Aldose Reductase Mediates Mitogenic Signaling in 
Vascular Smooth Muscle Cells.

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$The abbreviations used are: AR, aldose reductase; ARI, aldose reductase inhibitor(s); TNF-α, tumor necrosis factor-alpha; NF-κB, nuclear factor-kappa binding protein; VSMC, vascular smooth muscle cells.
ABSTRACT

Abnormal vascular smooth muscle cell (VSMC) proliferation is a key feature of atherosclerosis and restenosis, however, the mechanisms regulating growth remain unclear. Herein we describe that inhibition of the aldehyde-metabolizing enzyme aldose reductase (AR) inhibits NF-κB activation during restenosis of balloon-injured rat carotid arteries as well as VSMC proliferation due to tumor necrosis factor (TNF-α) stimulation. Inhibition of VSMC growth by AR inhibitors was not accompanied by increase in cell death or apoptosis. Inhibition of AR led to a decrease in the activity of the transcription factor NF-κB in culture and in the neointima of rat carotid arteries after balloon injury. Inhibition of AR in VSMC also prevented the activation of NF-κB by fibroblast growth factor (bFGF), Angiotensin-II (Ang-II) and platelet-derived growth factor (PDGF-AB). The VSMC treated with AR inhibitors showed decreased nuclear translocation of NF-κB, and diminished phosphorylation and proteolytic degradation of IκB-α. Under identical conditions, treatment with AR inhibitors also prevented the activation of protein kinase C (PKC) by TNF-α, bFGF, Ang-II, and PDGF-AB but not phorbol esters, indicating that AR inhibitors prevent PKC stimulation or the availability of its activator, but not PKC itself. Treatment with antisense AR, which decreased the AR activity by >80 %, attenuated PKC activation in TNF-α, bFGF, Ang-II, and PDGF-AB-stimulated VSMC and prevented TNF-α-induced proliferation. Collectively, these data suggest that inhibition of NF-κB may be a significant cause of the antimitogenic effects of AR inhibition and that this may be related to disruption of PKC-associated signaling in the AR-inhibited cells.
INTRODUCTION

Aldose reductase (AR) catalyzes the reduction of a wide range of aldehydes (1). The substrates of the enzyme range from aromatic and aliphatic aldehydes to aldoses such as glucose, galactose, and ribose. The reduction of glucose by AR is particularly significant during hyperglycemia and increased flux of glucose via AR has been etiologically linked to the development of secondary diabetic complications (1,2). However, recent studies showing that AR is an excellent catalyst for the reduction of lipid peroxidation-derived aldehydes and their glutathione conjugates (3-8) suggest that in contrast to its injurious role during diabetes, under normal glucose concentration, AR may be involved in protection against oxidative and electrophilic stress. The antioxidant role of AR is consistent with the observations that in a variety of cell types AR is upregulated by oxidants such as hydrogen peroxide (9), lipid peroxidation-derived aldehydes (10,11), advanced glycosylation end products (AGE; 12) and nitric oxide (13). The expression of the enzyme is also increased under several pathological conditions associated with increased oxidative or electrophilic stress such as iron overload (14), alcoholic liver disease (15), heart failure (16), myocardial ischemia (17), vascular inflammation (11) and restenosis (10). Together, these observations provide strong support to the view that AR protects against the toxicity of electrophilic aldehydes and their glutathione conjugates by reducing them to less toxic or metabolic inert alcohols.

Although glucose is a poor substrate of AR, the enzyme is recruited in renal tissues to generate sorbitol for balancing the osmotic gap during diureseis (18). The abundance and the transcription of the AR gene are dramatically enhanced by the activation of the transcription factor-TonE-binding protein (19,20). However, osmotic role of AR in non-renal tissues is unclear, and the high expression of the enzyme in tissues such as heart, blood vessels, skeletal
muscle or brain suggests that the enzyme may be involved in processes other than osmoregulation and glucose metabolism. Recent evidence shows that in addition to osmotic or oxidative stress, AR and its homologs are also upregulated by mitogenic stimuli. Stimulation of NIH 3T3 cells by FGF-1 (and to a lesser extent by FGF-2, EGF and phorbol esters) leads to a dramatic increase in the expression of an aldo-keto reductase-FR-1, (21) which is related to AR in structure and function (21,22). The AR protein itself is also increased by growth factors in the 3T3 fibroblasts (23), astrocytes (24) and the vascular smooth muscle cells (VSMC; 10). Although the quiescent VSMC of the tunica media do not express detectable levels of AR, the expression of the enzyme is markedly induced during vascular inflammation or growth (10,11). Moreover, we have previously shown that inhibition of AR prevents serum-induced VSMC growth in culture and neointima formation in balloon-injured rat carotid arteries (10). However, the mitogenic events regulated by AR have not been identified and the mechanism by which AR prevents VSMC growth remains unclear. We, therefore, examined the participation of AR in VSMC mitogenesis in response to TNF-α, which is the main mitogen driving neointima formation in vivo (25,26) and various growth factors.

