Oxidative stress in the pathogenesis of experimental mesangial proliferative glomerulonephritis

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Budisavljevic, Milos N., LeAnn Hodge, Kelli Barber, John R. Fulmer, Ramon A. Durazo-Arvizu, Sally E. Self, Martin Kuhlmann, John R. Raymond, and Eddie L. Greene. Oxidative stress in the pathogenesis of experimental mesangial proliferative glomerulonephritis. Am J Physiol Renal Physiol 285: F1138–F1148, 2003; 10.1152/ajprenal.00397.2002.—Reactive oxygen species (ROS) are increasingly believed to be important intracellular signaling molecules in mitogenic pathways involved in the pathogenesis of glomerulonephritis (GN). We explored the effects of the antioxidants α-lipoic acid and N-acetyl-L-cysteine on ERK activation in cultured mesangial cells and the role of ERK activation in the severity of glomerular injury in a rat model of anti-Thy 1 GN. In cultured mesangial cells, growth factors stimulated ERK phosphorylation by 150–450%. Antioxidants reduced this increase by 50–60%. Induction of anti-Thy 1 nephritis in rats led to a 210% increase in glomerular ERK phosphorylation. This increase in phosphorylated ERK was reduced by 50% in animals treated with α-lipoic acid. Treatment with α-lipoic acid resulted in significant improvement of glomerular injury. Cellular proliferation was reduced by 100%, and the number of proliferating cell nuclear antigen-positive cells was reduced by 64%. The increased expression of glomerular transforming growth factor-β1 protein and mRNA in rats with anti-Thy 1 nephritis was significantly attenuated and mesangial cell transformation into myofibroblasts was completely prevented by treatment with α-lipoic acid. The effects of α-lipoic acid were at least partially due to inhibition of oxidative stress. In rats with anti-Thy 1 nephritis, ROS production was increased 400–500%, and this increase was inhibited by 55% by treatment with α-lipoic acid. We suggest that ROS may mediate glomerular injury by inducing ERK phosphorylation. α-Lipoic acid should be considered a potential therapeutic agent in certain types of human GN.

α-lipoic acid; anti-Thy 1 nephritis; extracellular signal-regulated kinase; α-smooth muscle actin; transforming growth factor-β1

EARLY CELLULAR PROLIFERATION followed by subsequent fibrosis is a prominent hallmark of proliferative glomerulonephritis (GN), and it may ultimately lead to end-stage renal disease (17). The involvement of extracellular stimuli, such as growth factors, cytokines, activated complement, and immune complexes, in the pathogenesis of experimental and human GN has been known for many years. However, the intracellular mediators that transduce signals from noxious extracellular stimuli to unfettered cellular proliferation and accompanying excess extracellular matrix deposition are only recently being unraveled (35). Precise delineation of these intracellular mediators is essential for development of strategies that will ameliorate the progression of disease and prevent the inexorable loss of renal function. Experiments performed initially in cultured glomerular cells and, more recently, in certain models of experimental GN implicate the activation of ERK in glomerular cellular proliferation (6). ERK, a mitogen-activated protein kinase, is a member of the family of serine/threonine kinases that regulates the expression of many genes important for cellular growth, mainly by phosphorylating transcription factors, including c-myc, c-Jun, the STAT proteins, NFIL6, ATF2, ETS1, and ELK1. Binding of extracellular ligands to G protein- or tyrosine kinase-coupled receptors leads to a series of protein-protein interactions that culminate in phosphorylation of the ERK kinase (MEK) that is a specific activator of ERK. The activated (phosphorylated) ERK can then translocate to the nucleus, where it targets specific transcription factors, which, when activated, result in increased cellular proliferation and multiple other effects related to cell function (10, 34, 40).

Enhanced generation of reactive oxygen species (ROS) has also been demonstrated in human and experimental GN (14, 33, 36). However, many of the previous reports evaluating the effects of ROS have concentrated on the direct damaging effects of ROS on renal structural integrity, particularly those caused by...
formation of lipid peroxidation products. Because of more recent evidence implicating ROS as signaling molecules (37), we explored the roles of ROS as regulators of intracellular signaling in mitogenic pathways involved in the injury produced in an in vivo model of GN. In the present study, we extended our previous findings of ROS as major participants in ERK phosphorylation in cultured mesangial cells (12). We suggest that oxidative stress can cause glomerular injury, not only by inflicting direct damage on the glomerular structures but also by inducing ERK activation and cellular proliferation. Furthermore, suppression of oxidative stress results in inhibition of ERK activation, reduction of transforming growth factor-β1 (TGF-β1) mRNA transcription, and amelioration of glomerular injury.

METHODS

Unless otherwise specified, all drugs, chemicals, reagents, and antibodies were obtained from Sigma (St. Louis, MO). The studies were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina. All in vivo animal experiments were conducted in a humane manner in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Mesangial Cell Culture

Glomerular isolation and establishment of primary mesangial cell cultures were performed as described previously (31). Cells from passages 6–12 were used for all in vitro experiments. For experiments investigating ERK phosphorylation, cell quiescence was induced by placing cell cultures that were 60–70% confluent in RPMI 1640 medium containing 0.5% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin for 48 h before stimulation with growth factors. Equal numbers of quiescent cells (≈2 × 10^6 cells) in 12-well culture plates were treated with epidermal growth factor (EGF, 10 ng/ml), basic fibroblast growth factor (bFGF, 10 ng/ml), platelet-derived growth factor (PDGF, 10 ng/ml), thrombin (1 μM), or vehicle for 10 min in the presence or absence of 100 and 250 μM α-lipoic acid or 30 mM N-acetyl-l-cysteine (NAC).

