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GW274150, a potent and highly selective inhibitor of iNOS, reduces experimental renal ischemia/reperfusion injury

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GW274150, a potent and highly selective inhibitor of iNOS, reduces experimental renal ischemia/reperfusion injury.

Background. Generation of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) may contribute to renal ischemia/reperfusion (I/R) injury. The aim of this study was to investigate the effects of GW274150, a novel, highly selective, potent and long-acting inhibitor of iNOS activity in rat and mouse models of renal I/R.

Methods. Rats were administered GW274150 (5 mg/kg intravenous bolus administered 30 minutes prior to I/R) and subjected to bilateral renal ischemia (45 minutes) followed by reperfusion (6 hours). Serum and urinary indicators of renal dysfunction, tubular and reperfusion injury were measured, specifically, serum urea, creatinine, aspartate aminotransferase (AST) and *N*-acetyl-β-D-glucosaminidase (NAG) enzymuria. In addition, renal sections were used for histologic scoring of renal injury and for immunologic evidence of nitrotyrosine formation and poly [adenosine diphosphate (ADP)-ribose] (PAR). Nitrate levels were measured in rat plasma using the Griess assay. Mice (wild-type, administered 5 mg/kg GW274150, and iNOS−/−) were subjected to bilateral renal ischemia (30 minutes) followed by reperfusion (24 hours) after which renal dysfunction (serum urea, creatinine), renal myeloperoxidase (MPO) activity and malondialdehyde (MDA) levels were measured.

Results. GW274150, administered prior to I/R, significantly reduced serum urea, serum creatinine, AST, and NAG indicating reduction of renal dysfunction and injury caused by I/R. GW274150 reduced histologic evidence of tubular injury and markedly reduced immunohistochemical evidence of nitrotyrosine and PAR formation, indicating reduced peroxynitrite formation and poly (ADP-ribose) polymerase (PARP) activation, respectively. GW274150 abolished the rise in the plasma levels of nitrate (indicating reduced NO production). GW274150 also reduced the renal dysfunction in wild-type mice to levels similar to that observed in iNOS−/− mice subjected to I/R. Renal MPO activity and MDA levels were significantly reduced in wild-type mice administered GW274150 and iNOS−/− mice subjected to renal I/R, indicating reduced polymorphonuclear leukocyte (PMN) infiltration and lipid peroxidation.

Conclusions. These results suggest that (*1*) an enhanced formation of NO by iNOS contributes to the pathophysiology of renal I/R injury and (*2*) GW274150 reduces I/R injury of the kidney. We propose that selective inhibitors of iNOS activity may be useful against renal dysfunction and injury associated with I/R of the kidney.

Key words: rat, mouse, kidney, reperfusion injury, renal dysfunction, tubular injury, nitric oxide, inducible nitric oxide synthase, poly (ADP-ribose) polymerase, peroxynitrite, myeloperoxidase, malondialdehyde, oxidative stress.

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Nitric oxide (NO), generated by any one of the three isoforms of nitric oxide synthase (NOS) plays an important role in renal function, both under normal and pathophysiologic conditions [1, 2]. Although NO appears to play an important functional role within the proximal tubule, it can also contribute to pathophysiology of this part of the kidney [3, 4]. All three isoforms of NOS have been located in the kidney; the constitutive isoform [endothelial NOS (eNOS)] and neuronal NOS (nNOS)] isoforms have been identified in the renal vasculature and macula densa, respectively [1, 2] and inducible NOS (iNOS) can be located and induced in several renal cell types (e.g., in glomerular mesangial and proximal tubular cells) by cytokines, lipopolysaccharide (LPS), and during renal ischemia/reperfusion (I/R) [2–6], leading to renal cell injury [7]. Recently, it has been suggested that an imbalance between the expression and activity of iNOS and eNOS is an important contributor to the pathophysiology of acute renal failure (ARF) [8] and there is good evidence that NO is implicated in renal reperfusion-injury [6, 9, 10]. Several in vivo and in vitro investigations have demonstrated how inhibition of the expression or

activity of iNOS, or absence of iNOS itself, can ameliorate or prevent renal I/R injury or hypoxic injury to the proximal tubules demonstrating that NO, generated by iNOS, contributes to renal I/R injury [5, 6, 11–15]. Furthermore, it is now clear that the production of reactive oxygen species, such as hydrogen peroxide, superoxide and hydroxyl radicals, contribute significantly to renal I/R injury (and associated ARF) [16, 17]. Traditionally, reactive oxygen species have been considered to exert their effects through a direct toxic action on target cells, for example, reactive oxygen species cause DNA damage during renal I/R and oxidative stress [18] leading to the activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP), depletion of nicotinamide adenine dinucleotide (NAD^+), adenosine triphosphate (ATP), and ultimately cell death [19, 20]. Thus, various antioxidant strategies, such as the use of tempol or desferrioxamine have been shown to provide beneficial actions against renal dysfunction and injury mediated by I/R of the kidney [21]. However, reactive oxygen species can also cause indirect injury to cells. Superoxide anions react with NO to form peroxynitrite [22], which causes injury via protein tyrosine nitration [23] or via oxidant injury subsequent to its decomposition into hydroxyl radical and nitrous oxide [23, 24]. Specifically in the kidney, peroxynitrite generation has been implicated in the pathophysiology of renal I/R injury [6–8, 15].

Early studies have demonstrated that inhibition of all isoforms of NOS using nonselective NOS inhibitors such as N^G-monomethyl L-arginine (L-NMMA) causes a concomitant inhibition of eNOS activity, causing excessive vasoconstriction and hence, exacerbates renal ischemia, microvascular thrombosis and mortality [25]. This is supported by the finding that N^G-nitro-L-arginine methyl ester (L-NAME), a relatively selective inhibitor of eNOS activity, augments the degree of kidney injury (glomerular thrombosis) and reduces renal perfusion in a rodent model of endotoxic shock [26]. We have recently reported that L-N^6-(1-iminoethyl)lysine (L-NIL) and aminoethyl-isothiourea (AE-ITU) can significantly reduce renal dysfunction and injury caused by I/R of the rat kidney [6]. However, although both L-NIL and AE-ITU are relatively selective inhibitors of iNOS activity [27–29], recent studies have suggested that L-NIL may lose its isoform selectivity at higher doses [15] and that AE-ITU can exert nonspecific effects [30], including reduction of the formation of reactive oxygen species by activated polymorphonuclear leukocytes (PMNs), inhibition of cyclooxygenase activity and attenuation endothelin-1 formation to name but a few [30–32]. All of these pharmacologic effects may contribute to the anti-ischemic effects of AE-ITU in vivo.

GW274150 ([S]-2-amino-[1-iminoethylamino]-5-thioheptanoic acid) has recently been identified as a novel, highly selective, potent and long-acting inhibitor of iNOS activity [29, 33]. GW274150 is a sulfur-substituted acetamine amino acid derivative of L-lysine which, like 1400W, has a very high degree of selectivity for iNOS versus both eNOS (>250-fold) and nNOS (>80-fold) [29, 33]. As with 1400W, inhibition of iNOS activity by GW274150 is nicotinamide adenine dinucleotide phosphate (NADPH)-dependent and develops slowly, whereas inhibition of eNOS and nNOS is rapidly reversible [29, 33] and efficacy and selectivity of GW274150 is maintained in intact cells and tissues. Initial studies using GW274150 indicate that unlike 1400W (and several other nonamino acid NOS inhibitors), GW274150 does not produce acute toxicity [29]. The half-life of GW274150 in the rat is 5 hours and 3 hours in mice and a single injection of this iNOS inhibitor (3 mg/kg) abolishes the increase in nitrite and nitrate caused by an injection of endotoxin, even if endotoxin is injected 14 hours after administration of GW274150 (abstract; Alderton W et al, *Acta Physiol Scand* 167:11, 1999). GW274150 did not have any effect on conscious mouse blood pressure at 100 mg/kg or on rat brain nitrate at 20 mg/kg indicating high selectivity for iNOS (abstract; Alderton W et al, *Acta Physiol Scand* 167:11, 1999).

