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ORIGINAL PAPER

Clopidogrel protects from cell apoptosis and oxidative damage in a mouse model of renal ischaemia–reperfusion injury

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Abstract

Renal ischaemia-reperfusion injury (IRI) is consecutive to tissue oxidative damage and cell apoptosis that lead to acute renal failure (ARF) in renal allografts. The aim of this study was to investigate the beneficial effects of a pretreatment by clopidogrel on renal IRI in mice. IRI was induced by bilateral renal ischaemia for 45 min followed by reperfusion. Sixty-two healthy male BALB/c mice were randomly assigned to one of the following groups: PBS + ischaemia-reperfusion (IR); clopidogrel + IR; PBS + sham IR; clopidogrel + sham IR. Clopidogrel (25 mg/kg) or PBS was administered *per os* to the animals via a gastric cannula 24 h before operation. All mice were given a single dose of clopidogrel or PBS. Renal function histological damage, renal cell apoptosis, renal antioxidant activities, and CD41 expression were determined 24 h after reperfusion. The survival rates were evaluated over 7 days. Animals pretreated with clopidogrel had lower plasma levels of blood urea nitrogen (BUN) and creatinine, lower histopathological scores, and improved survival rates following IR. Renal cell apoptosis induced by IR was decreased in kidneys of mice pretreated by clopidogrel, with an increase in Bcl-2 and Bcl-xL expression and a decrease in caspase-3, caspase-8, and Bax expression. Renal reduced glutathione, superoxide dismutase, and catalase activities were unmodified by the pretreatment with clopidogrel. However, clopidogrel resulted in an increased total antioxidant capacity of the kidney. Furthermore, pretreatment by clopidogrel decreased the number of CD41-positive cells. Thus, clopidogrel exerts protective effects on renal IRI in mice by abrogating renal cell apoptosis as a consequence of improved renal antioxidant capacity and could be tried as a novel therapeutic tool in renal IRI.
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Keywords: kidney; ischaemia-reperfusion injury; apoptosis; oxidant damage; clopidogrel

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Introduction

Renal ischaemia–reperfusion injury (IRI) is associated with a high level of mortality and morbidity [1,2], and no specific treatment is presently available because of the poor understanding of the pathophysiolology. IRI is consecutive to a cascade of cellular events including the release of ROS, cell apoptosis, necrosis, infiltration by inflammatory cells, and the release of active mediators leading to tissue damage [3,4]. Restoring the blood flow, although critical to prevent ongoing injury, paradoxically potentiates the deleterious cascade and aggravates the pre-existing damage [5]. Among the various events that lead to IRI, apoptosis appears to be crucial [6]. Indeed, ischaemia induces cell apoptosis in various segments of the renal tubules [7,8], and the inhibition of apoptotic cell death abrogates the reperfusion-induced damage and can impact on the functional outcome independently of inflammation [6,9,10]. ROS plays a pivotal role in this apoptotic process [11–13] and in the production of pro-inflammatory cytokines that can further accelerate apoptotic signalling and cellular damage [14]. The organism protects itself through endogenous free radical scavenger systems. *In vivo* studies have shown that resistance to renal IRI can be induced by decreasing the oxidative stress and preserving antioxidant proteins [15,16]. IRI also involves platelet activation, since inhibiting thrombocyte activity reduces ischaemic tissue injury in the heart [17], lung [18], and pancreas [19]. Furthermore, the blockade of platelet P-selectin or its ligands shared with E- and L-selectins [20,21]

protects rats from ischaemic ARF [3]. Actually, platelet P-selectin, and not endothelial P-selectin, is the main determinant of the P-selectin-mediated renal injury.

Clopidogrel, a thioenopyridine-derived anti-platelet drug, has been used to prevent myocardial ischaemia–reperfusion damage [22] and acute ischaemia of the lower extremities [13]. The effect of the drug results from the antagonism between clopidogrel and a platelet ADP receptor, P2T. This antagonism is non-competitive, irreversible, and results in 50–70% of platelet fibrinogen binding. The protection offered by clopidogrel also results from the binding to a platelet adenosine diphosphate (ADP) P2Y12 purinergic receptor, which leads to inhibition of platelet activation and aggregation [23], and inhibition of free radical production [13]. The aim of the present study was to investigate whether clopidogrel can abrogate the harmful effects of IR in a murine model.

Materials and methods

Chemicals and reagents

Clopidogrel (Plavix) was obtained from Bristol-Myers Squibb/Sanofi Pharmaceuticals (Bridgewater, NJ, USA). The antibodies against caspase-3/-8, Bcl-2, Bcl-xL, Bax, Bad, and PARP were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). CD41 antibody was purchased from Abcam PLC (Cambridge Science Park, Cambridge, UK). A TUNEL assay kit was purchased from R&D Systems, Inc (Minneapolis, MN, USA). Total antioxidant assay kits were purchased from Sigma Inc (St Louis, MI, USA).

Animals

Healthy male BALB/c mice (Janvier SAS, Le Genest-St-Isle, France) weighing 20–25 g were used in all experiments. They were housed in macrolon cages under standard laboratory conditions (temperature 22 ± 2 °C and relative humidity 55%). The animals were given standard mouse chow and tap water *ad libitum*. This study was conducted in compliance with approved animal experimental procedures by the Animal Care and Use Committee at Paris Descartes University.

