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Antioxidant treatment induces transcription and expression of transforming growth factor β in cultured renal proximal tubular cells

Gunter Wolf[a,*], Tete Hannken[a], Regine Schroeder[a], Gunther Zahner[a], Fuad N. Ziyadeh[b], Rolf A.K. Stahl[a]

[a] *Department of Medicine, Division of Nephrology and Osteology, University Hospital Eppendorf, University of Hamburg, Pavilion 61, Martinistraße 52, D-20246 Hamburg, Germany*
[b] *The Renal-Electrolyte and Hypertension Division and the Penn Center for Molecular Studies of Kidney Diseases, University of Pennsylvania School of Medicine, Philadelphia, PA, USA*

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Abstract Transforming growth factor β (TGF-β) plays an important role in the development of tubulointerstitial fibrosis in chronic renal disease. We were interested whether interference with oxygen radicals may modulate TGF-β expression. Unexpectedly, we discovered that diphenylene iodine (DIP), an inhibitor of NADP(H) oxidase, induces a robust increase in TGF-β transcript expression in cultured mouse proximal tubular cells (MCT cells). A similar increase was seen with EUK-8, a synthetic salen–manganese complex with high oxyradical scavenger activities. This induction of TGF-β1 mRNA was paralleled by increasing protein expression. Transient transfection of MCT cells with a reporter construct in which murine TGF-β1 enhancer/promoter elements were cloned in front of the luciferase gene, revealed that DIP, EUK-8, and Tiron all stimulated transcription of the TGF-β1 gene whereas exogenous H_2O_2 suppressed transcription. Antisense oligonucleotides against p22phox, but not sense oligonucleotides, also increased transcriptional activity of TGF-β1. Mutagenesis of Sp1 binding sites in the mouse TGF-β1 enhancer/promoter abolished the stimulatory effect of the antioxidants. Gel shift experiments revealed that DIP as well as EUK-8 activated binding of nuclear proteins to Sp1 consensus sequence. Our data provide evidence that TGF-β1 transcription is negatively regulated in MCT cells under basal conditions by NADP(H) oxidase-mediated oxygen radicals. Thus, antioxidant therapy may increase local synthesis of TGF-β1 in the tubulointerstitium.

Key words: Reactive oxygen species; NAD(P)H oxidase; Transforming growth factor β; Fibrosis; Sp1

1. Introduction

Tubulointerstitial fibrosis and tubular atrophy are morphological correlates of endstage renal failure irrespective of the primary renal disease. One of the major profibrogenic cytokines implicated in this process is transforming growth factor β (TGF-β) [1]. TGF-β stimulates transcription and secretion of various extracellular matrix proteins and further increases their abundance by interfering with the activity of specific proteases that are responsible for normal matrix turnover [1]. More recently it has been proposed that tubular cells may transdifferentiate into tubulointerstitial fibroblasts under certain pathological conditions [2]. TGF-β appears to be a major cytokine involved in this process [3]. Increasing evidence suggests that TGF-β synthesis in the tubulointerstitial environment does not only occur in immune cells recruited from the circulation and local fibroblasts, but that tubular cells themselves are an important source for this profibrogenic cytokine [1,4]. High glucose, advanced glycation endproducts, and angiotensin II (ANG II) may all stimulate synthesis of TGF-β in tubular cells [2,4,5]. We have previously shown that ANG II directly stimulates transcription and expression of TGF-β1 in a mouse proximal tubular cell line [4,5]. Since we have also discovered that ANG II increases reactive oxygen species in cultured tubular cells, partly by up-regulating a membrane-bound NAD(P)H oxidase [6–8], we reasoned that these oxygen radicals may be involved in the expression of TGF-β1. However, surprisingly we learnt that antioxidant treatment rather than oxygen stress stimulates transcription and expression of TGF-β1 in cultured murine proximal tubular cells. These findings may have important implications for the remodeling of the tubulointerstitium after oxygen-dependent injury such as the reperfusion phase subsequent to ischemia.

2. Materials and methods

2.1. Cell culture

Mouse murine proximal tubular (MCT) cells are a well-characterized cell line with many properties of proximal tubular cells [9–11]. Although these cells are transformed with a non-capsid form of the SV 40 virus, they have been used by many investigators studying a broad range of topics from metabolic studies to ion transport and have been found to faithfully reflect many characteristics of proximal tubules [9–11]. MCT cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 450 mg/dl glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 2×10^{-3} mol/l supplemental glutamine, and 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL, Eggenstein, Germany) at 37°C in 5% CO_2. Cells were passaged every 72 h by light trypsination.