Similarly, in [3H]thymidine experiments designed to measure cellular proliferation, cell quiescence was induced when cells were 50% confluent by treatment of the cells with RPMI 1640 medium containing 0.1% fetal calf serum (FCS) and penicillin-streptomycin for 48 h. Cells were then stimulated with 10% FCS or PDGF (10 ng/ml), or vehicle for 5 min, the lysates were sonicated for 1 min and centrifuged at 4°C at 10,000 g for 30 min, and the supernatant was stored at −70°C before the induction of GN. The untreated group (n = 12) received intraperitoneal injections of vehicle according to the same protocol. Animals were killed 4 and 7 days after induction of GN. In each experiment, kidneys from normal animals, i.e., those without anti-Thy 1 nephritis, served as controls. The lower pole of one kidney from each animal was placed in Carnoy’s solution and processed for histology and immunohistochemistry. The remaining kidney tissue was used for isolation of glomeruli by selective sieving, as described previously (31). The purity of glomerular isolates (>90%) was routinely established by light microscopy. Isolated glomeruli were used for analysis of ERK activation by Western blot and TGF-β1 message induction by relative quantitative PCR (see below).

Western Blot Analysis for Total and Phosphorylated ERK

Cultured mesangial cells. This assay was performed as described previously (13). Briefly, 20 μl of cell lysates were loaded onto 4–20% Tris-glycine gradient miniature precast gels (Novex, San Diego, CA) for electrophoresis. Proteins were transferred onto Immobilon-P (Millipore, Bedford, MA) membranes and probed with a phosphorylation state-specific ERK1/2 antibody or a total ERK1/2 antibody that recognizes phosphorylated and nonphosphorylated ERK (New England Biolabs, Beverly, MA). After incubation with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody, bands were visualized using a CDP® Star kit (New England Biolabs). Band intensity was determined by densitometry.

Glomeruli from animals with anti-Thy 1 nephritis in vivo. Soluble lysates from isolated glomeruli were prepared as previously described with minor modifications (3). Briefly, isolated glomeruli were placed immediately in 500 μl of lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 0.1 mM sodium orthovanadate, and a proteinase inhibitor cocktail containing 150 nM aprotinin, 1 μM leupeptin, 500 μM 4-(2-aminoethyl)benzenesulfonyl fluoride HCl, 1 μM E-64, and 0.5 mM EDTA (Calbiochem, San Diego, CA) at 4°C. After incubation for 5 min, the lysates were sonicated for 1 min and then centrifuged for 15 min at 10,000 g at 4°C. A small aliquot of the supernatant was set aside for determination of total protein concentration using the bicinchoninic acid protein assay (Pierce, Rockford, IL), and the remainder was stored at −20°C until the day of assay. To detect total and phosphorylated ERK, the supernatant (20 μg of protein) was mixed 1:1 with 2× Laemmli buffer, boiled for 5 min at 95°C, and loaded onto 4–20% polyacrylamide gradient gels (Novex). After electrophoresis, proteins were processed for immunoblotting as described above.

TGF-β1 mRNA Expression

Total RNA was prepared from glomeruli isolated from control rats and rats with anti-Thy 1 nephritis. Briefly, glomeruli were washed in PBS, centrifuged at 400 g, and resuspended in TRIzol (GIBCO BRL, Gaithersburg, MD; 1 ml/100 mg of glomeruli). Subsequent steps were carried out as recommended by the manufacturer. The isolated RNA was stored at −70°C until the day of the assay.

Total RNA was used to synthesize first-strand cDNA using a SuperScript first-strand synthesis system for RT-PCR (GIBCO BRL) according to the manufacturer’s directions. Briefly, 1 μg of total RNA was incubated with a 10 mM dNTP mixture and 1 μl of random hexamers in nuclelease-free water for 5 min at 65°C and then chilled on ice for ≥1 min. A reaction mixture containing 10× RT buffer, 25 mM MgCl₂, 0.1 M DTT, and an RNase inhibitor was added, and the
samples were incubated for 2 min at room temperature. RT (50 U) was added, and the samples were incubated for 10 min at room temperature and then for 50 min at 44°C. For termination of the reaction, the sample was heated to 70°C for 15 min, placed on ice for 1 min, and treated with RNase H for 20 min at 37°C. Samples were stored at −20°C until they were used for PCR.

Relative semiquantitative PCR was done using a thermal cycler (model 9700, Perkin-Elmer) and a TGF-β1 gene-specific relative RT-PCR kit (Ambion, Austin, TX) according to the manufacturer’s directions. Briefly, the kit uses a multiplex reaction to coamplify a TGF-β1 target and an 18S ribosomal target, which is used as an internal standard. Samples were amplified using a hot start and 28 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s. PCR products were visualized on 1% agarose gels with ethidium bromide staining. Band intensities were measured using a UMAX 4.3 scanner, Adobe Photoshop 4.0, and Scananalysis 2.5 software (Biosoft 1995, Ferguson, MO).

