Aldose reductase regulates TNF-α-induced cell signaling and apoptosis in vascular endothelial cells

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Abstract In this study, we examined the role of aldose reductase (AR) in regulating the cytotoxic effects of TNF-α on human umbilical vein endothelial cells. Inhibition of AR by sorbinil or tolrestat prevented TNF-α-induced increase in Bax and Bak and the downregulation of Bcl-2. Inhibition of AR abrogated AP-1 DNA binding activity and prevented the activation of caspase-3, JNK, and p38 MAPK in cells stimulated by TNF-α. Exposure to TNF-α also induced apoptotic cell death, which was attenuated by AR inhibition or antisense ablation. These observations suggest that AR is a critical regulator of TNF-α-induced apoptotic signaling in endothelial cells.

Keywords: Aldose reductase; Apoptosis; TNF-α; Bcl-2; AP-1; Endothelial cell

1. Introduction

Cytokines such as TNF-α are expressed locally in the vessel wall induced growth arrest and apoptosis in endothelial cells [1,2]. Cytokine-induced endothelial dysfunction and apoptosis is of significance to the development of several pathophysiological disorders. Disseminated endothelial apoptosis contributes to endotoxin shock [3], acute allograft rejection [4], and negative ventricular remodeling during heart failure [5]. Moreover, focal loss of endothelium contributes to the erosion and rupture of advanced atherosclerotic plaques [6].

Signaling events leading to endothelial apoptosis are redox-sensitive. Apoptotic agents such as TNF-α increase the generation of reactive oxygen species (ROS) and the outcome of apoptotic signaling is critically modulated by the cellular redox state and glutathione levels [7,8]. However, the mechanisms by which changes in redox state contribute to apoptotic signaling remain poorly characterized. We have recently reported that aldose reductase (AR), a member of the aldo-keto reductase (AKR) superfamily, regulates TNF-α-induced NF-κB activation in smooth muscle cells [9], and that inhibition of the enzyme prevents smooth muscle cell growth in culture [10], neointimal hyperplasia in balloon-injured arteries [9] and the second window of ischemic preconditioning in heart [11]. Structure-activity studies indicate that AR is an efficient catalyst for removing the aldehyde products of lipid peroxidation [12,13] and that inhibition of the enzyme increases the accumulation of lipid peroxidation products such as 4-hydroxy-*trans*-2-nonenal (HNE) in hearts subjected to ischemia-reperfusion [11] or in the kidneys of animals subjected to chronic systemic inflammation [14]. In addition, we have also observed that inhibition of AR could prevent TNF-α-induced apoptosis in lens epithelial cells [15]. Other investigators have also suggested that AR is involved in regulating apoptosis [16]. However, the mechanisms by which inhibition of AR prevents apoptosis remain unclear. In the present study, we examined TNF-α-induced growth arrest and cell death in endothelial cells. Our results show that inhibition of AR prevents apoptosis by interrupting several different pathways of TNF-α signaling and suggest that AR is essential to the progression of the apoptotic program triggered by TNF-α.

2. Materials and methods

2.1. Reagents and cell culture

Antibodies against Bcl-2, Bak, Bax, Bcl-xl, phospho-JNK and JNK and phospho-p38 and p38 were obtained from Cell Signaling Inc. Consensus oligonucleotides for AP-1 transcription factor were obtained from Promega Corp. All other reagents used were of analytical grade and obtained from Sigma Chem. Co. Recombinant TNF-α was purchased from Research Diagnostics. Human umbilical vascular endothelial cells (HUVECs) were obtained from ATCC and were maintained and grown confluent in Ham's F12K medium supplemented with 2 M L-glutamate, 0.1 mg/ml heparin, and 0.05 mg/ml endothelial growth supplement (ECGS) and 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. The cells <12 passages were used for this study.

2.2. Measurement of cell viability and apoptosis

The cells were grown to confluency in Ham's F12K medium and harvested by trypsinization and were plated 5000 cells/well in a 96-well plate. Sub-confluent cells were growth-arrested in 0.1% FBS containing ECGS. After 24 h, TNF-α (2 nM) without or with AR inhibitors sorbinil or tolrestat were added to the media and the cells were incubated for another 24 h. Cells incubated with the AR inhibitors alone served as control. Cell viability was determined by cell counts, MTT-assay and thymidine incorporation as described earlier [9,15] and apoptotic cell death was quantified using "Cell Death Detection ELISA" Kit (Roche Inc.) according to the manufacturer's instructions. Caspase-3 activity was measured by using the caspse-3-specific substrate Z-DEVD-AFC, (CBZ–Asp–Glu–Val–Asp–AFC). The cells with fragmented and/or condensed nuclei were classified as apoptotic cells and were identified by using Hoechst 33342 dye.