EXPERIMENTAL PROCEDURES

Materials-Dulbecco's Modified Eagle's Medium (DMEM), Phosphahte buffered saline (PBS), penicillin/streptomycin solution, trypsin and fetal bovine serum (FBS) were purchased from GIBCO BRL Life Technologies (Grand Island, NY). Antibodies against IκB-α and p65 were obtained from Santa Cruz Biotechnology. Phospho-IκB-α (Ser32) antibody was purchased from New England BioLabs. Mouse anti-rabbit GAPDH antibodies were obtained from Research Diagnostics Inc., and anti-AR polyclonal antibodies against recombinant AR were
raised in rabbits. LipofectAMINE Plus and Opti-minimal essential medium were obtained from Life Technologies, Inc. Aldose reductase antisense oligonucleotide (5’-CCTGGGGCAGTCAATGTGG-3’) and mismatched control (scrambled) oligonucleotide (5’-GGTGATAGCTGACGCGGTCC-3’) were used for transfection in VSMC to prevent the translation of AR mRNA. Consensus oligonucleotide for NF-κB transcription factor (5’-AGTTGAGGGGACTTTCCCAGGC-3’) was obtained from Promega Corp. Mouse NF-κB monoclonal antibodies against p65 subunit that selectively binds to the activated form of NF-κB were obtained from Chemicon International Inc. Phorbol 12-myristate 13-acetate (PMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and other reagents used in the EMSA and western blot analysis were obtained from Sigma. All other reagents used were of analytical grade.

Immunohistochemistry of balloon-injured rat carotid arteries-The carotid arteries of adult male Sprague-Dawley rats were injured as described previously (10). Briefly, the rats were anesthetized by an intraperitoneal injection of ketamine (2 mg/kg) and xylazine (4 mg/kg). The left carotid artery was injured by balloon withdrawal 3 times, thus creating a denuded area. The right carotid artery was left uninjured and served as a control for each animal. Starting 1 day before injury and throughout the observation time, the animals were fed either the AR inhibitor-tolrestat (10 mg/kg/day) or PBS. There were no signs of toxicity related to drug exposure. Ten days after injury, the arteries were perfusion-fixed with 4% paraformaldehyde and stored in 70% ethanol. Five micron sections of formalin fixed, (fixation limited to 18 hours and tissues held in 70% alcohol until processed) paraffin embedded tissues taken from rat aorta, were stained with mouse monoclonal antibodies against activated RelA (p65) subunit of NF-κB from Chemicon (MAB 3026). Following deparaffinization and hydration, the sections were placed in a pressure
cooker in Target Retrieval Solution (Dako Cat # S1699) consisting of a citrate buffer (pH 6.0) for 27½ minutes. Slides were cooled rapidly and immunostained using the Dako Autostainer. The slides were washed in Tris buffer (Dako Cat # S1968), endogenous peroxidase was removed with 3% hydrogen peroxide. The slides were incubated in primary antibody, anti-NF-κB diluted at 1:100 (10 µg of the primary antibody) for 120 min. Slides were incubated in the detection system, (Dako Cat # K0609), link and label each for 20 minutes. Slides were then incubated in the chromogen-diaminobenzidine (Dako Cat # K3466) for 10 min. Nuclei were stained in Mayer’s hematoxylin at ½ the strength. Areas of positive reactivity are stained brown.

**Cell culture**-Rat VSMC were isolated from healthy rat aorta and characterized by smooth muscle cell specific α-actin expression. VSMC were maintained and grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

**Cytotoxicity assays**-The rat VSMC were grown in DMEM and were harvested by trypsinization and plated in a 96-well plate at a density of 2,500 or 5,000 cells/well. Cells were grown 24 h in the indicated media and were growth-arrested at 60 to 80% confluency for 24 h in media containing 0.1% FBS. Low serum levels were maintained during growth arrest to prevent slow apoptosis that accompanies complete serum deprivation of these cells. The growth-arrested cells were treated with TNF-α (10 pM to 10,000 pM), or AR inhibitors (0.5 µM to 20 µM), or medium containing both TNF-α and AR inhibitors for another 24 h. The rate of cell proliferation or apoptosis was determined by cell count, MTT assay or the incorporation of [³H]-thymidine.

**Cell number**-The loss of membrane integrity indicated by the inability of the cells to exclude trypan-blue was used to measure cell viability using a hemocytometer. Briefly, the cells
were harvested by trypsinization, washed and suspended in PBS, and incubated with equal amount of 0.1% trypan-blue. The percentage of trypan-blue positive cells was calculated and the values from 4 separate experiments for each treatment were used for statistical analysis.

**MTT assay**_ Twenty five microliters of 5 mg/ml MTT were added to each well of the 96-well plate plated with VSMC. The plate was incubated at 37°C for 2 h. The formazan granules generated by the live cells were dissolved in 100% DMSO and absorbance at 562 nm was monitored using a multiscanner ELISA autoreader.

