Inhibition of nuclear factor-\( \kappa \)B activation reduces cortical tubulointerstitial injury in proteinuric rats

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Inhibition of nuclear factor-\( \kappa \)B activation reduces cortical tubulointerstitial injury in proteinuric rats.

Background. Protein-induced chemokine expression in proximal tubular cells is mediated by the transcription factor nuclear factor-\( \kappa \)B (NF-\( \kappa \)B). We hypothesized that in vivo inhibition of renal NF-\( \kappa \)B activation would reduce interstitial monocyte infiltration in a rat model of nonimmune proteinuric tubulointerstitial inflammation.

Methods. Male Wistar rats received a single intravenous injection of doxorubicin hydrochloride [adriamycin (ADR)], 7.5 mg/kg and were studied 7, 14, 21, and 28 days later. In a second study, inhibitors of NF-\( \kappa \)B [N-acetyl cysteine (NAC; 150 mg/kg, b.i.d., i.p.), pyrrolidine dithiocarbamate (PDTC, 50 mg/kg, b.i.d., i.p.)] or vehicle were commenced on day 14 after the onset of proteinuria and were continued until day 30.

Results. Rats injected with ADR had increased proteinuria (\( U_{\text{V}} \), day 28, 247 \( \pm \) 57; control, 18 \( \pm \) 2 mg/day; \( P < 0.01 \)) and cortical tubulointerstitial injury [tubule cell atrophy, interstitial volume, and monocyte/macrophage (ED-1) infiltration]. Electrophoretic mobility shift assay of nuclear extracts from whole cortex of ADR rats demonstrated that NF-\( \kappa \)B activation (p50/65, p50/c-Rel) increased from day 7 (4.7 \( \pm \) 0.2 fold-increase above control; \( P < 0.01 \)) was maximal at day 28 (6.2 \( \pm \) 0.7; \( P < 0.01 \)) and correlated with \( U_{\text{V}} \) (\( r = 0.63 \); \( P < 0.05 \)) and interstitial ED-1 infiltration (\( r = 0.67 \); \( P < 0.01 \)). Chronic treatment of ADR rats with PDTC suppressed NF-\( \kappa \)B activation (by 73%; \( P < 0.05 \)) without any effect on \( U_{\text{V}} \). NF-\( \kappa \)B inhibition with PDTC was accompanied by a reduction in tubule cell atrophy (59%; \( P < 0.01 \)), interstitial volume (49%; \( P < 0.05 \)) and ED-1 infiltration (48%; \( P < 0.01 \)), and cortical lipid peroxidation (41%; \( P < 0.05 \)) compared with vehicle-treated ADR rats. In contrast NAC had no effect on NF-\( \kappa \)B activation, tubulointerstitial injury, or \( U_{\text{V}} \) in ADR rats.

Conclusion. The activation of NF-\( \kappa \)B may have an important role in mediating cortical interstitial monocyte infiltration and tubular injury in nonimmune proteinuric tubulointerstitial inflammation.

Experimental nephrotic syndrome can be induced in rats with injections of puromycin aminonucleoside, bovine serum albumin, or adriamycin (ADR) [1–3]. In their early stages, these models are defined by a nonimmune and noninflammatory chronic glomerulopathy in conjunction with heavy proteinuria and prominent tubulointerstitial injury [4–6]. The latter is characterized by the presence of tubular damage (atrophy, vacuolization) and a marked interstitial mononuclear inflammatory cell infiltrate composed largely of macrophages and T lymphocytes [4–7].

In vitro and in vivo studies strongly support the hypothesis that the excess plasma proteins that are present in the glomerular ultrafiltrate and their reabsorption by tubular cells are, in part, responsible for the induction of tubulointerstitial injury in proteinuric models [8–13]. Tubular cells, overloaded with proteins, secrete chemotactic and inflammatory mediators that could regulate the infiltration of inflammatory cells into the interstitium [8–13]. Recently, it was reported that protein-induced chemokine production in proximal tubular cells is mediated by the transcription factors belonging to the nuclear factor-\( \kappa \)B (NF-\( \kappa \)B) family [11, 14]. These ubiquitous transcription factors also up-regulate other inflammatory mediators, which are overexpressed in the kidneys of proteinuric animals [15–21]. Although the pathological significance of renal NF-\( \kappa \)B activation has been documented in experimental models of ureteric obstruction [22] and immune glomerular injury [23], its in vivo role in nonimmune proteinuric renal diseases is not known.

This study was designed to test the hypothesis that the activation of NF-\( \kappa \)B in the renal cortex mediates the infiltration of monocytes/macrophages into the cortical interstitium in a rat model of proteinuric renal disease, ADR nephrosis. The first aim of this study was to establish whether NF-\( \kappa \)B is activated in the renal cortex of ADR rats, as well as to determine the relationship, if any, of NF-\( \kappa \)B with proteinuria, monocyte/macrophage infiltration, and tubulointerstitial injury. The second aim was to evaluate the effect of NF-\( \kappa \)B inhibition on monocyte/macrophage infiltration and tubular injury. For this purpose, the antioxidant NF-\( \kappa \)B inhibitors [24–27] pyr-
 rolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC) were administered to ADR rats with established proteinuria.

METHODS

Animals

Inbred male Wistar rats (six to eight weeks old) were supplied by the Animal Care Facility, Westmead Hospital, Sydney, Australia, and were housed under controlled environmental conditions (12 hours of a dark/light cycle, 22°C). The animals were allowed free access to standard rat chow (19.0% protein; Glen Forrest Stock, Perth, Australia) and water unless otherwise specified in the experimental protocols. All animals were handled within the guidelines of the National Health and Medical Research Council of Australia, and protocols were approved by the Animal Care and Ethics Committee, Westmead Hospital.

Experimental design

In the first study, the time course of NF-κB activation in ADR nephrosis was examined. Rats (the body wt at the start was 222 to 296 g, and the mean was 261 g, \( N = 20 \)) received either a single intravenous injection of doxorubicin hydrochloride (ADR, 7.5 mg/kg; David Bull Laboratories, Victoria, Australia) or an equal volume of saline. The animals were anesthetized with ketamine/xylazine (intraperitoneal injection (i.p.i.)]] [3]. On days 7, 14, 21, and 28 after injection, animals from each group (\( N = 4 \)) were anesthetized, and a midline laparotomy was performed. The animals were killed by exsanguination, and both kidneys were removed, weighed, and processed for various analyses, as outlined later in this article. These time points were chosen because in previous studies, we found that proteinuria and tubulointerstitial injury were minimal at day 7, mild to moderate at day 14, and severe between days 21 to 28. Because proteinuria and other parameters were not significantly different between individual control animals, the data from this group were analyzed together. To assess the functional parameters of the model, non-fasting animals were placed in metabolic cages (Techniplast, Buguggiate, Italy) one day prior to sacrifice for determination of 24-hour urinary protein (\( U_pV \)) and creatinine excretion (\( U_GV \)). Venous blood for serum creatinine (\( S_C \)) and albumin (\( S_A \)) was collected at the time of sacrifice.

In the second study, the effect of NAC and PDTC on established ADR nephrosis was evaluated. Rats (the body wt at the start was 262 to 319 g, mean 283 g, \( N = 29 \)) were placed in metabolic cages on day 10 after ADR or saline for the determination of baseline \( U_pV \) and \( U_GV \). Venous blood (150 μl) was collected at the end of the clearance period for \( S_C \) and \( S_A \). Animals injected with ADR were stratified into three groups on day 14 according to body weight, baseline endogenous creatinine clearance (\( C_G \)), and \( U_pV \). Each group received either NAC (300 mg/kg/day, \( N = 8 \)), PDTC (100 mg/kg/day, \( N = 8 \)), or vehicle (\( N = 7 \)) in two divided doses (6 a.m. and 6 p.m.) via intraperitoneal injection from day 14 until day 30. Rats were monitored and weighed daily. At the end of the study on day 30, nephrectomies were performed as described earlier here approximately three hours after the final injection of NAC or PDTC.

