Osmotic Response Element Is Required for the Induction of Aldose Reductase by Tumor Necrosis Factor-α*

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Induction of aldose reductase (AR) was observed in human cells treated with tumor necrosis factor-α (TNF-α). AR protein expression increased severalfold in human liver cells after 1 day of exposure to 100 units/ml TNF-α. An increase in AR transcripts was also observed in human liver cells after 3 h of TNF-α treatment, reaching a maximum level of 11-fold at 48 h. Among the three inflammatory cytokines: TNF-α, interleukin-1, and interferon-γ, TNF-α (100 units/ml) gave the most induction of AR. Differences in the pattern of AR induction were observed in human liver, lens, and retinal pigment epithelial cells. A deletion of the osmotic response element (ORE) abolished the induction by TNF-α and osmotic stress. A point mutation that converts ORE to a nuclear factor-κB (NF-κB) sequence abolished the osmotic response but maintained the TNF-α response. Electrophoretic gel mobility shift assays showed two NF-κB proteins, p50 and p52, capable of binding ORE sequence, and gel shift Western assay detected NF-κB proteins p50 and p65 in the ORE complex. Inhibitors of NF-κB signaling, lactacystin, and MG132 abolished the AR promoter response to TNF-α.

Accumulation of osmolytes such as sorbitol, myoinositol, betaine, and glycerophosphorylcholine helps regulate osmotic pressure in renal medullary cells during antidiuresis. It has been shown that sorbitol accumulates in renal medulla cells and other cell types when cultured in hypertonic medium (8, 9). Elevation of AR activity and gene expression occur under these conditions. Recent reports describe the involvement of two AR promoters cis-elements, the osmotic response element (ORE) (10–12), similar to the tonicity-responsive enhancer element originally found in the dog betaine transporter gene (13), and the aldose reductase enhancer element (14) in regulating osmotic response and constitutive promoter activities. A signal transduction study revealed that the p38 and the c-Jun N-terminal kinase (SAPK/JNK) pathways are not necessary for the transcriptional regulation of the AR promoter through ORE (15). ORE differs from the NF-κB binding sequence by one base pair, but no detailed study has been reported concerning the functional relationship between these two elements. Recently, it was reported that the tonicity-responsive enhancer element forms DNA-protein complexes of 200 kDa by electrophoretic gel mobility shift assay (16).

Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine that has been classically studied as a molecule central to the pathogenesis of infectious, inflammatory, and autoimmune diseases. TNF-α is produced primarily by active macrophage and T-cells in response to various stimuli. It is functional in both the transmembrane and the secreted homotrimeric form (17, 18). In obese-induced insulin resistance (19–25), TNF-α causes the reduction of insulin-stimulated tyrosine phosphorylation of the insulin receptor and its substrate insulin receptor substrate-1 (IRS-1) (26). Insulin-sensitizing agents such as thiazolidinediones have been shown to reduce the inhibition of tyrosine phosphorylation of IRS-1 by TNF-α (27). TNF-α also activates nuclear factor-κB (NF-κB) by phosphorylation of the NF-κB inhibitor IκB, which releases NF-κB to the nucleus in homodimer or heterodimer forms (28, 29).

The striking similarity between ORE and the NF-κB binding sequence has led us to investigate the effect of the activators of NF-κB on AR transcription. In this paper we report a significant induction of AR by TNF-α, which is mediated by NF-κB binding to the previously described ORE. Deletion or mutation of this element has a significant effect on the response of the AR promoter to both TNF-α and hyperosmotic stress.

**Experimental Procedures**

Cell Culture and Cytokine Treatment—The human liver cell line (Chang liver; ATCC, Rockville, MD) was cultured in basal medium with Earle’s BSS (BME; Life Technologies, Inc.), 10% fetal bovine serum (FBS) (Life Technologies, Inc.), and 50 μg/ml gentamycin (Life Technologies, Inc.). Human lens cell line SRA01/04 (30) was cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% FBS.
and 50 μg/ml gentamycin. Fourth passage human retinal pigment epithelial (RPE) cells were cultured in Dulbecco’s modified Eagle’s medium with 15% FBS, 50 μg/ml gentamycin, and 0.5 μg/ml fungizone (Life Technologies, Inc.). TNF-α (Life Technologies, Inc.) was added to the medium at various concentrations between 20 and 500 units/ml. The culture was replaced every 2 days.