**Thymidine-incorporation**_[^H]-thymidine (10 μCi/ml) was added to the cells 6 h prior to the end of the growth-arrest protocol. After mitogenic stimulation, the cells were harvested on Millipore multiscreen system, 96-well filtration plates and were washed with PBS using multiscreen separation systems vacuum manifold. Filters were air-dried and the radioactivity was measured using a LS1801 Beckman counter.

**Apoptosis**_ Cell death was assessed by using “Cell Death Detection ELISA” kit (Roche Inc.) that measures cytoplasmic DNA-histone complexes, generated during apoptotic DNA fragmentation, and cell death detection was performed according to the manufacturer's instructions and monitored spectrophotometrically at 405 nm.

**Caspase-3 activity**_ The activity of caspase-3 was measured by using the specific caspase-3 substrate Z-DEVD-AFC, (CBZ-Asp-Glu-Val-Asp-AFC), which was incubated with cell lysate and the fluorescence (excitation: 400 nm, emission: 505 nm) released by the cleavage of substrate was measured by using fluorescence 96-well plate reader.

**Electrophoretic mobility gel shift assays (EMSA)**_—Cytosolic and nuclear extracts were prepared as described (27). Consensus oligonucleotide for NF-κB transcription factors was 5'-end labeled using T4 polynucleotide kinase. The assay procedure was as described before (27).
Briefly, nuclear extracts prepared from various control and treated cells were incubated with the labeled oligonucleotide for NF-κB for 15 min at 37°C, and the DNA-protein complex formed was resolved on 6.5% native polyacrylamide gels. The specificity of binding was examined by competition with excess of unlabeled oligonucleotide. Supershift assay was also performed to determine the specificity of NF-κB binding to its specific consensus sequence by using anti-p65 antibodies. After electrophoresis, the gels were dried by using a vacuum gel dryer and were autoradiographed on Kodak X-ray films. The radiolabeled bands were quantified by an Alpha Imager 2000 Scanning Densitometer equipped with the AlphaEase™ Version 3.3b software.

**Western blot analysis**—Equal amount of either cytoplasmic or nuclear extracts were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were electroblotted to nitrocellulose filters and probed with rabbit polyclonal antibodies against either IκB-α or IκB-α-phosphorylated at Ser-32 or p65. The antibody binding was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, NJ).

**Protein kinase C assay**—The VSMC, with or without mitogenic stimulation were washed twice with an ice-cold PBS, and sonicated with three10-second bursts in 1 ml of the extraction buffer (25 mM Tris-HCl, pH 7.5 containing 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM 2-mercaptoethanol, 1μg/ml leupeptin, 1μg/ml aprotinin and 0.5 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged at 100,000 g for 60 min at 4°C in a Beckman ultracentrifuge. The pellets containing the membrane fraction were solublized by suspending in the assay buffer containing 1% Triton X-100 and stirring at 4°C for 1 h. The PKC activity was measured by using the Promega Signa TECT PKC assay system. Aliquots of the reaction (25 mM Tris-HCl pH 7.5, 1.6 mg/ml phosphatidylserine, 0.16 mg/ml diacylglycerol,
and 50 mM MgCl₂) were mixed with [α⁻³²P] ATP (3,000 Ci/mmol, 10 µCi/µl) and incubated at 30°C for 10 min. To stop the reaction, 7.5 M guanidine hydrochloride was added and the phosphorylated peptide was separated on binding paper. After the paper was washed, the extent of phosphorylation was detected by determining the radioactivity. The incorporation of radioactivity was linear for 15 min, and the PKC activity was determined by subtracting the initial rate of protein kinase activity (in the absence of activators) from the rate of protein kinase activity in the presence of phosphatidylinerine, and diacylglycerol.

Antisense Ablation of AR _ VSMC grown to 60-70% confluency in DMEM supplemented with 10% FBS were washed with opti-minimal essential medium for four times, 60 min before the transfection with oligonucleotides. The cells were incubated with 1 µM AR antisense or scrambled control oligonucleotides using LipofectAMINE Plus (15 µg/ml) as the transfection reagent as suggested by the supplier. After 12 h, the medium was replaced with fresh DMEM (containing 10% FBS) for another 12 h followed by 24 h of incubation in serum free-DMEM (0.1% FBS) before TNF-α stimulation. Changes in the expression of AR were estimated by Western blot analysis using anti-AR antibodies and by measuring the AR activity in the total cell lysate.

