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# The tyrosine kinase inhibitor tyrphostin AG126 reduces renal ischemia/reperfusion injury in the rat

PRABAL K. CHATTERJEE, NIMESH S.A. PATEL, ESPEN O. KVALE, PAUL A.J. BROWN, KEITH N. STEWART, DOMENICO BRITTI, SALVATORE CUZZOCREA, HELDER MOTA-FILIPE, and CHRISTOPH THIEMERMANN

*Department of Experimental Medicine, Nephrology & Critical Care, William Harvey Research Institute, Queen Mary - University of London, London, United Kingdom; Departments of Pathology and Medicine & Therapeutics, University of Aberdeen, Aberdeen, United Kingdom; Department of Veterinary and Agricultural Sciences, University of Teramo, Teramo, Italy; Department of Clinical and Experimental Medicine & Pharmacology, University of Messina, Messina, Italy; and Laboratory of Pharmacology, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal*

**The tyrosine kinase inhibitor tyrphostin AG126 reduces renal ischemia/reperfusion injury in the rat.**

*Background.* We investigate the effects of tyrphostin AG126, an inhibitor of tyrosine kinase activity, on the renal dysfunction and injury caused by ischemia/reperfusion (I/R) of the kidney.

*Methods.* Tyrphostin AG126 (5 mg/kg intraperitoneally) was administered to male Wistar rats 30 minutes prior to bilateral renal ischemia for 45 minutes followed by reperfusion for up to 48 hours. Biochemical markers of renal dysfunction and injury were measured and renal sections assessed for renal injury. Expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and formation of nitrotyrosine and poly (ADP) ribose (PAR) were assessed using immunohistochemistry. Rat proximal tubular cells (PTCs) were incubated with interferon-$\gamma$ (100 IU/mL), bacterial lipopolysaccharide (10 $\mu$g/mL), and with increasing concentrations of tyrphostin AG126 (0.0001–1 mmol/L) for 24 hours. Nitric oxide production was measured in both plasma from rats subjected to I/R and in incubation medium from PTCs.

*Results.* After 6 hours of reperfusion, tyrphostin AG126 significantly reduced the increase in serum and urinary indicators of renal dysfunction and injury caused by I/R and reduced histologic evidence of renal injury. Tyrphostin AG126 also improved renal function (after 24 and 48 hours of reperfusion) and reduced the histologic signs of renal injury (after 48 hours of reperfusion). Tyrphostin AG126 reduced the expression of iNOS and nitric oxide levels in both rat plasma and in PTC cultures, as well as expression of COX-2. Tyrphostin AG126 also reduced nitrotyrosine and PAR formation, suggesting reduction of nitrosative stress and poly (ADP-ribose) polymerase (PARP) activation, respectively.

*Conclusion.* Taken together, these results show that tyrphostin AG126 significantly reduces the renal dysfunction and injury caused by I/R of the kidney. We propose that inhibition of tyrosine kinase activity may be useful against renal I/R injury.

**Key words:** kidney, reperfusion-injury, renal dysfunction, tubular injury, proximal tubular cells, inducible nitric oxide synthase, nitric oxide, cyclooxygenase-2, poly (ADP-ribose) polymerase, oxidative stress, peroxynitrite, nitrosative stress.

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Despite significant advances in critical care medicine, acute renal failure (ARF) remains a major clinical problem, producing considerable morbidity and mortality, which has not decreased significantly over the last 50 years [1–3]. Renal ischemia, whether caused by shock or during surgery or transplantation, is a major cause of ARF, initiating a complex and interrelated sequence of events, resulting in injury to, and eventual death of, renal cells [3, 4]. The prognosis is complicated by the fact that reperfusion, although essential for the survival of ischemic renal tissue, causes additional damage (reperfusion injury) [5, 6], which contributes to the renal dysfunction and injury associated with ischemia/reperfusion (I/R) of the kidney [3–6]. Within the kidney, the proximal tubule (PT) appears to be particularly susceptible to I/R injury, leading to acute tubular necrosis (ATN), which plays a pivotal part in the pathogenesis of ARF [4, 6]. Previous interventions against ARF have proved to be largely negative and dialysis still remains the only effective therapy [1, 2]. The development of novel therapies for the reduction of renal dysfunction and injury caused by I/R has, therefore, been a topic of intense research interest [1, 2].

Nitric oxide, 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 [7, 8]. Although nitric oxide appears to play an important functional role within the PT, it can also contribute to pathophysiology of this part of the kidney [9, 10]. 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 [7, 8] and inducible NOS (iNOS) can be induced in the kidney by cytokines, bacterial lipopolysaccharide (LPS) and during I/R [8–13], leading to renal cell injury [14]. Furthermore, both cyclooxygenase (COX)-1 and COX-2 are expressed in distinct but important areas of the kidney [15, 16]; however, the role of COX-2 induction in the development of renal I/R injury still remains unclear [11, 17, 18]. Induction of COX-2 results in an enhanced formation of metabolites of arachidonic acid (e.g., vasodilator prostaglandins), which exert proinflammatory effects [19], and elevated plasma levels of certain prostaglandins have been documented in rats and patients with renal injury and disease [20, 21]. Furthermore, it is now clear that the production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, and hydroxyl radicals contribute significantly to the development of renal I/R injury (and associated ARF) [5, 22]. Superoxide anions also react with nitric oxide to form peroxynitrite [23], which causes injury via oxidant injury and protein tyrosine nitration [24]. Specifically in the kidney, peroxynitrite generation has been implicated in the pathophysiology of both renal I/R and hypoxia-reoxygenation injury, respectively [11–14, 25–27]. Both ROS and peroxynitrite also cause DNA damage during renal I/R [27, 28], leading to the activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP), depletion of $NAD^+$ and adenosine triphosphate (ATP) and ultimately cell death [29, 30]. Thus, various strategies that reduce the formation or presence of ROS, such as the use of TEMPOL, TEMPONE, or desferrioxamine, have been shown to provide beneficial actions against renal dysfunction and injury caused by I/R of the kidney [31, 32].

Phosphorylation of proteins on tyrosine residues by protein tyrosine kinases plays an important role in the regulation of cell proliferation, cell differentiation, and cellular signaling processes [33, 34]. The receptor tyrosine kinases participate in transmembrane signaling, whereas the intracellular tyrosine kinases mediate signal transduction to the nucleus [33]. Enhanced tyrosine kinase activity has been implicated in the pathophysiology of many diseases states, including cancer, atherosclerosis, and psoriasis [35, 36], and several protein kinase inhibitors have been developed as novel therapeutics for the therapy of systemic inflammation, shock, and organ injury [37]. Many previous strategies aimed at reducing renal I/R injury have been limited to targeting a single mediator [11–13, 29–32]. In contrast, tyrosine kinase inhibitors act directly on cells rather than individual mediators [e.g., by reducing the activation of the transcription factor nuclear factor (NF)-κB] and thereby, expression of iNOS and COX-2 and the formation and/or effects of proinflammatory cytokines [e.g., tumor necrosis factor (TNF)-α and interleukin (IL)-1β] [38–40]. Tyrosine kinase inhibitors have therefore provided a promising approach for the modulation of diseases with abnormalities in protein kinase signaling pathways such as renal cell carcinoma [41]. A particular family of tyrosine kinase inhibitors, the tyrphostins, which are derivatives of benzylidene malononitrile, have been described as potential antiproliferative agents [42–44]. The expression of iNOS caused by inflammatory stimuli in cultured cells involves the phosphorylation of tyrosine residues in proteins and is prevented by the tyrosine kinase inhibitors genistein, erbstatin and tyrphostin AG126 [45–48]. The expression of COX-2 by endotoxin or proinflammatory cytokines is also mediated by tyrosine kinases [47] and can be inhibited by tyrphostin AG126 and erbstatin [49, 50].

The effect of inhibition of tyrosine kinase activity on renal I/R injury is not known. We propose here that inhibition of the activity of protein tyrosine kinases may represent a novel approach for the therapy of renal I/R injury and investigate the effects of tyrosine kinase inhibitor tyrphostin AG126 on the renal dysfunction and injury caused by I/R of the kidney of the rat. We also hypothesize here that tyrphostin AG126 reduces the activation of the transcription factor NF-κB, thereby reducing the expression of proinflammatory genes. We have therefore investigated the effects of tyrphostin AG126 on the expression of two such proinflammatory genes (iNOS and COX-2). Finally, we have investigated the effects of tyrphostin AG126 on the formation of nitric oxide and nitrotyrosine and subsequent activation of PARP, all of which are implicated in the development of renal I/R injury.

