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Nitric oxide protection against adriamycin-induced tubulointerstitial injury

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Abstract

It is well known that oxidative stress is related to the pathogenesis of adriamycin (ADR) nephropathy. However, it is unclear how nitric oxide (NO) is associated with the pathophysiological process after ADR administration. The NO level in a kidney homogenate was assayed by electron paramagnetic resonance (EPR) spectrometry using a direct *in vivo* NO trapping technique after ADR administration. N-(3-(aminomethyl)benzyl)acetamidine (1400W) was used as a specific, inducible nitric oxide synthase (iNOS) inhibitor. The levels of NO after ADR administration gradually increased for 6 h and then decreased until 24 h after ADR administration. The fractional excretion of Na (FE_{Na}) in the urine was elevated in the ADR group on day 1. Pre-treatment of the animals with 1400W attenuated the increase in NO levels despite further elevation of FE_{Na}. These findings suggest that iNOS-derived NO does not produce a harmful effect but rather protects the ADR-treated kidney against sodium excretion.

Keywords: *Nitric oxide, adriamycin-induced sodium excretion, electron paramagnetic resonance, in vivo spin trapping technique, N-(3-(aminomethyl)benzyl)acetamidine (1400W).*

Introduction

Adriamycin (ADR), an anthracycline antibiotic, is one of the most effective anti-tumour agents used to treat human malignancies. However, treatment of rats with ADR also induces nephrotic syndrome initially, followed by chronic renal dysfunction due to glomerulosclerosis. Many mechanisms—including those involving free radical production—have been proposed to explain the ADR-induced renal injury. Thus, several *in vitro* studies have shown that reactive oxygen species (ROS) are produced by ADR treatment. Because of its quinone structure, ADR can produce ROS by a one-electron reduction reaction catalysed by several endogenous enzymes, including cytochrome P450 [1] and NADH dehydrogenase [2]. Recently it was reported that reactive nitrogen oxide species act as redox messengers activated by oxidative stress [3].

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Nitric oxide (NO) is a small signalling molecule regulating a variety of diverse cellular functions including many physiological processes that range from regulation of vascular tonus to neuronal transmission and from apoptosis to inflammation. Most of the physiological actions are mediated by NO generated by constitutive isoforms of nitric oxide synthase (cNOS). On the other hand, NO produced by inducible nitric oxide synthase (iNOS) can be a cytotoxic agent, especially when present in large amounts for long periods of time [4–7]. In addition, it has been shown that various functions of three NOS isoforms are profoundly influenced by ADR treatment [8–12]. Administration of ADR caused endothelial nitric oxide synthase (eNOS)-dependent generation of superoxide, leading to acute endothelial dysfunction [8]. The increased generation of superoxide was followed by suppression of NO formation, implying that ADR inhibits the eNOS activity [9]. It was demonstrated that the reductase domain of neuronal nitric oxide synthase (nNOS) catalyses the one-electron reduction of ADR with a fairly low redox potential [10]. Moreover, ADR treatment affects iNOS induction and its mRNA expression [11,12]. Administration of 4′-epi-adriamycin to rats strongly inhibited the iNOS induction in lung, ascites and bone marrow previously induced by lipopolysaccharide [11]. The increased expression of iNOS mRNA and protein by IFN-gamma/IL-1beta in colorectal cancer cells, DLD-1, were completely blocked by ADR [12].

There are two conflicting reports on iNOS roles in ADR-induced nephropathy. One showed that over-production of NO derived from iNOS might contribute to proteinuria by enhancing glomerular damage through interaction with the superoxide anion [13]. In the other report, it was found that endogenous iNOS-derived NO played a protective role against tubulointerstitial injury and cytokine production in ADR-induced nephropathy [14]. It is a question whether iNOS is induced by ADR in the kidney or whether the amount of NO produced by iNOS influences the pathophysiological process in ADR-induced nephropathy. We have a tool to directly measure NO production in tissue, which is electron paramagnetic resonance (EPR) spectrometry combined with a spin trapping technique (EPR NO trapping technique) [15–18]. To answer the question, we have set up a rat model on the first day after ADR administration and measured NO production in the kidney using an *in vivo* EPR NO trapping technique with an iron-diethyldithiocarbamate (Fe-DETC) complex as an NO trapping agent [19–21]. To inhibit the effect of iNOS, we used the iNOS specific inhibitor, N-(3-(aminomethyl)benzyl)acetamidine (1400W) [22–24] and we confirmed the expression of the iNOS protein in the kidney by an immunohistochemical technique.

