multiple unsaturated centers, cellular lipids are particularly vulnerable to oxidation. Thus, in the absence of adequate quenching and detoxification, free radicals derived from oxygen and other redox metabolites initiate and sustain peroxidative reactions in the membrane. These reactions generate a variety of reactive metabolites, including peroxides, epoxides and aldehydes. Previous studies have shown that aldehydes are the major end products generated during the oxidation of lipoprotein and membrane lipids [5]. Several of these aldehydes, particularly those with  $\alpha,\beta$ -unsaturation, are highly reactive and react readily with cellular nucleophiles, of which glutathione is most abundant. In addition, these aldehydes also react with membrane lipids, cellular proteins and nuclear DNA to form covalent adducts, leading to persistent and long-term changes in cellular function and gene expression [5]. Our studies show that AR catalyzes the reduction and detoxification of these aldehydes and their glutathione conjugates [4,6], thereby protecting cells from aldehyde toxicity and diminishing the consequences of oxidative stress.

Recent investigations suggest that, in addition to their well recognized toxicological effects, free radicals and reactive oxygen species (ROS) are also essential mediators of cell signaling [7,8]. High concentrations of ROS are generated during cell growth, and increased quenching of these species and prevention of the associated changes in the cellular redox state inhibits mitogenic signaling and cell cycle progression. Interestingly, extensive evidence also suggests that the formation of ROS is increased during programmed cell death and apoptosis, and indicates that changes in the cellular redox state are important regulators of cell death. Thus, it appears that the generation of ROS and the associated redox changes play a critical role in cellular decisions of growth, survival and death.

Although, the ROS affect several cell constituents, membrane lipids are their most significant targets. Thus, part of the cellular effects of ROS may be mediated and sustained by lipid peroxidation reactions and their products. Since aldehydes are the major end-products of lipid peroxidation, it has been suggested that they are second messengers of ROS [5]. Indeed, our studies show that, like ROS, aldehydes

generated from lipid peroxidation cause specific alterations in ion transport, and energy metabolism [9], and stimulate cell growth [10]. Based on these observations, we hypothesized that AR, by regulating the cellular concentrations of these aldehydes, may be an important regulator of the redox cell signaling leading to either cell growth or cell death. To test this view, we examined the involvement of AR in vascular smooth muscle cell (VSMC) growth. Overwhelming evidence demonstrates a critical role of ROS in the VSMC growth *in vitro* and *in vivo* [7,8], providing a facile experimental paradigm for investigating the role of AR.

## 2. Upregulation of AR by mitogenic signaling

To delineate the role of AR in VSMC growth, we examined changes in the expression of AR in response to mitogenic signaling. Human aortic smooth muscle cells were purchased from Clonetics and cultured under conditions described previously [11]. To induce quiescence, the cells were cultured in DMEM containing 0.1% serum. After 24 h, the cells were stimulated with either thrombin (2 U/ml) or serum (10%) or FGF-2 (10 ng/ml), and changes in AR mRNA and protein were examined after 12 h of incubation with these mitogens. As shown in Fig. 1, exposure of the cells to these mitogens led to a two- to fourfold increase in the steady-state abundance of AR mRNA. A four- to sevenfold increase in immunoreactivity to anti-AR antibodies was also observed (data not shown). In parallel experiments, the AR activity was increased two- to threefold, 12 h after stimulation

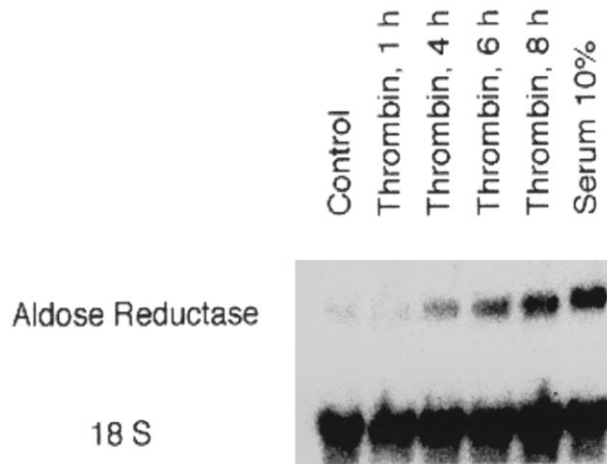


Fig. 1. Serum and thrombin enhance steady-state levels of aldose reductase mRNA. Growth-arrested human aortic smooth muscle cells were treated with either serum (10%) or thrombin (2 U/ml), and total mRNA was extracted from the cells after 8 h (for serum-treated cells) or as indicated (for thrombin-treated cells). Northern blot analysis was performed with  $^{32}\text{P}$ -labeled AR cDNA. Nitrocellulose filters were also hybridized with an  $^{18}\text{S}$  probe to correct for loading differences. Calculated fold-change was determined on the basis of corrected densitometric analysis.