2.2. Northern blots

Quiescent MCT cells were stimulated for 24 h with a single dose of 1–5 μM diphenylene iodine (DIP, Sigma, Deisenhofen, Germany), an inhibitor of NAD(P)H oxidase. In other studies, MCT cells were incubated in 0.5–8 μM EUK-8 (Calbiochem, Bad Soden, Germany), a synthetic salen–manganese complex with high superoxide dismutase, catalase, and oxyradical scavenger activities [12,13]. To test the direct effect of oxygen stress, MCT cells were treated with 0.1–10 μM H_2O_2

*Corresponding author. Fax: (49)-40-42803 5186.
E-mail: wolf@uke.uni-hamburg.de

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for 24 h. After washing in RNase-free phosphate-buffered saline (PBS), cells were directly lysed with acid guanidinium thiocyanate, and total RNA was isolated. Equal amounts of total RNA (15 μg per lane) were denatured in formamide–formaldehyde at 65°C and electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde. Blotting, hybridization, and washing conditions were exactly as previously described [4]. The cDNA probes used were an *Eco*RI fragment encoding mouse TGF-β and a 2.0 kb insert of the plasmid pMCI encoding the murine ribosomal 18S band. Exposed films were scanned with Fluor-S™ multiimager (Bio-Rad laboratories, Hercules, USA), and data were analyzed with the computer program Muli-Analyst™ from Bio-Rad. A ratio between the intensities of the hybridization signals for TGF-β receptors and 18S was calculated. Northern blots were repeated three times with qualitatively similar results.

2.3. Western blots

A total of 10^6 quiescent cells were treated for 24 h in serum-free DMEM with DIP. At the end of the stimulation period, cells were washed twice in PBS, and cell monolayers were directly lysed on ice in 150 μl of a buffer containing 2% sodium dodecyl sulfate (SDS) and 60 mM Tris–HCl (pH 6.8) supplemented with a cocktail of protease inhibitors (Complete™, Boehringer Mannheim, Germany, contains antipain–HCl, chymostatin, leupeptin, bestatin, pepstatin, phosphoramidon, aprotinin, EDTA). The protein content was measured by a modification of the Lowry method. Protein concentrations were adjusted to 80 μg/sample, and 5% glycerol/0.03% bromophenol blue/10 mM dithiothreitol (DTT) were added, then samples were boiled for 5 min. After centrifugation, supernatants were loaded onto a denaturing 12% SDS–polyacrylamide gel. Low molecular weight markers (Rainbow markers; Amersham), which comprise 2350–45 000 Da served as the molecular weight standards. After completion of electrophoresis, proteins were electroblotted onto a nitrocellulose membrane (High-bond-N, Amersham) in transfer buffer (50 mM Tris–HCl (pH 7.0), 380 mM glycine, 20% methanol). Filters were stained with Ponceau S to control for equal loading and transfer. The blots were blocked in 5% non-fat dry milk in PBS with 0.1% Tween 20 for 1 h at 22°C. For the detection of TGF-β1, a 1:1000 dilution of a polyclonal rabbit anti-mouse TGF-β1 antibody (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) was used. Washes, incubations with horseradish peroxidase-conjugated anti-rabbit secondary antibodies, and detection using the ECL reagent (Amersham) were performed according to the manufacturer's recommendations. Western blots were independently repeated three times with qualitatively similar results.