Materials and methods

Animal preparations

Male Wistar rats, weighing 180–220 g, were used in all the experiments. The animals were kept in individual cages at a controlled temperature (23°C) on a 12-h light and dark cycle. For 24 h before sacrifice, they were deprived of food but allowed free access to tap water. All procedures related to animal care described herein were in accordance with the criteria outlined in the Guideline for Animal Experimentation prepared by the Japanese Association for Laboratory Animal Science, 1987.

The rats were divided into control (18 animals), ADR (20 animals) and ADR + 1400W (6 animals) groups. The rats of the ADR group were administered ADR (5 mg/kg b.wt; Wako Pure Chemical Industries, Osaka, Japan) dissolved in 1 ml of 0.9% NaCl aqueous solution (saline) intravenously and the control group received an identical volume of saline. An iNOS-selective inhibitor, 1400W (5 mg/kg in saline; Cayman Chemical, Ann Arbor, MI), was administered intravenously to the rats 30 min before ADR and the effect of the inhibitor on NO generation was examined 6 h after the ADR administration.

Direct measurement of NO by EPR spectrometry

The NO produced in the kidneys of the rats was measured by using an NO trapping technique combined with EPR spectrometry [21]. This technique is a method for analysing NO production directly both *in vivo* and *in vitro*. Five rats were killed at each time point, 4, 6, 8 and 24 h after the ADR treatments and six rats were killed at 0, 6 and 24 h after the saline administration and 6 h after ADR + 1400W. Their kidneys were removed so that NO in their tissues could be measured by EPR spectrometry. We used Fe-DETC complex as an NO-trapping reagent to quantify NO levels in a kidney from each of the rats. A DETC·$3H_2O$ solution (400 mg/kg; Aldrich Chemical Co., Milwaukee, WI) and a Fe-citrate mixture (40 mg/kg of $FeSO_4 \cdot 7H_2O$ and 200 mg/ml of sodium citrate; Wako Pure Chemicals, Osaka, Japan) were injected intraperitoneally and subcutaneously, respectively. The Fe-DETC complex, thus formed, traps endogenously produced NO to yield an NO-Fe-DETC complex. Thirty minutes after the trapping agent was injected, the kidney was removed under deep anaesthesia. The matrix from cortex to medulla was cut and minced. Each sample, drawn by a 1 ml plastic syringe, was collected with a glass capillary tube (75 mm in length; 46-μl inside volume) and transferred into a quartz tube (outer diameter, 5 mm). EPR spectra were recorded at ambient temperature with a spectrometer (TE-200; JEOL, Tokyo, Japan). The instrument settings were as follows: Centre field, 331 mT; field scan, 4 mT; sweep

time, 4 min; time constant, 0.3 s; modulation amplitude, 0.32 mT; modulation frequency, 100 KHz; microwave power, 60 mW; microwave frequency, ~9.5 GHz. The amplitude of the signal, which was proportional to the amount of NO, was obtained by measuring the peak-to-peak height of the lower field side signal in a three-line spectrum that is characteristic of an NO adduct. The NO adduct concentration of the Fe-DETC complex was estimated by comparison with the signal height of a standard solution of a chemically synthesized NO complex. The concentration in tissues, estimated at 30 min after the injection of the NO trapping reagent, was expressed in nanomoles per gram of tissue per 30 min.

Measurement of urinary 8-OHdG

Two-millilitre samples of well-mixed 24 h urine were collected from the rats. The samples were deoxygenated by bubbling 100% nitrogen gas through them for 5 min at room temperature to prevent the artificial formation of 8-OHdG and stored frozen at -80°C until analysed. Urine samples were centrifuged at 1000 g for 40 min and, after proper dilution, the supernatants were used for the determination of 8-OHdG by a competitive enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Japan).