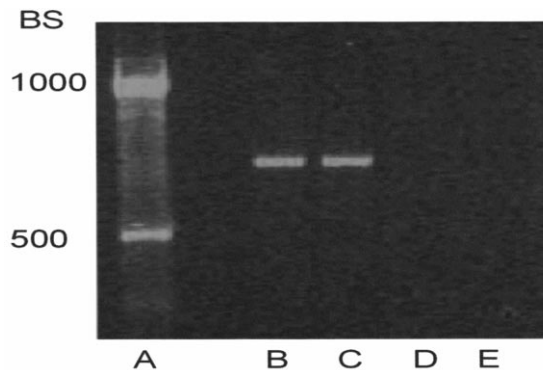


Fig. 2. Absence of aldose reductase mRNA in aorta. Strips of rat and rabbit aorta were dissected to remove the endothelium, and total RNA was extracted from the media. The transcripts were amplified by RT-PCR. Lane (A), markers; lane (B), rabbit glyceraldehyde 3-phosphate dehydrogenase (G3PDH) primer; lane (C), rat G3PDH primer; lane (D), rabbit AR primer; lane (E), rat AR primer.

with serum. These observations indicate that mitogenic stimulation of VSMC stimulates AR, and both AR transcript and protein are enhanced, suggesting a growth-related role of this enzyme. Moreover, stimulation of AR by both thrombin as well as serum suggests that processes regulating the cellular abundance of AR are common to several growth factor receptors, or that the upregulation of AR may be a general feature of VSMC growth.

To examine whether similar changes in AR expression occur *in vivo*, we measured the expression of AR in rat and rabbit aorta. Surprisingly, no expression of AR mRNA was detected in VSMC of the vessel wall. As shown in Fig. 2, reverse transcription-polymerase chain reaction (RT-PCR) failed to detect AR in medial sections of rat and rabbit aorta, although high levels of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcripts were measured. In agreement with this data, we also failed to detect AR immunoreactivity in cross-section of rat carotid arteries. As shown in Fig. 3A, AR was localized to the single layer of endothelium, with little or no staining of the media, although the surrounding adipocytes were intensely stained. Thus, the quiescent, contractile SMC of the media do not express measurable levels of AR. These observations are consistent with previous reports showing lack of expression of AR in normal human [12] and rat [13] arteries.

Under normal physiological conditions, the VSMC regulate the vascular tone and, because contractility is a major function of these cells, they express high levels of contractile proteins such as  $\alpha$ -actin, tropomyosin and smooth muscle myosin heavy chain [14]. Thus, the lack of expression of AR in these cells indicates that the enzyme is not essential for maintaining (housekeeping) functions of VSMC, and suggests that AR is specifically associated with proliferating cells. *In vivo*, the VSMC proliferate in response to injury or abnormal growth stimulus. During such growth, they modulate their phenotype to a less differentiated synthetic state, accompanied by a downregulation of contractile markers and upregulation of synthetic or fetal genes [14]. It is currently believed that the transition from the

contractile to the synthetic phenotype is a prerequisite for SMC growth and proliferation. Hence, to assess whether AR is enhanced during VSMC growth *in vivo*, we used a carotid restenosis model of VSMC growth. For this, we examined the expression of AR in carotid arteries after balloon injury. As described previously [11], the left internal carotid artery was injured by balloon withdrawal creating a denuded area. The right carotid artery remained uninjured and served as a control for each animal. Twenty one days after balloon injury, the carotid arteries were removed, sectioned and stained with anti-AR antibodies. As shown in Fig. 3, cross-sections of balloon-injured carotid arteries showed intense staining that was mainly localized to the proliferating neointima. In contrast, the quiescent cells of the media displayed little or no staining, indicating that the expression of AR protein is considerably enhanced in the neointimal SMC, whereas it remains low or non-existent in the media. Thus, the *in vivo* observations are consistent with the data obtained with mitogen-stimulated VSMC in culture and indicate that the expression of AR is enhanced both during *in vitro* and *in vivo* VSMC growth. Together, these data support the view that, in VSMC, AR is a growth-responsive gene product.

