Pargyline reduces renal damage associated with ischaemia–reperfusion and cyclosporin

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Abstract

Background. The slow deterioration of the kidney graft is characterized histologically by interstitial fibrosis and tubular atrophy (IFTA). Immunological and non-immunological stress is the main cause of progression towards IFTA. Our study focused on the non-immunological injuries induced by ischaemia–reperfusion (IR) and cyclosporin (CsA) toxicity, which remain the two stress factors putting a damper on the outcome of the renal graft. Endogenous reactive oxygen species (ROS) are essentially produced by mitochondria, and we have previously shown that the blockage of the mitochondrial enzymes monoamine oxidases (MAOs) prevents H2O2 production in the early reperfusion stage following IR.

Methods. We used a rat model of IFTA consisting in unilateral nephrectomy followed by IR and daily CsA administration. Four weeks after IR, we analysed renal function, histological alterations and a number of inflammatory and fibrotic genes.

Results. We observed, 28 days after pargyline-mediated blockade of MAO (before or after IR), improved renal function as well as a net decrease in renal inflammation associated to lower IL-1β and TNF-α gene expression. However, significant reduction in apoptosis, necrosis and fibrosis was only observed when pargyline was administered before IR. This protective effect was associated to decreased expression of TGF-β1, collagen types I, III and IV and also to the normalization of antioxidant (SOD1, catalase) and inflammatory (COX2, LOX5) gene expression.

Conclusion. It appears that the blockage of ROS produced by MAO and subsequent cell death might be an effective protective strategy against IFTA progression.

Keywords: IFTA; ischaemia–reperfusion; MAO; oxidative stress

Introduction

The number of cases of acute renal allograft rejection is decreasing [1]. Therefore, clinicians are now increasingly facing late allograft loss characterized by the progressive decline in renal function. This graft dysfunction is usually associated to tissue damage characterized by interstitial fibrosis and tubular atrophy (IFTA [2]). IFTA can be due to various immunological and non-immunological insults [3]. The immunological factors have been, and continue to be, under intensive investigation [4]. Among the non-immunological causes, ischaemia–reperfusion (IR) and renal toxicity induced by chronic administration of calcineurin inhibitors such as cyclosporin (CsA) are clearly two stress factors where no satisfactory countermeasures have been proposed.

Oxidative stress caused by the excessive production of reactive oxygen species (ROS) after reperfusion plays a central role in the mediation of IR injury leading to cell death by apoptosis and necrosis [5]. Oxidative stress is, in the majority of the cases, exacerbated by immunosuppressive therapies, mainly those using CsA [5]. In addition, oxidative stress and CsA stimulate macrophage infiltration and fibroblast activation responsible for interstitial fibrosis. Whereas exogenous sources of ROS originate from inflammatory cytokines, environmental toxins, chemotherapeutics and UV light, endogenous ROS are mainly produced by mitochondria [6]. In the kidney, the mitochondrial enzymes monoamine oxidases (MAOs) have been identified as an important source of intracellular ROS. Based on genetic criteria, substrate specificity and inhibition by selective compounds, these enzymes have been classified into two isoforms, MAO-A and MAO-B. However, in the rat kidney, the predominant active isoform is MAO-A. Indeed, renal MAO-A activity is higher than MAO-B [7]. In addition, we have previously reported that, in rat kidney cortex, H2O2, produced by MAO-A, plays a critical role in oxidative stress, apoptosis and necrosis in the early reperfusion...
stage following ischaemia [8]. Indeed, the blockade of MAO by the irreversible MAO inhibitor pargyline (Pg) prevented H₂O₂ production and decreased tubular apoptosis and necrosis [8]. Based on these previous studies and since the long-term outcome of kidney graft is influenced by events occurring within 48 h time period before and after IR injury, we questioned whether these early protective effects of MAO blockade could improve renal structure and function on the long term.

Therefore, the aim of the present work was to investigate the long-term impact of early MAO inhibition (before or after ischaemia) in a rat model mimicking the non-immunological insults involved in IFTA induced by the association of IR and CsA in uninephrectomized animals.

Materials and methods

Animals
Male Sprague–Dawley rats (8 weeks old, weighing ~250 g, Harlan ZI Du Malcourt, France) with free access to normal salt diet and water ad libitum were used. Experiments were conducted in accordance with the European Communities Council Directive (86/609/EEC) for experimental animal care and were approved by the local animal care and use committee.