In ADR nephrosis, the severity of the proteinuria and tubulointerstitial inflammation can be reduced by restricting dietary protein or by administering antioxidants prior to or just after disease induction [16, 28]. The latter is thought to be due to antioxidant-mediated protection of the glomerular podocytes against the effects of ADR [28]. To eliminate these factors as possible confounding variables, all ADR rats were pair fed, and NAC and PDTC were commenced on day 14, when proteinuria is well established [3].

N-acetylcysteine and PDTC (Sigma-Aldrich, Sydney, Australia) were dissolved in saline under sterile conditions 30 minutes prior to administration. NAC was neutralized to pH 7.4 with 6.0 m sodium hydroxide. Control groups received an equal volume of saline. The dose and route of administration of NAC and PDTC were determined from previous reports [26, 27] and pilot studies in ADR rats. Single intraperitoneal doses of NAC (between 200 and 1000 mg/kg) and PDTC (between 50 and 200 mg/kg) have been reported to suppress NF-κB activation in the lungs of endotoxin-treated rats [26, 27]. However, in pilot studies, total daily doses of NAC exceeding 300 mg/kg caused anorexia and weight loss in rats with ADR nephrosis, whereas with lower doses, rats remained well. With PDTC, a single intraperitoneal dose of 200 mg/kg was lethal in ADR rats, and between 75 and 150 mg/kg caused dose-dependent acute systemic toxicity manifested by generalized seizures, neuromuscular irritability, and hypersalivation. These effects were transient, lasting 10 minutes after injection and have been reported previously, although only at doses greater than 450 mg/kg in normal rats [29]. At lower doses of PDTC (as used in this study), rats developed mild hyper- salivation only, lasting 10 minutes after the injection, and the animals remained well in between doses. To administer total daily doses known to suppress NF-κB activation while minimizing the acute toxic effects of PDTC and ensuring constant drugs levels of NAC [26], both drugs were administered twice daily. The reasons for the increased sensitivity to the toxic effects of NAC and PDTC in ADR rats are unknown, but may relate to the use of repeated injections and altered pharmacokinetics in the nephrotic syndrome.
Assessment of renal function

All urine and blood specimens were analyzed by the Institute of Clinical Pathology and Medical Research, Westmead Hospital, using a BM/Hitachi 747 analyzer (Tokyo, Japan). Total protein was analyzed by the Biuret method [30] and creatinine by the Jaffe kinetic method without deproteinization [31]. The $C_\text{Cl}$ was calculated from the $U_{\text{Cr}}V$ and $S_{\text{Cr}}$, and was expressed as milliliter per minute or milliliter per minute normalized to kidney weight [32].

Histology and morphometric analysis

Coronal sections of renal tissue were immersion-fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections 4 $\mu$m thick were stained with periodic acid-Schiff (PAS). A computer-assisted image analysis system was used to quantitate glomerular and tubulointerstitial structure in the renal cortex, as described previously [32]. Sections were viewed with a microscope (Axioskop; Carl Zeiss Jena GmbH, Jena, Germany), and the images were digitalized onto a computer screen using a video camera linked to image analysis software (Optimas Image Analysis System, version 5.2; Optimas Corporation, Seattle, WA, USA). To quantitate tubular atrophy (tubular dilation and tubule cell atrophy), the cross-sectional diameter and tubule cell height of an individual cortical tubule were measured using line morphometric measurements ($\times 400$). The tubular diameter was defined as the length of a straight line that passes through the center of a symmetrically sectioned tubule and joins two points on the tubular circumference (lines A–C, Fig. 1). The shortest diameter was selected when the cross section of the tubule was elliptical in shape. The tubule cell height was defined as the distance of a perpendicular straight line joining two points on the base and apical surface of a tubule cell (lines B and C, Fig. 1). A total of 50 randomly selected cortical tubules in 10 nonoverlapping fields ($\times 400$) was measured, and the mean cross-sectional tubular diameter and tubule cell height were determined for each section.

The cortical interstitial volume was defined as the peritubular space and included the tubular basement membrane and peritubular capillaries. The latter were included because in preliminary studies we have noted that the interstitial volume expansion in this model is partly due to tubular basement membrane hypertrophy and neovascularization (unpublished observations). To quantitate this area, cortical fields ($\times 200$) were viewed on a video screen as described earlier here, and the region occupied by the interstitial space was carefully traced manually with a mouse. The area of the traced regions was determined with the image analysis software and was expressed as a percentage of the total area of the field. The mean percentage area for five nonoverlapping cortical fields was calculated for each section.

Fig. 1. Method to quantitate the cross-sectional tubular diameter (line A-C) and tubule cell height (line B-C). Photomicrograph of a proximal tubule from a periodic acid-Schiff stained section of a normal rat ($\times 1200$).

The glomerular capillary tuft area (GCA) was measured by tracing the outline of the capillary tuft ($\times 400$) with the mouse and then determining the area (in $\mu$m$^2$) as described earlier here. The mesangial matrix area (MA) was the PAS-positive intercapillary area divided by the GCA and expressed as a percentage. To determine the glomerular cellularity (GC), each clearly visible nucleus within the glomerular capillary tuft was marked on the video screen by point morphometry, and the total number was counted. A total of 20 randomly selected glomeruli was evaluated, and the mean GCA, MA, and GC were determined per section. All fields for morphometric analysis were randomly selected, and the examiner of the histological sections was blinded to the experimental groups.

Immunohistochemistry

Monocyte/macrophage infiltration was determined by immunohistochemical staining for the ED-1 antibody, which recognizes a glycoprotein present on the lysosomal membrane of monocytes and the majority of macrophages [33]. Immediately after nephrectomy, coronal slices of each kidney were placed in OCT embedding compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen, sealed with parafilm, and stored at $-70^\circ$C. Frozen sections, 6 $\mu$m thick, were cut with a cryostat (Microm HM 505E; Walldorf, Germany) and placed on poly-l-lysine–coated slides. The slides were air dried overnight at room temperature, wrapped in aluminum
Kidney wt: Body wt

S Cr, serum creatinine;
SA1b, serum albumin;
V, 24-hour urinary volume;
U p, 24-hour urinary protein excretion;
U p:cr, urinary protein:creatinine ratio;
CCr, endogenous creatinine clearance.

Values are expressed as mean ± sem. Abbreviations are: Body wt, final body weight; Kidney wt, kidney weight; S Cr, serum creatinine; S Alb, serum albumin; V, 24-hour urinary volume; U V, 24-hour urinary protein excretion; U p, urinary protein:creatinine ratio; CCr, endogenous creatinine clearance.

* P < 0.05 and ** P < 0.01 when compared to the control group.