Chemiluminescent Detection of O2·− Production in Glomerular Cells

The procedure of Ohara et al. (27) was used to measure O2·− production in glomerular fractions of rat kidney. Briefly, rats were killed 24 h after induction of anti-Thy 1 nephritis, and glomeruli were collected in a Krebs-HEPES (K-H) buffer consisting of (in mM) 99.01 NaCl, 4.69 KCl, 1.87 CaCl2, 1.20 MgSO4, 1.03 K2HPO4, 25.0 NaHCO3, 20.0 Na-HEPES, and 11.1 glucose. The glomerular fractions were centrifuged at 3,000 rpm for 7 min at 4°C, and the pellets were resuspended in 400 μl of K-H buffer. Assays were performed using 100 μl of this sample suspension in 2 ml of K-H buffer. After these samples were heated at 37°C for 30 min, lucigenin was added to the tubes for a final concentration of 0.25 mM. The tubes were left at room temperature for 15 min, and then chemiluminescence of each sample was measured over a period of 30–60 min using a luminometer (Femtomaster FB12, Zylux). A blank containing all components except sample suspension was read before each series of sample readings and was subtracted from the sample readings.

Immunohistochemistry of Proliferating Cell Nuclear Antigen

Slides with 3-μm sections of kidney tissue were prepared by heating at 60°C for 30 min. Sections were deparaffinized in three changes of xylene, gradually hydrated through multiple changes of ethanol, and rinsed in 1× TBSA-BSA (150 mM NaCl, 0.1% BSA, 5 mM Tris, 0.1% Triton X-100, and 0.05% sodium azide, pH 7.4). Endogenous peroxidase activity was quenched with 3% H2O2 in methanol for 10 min. The slides were then denatured in 4 N HCl for 20 min and stabilized in 100 mM sodium tetraborate for 5 min. Nonspecific staining was blocked using 10× TBSA-BSA at 30 min at room temperature. Sections were then incubated overnight at 4°C with a monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (Calbiochem), rinsed, incubated for 2 h at room temperature in a horseradish peroxidase-labeled goat anti-mouse IgG (Kirkegard and Perry, Gaithersburg, MD), developed using 3,3′-diaminobenzidine, and counterstained in eosin.

In initial experiments, before the rats were killed, they were injected with bromodeoxyuridine, and slides were stained with antibromodeoxyuridine antibodies in addition to anti-PCNA antibodies. We obtained nearly identical results. Therefore, we presented only data with anti-PCNA staining.

Immunofluorescent Studies

Staining for α-smooth muscle actin. Slides were deparaffinized and hydrated as described above, rinsed in PBS, and immersed in 10 mM sodium citrate solution (pH 6.0) for 5 min at 95°C. The solution was changed, and the process was repeated once. Slides were allowed to cool in the same solution for 20 min and rinsed in PBS. Nonspecific staining was blocked with PBS + 1% BSA + 10% normal goat serum for 1 h at room temperature. Slides were incubated overnight at 4°C in monoclonal anti-human smooth muscle actin (α-SMA) antibody (Dako, Carpinteria, CA). Antibodies were visualized using a goat anti-mouse FITC-labeled secondary antibody.

Staining for TGF-β1 protein. Slides were prepared as described for α-SMA and incubated with goat anti-TGF-β1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Staining was visualized using a donkey anti-goat FITC-labeled secondary antibody.

Complement 3 staining. Untreated or α-lipoic acid-treated animals were killed 1 h after induction of anti-Thy 1 nephritis. Kidneys were placed in liquid nitrogen and prepared for cryostat sections. Sections were rinsed three times in PBS + 1% BSA and incubated overnight at 4°C with sheep anti-rat complement 3 antibody (Biogenesis, Poole, UK). Antibodies were visualized using a donkey anti-sheep FITC-conjugated secondary antibody.

Scoring of Glomerular Injury

Kidney sections were stained with hematoxylin and eosin or periodic acid-Schiff and scored for cellularity and number of PCNA-positive cells, mitoses, and microaneurysms, as previously described (16). To assess cellular proliferation and the number of PCNA-positive cells, the cell count was determined in 30 glomeruli per kidney section, and the results were averaged. To assess cellularity, glomeruli were scored and normalized to control values. PCNA is reported as the number of positive cells per glomerulus. The numbers of mitoses and microaneurysms were also counted in 30 glomeruli. All glomerular scoring was done by a pathologist (S. E. Sell) who was unaware of the experimental design.

Statistical Analysis

Values are results of at least two separate experiments. The results between experiments were consistent, and data from typical experiments are presented in Figs. 1–3, 5, 6, 8, 10, and 11. Results from treated and untreated animals in which anti-Thy 1 nephritis was induced were normalized to results from healthy control animals. A two-sample two-sided t-test was used to evaluate treatment, i.e., with α-lipoic acid, for significance against the untreated group. Analyses were confirmed using nonparametric statistics, the traditional Mann-Whitney test, and approximated permutation test. Experiments involving repeated measures were analyzed using a generalized estimating equations approach. The generalized estimating equations approach provides model-based regression methods applicable for analysis of the correlated data that result from the repeated-measures experiments. Differences between treatments were considered statistically significant for P < 0.05. Values are means ± SE. Analyses were performed using the statistical software STATA.
RESULTS

Role of Antioxidants in Growth Factor-Induced ERK Phosphorylation in Cultured Rat Mesangial Cells