2.4. Transient transfections and reporter gene assays

The CAT reporter plasmids A406 (having a 406 bp regulatory region 5′ end upstream of the murine TGF-β1 gene transcriptional start site) and A113 (113 bp 5′ end fragment) were generous gifts from Dr. Andrew G. Geiser (National Cancer Institute, Bethesda, MD, USA) [14]. The TGF-β1 regulatory fragments were subcloned in front of the luciferase gene of the pGL2-basic vector (Promega, Madison, WI, USA). Three Sp1 binding sites in the construct A113 were progressively deleted using standard PCR technology resulting in the plasmids A113Δ-Sp1(IV), A113Δ-Sp1(IV)-Sp1(III), and A113Δ-Sp1(IV)-Sp1(III)-Sp1(II). All constructs were sequenced to confirm correct identity. To test for potential antioxidant-mediated transcriptional activity of the TGF-β1 gene, transient transfection of MCT cells was performed using Lipofectin™ (Gibco-BRL) according to the manufacturer's recommendations. Quiescent MCT cells (final density 10^5 cells) were co-transfected with 10 μg of the various TGF-β1 promoter/luciferase constructs, and the same amount of the plasmid pSV-β galactosidase in which the β-galactosidase gene is under control of SV 40 promoter and enhancer. As an additional control, MCT cells were transfected with pGL2-control plasmid in which the luciferase gene is under control of SV 40 enhancer/promoter. Other cells also received 1 μg/ml either p22phox, a subunit of membrane-bound NAD(P)H oxidase, sense (5′-GGTCCTCACCATGGGGCAGATC-3′) or antisense (5′-GATCTGCCCCATGGTGAGGACC-3′) phosphothioate-modified oligonucleotides [6,7]. These antisense oligonucleotides have previously been shown to attenuate NAD(P)H oxidase activity [6,7]. After 12 h, the serum-free medium was renewed and cells were incubated for another 24 h with control medium, H_2O_2, or the various antioxidants including 0.1 M Tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid, Sigma), another synthetic oxygen radical scavenger. At the end of the experiments, cells were washed three times in PBS, cell layers were lysed, and protein concentrations of supernatants were adjusted to equal concentrations. Luciferase and β-galactosidase activities were measured as previously described. A ratio between luciferase and β-galactosidase activities was calculated, and data from unstimulated control cells were considered as 100%. Transient transfections and reporter gene assays were independently performed at least six times for each experiment.

2.5. Gel shift assay

To investigate which DNA binding proteins may be induced by antioxidants in MCT cells, gel shift assays were performed. MCT cells were stimulated as appropriate for 24 h. Cells were washed directly in flask twice with ice-cold pBS and were scraped off the bottom using a rubber policeman. Cells were centrifuged, pellets were transferred to Eppendorf cups, and resuspended in a buffer A composed of 20 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml aprotinin. Cells were lysed by the addition of 10% Nonidet-P40 for 10 s, lysates were immediately centrifuged for 30 s at 13 000 rpm, and pellets were resuspended in buffer B (30 mM HEPES, 500 mM NaCl, 0.1 MD EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). After another centrifugation step at 13 000 rpm for 5 min, supernatants containing nuclear proteins were aliquoted, frozen, and stored in liquid nitrogen. A total of 3.50 pmol of double-strand consensus oligonucleotides for Sp1 (5′-ATTC-GATCGGGGCGGGGCGAGC-3′) and AP2 (5′-CGCTTGAT-GAGTCAGCCGGAA-3′) were end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) using T4 polynucleotide kinase. Binding reactions were performed in gel shift binding buffer (10 mM Tris–HCl (pH 7.5), 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 50 μg/ml poly [dI-dC]) with and without cold competitor oligonucleotides at room temperature for 30 min. Reactions were stopped by addition of loading buffer and samples were run on a non-denaturing 6% polyacrylamide gel. Gels were then exposed to X-ray films. Gel shift analysis was independently performed three times (separate cell stimulation, nuclear extract preparation, and binding reactions) with qualitatively similar results.

2.6. Cytotoxicity assay

To test for the potential cytotoxicity of the antioxidants DIP, and EUK-8 as well as H_2O_2, a quantitative lactate dehydrogenase (LDH) release assay adapted to a microtiter plate (CytoTox96®, Promega) was used. This assay is based on LDH-mediated conversion of a tetrazolium salt into a red formazan product, and the assay was exactly performed as recommended by the manufacturer. Percent cytotoxicity was calculated as experimental LDH release/maximum LDH release.

2.7. Statistical analysis

All data are presented as the means ± S.E.M. Statistical significance between different groups was first tested with the non-parametric Kruskal–Wallis test. Individual groups were subsequently tested using the Wilcoxon–Mann–Whitney test. A P value of < 0.05 was considered significant.