Study design

Mice were randomized into groups as follows: (1) IR group with clopidogrel ($n = 25$); (2) IR group with PBS ($n = 22$); (3) sham-operated group with clopidogrel ($n = 5$); (4) sham-operated group with PBS ($n = 9$); clopidogrel (25 mg/kg) or PBS was administered *per os* to the animals via a gastric cannula 24 h before operation for preventive treatment and (5) 3 h after operation for curative treatment. All mice were given a single dose of clopidogrel or PBS.

Mouse model of renal IRI

The mice were anaesthetized by intraperitoneal injections of 200 mg/kg avertin (0.04 M 2,2-tribromoethanol, 2.5% 2-methyl-2-butanol). Renal IRI was induced by bilateral clamping of renal arteries for 45 min, followed by reperfusion for 24 h. Briefly, a midline incision was made. Then the renal pedicles were exposed by abdominal incision and clamped bilaterally for 45 min. (Pilot studies were performed using 30 min and 35 min of ischaemia, but these periods of time did not lead to severe, reproducible increments in serum creatinine and BUN in our model.) For reperfusion, the clamps were released and the kidneys monitored for colour change to confirm blood reflow before suturing the incisions. After suturing, 0.3 ml of saline was given intraperitoneally. For the sham control groups, animals were operated on similarly without renal pedicle clamping. The surgery was conducted on a thermostatic stage and the body temperature was maintained at 35–37 °C with a heating pad. We used different sets of mice in the experiments for blood sample collections ($n = 14$; but 24 h after reperfusion two mice died in the IR + PBS group, so we included only the 12 remaining mice) and for the survival rate evaluation ($n = 11$; but a mouse died during the operation in the IR + PBS group, so only ten mice were included). For the curative treatment, only the survival rate was observed (IR + PBS, $n = 4$; IR + clopidogrel, $n = 8$). Except for mice whose survival was being monitored, animals were sacrificed 24 h after reperfusion and kidneys were isolated, quickly frozen in liquid nitrogen, and stored at −80 °C or at 4 °C in formol-acetic-alcohol (4% formol, 5% acetic acid, 75% absolute ethanol) until further analysis.

Assessment of renal function

Blood samples were obtained by puncture in the orbital angular vein 24 h after reperfusion and then centrifuged (2500 *g* for 10 min). The levels of serum creatinine and blood urea nitrogen (BUN) were measured.

Histopathological evaluation of kidneys

Kidneys were collected, cut coronally, fixed in 10% formaldehyde, and embedded in paraffin. Five-micrometre sections were prepared and stained with haemotoxylin and eosin (H&E). Sections were scored with a semi-quantitative scale designed to evaluate changes in the kidney 24 h after IR [24]. Specifically, one whole deep coronal section was examined under the microscope. The extent of tubular necrosis was graded based on the per cent involvement of the kidney: 0 = normal kidney; 1 = minimal necrosis (<5% involvement); 2 = mild necrosis (5–25% involvement); 3 = moderate necrosis (25–75% involvement); 4 = severe (>75% involvement).

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Immunofluorescence analysis

Five-micrometre sections of fixed kidney obtained 24 h after IR were deparaffinized in a xylene substitute (Histosol; Thermo-Shandon, Pittsburgh, PA, USA) and rehydrated through graded ethanol series. The sections were put in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and then heat-induced epitope retrieval was performed using a pressure cooker. The sections were rinsed in Tris-buffered saline (TBS) with 0.025% Triton X-100 and blocked for 2 h at room temperature in TBS with 5% normal mouse serum, 1% bovine serum albumin. Integrin alpha IIb (CD41) antigen was detected following incubation with 1 : 50 rabbit anti-CD41 antibody (Abcam PLC) overnight at 4 °C. After rinsing in TBS with 0.025% Triton, the sections were incubated with the corresponding secondary antibody conjugated with FITC (Sigma), diluted 1 : 160, for 60 min at room temperature. After a final wash, the sections were mounted with glycerol, coverslipped, and visualized using a fluorescence microscope. Immunostaining of kidneys sections was analysed using ImageJ 1.36 b software (National Institutes of Health; http://rsb.info.nih.gov/ij) for a quantitative assessment of the fluorescence intensity as described by Cooper Lab, Department of Cell Biology and Physiology, Washington University in St Louis (http://www.cooperlab.wustl.edu/).

Immunohistology analysis

Five-micrometre sections of fixed kidney obtained 24 h after IR were deparaffinized in Histosol (Thermo-Shandon) and rehydrated through graded ethanol series. The sections were put in 3% H_2O_2 and then proteinase K was used for proteinase digestion. The sections were rinsed in Tris-buffered saline (TBST) with 0.1% Tween 20 and blocked for 20 min at 4 °C in TBST with 5% bovine serum albumin. GR-1 antigen was detected following incubation with 1 : 50 rat anti-mouse Ly-6G and Ly-6C antibody (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at 4 °C. After rinsing in TBST, the sections were incubated with the corresponding secondary antibody conjugated with horseradish peroxidase (Zymed Laboratories, Carlsbad, Canada), diluted 1 : 100, for 30 min at 4 °C. A DAB (3,3′-diaminobenzidine tetrahydrochloride) Substrate Kit (Thermo-Fisher, Rockford, IL, USA) enabled chromogenic detection of horseradish peroxidase activity. After a final wash, the sections were mounted with Shandon-mount (Thermo-Shandon), coverslipped, and visualized using an Olympus microscope. Immunostaining of skin sections was analysed using ImageJ.