Here we investigate the effects of GW274150 on the renal dysfunction and injury caused by I/R of the kidney of the rat and mouse in vivo. In order to ensure that an enhanced formation of NO by iNOS does indeed contribute to renal I/R injury, we have compared the degree of injury/dysfunction caused by I/R in either wild-type mice treated with GW274150 or in mice in which the gene for iNOS is absent (iNOS−/−).

METHODS

Renal I/R (rat model)

This study involved 42 male Wistar rats (Tuck, Rayleigh, Essex, U.K.) weighing 220 to 330 g. Rats received a standard diet and water ad libitum and were cared for in accordance with both the Home Office *Guidance in the Operation of the Animals (Scientific Procedures) Act 1986*, published by Her Majesty's Stationery Office, London, U.K. and the *Guiding Principles in the Care and Use of Animals* published by the American Physiological Society. Rats were subjected to bilateral renal ischemia for 45 minutes followed by reperfusion for 6 hours as described previously [20], during which time their mean arterial blood pressures (MAP) and heart rates were constantly monitored. Upon completion of surgical procedures, rats were randomly allocated into the following four groups: (*1*) *I/R-only group*: rats were subjected to renal ischemia for 45 minutes followed by reperfusion for 6 hours ($N = 12$); (*2*) *I/R + GW group*: rats were administered GW274150 (5 mg/kg intravenous bolus) 30 minutes prior to commencement of I/R ($N = 12$); (*3*) *Sham + saline group*: rats were subjected to identical surgical procedures except for renal I/R, and were main-

tained under anesthesia for the duration of the experiment (45 minutes + 6 hours, $N = 12$); and (*4*) *Sham + GW group*: rats were treated identical to sham-operated rats except for the administration of GW274150 (5 mg/kg intravenous bolus) 30 minutes prior to commencement of the experimental period (45 minutes + 6 hours, $N = 6$).

The time of administration and dose of GW274150 used were based on those previously shown by us to provide beneficial actions against renal dysfunction in a model of hemorrhagic shock in the rat (abstract; Izumi M et al, *Br J Pharmacol* 135:320P, 2002) and by others to reverse delayed gastrointestinal transit in a rat model of postoperative ileus (abstract; Alderton W et al, *Acta Physiol Scand* 167:11, 1999). All rats received an infusion of saline (2 mL/kg/hour) throughout the I/R period.

Renal I/R injury (mouse model)

In vivo mouse studies were carried out using 36 male wild-type (C57B6) or iNOS−/−(129SV/C57BL/6) mice weighing 15 to 25 g. iNOS−/− mice were kindly supplied by Professor Fons A.J. Van de Loo (Department of Rheumatology, University Hospital Nijmegen, Nijmegen, The Netherlands). In iNOS−/− mice, a neocassette using homologous recombination was used to replace the first four exons of the iNOS gene as previously described [34]. Mice were allowed access to food and water ad libitum and were cared for in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with the European Economic Community regulations (O.J. of E.C. L358/1 12/18/1986).

Mice were anesthetized using chloral hydrate (125 mg/kg, intraperitoneally) and core body temperature maintained at 37°C using a homoeothermic blanket. Mice were then divided into the following six groups: (*1*) *I/R-only group*: mice were subjected to renal ischemia (30 minutes) followed by reperfusion for 24 hours ($N = 6$); (*2*) *I/R + GW group*: mice were administered GW274150 (5 mg/kg intraperitoneal bolus) 30 minutes prior to commencement of I/R and after 12 hours of reperfusion ($N = 6$); (*3*) *I/R + iNOS−/− group*: iNOS knockout mice were administered GW274150 (5 mg/kg intraperitoneal bolus) 30 minutes prior to commencement of I/R and after 12 hours of reperfusion ($N = 6$); (*4*) *Sham + saline group*: mice were subjected to identical surgical procedures except for renal I/R ($N = 6$); (5) *Sham + GW group*: mice were treated identical sham-operated mice except for the administration of GW274150 (5 mg/kg intraperitoneal bolus) 30 minutes prior to commencing I/R and after 12 hours of reperfusion ($N = 6$); and (*6*) *sham + iNOS−/− group*: iNOS knockout mice were subjected to identical surgical procedures except for renal I/R ($N = 6$). Mice which did not receive GW274150 were administered 20 mL/kg saline (vehicle for GW274150) at equivalent time points (30 minutes before sham operation and after 12 hours).

After performing a midline laparotomy, mice from the I/R groups were subjected to bilateral renal ischemia for 30 minutes, during which the renal arteries and veins were occluded using microaneurysm clamps. The time of ischemia chosen was based on that found to maximize reproducibility of renal functional impairment while minimizing animal mortality in these animals, which were not administered fluid intravenously [35]. After the renal clamps were removed, the kidneys were observed for a further 5 minutes to ensure reflow, after which 1 mL saline at 37°C was injected into the abdomen and the incision was sutured in two layers. Mice were then returned to their cages where they were allowed to recover from anesthesia and observed for 24 hours. Sham-operated mice underwent identical surgical procedures to I/R mice except that microaneurysm clamps were not applied.

The time of administration and dose of GW274150 used were based on those previously reported by us to provide beneficial actions in a murine model of splanchnic I/R [36] and by others to abolish the increase in nitrite and nitrate caused by an injection of endotoxin (abstract; Alderton W et al, *Acta Physiol Scand* 167:11, 1999). Due to the shorter half-life of GW274150 in the mouse (3 hours) compared to the rat (5 hours), a further dose of GW274150 (5 mg/kg intraperitoneally) was administered to mice at the mid-point of the reperfusion period (12 hours after renal ischemia).

Measurement of biochemical parameters

At the end of the reperfusion period, 1 mL blood samples were collected from rats undergoing renal I/R via the carotid artery into tubes containing serum gel. The samples were centrifuged (6000 rpm for 3 minutes) to separate serum. All serum samples were analyzed for biochemical parameters within 24 hours after collection (Vetlab Services, Sussex, U.K.). Serum urea and creatinine concentrations were used as indicators of renal (glomerular) function [20]. Aspartate aminotransferase (AST), an enzyme located in the proximal tubule, was used as an indicator of reperfusion injury [21]. Urine samples were collected throughout the reperfusion period and the volume of urine produced recorded. Urine flow was calculated based on the weight of each rat. The enzyme activity of urinary *N*-acetyl-β-D-glucosaminidase (NAG), a specific indicator of tubular damage [21], were also measured (Clinica Medica é Diagnóstico Dr. Joaquim Chaves, Lisbon, Portugal). Urinary NAG activity was standardized using urinary levels of creatinine to take account of urinary flow rate and expressed as IU/mmol creatinine.

Blood samples from anesthetized mice were obtained via cardiac puncture 24 hours after renal ischemia. Serum levels of urea and creatinine were measured using stan-

dard urease assay/conductivity and picric acid reactions, respectively.

Histologic evaluation

Renal sections were prepared as described previously and used for the assessment of renal I/R injury [21]. Briefly, 100 intersections were examined for each kidney and a score from 0 to 3 was given for each tubular profile involving an intersection: 0, normal histology; 1, tubular cell swelling, brush-border loss, and nuclear condensation, with up to 1/3 of tubular profile showing nuclear loss; 2, as for score 1, but greater than 1/3 and less than 2/3 of tubular profile shows nuclear loss; and 3, greater than 2/3 of tubular profile shows nuclear loss. The histologic score for each kidney was calculated by addition of all 100 scores with a maximum score of 300.

Immunohistochemical localization of iNOS, nitrotyrosine and poly(ADP-ribose)

Immunohistochemical localisation of iNOS, nitrotyrosine (indicating the formation of peroxynitrite) and PAR [indicating activation of poly(ADP-ribose) polymerase (PARP)] in kidney sections was performed as previously described [5, 21]. Sections were incubated overnight at 4°C with primary anti-iNOS, antinitrotyrosine or anti-PAR antibody (1:500 [v/v] in phospate-buffered saline (PBS) (DBA, Milan, Italy). Controls included kidney sections incubated with buffer alone or nonspecific purified immunoglobulin (IgG) (DBA). After blocking endogenous avidin and biotin, specific labeling of antigen-antibody complex was visualized using an avidin-biotin peroxidase complex immunoperoxidase technique using chromogen diaminobenzidine.

