Nephrol Dial Transplant (2001) 16: 32–38

Nephrology
Dialysis
Transplantation

Original Article

Association of nitric oxide production and apoptosis in a model of experimental nephropathy

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Abstract

Background. In recent studies increased amounts of nitric oxide (NO) and apoptosis have been implicated in various pathological conditions in the kidney. We have studied the role of NO and its association with apoptosis in an experimental model of nephrotic syndrome induced by a single injection of adriamycin (ADR).

Methods. The alteration in the NO pathway was assessed by measuring nitrite levels in serum/urine and by evaluating the changes in vascular reactivity of the isolated perfused rat kidney (IPRK) system. Rats were stratified into control groups and ADR-induced nephropathy groups. These two groups were then divided into: group 1, animals receiving saline; and group 2, animals receiving aminoguanidine (AG) which is a specific inhibitor of inducible-NO synthase. On day 21, rats were sacrificed after obtaining material for biochemical analysis.

Results. Histopathological examination of the kidneys of rats treated with ADR revealed focal areas of mesangial proliferation and mild tubulointerstitial inflammation. They also had significantly higher levels of proteinuria compared with control and treatment groups ($P<0.05$). Urine nitrite levels were significantly increased in the ADR-nephropathy group ($P<0.05$). In the IPRK phenylephrine and acetylcholine related responses were significantly impaired in the ADR-nephropathy group. Apoptosis was not detected in controls. However, in the ADR-nephropathy group, numerous apoptotic cells were identified in the tubulo-interstitial areas. Double staining revealed numerous interstitial apoptotic cells to stain for ED1, a marker for monocytes/macrophages. Treatment with AG prevented the impairment of renal vascular bed responses and reduced both urine nitrite levels and apoptosis to control levels.

Conclusion. We suggest that interactions between NO and apoptosis are important in the pathogenesis of the ADR-induced nephrosis.

Keywords: adriamycin-induced nephropathy; aminoguanidine; apoptosis; isolated perfused rat kidney; nitric oxide

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Introduction

Nitric oxide (NO) is a small signalling molecule regulating a variety of diverse cellular functions including many physiological and pathophysiological processes ranging from regulation of vascular tonus to neuronal transmission, from apoptosis to inflammation. Most of the physiological actions are mediated by NO bursts generated by constitutive isoforms of nitric oxide synthase (cNOS). On the other hand, when produced by inducible NOS (iNOS), in large amounts for long periods, NO can be a cytotoxic agent [1–4]. Under inflammatory conditions, glomerular mesangial cells, endothelial cells, macrophages, neutrophils and vascular smooth muscle cells can express iNOS. The NO produced by iNOS, may be one of the key elements in any inflammatory injury [5]. Indeed, iNOS has been implicated in the pathogenesis of glomerulopathies in animal models and in a few human diseases [6,7]. The actions of NO which support its inflammatory role in glomerulopathies are associated with NO's complex effects on many cell functions including its inflammatory effects and cytotoxicity [6]. Large amounts of NO may react with superoxide anion to produce strong oxidants such as peroxynitrite, which may mediate cell death [5,8]. NO also inhibits DNA synthesis, damages DNA and, hence, induces apoptosis. In the only human model studying the association of inducible NOS and apoptosis, Wang *et al.* [7] suggested that NO production played a role in the induction of apoptosis in lupus nephritis, and that this was in part modulated by p53.

Apoptosis is a programmed form of cell death. Its

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role in kidney disease is complex [9]. In certain experimental models of proliferative glomerulonephritis, apoptosis has been suggested to be a necessary process in the regulation of endothelial or mesangial cells in the repair process [10,11]. On the other hand, apoptosis has been implicated in the sclerotic process within the kidney; apoptotic bodies have been demonstrated in the sclerotic areas of experimental and human renal diseases [11,12].