## METHODS

### Renal ischemia/reperfusion (short-term model)

In vivo studies were carried out using 60 male Wistar rats (Tuck, Rayleigh, Essex, UK) weighing 220 to 310 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 of 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 [30, 31]. Upon completion of surgical procedures, rats were randomly allocated into the following five groups: (*1*) I/R + saline group, in which rats were administered 2 mL/kg saline intraperitoneally 30 minutes prior to renal I/R (45 minutes renal ischemia followed by reperfusion for 6 hours) ($N = 12$); (*2*) I/R + vehicle group, which was the same as I/R + saline group, but rats were administered vehicle

for tyrphostin AG126 [2 mL/kg 50% (vol/vol) dimethyl sulfoxide (DMSO) in 0.9% (vol/vol) saline, intraperitoneally] 30 minutes prior to I/R ($N$ = 12); (*3*) I/R + tyrphostin AG126 group, in which rats were administered tyrphostin AG126 (5 mg/kg, intraperitoneally) 30 minutes prior to I/R ($N$ = 12); (*4*) sham + saline group, in which rats were administered 2 mL/kg saline intraperitoneally 30 minutes prior to undergoing identical surgical procedures as the above groups except for renal I/R, and were maintained under anesthesia for the duration of the experiment (45 minutes + 6 hours, $N$ = 12); and (*5*) sham + tyrphostin AG126 group; which was identical to sham-operated rats except for the administration of tyrphostin AG126 (5 mg/kg, intraperitoneally) 30 minutes prior to undergoing identical surgical procedures as the above groups except for renal I/R, and were maintained under anesthesia for the duration of the experiment (45 minutes + 6 hours, $N$ = 12). All rats received an infusion of saline (2 mL/kg/hour) throughout I/R.

**Renal ischemia/reperfusion injury (long-term model)**

To assess the effects of tyrphostin AG126 administration on renal function and injury in the later stages of ischemic ARF, further studies were performed using 25 male Wistar rats (Charles River, Milan, Italy) weighing 200 to 260 g. Rats 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 (DM 116192), as well as with the European Economic Community regulations (OJ of EC L358/1, December 18, 1986).

Rats were subjected to bilateral renal ischemia for 45 minutes followed by reperfusion for up to 48 hours as described previously [51]. Rats were anesthetised using chloral hydrate (400 mg/kg, intraperitoneally) and core body temperature maintained at 37°C using a homeothermic blanket. Rats were then divided into the following four groups: (*1*) I/R + saline group, in which rats were subjected to renal ischemia (45 minutes) followed by reperfusion for 48 hours and were administered 2 mL/kg saline, intraperitoneal bolus 30 minutes before sham operation and after 12 hours ($N$ = 10); (*2*) I/R + vehicle group, in which rats were subjected to renal ischemia (45 minutes) followed by reperfusion for 48 hours and were administered 2 mL/kg 50% (vol/vol) DMSO/saline (vehicle for tyrphostin AG126), intraperitoneally 30 minutes before sham operation and after 12 hours ($N$ = 5); (*3*) I/R + tyrphostin AG126 group, in which rats were subjected to renal ischemia (45 minutes) followed by reperfusion for 48 hours; rats were administered tyrphostin AG126 (5 mg/kg intraperitoneal bolus) 30 minutes prior to commencement of I/R and after 12 hours of reperfusion ($N$ = 5); and (*4*) sham + saline group, in which rats were subjected to identical surgical procedures except for renal I/R ($N$ = 5).

After performing a flank incision, rats belonging to the I/R groups were subjected to bilateral renal ischemia for 45 minutes during which the renal arteries and veins were occluded using microaneurysm clamps. 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. Rats were then returned to their cages where they were allowed to recover from anesthesia and observed for 48 hours. Sham-operated rats underwent identical surgical procedures to I/R rats except that microaneurysm clamps were not applied. The time course and doses of tyrphostin AG126 used in this study were based on those previously shown by us, and others, to provide beneficial actions against endotoxic shock (which involves an element of renal I/R injury) or inflammation in the rat [50, 52–56].

**Measurement of biochemical parameters**

At the end of the reperfusion period (short-term model) or after 24 and 48 hours of reperfusion (long-term model), blood samples were collected via the carotid artery or tail vein into tubes containing serum gel. The samples were centrifuged (6000 rpm for 3 minutes) to separate serum from which biochemical parameters were measured (Vetlab Services, Sussex, UK; or by DB, University of Teramo, Italy). Serum creatinine concentrations were used as indicators of glomerular function [30, 31]. Aspartate aminotransferase (AST), an enzyme located in the PT, was used as an indicator of reperfusion injury [13, 31, 57]. Urine samples were collected throughout the reperfusion period and the volume of urine produced recorded. Urine concentrations of $Na^+$ were measured (Vetlab Services) at the end of the reperfusion period and used in conjunction with serum $Na^+$ concentrations to estimate fractional excretion of sodium ($FE_{Na}$) using standard formulas, and which was used as an indicator of tubular function [30, 31]. Concentrations of urinary *N*-acetyl-β-D-glucosaminidase (NAG), a specific indicator of tubular damage [13, 31], was also measured (Clínica Médica é Diagnóstico Dr. Joaquim Chaves, Lisbon, Portugal) and used as a marker of tubular injury.

**Histologic evaluation**

Renal sections were prepared as described previously and used for the assessment of renal I/R injury [13, 31]. 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, nuclear condensation, with up to one third of tubular profile showing nuclear loss; 2, as for score 1, but greater than one third and less than two thirds of tubular profile shows nuclear loss; and 3, greater than two thirds of tubular profile shows nuclear

loss. The total score for each kidney was calculated by addition of all 100 scores with a maximum score of 300.

### Immunohistochemical localisation of iNOS, COX-2, nitrotyrosine, and PAR

Immunohistochemical localisation of iNOS, COX-2, nitrotyrosine (indicative of peroxynitrite formation and/or nitrosative stress, see **Discussion** section), and poly (ADP-ribose) (PAR) (indicative of PARP activation, see **Discussion** section) in kidney sections was performed as previously described [11–13, 29–32]. Kidneys were fixed in 10% formalin buffered using phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.4) and 8 μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (vol/vol) hydrogen peroxide in 60% (vol/vol) methanol for 30 minutes. Sections were then permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 20 minutes and nonspecific adsorption was minimized by incubation in 2% (wt/vol) normal goat serum in PBS for 20 minutes. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 minutes with avidin and biotin (DBA, Milan, Italy). Sections were then incubated overnight at 4°C with primary anti-iNOS, anti-COX-2, anti-PAR or anti-nitrotyrosine antibody [1:500 (vol/vol) in PBS] (DBA) or with control solutions which included incubation with buffer alone or nonspecific purified rabbit IgG (DBA). Specific labeling of antigen-antibody complex was visualized using a biotin-conjugated goat antirabbit IgG (DBA) and avidin-biotin peroxidase complex (DBA) immunoperoxidase technique using chromogen diaminobenzidine.

### Isolation and culture of rat proximal tubular cells

Rat proximal tubular cells (PTCs) were isolated from kidneys obtained from 12 male Wistar rats (250 to 350 g) using collagenase digestion, differential sieving and Percoll density centrifugation as described previously [31]. Primary cultures of PTCs were cultured in minimum essential medium (MEM) containing 10% (vol/vol) fetal calf serum (FCS) in a humidified 5% $CO_2$/95% air atmosphere at 37°C and medium was changed every 48 hours until the cells reached confluence.

### In vitro experimental design

Confluent primary cultures of PTCs were incubated with 100 IU/mL interferon-γ (IFN-γ) and 10 μg/mL LPS in combination for 24 hours in the absence or presence of increasing concentrations of tyrphostin AG126 (0.0001 to 1 mmol/L). The range of tyrphostin AG126 concentrations used was determined from previous studies, which have shown tyrphostin AG126 to effectively inhibit nitric oxide production by J774.2 macrophages incubated with LPS for 24 hours [50]. Tyrphostin AG126 was added to incubation medium (MEM) 10 minutes before the addition of IFN-γ and LPS to provide final concentrations ranging from 0.0001 to 1 mmol/L. Upon completion of incubations (24 hours after the addition of IFN-γ/LPS), nitric oxide levels in incubation medium were measured as nitrite ($NO_2$)/nitrate ($NO_3$) after conversion of nitrate to $NO_2$ using $NO_3$ reductase as described below and previously [12, 13].