In situ TUNEL assays using a commercially available kit according to the manufacturer's recommendations

The terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL; R&D Systems Europe, Lille, France) staining assay was used to detect DNA strand-breaks. Fixed kidney sections obtained 24 h after renal IR were deparaffinized in xylene and rehydrated through a graded ethanol series. The number of TUNEL-positive nuclei per 200× field was evaluated in 25 fields per section.

Immunoblot analysis

Kidneys were crushed in ice-cold buffer [1 mM Tris–HCl buffer, pH 7.5, with a cocktail of protease inhibitors (Roche-Diagnostics, Basel, Switzerland), 25 mM NaF, 10 mM NaV, 0.5 mol/l EDTA, and 1% Triton X-100] and then centrifuged at 20 000 *g* for 20 min. Protein concentration was determined using the Bradford protein assay (Bio-Rad, Marnes-la-Coquette, France). Aliquots of 200 µg of protein extracts were separated on 10–15% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% milk in TBST buffer (10 mM Tris-base, 100 mM NaCl, 0.1% Tween 20, pH 8.0) and then probed overnight at 4 °C with either one of the following primary antibodies: rabbit polyclonal anti-mouse caspase-3, caspase-8, Bad primary antibodies; mouse polyclonal anti-mouse Bcl-2, Bcl-xL, Bax primary antibodies (1 : 200; Santa Cruz), or mouse monoclonal anti-β-actin (1 : 50 000; Sigma). Revelation was then performed with 1 : 1000 goat anti-rabbit or 1 : 1000 goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). Protein bands were detected using Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Images of blots were acquired for quantification using a Fujifilm LAS3000 digital imager and analysed with Fujifilm Multi-Gauge Imaging software (Fujifilm, Japan).

Determination of antioxidant enzymatic activities and total antioxidant capacity (TAC) in kidneys

The SOD activities of tissues were evaluated using the nitroblue tetrazolium reduction technique as previously described by Beauchamp and Fridovich [25]. The catalase activities of tissues were determined at 25 °C by UV spectroscopy at 240 nm according to Aebi [26]. Levels of GSH in tissues were measured by the method of Baker *et al* [27] and levels of TAC in tissues by the method of Erel [28]. Antioxidant enzyme activities and TAC in tissues were reported to the amount of proteins contained in each sample, measured with a Bradford Assay kit (Coomassie Plus Pierce-Perbio, Rockford, IL, USA).

Statistical analysis

The results were expressed as means ± SE. Data were analysed with one-way ANOVA plus the Tukey *post-hoc* multiple-comparisons test for comparisons of mean values among multiple groups. All analyses were carried out using the GraphPad Prism 5.0 statistical software package, and a p value of less than 0.05 was accepted as statistically significant.

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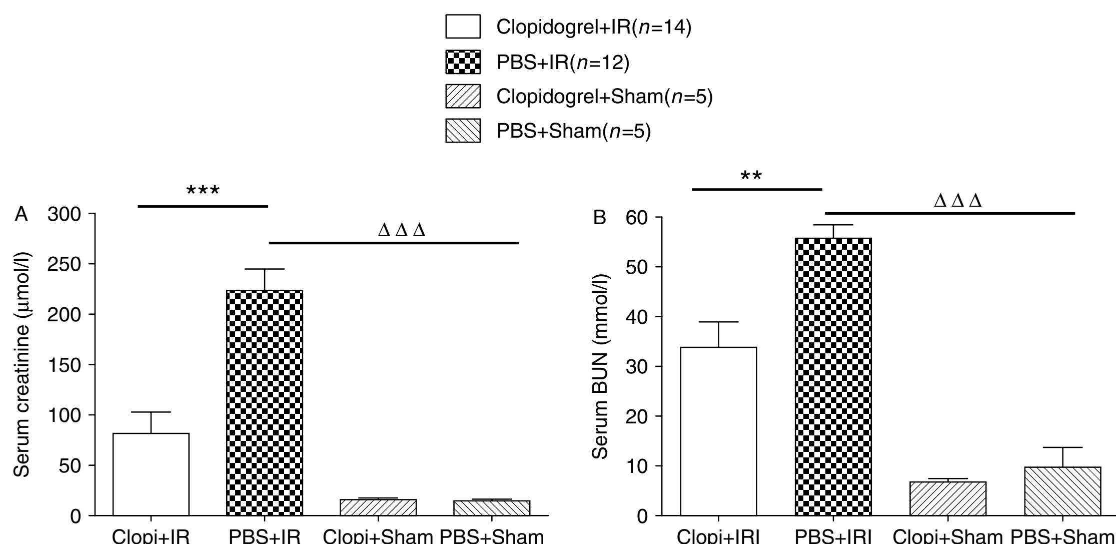

Figure 1. Clopidogrel protects mouse renal function following ischaemia reperfusion. Levels of serum creatinine (A) and BUN (B) were determined 24 h after reperfusion. Data shown are means ± SE. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\Delta\Delta\Delta}p < 0.001$.