3. Results

Incubation of quiescent MCT cells for 24 h with the NADP(H) oxidase inhibitor DIP induced a strong increase in TGF-β1 mRNA as detected by Northern blots (Fig. 1; controls: 1.0 ± 0.0, 1 μM DIP: 4.4 ± 0.5*, 3 μM DIP: 3.8 ± 0.4*, 5 μM DIP: 4.7 ± 0.7* relative changes in RNA expression normalized to 18S, $*P < 0.05$, $n = 3$). In addition, EUK-8, a synthetic salen–manganese complex with high superoxide dismutase, catalase, and oxyradical scavenger activities, also stimulated an increase in TGF-β1 transcripts (Fig. 2; controls: 1.0 ± 0.0, 0.5 μM EUK: 1.8 ± 0.2*, 1 μM EUK: 1.2 ± 0.3, 2 μM EUK: 0.9 ± 0.3, 5 μM EUK: 3.2 ± 0.3*, 8 μM EUK: 3.0 ± 0.4* relative changes in RNA expression normalized to 18S, $*P < 0.05$, $n = 3$). In contrast, TGF-β1 mRNA expression was reduced by treatment with H_2O_2 (data not shown). Fur-

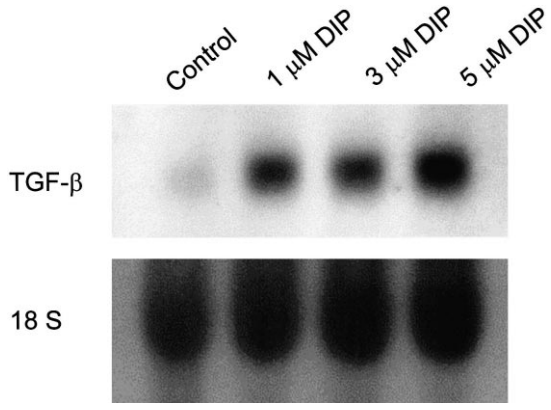

Fig. 1. Northern blot for TGF-β1. A single dose of 1–5 μM of DIP for 24 h, an inhibitor of membrane-bound NAD(P)H oxidase, significantly increased TGF-β1 mRNA expression. This blot is representative for three independent experiments with qualitatively similar results.

thermore, DIP also increased TGF-β1 protein expression as detected by Western blotting (Fig. 3). The used concentrations of DIP and EUK were not toxic for the cells, but rather reduced, at least in higher concentrations, the spontaneous apoptosis of MCT cells grown in serum-free medium (Table 1).

We next tested whether the antioxidants may directly influence transcriptional activity of the mouse TGF-β1 gene. To this end, a construct with 406 bp upstream the first transcriptional start site containing enhancer/promoter elements [14] was subcloned in a luciferase reporter plasmid. This construct has previously been characterized in detail and is activated after stimulation with various agents that induce TGF-β1 expression. As shown in Fig. 4, 1–3 μM DIP as well as 0.5–2 μM EUK significantly increased luciferase activity. However, DIP did not stimulate transcriptional activity of the pGL2-control construct (controls: 100 ± 0.0, 1 μM DIP: 77 ± 19, 3 μM DIP: 71 ± 11, 5 μM DIP: 101 ± 13% relative change in luciferase activity normalized to β-galactosidase, $n = 4$, not significant versus controls). In order to inhibit membrane-bound NAD(P)H oxidase by other means than with DIP, p22phox phosphothioate-modified antisense oligonucleotides were transfected into MCT cells. We and others have previ-

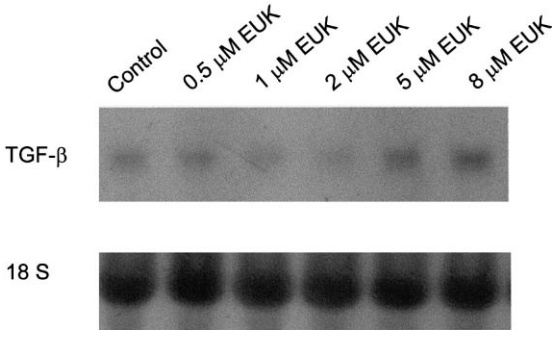

Fig. 2. EUK-8, a synthetic salen–manganese complex with strong oxyradical scavenger activities, also stimulated TGF-β1 transcripts in MCT cells. This blot is representative for three independent experiments with qualitatively similar results.