RESULTS

Inhibition of AR diminishes NF-κB activation _We have previously reported that inhibition of AR prevents serum-induced VSMC growth in culture and decreases neointima formation in balloon-injured carotid arteries (10). However, the mechanism by which AR facilitates VSMC growth was not examined. Because the transcription factor NF-κB plays a central role in VSMC mitogenesis (28-30) and activated NF-κB has been localized to
atherosclerotic lesions and restenotic vessels (31), we examined the effect of AR inhibition on NF-κB activity in balloon-injured arteries. Rat carotid arteries were injured as described before and were stained with antibodies that specifically recognize activated NF-κB. As shown in Fig. 1, no significant staining by antibodies directed against activated NF-κB was observed in control, uninjured carotid arteries. However, arteries obtained after 10 days of balloon injury displayed intense staining, and the intensity of staining was significantly lower in the arteries of rats fed tolrestat, indicating that inhibition of NF-κB activation could be one of the mechanisms by which AR inhibitors diminish neointimal hyperplasia. To further assess the significance of this finding and to delineate the processes in mitogenic signaling sensitive to AR inhibition, we examined the antimitogenic effects of AR inhibitors with VSMC in culture. For these experiments we tested the effects of AR inhibition on TNF-α–mediated VSMC growth, because cell growth in injured vessels has been shown to be to a large extent due to TNF-α (25,26).

*Attenuation of TNF-α-induced VSMC proliferation*—To investigate the role of AR in the signal transduction pathway of TNF-α leading to VSMC proliferation, we determined the effect of ARI, sorbinil or tolrestat. The extent of VSMC proliferation was determined by following VSMC cell number, MTT assay and DNA synthesis by following thymidine incorporation. The results shown in Fig. 2A demonstrate that the treatment of VSMC with several concentrations of TNF-α ranging from 1 to 12 pM for 24 h significantly stimulated VSMC growth. The increase in growth was attenuated by 10 µM sorbinil added to the incubation media under identical conditions (Fig. 2B). In the absence of TNF-α, increasing concentrations of sorbinil (from 0.1-10 µM) did not affect the growth, indicating that sorbinil by itself does not affect VSMC growth at the concentrations used (Fig. 2B). Similar results were obtained when the proliferation was estimated by counting cell number or by the MTT assay (data not shown). To rule out inhibitor-
specific effects, we also examined the effect of tolrestat, which is structurally different from sorbinil. Like sorbinil, tolrestat also inhibited VSMC proliferation caused by TNF-α (Fig. 2 C-E), but by itself had no effect on cell growth. Thus, inhibition of AR by two structurally-unrelated inhibitors prevents VSMC growth suggesting that AR is an obligatory mediator of TNF-α-induced VSMC growth.

*Attenuation of VSMC proliferation by inhibiting AR is not due to apoptosis*-To demonstrate that the sorbinil or tolrestat-mediated attenuation of TNF-α-induced VSMC proliferation is not due to apoptosis, we measured apoptosis as well as caspase-3 activity under identical conditions used to prevent TNF-α-induced VSMC proliferation by sorbinil or tolrestat. However, neither of these inhibitors caused apoptosis or the activation of caspase-3 (data not shown), indicating that inhibition of AR prevents cell proliferation, not by increasing cell death but by inhibiting VSMC growth.

*Attenuation of TNF-α-induced activation of NF-κB*-We next examined whether in cultured VSMC, inhibition of AR prevents TNF-α–mediated activation of NF-κB as observed in restenotic vessels (Fig 1). Upon stimulation of VSMC with TNF-α, a pronounced activation of NF-κB was observed as determined by EMSA. To examine the role of AR, we preincubated the VSMC for 24 h with different concentrations of sorbinil followed by incubation with TNF-α (0.1 nM) for 60 min at 37°C and determined NF-κB activity by EMSA. To ascertain that the gel-retarded band, observed with the TNF-α-treated cells was indeed due to NF-κB, we incubated the nuclear extract from TNF-α-activated cells with antibodies to p65 subunit followed by NF-κB determination by EMSA. Antibodies to p65 shifted the band to a higher molecular weight (Fig. 3, lane 6), at the same time, the preimmune serum had no effect on the mobility of NF-κB (Fig. 3, lane 7). In addition, excess (20 and 50 fold) cold NF-κB oligonucleotide completely
eliminated the band, indicating that it was specifically due to NF-κB (Fig. 3, lanes 8 and 9). These observations validate our measurement of NF-κB activity and substantiate that the specific activity reported by EMSA is entirely due to NF-κB activation. However, as shown in Fig. 4A almost 60% of the TNF-α-induced NF-κB activation was prevented by 10 µM sorbinil. The extent of inhibition by sorbinil was dose-dependent, although sorbinil by itself did not activate NF-κB even when added to a concentration of 100 µM. On the basis of these observations we conclude that inhibition of AR prevents TNF-α-induced activation of NF-κB.