We have set up a model of nephrotic syndrome in rats induced by adriamycin (ADR), to study the role of NO and its association with apoptosis. The alteration in the NO pathway in this experimental model was assessed by measuring nitrite levels in serum/urine and by evaluating the changes in vascular reactivity of the isolated perfused rat kidney. The isolated perfused kidney system enables us to study the role of NO, on the renal vascular tone. Since increased NO synthesis by iNOS is expected to be associated with the inflammatory response, we have then examined the effect of aminoguanidine (AG) treatment on the observed changes in NO-mediated responses and the induction of apoptosis. AG was chosen because it is a relatively selective inhibitor of iNOS, and not constitutive NOS [13]. We, thus, wished to confirm the effect of NO produced by iNOS by a specific inhibitor.

Materials and methods

Animals and experimental protocol

Wistar rats weighing 200–250 g were stratified into a control group and ADR-nephropathy group. ADR was administered as a single intravenous (i.v.) dose of 5 mg/kg and the control group received saline by the same route. Both the control group and the ADR-nephropathy group were then divided up again into two groups: group 1, animals receiving saline ($n=5$ and 6, respectively); and group 2, animals receiving AG (both $n=5$). AG was administered at a dose of 50 mg/kg/day intraperitoneally (i.p.) and was added daily to the drinking water at a concentration of 1 g/l. Drinking water was distilled water, free of nitrates. The amount of water but not the food consumed was measured daily. All animals were sacrificed on day 21 of treatment. It has been previously shown that proteinuria in this model reached a peak at day 21 [14]. One day before sacrifice, the rats were individually housed in metabolic cages and urine was collected for 24 h. Protein and nitrite levels in urine samples were measured.

Isolated perfused kidney experiments

Rats were anaesthetized with pentobarbital sodium (30 mg/kg) by i.p. injection. After laparotomy with a midline incision, heparin (200 U/kg) was injected into the aorta. The aorta was ligated distal to the right renal artery, and a polypropylene cannula was inserted into the left renal artery through an incision. The left kidney was removed and placed into the perfusion system with the renal artery cannula to study the vascular reactivity of renal vascular bed. In the meantime a blood sample was collected from the inferior vena cava for analysis of serum nitrite levels, and the right kidney was resected for histopathological and immunohistochemical studies.

The Ethics Committee of Ankara University, Faculty of Veterinary Medicine, Ankara, Turkey, approved the animal experimentation.

Biochemical evaluation

Serum creatinine (by standard autoanalyser technique), urine protein levels (by the Biuret method), plasma and urine nitrite levels were measured in each group of rats by standard autoanalyser techniques. Nitrite was assayed colorimetrically after reaction with Griess reagent as described previously [15].

Histopathological examination

After removal of the kidney, all tissues were fixed in 10% formalin, embedded in paraffin and examined in multiple consequent sections. The histological study was carried out using haematoxylin eosin (H&E) and periodic acid-Schiff (PAS). Glomerular and tubular cells were counted at a magnification of ×200. For quantification of glomerular cells and tubular cells, 20 glomeruli and 20 fields were counted, respectively, to take an average attributed to the animal study. For the quantitation of the interstitial cells 10 fields with ×400 magnification were counted by one person blinded to the specimen experimental code (D.O.)

Immunohistochemistry

Sections 5-μm thick were cut from paraffin blocks and mounted on poly-L-lysine coated slides. After deparaffinization, slides were put in a microwave oven in 10 mM sodium citrate at 700 W (4×5 min) for antigen retrieval. Immunohistochemical staining using the streptavidin–biotin peroxidase method was performed with monoclonal primary antibody against proliferating cell nuclear antigen (PCNA, Biogenex, 1/80 dilution). Negative control sections were incubated with phosphate-buffered saline (PBS) or non-immune globulin instead of the primary antibody. Human tonsil sections served as positive control. PCNA positive tubular and interstitial cells were counted in 10 fields at a microscopic magnification of ×400 (D.O.).