**Protein Extraction, SDS-Polyacrylamide Gel Electrophoresis (PAGE), and Immunoblotting—**Liver cells were washed with phosphate-buffered saline (Life Technologies, Inc.), and scraped, pelleted, and resuspended in sodium dodecyl sulfate (SDS) gel loading buffer (30 mM Tris-HCl, pH 6.8, 5% glycerol, 1% SDS, 2.5% β-mercaptoethanol, 0.05% bromophenol blue) for protein analysis. A total of 200,000 cells (approximate human liver cell equivalents) were added to each well of a 6-well plate. After incubation overnight, the medium was aspirated, and fresh medium with TNF-α (100 units/ml) was added. The cells were washed with PBS, resuspended in 200 μl of lysis buffer (50 mM HEPES, pH 7.9, 15% Glycerol, 1% DTT, 0.5% IGEPAL CA-630), and centrifuged at 13,000 g for 2 min. The supernatant was frozen at –70 °C until use. Samples were thawed and sonicated for 30 s. Western blot analysis using human AR antibodies was performed.

**Luciferase Promoter Constructs and Luciferase Assay—**The human AR promoter was isolated from a phage human genomic library (CLONTECH) using the human cDNA as a probe (37). The 5′ flanking sequence of the gene was sequenced up to 18 S ribosomal RNA.

**Quant software (Molecular Dynamics, Sunnyvale, CA) after normalizing the expression to 18 S ribosomal RNA.**

**Induction of Aldose Reductase by Tumor Necrosis Factor-α**

**RESULTS**

**Induction of AR by TNF-α—**Human liver cells were exposed to TNF-α (100 units/ml) for 1–5 days. AR protein expression increased in liver cells as early as 1 day (Fig. 1). This increase was observed throughout the 5 days of the experiment. Northern blot analysis of human AR in liver cells treated for various times with TNF-α indicates that AR mRNA increases by 3 h with a maximum 11-fold increase at 48 h (Fig. 2). The effect of interferon-γ on AR was also observed, with a 7-fold increase at 48 h (Fig. 2). The effect of interferon-γ on TNF-α treatment gave the highest increase in liver cells compared with other cytokines. No induction was observed with interferon-γ treatment.

**Northern analyses were performed on liver, lens, and RPE cells treated for 12 h with various concentrations of TNF-α from 0 to 500 units/ml (Fig. 4). In liver cells, a linear increase in
was observed with increasing TNF-α concentrations. In contrast, lens cells showed no induction until the TNF-α concentration reached 100 units/ml. This elevated level did not change with 500 units/ml TNF-α. In RPE cells, a sigmoidal pattern of induction was observed.

Identification of Region Important for Both TNF-α and Osmotic Response of the Human AR Promoter—Thirteen reporter constructs containing sequential deletions of the human AR promoter were transfected into human liver cells that were treated with TNF-α or stressed with hypertonic medium for 12 h (Fig. 5). TNF-α treatment increased the AR promoter activity 3.3–5.5-fold in constructs 1–8 compared with the constitutive activity of the control cells, while deletion of ORE in construct 9 abolished the TNF-α response. Osmotic stress increased the promoter activity 4.0–10.8-fold in constructs 1–8 compared with the constitutive activity of the control cells, while deletion of ORE in construct 9 abolished the osmotic response. This experiment indicates that the important promoter region for TNF-α response and osmotic stress response is between constructs 8 and 9.

ORE Is Required for the TNF-α Response of the AR Promoter—To further characterize the AR promoter region between constructs 8 and 9, a small deletion was made between sequences –1,160 bp to –1,150 bp of construct 8, which eliminated the entire ORE (Fig. 6). This deletion abolished the TNF-α response and osmotic response of the AR promoter. It also had an inhibitory effect on the constitutive promoter activity. A point mutation from adenine to guanine at position –1,157 bp in construct 8 was made to convert ORE to the NF-κB sequence (41). This change abolished the osmotic response but not the TNF-α response of the AR promoter. The construct with mutations of two 5′ guanines (–1,159 bp, –1,158 bp) and one 3′ cytosine (–1,150 bp) had the same effect as the deletion construct.

ORE Complex in EGMSA Contains NF-κB Proteins—Elec-
trophoretic gel mobility shift Western assays were performed to determine whether the ORE complexes contain NF-κB proteins (Fig. 7). EGMSA of ORE was performed using extracts of human liver cells that had been treated with TNF-α. The ORE complex was transferred to a nitrocellulose membrane and Western analysis was performed with NF-κB antibodies specific for p50, p52, p65, Rel-B, and c-Rel. The p50 antibody reacted with the fastest migrating ORE complex (Fig. 7, arrow 5), while the p65 antibody reacted with two other ORE complexes (Fig. 7, arrows 1 and 2).