To examine the mechanisms of inhibition of NF-κB, we tested whether the effect of sorbinil could be overcome by higher concentration of TNF-α. Sorbinil (10 µM)-pretreated or -untreated VSMC were incubated with various concentrations of TNF-α (0-10,000 pM) for 60 min, and the activation of NF-κB was measured. Although, compared to 0.1 nM, 10 nM TNF-α caused a more pronounced activation of NF-κB, the extent of inhibition by sorbinil was unaffected by the concentration of TNF-α (Fig. 4B). To determine the minimum duration of sorbinil exposure required to prevent TNF-α signaling, VSMC were incubated with 10 µM sorbinil for 0-48 h prior to stimulation by TNF-α for 60 min. A significant inhibition of TNF-α-mediated activation of NF-κB in cells pre-incubated with ARI for 12 h was observed. However, for maximal inhibition, 24 h pretreatment of VSMC was necessary (Fig. 4C). No significant inhibition of NF-κB activation was observed when sorbinil and TNF-α were added together for 60 min (Fig. 4, lanes 3-5). These results demonstrate that the extent of NF-κB inhibition by sorbinil is independent of the extent to which the pathway is activated, and that the inhibition requires prolonged pre-incubation, suggesting that changes in metabolism and/or gene expression may be necessary for sorbinil to disrupt TNF-α-signaling.
In addition to TNF-α, NF-κB is also activated by a variety of stimuli including growth factors such as PDGF-AB, bFGF, and Ang-II. We, therefore, tested whether inhibition of AR would also prevent activation of NF-κB caused by mitogens other than TNF-α. For this, untreated or sorbinil-treated VSMC were incubated with mitogenic concentrations of bFGF, PDGF-AB and the hypertrophic concentration of Ang-II, and the activation of NF-κB was measured by EMSA. In all instances, a pronounced increase in the activity of NF-κB was observed (Fig. 5), and preincubation of VSMC with sorbinil led to a decreased activation of NF-κB in FGF, PDGF or Ang-II stimulated cells. At the same time inhibition of AR did not attenuate NF-κB activation induced by the phorbol ester, PMA (Fig. 5). On the basis of these observations we conclude that inhibition of AR prevents NF-κB activation, regardless of the nature of the receptor involved in the process.

*Attenuation of TNF-α-induced phosphorylation and degradation of IκB-α and NF-κB nuclear translocation:* Extensive investigations show that phosphorylation, ubiquitination and proteolytic degradation of IκB-α precede the activation of NF-κB in the cytosol and the active dimer of NF-κB translocates to the nucleus, where it binds to specific DNA sequences and activates the transcription of inflammatory genes (32-34). We, therefore, investigated whether the inhibition of AR prevents the phosphorylation and degradation of IκB-α. We determined the effect of sorbinil on the cellular abundance and phosphorylation state of IκB-α protein by Western blot analysis using antibodies against IκB-α and phospho-IκB-α. Upon stimulation of VSMC with TNF-α, a partial IκB-α phosphophorylation in the VSMC was observed within 5 min and complete phosphorylation occurred by 15 min (Fig. 6A). However, when sorbinil-pretreated VSMC were stimulated with TNF-α, little or no phosphorylation of IκB-α was observed for 120
min (maximal observation time). Because the phosphorylated IκB-α is prone to proteolytic degradation, we next determined the effect of sorbinil on the degradation of IκB-α. Upon stimulation with TNF-α, a complete degradation of IκB-α was observed in 15 min and full resynthesis was achieved in 30 min. However, in sorbinil-pretreated cells, no degradation of IκB-α was observed for a total observation time of 120 min (Fig. 6B). Since transcriptional activation by NF-κB requires its nuclear translocation where it can bind to its specific consensus sequences and activate the transcription of target genes, we measured NF-κB activity by EMSA (Fig. 6C) in the nuclear extracts and further identified NF-κB translocation by Western blot analysis using p65 antibodies in the cytosolic and nuclear extracts, 60 min after stimulation with TNF-α. Exposure of VSMC to TNF-α for 30 min resulted in the translocation of NF-κB to the nucleus, which was maximal in 60 min. However, in the sorbinil-pretreated cells, we observed only a partial translocation of NF-κB in 60 min after exposure to TNF-α (Fig. 6D, 6E). From these results it is concluded that sorbinil inhibits the TNF-α-induced phosphorylation of IκB-α, prevents its proteolytic degradation, and attenuates active p65/p50 (NF-κB) dimer translocation from cytosol to nucleus.