### Measurement of nitrite/nitrate concentrations

Concentrations of $NO_2$/$NO_3$, the primary oxidation products of nitric oxide subsequent to reaction with oxygen, were measured and used as an indicator of nitric oxide synthesis. $NO_2$/$NO_3$ levels in rat plasma and incubation medium from PTCs were measured as previously described after enzymatic conversion of $NO_3$ to $NO_2$ using nitrate reductase [12, 13].

### 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 12 separate rats. For histologic scoring, each data point represents analysis of kidneys taken from six individual rats. For immunohistochemical analysis, the figures shown are representative of at least six experiments performed on different experimental days. For in vitro studies involving PTCs, measurements were taken from cultures obtained from 12 separate isolations using both kidneys from 12 separate rats. For the determination of $NO_2$/$NO_3$ levels in incubation medium, experiments were performed in triplicate. 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.

### Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company, Ltd. (Poole, Dorset, UK). All stock solutions were prepared using nonpyrogenic saline [0.9% (wt/vol) NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK]. LPS was obtained from *Escherichia coli* serotype 0.127:B8 (Sigma Aldrich Company).

## RESULTS

The mean ± SEM for the weights of the rats used in the short-term study was 252 ± 5 g ($N$ = 60) and for the long-term study was 220 ± 2 g ($N$ = 25). On comparison with sham + saline rats, renal I/R produced significant increases in serum, urinary, and histologic markers of renal dysfunction and injury as described in detail

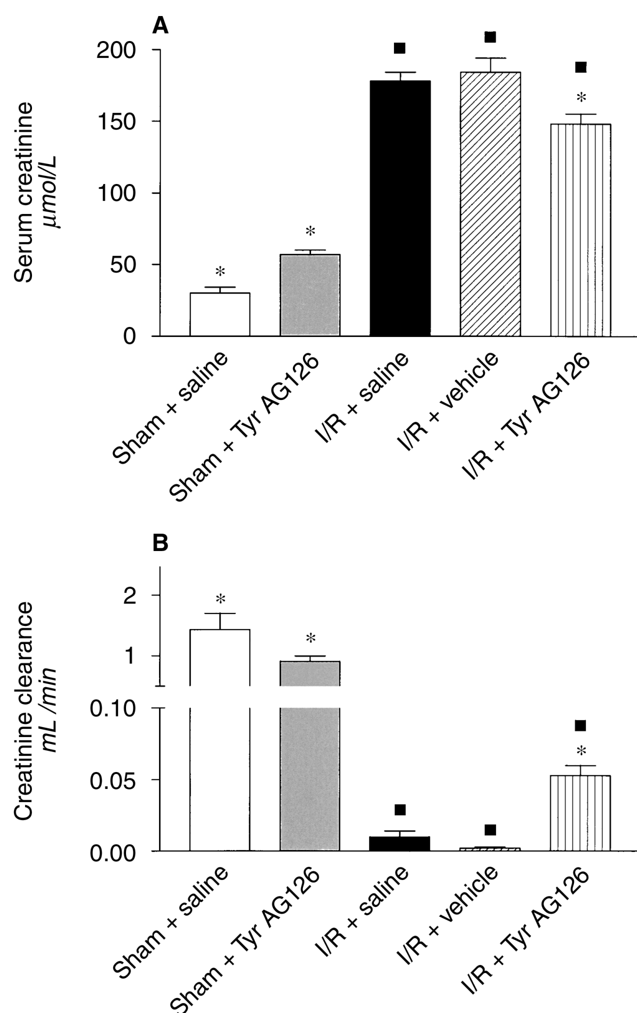



**Fig. 1. Effect of tyrphostin AG126 (Tyr AG126) on glomerular function**. (*A*) Serum creatinine levels and (*B*) creatinine clearance were measured subsequent to sham operation (sham + saline) or renal ischemia/reperfusion (I/R) (I/R + saline) in the absence or presence of tyrphostin AG126 (5 mg/kg administered intraperitoneally 30 minutes prior to I/R). A separate group of rats received vehicle for tyrphostin AG126 [50% (vol/vol) dimethyl sulfoxide (DMSO)/saline] 30 minutes prior to I/R (I/R + vehicle). *$P < 0.05$ vs. I/R + saline group, ■$P < 0.05$ vs. sham + saline group.

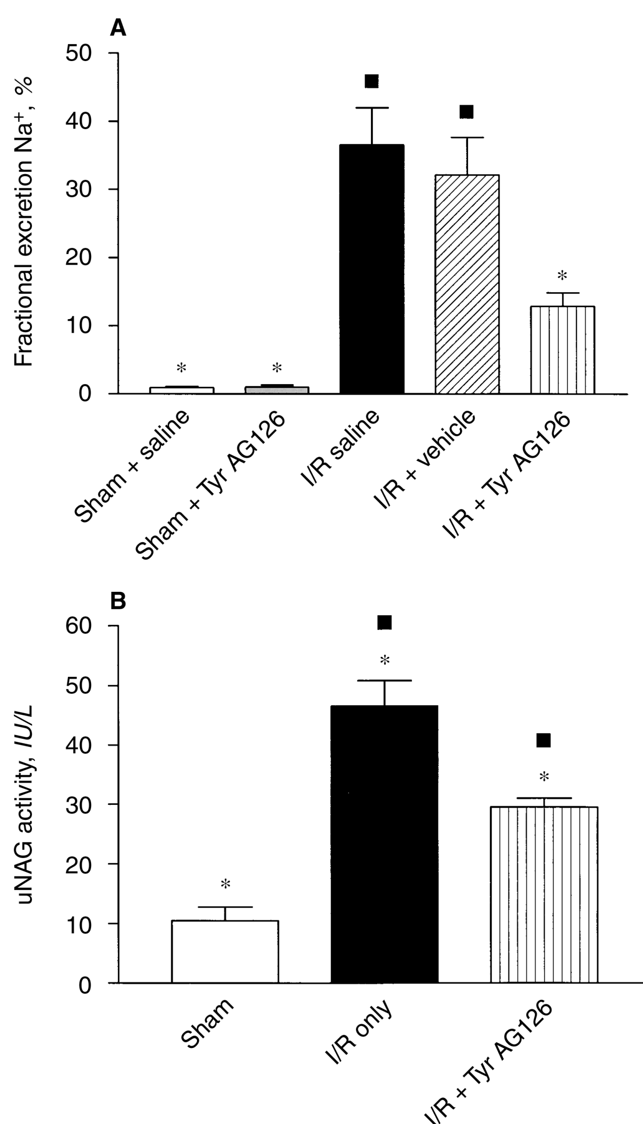



**Fig. 2. Effect of tyrphostin AG126 (Tyr AG126) on tubular function and injury.** (*A*) Fractional excretion of sodium ($FE_{Na}$) and (*B*) urinary levels of *N*-acetyl-β-D-glucosaminidase (NAG) were measured as respective markers of tubular function and injury, subsequent to sham-operation (sham + saline) or renal ischemia/reperfusion (I/R) (I/R + saline) in the absence or presence of tyrphostin AG126 (5 mg/kg administered intraperitoneally 30 minutes prior to I/R). A separate group of rats received vehicle for tyrphostin AG126 [50% (vol/vol) dimethyl sulfoxide (DMSO)/saline] 30 minutes prior to I/R (I/R + vehicle). *$P < 0.05$ vs. I/R + saline group, ■$P < 0.05$ vs. sham + saline group.

below (Figs. 1 to 4). When compared to rats used as shams, renal I/R [in the presence or absence of tyrphostin AG126 or its vehicle [50% (vol/vol) DMSO/50% (vol/vol) saline] did not have a significant effect on urine flow (0.015 ± 0.002 mL/min, $N = 60$).