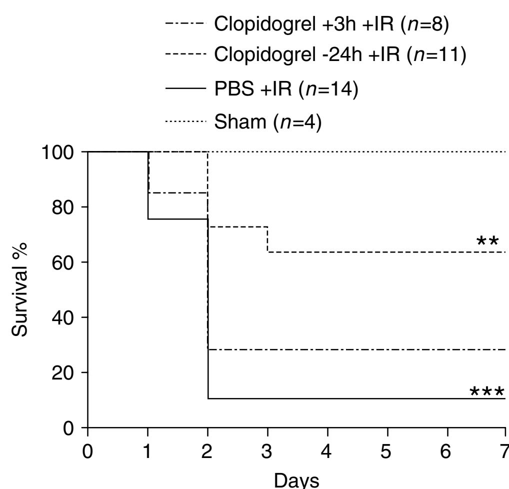

Figure 2. Clopidogrel protects against lethal renal IR. The survival rate of mice was observed over 7 days following IR with or without pretreatment by clopidogrel 24 h before IR or 3 h after IR. $^{**}p < 0.01$; $^{***}p < 0.001$.

Results

Pretreatment by clopidogrel decreased the levels of serum creatinine and BUN

Serum creatinine and BUN concentrations 24 h after reperfusion were significantly higher following IR than following sham operation ($p < 0.001$). The administration of clopidogrel significantly inhibited the increase in serum BUN and creatinine concentrations 24 h after reperfusion in comparison with the PBS-treated IR group (33.59 ± 5.19 and 55.53 ± 2.84 mmol/l, respectively, p ¡ 0.01 and 82.07 ± 20.63 and 223.20 ± 21.56μmol/l, respectively, $p < 0.001$) (Figure 1). These data suggest that clopidogrel protects renal function from the deleterious effects of IR.

Clopidogrel protected against lethal renal IRI mostly in a preventive manner

To investigate the protective effects of clopidogrel on lethal ARF, the survival rate of mice was observed for 7 days after reperfusion. Untreated mice developed severe renal failure following IR. As a result, animal death started 24 h after reperfusion. Forty-eight hours later, 86% of the untreated mice had died. Strikingly, in the preventive clopidogrel-treated group, 64% of the mice survived until 7 ($p < 0.01$) and in the curative clopidogrel-treated group, 25% survived until the third day ($p < 0.05$). No mouse was lost in the sham-operated group (Figure 2).

Pretreatment by clopidogrel decreased tubular damage

The functional changes induced by clopidogrel were associated with histological improvements. Clopidogrel prevented tubular dilatation, necrosis, congestion, and vacuolization that were constantly observed 24 h after reperfusion (Figures 3A and 3E). Scoring histopathological damage of the tubules confirmed the protective effect of clopidogrel (mean scores: 1.10 ± 0.07 in clopidogrel + IR mice versus 3.38 ± 0.06 in PBS + IR mice, $p < 0.001$) (Figure 3M). Sham-operated mice displayed no tubular injury (Figures 3C, 3D, 3G, and 3H). Thus, pretreatment by clopidogrel decreased renal histological damage after IR.

Pretreatment by clopidogrel decreased apoptotic cell death in kidneys

Using TUNEL staining, we examined the effects of clopidogrel on apoptosis following IR. As shown in Figure 3J, mice treated by PBS instead of clopidogrel displayed high numbers of TUNEL-positive cells. The apoptotic lesions predominated in the tubules of the outer medulla and were more severe in the PBS group

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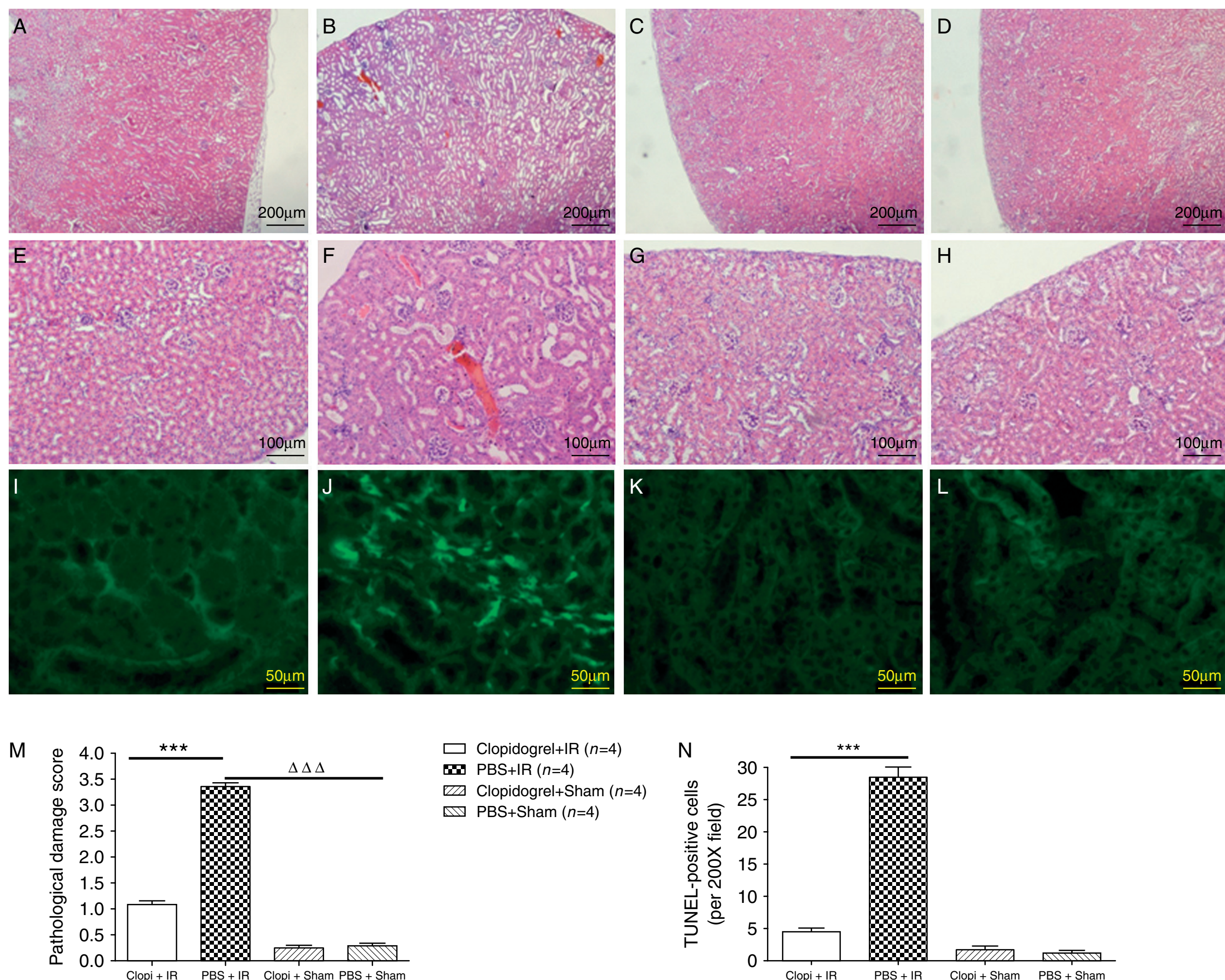