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2.3. Electrophoretic mobility gel shift assay

The cytosolic and nuclear extracts were prepared as described [9,15]. Briefly, after indicated treatments, HUVECs were washed with cold PBS, suspended in 0.4 ml of hypotonic lysis buffer (10 mM HEPES, pH 8.0, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, and 0.5 mg/ml benzamidine) and incubated on ice for 30 min. After incubation, 12.5 μl of 10% Nonidet P-40 was added and the tubes were vigorously vortexed for 10 s. The nuclei were pelleted by centrifugation for 30 s at 4000 rpm. The supernatant was collected and recentrifuged at 12 000 rpm for 2 min at 4 °C and used as the cytosolic extract. The nuclear pellet was suspended in 50 μl of cold nuclear extract buffer (20 mM HEPES, pH 8.0, 0.5 M KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, 0.5 mg/ml benzamidine, and 20% glycerol). Protein concentration in the extracts was determined using a Protein Assay Kit from Bio-Rad. Consensus oligonucleotides for AP-1 transcription factors were 5′-end labeled using T4 polynucleotide kinase. The EMSA were performed as described earlier [9].

2.4. Western blot analysis

To examine caspase-3 activation, JNK and p38 phosphorylation, Bcl-2, Bak, Bax and Bcl-xl upregulation, Western blot analyses were carried out as described earlier [9] using antibodies against PARP, JNK and phospho-JNK, p38 and phospho-p38, Bcl-2, Bak, Bax and Bcl-xl. The proteins were transferred to nitrocellulose filters, probed with the indicated antibodies and the antigen–antibody complex was detected by enhanced chemiluminescence (Amersham Pharmacia