### Effect of tyrphostin AG126 on renal dysfunction caused by I/R

Rats that underwent renal I/R exhibited significant increases in the serum concentrations of creatinine compared to sham-operated rats (Fig. 1A), suggesting a significant degree of glomerular dysfunction. Compared to rats subjected to I/R only (I/R + saline or I/R + vehicle groups), administration of tyrphostin AG126 to rats prior to renal I/R produced a modest, but significant reduction in the increase in serum levels of creatinine caused by renal I/R (Fig. 1A). In order to discount the possibility of a rapid increase in serum creatinine levels due to increased release of creatinine from muscle during I/R, creatinine clearance was also measured (Fig. 1B). Rats subjected to renal I/R demonstrated a significant reduction in creatinine clearance compared to sham-operated rats (Fig. 1B), suggesting significant glomerular dysfunc-

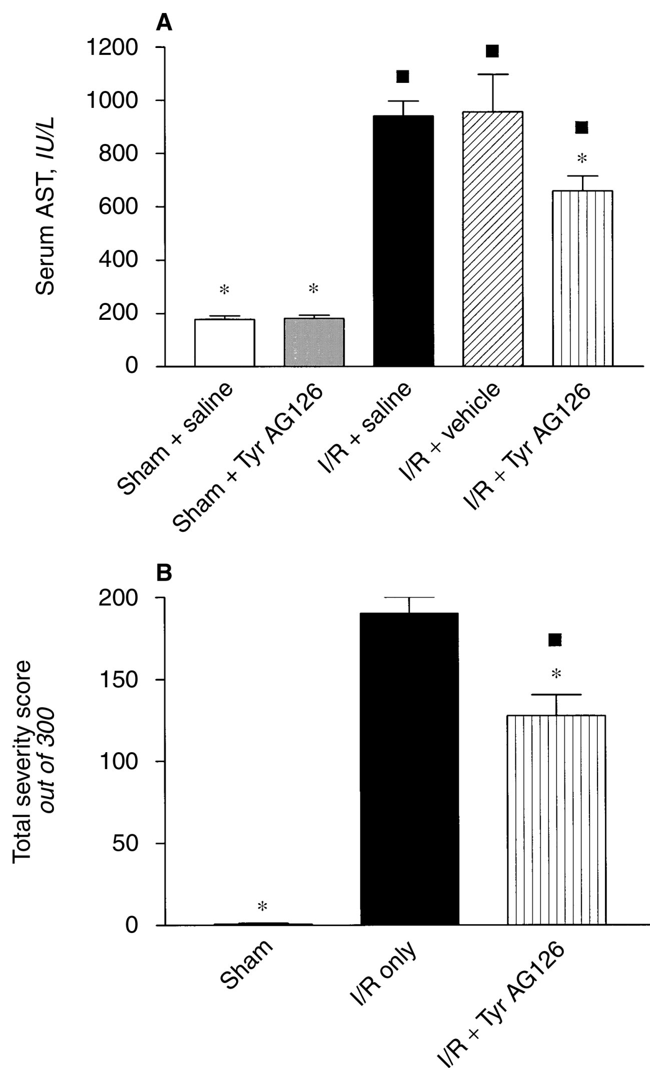
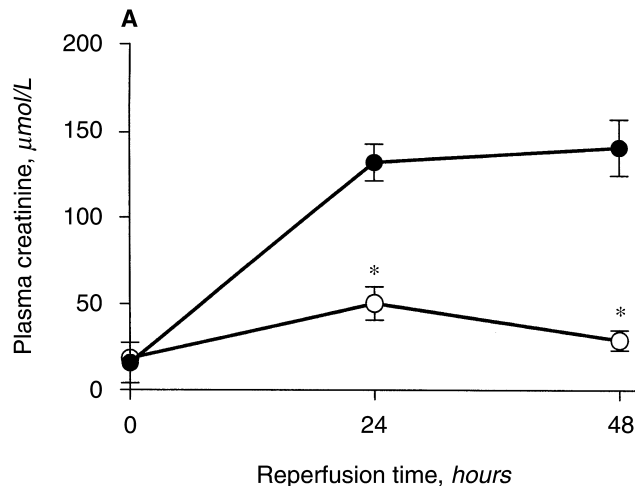
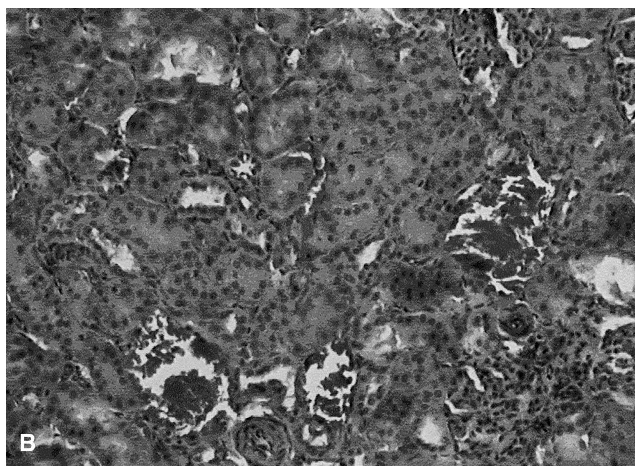



**Fig. 3. Effect of tyrphostin AG126 (Tyr AG126) on reperfusion injury and histologic scoring of renal injury.** (*A*) Serum levels of aspartate aminotransferase (AST) were measured as an indicator of reperfusion injury and (B) renal sections were scored for characteristic histologic signs of renal injury subsequent to sham operation (sham + saline) or renal ischemia/reperfusion (I/R) (I/R + saline) in the absence or presence of tyrphostin AG126 (5 mg/kg administered intraperitoneally 30 minutes prior to I/R). A separate group of rats received vehicle for tyrphostin AG126 [50% (vol/vol) dimethyl sulfoxide (DMSO)/saline] 30 minutes prior to I/R (I/R + vehicle). *$P < 0.05$ vs. I/R + saline group, ■$P < 0.05$ vs. sham + saline group.



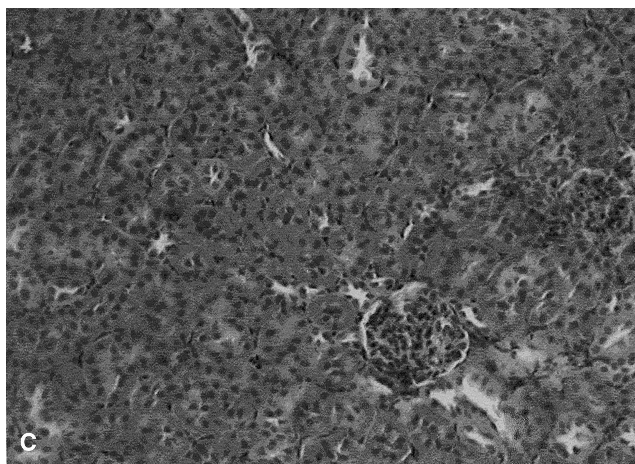




**Fig. 4. Effect of tyrphostin AG126 (Tyr AG126) on the course of renal ischemia/reperfusion (I/R) injury.** (*A*) Serum creatinine levels were measured 24 and 48 hours after renal ischemia in rats subjected to I/R and which were administered either saline [(●) = I/R + saline] or 5 mg/kg tyrphostin AG126 [(○)= I/R + Tyr AG126] 30 minutes prior to ischemia and after 12 hours of reperfusion. Data represent mean ± SEM for *N* observations. *$P < 0.05$ vs. I/R + saline group. Histologic examination of kidney sections prepared from rats subjected to ischemia followed by reperfusion for 48 hours and treated with saline (*B*) displayed more severe renal injury than rats subjected to I/R but which were administered tyrphostin AG126 (*C*). Hemotoxylin and eosin, original magnification ×125, figures are representative of at least three experiments performed on different days.

tion. Compared to rats subjected to I/R only, administration of tyrphostin AG126 produced a modest, but significant improvement in creatinine clearance (Fig. 1B).

Administration of vehicle for tyrphostin AG126 to rats (I/R + vehicle group) did not have a significant effect on the increased serum levels of creatinine or reduction in creatinine clearance associated with renal I/R (Fig. 1). Administration of tyrphostin AG126 to sham-operated rats (sham + tyrphostin AG126 group) did not have any effect on serum creatinine levels or creatinine clearance when comparison with sham-oper-

ated rats administered saline only (sham + saline group) (Fig. 1).

### Effect of tyrphostin AG126 on tubular dysfunction and injury caused by renal I/R

Renal I/R produced significant increases in $FE_{Na}$ (Fig. 2A) and urinary levels of NAG (Fig. 2B), suggesting a marked increase in tubular dysfunction and injury, respectively. On comparison with $FE_{Na}$ measured in rats subjected to I/R-only, administration of tyrphostin AG126 produced a significant reduction in the increase in $FE_{Na}$ associated with renal I/R (Fig. 2A), suggesting improvement in tubular function. Administration of tyrphostin AG126 also significantly reduced the increase in urinary NAG levels caused by renal I/R (Fig. 2B), suggesting reduction of tubular injury. Administration of the vehicle for tyrphostin AG126 to rats did not have a significant effect on the increase in $FE_{Na}$ (Fig. 2A) and administration of tyrphostin AG126 to sham-operated rats did not have any effect on $FE_{Na}$ when compared with sham-operated rats administered saline only (Fig. 2A).