Drug treatments
Pg was purchased from Sigma-Aldrich Co, Ltd (Ilkirch, France), and cyclosporin (CsA) was provided by Novartis Pharmaceutical (Néoral® Basel, Switzerland). As previously described [9], Pg was dissolved in water and administered i.v. at a single dose (6 mg/kg). Pargyline was administered 15 min either before ischaemia (group +PgB) or 30 min after reperfusion (group +PgA). CsA was diluted in olive oil and was administered by gavage at a dose of 15 mg/kg/day (group +CsA).

Surgery protocols
Unilateral nephrectomy. Under anaesthesia with isoflurane/oxygen inhalation (3%/97%), an incision was made in the left flank, and the kidney was isolated (adrenal gland remained intact), ligated with 4–0 silk suture, and excised. The flank incision was sutured and cleaned with antimicrobial agent (Betadine®). Renal ischaemia–reperfusion. Seven days after unilateral nephrectomy, rats were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.), and ischaemia was induced by clamping the right renal pedicle for 45 min using atraumatic vascular microclamps (Arenx, France). After clamp removal, the right kidney was inspected for restoration of blood flow. Twenty-four hours after reperfusion, all rats were treated daily with CsA for 7 or 28 days. Control-operated animals were subjected to the same surgical procedure without clamping the renal pedicle. Thus, animals were divided into six groups for each treatment time period. Three control groups: CsA+vehicle (CsA); CsA+PgB (CsA+ Pg after (CsA+PgB); CsA+Pg after (CsA+PgA) and three IR groups: IR+CsA+vehicle (IR+CsA); IR+CsA+PgB (IR+CsA+PgB); IR+CsA+Pg after (IR+CsA+PgA). Each group consisted of six animals.

Rats were weighed then euthanized and kidneys removed and weighed. Blood samples were collected with heparinized syringes from aortic abdominal artery and centrifuged for 10 min at 8000 rpm, and plasma was stored at −80°C. The right kidney was isolated, dissected longitudinally into two parts: one was fixed in Carnoy’s solution for histopathological analysis and the other divided into three sagittal parts, snap frozen in liquid nitrogen and stored at −80°C for RNA, protein, and enzymatic activity measurements.

Monoamine oxidase-A activity
The monoamine oxidase-A activity was determined using a specific radioactive substrate 14C-[5HT] (NEN® radio-chemicals) as previously described [8]. Results are expressed as picomole of substrate oxidized per minute per milligram of protein.

Renal function
Plasma creatinine (µM) and blood urea nitrogen (mM) were measured with a Luminex 100 IS system (Luminex Corporation, USA).

Renal morphology
For light microscopic investigations, renal tissue specimens were fixed in Carnoy’s solution and embedded in paraffin. Paraffin sections (4 µm) were stained with haematoxylin/eosin (H&E), periodic acid–Schiff (PAS) and Sirius red. All tissue sections were semi-quantified microscopically by an expert pathologist blinded to the treatment conditions. Chronic tubular injury was defined by tubular atrophy with irregular thickening of the tubular basement membrane (stained by PAS: ×200). The percentage of fibrosis was estimated by Sirius red (×200). The percentage of tubular necrosis in all kidneys was defined by brush border loss and desquamation of tubular epithelial cells, cellular debris in the tubular lumen with cast formation and tubular dilatation (revealed by H&E: ×20). Percentage of tubulointerstitial inflammation was estimated by infiltration of mononuclear cells in the interstitium of all kidney parenchyma (H&E: ×400).

Oxidative DNA damage was evaluated by immunodetection of 8-hydroxy-2’-deoxyguanosine (8-OHdG). Kidney sections were incubated with an 8-OHdG monoclonal antibody (1:200, AbCys S.A) for 1 h at 37°C. After washing with TBS-Tween, 0.01%, the sections were incubated with a secondary anti-mouse HRP antibody (Dako Cytomation) for 30 min at room temperature. Peroxidase activity was measured using DAB (Dako Cytomation) as a substrate. Representative image showed positive stained nuclei at ×400 magnification.

Gelatin zymography
Latent and active forms of MMP-2 (gelatinase A) were analysed by gelatin zymography as described previously [10]. The per cent of intensity of the active forms (A) and latent forms (L) was quantified by gel densitometry using the ‘Image J’ software.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling assay
Apoptosis was evaluated with the DeadEnd Fluorometric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) system according to the manufacturer’s instructions (Promega, USA) as described previously [9]. Values are expressed by the number of fluorescent cells counted in 10 fields at ×200 magnification.