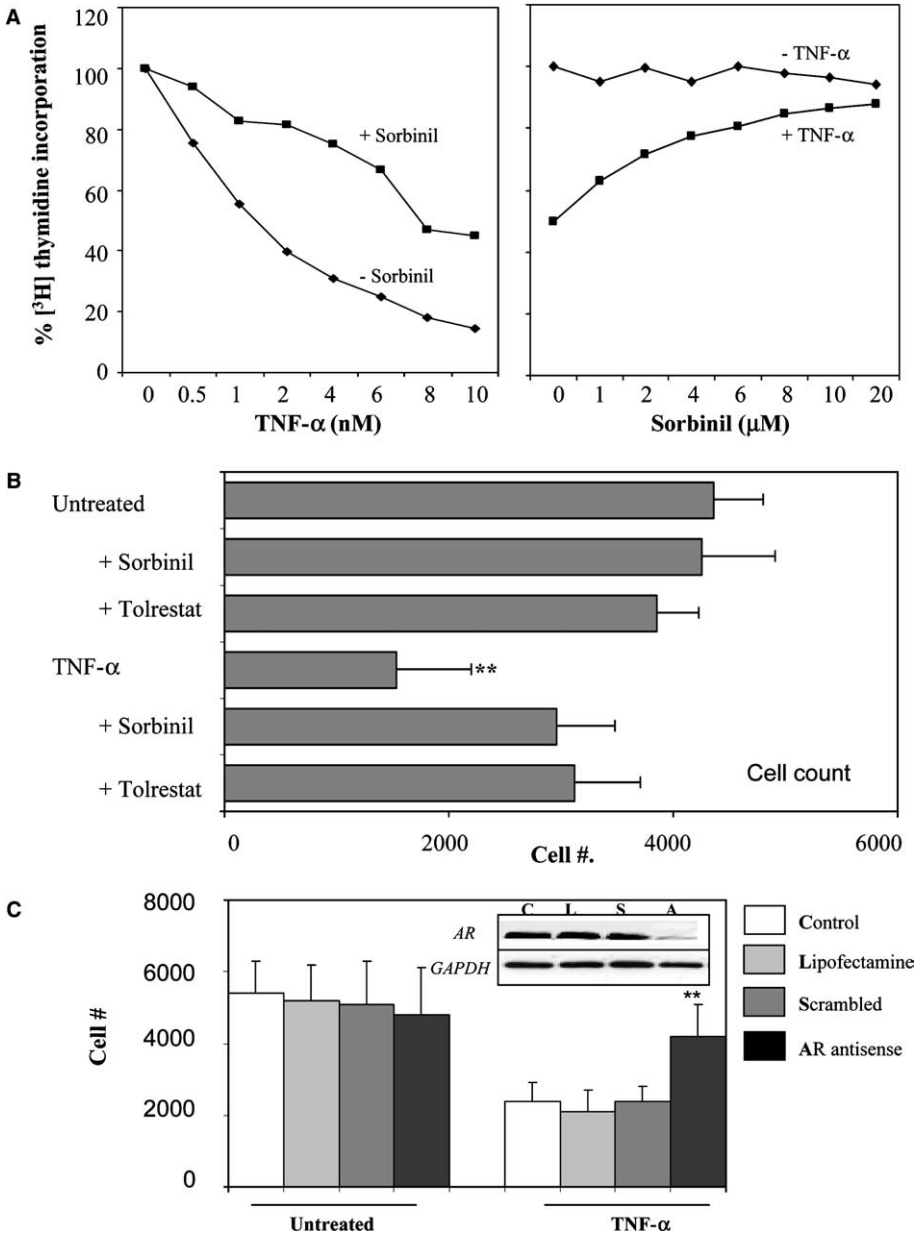

Fig. 1. Inhibition of AR prevents TNF-α cytotoxicity. (A) Serum-starved HUVECs were stimulated for 24 h with 0–10 nM TNF-α without or with 10 μM sorbinil or with 2 nM TNF-α in the presence of 0–20 μM sorbinil. Cell growth was determined by [^{3}H]thymidine incorporation. (B) Viability of HUVECs that were either left untreated or treated with 10 μM sorbinil or tolrestat alone or in the presence of 2 nM TNF-α. (C) Serum-starved cells were either left untreated (control; C) or treated with AR antisense (A), scrambled (S) oligonucleotides, or lipofectamine (L) alone and then cultured with 2 nM TNF-α for 24 h. The inset shows Western blots of protein extracts of cells using anti-AR antibodies. The number of viable cells in (B) and (C) was determined by trypan blue exclusion. Bars represent means ± S.E.M. ($n = 4$), **, $P < 0.001$ versus untreated cells (C) or cells transfected with scrambled oligonucleotides (S).

Biotech, NJ). Immunopositive bands were quantified by Kodak Alpha Imager 2000 Scanning Densitometer equipped with the AlphaEase™ Version 3.3b software and the average change in fold-intensity was calculated.

2.5. Statistical analysis

Data are presented as means ± S.E.M. and P values were determined by unpaired Student's t-test.

3. Results

To delineate the role of AR in TNF-α-induced HUVECs apoptosis, we determined the effect of the AR inhibitor – sorbinil. As shown in Fig. 1A, treatment of serum-starved HUVECs with TNF-α (1–10 nM) for 24 h decreased [^{3}H]thymidine incorporation in a concentration-dependent manner. The decrease in [^{3}H]thymidine incorporation by increasing concentration of TNF-α was attenuated by sorbinil. In the absence of TNF-α, 1–20 μM sorbinil did not affect [^{3}H]thymidine incorporation, indicating that inhibition of AR by itself does not affect DNA synthesis under these conditions. However, inhibition of thymidine incorporation by TNF-α was prevented by sorbinil in a concentration-dependent fashion. At 10 μM sorbinil, the extent of thymidine incorporation in TNF-α-treated cells was >80% of the untreated cells. From these data, we conclude that sorbinil abrogates TNF-α-induced inhibition of DNA synthesis.

Inhibition of AR also prevented TNF-α-induced growth arrest. As shown in Fig. 1B, treatment with TNF-α decreased cell density, which was prevented upon pretreatment with AR inhibitors, sorbinil and tolrestat. In the absence of TNF-α, these inhibitors did not affect cell growth. Because sorbinil and tolrestat are structurally unrelated compounds, the observation that inhibition of TNF-α-induced growth arrest is prevented by either of these drugs provides strong evidence supporting a role of AR in mediating the effects of TNF-α on cell growth. Nonetheless, the possibility that these drugs have non-specific effects is not rigorously excluded. Therefore, we tested whether inhibition of AR by antisense oligonucleotides will have similar effects. Transient transfection of HUVECs with AR antisense decreased the abundance of AR protein by ~90% as compared with scrambled oligonucleotide-transfected cells (Fig. 1C, inset). When cultured with TNF-α for 24 h, <50% of the cells treated with lipofectamine or scrambled