Detection of apoptotic cells with TUNEL staining

DNA fragmentation was detected *in situ* by 3′-end labelling using a kit containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP according to the manufacturer's recommendations (ApopTagR, Intergen, NY, USA). Briefly, formalin-fixed and paraffin-embedded 5-μm thick tissue sections were deparafinized and dehydrated. Nuclear proteins were stripped from the DNA by incubating in proteinase K for 30 min, and endogenous peroxidase was blocked with H_2O_2. Sections were incubated in a buffer containing TdT and digoxigenin-labelled dUTP, followed by digoxigenin conjugated peroxidase treatment. Diaminobenzidine (DAB) was used as chromogen and the background was stained with methyl green. Positive and negative controls were included in every set of experiments. Brown labelled TUNEL positive apoptotic cells in the glomeruli and tubulointerstitium were counted independently by two investigators under ×400 magnification. Apoptotic cells were identified by the presence of various types of chromatin

condensation (perinuclear ring formation, patches or apoptotic bodies), while cells showing diffuse cytoplasmic labelling considered as necrotic cells were not counted.

Double staining for both apoptosis and ED1 or α-SMA

In order to identify the type of apoptotic cells, double immunohistochemical staining was used on paraffin sections for both ISEL (*in situ* end labelling) and ED1 (detecting the antigen on rat monocytes /macrophages; 1 : 50, Serotec, UK) or α-SMA (smooth muscle actin) (detecting myofibroblasts; 1 : 250, DAKO, Denmark). Briefly, 4 mm sections of tissue were dewaxed, and hydrated according to standard protocols. Sections were then digested with proteinase K, endogenous peroxidase was blocked with 3% H_2O_2, and then taken through the ApopTag protocol from equilibration buffer to stop/wash as in the ApopTag method. Sections were preincubated with blocking horse serum for 30 min, labelled with the anti-ED1 or α-SMA antibody at 40°C overnight. Sections were labelled with biotinylated secondary antimouse IgG at 37°C for 30 min with alkaline phosphatase–streptavidin for another 30 min and developed with Fast Red TR/Naphthol AS-MX solution to produce the bright pink colour. Subsequently, anti-digoxigenin peroxidase antibody was applied on the sections and the development was achieved by the solution of DAB, in which a yellow-brown colour indicates a positive staining.

Isolated perfused kidney

Perfusion of the isolated left kidney was carried out with oxygenated (95% O_2, 5% CO_2) and warmed (37°C) Krebs–Henseleit solution, using a Harvard peristaltic pump (model 1203a) (Newport Beach, CA, USA) at a constant flow of 6 ml/min. Krebs–Henseleit solution had the following composition (mM): NaCl, 95; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.6. Mean perfusion pressure was measured via a pressure transducer and monitored continuously on an IBM-compatible personal computer by 'transducer data acquisition system (MAY 95) (Commat, Ankara, Turkey).

The basal perfusion pressure of the isolated kidneys were stabilized within 30 min. Each kidney preparation from the control and ADR-nephropathy group of rats was used to study the following parameters. (i) Phenylephrine-induced pressor response: phenylephrine from 10^{-7} to 10^{-5} M concentration was added into the perfusion reservoir in a cumulative manner. The increase in perfusion pressure was determined and expressed as 'mmHg'. (ii) Acetylcholine-induced vasodilator response: perfusion pressure was increased to 70–80% of the maximum pressor response elicited by phenylephrine and acetylcholine at the dose of 0.05 and 0.1 μg was added by injection to a volume of 0.01 ml, through a rubber tube distal to the perfusion pump. The involvement of NO in the acetylcholine-induced vasodilator response was also tested. For this purpose, *N*-nitro-L-arginine methyl ester (L-NAME) (3×10^{-5} M) was added into the perfusion reservoir and acetylcholine response was re-evaluated in the control group. (iii) Papaverine and sodium nitroprusside-induced vasodilator response: the vasodilator response elicited by papaverine (2.5 and 5 μg) and sodium nitroprusside (2.5 and 5 μg) were evaluated as described above for acetylcholine. The decrease in perfusion pressure by acetylcholine, papaverine and sodium nitroprusside was expressed as percentage of phenylephrine-induced pressor response.