Measurement of nitrite/nitrate concentrations

Concentrations of nitrite (NO_2) and nitrite (NO_3), the primary oxidation products of NO subsequent to reaction with oxygen, were measured and used as an indicator of NO synthesis. NO_2/NO_3 levels in rat plasma and incubation medium from proximal tubule cells were measured as previously described [6] after enzymatic conversion of NO_3 to NO_2 using nitrate reductase.

Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) activity in kidneys was used as an indicator of PMN infiltration into renal tissues using a method previously described [21]. Briefly, at the end of the experiments, kidney tissue was weighed and homogenized in a solution containing 0.5% (w/v) hexadecyltrimethylammonium bromide dissolved in 10 mmol/L potassium phosphate buffer (pH 7.4) and centrifuged for 30 minutes at 20,000 g at 4°C. An aliquot of supernatant was then removed and added to a reaction mixture containing 1.6 mmol/L tetramethylbenzidine and 0.1 mmol/L hydrogen peroxide. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme required to degrade 1 μmol hydrogen peroxide at 37°C and was expressed in milliunits/100 mg wet tissue.

Determination of malondialdehye levels

Levels of malondialdehyde (MDA) in kidneys were determined as an indicator of lipid peroxidation following a protocol described previously [21]. Briefly, kidney tissue was weighed and homogenized in a 1.15% (w/v) KCl solution. A 100 μL aliquot of homogenate was then removed and added to a reaction mixture containing 200 μL 8.1% (w/v) lauryl sulfate, 1.5 mL 20% (v/v) acetic acid, 1.5 mL 0.8% (w/v) thiobarbituric acid, and 700 μL distilled water. Samples were then boiled for 1 hour at 95°C and centrifuged at 3000 g for 10 minutes. The absorbance of the supernatant was measured spectrophotometrically at 650 nm. MDA levels were expressed as μmol/L MDA/100 mg wet tissue.

Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company, Ltd. (Poole, Dorset, U.K.). All stock solutions were prepared using nonpyrogenic saline (0.9% [w/v] NaCl; Baxter Healthcare, Ltd., Thetford, Norfolk, U.K.). GW274150 was obtained from Alexis Biochemicals (Bingham, Nottingham, U.K.). LPS was obtained from *Escherichia coli* serotype 0.127:B8 (Sigma-Adrich Co.).

Statistical analysis

All values described in the text and figures are expressed as mean ± standard error of the mean (SEM) for N observations. Each data point represents biochemical measurements obtained from up to 12 separate animals. For histologic scoring, each data point represents analysis of kidneys taken from 6 individual animals. For immunohistochemical analysis, the figures shown are representative of at least three experiments performed on different experimental days. Statistical analysis was carried out using GraphPad Prism 3.02/Instat 1.1 (GraphPad Software, San Diego, CA, USA). Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test and a P value of less than 0.05 was considered to be significant.

RESULTS

The mean ± SEM for the weights of the rats used in the in vivo part of this study was 257 ± 6 g (N = 42) and for mice it was 23 ± 1 g (N = 36). On comparison with sham animals, renal I/R produced significant increases in serum, urinary, and histologic markers of renal dysfunction and injury as described in detail below (Figs.

1 and 2). GW274150 did not have a significant effect of MAP or heart rate when administered to rats undergoing sham-operation or renal I/R (data not shown).

Effect of GW274150 on renal dysfunction mediated by I/R (rat)

Rats, which underwent renal I/R, exhibited significant increases in the serum concentrations of urea and creatinine compared to sham-operated animals (Fig. 1 A and B), suggesting a significant degree of renal (glomerular) dysfunction. Compared to control animals (I/R only), administration of GW274150 produced modest, but significant, reductions in serum levels of urea and creatinine (Fig. 1 A and B).

When compared to rats used as shams, renal I/R did not have a significant effect on urine flow (Fig. 1C). However, rats subjected to renal I/R, which were administered GW274150, produced a significantly greater volume of urine (Fig. 1C). Administration of GW274150 to sham-operated rats also increased urine flow, however, when compared to sham-operated rats administered saline only, this increase was not significant (Fig. 1C).

Effect of GW274150 on renal injury caused by renal I/R (rat)

Renal I/R produced a significant increase in urinary NAG enzymuria (Fig. 2A), suggesting a marked increase in tubular injury. On comparison with urinary NAG enzymuria in control (I/R only) rats, administration of GW274150 produced a significant reduction of NAG enzymuria (Fig. 2A), suggesting reduction of tubular injury.

On comparison with values obtained from sham-operated animals, renal I/R produced a significant increase in serum concentrations of AST (suggesting significant reperfusion injury, Fig. 2B). Administration of GW274150 prior to renal I/R caused a significant reduction in serum AST levels (Fig. 2B), suggesting a marked reduction in reperfusion injury.

On comparison with the histologic score measured from kidneys obtained from sham-operated animals, renal I/R produced a significant increase in histologic score (Fig. 2C). Administration of GW274150 significantly reduced histologic score when compared to that obtained from rats subjected to renal I/R only (Fig. 2C), indicating a reduction in renal injury.

Effects of GW274150 on iNOS expression and plasma NO levels

When compared to kidney sections obtained from sham-operated rats, immunohistochemical analysis of sections obtained from rats subjected to renal I/R revealed positive staining for iNOS, which was unaffected by administration of GW274150 prior to I/R (data not shown).