### Extractions of Nuclear Proteins from Renal Cortical Homogenates

Cortical renal tissue was carefully dissected from the medulla, immediately snap frozen in liquid nitrogen, and stored at −70°C until further processing. Nuclear protein extracts from cortical tissue were prepared according to a previous method [27], with some modifications. One hundred milligrams of cortical tissue was homogenized with 20 even strokes of a glass Teflon homogenizer in distilled water followed by 0.01 M dithiothreitol, 0.1 M potassium phosphate buffer (pH 7.9), 10 mM KCl, 2 mM MgCl2, and 0.1 mM ethylenediaminetetraacetic acid (EDTA), with a cocktail of protease inhibitors [0.5 mM dithiothreitol, 0.1 mM pepstatin A, 1 μM phenylmethylsulfonyl fluoride (PMSF), 0.05 μg/ml leupeptin, and 0.01 mM aprotinin]. Sixty-five microliters of detergent Nondiet-P40 (2%) were added. The mixture was vortexed 10 times for one minute each, and was centrifuged at 13,000 g for five minutes. The supernatant was removed, and the pellet was resuspended in 60 μl of Buffer B, containing 50 mM HEPES, 10% (vol/vol) glycerol, 300 mM NaCl, 50 mM KCl, and the protease inhibitor cocktail. It was then vortexed vigorously five times for two minutes each. The mixture was centrifuged at 13,000 g for 10 minutes at 4°C. The supernatant was nuclear protein, and it was diluted to a standard concentration of 3 μg/μl and stored in aliquots at −70°C. The protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA, USA) [34].

### Electrophoretic Mobility Shift Assay and Densitometry

Double-stranded NF-κB consensus oligonucleotides (5'-AGTTGAGGGGACCTTCCAGG-3'; Promega, Madison, WI, USA) were end labeled with [32P]γATP (American Life Science, Sydney, Australia). Unincorporated label was removed with a G-50 sephadex spin column. The binding reaction was performed for 30 minutes at room temperature and contained 5 μg of nuclear protein extract, 2 μl of binding buffer (5 mM MgCl2, 50 mM Tris-HCl, 250 mM NaCl, 20% glycerol, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI.dC), 1 μl 32P-labeled NF-κB probe (50,000 cpm, Cherenkov counting) to a total volume of 10 μl in distilled water. The reaction was

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**Table 1. Functional parameters in rats injected with adriamycin (ADR)**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Body wt (g)</th>
<th>Kidney wt (g)</th>
<th>Kidney wt/Body wt</th>
<th>S Cr (μM/liter)</th>
<th>S Alb (g/liter)</th>
<th>V (ml/24 hr)</th>
<th>U V (mg/24 hr)</th>
<th>U p (mg/μM)</th>
<th>U p:cr</th>
<th>CCr (ml/min/g kidney wt)</th>
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<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>354 ± 25</td>
<td>0.89 ± 0.03</td>
<td>0.25 ± 0.02</td>
<td>51 ± 1</td>
<td>28 ± 1</td>
<td>31 ± 3</td>
<td>18 ± 2</td>
<td>0.19 ± 0.01</td>
<td>2.13 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>ADR day 7</td>
<td>6</td>
<td>285 ± 7</td>
<td>1.10 ± 0.04</td>
<td>0.39 ± 0.00</td>
<td>42 ± 1</td>
<td>20 ± 1</td>
<td>9 ± 3</td>
<td>182 ± 44</td>
<td>2.85 ± 0.42</td>
<td>1.36 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>ADR day 14</td>
<td>6</td>
<td>296 ± 6</td>
<td>1.26 ± 0.06</td>
<td>0.42 ± 0.02</td>
<td>45 ± 1</td>
<td>20 ± 1</td>
<td>20 ± 2</td>
<td>444 ± 63</td>
<td>5.36 ± 0.37</td>
<td>1.47 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>ADR day 21</td>
<td>6</td>
<td>323 ± 19</td>
<td>1.17 ± 0.05</td>
<td>0.36 ± 0.01</td>
<td>43 ± 1</td>
<td>18 ± 1</td>
<td>20 ± 2</td>
<td>468 ± 58</td>
<td>5.29 ± 1.17</td>
<td>1.56 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>ADR day 28</td>
<td>6</td>
<td>269 ± 9</td>
<td>1.33 ± 0.06</td>
<td>0.50 ± 0.03</td>
<td>73 ± 10</td>
<td>16 ± 1</td>
<td>14 ± 2</td>
<td>474 ± 57</td>
<td>6.88 ± 0.33</td>
<td>0.61 ± 0.13</td>
<td></td>
</tr>
</tbody>
</table>

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foil, and stored at −70°C until immunostaining. Slides were fixed with acetone at 4°C for 15 minutes and air dried for 10 minutes. Nonspecific staining was blocked with normal rabbit serum (dilution 1:5; Dako Australia, Sydney, Australia) and blocking buffer (Pierce, Rockford, IL, USA) for 30 minutes each. Sections were incubated with the primary antibody (MCA341, ED-1; dilution, 1:400; Serotec, Oxford, UK) for one hour. As a negative control, some sections were incubated with a mouse IgG1 negative control antibody (MCA1209; Serotec) at a protein concentration equal to that of the diluted ED-1 antibody. Slides were rinsed in 0.01 M phosphate-buffered saline (PBS) for 10 minutes in between incubations. Endogenous peroxidase was blocked with 3% H2O2 for three minutes, after which, slides were rinsed in distilled water followed by 0.01 M PBS. Antimouse rabbit antibody (P0850, 1:50; Dako Australia) in 1% normal rat serum (X0912; Dako Australia) and mouse peroxidase-antiperoxidase complex (Z0259, 1:100; Dako) were applied for 25 minutes each. The slides were incubated with 3,3-diaminobenzidine tetrahydrochloride (7.5 mg in 15 ml 0.01 M PBS, 15 μl of 30% H2O2) for 10 minutes to produce a dark brown/black-colored end-product. Sections were counterstained with Harris hematoxylin for one minute and dehydrated with graded ethanol and xylene, and the cover slip was mounted with an automatic processor (Tissue-Tek).

The number of ED-1-positive interstitial cells was quantitated in 10 nonoverlapping cortical fields (×400, measuring 0.075 mm2 each). Images were transferred onto a video screen with the computer-assisted image analysis as described earlier in this article. Interstitial cells with chromogen-labeled cytoplasm and a clearly visible nucleus were marked on the screen, and the total number per field was counted. The mean number of ED-1-positive cells per interstitial field was calculated for each section and expressed as cells per mm2. Similarly, the number of ED-1-positive cells per glomerulus was counted (×400), and the mean of 20 randomly selected glomeruli was determined.
stopped by the addition of 1 μl of gel-loading buffer (250 mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, 40% glycerol). The DNA-protein complexes were resolved by electrophoresis on a 10 × 12 cm 7% polyacrylamide gel (TBE buffer) [35]. The gel was run at 10 V/cm for 75 minutes and then dried at 80°C with a gel drier. Autoradiographs were prepared by exposing the dried gel to x-ray film (Hyperfilm HP film; Amersham Life Science) with intensifying screens for three to six hours at −70°C. The specificity of the NF-κB electrophoretic mobility shift assay (EMSA) was determined with reactions that contained either a negative control (no extract), positive control (extract), specific competitor (100-fold excess of unlabeled NF-κB probe), and a nonspecific competitor.

Fig. 2. Cortical tubulointerstitial injury during adriamycin (ADR) nephrosis. Photomicrographs of periodic acid-Schiff stained cortical sections obtained from control animals (A) and rats injected with ADR after 7 (B), 14 (C), 21 (D), and 28 days (E; ×250).
to supershift or reduce the intensity of the complexes were determined in preliminary experiments.