Figure 3. Effects of pretreatment with clopidogrel on renal tissue damage and apoptosis 24 h after ischaemia–reperfusion. Panels A–D (original magnification × 100) and E–H (original magnification × 200) are representative pictures of kidney sections (H&E). Severe tubular dilatation and necrosis were observed in IR mice pretreated with PBS (B, F) but not in sham-operated mice (C, D, G, H) treated by PBS or clopidogrel, whereas pretreatment with clopidogrel was associated with less histological damage (A). (I–L) Distribution of TUNEL-positive nuclei in the kidneys of mice pretreated with clopidogrel + IR (I), PBS + IR (J), and sham-operated mice treated or not with clopidogrel (K and L, respectively). (M) Histological scores and (N) quantification of TUNEL-positive apoptotic cells in kidneys (original magnification × 200). $^{***}p < 0.001$; $^{\Delta\Delta\Delta}p < 0.001$.

(positive cells per 200× field: 25.10 ± 1.48) than in the clopidogrel group (positive cells per 200× field: 3.85 ± 0.57, $p < 0.001$) (Figure 3N). Sham-operated mice showed no signs of apoptosis (Figures 3K and 3L). Thus, clopidogrel abrogated the apoptosis of renal cells following IR.

Pretreatment by clopidogrel down-regulated the expression of cleaved caspase-3 and caspase-8 proteins in kidneys

Caspase-3 and caspase-8 are key proteases involved in the caspase-dependent pathway of apoptosis. To ascertain the role of clopidogrel in suppressing apoptosis following IR, we examined renal caspase-3 and caspase-8 protein expression by western blotting using antibodies to active or inactive caspase-3 and caspase-8 24 h after reperfusion. There was no significant difference between pro-caspase-3 and pro-caspase-8 protein expression among the tested groups ($p > 0.05$). However, the expression of caspase-8 P20 fragments and caspase-3 P11 fragments was significantly higher in mice submitted to IR than in sham-operated mice ($p < 0.05$). Oral administration of clopidogrel decreased the levels of caspase-8 P20 fragments (density relative to β-actin: 0.05 ± 0.04 in clopidogrel + IR mice and 0.61 ± 0.33 in PBS + IR mice, $p < 0.05$). Caspase-3 P11 fragments were also present in smaller amounts in the kidneys from clopidogrel-treated mice submitted to IR than in controls (Figure 4) (density relative to β-actin: 0.18 ± 0.16 in clopidogrel + IR mice and 0.84 ± 0.19 in PBS + IR mice, $p < 0.05$).

Pretreatment by clopidogrel up-regulated the expression of Bcl-2 and Bcl-xL proteins and down-regulated the expression of Bax protein in kidneys

Early intracellular events occurring during the apoptotic process comprise mitochondrial changes mediated by protein members of the Bcl-2 family, including pro-apoptotic Bax and Bad proteins and anti-apoptotic