### Effect of tyrphostin AG126 on reperfusion injury caused by renal I/R

On comparison with values obtained from sham + saline rats, renal I/R produced significant increases in serum concentrations of AST (suggesting significant reperfusion injury) (Fig. 3A). Administration of tyrphostin AG126 prior to I/R caused a significant reduction in the increase in serum AST levels caused by renal I/R (Fig. 3A). Administration of the vehicle for tyrphostin AG126 to rats did not have a significant effect on the increase in AST levels associated with renal I/R (Fig. 3A) and administration of tyrphostin AG126 to sham-operated rats did not have any effect on serum AST levels on comparison with sham-operated rats administered saline only (Fig. 3A).

### Effects of tyrphostin AG126 on scoring of renal histopathology

On comparison with the total severity score measured from kidneys obtained from sham + saline rats, renal I/R produced a significant increase in total severity score (Fig. 3B). Administration of tyrphostin AG126 to rats subjected to I/R only (I/R + tyrphostin AG126 group) significantly reduced the increase in total severity score associated with renal I/R when compared to that obtained from rats subjected to renal I/R only (I/R + saline group) (Fig. 3B).

### Effect of tyrphostin AG126 on the course of renal I/R injury

In the long-term model of ischemic ARF, renal function (assessed by serum creatinine levels) was significantly improved after administration of tyrphostin AG126 to rats subjected to renal ischemia followed by reperfusion for 24 and 48 hours (Fig. 4A). Histologic examination of kidney sections prepared from rats subjected to ischemia followed by reperfusion for 48 hours and treated with vehicle for tyrphostin AG126 (I/R + vehicle group) (Fig. 4B) demonstrated greater renal injury than rats subjected to I/R but which were administered tyrphostin AG126 (I/R + tyrphostin AG126 group) (Fig. 4C).

### Effects of tyrphostin AG126 on iNOS expression and plasma nitric oxide levels

When compared to kidney sections obtained from sham + saline rats (Fig. 5A), immunohistochemical analysis of sections obtained from rats subjected to renal I/R only revealed positive staining for iNOS (Fig. 5B). In contrast, reduced staining was observed in the kidney sections obtained from rats administered tyrphostin AG126 prior to renal I/R (Fig. 5C). Administration of (*1*) tyrphostin to sham-operated rats or (*2*) vehicle for tyrphostin AG126 to rats subjected to I/R did not have a significant effect on the expression of iNOS caused by renal I/R (data not shown).

Renal I/R resulted in a significant increase in the plasma levels of $NO_2/NO_3$ (an indicator of the formation of nitric oxide) on comparison with values obtained from the plasma of sham + saline rats (Fig. 5D). Increased plasma $NO_2/NO_3$ levels caused by renal I/R were reduced by administration of tyrphostin AG126 to rats prior to renal I/R (Fig. 5D). Administration of the vehicle for tyrphostin AG126 to rats did not have a significant effect on the increased plasma levels of $NO_2/NO_3$ associated with renal I/R (Fig. 5D) and administration of tyrphostin AG126 to sham-operated rats did not have any effect on plasma $NO_2/NO_3$ levels when compared with sham-operated rats administered saline only (Fig. 5D).

### Effect of tyrphostin AG126 on nitric oxide production by rat PTC cultures

Incubation of primary cultures of rat PTCs with IFN-$\gamma$ and LPS in combination for 24 hours produced a significant increase in nitric oxide production as determined by measurement of $NO_2/NO_3$ levels (Fig. 5E). Incubation of PTCs with IFN-$\gamma$ and LPS in the presence of increasing concentrations of tyrphostin AG126 (0.0001 to 1 mmol/L) resulted in a significant, concentration-dependent inhibition of nitric oxide production (Fig. 5E).

### Effects of tyrphostin AG126 on COX-2 expression

When compared to kidney sections obtained from sham-operated rats (Fig. 6A), immunohistochemical analysis of sections obtained from rats subjected to renal I/R revealed positive staining for COX-2 (Fig. 6B). In contrast, reduced staining for COX-2 was observed in the kidney sections obtained from rats administered tyrphostin AG126 prior to renal I/R (Fig. 6C). Administration