Statistical analysis

Data are expressed as mean ± SEM. The statistical analysis of data was performed by analysis of variance followed by Tukey HSD test. *P* values <0.05 were considered significant. All the statistical analysis were made using SPSS software.

Results

General observations

Treatment with ADR and AG were well tolerated. Only two rats from the ADR-nephropathy group died during the study and were not included in the analysis. ADR was effective in inducing nephropathy in all injected rats. The weight gain of the animals in the ADR-nephropathy group (+40.8 ± 4.9 g) at the end of 21 days, was significantly higher than those of the control group and the groups receiving further therapy with AG ($P<0.05$). The amount of water consumed by the control group who received no treatment and who received AG were 28.5 ± 5.0 and 27.3 ± 5.3 ml, respectively, whereas water consumed in the ADR-nephropathy group without further treatment, and with aminoguanidine treatment were 29.7 ± 4.7 ml and 26.6 ± 3.9 ml, respectively. The differences between all groups were insignificant ($P>0.05$).

Biochemical analysis

Rats treated with ADR had significantly higher levels of proteinuria compared with control groups ($P<0.05$). Treatment with AG significantly reduced proteinuria of rats with ADR-induced nephropathy (Table 1). Serum creatinine levels remained normal in all.

Serum nitrite levels were comparable in the controls (9.5 ± 5.1 μM) and ADR-nephropathy (13.0 ± 5.4 μM) groups. However, urine nitrite levels were significantly increased in the ADR-nephropathy group (Table 1). Moreover, treatment with AG reduced urine nitrite levels significantly although they did not reach the levels of untreated controls (Table 1).

Renal vascular bed responses

In isolated rat kidneys baseline perfusion pressure was 85.2 ± 5.4 mmHg in the control group ($n=5$). Perfusion pressure in the ADR-nephropathy group was 91.4 ± 6.3 mmHg ($n=6$) and was not significantly different from that in the control group ($P>0.05$).

Phenylephrine (from 10^{-7} to 10^{-5} M) evoked a concentration-dependent increase in the perfusion pressure with a maximal pressor response of 172.4 ± 17.1 mmHg in the control group. This response was significantly attenuated in the ADR-nephropathy group and the maximum pressor response reduced to 130.6 ± 16.1 mmHg ($P<0.05$) (Fig. 1). Treatment with AG did not significantly alter either the baseline perfusion pressure or the pressor response elicited by phenylephrine in the control group; the maximum response was 189.8 ± 16.4 mmHg. On the other hand, in the

Table 1. Proteinuria, urine nitrite, tubular and interstitial apoptosis in control, and ADR-nephropathy groups of rats that were treated with AG or saline

	Control	Control + AG	ADR-nephropathy	ADR-nephropathy + AG
Proteinuria (mg/dl)	131.25 ± 28.67	183.75 ± 42.39	355.01 ± 34.20*	169.60 ± 96.25**
Urine nitrite (μM)	3.24 ± 1.81	7.37 ± 2.51	31.25 ± 6.54*	11.09 ± 1.83**
Tubular apoptosis	—	—	23.15 ± 2.30***	—
Interstitial apoptosis	—	—	47.6 ± 15.90	—

*Significantly more than all groups ($P<0.05$); **significantly less than ADR-nephropathy group ($P<0.05$); ***ratio of apoptotic cells to the tubular cells was at a median of 0.0212.

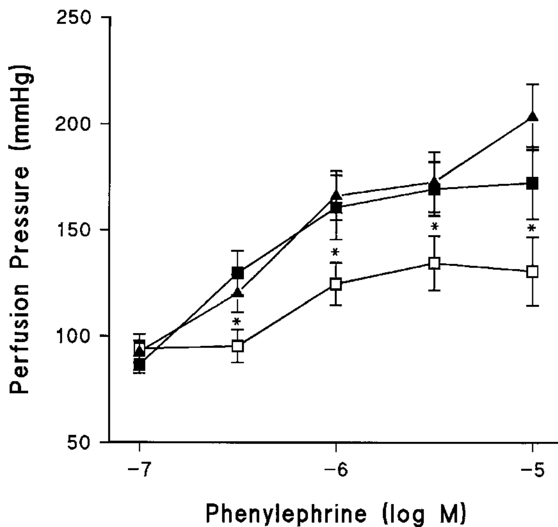

Fig. 1. Mean (SEM) increase in perfusion pressure elicited by phenylephrine in isolated perfused rat kidney from control (■), ADR-nephropathy (□) and AG ADR-nephropathy (▲) groups. The data are expressed as mmHg ($n=5$–6). *Significantly different from control group ($P<0.05$).