Measurement of renal cortical malondialdehyde

Reactive oxygen species (ROS) are increased in the renal cortex of rats with ADR nephrosis [37]. Lipid peroxidation, as determined by malondialdehyde (MDA), has been used as a measure of ROS generation in this model [37]. Because NAC and PDTC are potent antioxidants [38], their effect on cortical MDA was determined [39]. The kidney cortex was dissected from the medulla and weighed and stored on ice. The tissue was homogenized in 10% wt/vol containing 1.15% KCl solution and centrifuged at 3,000 r.p.m. for 10 minutes at 4°C. Four hundred microliters of the supernatant was mixed with 100 μl 8.1% sodium dodecyl sulfate (SDS), 750 μl 20% aqueous acetic acid, 750 μl 0.8% thiobarbituric acid, and 100 μl distilled water. The mixture was heated in a water bath at 90°C for 60 minutes and then cooled under tap water. Five hundred microliters of distilled water and 2.5 ml of N-butanol:pyridine (15:1 vol/vol) were added, the mixture vortexed, and then centrifuged at 3000 g for 10 minutes at 4°C. The absorbance of the supernatant was measured using a spectrophotometer (Beckman DU68; Beckman Instruments, Fullerton, CA, USA) at a wavelength of 532 nm. Samples were measured in duplicate, and 1,1,3,3-tetramethoxypropane was used as an external standard. MDA is expressed as nmol per 100 mg protein. Total protein was assayed by the Biuret method [30], using bovine serum albumin as an external standard (Sigma-Aldrich, Sydney, Australia).

Statistics

All data are expressed as mean and the SEM. Comparisons between two experimental groups were performed using the independent t-test and Mann–Whitney U-test for parametric and nonparametric data, respectively. Multiple parametric and nonparametric comparisons were performed using one-way analysis of variance and the Kruskal–Wallis test. The Shapiro–Wilk test was used to determine the distribution of the data. The Spearman rank correlation coefficient was used to determine the relationship between two variables. Calculations were performed using statistical software (JMP; SAS Institute, Cary, NC, USA) with a Macintosh computer. A P value of less than 0.05 was used to indicate statistical significance.

RESULTS

Time course of proteinuria and renal function in adriamycin nephrosis

All rats injected with ADR developed hypoalbuminemia and heavy proteinuria (Table 1). Ascites was also present in rats examined at day 28. UPV increased tenfold at day 7 and reached a peak of 26-fold between day 14 to day 28 compared with normal rats (Table 1). The Scr was reduced slightly in ADR rats between day 7 and
day 21, and this may have been due to a reduction in muscle mass caused by protein loss and catabolism. CCl4 was approximately 35% lower between day 7 to day 21 and 70% lower on day 28 in ADR rats compared with control animals (Table 1).

**Time course of renal histology and ED-1 infiltration in adriamycin nephrosis**

By light microscopic analysis, the histological changes in ADR rats were dominated by tubulointerstitial injury, the severity of which increased progressively in the renal cortex from day 7 to day 28 (Fig. 2). The tubular changes consisted of tubule cell brush border loss, vacuolization, cellular atrophy, and basement membrane hypertrophy. Focal areas of proteinaceous casts and frankly dilated tubules were also present. The interstitial space was expanded, and at day 14, this was due to an increase in mononuclear inflammatory cell infiltration and interstitial edema (Fig. 2C). By day 28, the cellular contribution was more marked. Thickening of tubular basement membranes, neoangiogenesis (not shown), and focal areas of matrix deposition also added to the expanded interstitium at this time point (Fig. 2E).

By morphometric analysis, the diameter of cortical tubules was not different at any time point in ADR rats and when compared with control animals (control, 42.2 ± 1.4; day 7, 41.8 ± 1.9; day 14, 42.1 ± 1.6; day 21, 44.2 ± 3.2; day 28, 38.5 ± 1.9 microns, P = NS; Fig. 3A). The cortical tubule cell height fell progressively in ADR rats and, by day 14, was significantly less than in control animals (P = 0.03). By day 28, tubule cell height was only 37% (P = 0.0001) of control values (control, 34.8 ± 1.2; day 7, 32.7 ± 2.7; day 14, 27.6 ± 1.9; day 21, 20.4 ± 1.4; day 28, 12.8 ± 1.0 microns, P < 0.0001, one-way analysis of variance; Fig. 3B). The reduction in tubule cell height was due to a combination of brush border loss and cellular atrophy. In control animals, the mean percentage cortical interstitial volume was approximately 2%. This value is much lower than that previously estimated for relative cortical interstitial volume in rats [40], a difference that may be due to the different method of quantitation and definition of interstitial volume used in this study. In rats injected with ADR the percentage cortical interstitial volume increased from day 14 (P = 0.001) and reached a peak at day 28 (P = 0.002) when compared with control animals (control, 1.9 ± 0.4; day 7, 2.1 ± 0.2; day 14, 7.1 ± 1.1; day 21, 12.8 ± 2.5; day 28, 19.9 ± 2.6%, P = 0.0001, one-way ANOVA; Fig. 3C).

The number of interstitial monocytes was quantitated with ED-1 immunostaining. Sections from normal and ADR rats that were stained with the negative control antibody did not react with chromogen (data not shown). In the interstitium of normal animals, no cells or an occasional cell stained positively for the ED-1 antigen (data not shown). In rats injected with ADR, the number of ED-1-positive cells increased dramatically in the cortical interstitium (Fig. 3D). This increase occurred as early as day 7 (P = 0.02) and reached a peak at day 28 (P = 0.004) when compared with control animals (control, 36.3 ± 7.6; day 7, 71.7 ± 6.1; day 14, 117.1 ± 6.9; day 21, 225.4 ± 21.6; day 28, 410.8 ± 63.4 cells/mm², P < 0.0001, one-way ANOVA; Fig. 3D). Interstitial ED-1-positive cells were particularly increased around cortical tubules that had evidence of tubule cell atrophy and luminal dilatation, and at day 28, focal collections of ED-1-positive interstitial cells were present (data not shown).

The glomerular changes in ADR rats were minimal and characterized by a mild increase in mesangial matrix and occasional dilated capillary loops. There was no evidence of glomerulosclerosis (data not shown). The mean GCA was similar in all groups (control, 12,236 ± 998; day 7, 12,220 ± 580; day 14, 12,340 ± 725; day 21, 12,060 ± 526; day 28, 11,052 ± 649 μm², P = NS, one-way ANOVA) as was the mean GC (control, 53 ± 1.4; day 7, 55 ± 5; day 14, 57 ± 5; day 21, 54 ± 1; day 28, 50 ± 3 nuclei per glomerulus, P = NS, one-way ANOVA). The mean MA was increased in ADR rats (control, 1.7 ± 0.4; day 7, 2.7 ± 0.7; day 14, 2.3 ± 0.7; day 21, 3.6 ± 0.7; day 28, 5.4 ± 1.0%, P = 0.03, one-way ANOVA), but this was significant at only day 28 when compared with the control group (P = 0.03). The mean number of ED-1-positive glomerular cells was not different in ADR rats when compared with control animals (control, 1.7 ± 0.3; day 7, 1.8 ± 0.1; day 14, 1.7 ± 0.4; day 21, 2.1 ± 0.1; day 28, 1.5 ± 0.2 cells per glomerular cross-section, P = NS, one-way ANOVA).