Quiescent cultured mesangial cells were stimulated with EGF, bFGF, PDGF, thrombin, or vehicle, and ERK phosphorylation was determined (Fig. 1). EGF, bFGF, PDGF, and thrombin induced ~350, 250, 150, and 250% increases in phosphorylated ERK, respectively, compared with treatment with vehicle alone. Conversely, when cells were treated with these growth factors in the presence of the thiol-derived antioxidants α-lipoic acid (250 μM) and NAC (30 mM), ERK phosphorylation was reduced significantly by 50–60% for each growth factor tested. Treatment with 100 μM α-lipoic acid modestly decreased phosphorylated ERK by 20–30%. Growth factors had no effect on total ERK, and neither α-lipoic acid nor NAC, when used alone, led to a significant change in ERK phosphorylation compared with vehicle (data not shown). These data demonstrate the ability of two chemically distinct antioxidants to significantly reduce growth factor-induced ERK phosphorylation in cultured mesangial cells. Our data suggest that ERK phosphorylation induced by growth factors is at least partly mediated through the generation of ROS.

ERK Phosphorylation Is Involved in Growth Factor-Stimulated Mesangial Cellular Proliferation

We, as well others, previously showed that an MEK inhibitor, PD-98059, attenuates increased ERK phosphorylation in agonist-stimulated mesangial cells. We wanted to determine whether this inhibition has effects on cellular proliferation. Cultured rat mesangial cells were stimulated with PDGF (10 ng/ml) alone or in the presence of PD-98059 or vehicle. PDGF stimulation resulted in a twofold increase in [3H]thymidine incorporation that was regularly attenuated by 5–15% in the presence of 2 μM PD-98059 (Fig. 2). However, coincubation of mesangial cells with 10 μM PD-98059 resulted in a significant 60% reduction in [3H]thymidine incorporation. PD-98059 or vehicle alone did not have effects on cellular proliferation (not shown). This experiment demonstrated that ERK phosphorylation has important roles in growth factor-induced mesangial cellular proliferation.

α-Lipoic Acid Attenuates Proliferation of Growth Factor-Stimulated Cultured Rat Mesangial Cells

The effects of α-lipoic acid on cellular proliferation were tested in primary cultures of rat mesangial cells. Cells were stimulated with PDGF or 10% FCS, and cellular proliferation was assessed by [3H]thymidine incorporation. PDGF at 10 ng/ml consistently induced at least a twofold increase in thymidine incorporation (Fig. 3). Coincubation of PDGF-stimulated mesangial cells with 100 μM α-lipoic acid resulted in a significant 28% reduction in [3H]thymidine incorporation. The vehicle in which α-lipoic acid was dissolved had no effect on PDGF-induced cellular proliferation. Similarly, 100 μM α-lipoic acid caused a significant decrease in thy-
midine incorporation in mesangial cells stimulated with FCS (data not shown). Incubation of mesangial cells with \[\text{H9251}\]-lipoic acid in the absence of growth factors induced a small, insignificant inhibition of cellular proliferation (data not shown). These studies suggest that treatment of mesangial cells with \[\text{H9251}\]-lipoic acid can attenuate growth factor-induced cellular proliferation.

To exclude the possibility that inhibition of thymidine incorporation was the result of cell death, we tested the viability of mesangial cells after exposure to \[\text{H9251}\]-lipoic acid, PD-98059, or vehicle. Mesangial cells from some plates were not recovered for counting after incubation with the inhibitors and \[^{[3]}\text{H}\]thymidine but were left to grow after the medium was changed. We found no difference in cell appearance and, in particular, nuclear morphology between cells that were exposed to \[\text{H9251}\]-lipoic acid and control cells that were never exposed to \[\text{H9251}\]-lipoic acid and \[^{[3]}\text{H}\]thymidine. Finally, we stained cells exposed to \[\text{H9251}\]-lipoic acid or PD-98059 with trypan blue and found no difference in mortality compared with cells exposed to vehicle.

**Effects of \[\text{H9251}\]-Lipoic Acid on ERK Phosphorylation and Pathological Changes in Anti-Thy 1 Nephritis**

After demonstrating that \[\text{H9251}\]-lipoic acid could decrease ERK phosphorylation and cellular proliferation in cultured mesangial cells, we wanted to explore the relevance of this finding in vivo. For in vivo experiments, we employed a rat model of mesangial proliferative GN, i.e., anti-Thy 1 nephritis. We first established that treatment of animals with \[\text{H9251}\]-lipoic acid does not interfere with the induction of nephritis. Complement binding to the glomerular mesangium, a crucial step in the development of anti-Thy 1 nephritis, was not impeded by treatment with \[\text{H9251}\]-lipoic acid (Fig. 4). This finding allowed us to proceed with experiments in which effects of \[\text{H9251}\]-lipoic acid on the pathogenesis and severity of glomerular injury in anti-Thy 1 nephritis could be tested.