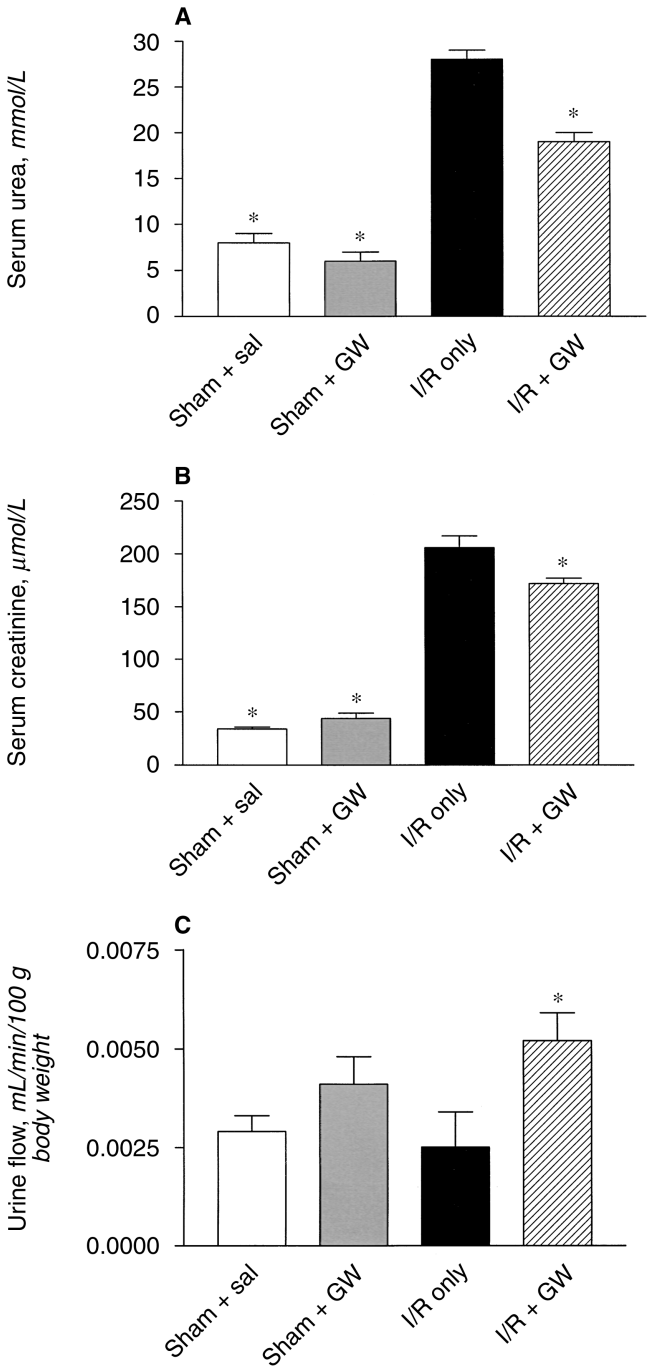

Fig. 1. Effect of GW274150 on renal function in the rat. Serum urea *(A)*, creatinine *(B)* levels, and *(C)* urine flow were measured subsequent to sham operation (Sham + sal, $N = 12$ for all determinations) or renal ischemia/reperfusion (I/R only, $N = 12$ for all determinations). Rats were administered GW274150 (GW) (5 mg/kg intravenous bolus) 30 minutes prior to renal I/R (I/R + GW, $N = 12$ for all determinations) or sham operation (Sham + sal, $N = 6$ for all determinations). Data represent mean ± SEM for number of observations. $^*P < 0.05$ vs. I/R only group.

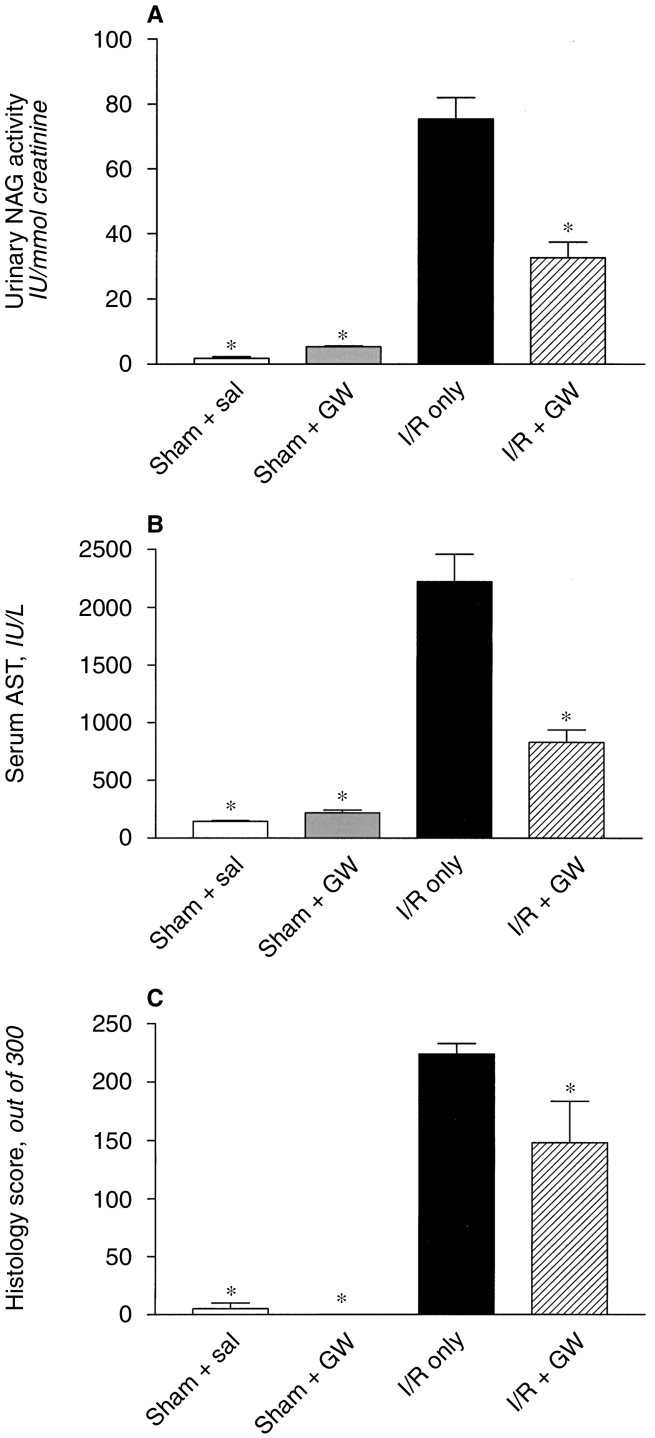

Fig. 2. Effect of GW274150 on tubular and reperfusion-injury. Urinary *N*-acetyl-β-D-glucosaminidase (NAG) activity *(A)* and serum levels of aspartate aminotransferase (AST) *(B)* were measured subsequent to sham-operation (Sham +sal, $N = 6$ for NAG activity; $N = 12$ for AST levels) or renal ischemia/perfusion (I/R only, $N = 6$ for NAG activity; $N = 12$ for AST levels). Rats were administered GW274150 (GW) (5 mg/kg intravenous bolus) 30 minutes prior to renal I/R (I/R + GW, $N = 6$ for NAG activity; $N = 12$ for AST levels) or sham operation (sham + GW, $N = 6$ for both determinations). *(C)* Renal sections were also assessed for scored for characteristic histologic signs of renal injury (see **Methods** section). Each data point represents analysis of kidneys taken from six individual animals. Data represent mean ± SEM for number of observations. $^*P < 0.05$ vs. I/R only group.

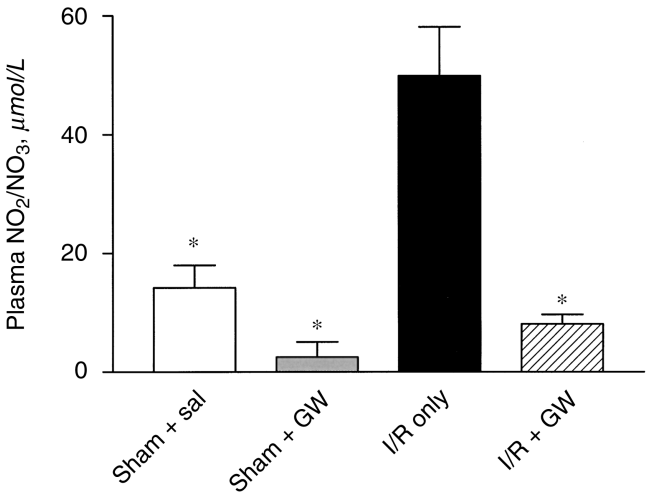

Fig. 3. Effect of GW274150 on nitric acid (NO) production during renal ischemia/reperfusion (I/R). Plasma levels of NO_2/NO_3 measured subsequent to sham operation (Sham + sal, $N = 12$) or renal I/R (I/R only, $N = 12$). Rats were administered GW274150 (GW) (5 mg/kg intravenous bolus) 30 minutes prior to renal I/R (I/R + GW, $N = 12$) or sham operation (Sham + GW, $N = 6$). Data represent mean ± SEM for number of observations, $^*P < 0.05$ vs. I/R only group.

Renal I/R resulted in a significant increase in the plasma levels of NO_2/NO_3 (an indicator of the formation of NO) on comparison with values obtained from the plasma of sham-operated animals (Fig. 3). Increased plasma NO_2/NO_3 levels caused by renal I/R were significantly reduced by administration of GW274150 to rats prior to renal I/R (Fig. 3).

Effect of GW274150 on nitrotyrosine formation during renal I/R

The formation of nitrotyrosine was used as an indicator of nitrosative stress [6]. In comparison to renal sections obtained from sham-operated rats, which were administered saline only (Fig. 4A), immunohistochemical analysis of renal sections obtained from rats subjected to renal I/R revealed positive staining for nitrotyrosine (Fig. 4B). In contrast, substantially reduced staining was observed in the kidney sections obtained from rats, which were administered GW274150 (Fig. 4C). No evidence of staining for nitrotyrosine was observed in kidney sections obtained from sham-operated rats, which were administered GW274150 (data not shown).