Immunohistochemistry

Dissected kidneys were fixed in 10% buffered formalin and embedded in paraffin. Serial sections cut from paraffin blocks were mounted on glass slides, dewaxed and dehydrated in xylene and washed in alcohol and phosphate-buffered saline (PBS). The sections were then incubated in 3% hydrogen peroxide for 15 min to block endogenous peroxidase. Sections were then incubated overnight at 4°C with polyclonal antibodies against iNOS (NOS2, M-19; Santa Cruz Biotechnology, Santa Cruz, CA). After washing with PBS and tap water for 15 min, they were incubated in HISTOFINE simplestain MAX-PO(R) (Nichirei, Tokyo, Japan) for 30 min, after which they were washed in PBS three times followed by diaminobenzine tetrahydrochloride for 2 min. The sections were then rinsed with deionized and filtered tap water. To ensure the specificity of the immunohistochemical staining, one specimen was processed without the primary antibody.

Miscellaneous tests

Urine was collected for 24 h. Urine volume, plasma and urine concentrations of creatinine and sodium were measured using standard laboratory methods. Fractional excretions of sodium were calculated by dividing their respective clearances by the creatinine clearance. Urinary NOx (nitrite plus nitrate), the oxidative metabolites of NO, were measured by the Griess reaction. The assay for urinary N-acetyl-β-D-glucosaminidase (NAG) activity was based on the enzymatic hydrolysis of sodio-3, 3′-dichlorophenol-sulphonphthaleinyl N-acetyl-β-D-glucosaminide and the subsequent detection of liberated chlorophenol red was conducted at 575 nm by spectrophotometry.

Statistical analyses

Data are expressed as the mean $\pm$ SD of the values in each group. Statistical analyses were performed using one-way analysis of variance followed by the Bonferroni correction for multiple comparisons and Student's *t*-test as appropriate. *P*-values less than 0.05 were considered significant.

Results

In vivo *NO level in the kidney tissue*

NO produced in the kidneys of rats were measured 4, 6, 8 and 24 h after ADR and 0, 6 and 24 h after saline administration. NO levels in nanomoles per gram of tissue per 30 min increased gradually after ADR administration, reached a maximum at 6 h and then decreased thereafter (Figure 1A). The NO levels were almost the same after saline administration. The increase in NO levels 6 h after ADR administration was significantly suppressed by pre-administration of a selective iNOS inhibitor, 1400W (Figure 1B).

FE_{Na} and NAG excretion

The ADR group exhibited a significantly higher FE_{Na} than did the control group on day 1. Furthermore, FE_{Na} was significantly higher in the ADR + 1400W group than in the ADR group on day 1 (Figure 1C). Urinary NAG excretion obtained from the 24 h urine in the ADR group was significantly higher than that in the control group on day 1, but not significantly different from that in the ADR + 1400W group (Figure 1D).

Urinary 8-OHdG, NOx and urine volume

Urinary 8-OHdG (Figure 2A), urine volume (Figure 2B) and NOx (Figure 2C) were increased in the ADR group compared to the values in the control group on day 1 and all differences were statistically significant.

Renal function

Creatinine clearance (Figure 2D) in rats receiving ADR was decreased compared to that of controls (183.15 ± 31.72 ml/min vs 207.36 ± 49.97 ml/min) on day 1, but the difference was not statistically significant.