**Attenuation of ERK phosphorylation in glomeruli of rats with anti-Thy 1 nephritis by \[\text{H9251}\]-lipoic acid.** To study ERK phosphorylation, anti-Thy 1 nephritis was induced in male Wistar rats (Fig. 5). The treated group received daily intraperitoneal injections of \[\text{H9251}\]-lipoic acid, while the untreated group received vehicle according to the protocol described in METHODS. Total and phosphorylated ERK were measured in isolated glomeruli from each animal. Four days after induction of GN, phosphorylated ERK was increased by 210% in glomeruli of untreated animals compared with healthy control animals in which anti-Thy 1 nephritis was not induced. We observed a significant, 50% reduction in phosphorylated ERK in rats treated with \[\text{H9251}\]-lipoic acid compared with anti-Thy 1 nephritis animals treated with vehicle alone. The levels of total ERK did not change during the course of nephritis and were not influenced by treatment with \[\text{H9251}\]-lipoic acid. Thus treatment with the antioxidant \[\text{H9251}\]-lipoic acid blunts ERK phosphorylation in glomeruli of rats with anti-Thy 1 nephritis treated with \[\text{H9251}\]-lipoic acid. Kidney tissue was snap-frozen in liquid nitrogen, and the cryostat section was incubated with sheep anti-rat complement 3 antibody. Binding was visualized using a donkey anti-sheep FITC-conjugated secondary antibody.

**Fig. 4.** Complement binding to glomerular mesangium in rats with anti-Thy 1 nephritis treated with \[\text{H9251}\]-lipoic acid. Kidney tissue was snap-frozen in liquid nitrogen, and the cryostat section was incubated with sheep anti-rat complement 3 antibody. Binding was visualized using a donkey anti-sheep FITC-conjugated secondary antibody.

**Fig. 5.** A: representative samples of total and phosphorylated ERK in glomeruli of control rats and rats with anti-Thy 1 nephritis with and without \[\text{H9251}\]-lipoic acid. Amount of total ERK was not different between control rats and rats with anti-Thy 1 nephritis with or without \[\text{H9251}\]-lipoic acid. Each lane represents a sample obtained from an individual rat. \*P < 0.05 vs. untreated.
activation in this model of GN. These experiments demonstrate that ERK is activated during the proliferative phase of this model of experimental GN. They further support our data obtained in cultured mesangial cells that ROS are involved in ERK activation.

Amelioration of glomerular injury with α-lipoic acid in rats with anti-Thy 1 nephritis. The effects of α-lipoic acid on the severity of glomerular injury were evaluated by assessing glomerular damage in the course of anti-Thy 1 nephritis. We evaluated cellular proliferation and the number of mitoses and PCNA-positive cells in the glomeruli of control animals and animals with anti-Thy 1 nephritis with and without α-lipoic acid treatment. At 4 days after induction of anti-Thy 1 nephritis, untreated animals developed the typical features of mesangial proliferative GN, with an increase in glomerular cellularity (Fig. 6A) compared with control animals as well as a ninefold increase in the number of PCNA-positive cells (Fig. 6B). Treatment with α-lipoic acid significantly reduced cellular proliferation and the number of PCNA-positive cells by 100 and 64%, respectively. Mitoses were very rarely seen in normal glomeruli. At 4 days after induction of anti-Thy 1 nephritis, we observed ~1 mitosis per 60 screened glomeruli in the untreated group. Although the difference did not reach statistical significance, treatment with α-lipoic acid decreased the incidence of mitoses by 50% to ~1 in 120 screened glomeruli (data not shown). From the data presented in Figs. 5 and 6, we can suggest that glomerular cellular proliferation correlated with ERK activation and that α-lipoic acid treatment not only blunted ERK activation but also significantly reduced glomerular cellular proliferation.

We did not investigate the cellular origins of ROS, but this issue was studied by Nakamura et al. (24) using cell-specific monoclonal antibodies. Their findings suggest that, in the early phase of anti-Thy 1 nephritis, ROS are mainly generated by infiltrating macrophages, but by 4 days after induction of anti-Thy 1 nephritis, mesangial cells appear to produce a substantial amount of ROS.

Antioxidant α-Lipoic Acid Prevents Phenotypic Transformation of Glomerular Mesangial Cells in the Course of Anti-Thy 1 Nephritis

In the course of experimental and human GN, proliferating mesangial cells undergo phenotypic changes while they acquire the characteristics of myofibroblasts (1, 17). This is typically demonstrated by positive staining of kidney sections with antibodies against α-SMA. Normal glomeruli did not stain positive for α-SMA (Figs. 7A and 8). Staining was detected only in the media of blood vessels. Four days after GN induction, α-SMA staining became positive in ≥75% of glomeruli (Figs. 7B and 8). In animals treated with α-lipoic acid, <3% of glomeruli expressed positive staining for α-SMA (Figs. 7C and 8). These experiments demonstrate that preventing the production of ROS and/or removing ROS with α-lipoic acid prevents the phenotypic changes in proliferating resident mesangial cells.