**Time course of cortical nuclear factor-κB activation in adriamycin nephrosis**

Nuclear factor-κB DNA-binding activities were assessed in whole cortical nuclear extracts from normal and ADR rats using EMSA and labeled consensus NF-κB oligonucleotide. The autoradiographs of the polyacrylamide gels are shown in Figure 4A. The results shown were performed during a single assay from the same gel and are representative of results obtained in four individual animals per time point with assays performed in duplicate. In normal rats, the incubation of cortical nuclear extracts with labeled consensus NF-κB oligonucleotide produced a slower and faster migrating complex I and II, respectively (Fig. 4A, lane 1). The density of both complex I and II increased in parallel in ADR rats (Fig. 4A, lanes 2 through 5) beginning from day 7 (lane 2) and reaching near maximal intensity between day 14 to day 28 (Fig. 4A, lanes 3 through 5). The mean densitometry (sum of complex I and II) of each group is shown in Figure 4B. The loading of a labeled NF-κB probe alone without any sample produced no bands (Fig. 4B, lane 1). The incubation of renal cortical nuclear
Fig. 3. Time course of tubulointerstitial morphometric parameters and ED-1 infiltration during adriamycin nephrosis (days 7, 14, 21 and 28). (A) Mean cross-sectional tubular diameter. (B) Mean cross-sectional tubule cell height. (C) Mean interstitial volume. (D) Mean number of ED-1-positive interstitial cells. *P < 0.05 and **P < 0.01, respectively, when compared with the control group; #P < 0.05 when compared to day 14 (N = 4 per group).

Fig. 4. Nuclear factor-κB activation during adriamycin (ADR) nephrosis. Nuclear extracts from renal cortical homogenates were subjected to EMSA using a 32P-labeled consensus NF-κB oligonucleotide. (A) Autoradiographs showing the time course of NF-κB activation in ADR nephrosis and normal rats (lane 1, control; lane 2, day 7 after ADR; lane 3, day 14 after ADR; lane 4, day 21 after ADR; lane 5, day 28 after ADR). The samples are from the same gel and had identical exposure times. The results are representative of four individual samples at each time point, and all assays were performed in duplicate. (B) Competition study (lane 1, NF-κB probe without nuclear extract; lane 2, nuclear extract from cortical tissue obtained seven days after injection with ADR; lane 3, same sample as that in lane 2 plus 100-fold molar excess of unlabelled NF-κB probe.)
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could be seen in some samples incubated with the p65 antisera but not at all with the c-Rel antisera. The results were the same when higher concentrations of p65 and c-Rel antisera were used or when the incubation period was increased (overnight at 4°C). In comparison, complex II was unaffected by p50-specific antisera but was partially depleted by the addition of p65-antisera (compare lanes 5 and 7, complex II; Fig. 6). The same result was obtained for animals at day 14 after ADR (data not shown). At day 21, the composition of complex I was the same as that for days 7 to 14 after ADR. In contrast, complex II also contained c-Rel dimers, because incubation with c-Rel antisera partially reduced the density of this band at this time point (Fig. 6, lane 12). The partial reduction in the density of complex II with p50 antisera at the day 21 time point (lane 10) was not seen in repeated assays, and was probably due to a slight reduction in either the amount of protein or probe that was loaded in that particular lane. At day 28, the pattern of subunit activation was the same as that for day 21 (data not shown). The results were representative of two to three individual animals per time point, with experiments repeated in duplicate. In summary, NF-κB activation in ADR rats was due to an increase in the nuclear translocation of p50/65 and p50/c-Rel heterodimers that account for complex I. Complex II was partially due to an increase in p65- and c-Rel-containing dimers.

Correlation between functional parameters, tubulointerstitial histology, and NF-κB activation

The protein excretion rate correlated positively with interstitial volume, interstitial ED-1 cell infiltration, and NF-κB activation (Table 2). In contrast, there was a negative correlation between protein excretion rate and tubule cell height (Table 2). CCl correlated positively with mean tubule cell height and correlated negatively with NF-κB activation, percentage interstitial volume, and interstitial ED-1 infiltration. Interstitial ED-1 infiltration correlated strongly with NF-κB activation, interstitial volume, and tubule cell height.

Effect of NAC and PDTC on functional parameters and renal lipid peroxidation in ADR rats at day 30

To determine whether the inhibition of NF-κB could attenuate interstitial monocyte/macrophage infiltration, NAC and PDTC were administered to rats with ADR nephrosis. ADR rats had similar food intake and weight gain during the study period because they were pair fed (data not shown). No abnormalities were noted in the animals treated with NAC and PDTC, and there was no mortality in any group during the study period. By de-
Fig. 6. Supershift assay of EMSA performed from cortical nuclear extracts. Lanes 1, 5, and 9: nuclear protein extracts obtained from control animals and animals day 7 and 21 after adriamycin (ADR), respectively, that were incubated with the negative control, normal rabbit serum (NRS). Lanes 2, 6, and 10: incubation with the p50 antibody. Lanes 3, 7, 11: incubation with the p65 antibody. Lanes 4, 8, and 12: incubation with the c-Rel antibody. The arrow is in alignment with the supershifted p50 band. The results are representative of two to three animals per time point, and all assays were performed in duplicate.

Table 2. Correlation between functional parameters, tubulointerstitial histology and NF-κB activation

<table>
<thead>
<tr>
<th></th>
<th>UPV</th>
<th>CCl</th>
<th>Tubule cell height</th>
<th>Interstitial ED-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB densitometry</td>
<td>0.63*</td>
<td>-0.53*</td>
<td>-0.51</td>
<td>0.67*</td>
</tr>
<tr>
<td>Tubule cell height</td>
<td>-0.70p</td>
<td>0.71b</td>
<td>-</td>
<td>-0.89p</td>
</tr>
<tr>
<td>Interstitial volume</td>
<td>0.80p</td>
<td>-0.66b</td>
<td>-0.92*</td>
<td>0.933</td>
</tr>
<tr>
<td>Interstitial ED-1</td>
<td>0.81p</td>
<td>-0.70p</td>
<td>-0.89p</td>
<td>-</td>
</tr>
</tbody>
</table>

The correlation data were determined using the Spearman’s ranked order correlation test in a total of 20 individual animals. Abbreviations are: UPV, 24-hour urinary protein excretion; CCl, endogenous creatinine clearance.

*P < 0.05

Table 3. Effect of N-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC) or vehicle on functional parameters in rats injected with adriamycin (ADR) or saline (control)

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>Control (N = 6)</th>
<th>ADR + vehicle (N = 7)</th>
<th>ADR + NAC (N = 8)</th>
<th>ADR + PDTC (N = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt g</td>
<td>10</td>
<td>328 ± 12</td>
<td>270 ± 4</td>
<td>269 ± 4</td>
<td>274 ± 3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>396 ± 11</td>
<td>274 ± 5</td>
<td>281 ± 4</td>
<td>276 ± 6</td>
</tr>
<tr>
<td>Scr μmol/liter</td>
<td>10</td>
<td>48 ± 1</td>
<td>46 ± 2</td>
<td>49 ± 2</td>
<td>46 ± 1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>52 ± 1</td>
<td>56 ± 3</td>
<td>64 ± 5</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>S Alb g/liter</td>
<td>10</td>
<td>52 ± 1</td>
<td>56 ± 3</td>
<td>64 ± 5</td>
<td>54 ± 2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>31 ± 2</td>
<td>17 ± 1*</td>
<td>16 ± 1*</td>
<td>17 ± 1*</td>
</tr>
<tr>
<td>V ml/24 hr</td>
<td>10</td>
<td>20 ± 2</td>
<td>24 ± 4</td>
<td>23 ± 1*</td>
<td>23 ± 1*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>17 ± 3</td>
<td>21 ± 3</td>
<td>23 ± 1</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>UPV mg/24 hr</td>
<td>10</td>
<td>20 ± 3</td>
<td>249 ± 27*</td>
<td>247 ± 26*</td>
<td>226 ± 21*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>18 ± 3</td>
<td>585 ± 39*</td>
<td>576 ± 23*</td>
<td>543 ± 55*</td>
</tr>
<tr>
<td>U_pur mg/μmol</td>
<td>10</td>
<td>0.25 ± 0.03</td>
<td>3.62 ± 0.41*</td>
<td>3.44 ± 0.29*</td>
<td>3.21 ± 0.27*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.17 ± 0.03</td>
<td>6.66 ± 0.50*</td>
<td>7.00 ± 1.28*</td>
<td>7.83 ± 1.00*</td>
</tr>
<tr>
<td>CCl ml/min</td>
<td>10</td>
<td>1.68 ± 0.11</td>
<td>1.52 ± 0.07</td>
<td>1.51 ± 0.13</td>
<td>1.53 ± 0.07</td>
</tr>
<tr>
<td>ml/min/g kidney wt</td>
<td>30</td>
<td>1.61 ± 0.06</td>
<td>0.92 ± 0.13</td>
<td>0.84 ± 0.08</td>
<td>1.06 ± 0.10</td>
</tr>
</tbody>
</table>