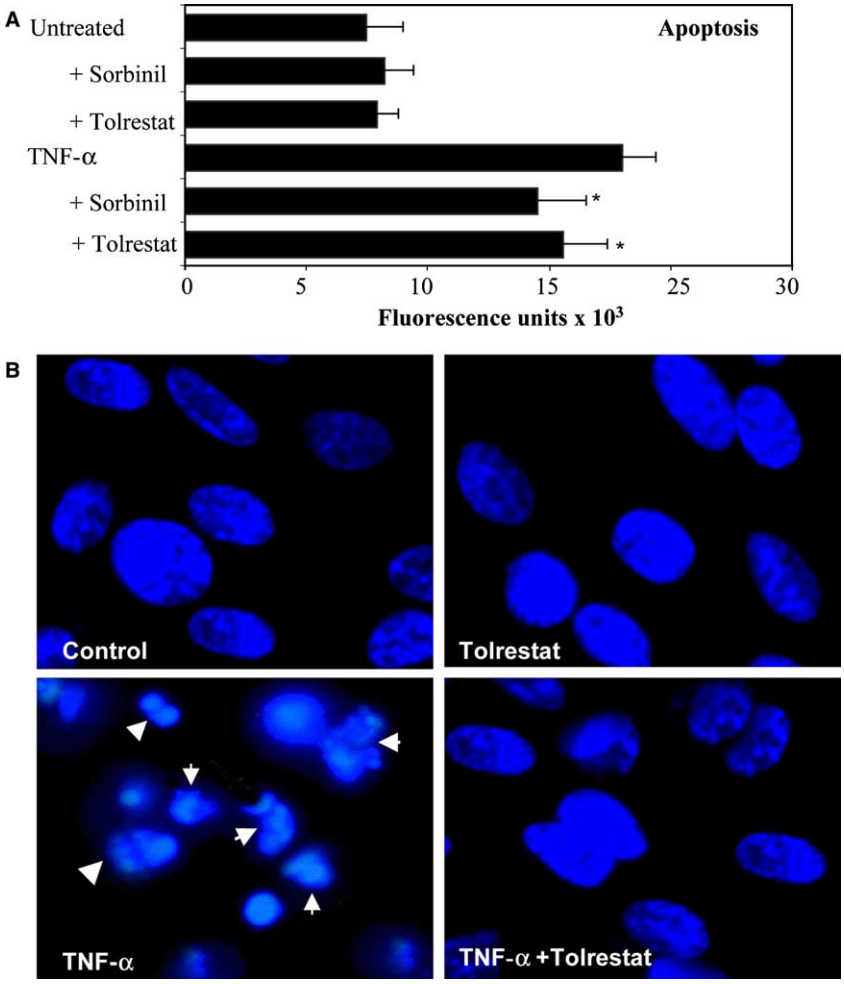

Fig. 2. Inhibition of AR prevents HUVECs apoptosis. Serum-starved cells were pre-cultured without or with 10 μM sorbinil or tolrestat and then stimulated with 2 nM TNF-α for 24 h. Apoptosis was determined by using the (A) Cell Death ELISA Kit. Bars represent means ± S.E.M. ($n = 4$), *, $P < 0.01$ versus TNF-α-treated cells, and (B) Hoechst 33342 nuclear dye. After treatment, the cells were incubated with 5 μg/ml of Hoechst 33342 for 30 min at 4 °C. Apoptotic cells with fragmented nuclei and with buds are indicated by arrowheads. Cells either left untreated (control) or treated with TNF-α (2 nM) without or with (10 μM) tolrestat.