Glomerular Expression of TGF-β1 Protein and mRNA in the Course of Anti-Thy 1 Nephritis

Proliferating mesangial cells upregulate genes important in production of extracellular matrix components (7). The generation of ROS has previously been shown to upregulate TGF-β1 mRNA and activity in cultured mesangial cells (13). TGF-β1 message induction contributes to the increase in mesangial matrix in a number of experimental and human glomerulonephritides. To explore the effects of α-lipoic acid on glomerular TGF-β1 expression, we used immunohistochemical methods to assess the glomerular expression of TGF-β1 in the course of anti-Thy 1 nephritis. In normal glomeruli, we did not observe any significant

![Fig. 6. Severity of glomerular injury in rats with anti-Thy 1 nephritis with or without α-lipoic acid compared with control rats (without anti-Thy 1 nephritis). A: 4 days after induction of anti-Thy 1 nephritis, rats developed increased glomerular cellularity compared with control animals. Treatment with α-lipoic acid resulted in 100% attenuation of glomerular hypercellularity. B: 9-fold increase in number of proliferating cell nuclear antigen (PCNA)-positive cells is significantly reduced 65% by treatment with α-lipoic acid. *P < 0.05 vs. untreated.](https://ajprenal.physiology.org/ on February 29, 2008)
staining with antibodies against TGF-β1 (Fig. 9A). A minimal quantity of scattered staining could be seen in some glomeruli early (by 4 days after induction of nephritis) in the course of glomerular injury (Fig. 9B). However, by 7 days after induction of anti-Thy 1 nephritis, virtually all glomeruli demonstrated pronounced immunoreactivity for TGF-β1 (Fig. 9C). In glomeruli from α-lipoic acid-treated rats with anti-Thy 1 nephritis, TGF-β1 expression was greatly attenuated 7 days after induction of nephritis (Fig. 9D). To assess glomerular TGF-β1 expression in the course of anti-Thy 1 nephritis and the effects of α-lipoic acid on this expression in more quantitative terms, we analyzed TGF-β1 mRNA expression. Total RNA was isolated from glomeruli of individual animals, and a relative quantitative RT-PCR was performed. Figure 10 demonstrates the relative abundance of TGF-β1 mRNAs in the glomeruli of control rats and in untreated or α-lipoic acid-treated anti-Thy 1 nephritis rats. Four days after induction of anti-Thy 1 nephritis, a small increase in TGF-β1 mRNA expression was noted in anti-Thy 1 compared with control animals without anti-Thy 1 nephritis. In α-lipoic acid-treated rats with anti-Thy 1 nephritis, a small decrease in glomerular TGF-β1 mRNA expression was demonstrated. At 7 days after induction of nephritis, TGF-β1 mRNA expression was increased ≥10-fold. Treatment of anti-Thy 1 rats with α-lipoic acid resulted in a significant 65% attenuation of TGF-β1 mRNA expression. These findings suggest that treatment with the antioxidant α-lipoic acid can significantly attenuate the increase in glomerular TGF-β1 mRNA expression in the anti-Thy 1 model of GN.

Fig. 7. Immunofluorescent staining of kidney tissue for α-smooth muscle actin (α-SMA) in control rats and untreated and α-lipoic acid-treated anti-Thy 1 nephritis rats. A: in control rats, i.e., without anti-Thy 1 nephritis, positive staining for α-SMA was seen only in media of blood vessels, while glomeruli are uniformly negative. B: in rats with anti-Thy 1 nephritis, positive staining for α-SMA was observed within glomeruli. C: in α-lipoic acid-treated anti-Thy 1 nephritis rats, glomerular staining became negative.

Fig. 8. Percentage of glomerular staining for α-SMA. At 4 days after induction of anti-Thy 1 nephritis, >75% of glomeruli expressed positive staining for α-SMA. Treatment with α-lipoic acid reduced staining to <3% of glomeruli. *P < 0.01 vs. untreated.
Effects of α-Lipoic Acid on ROS Expression in Anti-Thy 1 Nephritis

To determine whether the effects of α-lipoic acid were attributable to the attenuation of oxidative stress, we assayed glomerular ROS expression in control animals and in untreated and α-lipoic acid-treated animals with anti-Thy 1 nephritis. Glomeruli were isolated in K-H buffer, and glomerular ROS production was determined by measuring lucigenin-induced chemiluminescence. Development of anti-Thy 1 nephritis was associated with a fivefold increase in glomerular ROS production compared with control animals without anti-Thy 1 nephritis (Fig. 11). In α-lipoic acid-treated animals with anti-Thy 1 nephritis, there was a significant 55% decrease in glomerular ROS production. These experiments suggest that the effects of α-lipoic acid on mesangial cellular proliferation, in vitro and in vivo ERK phosphorylation, glomerular TGF-β1 expression, and mesangial cell phenotypic transformation are mediated through attenuation of ROS generation.

DISCUSSION

The role of oxidative stress as a major participant in the pathogenesis of human and experimental GN has been increasingly investigated (14, 18, 25, 26, 32, 33, 36). Our previous work in cultured rat mesangial cells demonstrated that ROS generation is involved in the signal transduction pathway linking activated cell surface receptors to ERK phosphorylation and that this effect was attenuated by coincubation of cells with α-lipoic acid (12). In addition, we have shown that receptor stimulation produces measurable amounts of H₂O₂ and O₂⁻ in a time scale similar to that of ERK phosphorylation and that three structurally different oxidants applied to mesangial cells were able to phosphorylate ERK to a similar degree. That work mapped ROS activity downstream from the PKC and proximal to the MEK in the ERK cascade. In the present studies, we suggest a role(s) for ROS in intracellular signal transduction pathways that lead to ERK activation in the anti-Thy 1 model of GN.