Effect of GW274150 on PAR formation during renal I/R

The formation of PAR was used as an indicator of the activity of the nuclear enzyme PARP as previously described [19, 20] and therefore, as an indicator of reactive oxygen species formation and oxidative stress [19, 20]. In comparison to renal sections obtained from sham-operated rats which were administered saline only (Fig. 5A), immunohistochemical analysis of renal sections ob-

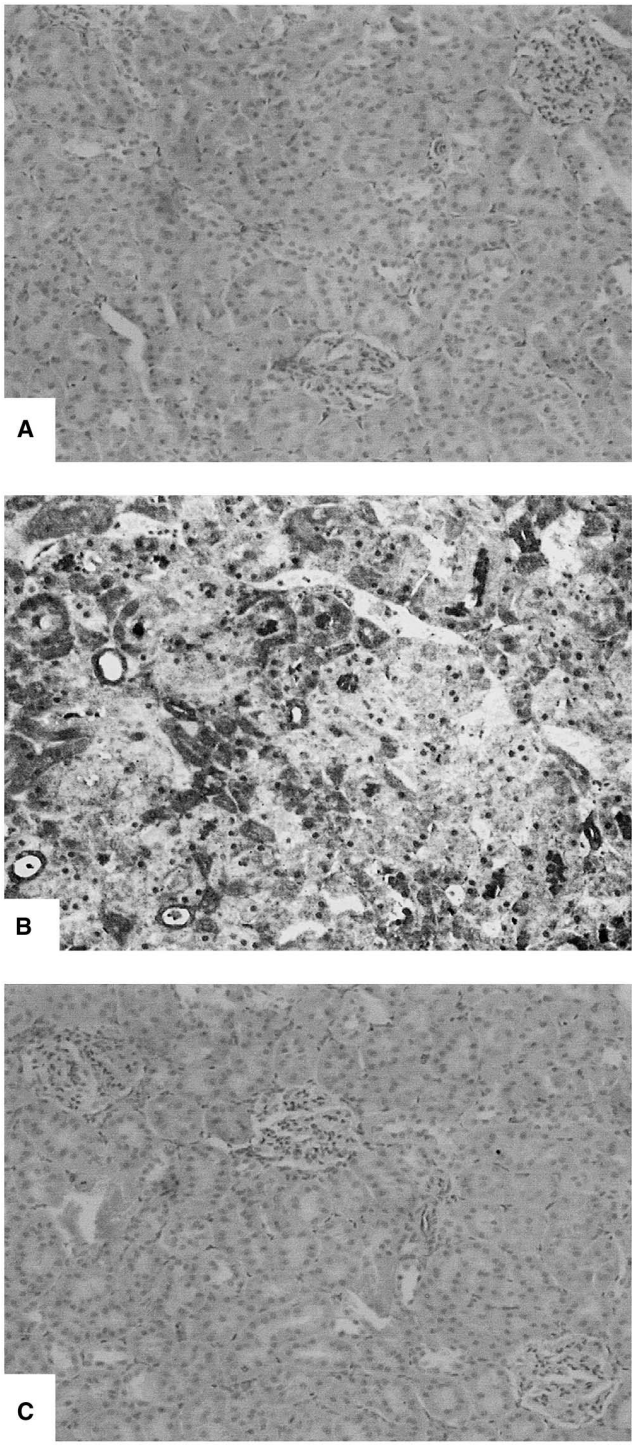

Fig. 4. Effect of GW274150 of peroxynitrite formation during renal ischemia/reperfusion (I/R). Immunohistochemical localization of nitrotyrosine formation (indicative of peroxynitrite formation and nitrosative stress) in rat kidney sections incubated overnight with 1:500 dilution of primary antibody directed against nitrotyrosine. (*A*) Sham-operated group. (*B*) I/R + saline group. In comparison, the nitrotyrosine immunoreactivity of kidneys from rats treated with GW274150 (5 mg/kg) was markedly reduced (*C*). Original magnification, ×150, figures are representative of at least three experiments performed on different experimental days ($N = 12$ for all groups).

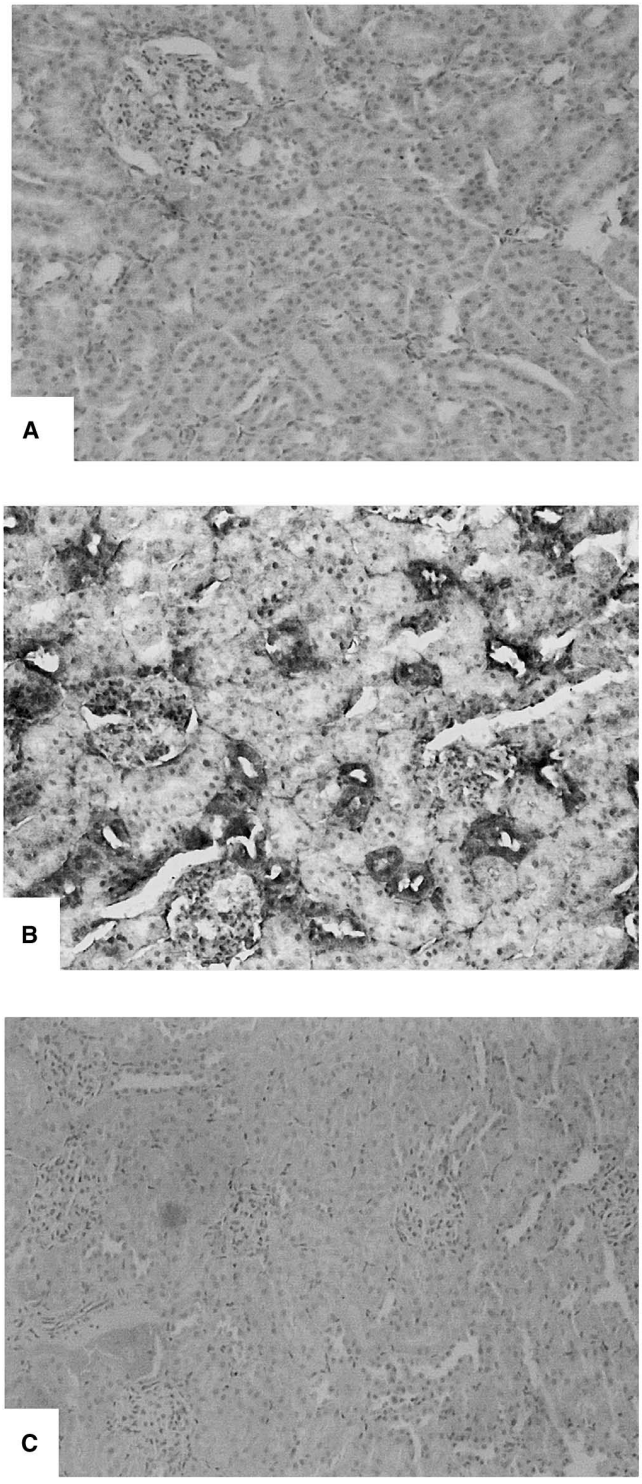

Fig. 5. Effect of GW274150 on poly ADP-ribose polymerase (PARP) activation during renal ischemia/reperfusion (I/R). Immunohistochemical localisation of poly (ADP-ribose) PAR activation (indicative of PARP activation and oxidative stress) in rat kidney sections incubated overnight with primary antibody directed against PAR (1:500 dilution). (*A*) Sham-operated group. (*B*) I/R + saline group. In comparison, the PAR immunoreactivity of kidneys from rats treated with GW274150 (5 mg/kg) was markedly reduced (*C*). Original magnification, ×150, figures are representative of at least three experiments performed on different experimental days ($N = 12$ for all groups).