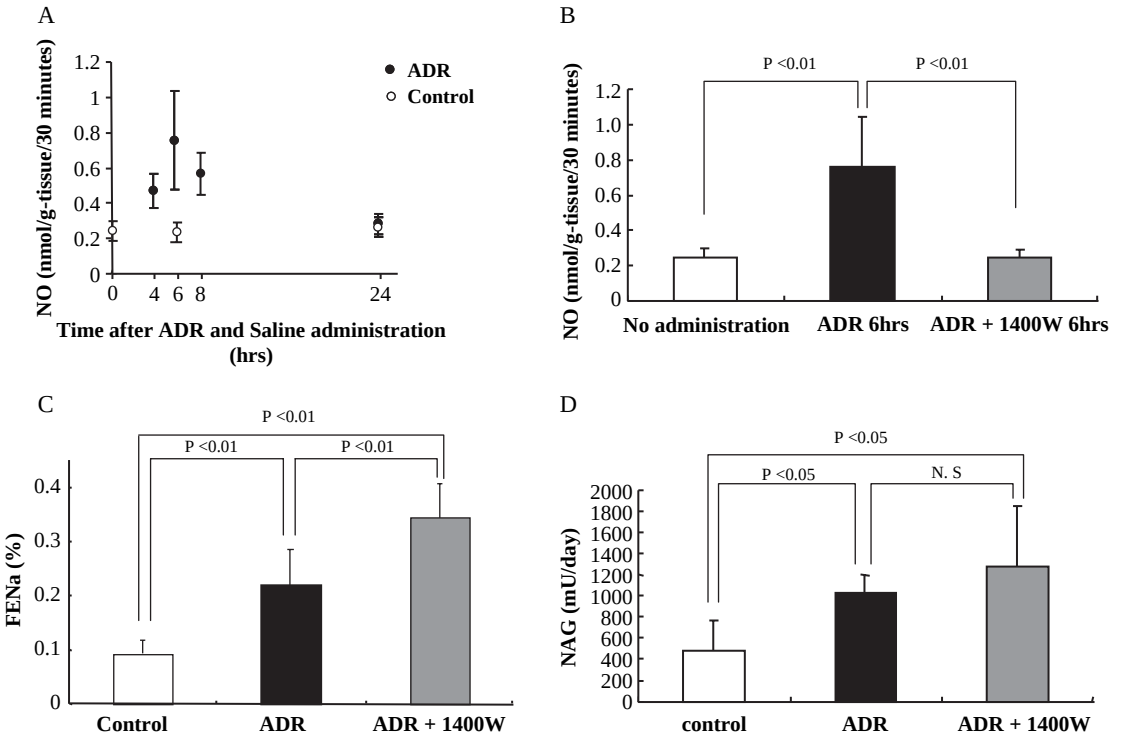

Figure 1. (A) Time course of NO concentration in the kidney after ADR and saline administrations. (B) Effect of the iNOS-specific inhibitor 1400W on the nitric oxide concentration in the kidney. (C) Fractional excretion of sodium (FE_{Na}). (D) NAG in the ADR and control groups.