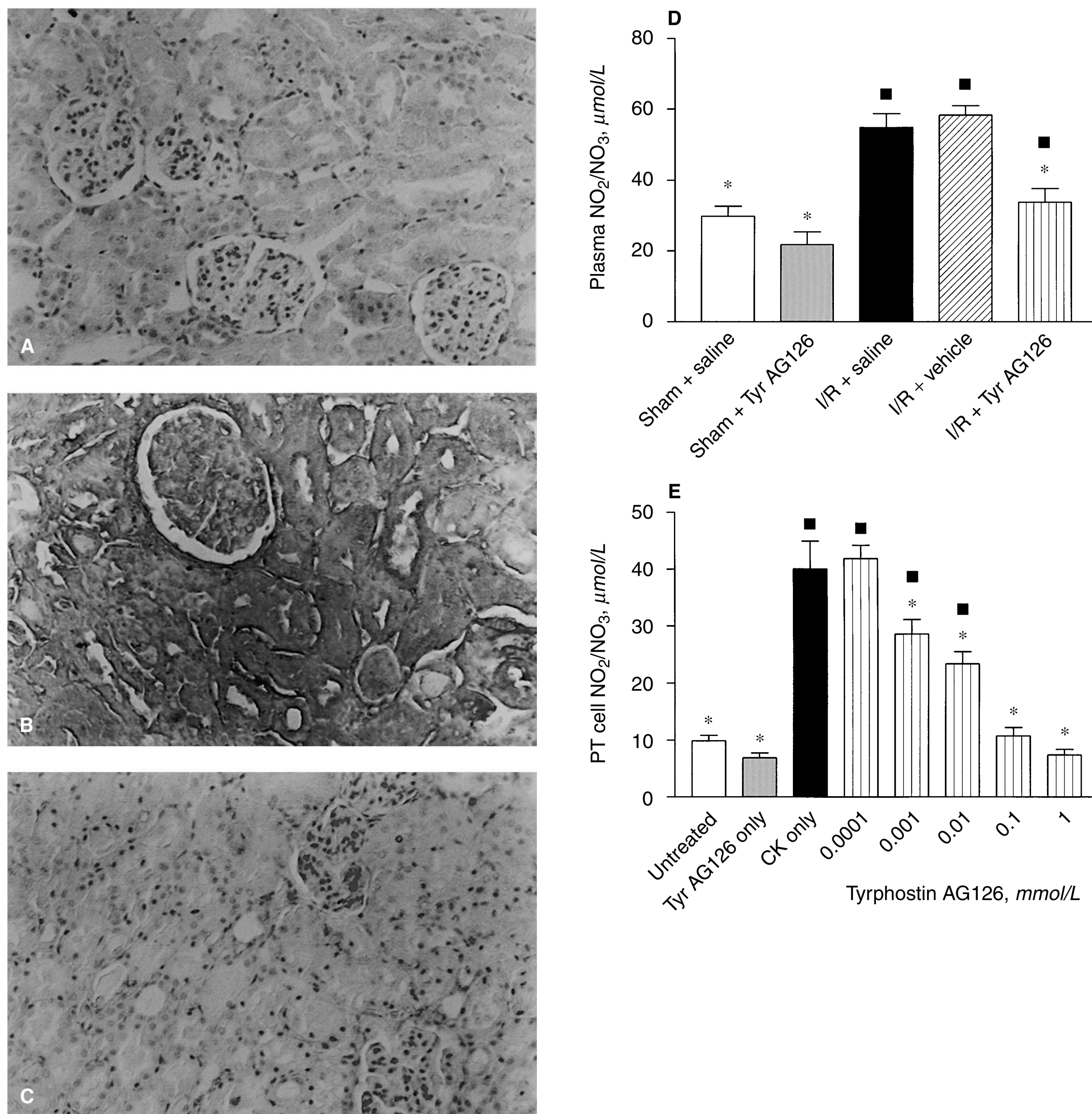



**Fig. 5. Effect of tyrphostin AG126 (Tyr AG126) on inducible nitric oxide synthase (iNOS) expression and nitric oxide production during renal ischemia/reperfusion (I/R).** Immunohistochemical localization of iNOS expression in rat kidney sections incubated overnight with primary antibody directed against iNOS [1:500 dilution with phosphate-buffered saline (PBS)] in (*A*) sham + saline group and (*B*) I/R + saline group. In comparison, the iNOS expression in kidneys from rats treated with tyrphostin AG126 (5 mg/kg intraperitoneally administered 30 minutes prior to I/R) was markedly reduced (*C*). Original magnification, ×125/150, figures are representative of at least 3 experiments performed on different experimental days. (*D*) Effect of tyrphostin AG126 (5 mg/kg administered intraperitoneally 30 minutes prior to I/R) on plasma $NO_2/NO_3$ levels subsequent to sham operation (sham + saline) or renal I/R (I/R + saline). A separate group of rats received vehicle for tyrphostin AG126 [50% (vol/vol) dimethyl sulfoxide (DMSO)/saline] 30 minutes prior to I/R (I/R + vehicle). *$P < 0.05$ vs. I/R + saline group, ■$P < 0.05$ vs. sham + saline group. (*E*) Primary cultures of rat proximal tubular (PT) cells were incubated with incubation medium only (untreated) or with 1 mmol/L tyrphostin AG126 only (Tyr AG126 only) or a combination of interferon-γ (IFN-γ) (100 IU/mL) and lipopolysaccharide (LPS) (10 μg/mL) (cytokine only) for 24 hours. PT cells were also incubated with IFN-γ and LPS in the presence of increasing concentrations of tyrphostin AG126 (0.0001 to 1 mmol/L). After 24 hours, levels of $NO_2/NO_3$ were measured in the incubation medium. *$P < 0.05$ vs. CK only group, ■$P < 0.05$ vs. untreated group.

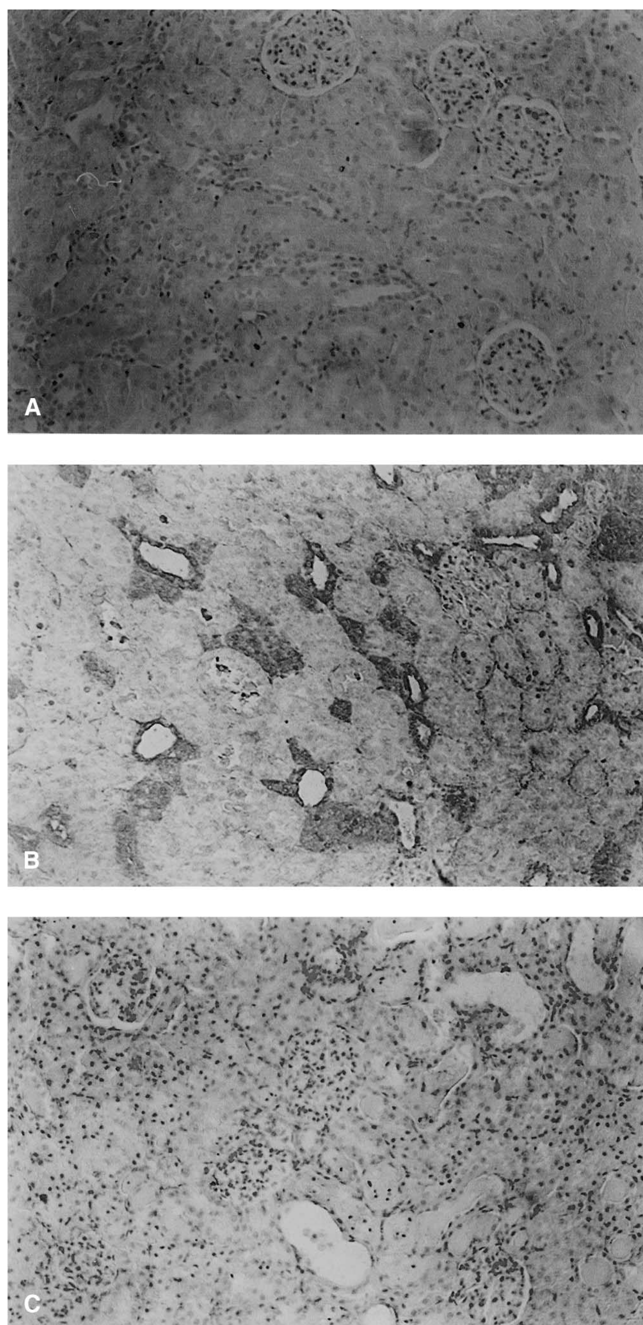



**Fig. 6. Effect of tyrphostin AG126 (Tyr AG126) on cyclooxygenase-2 (COX-2) expression during renal ischemia/reperfusion (I/R).** Immunohistochemical localization of the expression of COX-2 in rat kidney sections incubated overnight with primary antibody directed against COX-2 [1:500 dilution with phosphate-buffered saline (PBS)] in (*A*) sham + saline group and (*B*) I/R + saline group. In comparison, COX-2 expression in kidneys from rats treated with tyrphostin AG126 (5 mg/kg intraperitoneally administered 30 minutes prior to I/R) was markedly reduced (*C*). Original magnification, ×125, figures are representative of at least three experiments performed on different experimental days.

of tyrphostin AG126 to sham-operated rats or its vehicle to rats subjected to I/R did not have a significant effect on the expression of COX-2 caused by renal I/R (data not shown).

### Effect of tyrphostin AG126 on PAR formation during renal I/R

Formation of PAR was used as an indicator of the activity of the nuclear enzyme PARP as previously described [30] and, therefore, as an indicator of ROS formation and oxidative stress [29–32]. In comparison to renal sections obtained from sham-operated rats that were administered saline only (Fig. 7A), immunohistochemical analysis of renal sections obtained from rats subjected to renal I/R revealed positive staining for PAR (Fig. 7B). In contrast, substantially reduced staining was observed in the kidney sections obtained from rats, which were administered tyrphostin AG126 (Fig. 7C). Administration of (*1*) tyrphostin AG126 to sham-operated rats or (*2*) vehicle for tyrphostin AG126 to rats subjected to I/R did not have a significant effect on the formation of PAR caused by renal I/R (data not shown).

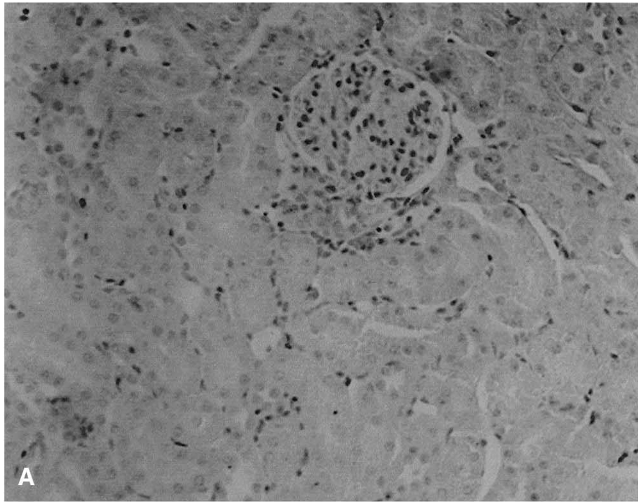
### Effect of tyrphostin AG126 on nitrotyrosine formation during renal I/R

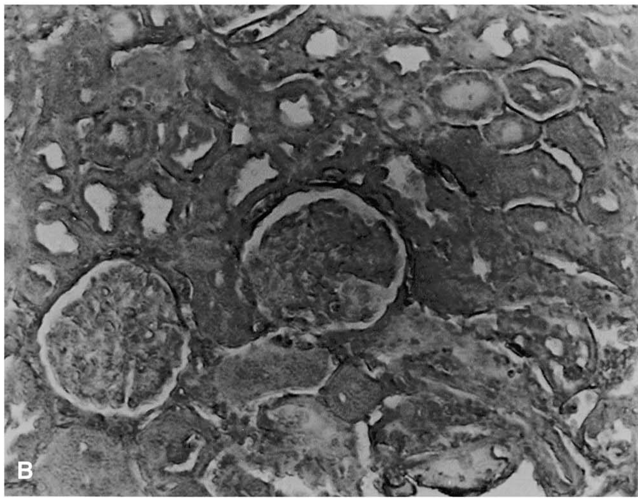
The formation of nitrotyrosine was used as an indicator of nitrosative stress [11–13, 31, 32]. In comparison to renal sections obtained from sham-operated rats that were administered saline only (Fig. 8A), immunohistochemical analysis of renal sections obtained from rats subjected to renal I/R revealed positive staining for nitrotyrosine (Fig. 8B). In contrast, substantially reduced staining was observed in the kidney sections obtained from rats that were administered tyrphostin AG126 prior to renal I/R (Fig. 8C). Administration of tyrphostin AG126 to sham-operated rats or its vehicle to rats subjected to I/R did not have a significant effect on nitrotyrosine formation (data not shown).