Determination of mRNA expression
Total RNA was isolated from rat frozen tissue using Qiagen RNasey Plus Mini kit (Qiagen, Valencia, CA, USA). RNA content was measured by a ND-1000 spectrophotometer. RNA quality was evaluated by Experion TM RNA HighSens Analysis Kit (BIO-Rad, US). cDNA was synthesized from similar amounts of RNA (2 μg) using the SuperScriptTM II Reverse Transcriptase kit (Invitrogen, CA). Real-time PCR was performed using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, USA). PCR amplification was performed with 15 ng of cDNA sample, 300 nM of forward and reverse primers (Table 1) in a final volume of 20 μL of Power SYBR Green qPCR Master Mix (DyNanoTM Flash SYBER Green qPCR kit, FINNZYMES). The reaction mixture was preheated at 95°C for 10 min, followed by 40 cycles at 95°C/15 s and 60°C/1 min. Relative gene expression was calculated by the comparative Ct method (2 ΔΔCt) using HPRT as reference gene.

Western Blot analysis
Kidney samples were homogenized in lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 0.1% SDS) with a protease inhibitor cocktail (Complete, Mini, Roche) and separated as previously reported [11]. Nitrocellulose membranes were blocked with
TBS-0.1% Tween +5% BSA at room temperature for 2 h and probed with monoclonal mouse anti-Bax (1:500), polyclonal rabbit anti-Bcl2 (1:500) overnight at 4°C. The membranes were then incubated for 1 h with the appropriate secondary antibody (1:100 000) conjugated with peroxidase (ECL® anti-rabbit or ECL® anti-mouse or ECL® anti-goat, GE Healthcare-little chalfont-GB). Detection was performed with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, USA) and analysed by the photon detector Gene Gnome® (Syngene Bio Imaging). Membranes were reprobed with polyclonal goat anti-GAPDH (1:500). Protein bands were quantified by densitometry using ImageJ software and results expressed as protein of interest/GAPDH ratio. All primary antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Thiobarbituric acid reagent substance assay
As an index of lipid peroxidation and oxidative stress, thiobarbituric acid reactive substances (TBARS) were determined according to the method of Ohkawa et al.[12]. Briefly, 50 μL of kidney homogenates or malone dialdehyde (MDA) standards were mixed with 50 μL of 0.8% thiobarbituric acid (TBA, w/v) and 5 μL of HCl 0.5 N (v/v). These mixtures were incubated at 95°C for 10 min and extracted, after cooling, with 200 μL of butanol-1 by centrifugation at 2000 g for 10 min. The absorbance of the pink organic phase formed was spectrophotometrically evaluated at 532 nm. TBARS concentrations were quantified by reference to MDA absorbance. Results were expressed as nanomoles TBARS per milligram of protein.

Statistical analysis
All data were expressed as means ± SEM. Groups of data were compared with an analysis of variance (ANOVA) followed by post hoc tests. A value of P < 0.05 was regarded as significant.

Results
MAO-A activity after pargyline treatment
As previously reported [9], a single injection of Pg inhibited MAO-A activity 1 h after reperfusion. This inhibition is short term as MAO-A activity returned to basal levels at 7 days post-reperfusion (Figure 1). Therefore, the effects observed in this study 28 days post IR are independent of a prolonged effect of MAO-A inhibition.