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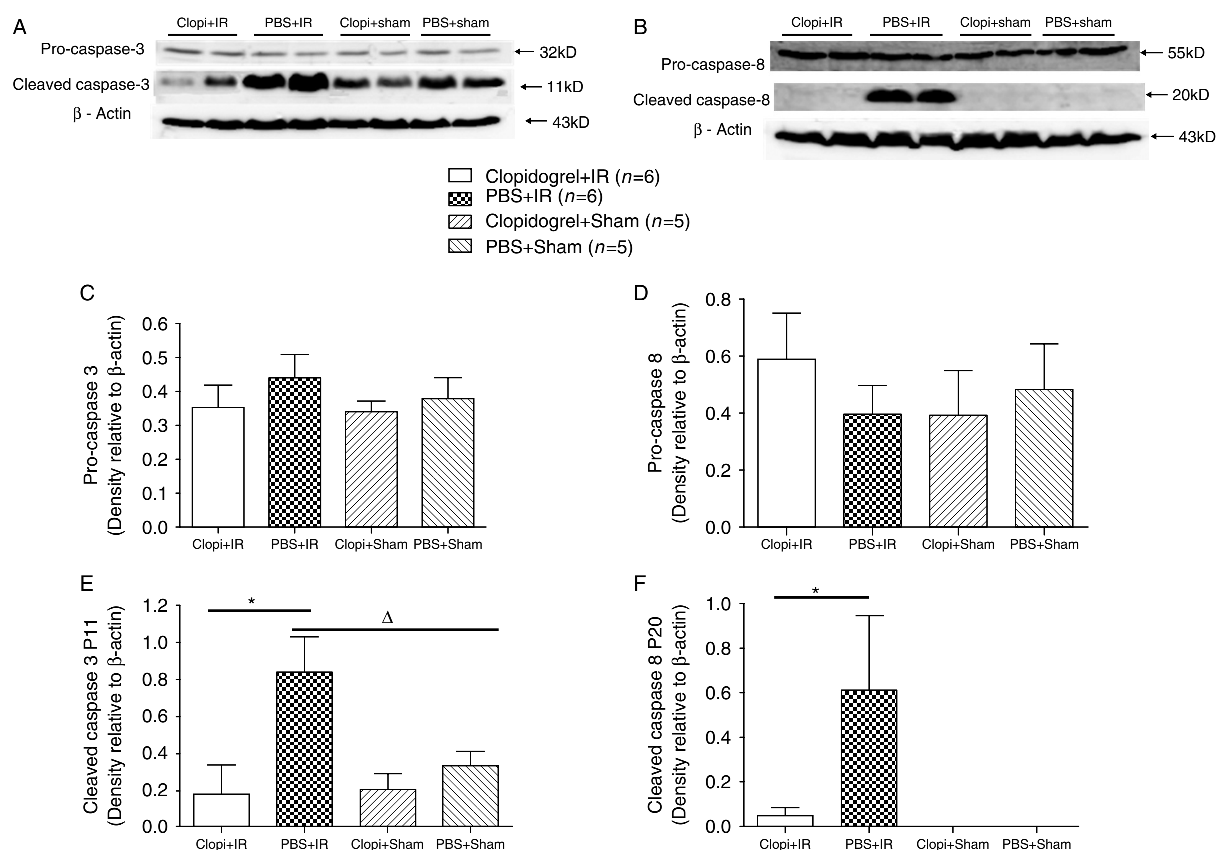

Figure 4. Effects of clopidogrel on caspase-3 and caspase-8 expression in kidneys. The expression of caspase-3 and caspase-8 in kidneys was measured by western blot analysis 24 h after reperfusion. Cleaved caspase-8 P20 fragments and caspase-3 P11 fragments were down-regulated in IR mice pretreated with clopidogrel (A, B). Bands were quantified by Fujifilm Multi-Gauge Imaging software and normalized to β-actin. Values are expressed as means ± SE (C–F). $^{*}p < 0.05$; $^{\Delta}p < 0.05$.

Bcl-2 and Bcl-xL. Therefore, Bax, Bad, Bcl-2, and Bcl-xL were evaluated by western blot analysis in order to elucidate how clopidogrel suppresses apoptosis following IR. Bax expression (density relative to β-actin: 0.25 ± 0.10 in clopidogrel + IR mice and 0.85 ± 0.20 in PBS + IR mice, $p < 0.05$) was significantly reduced after pretreatment with clopidogrel (Figure 5G, $p < 0.05$). In contrast, the expression of Bad remained unaltered in all the experimental and sham-operated groups (Figure 5H, $p > 0.05$).

The immunoreactivity to Bcl-2 (density relative to β-actin: 0.67 ± 0.19 in clopidogrel + IR mice versus 0.20 ± 0.08 in PBS + IR mice, $p < 0.05$) and Bcl-xL (density relative to β-actin: 1.39 ± 0.22 in clopidogrel + IR mice versus 0.62 ± 0.13 in PBS + IR mice, $p < 0.05$) was markedly increased (Figures 5E and 5F).

Pretreatment by clopidogrel decreased the expression of integrin alpha IIb (CD41) in kidneys

The expression and localization of the integrin alpha IIb (CD41) protein in kidneys were visualized by immunofluorescence. Semi-quantitative evaluation of the fluorescence by ImageJ (Figure 6I) showed that the kidneys in PBS-treated IR mice present widespread positive fluorescence for CD41 that mainly localized in small renal vessels, glomerular capsules, and tubules. Pretreatment by clopidogrel induced a remarkable decrease in fluorescence. In sham-operated mice, the kidneys did not express CD41. Thus, the pretreatment by clopidogrel inhibited the activation and aggregation of platelets in kidneys that usually followed IR.