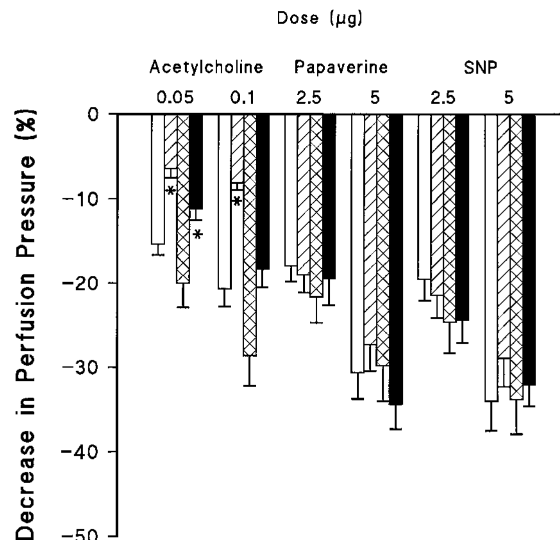

Fig. 2. Vasodilatory response to acetylcholine, papaverine and sodium nitroprusside (SNP) in isolated perfused rat kidney from control (open column); ADR-nephropathy (hatched column); AG-treated control (cross-hatched column) and AG-treated ADR-nephropathy (solid column) groups. The data are expressed as mean (SEM) percentage of phenylephrine-induced pressor response ($n=$ 5–6). *Significantly different from control group ($P<0.05$).

ADR-nephropathy group AG treatment completely reversed the attenuated phenylephrine response ($P<0.05$) (Fig. 1).

Administration of acetylcholine (0.05 and 0.1 μg) at the raised tone decreased the perfusion pressure in the control group. This vasodilator response was mediated by NO as it was significantly inhibited by L-NAME (3×10^{-5} M) ($n=5$) ($P<0.05$); acetylcholine-induced (0.05 μg) decrease in perfusion pressure in the absence and presence of L-NAME were 15.4 ± 1.3 and 4.7 ± 1.9%, respectively ($P<0.05$). Acetylcholine-induced vasodilation was found to be significantly attenuated in the ADR-nephropathy group when compared with that of the control group ($P<0.05$) (Fig. 2). In the control group, treatment with AG did not alter the acetylcholine response (Fig. 2). On the other hand, in the ADR-nephropathy group, AG treatment prevented the attenuation of acetylcholine-induced vasodilation (Fig. 2). This reversal by AG treatment was partial at 0.05 μg dose of acetylcholine since it remained reduced when compared with the control response ($P<0.05$) (Fig. 2).

Endothelium-independent vasorelaxants, papaverine and sodium nitroprusside were also tested. Papaverine (2.5 and 5 μg) and sodium nitroprusside (2.5 and 5 μg) decreased the perfusion pressure but these responses were not significantly different between the control and the ADR-nephropathy groups (Fig. 2).

Histopathological examination

Histopathological examinations of the kidneys isolated from the ADR-nephropathy group of rats showed focal areas of mesangial proliferation and mild tubulo-interstitial inflammation at the time of sacrifice. The number of tubulo-interstitial cells was significantly increased in the ADR-nephropathy group (90.18 ± 5.86/field) when compared with all the other groups ($P<0.01$). the mean number of interstitial cells in the control group who received saline was 48.60 ± 4.25/field. The mean number of interstitial cells in animals who received AG following ADR-nephropathy had decreased to 65.74 ± 2.36/field. In the control group animals that received AG this number was 64.65 ± 7.07/field.