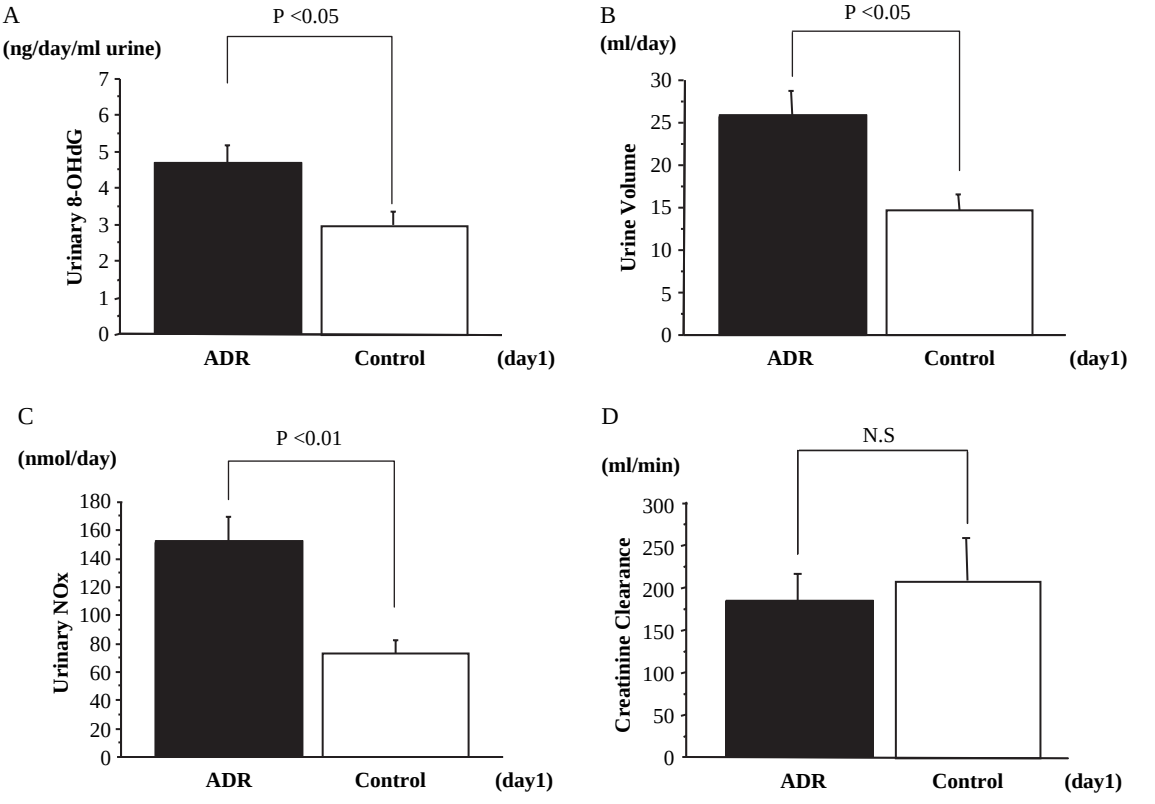

Figure 2. Summary of renal function and urinary oxidants on the first day after ADR and saline administration. (A) Urinary 8-OHdG. (B) Urine volume. (C) Urinary NOx. (D) Creatinine clearance.

Renal iNOS expression after ADR administration

Administration of ADR induced an increase in expression of iNOS in the proximal intraepithelial and apical side of the cells compared to that of saline-treated control rats (Figures 3A and B). Pre-administration of 1400W did not modify iNOS expression 6 h after ADR administration (Figure 3C).

Discussion

Our results show the following: (1) Urinary 8-OHdG, urinary NOx and urine volume increased significantly on the first day after ADR administration compared to the values in control animals, (2) there was a slightly increased NO production in the kidney after ADR administration, as shown by an EPR NO trapping technique, (3) the specific iNOS inhibitor, 1400W, inhibited the increase in NO level in the kidney after ADR administration without normalizing the NAG and FE_{Na} levels, (4) there was a greater elevation of the FE_{Na} level in the ADR + 1400W group than in the ADR-alone group and (5) iNOS was expressed in the renal cortex 6 h after ADR and ADR + 1400W administration, as proved by immunohistochemistry, which revealed staining of the intraepithelial cells on their apical sides along the proximal tubules.

The EPR NO trapping technique enables one to conduct *in vitro* and *in vivo* NO measurements in biological systems [25–28]. At present, NO trapping agents applicable to direct NO assay or *in vivo* NO measurements are limited to iron-dithiocarbamate (Fe-DTC) complexes. Several Fe-DTCs that selectively react with and trap NO have been developed and applied to biological NO measurements. Among them, the Fe-DETC complex is essentially insoluble in water but is lipid-soluble and able to travel through membranes [29,30], so it is suitable for the detection of intracellular and intramembrane NO in the kidney tissues after ADR administration. Thus, EPR signals observed in the kidney appear to be due to NO originating in the kidney alone.

The application of iNOS inhibitors with different selectivity has produced controversial results in several studies [31–33]. An iNOS inhibitor, 1400W, has been demonstrated to have markedly high selectivity for iNOS and to be a useful tool in understanding the role of iNOS [22]. In this study, accordingly, we used 1400W as a selective iNOS inhibitor.