Values are express as mean ± sem. Abbreviations: Body wt, body weight; Scr, serum creatinine; S Alb, serum albumin; V, 24-hour urinary volume; UPV, 24-hour urinary protein excretion; U_pur, urinary protein:creatinine ratio. There were no differences in these parameters in ADR rats treated with either vehicle, NAC and PDTC.

*P < 0.01 when compared to the control group

sign, baseline body weight, UPV, Scr, S Alb, and CCl were similar among all groups with ADR nephrosis (Table 3). The administration of NAC and PDTC did not alter these parameters at day 30 in comparison to the vehicle-treated ADR group (Table 3).

Cortical MDA was increased 3.7-fold in rats with ADR nephrosis in comparison to normal animals (control, 38.2 ± 4.3; ADR ± vehicle, 139.6 ± 23.9 nmol/100 mg protein, P = 0.0004; Fig. 7). Treatment with NAC had no effect on lipid peroxidation (138.5 ± 19.1, P = NS),
whereas PDTC reduced it by 40% (82.8 ± 4.0, P = 0.02) in comparison to vehicle-treated ADR rats (Fig. 7).

**Effect of NAC and PDTC on renal histology and ED-1 infiltration in ADR rats at day 30**

As described earlier in this article, ADR rats had increased kidney weight and tubulointerstitial injury compared with control animals (Table 4). The treatment of ADR rats with daily injections of NAC had no effect on kidney weight, tubulointerstitial histology in comparison to the vehicle treated rats (Fig. 8A, C and Table 4). In contrast, PDTC-treated ADR rats had reduced tubulointerstitial injury (Fig. 8E). With morphometric analysis, PDTC partially prevented the reduction in cross-sectional tubule cell height and increase in interstitial volume that occurred in ADR rats (Table 4).

The number of interstitial cells positive for ED-1 antigen was increased ninefold in ADR rats compared with normal controls (Table 4 and Fig. 8B). In ADR rats treated with NAC, the number of interstitial ED-1 positive cells appeared to be greater than in vehicle-treated ADR rats, but this was not significant (Table 4). In contrast, PDTC partially prevented the cortical interstitial infiltration with ED-1-positive cells that occurred in ADR rats (Table 4 and Fig. 8F).

The treatment with PDTC or NAC did not affect the mean GCA (control, 11.875 ± 1084 µm²; ADR ± vehicle, 12.216 ± 448 µm²; ADR ± NAC, 11142 ± 375 µm²; ADR ± PDTC, 12.185 ± 299 µm², P = NS, one-way ANOVA) or mean GC (control, 54 ± 3; ADR ± vehicle, 54 ± 3; ADR ± NAC, 57 ± 2; ADR ± PDTC, 56 ± 4 nuclei per glomerular cross-section, P = NS, one-way ANOVA). NAC caused a slight increase in the MA (ADR ± vehicle, 5.7 ± 0.5; ADR ± NAC, 7.0 ± 0.4; ADR ± PDTC, 5.2 ± 0.5%; P = 0.05, one way ANOVA). The mean number of ED-1-positive glomerular cells was not affected by NAC or PDTC (control, 2.0 ± 0.3; ADR ± vehicle, 1.9 ± 0.3; ADR ± NAC, 2.2 ± 0.7; ADR ± PDTC, 2.2 ± 0.6 ED-1-positive cells per glomerular cross-section; P = NS, Kruskal Wallis test).

**Effect of NAC and PDTC on NF-κB activation in adriamycin rats at day 30**

At day 30, vehicle-treated ADR rats had increased NF-κB activation compared with control animals (Fig. 9A). By densitometric analysis (sum of complex I and II), this represented a fourfold increase in renal cortical NF-κB (control, 1.0 ± 0.2; ADR ± vehicle, 4.1 ± 0.9-fold increase above control, P = 0.02, N = 6 to 7; Fig. 9B). In ADR rats treated with NAC, there was a tendency for NF-κB activation to be increased further, but the mean densitometry was not statistically significant when compared with the vehicle-treated group (ADR ± NAC, 6.5 ± 1.5-fold-increase above control, P = 0.22 when compared with ADR + vehicle group, N = 8; Fig. 9). In contrast, PDTC-suppressed NF-κB activation and the mean densitometry were similar to control animals (1.1 ± 0.3 fold-increase above control, P = 0.005 when compared with ADR + vehicle group, N = 8; Fig. 9).

**DISCUSSION**

The NF-κB transcription factors are critical for the activation of genes involved in immunological, inflammatory, and growth-related processes [20, 21]. The results of this article provide evidence that the activation of NF-κB could play an important role in the progression of cortical tubulointerstitial injury in nonimmune chronic glomerular disease. NF-κB was increased in cortical nuclear extracts derived from ADR rats for up to four weeks after disease induction. This activation was correlated with the degree of proteinuria and interstitial ED-1 infiltration and preceded the development of tubule cell atrophy. The inhibition of NF-κB with PDTC was associated with significantly reduced tubular injury and interstitial ED-1 infiltration. In contrast, treatment with NAC (which did not alter NF-κB activation) had no effect on cortical tubulointerstitial injury in ADR nephropsis.

Nuclear factor-κB proteins are activated in a variety of experimental models of renal inflammatory disease, including antiglomerular basement membrane nephritis [41], ureteric obstruction [22, 42], endotoxemia [43], and immune complex nephritis [23]. Our study shows that this also occurs in rats with nonimmune proteinuric tubulo-interstitial inflammation. As described previously [22] and confirmed in this study, the EMSA of cortical nuclear extracts from normal rats had two specific DNA protein complexes on gel autoradiographs. In order to characterize
the subunit specificity of these DNA-protein complexes, the gel supershift assay was performed. NF-κB proteins are composed of at least seven members: NFKB1 (p50/105), NFKB2 (p52/100), c-rel (c-Rel), relA (p65), relB (RelB), v-Rel, and the Drosophila Rel proteins [20, 21, 38]. We have focused on p50, p65, and c-Rel because of their known importance as positive transcriptional regulators and role in the production of proinflammatory mediators [20, 21, 44]. In our study, the slower migrating band in the EMSA from normal rats could be depleted or supershifted with either the p50, p65, and c-Rel antibodies, whereas the faster migrating band was unaffected by these antisera. It is possible that the faster migrating complex could be composed of other known members of the NF-κB family. However, in a previous study [22], this DNA-protein complex could not be depleted with p52 or RelB-specific antisera either, and it was speculated that it may contain novel NF-κB proteins [22].