## DISCUSSION

In the present study, we have shown that renal I/R of the rat kidney results in a reduction in renal function as was demonstrated by increased serum levels of creatinine. This glomerular dysfunction correlated with increased $FE_{Na}$ indicating dysfunction of the PT. Tubular injury was also confirmed by an increase in NAG enzymuria and increased serum levels of AST confirmed reperfusion injury caused by renal I/R. All these data, together with increased expression of iNOS and COX-2, as well as peroxynitrite formation and PARP activation, confirmed a well-known pattern of renal dysfunction and injury associated with I/R of the kidney [6, 58, 59] and are in agreement with the notion that renal I/R causes both glomerular and tubular dysfunction [60]. Characteristic histologic signs of tubular injury were also observed in kidneys subsequent to renal I/R, in keeping with the observation that the $S_3$ segment of the PT is particularly susceptible to renal I/R injury [61].

The main findings of the current study can be summa-



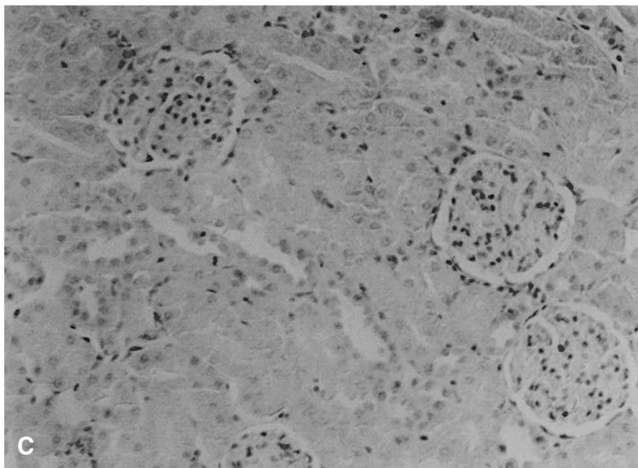
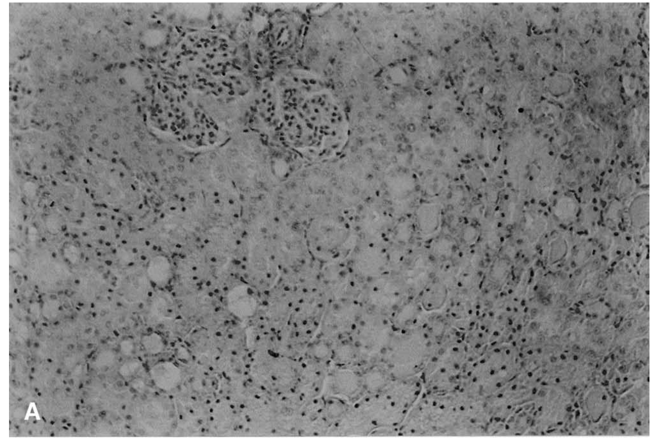
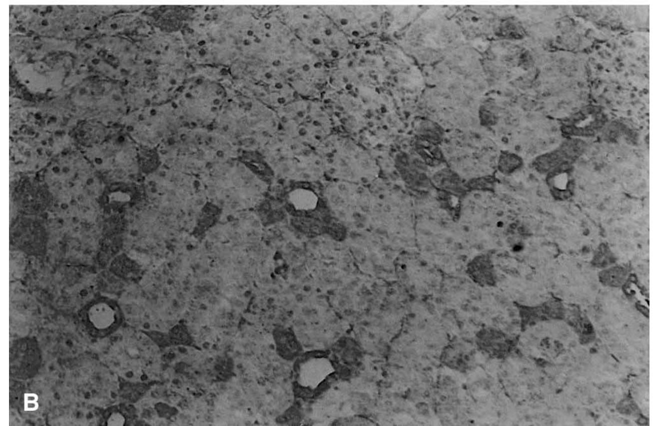




**Fig. 7. Effect of tyrphostin AG126 (Tyr AG126) on nitrotyrosine formation during renal ischemia/reperfusion (I/R).** Immunohistochemical localization of nitrotyrosine as an indicator of peroxynitrite formation and/or nitrosative stress (see **Discussion** section) in rat kidney sections incubated overnight with 1:500 dilution of primary antibody directed against nitrotyrosine in (*A*) sham + saline group and (*B*) I/R + saline group. In comparison, the nitrotyrosine immunoreactivity of kidneys from rats treated with tyrphostin AG126 (5 mg/kg administered intraperitoneally 30 minutes prior to renal I/R) was markedly reduced (*C*). Original magnification, ×150, figures are representative of at least three experiments performed on different experimental days.



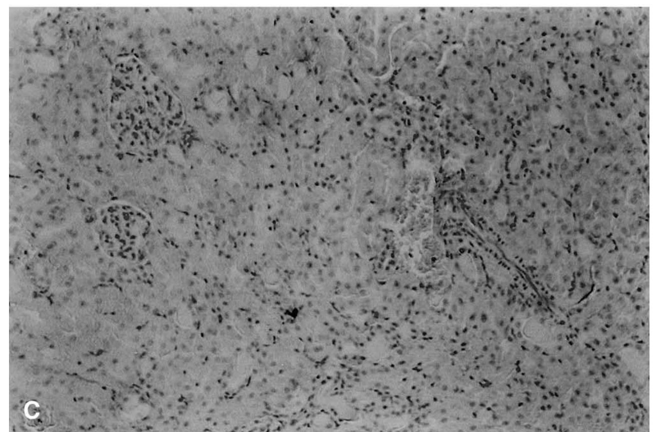




**Fig. 8. Effect of tyrphostin AG126 (Tyr AG126) on poly (ADP-ribose) (PAR) formation during renal ischemia/reperfusion (I/R).** Immunohistochemical localization of PAR formation as an indicator of poly (ADP-ribose) polymerase (PARP) activation in rat kidney sections incubated overnight with primary antibody directed against PAR (1:500 dilution) in (*A*) sham + saline group and (*B*) I/R + saline group. In comparison, the PAR immunoreactivity of kidneys from rats treated with tyrphostin AG126 (5 mg/kg administered intraperitoneally 30 minutes prior to renal I/R) was markedly reduced (*C*). Original magnification, ×125, figures are representative of at least three experiments performed on different experimental days.

rized as follows: (*1*) tyrphostin AG126 reduces the development glomerular and tubular dysfunction caused by renal I/R (serum creatinine levels, $FE_{Na}$), (*2*) tyrphostin AG126 reduces tubular and reperfusion injury (urinary NAG, AST) caused by renal I/R and the histologic evidence of renal injury, (*3*) tyrphostin AG126 reduces nitric oxide production subsequent to iNOS expression caused by renal I/R (as determined using immunohistochemistry), (*4*) tyrphostin AG126 reduces immunohistochemical evidence for the expression of COX-2, (*5*) tyrphostin AG126 reduced the formation of nitrotyrosine during renal I/R (indicating attenuation of peroxynitrite production and/or nitrosative stress, see below), and (*6*) tyrphostin AG126 reduced PAR formation (suggesting attenuation of PARP activation, see below). All of these findings support the view that tyrphostin AG126 attenuates the degree of renal dysfunction and injury caused by I/R of the kidney of the rat. The effects of tyrphostin AG126 on renal dysfunction and injury were also investigated in a longer-term model of ARF in which renal function was assessed after 24 and 48 hours after ischemia. The data obtained indicate that administration of tyrphostin AG126 can improve renal function at the later stages of reperfusion following ischemia, which was reflected by a marked reduction in the histologic evidence of renal injury after 48 hours of reperfusion.

What, then, are the mechanisms by which tyrphostin AG126 protects the kidney against I/R? Tyrphostin AG126, a derivative of benzylidene malononitrile, is a potent inhibitor of protein tyrosine kinases in vitro and in vivo [35, 42], which (among other effects) reduce the expression of iNOS and COX-2 in models of endotoxic and Gram-positive shock (which contains an element of renal ischemia and dysfunction) [38, 39, 50]. In this study, tyrphostin AG126 was able to markedly reduce the expression of iNOS caused by renal I/R and subsequently, nitric oxide levels in vivo; an effect which was reflected by the ability of tyrphostin AG126 to significantly reduce nitric oxide production by rat PTCs in a dose-dependent manner subsequent to the induction of iNOS by LPS/IFN-γ. There is now good evidence that nitric oxide, derived from NOS, plays an important role in renal function, both under normal and pathophysiologic conditions [7, 8] and that high levels of nitric oxide produced by iNOS are implicated in the renal dysfunction/injury associated with either renal I/R [10–14, 26, 27, 62–64] or during endotoxic and hemorrhagic shock [65, 66]. Several in vivo and in vitro investigations have demonstrated that inhibition of iNOS expression (e.g., using inhibitors of NF-κB activation), iNOS activity (e.g., using inhibitors of iNOS activity), absence of iNOS itself (using iNOS knockout mice), or scavenging of nitric oxide (e.g., using hemoglobin) can ameliorate or prevent nitric oxide–mediated renal injury [11–13, 25, 64–70] suggesting that nitric oxide, generated by iNOS, contributes to renal I/R injury. Thus, the inhibition of the expression of iNOS and subsequent reduced levels of nitric oxide contribute to the beneficial actions of tyrphostin AG126 against renal I/R injury.