Immunohistochemistry

There were no apoptotic cells in the kidney sections of animals in the control groups. In animals with ADR-nephropathy, apoptosis was not noted in the glomeruli. However, in this group, numerous apoptotic cells were identified in the tubulo-interstitial area (Table 1) (Fig. 3). The ratio of apoptotic cells to the tubular cells was at a median of 0.0212. On the other hand, animals with ADR-nephropathy that received AG treatment, did not show any apoptosis (Table 1). Apoptotic cells were to a large extent localized in areas of interstitial inflammation. Double immunostaining identified a large number of apoptotic monocytes/macropahages (ED1 + cells) in the animals with ADR-nephropathy (Fig. 4). On the other hand in animals treated with AG, there were hardly any double-stained cells. Very few cells double-stained for α-SMA (myofibroblasts) and apoptosis in all groups.

Cells staining for PCNA were identified in all groups in the tubulo-interstitial area. In the control group animals receiving only saline and those receiving AG, PCNA+ cells in the tubulo-interstitial areas were 11.50 ± 8.02/field and 5.80 ± 2.78/field, respectively, whereas in the ADR nephropathy groups the mean was 20.75 ± 3.86/field. In the ADR-group treated with AG, PCNA+ cells decreased to 9.20 ± 2.16/field, respectively. The differences between the ADR nephropathy group and all the other groups were significant (all $P < 0.05$).

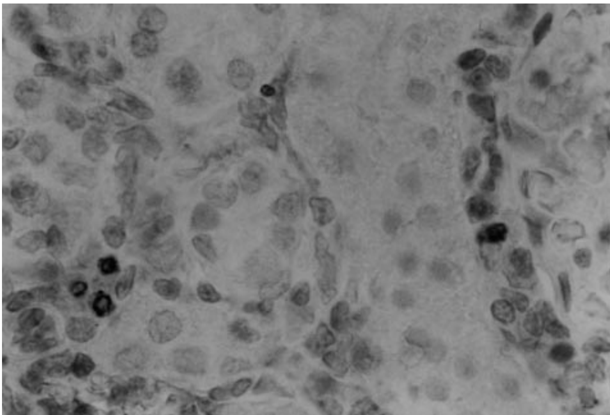

Fig. 3. Photomicrograph (original magnification ×400) of double immunostain for ED1 and apoptosis (ISEL) in ADR treated rat kidney. Note the presence of numerous interstitial ED1+ cells (monocytes/macrophages) with many showing apoptotic nuclei.

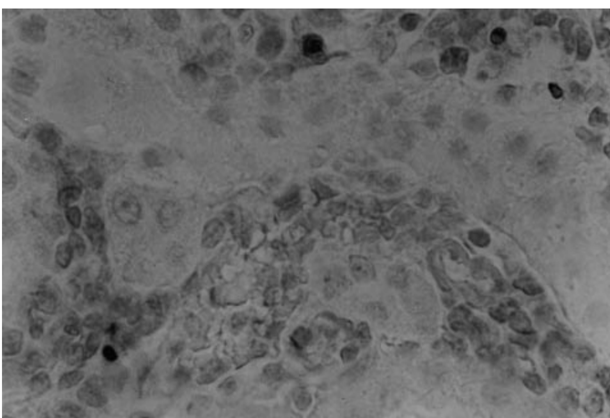

Fig. 4. Photomicrograph (original magnification ×400) of double immunostain for myofibroblasts (α-SMA+ cells) and apoptosis (ISEL) in ADR treated rat kidney. Note the separate staining of myofibroblasts and apoptotic cells.