Attenuation of PKC activation-TNF-α and other VSMC mitogens are known to activate the PKC family of kinases possibly by first activating phospholipase A2. We therefore, incubated the VSMC without or with sorbinil or tolrestat for 24 h followed by the addition of TNF-α, PDGF-AB, bFGF, Ang-II and PMA. All these agents led to the activation of the total membrane bound PKC activity. The activation of PKC by all the agents except PMA was strongly abrogated by sorbinil as well as tolrestat (Fig. 7A). The PMA-induced PKC activation was not affected by inhibiting AR (Fig.7A) under similar conditions, the activation of cytosolic PKC was not affected by the AR inhibitors themselves (data not shown). Although we used two
structurally-unrelated compounds that selectively inhibit AR (35, 36), the non-specific effects of these drugs could not be rigorously excluded. Therefore, we transfected VSMC with antisense AR oligonucleotides that decreased AR protein expression by >80% (Fig. 7B inset) and also the enzyme activity (data not shown). In contrast to the cells transfected with scrambled oligonucleotides, cells transfected with antisense AR displayed markedly attenuated activation of PKC upon stimulation with TNF-α, bFGF, PDGF-AB or Ang-II (Fig. 7B), indicating that similar to pharmacological inhibition, antisense ablation of AR prevents PKC activation. Moreover, consistent with the pharmacological data, transfection with antisense, but not scrambled oligonucleotides, attenuated TNF-α-induced proliferation as assessed by cell count and MTT assay (Fig. 8). Together, these observations suggest that the anti-mitogenic effects of tolrestat and sorbinil are not a reflection of their non-specific toxicity, but are specific to the inhibition of AR and that reaction product(s) of AR catalysis may be involved in this signaling process.

DISCUSSION

Despite its well-studied role in diabetic complications, the physiological function of AR remains unclear. Extensive investigations show that AR is a broad specificity enzyme that catalyzes the reduction of a wide range of aldehydes. Its kinetic properties are optimized such that the energetics of substrate binding do not contribute to the overall catalytic efficiency of the enzyme (1,6). As a result AR does not display predominant specificity for a unique substrate-product pair, rather it can reduce several types of aldehydes with nearly equal efficiency. Such behavior confers ideal properties to AR for the reduction of multiple aldehydes, such as the aldo-keto sugars and the aldehydes generated by lipid peroxidation (3-8). The broad substrate specificity of
the enzyme precludes its ready implication in a unique metabolic pathway, and suggests that it may be recruited for tissue specific-use under a variety of metabolic contexts.

Additional insights into the physiological functions of the enzyme are provided by recent studies showing that AR is upregulated during cell growth and proliferation (see Introduction). These observations suggest that AR is a growth-responsive protein and may be involved in facilitating metabolic changes that accompany growth. Consistent with this view, we have reported that the expression of the enzyme, while minimal in quiescent VSMC, is markedly enhanced in the neointima (10). A similar increase in the expression of AR was observed upon mitogenic stimulation of VSMC in culture. Moreover, we have shown that inhibition of the enzyme prevents VSMC growth in culture and decreases neointimal hyperplasia in balloon-injured carotid arteries. Hence, to further elucidate the role of this enzyme in VSMC growth, we examined changes in mitogenic signaling elicited by inhibition of AR.

In view of the previous reports demonstrating a central role of NF-κB in VSMC growth (28-30), we examined whether treatment with AR inhibitors prevented the activation of this transcription factor in proliferating arterial lesions. As shown in Fig. 1, inhibition of AR was indeed found to significantly decrease the expression of activated NF-κB in restenotic vessels, indicating that inhibition of NF-κB - mediated signaling may be an important cause of the antimitogenic effects of these drugs. Furthermore, because NF-κB plays a central role in the development of the inflammatory response, and AR is upregulated by cytokines and inflammation, it appears likely that the interaction between AR and NF-κB may be a critical determinant of the inflammatory response. Additionally, since hyperglycemic injury during diabetes has been linked to the activation of NF-κB (37-39), we speculate that the protective
effects of AR inhibitors against diabetic complications may be related to inhibitors ability to inhibit NF-κB activation and resultant inflammation.

The inhibitory effect of AR inhibition on NF-κB activation in situ was faithfully reproduced in culture. As reported before, we found that stimulation by TNF-α led to a pronounced activation of NF-κB in VSMC in culture and this was accompanied by increased VSMC growth. Our observation that pretreatment with two structurally unrelated compounds (sorbinil and tolrestat) prevent the TNF-α– induced activation of NF-κB and cell growth suggests that AR may be an obligatory mediator of TNF-α-signaling. However, the observation that the activation of NF-κB by FGF, PDGF-AB and Ang-II was also inhibited by the AR inhibitors suggests that the inhibition was not dependent on the nature of the initial trigger and that events downstream from receptor activation but proximal to NF-κB may be dependent on AR activity.

To further identify the locus of inhibition, we examined signaling events upstream to NF-κB activation. Our results showing that IkB-α-phosphorylation was abrogated by inhibiting AR, suggest a possible involvement of a specific kinase that is responsible for phosphorylation of IkB-α. The results from Fig.5 suggest that PKC may be a potential candidate for the activation of NF-κB through IkB-α phosphorylation because inhibition of AR did not prevent PMA induced NF-κB activation. Hence we examined whether inhibition of IkB-α-phosphorylation was due to alterations in PKC signaling. Indeed, as demonstrated by our results shown in Fig. 7A, inhibition of AR prevented the activation of PKC and as with NF-κB, the extent of inhibition was not significantly affected by the nature of the PKC activator, and comparable inhibition was observed when PKC was activated by either TNF-α, FGF, PDGF-AB or Ang-II. Interestingly, the activation of PKC by PMA was also insensitive to sorbinil or tolrestat. These observations
suggest that AR is essential for PKC activity. Furthermore, because the AR inhibitors did not affect the PMA-mediated activation of PKC, these results suggest that the anti-mitogenic effects of tolrestat and sorbinil are not due to their non-specificity. This is further corroborated by experiments showing that antisense ablation of AR prevents PKC activation by TNF-α and growth factors, but not PMA and that ablation of AR also inhibits TNF-α-induced growth.