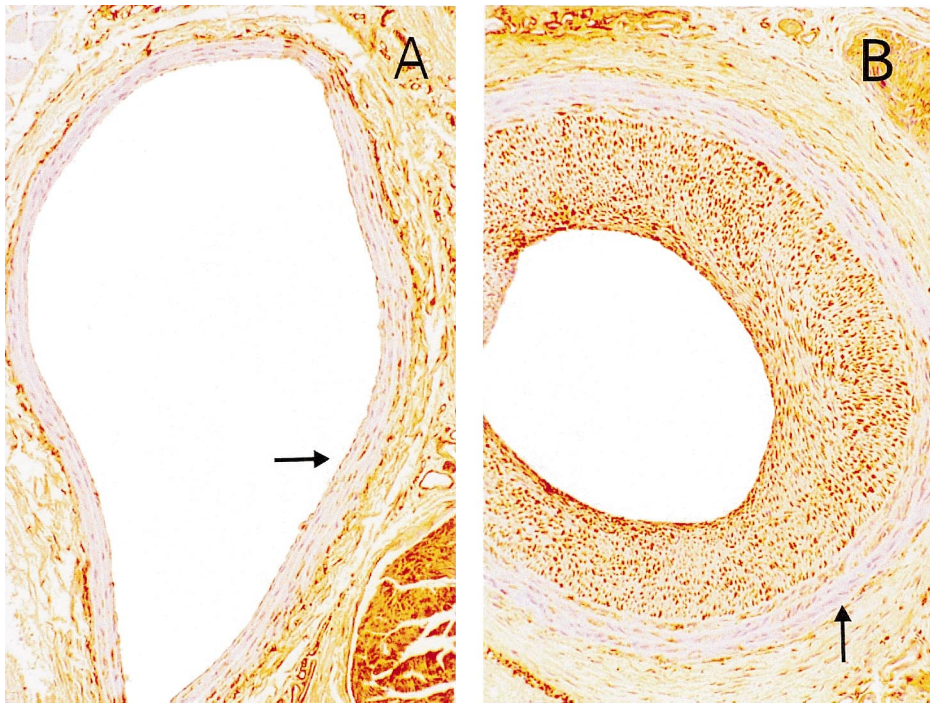


Fig. 3. Expression of AR in rat carotid arteries. Cross-sections of control, uninjured arteries (left panel) and of arteries after 21 days (right panel) of balloon injury were stained with anti-AR antibodies. The immunoreactivity of anti-AR antibodies appears as a dark brown stain, whereas the non-reactive cells show only background hematoxylin and eosin (light blue) staining. Arrows indicate the unstained medial layer.

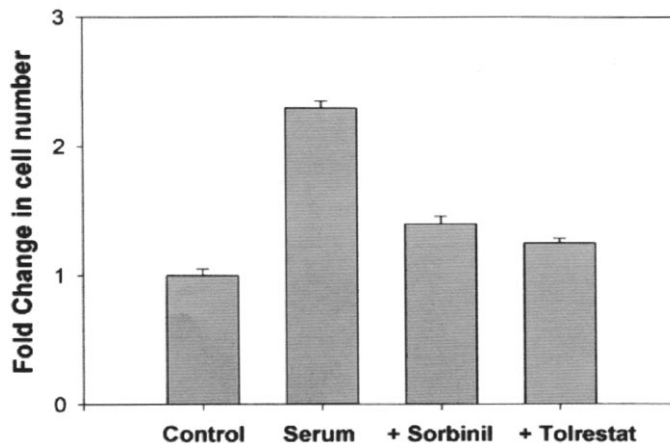


Fig. 4. Effect of aldose reductase inhibitors on vascular smooth muscle cell (VSMC) growth. Growth-arrested VSMC were treated with 10% serum and 10  $\mu$ M sorbinil or tolrestat. The control represents serum-induced cell growth in the absence of AR inhibitors. For assaying proliferation, the cells were counted with a hemocytometer after 72 h of exposure to the indicated culture condition.