Discussion

In this study, we have attempted to define the role of the NO pathway and its relationship with apoptosis in the acute phase of experimental ADR-nephropathy. ADR-induced nephrosis serves as an experimental model for idiopathic nephrotic syndrome with proteinuria reaching the maximum at 21 days [14]. The rats had increased urine nitrite excretion at the time of marked proteinuria at 21 days. Proteinuria was markedly decreased in the rats treated with AG, along with a decreased nitrite production. This was suggestive that increased production of NO might be responsible for proteinuria. Increased amounts of NO production may be expected to contribute to proteinuria through a number of mechanisms. Cattel [6] has suggested that NO may be damaging glomerular basement membrane through complexing with structural proteins. The damaged membrane would thus be expected to spill proteins. Overproduction of NO may also be contributing to proteinuria by enhancing glomerular damage through interaction with superoxide anion [5].

On the other hand proteinuria *per se*, has a direct effect on cellular infiltration, as mentioned above. Protein overload has been suggested to induce functional alterations of tubular cells, overexpressing pro-inflammatory mediators [16]. In the presented study, the tubulointerstitial inflammation of the rats corresponded to the time of overt proteinuria. Thus, one may speculate that the attenuation of interstitial inflammation is all secondary to the inhibition of proteinuria by the medication. However, AG is a well-known inhibitor of iNOS. Thus, we may suggest that the inhibition of NO has suppressed the inflammatory response. In fact in animals treated with AG both the number of cells and the double-stained cells representing the macrophage/monocytes, have significantly decreased. This finding may imply that AG has inhibited infiltration of the macrophage series. On the other hand, AG also has NO-independent effects which could contribute to the inhibition of inflammation, such as inhibition of AGE formation [17].

Our histopathological findings showed mild but clear tubulointerstitial changes with cellular infiltrates, by light microscopical examination. Interstitial inflammation maybe an important determinant of the outcome of glomerular inflammation [18,19]. In a number of studies interstitial inflammatory cells were considered to be more important to renal function than cells in the glomeruli. Goumenos *et al.* [19] have suggested that myofibroblasts of the interstitium may be playing

a crucial role in the pathogenesis of fibrosis in glomerular diseases. The tubulointerstitial cellular response and the localization of apoptotic changes in our rats with nephrosis emphasizes the importance of the tubulointerstitium in nephropathies [18]. It has been suggested that the interstitial changes are mainly produced by downstream diffusion of certain inflammatory mediators [18]. NO may well be one of the mediators concerned. An important feature of this study was the significant increase in interstitial cells and PCNA staining, along with the increased urine nitrite levels in the diseased animals, and there was a decrease in these cells after AG was administered. Furthermore, ED1 stained cells have also decreased after AG treatment. AG is a relatively specific inhibitor of iNOS, which is responsible of production of pathological amounts of NO. The alterations in the renal vascular responses further confirm the increased production of NO in this model. We suggest that these findings may be indicating the role of NO in the process.

The inhibition of NO production through AG has led to a decrease in the number of the cells in the interstitial area, along with a decline in urine nitrite levels and change in renal vascular bed responses. Cattel [6] has suggested that blood borne macrophages were the major source of glomerular NO. However, the role of intrinsic glomerular cells cannot be excluded, either acting subsequently or concomitantly. A limitation of our study was that we are unable to comment on whether the source of iNOS was resident mesangial cells or just infiltrating macrophages. Further immunohistochemical studies with iNOS staining would be required to clarify this point.

Physiological levels of NO, produced by constitutive NOS have a crucial role in the maintenance of renal haemodynamics [5]. NO has beneficial effects relating to its vasodilator and antithrombogenic properties. However, large amounts of NO, produced by iNOS, have been associated with inflammation and NO levels have correlated with the presence of infiltrating macrophages [6]. Weinberg *et al.* [20] have shown greater NO production and urine nitrite excretion to be associated with glomerulonephritis in their experimental model. On the other hand contradicting reports are present, assigning NO a beneficial role in the pathophysiology of glomerular diseases as well [6].