In our initial experiments, we examined the role of ROS in growth factor-induced ERK phosphorylation in cultured rat mesangial cells. To demonstrate that the role of ROS in ERK activation is general for growth factors, we stimulated cells with PDGF, EGF, bFGF, and thrombin. We demonstrated that two chemically distinct antioxidants significantly blunted growth factor-induced ERK phosphorylation in cultured mesangial cells. We obtained consistent reductions in ERK phosphorylation with both antioxidants in the presence of all growth factors tested. Previous data from our laboratory showed that the effects of antioxidants on mesangial cell ERK phosphorylation were abolished in the presence of buthionine sulfoximine, a potent inhibitor of glutathione synthase (12). Our previous study supports an intracellular location for the action of an antioxidant on ERK, because glutathione is the most abundant and one of the most important intracellular antioxidants.

We next demonstrated that the MEK inhibitor PD-98059 and α-lipoic acid attenuated PDGF-stimulated mesangial cellular proliferation in vitro. To induce cellular proliferation, we employed PDGF, because this growth factor has been widely implicated in the pathogenesis of the proliferation involved in glomerular injury in human disease and experimental models (17). Furthermore, interference with PDGF signal transduction pathways and inhibition in ERK phosphorylation have been shown to ameliorate the injury in experimental models of GN (5, 11). Although statistically significant, the in vitro inhibition of proliferation of PDGF-stimulated cultured mesangial cells by α-lipoic...
The potential clinical and pathophysiological relevance of the in vitro findings was tested in an in vivo animal model of mesangial proliferative GN, i.e., anti-Thy 1 nephritis. First, we demonstrated that the augmentation in ERK phosphorylation correlates with the cellular proliferation phase of anti-Thy 1 nephritis. A pathogenic role for ERK phosphorylation in GN has been previously suggested in the antiglomerular basement membrane model (3) and, more recently, in the anti-Thy 1 model of GN (4). Furthermore, Bokemeyer et al. (5) demonstrated that the ERK kinase inhibitor U-0126 decreased phosphorylated ERK expression and cellular proliferation in this model of experimental GN. Our results not only support the contention that ERK activation is likely a common pathophysiological mechanism underlying cellular proliferation in the course of experimental GN but also suggest that ROS play important roles in its activation.

Second, we have demonstrated that a 400–500% increase in oxidative stress occurs during the course of the anti-Thy 1 nephritis and that this increase precedes measurable increases in ERK phosphorylation. Our contention of a temporal relation between ROS generation and ERK phosphorylation in the course of anti-Thy 1 nephritis is supported by recent work by Gaertner et al. (9) and Bokemeyer et al. (4). They demonstrated that increases in glomerular ROS occur within several hours after induction of the disease, peak at 24 h, and remain elevated for ≥5 days. The assays of glomerular phosphorylated ERK detected a slight increase at 2 h, with further rises to a peak 6 days after induction of anti-Thy 1 nephritis.

The present study further demonstrates that treatment of rats with experimental GN with an antioxidant (α-lipoic acid) results in a significant decrease in oxidative stress and ERK phosphorylation. Thus it appears that ROS participate in ERK phosphorylation during the course of GN in a fashion similar to that observed in cultured mesangial cells stimulated by growth factors. The present studies also demonstrate acid was less pronounced than that by PD-98059 or α-lipoic acid in the course of anti-Thy 1 nephritis. Although we do not know the exact mechanism for this finding, we can speculate that α-lipoic acid may interfere with ROS production induced by other factors in the course of experimental nephritis and that PDGF effects on cellular proliferation may be only partially ROS dependent. Furthermore, the concentration of α-lipoic acid employed in cellular proliferation experiments was lower than that used in ERK phosphorylation assays (see METHODS). Nevertheless, our results are consistent with data from the literature demonstrating that modification of oxidative stress alters renal cellular proliferation and differentiation. For example, Kitamura (19) showed, in cultured rat mesangial cells, that a reducing agent (NAC) induced a cell phenotype associated with suppressed mitogenesis, whereas the oxidizing agents diamide and menadione had the opposite effect. It has also been shown that ROS are involved in angiotensin II-induced ERK activation and subsequent hypertrophy of renal tubular cells (15).

The present study further demonstrates that treatment of rats with experimental GN with an antioxidant (α-lipoic acid) results in a significant decrease in oxidative stress and ERK phosphorylation. Thus it appears that ROS participate in ERK phosphorylation during the course of GN in a fashion similar to that observed in cultured mesangial cells stimulated by growth factors. The present studies also demonstrate

Fig. 10. A: representative samples of glomerular expression of TGF-β1 mRNA (T) and 18S RNA (18) in control rats (C), untreated rats 4 and 7 days after induction of anti-Thy 1 nephritis (D4 and D7, respectively), and α-lipoic acid-treated rats 4 and 7 days after induction of anti-Thy 1 nephritis (D4LA and D7LA, respectively). Each lane represents a sample obtained from an individual rat. B: densitometric analysis of TGF-β1 mRNA expression. By 4 days after induction of anti-Thy 1 nephritis, there was a small increase in TGF-β1 mRNA expression compared with control rats without anti-Thy 1 nephritis (D4). Decrease in TGF-β1 mRNA expression was insignificant in α-lipoic acid-treated rats 4 days after induction of anti-Thy 1 nephritis (D4LA). At 7 days after induction of anti-Thy 1 nephritis (D7), glomerular TGF-β1 mRNA expression was increased 10-fold compared with control rats. Treatment with α-lipoic acid resulted in 68% reduction in TGF-β1 mRNA expression 7 days after induction of anti-Thy 1 nephritis (D7LA). Values are means ± SE from 6 individual rats in each experimental group. *P < 0.05 vs. D7.