Most physiological actions ranging from vascular tonus to neuronal transmission are mediated by a small amount of NO generated by constitutive NOS. On the other hand, when produced by iNOS, in large amounts for long periods of time, NO can be a cytotoxic agent [4–7]. Thus, we have realized the role of NO produced in tissues and organs by identifying the isoforms of NOS but not by measuring the amount of NO produced, because it could not easily be measured directly *in vivo*. Recently it was reported that iNOS-derived NO has a protective role against tubulointerstitial injury and cytokine production in ADR nephropathy [14]. Additionally, it was shown that iNOS-derived NO is an important molecule in the protection of cardiomyocytes from ADR-induced mitochondrial toxicity [34]. Both reports showed that

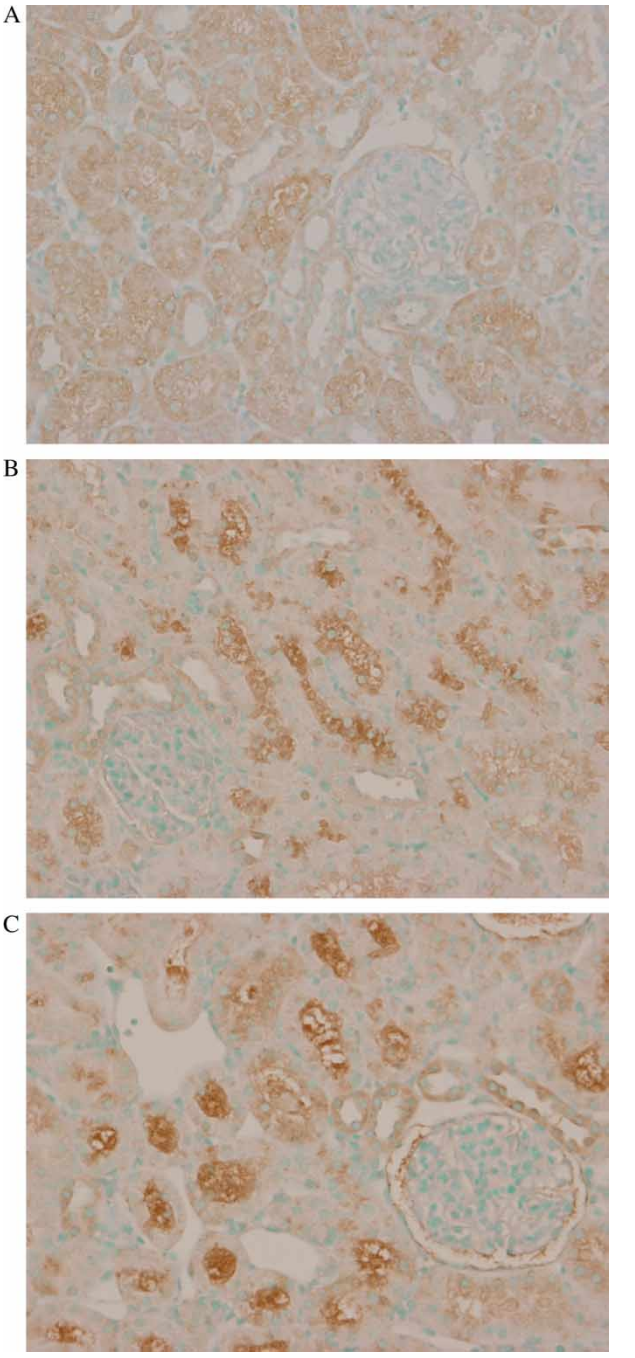

Figure 3. Immunohistochemistry of iNOS protein in the renal cortex (original magnification × 200). (A) Immunostaining for iNOS 6 h after exposure to saline. (B) ADR treatment. (C) ADR treatment plus 1400W. A stronger positive immunostaining was seen along the apical side at the proximal tubules than on the intraepithelial cells in the ADR-treated and ADR-treated plus 1400W groups.