Several investigators have linked PKC activation to AR activity (39-42). Both PKC and AR are coordinately upregulated under similar conditions and recent proteomic analysis shows that at least in the heart, PKC and AR are part of the same signaling complex. The AR-dependence of PKC is not entirely clear. However, it has been suggested that AR activity is required for the synthesis of diacylglycerol, which is an obligatory cofactor for the activation of classical and novel PKC isoforms. This view is consistent with our observation that pre-incubation with AR inhibitors was essential for inhibiting both the PKC and NF-κB signaling, suggesting that the lack of AR activity imposes a metabolic deficiency that interferes with optimal activation of PKC.

In conclusion, the most interesting and novel finding in the present study is that inhibition of AR activity by AR inhibitors or AR translation, diminishes both the TNF-α-induced activation of NF-κB and the VSMC proliferation. The preventive effect of sorbinil on the TNF-α-induced hyperproliferation was comparable to that with the phosphorylation of IκB-α, indicating that the inhibitory action of AR inhibitor on the VSMC growth may be related to disruption of the NF-κB pathway. Our studies further indicate that the inhibition of NF-κB may be related to abrogation of PKC signaling and that AR catalysed reaction product(s) may be an obligatory requirement for the activation of PKC. Further evaluation of this pathway and
assessment of its significance in cell growth will help in understanding the metabolic processes
that underlie signaling events culminating in cell growth.
LEGENDS

Fig. 1: Inhibition of AR prevents NF-κB activation in balloon-injured arteries. Cross sections of balloon-injured arteries were obtained from uninjured rat carotid arteries (A), and after 10 days of injury from rat that were treated with the vehicle (B) or 10 mg/kg/day tolrestat (C) and were stained with antibodies directed against activated NF-κB. Immunoreactivity of the antibodies is evident as a dark brown stain, whereas the non-reactive areas display only the background color. The extent of immunoreactivity was quantified by image analysis and is shown in Panel D. The bars represent mean immunoreactivity in the neointima of 5 animals ± SEM. * P<0.05 compared to control (untreated) rats.

Fig. 2: Inhibition of AR prevents TNF-α-induced proliferation. Growth-arrested rat VSMC were stimulated with the indicated concentrations of either TNF-α or sorbinil for 24 h. Cell proliferation was determined by measuring the incorporation of [3H]-thymidine (10 µCi/ml), added 6 h prior to the end of the experiment. The extent of proliferation is expressed a percent increase compared to serum-starved cells stimulated with the vehicle alone. (A) The dependence of VSMC proliferation on TNF-α concentration in the absence and the presence of 10 µM sorbinil. (B) Inhibition VSMC growth by different concentration sorbinil in the absence and the presence of 2 nM TNF-α. To examine the effect of AR inhibitors the VSMC were incubated with 10 µM sorbinil or tolrestat for 24 h without or with 2 nM TNF-α and the number of cells (C), MTT reactivity (D) and (E) [3H]-thymidine incorporation were measured as described in the text. Control dishes were stimulated with the vehicle alone. Bars represent mean ± SEM (n = 4), * P<0.05, **P<0.01 compared with treatment without the inhibitor.
Fig. 3: Pre-treatment with AR inhibitors prevents TNF-α-induced activation of NF-κB.

Rat VSMC were serum-starved and then left untreated or stimulated under the indicated conditions. Nuclear extracts were prepared and EMSA assay was performed as described in the text. Nuclear extracts of untreated cells (lane 1), cells pretreated with 10 µM sorbinil for 24 h (2), after stimulation with 0.1 nM TNF-α for 1 h (3), after combined treatment with 0.1 nM TNF-α and 10 µM sorbinil for 1 h (4), or 0.1 nM TNF-α and 10 µM tolrestat for 1 h (5). Lanes 6 and 7 show the supershift with p65 antibody or the preimmune sera. Lanes 8 and 9 show competition with 20x and 50x unlabeled oligonucleotide probe. The fold change in the NF-κB activity, determined by densitometric scanning, is indicated at the bottom of each lane.