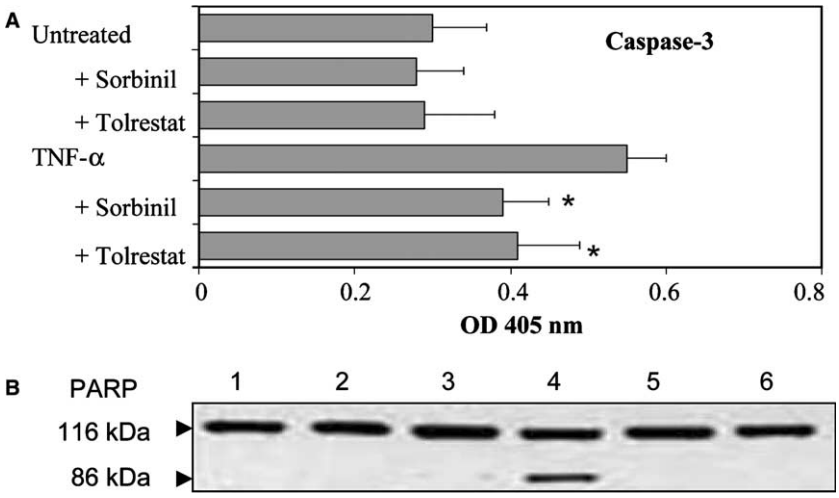

Fig. 3. Inhibition of AR prevents TNF-α-induced caspase-3 activation. Serum-starved cells were cultured without or with 10 μM sorbinil or tolrestat and then stimulated with 2 nM TNF-α for 24 h. (A) Caspase-3 activity and (B) Western blot analysis using anti-PARP antibodies. Bars represent means ± S.E.M. ($n = 4$), *, $P < 0.01$ versus TNF-α-treated cells. Panel B shows a representative Western blot prepared from extracts of cells that were either left untreated (lane 1), or treated with sorbinil (2), tolrestat (3) TNF-α (4), TNF-α + sorbinil (5), and TNF-α + tolrestat (6) using anti-PAPR antibodies.

oligonucleotides were viable as compared to respective controls without TNF-α. Ablation of AR by antisense significantly prevented the TNF-α-induced cell death (Fig. 1C). Because similar results were obtained with AR-antisense and inhibitors, AR inhibitors were used for most of the subsequent experiments.

To determine the effects of AR inhibition of apoptosis, we measured the free histones released upon nucleosomal degradation during apoptosis and Hoechst 33342 staining. As shown in Fig. 2A, stimulation with TNF-α caused the degradation of nucleosomal histones. Pretreatment of HUVECs with either sorbinil or tolrestat inhibited TNF-α-induced apoptosis by 40%. Additionally, cells treated with TNF-α displayed nuclear fragmentation and condensation. However, treatment with tolrestat prevented these changes (Fig. 2B).

To examine the effects of AR inhibition on known mediators of apoptosis, we measured TNF-α-induced caspase-3 activation in HUVECs by caspase-3-specific substrates as well as PAPR cleavage. As shown in Fig. 3, stimulation of HUVECs with TNF-α caused activation of caspase-3 and PARP cleavage. However, both sorbinil and tolrestat prevented the TNF-α-induced caspase-3 activation as well as PARP cleavage. Next, we determined the effect of AR inhibition on the expression of Bcl-2 family proteins. Stimulation with TNF-α down regulated the expression of Bcl-2 (Fig. 4A) and upregulated the expression of Bax and Bak (Fig. 4C and D). Stimulation with TNF-α did not affect Bcl-xl levels (Fig. 4B). Tolrestat completely prevented the downregulation of Bcl-2 and upregulation of Bak and Bax by TNF-α, but it did not affect basal levels of these proteins in the absence of TNF-α (Fig. 4F).

To determine how inhibition of AR prevents TNF-α signaling, we tested the effects of AR inhibition on JNK and p38 MAPK activation. Stimulation with TNF-α led to a marked increase in the phosphorylated forms of JNK and p38 MAPK (Fig. 5A and C), which was prevented in cells treated with sorbinil. However, total JNK and p38 MAPK were not affected by TNF-α (Fig. 5B and D). In addition to inhibiting TNF-α-induced activation of JNK and p38 MAPK, treatment