### Table 1. Primer sequences

<table>
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<tr>
<th>Gene (Rat) Name</th>
<th>GenBank accession numbers</th>
<th>Forward primer 5′-3′</th>
<th>Reverse primer 5′-3′</th>
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<tr>
<td>IL-1β Interleukine 1 beta</td>
<td>NM_031512</td>
<td>CTGACAGACCCAAAAGATTAAGG</td>
<td>CTTTGTGAGATGCTGTGTA</td>
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<tr>
<td>MMP-2 Matrix Metalloproteinase 2 Transforming Growth Factor β1</td>
<td>NM_031054</td>
<td>AGTAGAGCTGGCTTTAATCAGAGT</td>
<td>TCCTGCAAGCTTAAAGAGAAAC</td>
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<tr>
<td>TNF-α Tumour Necrosis Factor alpha</td>
<td>NM_012675</td>
<td>ACGGAAAGCATGATGAGATG</td>
<td>GAAGAGGCTGAGCAGCAGCA</td>
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<tr>
<td>Coll III Collagen III</td>
<td>NM_032085</td>
<td>ACAGCAGTCAAATGATTAGAATT</td>
<td>CCCCAGTCGACACATATT</td>
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<tr>
<td>Coll I Collagen I</td>
<td>NM_053394</td>
<td>GCCTGATCTGATCTGCCCAC</td>
<td>CATCGCCCTCCGTGGTTT</td>
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<tr>
<td>Coll IV Collagen IV</td>
<td>NM_001135759</td>
<td>ATTCCTTGTGATGACACCCAG</td>
<td>AAGCTGTTAAGTCCTGAGTA</td>
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<tr>
<td>SOD1 CuZnSOD</td>
<td>NM_017050</td>
<td>TGGTCTGATGAGATGCTGTG</td>
<td>CTTCACAGCTTACCTGTG</td>
</tr>
<tr>
<td>Catalase</td>
<td>NM_012520</td>
<td>CAAGCCTGAATGAGGAAGTG</td>
<td>TTGGAAAGATCTGAGGAGCC</td>
</tr>
<tr>
<td>SOD2 MnSOD</td>
<td>NM_017051</td>
<td>TGAGCAAATCTGACAGTCCAGG</td>
<td>CTACGAGCAGGGTTTGG</td>
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<tr>
<td>GPX1 Glutathione peroxidase 1</td>
<td>NM_030826</td>
<td>AATCAGTTCCGGACATCGAGG</td>
<td>GAAGGTAAGAGCGGTG</td>
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<tr>
<td>COX2 prostaglandin-endoperoxide synthase 2</td>
<td>NM_017232</td>
<td>CTGTGCGATGCTTTCCAGA</td>
<td>AAGGATTTCTGTCATGGC</td>
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<tr>
<td>LOX 5 Arachidonate 5-lipoxygenase</td>
<td>NM_012822</td>
<td>AGAGTTCCCTTGTATGTTG</td>
<td>GTGTTTTCCAGTTCTT</td>
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<tr>
<td>HPRT hypoxanthine phosphoribosyltransferase</td>
<td>NM_012583</td>
<td>TGACACTGTTAAACCAATGCAGACT</td>
<td>AGAGGTCCTTTTACCAAG</td>
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Fig. 1. Effect of pargyline administration on MAO-A activity in rats after IR. We analysed the efficiency of pargyline administration on MAO-A activity in our model of unilateral nephrectomy followed by ischaemia–reperfusion (IR) on male Sprague–Dawley rats receiving a daily treatment of cyclosporin (CsA). Rats are treated by vehicle (veh) or by pargyline 15 min before IR (+PgB) and 30 min after reperfusion (+PgA). MAO-A activity was detected by the use of a specific radioactive substrate [14C]-[5HT] in rats 1 h or 7 days post-reperfusion. Results are expressed as picomoles of substrate oxidized per minute per milligram of protein. Values are expressed as mean ± SEM. **P < 0.01 (vs Control veh) and #P < 0.05, ##P < 0.05 (vs IR veh).
Kidney function

After 28 days, control animals receiving either CsA alone or CsA ± Pg before or after sham surgery exhibited normal renal function (Table 2). Conversely, in the IR groups, kidney function was altered as early as 7 days after the insult (not shown) and remained significantly altered at 28 days as shown by the increased plasma creatinine and blood urea nitrogen levels. Interestingly renal function was significantly improved by Pg, independently of the time of administration. In addition, the increased ratio kidney/body weight induced by IR was significantly attenuated.

### Table 2. Effect of pargyline treatment on the kidney/body weight, plasma creatinine and BUN

<table>
<thead>
<tr>
<th>Day 28</th>
<th>Unx</th>
<th>CsA</th>
<th>CsA+PgB</th>
<th>CsA+PgA</th>
<th>IR+CsA</th>
<th>IR+CsA+PgB</th>
<th>IR+CsA+PgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Kidney/body weight) × 100</td>
<td>3.47 ± 0.10</td>
<td>3.88 ± 0.2</td>
<td>4.03 ± 0.3</td>
<td>8.19 ± 1.37**</td>
<td>4.80 ± 0.16***</td>
<td>5.04 ± 0.19***</td>
<td></td>
</tr>
<tr>
<td>Plasma creatinine (μM)</td>
<td>44.25 ± 1.49</td>
<td>40.7 ± 3.18</td>
<td>40.78 ± 4.0</td>
<td>145.9 ± 38*</td>
<td>46.75 ± 5.4****</td>
<td>54.5 ± 9.86***</td>
<td></td>
</tr>
<tr>
<td>BUN (mM)</td>
<td>7.94 ± 1.04</td>
<td>7.86 ± 1.84</td>
<td>6.34 ± 3.79</td>
<td>18.69 ± 4.7*</td>
<td>9.4 ± 0.8***</td>
<td>9.37 ± 2.61***</td>
<td></td>
</tr>
</tbody>
</table>