After the induction of ADR nephrosis, a significant increase in the activation of NF-κB occurred. This is unlikely to be due to a direct effect of ADR because the concentrations of anthracyclines that induce the cellular activation of NF-κB are not present in renal tissue beyond 72 hours after an in vivo injection [45, 46]. As in normal rats, the slower migrating band in the EMSA from ADR rats consisted of p50/65 and p50/c-Rel heterodimers. However, by day 21 and 28, the faster migrating band could also be partially depleted with p65- and c-Rel-specific antisera. These results contrast to the pattern of NF-κB activation in other renal models. In rats with ureteral obstruction, the p65 subunit appeared to be less prevalent [22], whereas in rats with immune complex nephritis, c-Rel was not present in the activated NF-κB [23]. Such differences may be important because in vitro studies suggest that variations in the subunit specificity (which may due to etiological stimulants responsible for NF-κB activation in the various models) are likely to determine the selection of genes that are activated [44].

Multiple signals can activate NF-κB in cells. In vitro studies from our laboratory [14] and work by Zoja et al support the hypothesis that proteinuria could directly induce NF-κB activation in proximal tubular cells [11]. This might be the case in ADR nephrosis because there was a positive nonlinear correlation between urinary protein excretion and the degree of cortical NF-κB activation. Reactive oxygen intermediates, particularly peroxides, are potent stimulants of NF-κB activation [38], and in this article, the cortical production of lipid peroxides was increased approximately fourfold in ADR rats, as has been demonstrated previously [37]. Inhibition of NF-κB with PDTC was associated with a 41% reduction in renal lipid peroxidation. The increased local production of tumor necrosis factor-α (TNF-α) and platelet-activating factor in ADR rats could also be involved in the renal activation of NF-κB in this model [15, 20, 21]. Mechanical stretch can activate NF-κB in vitro [47], and hence, tubular dilation could contribute to transcription factor activation in chronic renal disease models. However, this is probably of lesser importance in ADR nephrosis (during the time points of this study) and possibly of greater significance in rats with obstructive uropathy [22, 42].

The sensitive and reproducible method of computer-assisted image morphometric analysis was used to quantify glomerular and tubulointerstitial histology. Using this method, a time-dependent increase in tubule cell atrophy, as determined by the cross-sectional tubule cell height, was found in ADR rats. Interestingly, although there were focal areas of frankly dilated tubules, the reduction in tubule cell height was not accompanied by an increase in mean cortical tubular diameter. However, we have observed significant tubular dilation with longer term follow-up in an accelerated model of ADR nephrosis [32]. Another important finding of the study was the strong correlation between tubule cell atrophy and interstitial ED-1 infiltration, interstitial volume, proteinuria, and Ccr. Although it is not possible to infer a cause-effect relationship between these parameters, the results highlight the importance of tubular injury as an effecter of disease progression in proteinuric tubulointerstitial disease. This is corroborated by the extensive data presented by Bohle, Mackensen-Haen, and von Gise in human chronic renal disease [48].

Table 4. Effect of N-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC) or vehicle on cortical tubulointerstitial morphometric parameters in rats with adriamycin (ADR) nephrosis

<table>
<thead>
<tr>
<th>Group</th>
<th>KW g</th>
<th>KW:BW g/100 g</th>
<th>IV %</th>
<th>Mean tubular cell height μm</th>
<th>Mean tubule cell height μm</th>
<th>Number of ED-1 positive cells in the interstitium/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>1.28 ± 0.05</td>
<td>0.31 ± 0.01</td>
<td>3.6 ± 0.6</td>
<td>42.7 ± 1.6</td>
<td>28.0 ± 1.4</td>
</tr>
<tr>
<td>ADR</td>
<td>7</td>
<td>1.84 ± 0.14a</td>
<td>0.65 ± 0.05b</td>
<td>18.5 ± 2.3c</td>
<td>46.8 ± 2.6</td>
<td>14.6 ± 2.3c</td>
</tr>
<tr>
<td>ADR + NAC</td>
<td>8</td>
<td>1.65 ± 0.07b</td>
<td>0.57 ± 0.02b</td>
<td>23.8 ± 2.5b</td>
<td>44.6 ± 2.9</td>
<td>15.6 ± 1.2b</td>
</tr>
<tr>
<td>ADR + PDTC</td>
<td>8</td>
<td>1.31 ± 0.10b</td>
<td>0.44 ± 0.03bd</td>
<td>9.4 ± 1.9b</td>
<td>40.6 ± 4.7</td>
<td>23.2 ± 1.4d</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.
Abbreviation: IV, percentage interstitial volume.
+ P < 0.05 and * P < 0.01, respectively, when compared to the control group.
Fig. 8. Effect of vehicle, N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) on cortical tubulointerstitial pathology and interstitial ED-1 infiltration at day 30 after injection with adriamycin (ADR). Photomicrographs of periodic acid-Schiff stained cortical sections (A, C, E) and interstitial ED-1 immunostaining (B, D, F) obtained from rats injected with ADR and treated with vehicle (A, B), NAC (C, D), or PDTC (E, F; ×250).
Fig. 9. (A) Autoradiographs of electrophoretic mobility shift assays showing NF-κB activation in nuclear cortical extracts at day 30 after injection with saline (control) or adriamycin (ADR). Rats with ADR nephrosis were treated either with vehicle, N-acetylcysteine (NAC), or pyrrolidine dithiocarbamate (PDTC). Each lane represents a sample from an individual animal, and assays for all animals from each group were performed simultaneously. The results shown are representative of experiments performed in six to eight individual samples per group, which were done in duplicate. (B) Densitometric analysis of NF-κB bands (sum of complexes I and II; Fig. 4) in the experimental groups. Densitometry was performed on autoradiographs of simultaneously performed mobility shift assays that were developed for same period of time. The individual bar values are the mean of each group. *P < 0.05 when compared with the control group; **P < 0.05 when compared to the ADR + vehicle group (N = 6 to 8 per group).

ADR rats. For this purpose, we used NAC and PDTC, which inhibit NF-κB activation both in vitro and in vivo [25–27].

Chronic treatment of ADR rats with PDTC dramatically reduced the cortical activation of NF-κB such that the mean densitometry was similar to normal control animals. This complete inhibition was achieved despite PDTC being administered beyond a time point when NF-κB was already activated, demonstrating that this agent can be effective in both the prevention and termination of NF-κB activation in chronic disease. To our knowledge, this is the first time that this has been demonstrated with PDTC in an in vivo model.

The mechanisms by which PDTC inhibited NF-κB in this model are not known. The critical step that allows NF-κB dimers to enter the nucleus is the degradation of IkB (a family of inhibitory proteins bound to NF-κB within the cytosol), an event that may be triggered by the generation of intracellular reactive oxygen intermediates, depending on the type of cell and stimulus [20, 21, 38, 49]. PDTC is a potent antioxidant and, in this study, reduced renal lipid peroxidation [38]. However, it remains uncertain whether this and/or other effects are responsible for its ability to inhibit NF-κB. For example, PDTC can inhibit NF-κB DNA binding through a prooxidant effect [50]. Moreover, the disparity between the reduction in lipid peroxidation and NF-κB activation in this study also suggests that antioxidant-independent mechanisms could be involved in this model. PDTC is a potent metal chelator [24, 38, 50], can interact with thiol groups [50], and is capable of affecting the activity of other transcription factors [51]. The interactions between these effects and the inhibition of NF-κB have not been investigated in this study. Therefore, it is not possible to infer from the our data that PDTC was a direct and specific inhibitor of NF-κB in this model.