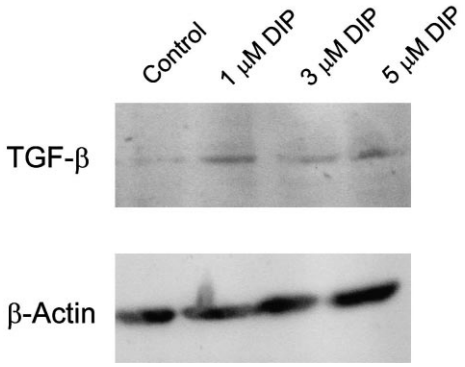

Fig. 3. Western blot for TGF-β1. DIP also increased TGF-β1 protein expression. This blot is representative for three independent experiments with qualitatively similar results.

ously shown that this antisense oligonucleotide inhibited NAD(P)H oxidase activity [6,7]. Transfection of MCT cells with p22phox antisense, but not sense oligonucleotides, increased TGF-β1 transcriptional activity (Table 2). In contrast, exogenously added H_2O_2 reduced TGF-β1 transcription (Table 2). Moreover, another oxygen radical scavenger (Tiron) also stimulated TGF-β1 transcription (Table 2). Analysis of the 406 bp sequence containing the TGF-β1 enhancer/promoter elements revealed the presence of four Sp1 consensus sequences [14]. The construct A113 in which the first Sp1 site was deleted [14] exhibited less transcriptional activity after challenge with DIP than the parental A406 reporter plasmid (Table 3). Moreover, progressive mutations of all remaining Sp1 sites lead to a complete abolishment of DIP-mediated TGF-β1 transcriptional activity (Table 3). We finally tested whether Sp1 proteins bind to their putative sequences using gel shift analysis. Fig. 5 shows an example of such a gel shift assay. There is base-line expression of Sp1 binding proteins in MCT cells grown in serum-free medium (control). This band is specific for Sp1 because it is abolished in the presence of cold competitor but not by addition of non-competing AP2 oligonucleotides. Stimulation of cells with 1–3 μM DIP, or 2–5 μM EUK induced an increase in Sp1 binding proteins. In contrast, H_2O_2 and ANG II, known to activate oxygen radical production in these cells [6], reduced the base-line expression of nuclear Sp1 proteins.

Table 1
Cytotoxicity assay

	Cytotoxicity (%)
Controls (serum-free)	3.4 ± 0.26
Controls (10% FCS)	3.3 ± 0.59
1 μM DIP (serum-free)	2.5 ± 0.13*
3 μM DIP (serum-free)	2.5 ± 0.19*
1 μM EUK (serum-free)	3.3 ± 0.23
3 μM EUK (serum-free)	3.3 ± 0.11
5 μM EUK (serum-free)	2.3 ± 0.09*
8 μM EUK (serum-free)	2.6 ± 0.07*
0.1 μM H_2O_2 (serum-free)	2.5 ± 0.19*
1 μM H_2O_2 (serum-free)	2.4 ± 0.10*
10 μM H_2O_2 (serum-free)	2.9 ± 0.10

*$P < 0.05$ versus controls (serum-free), $n = 4$.