To evaluate the role of NO in the renal vascular changes induced by ADR-nephropathy, isolated perfused rat kidney was used. Renal vascular bed responses have not been previously studied in nephrotic syndrome. We have observed clear changes in the renal vascular bed, which we have interpreted to be due to excess NO production. The endothelium-dependent vasodilatory response elicited by acetylcholine was significantly attenuated in the ADR-nephropathy group whereas papaverin and sodium nitroprusside-induced vasodilatory responses that are independent of endothelium were preserved. NO produced in large amounts has been suggested to cause the down-regulation of endothelial cNOS or has direct cytotoxic effects on the endothelium [7]. Thus, the induction of iNOS in renal vascular bed may explain the impairment of endothelial response in our experimental nephropathy. The protection of the acetylcholine response by AG treatment supports this proposal and reflects its selective inhibitory potency on iNOS. The attenuated phenylephrine-induced contractile response in the ADR-nephropathy group and its prevention by AG treatment further suggests the increased production of NO in the renal vascular bed. Thus, the renal vascular reactivity studies have validated the effect of NO produced by iNOS, in ADR-nephropathy.

Another adverse effect of NO maybe through inducing cytotoxicity and DNA damage. The interaction of NO with superoxide anion generates peroxinitrite, which induces lipid peroxidation and cytotoxicity [5]. iNOS mediated activation of endonuclease is suggested to play a role into nucleosomal fragments and apoptosis [7]. Again NO-induced inactivation of glutathione peroxidase and oxidative stress may also explain the cytotoxic and apoptotic effects of NO [7]. The association of NO production and apoptosis has been previously demonstrated in a number of cell types such as cortical neurons and vascular smooth muscle cells [8]. Wang *et al.* [7] have recently demonstrated this relationship in the model of human lupus nephritis as well. The authors have suggested that induction of iNOS, detected by immunohistochemical staining, played a role in the occurrence of apoptosis in the glomeruli of lupus nephritis. We have shown a relationship between iNOS and apoptosis in an experimental kidney disease as well. The association of NO pathway and apoptosis was strengthened by the disappearance of apoptosis in rats treated with AG.

Apoptosis has contradictory effects in kidney diseases. Apoptosis has been assigned a beneficial role in proliferative glomerulopathy induced by anti-Thy-1.1 antibody administration; apoptosis has been suggested to be necessary for regulating the number of intrinsic endothelial cells and for removing the 'unwanted cells' of inflammation [10,11]. On the other hand, Makino *et al.* [12] have observed TUNEL-positive cells mainly in the sclerotic lesions. Again the role of apoptosis in sclerosis was evident in the rat remnant kidney model [12]. Thus, although apoptosis may initially be a regulatory mechanism, 'uncontrolled' apoptosis seems to be involved in the sclerotic process. In our study model the late phase has not been studied since association with sclerosis was not an aim of this study. However, we have identified the significance of apoptosis in the acute phase of this experimental nephrosis and its association with the NO pathway. An important feature of apoptosis in this study was the tubulo-interstitial localization of the TUNEL-positive cells. Double staining revealed that these cells were negative for α-SMA, which characterizes myofibroblasts. Thus, apoptosis was evident in the infiltrating cells in this disease model and may be expected to serve a beneficial role. On the other hand since apoptotic cells have outgrown the proliferative response, it may be speculated that fibrosis may be favoured. Thus, late phase assessments of adriamycin nephrosis are

necessary to comment on the prognostic role of apoptosis in this disease model.

We suggest that marked NO production maybe important in the pathogenesis of ADR-nephropathy. Animals with ADR-nephropathy presented with increased nitrite levels and evidence of increased NO production in the renal vasculature along with (i) proteinuria, reflecting the clinical disease activity; (ii) interstitial cell increment, reflecting inflammation; and (iii) apoptosis. These effects including cellular infiltration, were suppressed with the administration of an iNOS inhibitor, AG, which is suggestive of the role of NO in the aforementioned features. The double staining of the inflammatory cells suggested to us that the increased cells undergoing apoptosis were mainly blood-borne. Further studies are needed to enlighten the localization of iNOS formation.

Acknowledgement. This study was partially supported by a grant from 'The Scientific and Technical Research Council of Turkey', project no. SBAG-AYD-165.

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Received for publication: 6.5.99
Accepted in revised form: 16.6.00