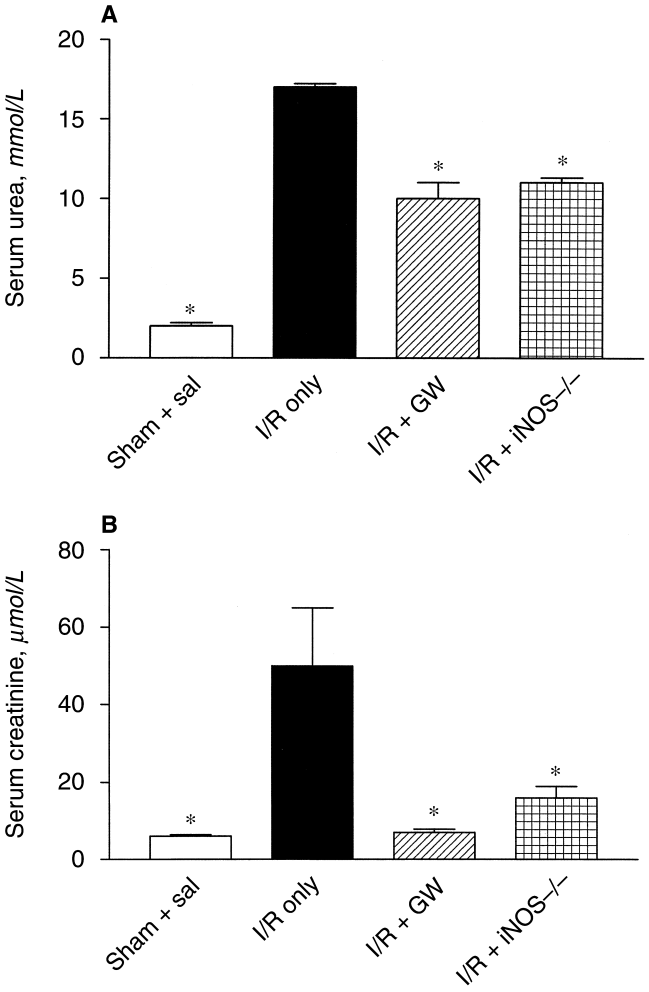

Fig. 6. Effect of GW274150 on renal function in the mouse. Serum urea (*A*) and creatinine (*B*) levels were measured subsequent to sham operation (Sham + sal, $N = 6$ for both determinations) or renal ischemia/reperfusion (I/R only, $N = 6$ for both determinations) in the absence or presence of GW274150 (5 mg/kg intravenous bolus administered 30 minutes prior to I/R and after 12 hours of reperfusion, $N = 6$ for both groups and determinations). Serum urea (A) and creatinine (B) levels were also measured in inducible nitric oxide synthase (iNOS) knockout mice subjected to I/R (I/R + iNOS−/−, $N = 6$ for both determinations). Data represent mean ± SEM for number of observations, $^*P < 0.05$ vs. I/R only group.

tained from rats subjected to renal I/R revealed positive staining for PAR (Fig. 5B). In contrast, substantially reduced staining was observed in the kidney sections obtained from rats, which were administered GW274150 (Fig. 5C). No evidence of staining for PAR was observed in kidney sections obtained from sham-operated rats, which were administered GW274150 (data not shown).

Effect of GW274150 on renal dysfunction mediated by I/R (mice)

Mice, which underwent renal I/R, exhibited significant increases in the serum concentrations of urea and creatinine compared to sham-operated animals (Fig. 6), suggesting a significant degree of renal (glomerular) dysfunction. Compared to control rats (I/R only), administration of GW274150 produced significant reductions in serum levels of urea and creatinine (Fig. 6), which in the case of serum creatinine levels, was similar to values obtained from sham-operated mice (Fig. 6B).

When compared to control (I/R only) mice, serum levels of urea and creatinine and, therefore, renal dysfunction, were significantly lower in iNOS-knockout mice subjected to renal I/R (Fig. 6). Serum urea and creatinine levels obtained from iNOS-knockout mice were not significantly different from those obtained from wild-type mice subjected to renal I/R (Fig. 6).

Effect of GW274150 on kidney MPO activity and MDA levels

Compared to sham-operated animals, mice subjected to renal I/R exhibited a substantial increase in kidney MPO activity (Fig. 7A), suggesting increased PMN infiltration into renal tissues. However, administration of GW274150 produced a significant reduction of MPO activity on comparison with that obtained from control (I/R only) mouse kidneys (Fig. 7A). Mice subjected to renal I/R exhibited a substantial increase in kidney MDA levels on comparison with sham operated mice (Fig. 7B), suggesting increased lipid peroxidation subsequent to the formation of reactive oxygen species. However, administration of GW274150 produced a significant reduction in MDA levels on comparison with MDA levels obtained from control (I/R only) mouse kidneys (Fig. 7B).

When compared to mice subjected to I/R only, renal MPO activity and MDA levels in iNOS-knockout mice subjected to renal I/R were significantly lower (Fig. 7) and were not significantly different from values obtained from sham-operated animals (Fig. 7). The values obtained for MPO activity and MDA levels in iNOS-knockout mice subjected to renal I/R were not significantly different from those obtained from wild-type mice administered GW274150 and subjected to renal I/R (Fig. 7).

DISCUSSION

In the present study, we have shown that renal I/R of both rat and mouse kidney results in a reduction in renal function as demonstrated by increased serum levels of urea and creatinine. In the rat, glomerular dysfunction correlated with the increased tubular injury suggested by increased urinary excretion of NAG and confirmed using histologic analysis. Increased serum levels of AST suggested reperfusion injury caused by renal I/R. The significant increase in NAG enzymuria observed during renal I/R correlated positively with corresponding increases in AST levels ($r = 0.91$) and with histologic scoring ($r = 0.89$). The increase in AST levels also correlated positively with histologic scoring ($r = 0.87$). All

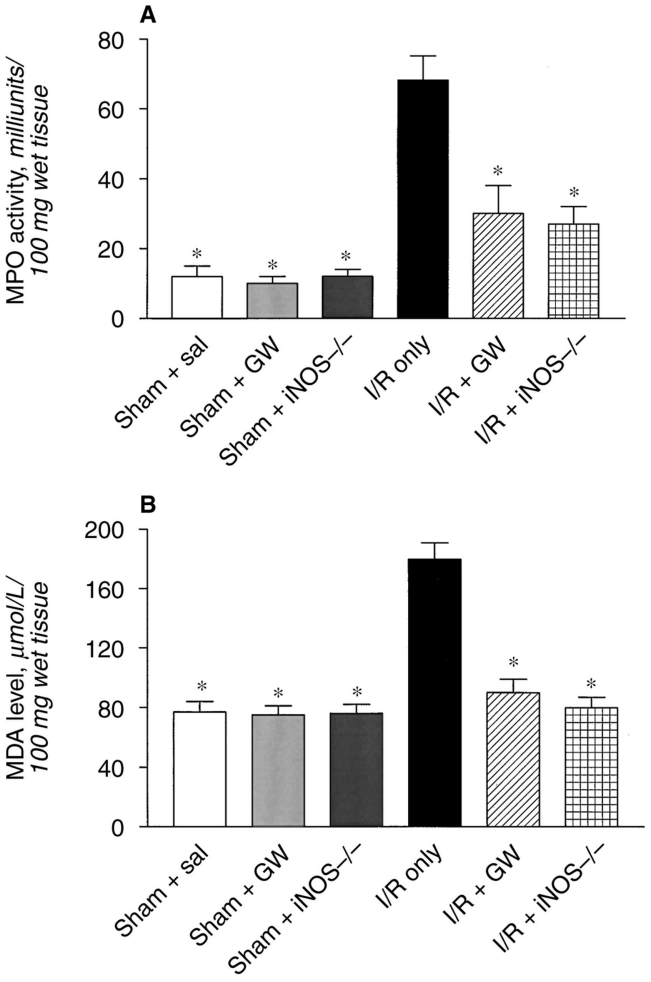

Fig. 7. Effect of GW274150 on kidney myeloperoxidase (MPO) activity and kidney malondialdehyde (MDA) levels. MPO activity *(A)* and MDA levels *(B)* were measured subsequent to sham operation (Sham + sal, $N = 6$ for both determinations) or renal ischemia/reperfusion (I/R only, $N = 6$ for both determinations) in the absence or presence of GW274150 (5 mg/kg intravenous bolus administered 30 minutes prior to I/R and after 12 hours of reperfusion, $N = 6$ for both groups and determinations). MPO activity (A) and MDA levels (B) were also measured in inducible nitric oxide synthase (iNOS) knockout mice subjected to I/R (I/R + iNOS−/−, $N = 6$ for both determinations). Data represent mean ± SEM for number of observations, $^*P < 0.05$ vs. I/R only group.