Pyrrolidine dithiocarbamate reduced the accumulation of ED-1-positive cells within the cortical interstitial space by 48% in nephrotic rats. Similar to these results, the continuous administration of PDTC reduced leukocyte-induced chemotaxis in alcohol-induced cerebral inflammation [52]. Many NF-κB-regulated genes play an important role in mediating leukocyte chemotaxis and activation [20, 21]. The inhibition of NF-κB with PDTC reduced albumin and IgG-induced RANTES (a CC chemokine) expression in LLC-PK1 cells [11]. PDTC also suppressed hypoxia-stimulated intercellular adhesion molecule-1 (ICAM-1) expression in cultured
human proximal tubular cells [53]. In vivo, PDTC reduced the glomerular expression of monocyte chemotactic protein-1 (MCP-1), interleukin-1β (IL-1β), and ICAM-1 in experimental anti-glomerular basement membrane nephritis [41]. Diethyldithiocarbamate (a di-thiocarbamate related to PDTC) reduced NF-κB and the transcription of IL-1β, TNF-α, IL-6, and inducible nitric oxide synthase (iNOS) in rats with ischemic myocardium [54]. However, the effects of PDTC on NF-κB–regulated genes are complex. In endothelial cells, PDTC reduced vascular cell adhesion molecule-1 but not ICAM-1, although both genes are regulated by NF-κB [20, 55]. Moreover, in lipopolysaccharide (LPS)-stimulated rabbit alveolar macrophages, PDTC paradoxically increased TNF-α, despite inhibiting NF-κB [56]. In ADR nephrosis, we have previously shown that there is a time-dependent increase in the transcription of MCP-1, TNF-α, and IL-10 in whole renal cortex (unpublished observation), yet in separate studies, we found that PDTC failed to attenuate whole cortical expression of MCP-1 and TNF-α but reduced IL-10. In vitro PDTC reduced LPS-induced MCP-1, TNF-α, and IL-10 in rat proximal tubular cells, suggesting that these effects may be cell and stimulus specific (manuscript submitted for publication).

The reduction in ED-1–positive interstitial cells in ADR rats by PDTC could also be explained by an increase in macrophage apoptosis, as has been demonstrated in vitro [57]. In contrast, when cultured rat proximal tubular cells are exposed to PDTC for 24 hours at NF-κB inhibitory concentrations, an increase in the rate of apoptosis was not observed (unpublished observation). Additionally, we have not investigated in this study whether PDTC could affect monocyte differentiation and proliferation. The latter could be mediated by inhibition of NF-κB–regulated colony-stimulating factors [20, 21]. Finally, NF-κB–independent mechanisms are also likely to play a role in monocyte chemotaxis, differentiation, and survival in ADR nephrosis because interstitial ED-1 infiltration was still increased fourfold in PDTC-treated rats compared with normal control animals.

Pyrrolidine dithiocarbamate partially attenuated the morphological features of tubular injury in ADR nephrosis. This may have been due to a reduction in macrophage infiltration because in the time course study, the onset of tubule cell atrophy was preceded by interstitial ED-1 infiltration. PDTC could also have directly attenuated tubular damage through its effects as an antioxidant [38], metal chelator [24], and possible inhibition of NF-κB–regulated proto-oncogenes such as c-myc [58]. That focal areas of tubular cells still had reduced brush border height following PDTC treatment may also account, in part, for the absence of a significant change in the urinary protein excretion in these rats [59].

Surprisingly, despite the attenuation in tubulointerstitial injury, PDTC treatment was not accompanied by an improvement in CCl₃. In micropuncture studies, O’Donnell et al showed that the single-nephron glomerular filtration rate was reduced in ADR nephrosis as result of a decrease in renal plasma flow and glomerular ultrafiltration coefficient (Kₐ) [60]. The reasons for the reduction in Kₐ in ADR nephrosis are not known but could include altered plasma protein concentration [61], glomerular structure [62], and tubuloglomerular feedback [48]. The persistence of these abnormalities in PDTC-treated ADR rats may explain why CCl₃ was not normalized. It remains to be determined whether long-term PDTC treatment could reduce interstitial fibrosis and prevent a further decline in renal function in this model.

Like PDTC, the effect of NAC may be due to its antioxidant properties as well as other, possibly unique, distal effects on the NF-κB signal transduction pathway [25, 26, 63]. However, in this study, twice daily intraperitoneal injections of NAC for 16 days failed to inhibit NF-κB in ADR rats. Moreover, in NAC-treated ADR rats, there was a tendency for increased cortical activation of NF-κB, interstitial ED-1 infiltration, and mesangial matrix deposition, although this was not statistically significant. Similar to these results, NAC, when administered once daily as an intraperitoneal injection for 14 days, worsened alveolar epithelial cell damage in rats with chronic hyperoxia [64]. NAC is also known to increase NF-κB activation in vitro in some cell types, an effect that may be due to auto-oxidation [65]. However, in contrast, NAC is protective in experimental models of acute tubular necrosis [66, 67, and Note Added in Proof] and suppressed renal NF-κB activation in LPS-induced glomerulonephritis. Furthermore, 1% NAC in drinking water provided ad libitum (for up to 5 days) to rats with nonproteinuric tubulointerstitial injury significantly reduced cortical NF-κB, activation, MCP-1 transcription, and interstitial monocyte infiltration and (abstract; Morrissey, Proceedings of the Extramural Grant Program Annual Meeting, p74, 1998). The reasons for the discrepancy with our study are not certain, but may include differences in the dose and route of drug administration, the duration of treatment, and finally that distinct pathways are responsible for NF-κB activation in ADR nephrosis and these may be NAC-resistant.

The cellular origin of the activated NF-κB proteins in ADR nephrosis is not known. Infiltrating macrophages may be a potential source because NF-κB activation was correlated with the number of ED-1–positive interstitial cells. However, NF-κB was already increased approximately fourfold by day 7 (when there was only a mild increase in the number of interstitial cells), suggesting that resident renal cells could also be a contributing factor. In vitro, NF-κB can be induced in mesangial, proximal tubular, and endothelial cells [11, 14, 49, 55]. We postulate that proximal tubular cells are likely to be a major source during the progressive stages of ADR nephrosis because of their abundance in the renal cortex, their
pathogenetic significance in proteinuric tubulointerstitial inflammation [9], and the absence of glomerular inflammation in this model. Furthermore, in rats with obstructive uropathy, the p50 subunit was mainly localized to the nuclei of tubular cells [22].

In conclusion, the results of this study show that the activation of NF-κB could play a pathogenic role in mediating monocyte/macrophage infiltration and tubular injury in nonimmune proteinuric renal disease. Future studies need to address the mechanisms by which PDTC-induced NF-κB inhibition caused these effects. In addition, experiments using decoy antisense oligodeoxy-nucleotides are required to confirm the role of NF-κB activation in this model [68].

NOTE ADDED IN PROOF


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APPENDIX

Abbreviations used in this article are: ADR, adriamycin; C5c, creatine clearance; EMSA, electrophoretic mobility shift assay; GC, glomerular cellularity; GCA, glomerular capillary area; iNOS, inducible nitric oxide synthase; ICAM, intercellular adhesion molecule; IL, interleukin; KI, glomerular ultrafiltration coefficient; LPS, lipopolysaccharide; NAC, N-acetylcysteine; NF-κB, nuclear factor-kappa B; MCP-1, monocyte chemoattractant protein-1; MA, mesangial matrix area; MDA, malondialdehyde; PAS, periodic acid-Schiff; PDTC, pyrrolidine dithiocarbamate; S\textsubscript{a}, serum albumin; S\textsubscript{c}, serum creatinine; TNF, tumor necrosis factor; U\textsubscript{12}V, 24-hour urinary protein excretion; U\textsubscript{2}V, 24-hour urinary creatinine excretion.

REFERENCES

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43. *O'Donnell MP*, *Michels L*, *Kasimak B*, *Rai L*, *Keane WF*: Adria-