Fig. 11. Effects of α-lipoic acid on oxidative stress in the course of anti-Thy 1 nephritis. One day after induction of anti-Thy 1 nephritis, glomerular lucigenin-induced chemiluminescence was increased 5-fold compared with control animals without anti-Thy 1 nephritis. This increase was reduced by 55% in α-lipoic acid-treated rats with anti-Thy 1 nephritis. Values are means ± SE from 6 individual rats in each experimental group. *P < 0.05 vs. untreated.
that decreases in oxidative stress and ERK phosphorylation are associated with the amelioration of glomerular injury in anti-Thy 1 nephritis. We found that exuberant cellular proliferation judged by total glomerular cellularity and the number of PCNA-positive cells was significantly reduced by treatment with α-lipoic acid, similar to that obtained by use of ERK kinase inhibitor by Bokemeyer et al. (5). It thus appears that the effects of the antioxidant α-lipoic acid parallel the effects of ERK kinase inhibition in the course of anti-Thy 1 nephritis. However, it is obvious that ROS are not the only factors involved in ERK phosphorylation in the course of this model of GN.

Treatment with α-lipoic acid also prevented phenotypic changes in the proliferating mesangial cells. It is interesting that mesangial cell expression of α-SMA in patients with IgA nephropathy directly correlates with progression to end-stage renal disease (38). Experimental evidence elsewhere suggests that the acquisition of the expression of α-SMA by cultured mesangial cell appears to be modulated by redox-sensitive signal pathways (19). Although we do not have direct proof, several lines of evidence suggest that ERK phosphorylation could induce phenotypic changes in the glomerulus and that the effect of α-lipoic acid in our study was at least partially the result of inhibition of ERK phosphorylation. For example, constitutive ERK activation has been associated with a transformed phenotype in other cell types (8, 21, 28). Treatment of rats with anti-Thy 1 nephritis with the ERK kinase inhibitor U-0126 resulted in decreased staining against α-SMA (5).

Phenotypic transformation of mesangial cells is associated with the increased production of TGF-β1 (39). We previously showed that TGF-β1 production by agonist-stimulated cultured mesangial cells was attenuated by ERK inhibition and antioxidants (13). Here we have demonstrated that treatment of anti-Thy 1 nephritis animals with an antioxidant resulted in attenuated TGF-β1 expression in a fashion similar to that observed in cultured mesangial cells, although it is clear that mesangial cells may not be the only source of glomerular TGF-β1 in the course of GN (20). A corresponding effect of α-lipoic acid, decreased production of extracellular matrix material, has recently been demonstrated in a rat model of diabetic nephropathy (23). It is interesting that a tendency for a decrease in matrix accumulation was also demonstrated in anti-Thy 1 nephritis by the ERK kinase inhibitor U-0126 (5).

It is highly unlikely that the effects observed with α-lipoic acid were the result of interference with the induction of GN for several reasons. First, all rats with anti-Thy 1 nephritis, regardless of therapeutic intervention, expressed similar degrees of proteinuria when tested after induction of GN (data not shown). Furthermore, staining of kidney sections of animals with anti-Thy 1 nephritis with anti-rat complement 3 antibodies demonstrated that treatment with α-lipoic acid did not alter complement binding to the glomeruli. Complement binding and resulting mesangiolyis are crucial initial steps in the generation of anti-Thy 1 nephritis. Finally, the extent of mesangiolyis was not significantly altered by treatment with α-lipoic acid (data not shown), suggesting that α-lipoic acid blocked specific pathways of glomerular damage.

α-Lipoic acid is a naturally occurring compound that serves as a prosthetic group to multiple enzyme complexes in the mitochondria (30). Abundant experimental evidence has demonstrated that α-lipoic acid is also a potent scavenger of free radicals and an important factor in maintaining antioxidant status in the cell by replenishing antioxidants such as glutathione (2, 29). It may also exert an antioxidant effect by chelation of transition metals (30). Previous studies have shown that treatment of rats with experimental diabetic nephropathy with α-lipoic acid resulted in increased renal cortical glutathione levels and decreased malondialdehyde, which is an indicator of significant lipid peroxidation (23).

On the basis of the data presented here, we suggest that the pathophysiological pathway depicted in Fig. 12 may play a role in the pathogenesis of GN. Different stimuli, such as activated complement, deposition of immune complexes, or local release of growth factors or cytokines, induce formation of ROS by intrinsic glomerular cells or infiltrated leukocytes. Generated ROS can then activate the ERK cascade, culminating in a sustained ERK phosphorylation. Phosphorylated ERK subsequently translocates into the nucleus and induces cellular proliferation, phenotypic transformation, and TGF-β1 production, resulting in a clinical picture of GN.

In summary, we have demonstrated that ROS have a prominent role in ERK activation in a model of proliferative GN and that suppression of ROS production results in blunted ERK activation, decreased TGF-β1 mRNA transcription, and amelioration of glomerular injury. On the basis of data from the present studies in an experimental model of mesangial proliferative GN and because of the low toxicity potential for α-lipoic acid, we propose that α-lipoic acid should be considered a potential therapeutic agent in certain types of human proliferative glomerular injury such as IgA nephropathy or mesangioproliferative GN, where current therapeutic regimens remain suboptimal in preventing progressive renal disease.

DISCLOSURES

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REFERENCES