Here we also demonstrate that the increase in the expression of COX-2 caused by renal I/R is abolished in the kidneys of rats treated with tyrphostin AG126. The promoter region of the murine and human COX-2 gene contains binding sites for NF-κB [71, 72]. The expression of the COX-2 gene is caused by oxidant stress [73], and ROS cause the activation of NF-κB [74], suggesting that NF-κB is one of the transcription factors involved. There is good evidence that an enhanced formation of prostanoids following the induction of COX-2 contributes to the pathophysiology of associated with I/R and inflammation [75, 76].

We report here that administration of tyrphostin AG126 markedly reduced the expression of both iNOS and COX-2 protein in kidneys obtained from rats subjected to I/R. These findings are in keeping with studies demonstrating that tyrphostin AG126 can reduce the expression of iNOS and COX-2 genes in shock and inflammation [38–40, 48–50, 55–57, 77]. We hypothesize here that tyrphostin AG126 mediates this effect via inhibition of the expression of NF-κB. There is now good evidence that certain protein tyrosine kinases play a pivotal role in the activation of NF-κB [78]. NF-κB is a member of a family of dimers belonging to the Rel/NF-κB family of polypeptides and the most frequently observed form of NF-κB is a dimer composed of two DNA-binding proteins, namely NF-κB (or p50) and RelA (or p65), although other dimeric combinations also exist [79]. Our hypothesis is supported by evidence obtained from in vitro studies this laboratory and by others, demonstrating that tyrphostin AG126 inhibits expression of iNOS and COX-2 in macrophages and pulmonary epithelial cells, respectively, via inhibition of the activation of NF-κB [48, 50].

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 [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 [80] 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 [81]. 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 both renal I/R and hypoxia-reoxygenation injury, respectively [25, 26]. Several studies have now demonstrated that reduction of the generation of peroxynitrite (e.g., by reduction of nitric oxide and superoxide levels) or the presence of peroxynitrite (e.g., using the peroxynitrite scavenger ebselen) can produce beneficial effects against renal I/R injury [25, 27]. We report here that tyrphostin AG126 reduces staining for nitrotyrosine residues in kidneys subjected to I/R injury and thus, reduces nitrosative stress. This is likely to be mediated by a reduction in nitric oxide levels subsequent to a reduction in expression of iNOS due to inhibition of the activation of NF-κB by tyrphostin AG126.

Peroxynitrite produces cellular injury and necrosis via several mechanisms, including peroxidation of membrane lipids, protein denaturation, and DNA damage [82–84]. Strand breaks in DNA triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARP, resulting in the depletion of its substrate $NAD^+$ and a subsequent reduction in the rate of glycolysis [82–84]. As $NAD^+$ functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed the *"PARP Suicide Hypothesis"* [82–84]. There is recent evidence that the activation of PARP may also play an important role in renal I/R and the development of ARF and that inhibition of PARP activity is beneficial against renal I/R injury [29, 30, 85]. We demonstrate here that tyrphostin AG126 attenuates the increase in PARP activity caused by renal I/R. This is also likely to be have been mediated by a reduction in peroxynitrite levels subsequent to inhibition of the activation of NF-κB by tyrphostin AG126 followed by a reduction in nitric oxide levels subsequent to a reduction in the expression of iNOS.

Phosphorylation of proteins on tyrosine residues by protein tyrosine kinases plays an important role in the regulation of cell proliferation, cell differentiation, and signaling processes in cells of the immune system [33, 34]. The receptor tyrosine kinases participate in transmembrane signaling, whereas the intracellular tyrosine kinases take part in the signal transduction to the nucleus [33, 34]. Enhanced activity of tyrosine kinases has been implicated in the pathophysiology of many diseases [35] and there is now good evidence that an increase in tyrosine kinase activity (secondary to genomic rearrangements, point mutations in-frame deletions or insertions, overexpression, and ectopic or unscheduled expression of growth factors) plays a pivotal role in cancer growth (e.g., chronic myeloid leukemia as well as certain solid tumors) [36, 42]. In addition to cancer, the excessive activation of tyrosine kinase is common in conditions associated with local inflammation (e.g., rheumatoid arthritis, atherosclerosis, psoriasis, diabetic retinopathy) or systemic inflammation (e.g., sepsis and septic shock) [35, 42]. Thus, it is not surprising that the identification and the development of novel therapeutic approaches for disease states linked to an enhanced activation of tyrosine kinase has become a major goal for scientists in both academia and in the pharmaceutical industry. In the past few years, more than 30 tyrosine kinase inhibitors have entered preclinical and clinical development and several ATP site–directed tyrosine kinase inhibitors are now in advances stages of clinical trials. Most notably, some of these small molecule tyrosine kinase-inhibitors (Gleevec™, Iressa™, Tarceva™) have already been approved for the use in patients with chronic myeloid leukemia [42]. The isoflavone genistein or specific tyrphostins (AG126, AG490, AG556, AG1641, or A1) have been used to inhibit the activity of tyrosine kinase in animal models of disease. Genistein inhibits a wide range of tyrosine kinases by acting as a competitive inhibitor of the binding of ATP and as a noncompetitive inhibitor of the substrate binding to tyrosine kinase [33]. The tyrphostins, on the other hand, act as competitive inhibitors of the substrate binding to tyrosine kinase [35]. To date, although there has only been one study demonstrating the beneficial action of AG556 against myocardial I/R [86], we report here for the first time, the reduction of renal I/R injury by the tyrosine kinase inhibitor tyrphostin AG126.

The recent sequencing of the human genome revealed that the kinome contains ~500 human protein kinase genes, of which more than 90 are tyrosine kinases. Thus, it is not surprising that the identification of a single agent that specifically targets a single kinase out of the whole human kinome has thus remained elusive. Thus, it is possible that the beneficial effects arising from the inhibition of a specific tyrosine kinase (or a group of tyrosine kinases) is offset by side-effects arising from the inhibition of other tyrosine kinases. As there is little information about the specificity and selectivity (toward different tyrosine kinases) of tyrphostin AG126 (or other tyrphostins), it is difficult to point out which of the many known tyrosine kinases play a crucial role in the pathophysiology of I/R injury. For example, although tyrphostin AG126 has been shown to inhibit the mitogen-activated protein (MAP) kinase p42 (ERK2), it is also possible that tyrphostin AG126 attenuates the activation of the p38 MAP kinase pathway, which is known to play an important role in the development of renal I/R injury [87]. Further studies aimed at elucidating which tyrosine kinase(s) is/are inhibited by tyrphostin AG126 are warranted. Nevertheless, our results support the view that inhibitors of (some) tyrosine kinases (including tyrphostin AG126) may be useful in the therapy of renal I/R injury.

Thus, in the present study we demonstrated for the first

time that the protein tyrosine kinase inhibitor tyrphostin AG126 reduces renal dysfunction and injury caused by renal I/R. The mechanisms by which tyrphostin AG126 provides these beneficial effects have still to be clearly defined but in this study we demonstrate that tyrphostin AG126 reduces the expression of iNOS and nitric oxide production and the renal expression of COX-2. We propose that tyrphostin AG126 reduces the activation of the transcription factor NF-κB, thereby reducing the expression of proinflammatory genes. Furthermore, we demonstrate here that tyrphostin AG126 reduces peroxynitrite formation and PARP activation, both of which are associated with the development of renal I/R injury. The data presented here suggest that clinical studies could be considered for the evaluation of the therapeutic potential of tyrphostin AG126 (or similar agents) in improving the course of ischemic ARF.

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*Reprint requests to Professor Christoph Thiemermann, Department of Experimental Medicine, Nephrology & Critical Care, William Harvey Research Institute, Queen Mary – University of London, Charterhouse Square, London, EC1M 6BQ, UK.*
*E-mail: c.thiemermann@qmul.ac.uk*

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