iNOS-derived NO protects against cytokine injury and oxidative stress under physiological conditions. However, the amounts of NO produced in the kidney and cardiomyocytes were not evaluated in these reports. Using the EPR NO trapping technique, we confirmed that the concentration of NO was sensitively altered in response to inactivation of iNOS and that attenuation of elevated NO levels with a specific iNOS inhibitor, 1400W, increased ADR-induced renal injury. These findings demonstrate that iNOS-derived NO in our model plays a protective role. In ADR treatment, the increase in urinary 8-OHdG, a marker of oxidation, in response to higher levels of NAG and FE_{Na} would reflect acute renal injury due to hydroxyl radicals derived from ADR, suggesting the participation of oxidative stress in the nephrotoxicity similar to that induced by mercuric chloride [35]. In addition, oxygen radicals (probably superoxide anion) formed from ADR may react with NO to reduce its concentration and consequently exacerbate tubulointerstitial injury. We recently proved, using *in vivo* EPR spectroscopy, that renal reducing ability declined on day 7 after ADR administration [36]. Treatment with antioxidative drugs during the early phase after ADR administration improved renal reducing ability on day 7 [37], indicating that the kidney suffered from continuous oxidative stress resulting from an imbalance between the production of oxidants and the respective defense systems of an organism. The renal reducing ability was not impaired on the first day after ADR administration (data not shown) and urinary 8-OHdG increased, indicating that the kidney suffered oxidant-dominant oxidative stress. Altogether, adequate and physiological amounts of NO generated in the proximal tubules by ADR-induced oxidative stress may have a protective effect under physiological conditions. It is likely that NO might have been a regulator of ADR-induced oxidative stress and would never exacerbate tubulointerstitial injury at all.

The expression of iNOS in the kidney after ADR administration has been found in the renal cortex of a rat model of unilateral nephrectomy, in which the increased expression was observed 6 weeks after ADR administration [38]. In this study, the induction of iNOS could be observed in the proximal epithelial cells in an early phase after ADR administration by means of immunohistochemistry and the production of NO in the kidney could be detected by EPR NO trapping technique. We propose as a possible mechanism for iNOS induction in the proximal tubule that ADR-derived oxidative stress to the vascular endothelium leads to poor circulation and/or hypoxia, which causes the production of inflammatory cytokines. Further studies will be required to elucidate this mechanism.

Intrarenal NO serves as a major regulator of sodium and water excretion and functions as an endogenous diuretic [39–41]. It was reported that NO produced by iNOS in tubular epithelial cells promotes natriuresis by inhibiting sodium reabsorption [42]. It is noteworthy that iNOS-derived NO has been shown to markedly reduce Na-K-ATPase activity in cultured thick ascending limb cells [43]. In this study, however, FE_{Na} in the ADR + 1400W group was more elevated than that in the ADR alone group. Thus it is probable that NO produced in our model contributes more to the neutralization of ADR-induced superoxide production than it functions as an endogenous diuretic. Therefore, such elevation in FE_{Na} in the presence of iNOS inhibitor may be due to ADR-induced oxidative injury to the tubulointerstitial area.

Interestingly, the immunohistochemistry revealed a stronger positive immunostaining for iNOS on the apical side of the proximal tubular cells than on the epithelial cells 6 h after ADR administration. In another animal model, the spontaneously hypertensive rat kidney, it was reported that immunostaining was seen in the proximal tubules of the cortex and in the medullary collecting ducts [44] but not on the apical side of the epithelial cells. It is likely that the expression of iNOS in the brush border area increases under stimulation by ADR. This iNOS may be partly responsible for the lack of ADR accumulation in the proximal tubules treated with ADR [45]. In addition, LPS-induced iNOS has been shown to be expressed along the brush border of the intestinal mucosa [46] and it has been reported that iNOS is constitutively expressed in the villous epithelium and that iNOS-derived NO is a key mediator of early villous reepithelialization following acute mucosal injury [47]. Until now, little attention has been given to the protective role of iNOS in acute renal injury in the renal tubules.

In summary, we examined the involvement of oxidative stress and iNOS induction in the pathogenesis of ADR-nephropathy. The increased urinary 8-OHdG, NAG, FE_{Na} and urine volume after ADR administration demonstrated that the kidney suffers from oxidative stress. Pre-treatment with 1400W (an iNOS inhibitor) attenuated the NO production in the kidney and increased FE_{Na}, indicating that iNOS-derived NO protects tubular epithelial cells after ADR administration. Moreover, we proved the induction of iNOS in the proximal tubules by immunohistochemistry and the production of NO in the kidney by EPR spectrometry combined with an NO spin trapping technique at an early phase after ADR administration. We confirmed that the amount of NO produced as well as the particular isoform of NOS are pivotal factors in the pathophysiological effect of NO.

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