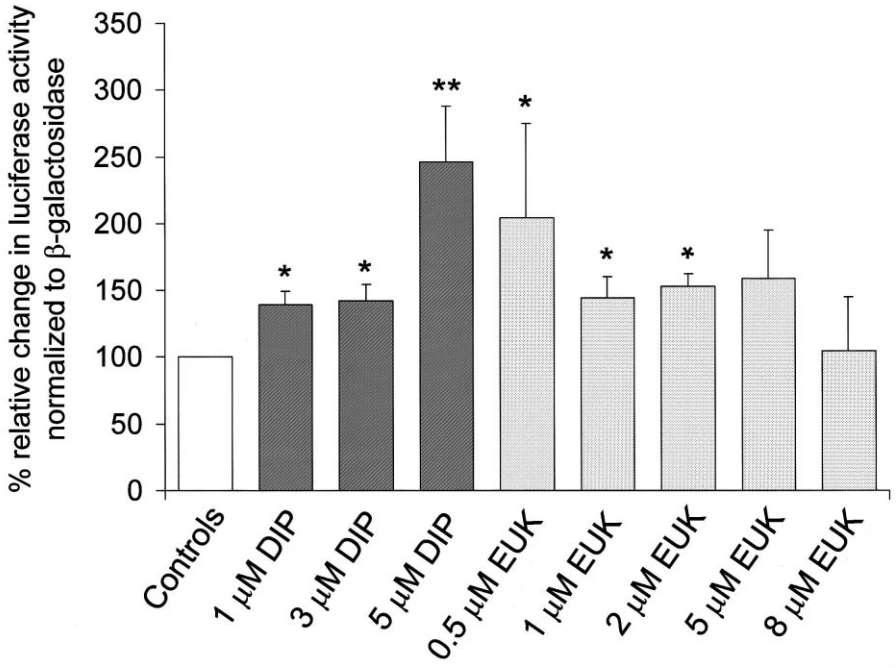

Fig. 4. Results of TGF-β1 transcriptional activation studies using the construct A406. Luciferase activity was normalized to β-galactosidase expression. Controls were assigned a relative value of 100%. Stimulation for 24 h with 1–5 μM DIP or 0.5–2 μM EUK induced a significant increase in TGF-β1 transcription. *$P < 0.05$ versus controls, **$P < 0.01$ versus controls, $n = 8$.

4. Discussion

The present data convincingly show that inhibition of membrane-bound NAD(P)H oxidase either pharmacologically with DIP or using antisense oligonucleotides stimulates transcription of TGF-β1 in cultured mouse proximal tubular cells. To the best of our knowledge, this is the first demonstration that TGF-β1 transcription is stimulated by antioxidants. The increase in transcriptional activity results in an increase in steady-state mRNA as well as TGF-β1 protein expression. Furthermore, the antioxidant EUK exerted similar effects indicating that these observed effects are not due to some particular properties of DIP.

These findings indicate that base-line TGF-β1 transcription is negatively regulated by the intracellular generation of reactive oxygen species. There is increasing evidence that the membrane-bound NAD(P)H oxidase plays an important role in the generation of oxygen radicals and these species are generated in much lower concentrations than those during the 'respiratory burst' of neutrophils [15–17]. The membrane-bound NAD(P)H oxidase is activated by various hormones and the generated reactive oxygen radicals may serve as signal intermediates rather than exerting toxic activities [16,17].

Table 2
Transcriptional activity of A406 luciferase construct

	Relative change in luciferase normalized to β-galactosidase (%)
Without oligos	100 ± 0.0
p22phox sense oligos	93 ± 12
p22phox antisense oligos	160 ± 10*
Controls	100 ± 0.0
0.1 μM H_2O_2	79 ± 11
1 μM H_2O_2	67 ± 26
2 μM H_2O_2	64 ± 17*
0.1 μM Tiron	151 ± 14*

*$P < 0.05$ versus controls, $n = 3$–5.

Our gel shift experiments clearly demonstrate that more Sp1 protein binds to target sequences when the generation of reactive oxygen radicals is inhibited by either DIP or EUK. Furthermore, binding of Sp1 is reduced by exogenous H_2O_2 as well as by ANG II. We have previously demonstrated that ANG II stimulates reactive oxygen formation in MCT cells through activation of membrane-bound NAD(P)H oxidase. The mutagenesis experiments revealed that the simulated increase in TGF-β1 transcription depends on the presence of Sp1 binding sites in the enhancer/promoter region. Indeed, repression of transcription by oxidative stress through a reduction in Sp1 binding has been characterized for various genes [15]. Sp1 that binds to GC-rich DNA sequences is particularly sensitive to thiol-oxidizing reagents [15]. The DNA binding activity of Sp1 is reversibly impaired by H_2O_2 in vivo [15]. In addition, low intracellular concentrations of antioxidant substances such as glutathione also reduced binding of Sp1 to target sequences [15]. In addition to Sp1, it has been reported in endothelial cells that the transcription factor AP-1 is activated by the antioxidant pyrrolidine dithiocarbamate[18]. However, although the TGF-β1 enhancer/promoter has a single AP-1 consensus site adjacent to the transcriptional start [14], this site remains intact in the mutagenesis experiments. Yet, DIP-mediated transcription was significantly reduced in the constructs with mutated Sp1 sites but intact AP-1 sequence. Thus, we believe that the induced transcription of TGF-β1 by antioxidants is primarily mediated through increased Sp1 binding and that AP-1 does not play a major role in our system.