We determined the per cent of kidney vs body weight, plasma creatinine (μM) and blood urea nitrogen (BUN; mM) of control (CsA), CsA treated with Pg before or after surgery (CsA+PgB, CsA+PgA); ischaemia–reperfusion (IR+CsA); pargyline-treated rats 15 min before IR (IR+CsA+PgB) and 30 min after reperfusion (IR+CsA+PgA) at 28 days post IR (n=6 for each group).

Results are expressed as means ± SEM.

*P < 0.01, **P < 0.001 (vs CsA) and ***P < 0.05, ****P < 0.01 (vs IR+CsA).

### Kidney function

Fig. 2. Effect of pargyline treatment on fibrosis, tubular atrophy, necrosis and inflammation at 28 days post IR. A. Representative photomicrographs of kidney sections stained with Sirius red (magnification ×200) from control (CsA), ischaemia–reperfusion (IR+CsA) and pargyline-treated before IR (IR+CsA+PgB) and after IR (IR+CsA+PgA) rats at 28 days post surgery. Semi-quantitative results are expressed by per cent Sirius red staining in kidney sections. B. Representative photomicrographs of kidney sections stained with PAS (magnification ×200) from the same groups. Kidney from IR+CsA showed a dramatic tubular dilatation and atrophy with thick basement membrane (black arrows) compared to CsA. These pathological features are reduced significantly in IR+CsA+PgB compared to IR. Semi-quantification data for tubular atrophy expressed as per cent of tubular atrophy in kidney sections was represented 28 days after IR. C. Representative photomicrographs and semi-quantification of tubular necrosis. Kidney sections were stained with haematoxylin/eosin (H&E, magnification ×20). Necrosis was defined by brush border loss and desquamation of tubular epithelial cells, cellular debris in the tubular lumen with cast formation (black arrows) and tubular dilatation. D. Inflammation was estimated by the infiltration of mononuclear cells that appeared in the interstitium at 28 days. Semi-quantification data for inflammatory cells expressed as per cent of inflammatory cells in kidney sections was represented (H&E, magnification ×400). Data are expressed as means ± SEM (n= 6 for each group). *P < 0.05, **P < 0.01, ***P < 0.01 (vs CsA) and # P < 0.05, ## P < 0.01 (vs IR+CsA).
by Pg administration (Table 2). As described previously [13,14], sham animals receiving CsA under a normal salt diet for 28 days had no detectable morphological, IFTA changes, and Pg administration was without effect (CsA +PgB, CsA+PgA not shown).

**Kidney morphology**

At 7 days post IR, kidneys had no IFTA (not shown). As expected, the combination of IR injury and CsA administration led to a dramatic increase in interstitial fibrosis tubular atrophy and necrosis 28 days post surgery (Figure 2A–C, respectively). This increase in IFTA and necrosis was significantly reduced only when Pg was administered before IR.

Because IFTA is most of the time accompanied by chronic inflammatory cell infiltration, we have scored the mononuclear cell infiltrate in the interstitium and found that Pg administration either before or after IR induced a significant reduction of this inflammatory infiltrate (Figure 2D).

**Extracellular matrix accumulation**

Fibrosis is characterized by extracellular matrix (ECM) protein (mainly collagens) accumulation resulting from an imbalance between its synthesis and degradation. To understand the protective effects of Pg, we have analysed the expression of ECM compounds known to be accumulated under pathological conditions. We found that administration of Pg before IR reduced collagen type I, III and IV mRNA expression (Figure 3A) 28 days post IR. To investigate the degradation process, we looked at matrix metalloproteinase-2 (MMP2) expression and activity and found a significant reduction of mRNA expression and enzyme activity (Figure 3B). Consistent with the absence of the protective effect of Pg administration after IR on interstitial fibrosis (Figure 2A), no effect was observed on collagen mRNA expression as well as MMP2 expression and activity.