Common ORE complexes were observed between control, osmotically stressed, and TNF-α-treated cell extracts (Fig. 7A). ORE complex 1 was observed specifically with TNF-α-treated cell extracts, while ORE complex 2 was observed in the TNF-α-treated and osmotically stressed cell extracts. ORE complex 3 was observed in control and TNF-α-treated cell extracts. ORE complex 4 was observed only in the osmotically stressed cell extracts. ORE complex 5 was observed in all cell conditions.

**FIG. 4.** Northern analyses of AR expression in liver, lens, and RPE cells treated with various TNF-α concentrations. Human liver, lens, and RPE cells were exposed to different concentrations of TNF-α (0–500 units/ml) for 12 h. A, Northern blot of human AR. B, AR induction with increasing TNF-α concentrations is indicated as a ratio based on control (0 units/ml) as 1. C, Northern blot of human 18 S ribosomal RNA. D, ethidium bromide staining of total RNA separated on agarose gel.

**FIG. 5.** Comparison of human AR promoter activity in transfected human liver cells by TNF-α treatment and hyperosmotic stress. A, scheme of 13 luciferase (LUC) reporter constructs of the human AR promoter between –3.7 kilobase pairs and +31 bp used for transfection in human liver cells. B, graph of relative luciferase activity of TNF-α-treated cells (black bars) and control cells (white bars) based on calculation in C. C, relative luciferase activity of TNF-α-treated and control cells. T/C indicates the ratio of the promoter activity of TNF-α-treated cells over control cells. D, graph of relative luciferase activity of osmotically stressed hypertonic (black bars) and control cells (white bars). E, relative luciferase activity of osmotically stressed hypertonic cells and control cells. H/C indicates the ratio of the promoter activity of osmotically stressed cells over control cells (+). Bar graphs represent the mean ± standard deviation (error bar); n = 3. n indicates the number of wells used for this particular experiment.
An additional EGMSA for ORE was performed with commercially available recombinant pure p50 and p52 proteins (Fig. 8). Probe P1, which contains the wild type sequence of ORE, bound both NF-κB p50 and p52. Probe P2, which contains a point mutation converting ORE to the NF-κB sequence at position –1,157 bp, also bound NF-κB p50 and p52 at the same binding strength. No binding for probe P3, which contains a random 4-bp mutation, was observed.

Inhibition of NF-κB Signaling Abolished TNF-α Response of AR—NF-κB signaling inhibitors lactacystin and MG132 were added to transfected liver cells exposed to TNF-α in order to determine the response of the human AR promoter. Both inhibitors had no effect on the constitutive AR promoter activity but abolished the TNF-α response (Fig. 9).

**DISCUSSION**

In this paper we have reported that the inflammatory cytokine TNF-α is an inducer of human AR. Recent studies indicate that TNF-α may be involved in obese related diabetic complications (19–25). TNF-α is higher in obese patients, and it has
been shown to block phosphorylation of IRS-1 (26). AR induction by TNF-α may contribute to the severity of diabetic complications in obese patients. Further studies on the relationship between obesity, TNF-α, and severity of diabetic complications are being investigated.

Induction of AR by TNF-α was observed in several cell types including human liver, lens, and retinal pigment epithelial cells. The only difference in AR induction between the cell types was observed with increasing concentrations of TNF-α, where a linear, threshold, or sigmoidal response was observed for liver, lens, or RPE cells, respectively. As observed with liver cells, AR in lens and retinal pigment epithelial cells was induced the most by TNF-α compared with treatment with INF-γ or interleukin-1α (data not shown).

We (14) and others (10–12) have identified a cis-element (ORE) required for the osmotic response of AR in several species. This element is similar to a previously reported tonicity-responsive enhancer element (13) and to an element in the osmoregulating myoinositol transporter gene recently reported by Zhou et al. (39). The ORE sequence differs by only one nucleotide from the NF-κB binding sequence (40). In our study, we evaluated the relationship between ORE and NF-κB during induction of AR protein and gene expression. The time required for the up-regulation of AR transcription by the NF-κB activator TNF-α was similar to the previously described osmotic response (9); however, AR protein expression was much faster for the TNF-α response compared with the osmotic response. This result suggests the possibility of a common transcriptional regulation for both TNF-α and osmotic response but a different regulation for translation of AR.