Pretreatment by clopidogrel decreased the infiltrate of granulocytes and monocytes (GR-1) in kidneys

The expression and localization of Ly-6G and Ly-6C (GR-1) in the kidneys of the mice of the different groups were measured. The kidneys in PBS-treated IR mice showed widespread positive brown-coloured sites that mainly localized in glomerular capsules and tubules. Pretreatment by clopidogrel induced a remarkable decrease of the number of stained cells. In sham-operated mice, the kidneys did not express GR-1. Thus, the pretreatment by clopidogrel inhibited the granulocytes and macrophage infiltration that may be implicated as key mediators of very early inflammatory responses after renal IR injury.

Pretreatment by clopidogrel increased the total antioxidant capacity in kidneys

To determine whether the protection afforded by clopidogrel was related to abrogation of the oxidative stress, we measured antioxidant enzymatic activities and the total antioxidant capacity (TAC) in kidneys 24 h after reperfusion. The activities of SOD, CAT, and GSH

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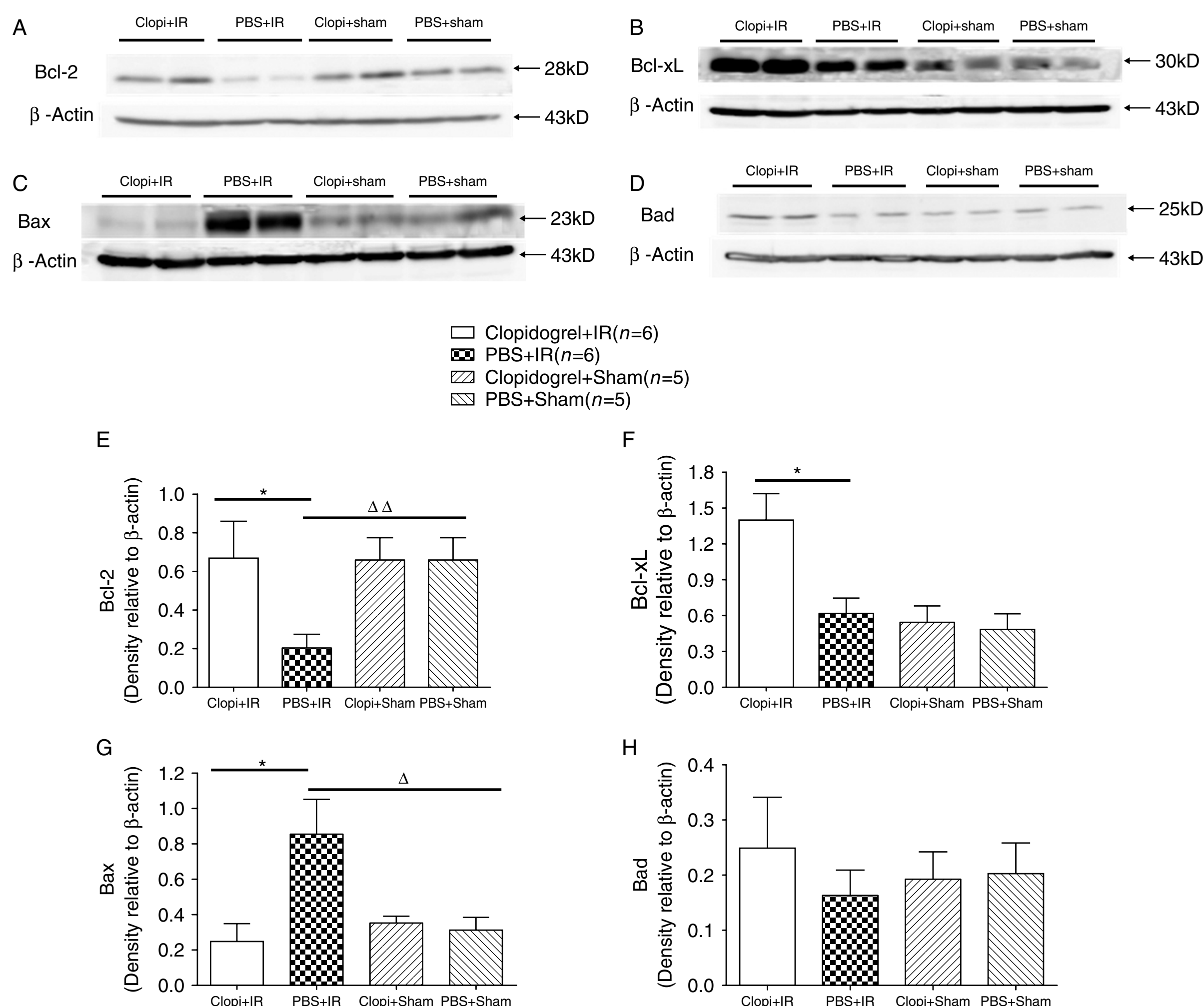

Figure 5. Effects of clopidogrel on the expression of the proteins of the Bcl-2 family in kidneys. The expression of Bcl-2, Bcl-xL, Bax, and Bad was determined by western blot analysis 24 h after reperfusion. Bcl-2 and Bcl-xL were up-regulated and Bax was down-regulated in IR mice pretreated with clopidogrel (A–D). The density of western blot bands was quantified by Fujifilm Multi-Gauge Imaging software and normalized to β-actin. Values are expressed as means ± SE (E–H). $^{*}p < 0.05$; $^{\Delta\Delta}p < 0.01$.

in kidneys were unchanged, whatever the group of animals (Figures 7A–7C). However, renal IR significantly decreased the TAC in kidneys versus the sham-operated group ($p < 0.01$). Pretreatment by clopidogrel prior to IR resulted in a higher TAC level than PBS (0.74 mmol/mg protein ± 0.16 versus 0.38 mmol/mg protein ± 0.02, $p < 0.01$) (Figure 7D).