### 3. Inhibition of SMC growth by AR inhibitors

Stimulation of AR by mitogenic signaling suggests that AR may be required for cell growth. To test this, the effects of AR inhibitors on VSMC growth in culture and in vivo were examined. For proliferation assay, human aortic smooth muscle cells were cultured in 24-well plates. The cells were treated with 10 or 0.1% fetal calf serum in the presence and absence of AR inhibitors for 72 h, trypsinized and counted in a hemocytometer [11]. The cells were exposed to either 10  $\mu$ M sorbinil or tolrestat. At these concentrations, the AR inhibitors led to a 60–80% decrease in VSMC proliferation (Fig. 4), but did not affect cell viability in the presence of 0.1% serum. Inhibition of VSMC growth by two structurally unrelated AR inhibitors indicates that AR may be an essential component of VSMC growth.

To investigate whether inhibition of AR also prevents VSMC growth in vivo, we examined the effects of sorbinil on the neointima formation following balloon injury. For these experiments, we used sorbinil because previous studies show that the drug is well tolerated by rats [15]. Sorbinil was administered by oral gavage at a concentration of 40 mg/kg/day, 1 day before balloon injury, and was maintained at this dose for the duration of the experiment. Neointima formation was monitored in cross-section of the carotid arteries. Four days after balloon injury, the formation of the neointima was minimal in both the treatment groups, but a marked intimal hyperplasia was observed, 4 and 21 days post-injury (Fig. 5). However, the lesion size, quantified as the ratio of the neointima to the media, was significant but smaller in sorbinil-treated animals. These observations clearly indicate that inhibition of AR diminishes neointimal hyperplasia in balloon-injured carotid arteries, and are consistent with the view that AR is an essential component of VSMC growth.

#### 4. Contribution of AR to VSMC growth

Although our experiments show that AR is upregulated during VSMC growth and that inhibition of the enzyme prevents VSMC proliferation in culture and neointimal hyperplasia in vivo, these data do not provide additional insights into the mechanisms by which AR participates in mitogenic signaling and mediates cell growth. However, our studies on the kinetic and metabolic properties of AR suggest that a critical involvement of the enzyme in the detoxification of lipid-peroxidation-derived aldehydes and their glutathione conjugates [4,6]. Based on these observations, we speculate that the involvement of AR in VSMC growth may be related to its ability to metabolize reactive end products of ROS signaling and to restore the cellular redox state altered by oxidative components of mitogenic signaling.

Several studies demonstrate that mitogens such as PDGF, thrombin and FGF stimulate ROS formation in VSMC [7,8]. That the increased generation of ROS is essential for cell growth is suggested by the observations that interventions which prevent the ROS formation (e.g. increased cellular concentration of catalase) or diminish oxidative stress (e.g. administration of *N*-acetyl cysteine or probucol) diminish cell growth. We propose that the ROS generated by mitogenic stimuli initiate lipid peroxidation reactions, which lead to the formation of several reactive end-products. Because these products are more stable than their free radical precursors and can diffuse to sites distal from their site of origin, they can mediate

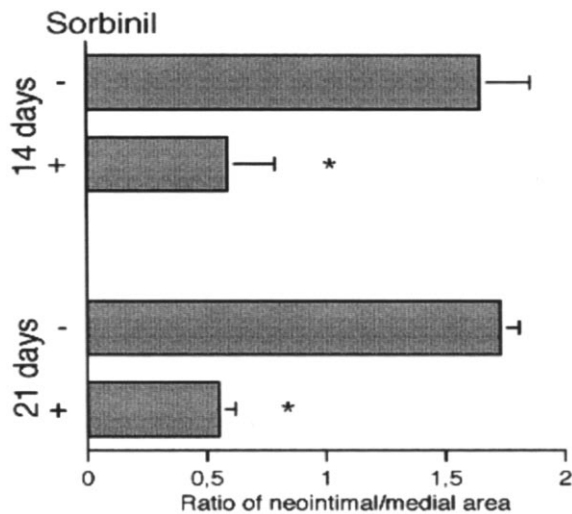
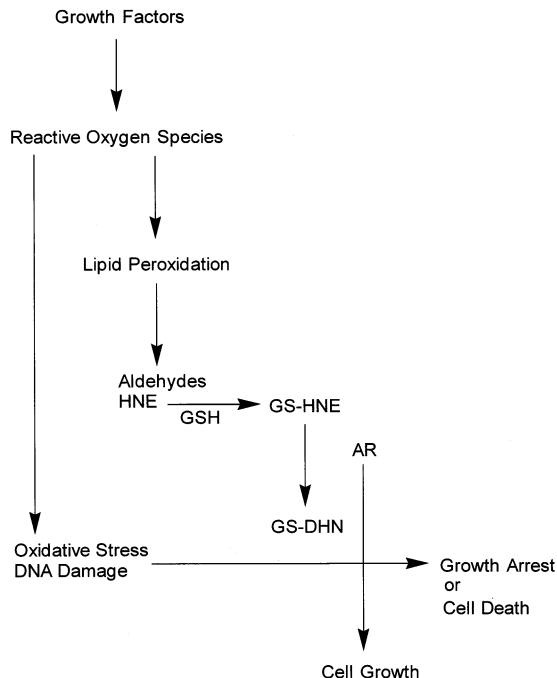