**Inflammatory and profibrotic cytokines**

To gain further insight in the mechanism of action of Pg, we studied the expression of important cytokines known to be involved in the fibrotic process. A dramatic reduction in the mRNA expression of the profibrotic cytokine TGF-β was only observed in the group receiving Pg before IR insult (Figure 4). Interestingly, Pg administration (before or after IR) was associated with a significant reduction in mRNA expression of inflammatory cytokines IL-1β and TNF-α.

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**Fig. 3.** Effect of pargyline treatment on fibrosis markers at 28 days post IR. **A.** Renal mRNA expression of collagen I, collagen III and collagen IV studied by qRT–PCR 28 days post IR. The results are normalized to reference gene HPRT and referred to the CsA expression levels. B. Renal gene expression of MMP2 and MMP2 activity in non-treated and treated rats subjected to IR+CsA were studied. Gelatin zymograms showing the latent (L) and active (A) forms of MMP2 was quantified by ‘Image J’ software, and the per cent of activity was represented for each form. Data are expressed as means ± SEM (n= 6 for each group). *P < 0.05, **P < 0.01, ***P < 0.001 (vs CsA) and # P < 0.05, ## P < 0.01 (vs IR+CsA).
Apoptosis

An important signalling pathway related to tubular atrophy, induced by oxidative stress following IR, is apoptosis. The number of apoptotic cells, mainly located in proximal tubule at the cortico-medullary junction, was significantly increased in the IR group, and Pg administration before IR significantly attenuated apoptosis (Figure 5A). This effect was associated to a decreased Bax expression and an increased Bcl2 expression (Figure 5B).

SOD1, catalase, COX2, LOX5 mRNA expression

To obtain further details on the beneficial antioxidant effects of Pg administration on kidney damage induced by both IR+CsA administration, we analysed mRNA expression of some of the important genes involved in oxidative stress and inflammation. As shown in Figure 6, injection of Pg before IR clearly up-regulated antioxidant genes (SOD1 and catalase) and down-regulated COX2 and LOX5 gene expression, which are both involved in the production of ROS [15]. This antioxidant effect appeared to be specific since no effect was observed on SOD2 and glutathione peroxidase (GPX1) (not shown). Administration of Pg after IR was without significant effects on SOD1, catalase and COX2 expression but reduced LOX5 expression.

Oxidative stress markers

Oxidative DNA damage was evaluated by 8-OHdG immunostaining in all kidney sections [16]. At 28 days post IR, tubular cells were highly 8-OHdG positive. In addition, in the interstitium, these 8-OHdG-positive cells were associated to the fibrotic areas (Figure 7A). Interestingly, Pg-treated rats before IR reduced significantly the number and the intensity of stained cells. To confirm this histological assessment, we evaluated TBARS, an index of lipid peroxidation. As shown in Figure 7B, TBARS was increased in IR groups and significantly reduced in the group treated with Pg before IR.

Discussion

The main result of our study is that pre-treatment with a single dose of Pg, an irreversible MAO inhibitor, significantly ameliorates long-term kidney function and structure in a rat model of IFTA progression.

It is now well accepted that IR injury and chronic calcineurin inhibitor administration such as CsA following kidney transplantation can lead to IFTA [3,17]. In our model, the animals are uninephrectomized followed by IR and, to mimic better the human situation, the animals received a daily administration of CsA for 28 days. The worsening effect of CsA is not related to direct tubular injury but has been attributed to additional vasoconstriction following the IR insult [18]. Consistent with other studies using a similar experimental model [19,20], we found that compared to IR alone, CsA aggravated the long-term outcome of the pathology as stated by the dramatic decline in the renal function characterized by an increase in creatininaemia and blood urea nitrogen and also histologically as demonstrated by the increase in IFTA. In addition, this model is associated with an increase in apoptosis and necrosis, two well-known mechanisms related to the deleterious effects of IR injury and CsA [21,22].

In this pathological context, an important event associated to CsA administration and IR injury, more precisely to reperfusion, is the excessive production of ROS (hydrogen peroxide, superoxide anions, hydroxyl radical) above the physiological scavenging capacity of the kidney leading thus to renal tissue damage [5,23]. Whereas in theory, targeting oxidative stress by antioxidants should have been an efficient therapy, mitigated and even controversial data have been obtained. Indeed, in the IR injury animal models, vitamin E [24] and the combination of a xanthine oxidase inhibitor and a hydroxyl radical scavenger [25] have no apparent beneficial effect, whereas a nonpeptidyl mimic of superoxide dismutase [26], N-acetylcysteine [27] and pargyline [8] have protected against tissue damage. However, those
studies only reported short-term effects of the antioxidant therapy. As all studies underlined the fact that excessive ROS production following reperfusion might be a major and determinant event in the appearance of IFTA, we hypothesized that the early blockage of ROS production could exert a long-term protective effect.