We have previously demonstrated that ANG II stimulates TGF-β1 transcription [5]. We also found that ANG II upregulated membrane NAD(P)H oxidase and increased the

Table 3
Effects of deletion/mutation of Sp1 binding sites on transcriptional activity

	Relative change in luciferase normalized to β-galactosidase (%)
A406 (all five Sp1 sites)+controls	100 ± 0.0
A406 (all five Sp1 sites)+5 μM DIP	246 ± 41**
A113 (four Sp1 sites)+controls	100 ± 0.0
A113 (four Sp1 sites)+5 μM DIP	233 ± 19**
A113Δ-Sp1(IV) (three Sp1 sites)+controls	100 ± 0.0
A113Δ-Sp1(IV) (three Sp1 sites)+5 μM DIP	110 ± 26
A113Δ-Sp1(IV)-Sp1(III) (two Sp1 sites)+control	100 ± 0.0
A113Δ-Sp1(IV)-Sp1(III) (two Sp1 sites)+5 μM DIP	86 ± 15
A113Δ-Sp1(IV)-Sp1(III)-Sp1(II) (one Sp1 site)+control	100 ± 0.0
A113Δ-Sp1(IV)-Sp1(III)-Sp1(II) (one Sp1 site)+5 μM DIP	65 ± 11*

*$P < 0.05$ versus controls, **$P < 0.01$ versus control, $n = 3–6$.

synthesis of intracellular reactive oxygen species [6]. However, our current results clearly suggest that the previously reported ANG II stimulation of TGF-β1 transcription in MCT cells is rather inhibited by concomitant induction of oxygen radicals and may involve transcription factors other than the Sp1 binding sequence in the TGF-β1 enhancer/promoter. Further experiments are necessary to address whether the ANG II-induced TGF-β1 expression is further enhanced in the presence of antioxidants uncoupling the two signal transduction pathways.

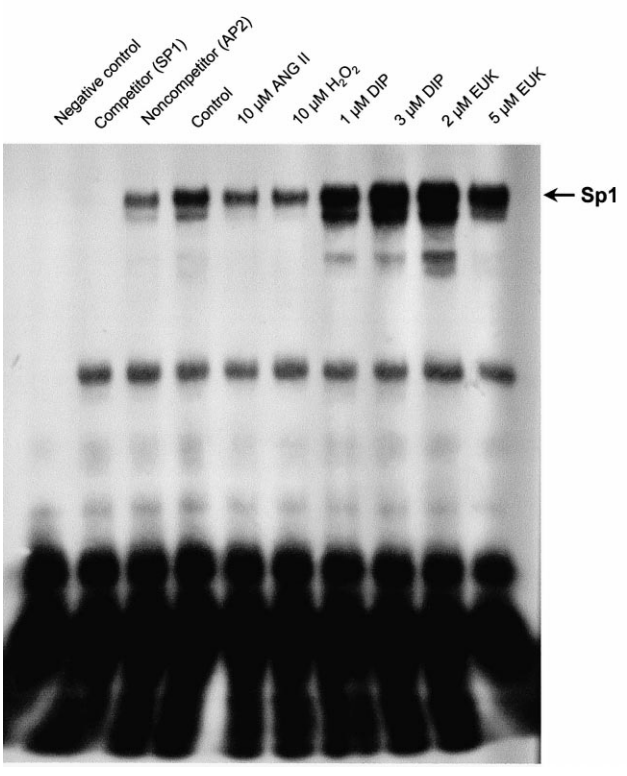

Fig. 5. Gel shift assay for Sp1. Nuclear proteins were isolated from MCT cells treated for 24 h with the various mediators. Proteins were incubated with radioactively labeled Sp1 consensus oligonucleotides. No retention of band with nuclear proteins (negative control). The specific Sp1 band was attenuated by cold competitor, but not non-competitor (AP2 oligonucleotides) indicating specificity of the binding. ANG II as well as H_2O_2 significantly reduced binding of proteins to Sp1 oligonucleotides. In contrast, 1–3 μM DIP and 2–5 μM EUK considerably enhanced the amount of binding. This blot is representative for three independent experiments with qualitatively similar results.

Reperfusion of the kidney after transient ischemia is a typical situation with enhanced generation of oxygen radicals [19], though this occurs through the xanthine oxidase system and not by activation of membrane-bound NAD(P)H oxidase. This situation is also characterized by tubular cell proliferation and migration along the basement membrane to reconstitute destroyed renal tissue [20,21]. Since TGF-β inhibits proliferation of tubular cells by induction of inhibitors of cyclin-dependent kinases [22,23], a down-regulation of this cytokine by oxygen radicals may facilitate regeneration of tubules after renal ischemia.

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