Thirteen reporter constructs of the human AR promoter were transfected into human liver cells to determine the critical promoter region for TNF-α response. Deletion of constructs 8 and 9 totally abolished the TNF-α response, which shares the same critical region as the osmotic response. The AR response to TNF-α treatment in all reporter constructs was similar to those of the osmotic stress, which suggests a possible common transcriptional mechanism for both responses. Inhibition of
FIG. 7. Identification of NF-κB proteins complexed with ORE. EGMSA was performed on cell extracts from cells stressed with hypertonic medium or TNF-α exposure, followed by Western blot analysis with NF-κB antibodies to p50, p52, p65, Rel-B, or c-Rel. A, EGMSA of cell extracts from human liver cells treated with hypertonic medium or TNF-α were mixed with ORE probe. C, control; O, hypertonic; T, TNF-α. Numbered arrows indicate the complexes formed. B, ORE complexes formed in cell extracts from TNF-α-treated cells were separated on native PAGE followed by Western blot analysis with NF-κB antibodies. Numbered arrows indicate bands detected by NF-κB antibodies, which match the location of the complexes detected by EGMSA.

| Probe | P1 | P2 | P3 |
|-------|----|----|----|
| NF-κB Protein | + | + | + |
| Competition P1 | - | - | - |
| Competition P2 | - | + | - |
| Competition P3 | - | - | + |
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

FIG. 8. EGMSA of recombinant NF-κB proteins p50 and p52. EGMSA was performed with two recombinant NF-κB proteins (p50, p52) and three probes. Probe P1: wild type sequence of ORE; probe P2, point mutation of probe P1 which converts ORE sequence to NF-κB binding sequence; probe P3, 4-bp mutation of probe P1.

Probes:
- Probe P1: 5′-AAA TGG AAA ATC ACC GGC-3′
- Probe P2: 5′-AAA TGG GAA ATC ACC GGC-3′
- Probe P3: 5′-AAA TTT AAA AAA ACC GGC-3′

Free Probe
both TNF-α and osmotic response by the deletion of ORE in construct 8 confirmed the involvement of this element as well as the importance of this element in both responses. A point mutation in construct 8, which converts the ORE sequence to the NF-κB sequence, abolished the osmotic response as previously reported (10) but did not effect the TNF-α response as shown in this study. This result indicates that ORE requires adenine instead of guanine at position –1,157 bp in the human AR promoter for osmotic response, but does not require this nucleotide for the TNF-α response. Two 5’ guanines and one 3’ cytosine in ORE, which match the critical nucleotides of NF-κB binding sequence, were mutated to confirm the binding of NF-κB protein. This 3-bp mutation construct had the same effect as the deletion construct, which not only confirms the binding of NF-κB protein but also confirms the importance of this binding site. A summary of the NF-κB/ORE site is shown in Fig. 10. Binding of two commercially available NF-κB proteins, p50 and p52, was not affected by the point mutation converting ORE to an NF-κB sequence. The evidence for common and different ORE complexes found between control, osmotic stress, and TNF-α in EGMSA suggests that a rearrangement of transcription factors on the ORE are required for the initiation of transcription by different stimuli. Inhibitors MG-132 and lactacystin used for suppression of NF-κB activity inhibit proteasome degradation of IκB when phosphorylated by IκB kinase. The inhibition of TNF-α response by both inhibitors support the involvement of NF-κB as a signaling molecule for the TNF-α response of the AR promoter through ORE. This is the first study to identify specific transcription factor binding to ORE. Our results clearly indicate that NF-κB proteins p50 and p65 bind to ORE and that ORE responds to multiple signaling for AR transcription. The similarity of ORE complexes found between osmotic and TNF-α-treated cells may suggest involvement of NF-κB in the osmotic response of AR.

![Fig. 9. Effect of NF-κB inhibitors on AR promoter response induced by TNF-α.](image)

![Fig. 10. Schematic summary of promoter region required for TNF-α response of human AR.](image)
recent report showed that osmotic stress of HeLa cells causes the tumor necrosis factor receptor to cluster on the surface of the cell membrane (41). Together, these observations suggest a possible relationship between cytokine and osmotic response in the cell. However, abolishment of osmotic response by conversion of ORE sequence to NF-κB sequence rules out the possibility that the NF-κB transcription factor is directly responsible for ORE activation in human cells under osmotic stress.

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