Our previous studies [8,9] demonstrated that renal MAOs were clearly involved in the production of ROS following kidney IR injury. In the present study, we provided evidence that this early blockade also significantly decreases long-term renal damage. We first looked at interstitial fibrosis and tubular atrophy. Compared to untreated animals, a single injection of Pg, before performing IR injury, significantly reduced both IF and TA. Because IF results from an imbalance between synthesis and degradation of ECM proteins [28], these two processes were studied. We found that Pg injection, before IR injury, was associated with a decreased expression of ECM components and also with decreased MMP2 expression and activity, suggesting a reduction in the synthesis of collagens rather than activation of ECM degradation. In addition and consistent with a decreased synthesis of the ECM, we observed that Pg administration was associated with a reduced TFG-β1 expression. Our data are in line with recent in vitro work showing the close interplay between ROS and TGF-β1 expression in the mechanism of epithelial to mesenchymal transition [29,30]. Those data were confirmed in vivo in a kidney transplant model where ROS production was associated to the over-expression of collagens I and III [31].

IR injury aggravated by CsA results in an intense inflammatory response characterized by inflammatory cell infiltration [17,32]. As expected, we found an increased infiltration of mononuclear cells in the tubulointerstitium associated to an increased expression of inflammatory cytokines IL-1β and TNFα. This inflammatory event was significantly attenuated by Pg injection either before or after the IR insult. This effect is consistent with the already described anti-inflammatory effect of other antioxidants [26,27]. Surprisingly, in the animals receiving Pg after IR, we observed that, despite a lower inflammation, IF remained unaffected suggesting that in this animal model inflammation is a secondary event compared to the oxidative stress in the fibrotic process. This dissociation between kidney inflammation and fibrosis has already been
described in a ureteral obstruction-induced renal fibrosis model where a significant increase in interstitial fibrosis was observed in angiotensin type 2 receptor knockout mice compared to wild type without modification of macrophage infiltration [33]. Similarly, we observed a significant increase in interstitial fibrosis in bradykinin B2-receptor knockout mice, whereas tissue inflammation assessed by the macrophage infiltrate was clearly decreased [10]. An explanation of this dissociation might reside in macrophage polarization, which has been shown to play an important role in inflammation and tissue fibrosis [34]. Indeed it is possible that, although total inflammation is reduced, the remaining macrophages are polarized towards the M2 subtype that can produce large amounts of TGF-β, thereby promoting fibrosis. Further studies are necessary to clarify this point.

There is now a large body of evidence showing that renal tissue damage following the IR insult involves cell death [35]. We thus examined cell apoptosis and necrosis in our animal model. Consistent with our previous work [8], we found that pre-treatment with Pg before IR strongly reduced apoptosis and necrosis even 28 days after the IR insult and continuous CsA treatment. The anti-apoptotic effect was associated with reduced expression of Bax and over-expression of Bcl2. No effect was observed when Pg was administrated after IR, which is consistent with the absence of protective effect on kidney structure.

Fig. 6. Effect of pargyline on renal gene expression profile. Renal expression of genes involved in oxidative stress (SOD1, catalase, COX2, LOX5) was studied by qRT–PCR at 28 days post IR. The results of the qRT–PCR are normalized to reference gene HPRT and referred to CsA expression levels. Data are expressed as means ± SEM (n= 6 for each group). *P < 0.05, **P < 0.01 and (vs CsA) and #P < 0.05, ## P < 0.01 (vs IR+CsA).