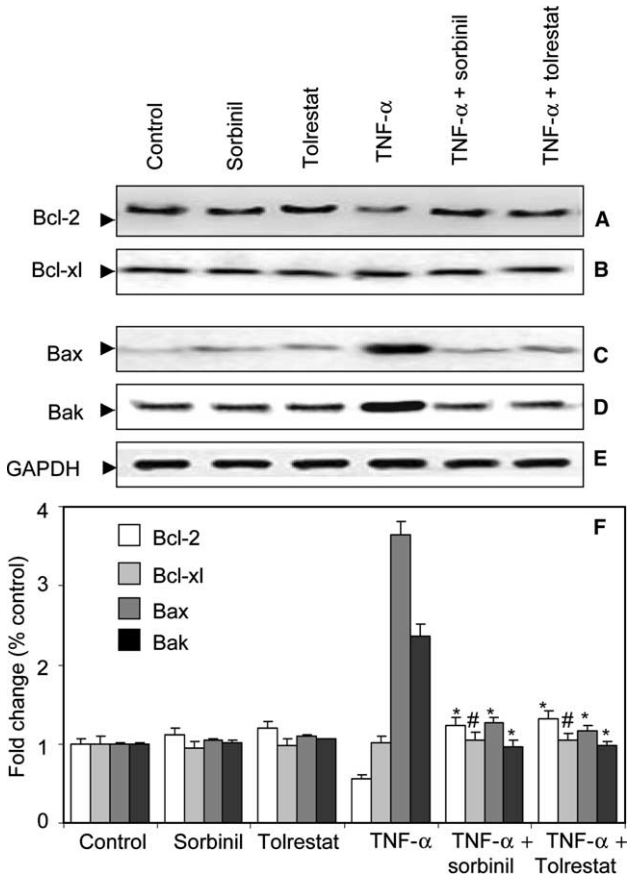

Fig. 4. Inhibition of AR regulates the expression of Bcl-2 family of proteins. Serum-starved HUVECs were either left untreated or pre-incubated with sorbinil or tolrestat (10 μM) for 24 h and were then stimulated with TNF-α (2 nM) for 24 h. Western blot analysis was performed from pooled samples of three independent experiments and were developed using antibodies against Bcl-2 (A), Bcl-xl (B), Bax (C) and Bak (D) proteins. (E) Data represent means ± S.E.M. of three independent analyses. $P < 0.001$ versus TNF-α treated cells without inhibitors.

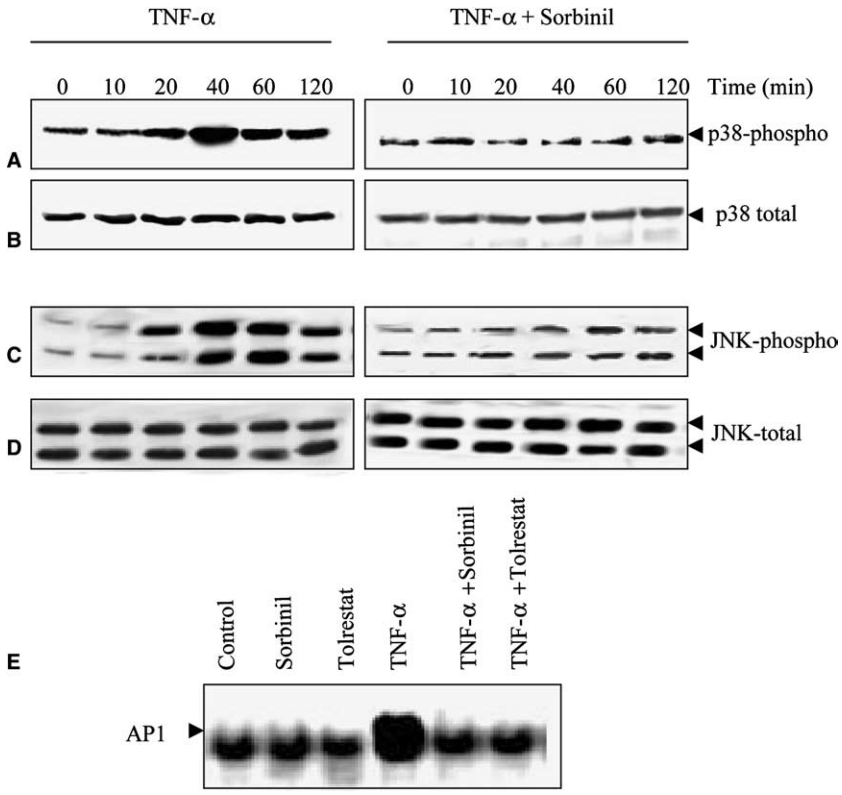

Fig. 5. Inhibition of AR prevents phosphorylation of JNK and p38 activation of AP-1. Serum-starved HUVECs were either left untreated or pre-incubated with tolrestat (10 μM) for 24 h and then stimulated with TNF-α (0.1 nM). Western blot analysis was performed from pooled samples of three independent experiments and developed using anti-phosphop38 (A), p38 (B), phosphoJNK (C) and JNK (D) antibodies. (E) Serum-starved HUVECs were pre-incubated without or with sorbinil and tolrestat (10 μM) for 24 h, and were then stimulated with 0.1 nM TNF-α for 1 h.

with AR inhibitors also prevented downstream activation of transcription factor AP-1 (Fig. 5E), suggesting that the inhibition of AR abolishes the cytotoxic effects of TNF-α.