these data, together with increased expression of iNOS (data not shown), NO, reactive oxygen species, and peroxynitrite formation, PARP activation, lipid peroxidation and infiltration of PMNs, confirmed a well-known pattern of renal dysfunction and injury caused by I/R of the kidney and are in keeping with the notion that renal I/R causes both glomerular and tubular dysfunction [37, 38]. Marked tubular damage in models of renal I/R such as the one used here has been demonstrated previously [5, 6, 20, 21, 37, 38] and characteristic histologic signs of marked tubular injury were observed in kidneys subsequent to renal I/R, in keeping with the observation that the S3 segment of the proximal tubule is particularly susceptible to renal I/R injury [39]. Although we have recently discovered the beneficial effects of GW274150 against renal dysfunction in hemorrhagic shock (abstract; Izumi M et al, *Br J Pharmacol* 135:320P, 2002), the ability of GW274150 to reduce renal dysfunction and injury caused by I/R of the kidney (or to our knowledge, I/R injury in any other organ) have not been investigated.

We demonstrate here, for the first time, that administration of a single dose of GW274150 (5 mg/kg), prior to I/R produces a significant reduction of renal dysfunction and injury caused by I/R of the kidney of the rat in vivo. This conclusion is supported by the following specific, key findings that, in a rat model of renal I/R, GW274150 reduced the increases in (*1*) serum levels of urea and creatinine, (*2*) urinary NAG activity, (*3*) serum levels of AST, and (*4*) plasma levels of NO caused by renal I/R. GW274150 also significantly reduced the histologic evidence of I/R-mediated tubular injury and substantially reduced the immunohistochemical evidence of PAR and nitrotyrosine formation. GW274150 also significantly inhibited cytokine-stimulated NO production by primary cultures of rat proximal tubule cells. Finally, using an in vivo model of renal I/R in the mouse, GW274150 significantly reduced serum levels of urea and creatinine to levels comparable to those obtained iNOS-knockout mice subjected to I/R of the kidney. In these kidneys, GW274150 also reduced MPO activity and MDA levels significantly, indicating reduced PMN infiltration and lipid peroxidation, respectively. In this study, the effects of GW274150 were only investigated after administration prior to I/R. Thus, beneficial effects observed in this study suggest that GW274150 could be given prophylactically in circumstances where the occurrence of renal I/R is foreseeable (e.g., during surgery for supra- or juxtarenal abdominal aortic aneurysms or renal transplantation or in patients subjected to cardiovascular anesthesia) [40–42]. Investigations are currently underway in this laboratory to investigate the effects of administration of GW274150 during the reperfusion period.

It is interesting to consider the mechanisms by which GW274150 provided the beneficial effects reported here. First, GW274150 is readily transported into macrophages via a broad-spectrum amino acid carrier capable of transporting both cationic and neutral amino acids (abstract; Baydoun AR et al, *Br J Pharmacol* 136:33P, 2002). Thus, it is highly likely that GW274150 will accumulate in renal cells, especially in proximal tubule cells, which express amino acid transporters and are responsible for the uptake of amino acids [43]. Second, unlike GW273629 ([R]-2-amino-6-[1-iminoethylamino]-4,4 dioxo-4-thiahexanoic acid), which is a highly selective, but short-acting iNOS inhibitor (abstracts; Beswick P et al, *Acta Physiol Scand* 167:70, 1999; Alderton W et al, *Acta Physiol Scand* 167:11, 1999; and Knowles R et al, *Acta Physiol Scand*

167:12, 1999), the half-life of GW274150 in the rat is 5 hours and 3 hours in mice and a single injection of this iNOS inhibitor (3 mg/kg) abolishes the increase in NO_2 and NO_3 caused by a single injection of endotoxin, even if the endotoxin is injected 14 hours after administration of GW274150 (abstract; Alderton W et al, *Acta Physiol Scand* 167:11, 1999). There is now good evidence that NO, derived from NOS, plays an important role in renal function, both under normal and pathophysiologic conditions [1, 2] and that high levels of NO produced by iNOS are implicated in the renal dysfunction injury associated with either renal I/R [5–10, 44] or during endotoxic and hemorrhagic shock [45, 46]. Several in vivo and in vitro investigations have demonstrated that inhibition of iNOS expression (e.g., using inhibitors of nuclear factor-κB or tyrosine kinase activation), iNOS activity (e.g., using inhibitors of iNOS activity), absence of iNOS itself (using iNOS knockout mice), or scavenging of NO (e.g., using hemoglobin) can ameliorate or prevent NO-mediated renal injury [11–15, 44, 46], suggesting that NO, generated by iNOS, contributes to renal I/R injury. Furthermore, the generation and action of reactive oxygen species plays an important role in pathophysiology of renal I/R [17–21] and the combination of superoxide and NO leads to the formation of peroxynitrite, which also contributes to renal I/R injury [6, 15, 21]. Together, along with formation of proinflammatory cytokines [e.g., tumor necrosis factor-α, interleukin (IL)-1β, IL-6 or IL-8], the expression on endothelium and neutrophils of adhesion molecules [e.g., vascular cell adhesion molecule-1 or intercellular cell adhesion molecule-1 (ICAM-1)], and the overproduction of other vasoactive mediators (e.g., eicosanoids via cyclooxygenase-2), formation of both reactive oxygen species and reactive nitrogen species contribute significantly to ischemic acute tubular necrosis and ARF [7, 16, 17, 47].

Pathophysiologic conditions such as renal I/R and endotoxemia cause the expression of iNOS mRNA and protein within the kidney [7, 48] and increase eNOS activity [49], but do not affect expression of nNOS [50]. Although endogenous NO plays an important role in the control of regional blood flow and intrarenal hemodynamics (e.g., in supporting medullary reperfusion) [51] and supports renal blood flow after I/R [52], the contribution of NO to changes in renal function following injury is still relatively poorly understood. Early studies suggested that inhibition of all isoforms of NOS by nonselective NOS inhibitors by, for example, L-NMMA, causes a concomitant inhibition of eNOS activity, causing excessive vasoconstriction and, hence, exacerbates organ ischemia, microvascular thrombosis and mortality [25]. This is supported by the finding that a high dose of L-NAME, a relatively selective inhibitor of eNOS activity, reduces renal function in rats with or without endotoxemia, augments the degree of kidney injury (glomerular thrombosis), and reduces perfusion in a rodent model of endotoxic shock [26, 53]. Thus, the beneficial hemodynamic effects of nonselective NOS inhibitors may well be due to inhibition of iNOS activity, while the reported adverse effects may be due to inhibition of eNOS activity [54, 55].

In this study, administration of GW274150 significantly increased urine flow in rats subjected to I/R (Fig. 1C) by 123% (compared to rats subjected to I/R only). This was comparable to the increase in urine flow observed in rats subjected to I/R which were administered inhibitors of NOS activity [L-NIL (115% increase) and AE-ITU (109% increase), unpublished results] and which also reduced renal I/R injury [6]. The mechanism involved in the ability of NOS inhibitors to increase urine flow is not entirely clear. Booke et al [56] have previously shown that inhibition of NOS activity by L-NMMA markedly increased urine flow (and attenuated renal dysfunction) in a sheep model of endotoxic shock without influencing blood pressure or total renal blood flow. It has been proposed that several factors could contribute to this phenomenon (e.g., an increased in renal perfusion pressure without alteration in renal blood flow) [57]. Furthermore, L-NAME has a differential effect on the renal vasculature where it causes a greater constriction of the efferent renal arteriole than the afferent vessel, causing renal filtration pressure to increase [58], however, in view of the high selectivity for GW274150 for iNOS over eNOS [29], this is not likely to play a significant role. Furthermore, a direct diuretic effect of GW274150 cannot be discounted as an increase in urine flow was also observed in sham-operated rats administered GW274150 (although this increase did not differ significantly from urine flow measured in sham-operated rats administered saline only). Thus, the mechanism(s) underlying the ability of GW274150 to modulate urine flow during renal I/R certainly warrants further investigation.