Fig. 7. Effect of pargyline on oxidative stress and lipid peroxidation. A. Panels show representative immunohistological staining for 8-OHdG in kidney sections from CsA, IR+CsA, IR+CsA+PgB and IR+CsA+PgA at 28 days post IR. As expected, in the CsA panel, endothelial cells were stained by 8-OHdG (black arrows). A dramatic increase in 8-OHdG staining was observed in the IR+CsA panel. Administration of Pg before IR strongly reduced 8-OHdG staining (panel IR+CsA+PgB), whereas administration of Pg after IR was without effect (panel IR+CsA+PgA). Dotted arrows show the positive nuclei stained by 8-OHdG (magnification ×400). B. TBARS, as an index of oxidative stress and lipid peroxidation, was evaluated in kidneys of all experimental groups at 28 days post IR. The TBARS concentration was expressed as nanomoles TBARS per milligram of protein. Data are expressed as means ± SEM (n= 6 for each group). *P < 0.05 (vs CsA) and ## P < 0.01 (vs IR+CsA).
Pargyline reduces renal damage associated with IR and CsA

Importantly, whereas the blockade of MAO activity induced by a single injection of Pg only lasted a few days [9], this was sufficient to induce significant antioxidative and anti-inflammatory effects as stated by, on the one hand, the decreased DNA oxidative stress and lipid peroxidation (assessed by 8-OHdG and TBARS) and, on the other hand, the normalization of antioxidant (SOD1 and catalase) and inflammatory (COX2 and LOX5) gene expression, which were, respectively, down- and up-regulated in this animal model of IR injury aggravated by CsA. This antioxidative effect is of particular interest since it was shown that the combination of SOD and catalase mimetics exerts beneficial effect, whereas SOD mimetics alone are without effect [36]. Although Pg administration before or after IR injury was accompanied by improved renal function, the protective effect on kidney tissue was only clearly observed when Pg was injected before IR injury and CsA administration, suggesting that, to be fully efficient at the long term, it is necessary to block, in advance, the systems responsible for the overproduction of ROS and subsequent cell death. The limited protective effects on IFTA observed by Pg administration after IR injury have to be related to the restricted inhibition of inflammation without effect on TGF-β expression, apoptosis and probably unknown post IR mechanisms that need further investigation.

In conclusion, we have shown that pre-treatment with Pg, most probably preventing ROS flooding during the reperfusion phase, has long-lasting effects on preservation of renal structure in a model of IFTA. The prevention of the oxidative stress by Pg leads to a strong decrease in inflammatory cytokine expression (IL-1β, TNFα) and an important decreased TGF-β expression which is the most profibrotic cytokine. This was paralleled by a significant decrease in the accumulation of the ECM as well as apoptosis and necrosis leading to significantly less IFTA at the long term.

On a putative therapeutic point of view, one should recall that the use of irreversible MAO inhibitors was abandoned because of severe secondary effects due to the accumulation of the tyramine pressor effect called ‘the cheese effect’ [37]. We can imagine that the therapeutic use of this compound would be devoid of this secondary effect because, in the present study, kidney protection was observed after a unique dose of pargyline. Therefore, Pg pre-treatment might not only be beneficial in the acute phase of IR in renal transplantation but also in the later phase which is currently the major clinical challenge.

Conflict of interest statement. None declared.

References


The PI3K/Akt/mTOR pathway is activated in murine lupus nephritis and downregulated by rapamycin

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Abstract

Background. The mammalian target of rapamycin (mTOR) inhibitor, rapamycin, has been shown to inhibit the progression of murine lupus nephritis by virtue of its potent immunosuppressive properties. The phosphoinositide-3-kinase (PI3K)/Akt pathway is a major upstream activator of mTOR and has been implicated in the propagation of cancer and autoimmunity. However, the activation status of the PI3K/Akt/mTOR pathway in lupus nephritis has not been studied so far.

Methods. In NZBW/F1 female mice, we examined the glomerular expression of Akt and mTOR by immunofluorescence and western blot. We also searched for specific phosphorylations of these kinases known to ensue during activation of the PI3K/Akt/mTOR pathway. In parallel, we examined the therapeutic role of rapamycin either before or after the development of overt lupus nephritis.

Results. We found that in untreated mice, as opposed to healthy controls, Akt and mTOR were over-expressed and phosphorylated at key activating residues. Rapamycin prolonged survival, maintained normal renal function, normalized proteinuria, restored nephrin and podocin levels, reduced anti-dsDNA titres, ameliorated histological lesions, and reduced Akt and mTOR glomerular expression activation.

Conclusions. These results suggest that: (i) the PI3K/Akt/mTOR pathway is upregulated in murine lupus nephritis, thus justifying treatment with rapamycin; (ii) rapamycin not only blocks mTOR but also negatively regulates the PI3K/Akt/mTOR pathway; and (iii) rapamycin is an effect-