Fig. 5. Inhibition of aldose reductase decreases intimal hyperplasia. Rat carotid arteries were injured by balloon angioplasty and cross-sections of the arteries were obtained 10 and 21 days after balloon injury. The animals received either the vehicle (-) or 40 mg/kg sorbinil per day (+) by oral gavage. Sections were stained with hematoxylin and eosin, and changes in neointimal/media ratio were calculated at indicated days after balloon injury. Data are presented as mean  $\pm$  S.E.M. \*  $P < 0.05$  as compared with control.



Scheme 1. Proposed role of aldose reductase in vascular smooth muscle cell growth.

and sustain long-term oxidative stress and redox modifications, thereby profoundly affecting the expression and the activity of multiple transcription factors and growth mediators. That products of lipid peroxidation are capable of sustaining and initiating growth is suggested by the observations that two of the most abundant products of lipid peroxidation, 4-hydroxy-trans-2-nonenal (HNE) [10] and F2-isoprostanes [11], are potent mitogens, and could be endogenous modulators of cell growth. Metabolism of these aldehydes by AR is, therefore, expected to diminish their cellular concentrations and lifetimes, thereby altering their growth promoting effects. However, in addition to their mitogenic effects at low concentrations, at high concentrations these aldehydes are cytotoxic and cause growth arrest or even cell death. Hence, a likely mechanism of growth arrest induced by AR inhibitors may be the toxicity of lipid peroxidation products accumulating in the absence of AR. In support of this view, it has been recently demonstrated that inhibition of AR enhances HNE accumulation and apoptosis in inflamed arteries [12], further supporting a critical role of AR in regulating the cellular effects of endogenously generated products of lipid peroxidation.

A likely mechanism by which AR could regulate cell growth is shown in Scheme 1. By participating in the metabolism and removal of reactive aldehydes derived from lipid peroxidation, AR could facilitate cell growth by regulating the cellular consequences of ROS and their products, and minimizing oxidative stress generated by growth factors and cytokines. Because conditions of oxidative stress prevent cell

cycle progression, growth arrest by inhibiting AR indicates that inefficient removal of oxidants prevents cell growth. Further experiments to test this view are underway.

Our current studies indicate that inhibition of AR may be a potentially useful approach for minimizing abnormal VSMC growth. Increased proliferation of VSMC is the critical feature of atherosclerosis, restenosis and hypertension. Together, these diseases, which can lead to myocardial infarction and stroke, are responsible for up to one-half of the mortality in the industrialized world [16]. Even though many strategies have been devised to prevent abnormal VSMC growth, most have been targeted to inhibit individual growth factors or signaling mechanisms that lead to cell growth. However, the redundancy of growth factors within the vessel wall [16] limits the efficacy of interrupting specific mitogens. Furthermore, the second messengers of growth factors (e.g. tyrosine kinases and G-proteins) as well as the immediate–early responses they elicit, are common to a variety of stimuli and regulate multiple cellular functions. Because of these pleiotropic effects, the toxicity associated with inhibitors of such signaling (e.g. tyrosine kinase inhibitors) is expected to be high [17]. In contrast, inhibition of AR, which appears to prevent growth by both G-protein and tyrosine kinase receptors, may be a more general approach to inhibiting abnormal VSMC growth. Several relatively specific AR inhibitors have been synthesized and are currently under clinical trial for the treatment of secondary diabetic complications. Moreover, the structure of AR is known in detail [1,4], so that, if necessary, even more selective inhibitors could be designed.

### Acknowledgements

This work was supported in part by NIH grants HL55477 and HL59378 (A.B.); a grant from the American Heart Association–Ohio Valley Affiliate (S.Q.L.) and a research scholarship Ru 620/1-1 from the German Research Foundation DFG (J.R.).

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