GW274150 is a novel inhibitor of iNOS activity, which has a very high degree of selectivity for iNOS versus both eNOS (>250-fold) and nNOS (>80-fold) [29, 33] (abstract; Alderton W et al, *Acta Physiol Scand* 167:11, 1999). In this study, GW274150 did not have any effect on MAP (or heart rate) measured from sham-operated rats or those undergoing renal I/R. Renal I/R resulted in an increase in the plasma levels of NO, indicating increased iNOS protein expression and subsequent activity. Pretreatment of rats with a single bolus injection of GW274150 abolished the increase in iNOS activity caused by I/R in vivo without affecting the expression of iNOS protein. One could argue that the observed beneficial effects of GW274150 in our rat model of renal I/R are due to effects that are not related to inhibition of iNOS activity (nonspecific effects). However, we also show here that the renal dysfunction caused by I/R is significantly reduced in iNOS−/− mice when compared to their wild-type littermates, confirming the finding of previous in

vitro and in vivo studies that absence of iNOS reduces renal I/R injury in rodents [11, 12]. We also report that pretreatment of wild-type mice with GW274150 reduces the renal dysfunction caused by I/R and, interestingly, the degree of protection against renal dysfunction was similar in both wild-type mice treated with GW274150 and in iNOS−/− mice.

In this study, renal I/R caused an increase in the immunologic evidence of the generation of peroxynitrite, which was identified as increased nitration of proteins. Generation of peroxynitrite causes renal injury via direct oxidant injury and protein tyrosine nitration [23, 24]. Nitrotyrosine formation, along with its detection using immunohistochemical staining, was initially proposed as a relatively specific marker for the detection or "footprint" of the endogenous formation of peroxynitrite [59], although there is more recent evidence that certain other reactions can also induce tyrosine nitration (e.g., the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide) can lead to the formation of nitrotyrosine [60]. Therefore, increased nitrotyrosine staining is now considered to act as an indicator of "increased nitrosative stress" rather than a specific marker of the generation of peroxynitrite. Respective in vivo and in vitro studies have demonstrated that formation of peroxynitrite contributes to the pathophysiology of renal I/R [6–8, 15]. Furthermore, peroxynitrite impairs the adhesion of the tubular membrane to the basement membrane leading to the tubular obstruction often observed in ARF [61]. We report here that the selective iNOS-inhibitor GW274150 reduces staining for nitrotyrosine residues in kidneys subjected to I/R injury. Thus, we propose that the inhibition of iNOS activity afforded by GW274150 results in the attenuation of the nitration of proteins, possibly secondary to a reduced formation of peroxynitrite.

Reactive oxygen species (and peroxynitrite) produce cellular injury and necrosis via several mechanisms, including peroxidation of membrane lipids, protein denaturation and DNA damage. Both produce strand breaks in DNA, activating the nuclear enzyme PARP, resulting in the depletion of its substrate NAD^+. As NAD^+ functions as a cofactor in glycolysis and the tricarboxylic acid cycle, its depletion leads to a rapid fall in intracellular ATP a phenomenon termed "the PARP Suicide Hypothesis" [62]. There is recent evidence that the activation of PARP may also play an important role in the injury and dysfunction caused by I/R and inflammation of the kidney and other organs and tissues [19, 20, 62]. We demonstrate here that inhibition of iNOS activity attenuates the increase in PARP activity in the kidneys of rats subjected to I/R injury of the kidney.

GW274150 significantly reduced the increase in renal MPO activity caused by I/R of the mouse kidney, suggesting a significant reduction in PMN infiltration into renal tissues. This finding is in keeping with previous studies reporting that depletion of PMN activity or numbers reduces renal I/R injury [63, 64]. Activated PMNs are generally considered to be the principal effectors of renal I/R injury as they can release superoxide, which can be converted to hydroxyl radicals, and NO, which can combine with superoxide to form peroxynitrite [60, 64]. Hydroxyl radicals and peroxynitrite are highly reactive and cause tissue injury (e.g., via lipid peroxidation, DNA damage, and activation of poly (ADP-ribose) polymerase) [19–21, 37, 38, 64]. Furthermore, PMNs release MPO, which catalyses the formation of another potent oxidant, hypochlorous acid [60] and also generates cytokines, which, via induction of iNOS and production of NO among other mechanisms, leads to further renal pathophysiology [65]. Although there is still some debate as to the role of iNOS and NO in the regulation of PMN recruitment during inflammation [66], our study suggests that inhibition of iNOS using GW274150 reduces PMN infiltration into renal tissues and reduces renal I/R dysfunction. These findings are supported by the results obtained from iNOS−/− mice, which demonstrated renal MPO levels similar to that observed in GW274150-treated mice subjected to renal I/R and which displayed a similar magnitude of renal dysfunction subsequent to renal I/R. These findings confirm our previous findings that inhibition of iNOS and subsequent reduction of NO also attenuates the expression of adhesion molecules (P-selectin and ICAM-1) with consequent reduction of PMN infiltration and related oxidative and nitrosative stress, thereby reducing splanchnic I/R [36] and support the finding of Tassiopoulos et al [67] who demonstrated that inhibition of iNOS using aminoguanidine can decrease PMN chemotaxis and sequestration and attenuate lung injury subsequent to acute aortic occlusion.

Renal I/R also caused a significant increase in MDA levels, which is a secondary product of oxidative stress formed during lipid peroxidation. During I/R, the formation of reactive oxygen species and the decomposition of peroxynitrite into hydroxyl radical and nitrogen dioxide (both of which are potent oxidant species) lead to the initiation and propagation of lipid peroxidation. Administration of GW274150 prior to renal I/R caused a significant reduction in MDA levels in murine kidney tissues, suggesting a significant reduction in lipid peroxidation (subsequent to a reduction in the production of NO, peroxynitrite, and reactive oxygen species). The MDA levels measured in these mice were comparable to those measured in the renal tissues obtained from iNOS−/− mice subjected to I/R, confirming the role of iNOS and subsequent production of NO, peroxynitrite and ROS in the development of renal I/R injury.

CONCLUSION

In conclusion, we demonstrate that the novel, potent, and selective iNOS-inhibitor GW274150 reduces the degree of renal dysfunction and injury caused by I/R of the rat and mouse kidney in vivo, reduces NO production during renal I/R, and reduces the evidence of I/R-mediated peroxynitrite formation in vivo. In addition, we report that the degree of renal dysfunction caused by I/R in wild-type mice treated with GW274150 is similar to that observed in iNOS−/− mice. Taken together, these findings support the conclusions that an enhanced formation of NO by iNOS contributes to the pathophysiology of renal I/R injury and selective inhibitors of iNOS activity such as GW274150 may be of therapeutic benefit in conditions associated with renal I/R and ischemic ARF.

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APPENDIX

Abbreviations used in this article are: ARF, acute renal failure; AST, aspartate aminotransferase; cNOS, constitutive nitric oxide synthase; eNOS, endothelial nitric oxide synthase; ICAM, intercellular adhesion molecule; I/R, ischemia/reperfusion; iNOS, inducible nitric oxide synthase; IL, interleukin; LPS, lipopolysaccharide; MAP, mean arterial (blood) pressure; MDA, malondialdehyde; MPO, myeloperoxidase; NAG, *N*-acetyl-β-D-glucosaminidase; NO, nitric oxide; NO_2, nitrite; NO_3, nitrate; nNOS, neuronal nitric oxide synthase; PAR, poly (ADP-ribose); PARP, poly (ADP-ribose) polymerase; PBS; phosphate-buffered saline, PMN; polymorphonuclear leukocytes; SEM, standard error of the mean.

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