Fig. 4: Concentration and time-dependence of sorbinil inhibition of TNF-α-stimulated NF-κB activity. (A) Quiescent VSMC were preincubated without or with the indicated concentrations of sorbinil for 24 h, and then stimulated with 0.1 nM TNF-α for 1 h, (B) The VSMC were preincubated without or with 10 µM sorbinil for 24 h followed by stimulation with the indicated concentration of TNF-α for 1 h, (C) VSMC were preincubated without or with sorbinil 10 µM, for indicated time periods and then stimulated with 0.1 nM TNF-α for 1 h. After the different treatment protocols, the nuclear extracts were prepared and NF-κB activity was measured by EMSA as described in the text. Fold change in the NF-κB activity, determined by densitometric scanning, is indicated at the bottom of each lane.

Fig. 5: Inhibition of AR decreases the stimulation of NF-κB by bFGF, PDGF-AB and Ang-II. Quiescent VSMC were preincubated without or with 10 µM sorbinil 24 h, followed by
stimulation with either bFGF (5 ng/ml), PDGF-AB (5 ng/ml), Ang-II (2 µM) or PMA (10 nM) for 2 h as indicated. The nuclear extracts were prepared and the NF-κB activity was measured by EMSA.

Fig. 6: Inhibition of AR prevents phosphorylation and degradation of IκB-α and the resultant nuclear translocation of NF-κB. Quiescent VSMC were left either untreated (left panel) or pre-incubated with 10 µM sorbinil for 24 h (right panel), and then stimulated with 0.1 nM TNF-α. After the indicated duration of exposure, the cells were harvested, lysed and their nuclear and cytosolic extracts were prepared as described in the text. The cytosolic extracts were separated by SDS-PAGE by loading equal amounts of protein in each lane. Western blots were developed using antibodies directed against phospho-IκB-α protein (A) and IκB-α (B). Panel C shows the activity of NF-κB determined by EMSA. The translocation of p65/p50 dimer was determined in the cytosolic extracts (CE) as well as nuclear extracts (NE) by western blot analysis using antibodies against p65 (C), in the presence of TNF-α alone (D) and TNF-α and with sorbinil (E).

Fig. 7: Inhibition of AR abrogates PKC activation. (A) Quiescent VSMC were preincubated with 10 µM sorbinil or tolrestat for 24 h, (B) the VSMC were transiently transfected with AR antisense or scrambled control oligonucleotide as described in the experimental procedures, subsequently the cells were stimulated with TNF-α (0.1 nM), bFGF (5 ng/ml), PDGF-AB (5 ng/ml), Ang-II (2 µM) or PMA (10 nM) for 4 h and the membrane-bound PKC activity was determined as described in the text. In (A) Bars represent mean ± SEM (n= 4). ** P<0.01, ***P<0.001 and ## non significant, compared with the activity without the inhibitor. In (B) Bars
represent mean ± SEM (n=4). * P<0.01, **P<0.001 compared with the activity in the Scrambled control oligonucleotide transfected cells. The inset in B shows the AR expression as determined by Western blot analysis in VSMC transfected with antisense AR.

**Fig. 8: Transient transfection of antisense AR prevents TNF-α-induced proliferation of VSMC.** Quiescent VSMC were either left untreated or preincubated with AR antisense or scrambled oligonucleotides as described in the text. After 24 h of treatment, the cells were stimulated with 2 nM TNF-α or medium and the number of cells (A) and MTT reactivity (B) were measured. Bars represent mean ± SEM (n = 4).
REFERENCES


Figure 2

A. Proliferation (%) vs. TNF-α (nM)

B. Proliferation (%) vs. Sorbinil (µM)

C. Cell count

D. MTT assay

E. [³H]-Thymidine Incorporation

- Control
- Tolrestat
- Sorbinil
- TNF-α
- Tolrestat + TNF-α
- Sorbinil + TNF-α
Figure 4

(A) Sorbinil µM, -TNF-α and +TNF-α

(B) TNF-α pM, -Sorbinil and +Sorbinil

(C) Time hr, Sorbinil and Sorbinil + TNF-α
Figure 5

Control
TNF-α
TNF-α + Sorbinil
TNF-α + Tolrestat

Control
bFGF
bFGF + Sorbinil
PDGFab
PDGFab + Sorbinil
Ang II
Ang II + Sorbinil

PMA
PMA + Sorbinil
PMA + Tolrestat
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Figure 7A

PKC activity (pmol/µg protein/min)

Control
TNF-α
TNF-α + Sorbinil
TNF-α + Tolrestat
Control
bFGF
bFGF + Sorbinil
bFGF + Tolrestat
Control
PDGF-AB
PDGF-AB + Sorbinil
PDGF-AB + Tolrestat
Control
Ang -II
Ang-II + Sorbinil
Ang-II + Tolrestat
Control
PMA
PMA + Sorbinil
PMA + Tolrestat
Figure 8

A. Cell counting

- Control
- Lipofectamine
- Scrambled oligo
- AR antisense

B. MTT assay

- Untreated
- TNF-α
Aldose reductase mediates mitogenic signaling in vascular smooth muscle cells
Kota V. Ramana, Deepak Chandra, Sanjay Srivastava, Aruni Bhatnagar, Bharat B.
Aggarwal and Satish K. Srivastava

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