4. Discussion

The present study shows that inhibition of AR prevents TNF-α-mediated growth arrest and apoptosis in vascular endothelial cells. Decreased cytotoxicity of TNF-α appears to be due to attenuation of apoptotic signaling, inhibition of stress-activated kinases (JNK and p38), and diminished stimulation of caspase-3. Inhibition of AR also prevented the TNF-α-induced decrease in the abundance of Bcl-2 and the upregulation of Bax and Bak, indicating that inhibition of AR profoundly affects the apoptotic signaling program of TNF-α.

Binding of TNF-α to its receptor triggers multiple signaling events that initiate both cell death and cell survival pathways [17,18]. Post-receptor events diverge into distinct signaling pathways leading to the activation of JNK-AP-1 and NF-κB and the induction of cell death. The outcome of this signaling is determined by several regulatory mechanisms including generation of ceramide from sphingomyelin and changes in the cellular redox state [17,18]. We have shown earlier that inhibition of AR blocks NF-κB activation and prevents proliferation of vascular smooth muscle cells [9]. Our current observations that inhibition of AR prevents TNF-α-induced JNK and AP-1 activation and apoptotic cell death in endothelial cells suggest that in addition to cell survival signaling (NF-κB and p38 activation), AR is essential also for other consequences of TNF-α stimulation that result in JNK activation and apoptosis. Collectively, these results provide comprehensive support to the notion that AR-sensitive events are upstream to the dichotomy between JNK and NF-κB activation and that AR-catalysis is essential for the manifestation of the pleiotrophic effects of TNF-α, including TNF-α-induced cell death. We propose that inhibition of AR prevents signaling events common to both JNK activation and cell death, even though the cell survival signaling, e.g., activation of p38 is prevented. The net effect is inhibition of apoptosis, presumably because the cell death pathways are more strongly suppressed than the signaling events leading to cell survival.

The mechanisms by which inhibition of AR promotes cell survival remain unclear, but may relate to AR-induced changes in the cellular redox state. Previous studies have shown that the activation of caspase-3 by TNF-α in endothelial cell is in part due to increased generation of ROS in the mitochondria [7]. Similarly, ROS could be mediators of TNF-α signaling leading to the stimulation of Bax and Bak and the downregulation of Bcl-2. The critical role of redox changes in TNF-α signaling is suggested by data showing that treatment with antioxidants simultaneously blocks TNF-α-induced NF-κB, AP-1, and stress kinase activation [19], as well as the downregulation of Bcl-2 and apoptosis in HUVECs [20]. Thus, inhibition of AR may be concurrently preventing multiple pathways of TNF-α signaling by minimizing

oxidative changes. Indeed, inhibition of AR catalysis that consumes NADPH has been thought to improve the intracellular reducing environment [20,21]. The NADPH-sparing effect of AR inhibition could prevent oxidative stress by supporting reductive catalysis mediated by oxidoreductases such as glutathione reductase. Nevertheless, a direct antioxidant effect of AR inhibition during TNF-α signaling seems unlikely because pre-incubation with AR inhibitors was required to extinguish TNF-α-signaling. This observation suggests that pre-inhibition of AR establishes a metabolic state that is not conducive to TNF-α signaling and that AR plays a permissive rather than a mediatory role in TNF-α signaling. Clearly, extensive future investigations will be required to elucidate how cellular responses to AR inhibition prevent cytokine signaling.

The demonstration that inhibition of AR prevents the activation of stress kinases and apoptotic signaling provides new avenues for understanding and managing vascular inflammation. Because of its well-studied role in the development of secondary diabetic complications, several AR inhibitors have been developed to treat and prevent long-term diabetic complications such as retinopathy, neuropathy, and renal failure [21,22]. The observation that inhibition of AR prevents endothelial apoptosis suggests that AR inhibitors could be useful in preventing endothelial injury and dysfunction associated with several pathophysiological conditions, including sepsis and atherogenesis.

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