Discussion

We have shown that clopidogrel, an antagonist of platelet ADP receptors used in cardiovascular diseases, protects kidneys from IRI through its anti-apoptotic and antioxidant properties that target various steps of the pathophysiological pathways leading to IRI. We were prompted to investigate those mechanisms by our observation that the increased survival rate of mice pretreated by clopidogrel was associated with an improvement in all the histopathological and biological parameters tested in mice submitted to IR and pretreated by clopidogrel, versus control mice.

The inhibition of tubular cell apoptosis by clopidogrel following IR is in line with previous reports that some pharmacological compounds that can decrease apoptosis can also abrogate renal injury [7,29–31]. Clopidogrel impacts the apoptotic pathways at several levels. First, clopidogrel prevents caspase-8 and caspase-3 cleavage in the kidney. Several studies have documented caspase activation in the kidney following IR. The key caspase is caspase-8, which initiates the cascade leading to the cleavage of caspase-3 [32]. The activation of death receptors during IR involves both the Fas and the TNF pathways [6,33]. However, the apoptotic pathways involved in renal IRI are not restricted to those triggered by death receptors. IRI also depends on apoptotic signals triggered by DNA damage, perturbations in redox balance, or growth factor deprivation that determine the mitochondrial release of pro-apoptotic molecules [34]. Members of the Bcl-2 family control the permeability of the mitochondrial outer membrane to release various pro-apoptotic proteins [35]. In our hands, clopidogrel exerts its powerful cytoprotective effect by modulating the expression of the Bcl-2 gene family. Bcl-2

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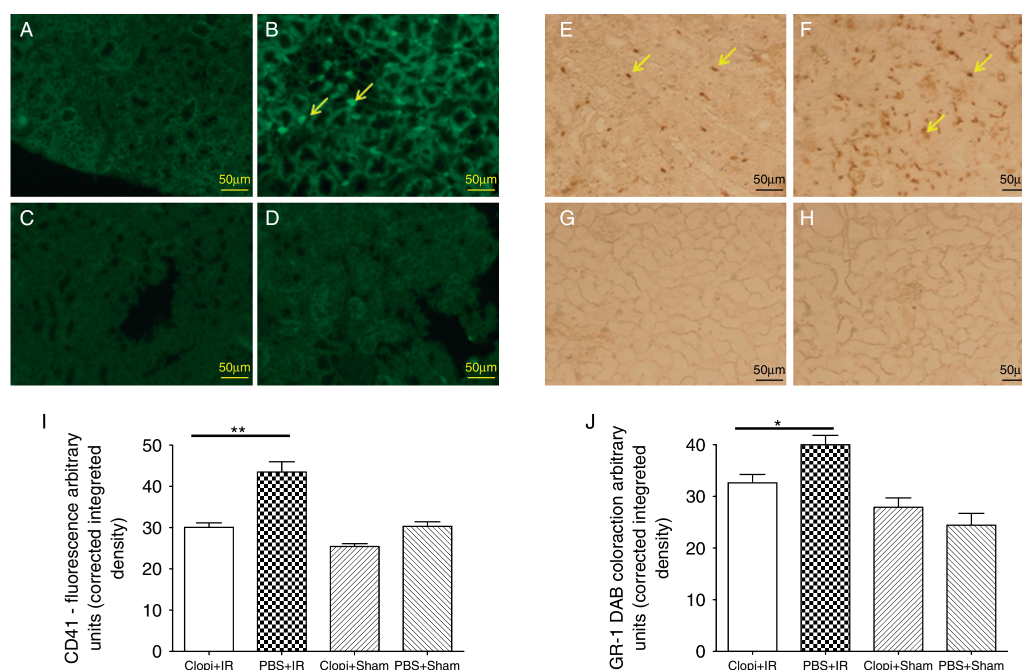

Figure 6. (A–D) Expression and localization of CD41 in kidneys. FITC staining of CD41 was less marked in the kidneys of IR mice pretreated with clopidogrel (A) than in the kidneys of IR mice pretreated with PBS (B). No CD41 expression was observed in sham-operated mice (C, D). Arrows: positive fluorescence. Original magnification: × 400. (I) Immunostaining of kidney sections was analysed using ImageJ 1.36 b software (National Institutes of Health; http://rsb.info.nih.gov/ij) for a semi-quantitative assessment of the fluorescence intensity. (**$p < 0.01$). (E–H) Expression and localization of GR-1 epitopes Ly-6G and Ly-6C in kidneys. The dark brown staining due to DAB coloration was less marked in the kidneys of IR mice pretreated with clopidogrel (E) than in those of IR mice pretreated with PBS (F). No CD41 expression was observed in sham-operated mice (G, H). Original magnification: × 400. (J) Immunostaining of kidney sections was analysed using ImageJ 1.36 b software (National Institutes of Health; http://rsb.info.nih.gov/ij) for a semi-quantitative assessment of the staining intensity. *$p < 0.05$.

and Bcl-xL, two anti-apoptotic molecules, are induced by clopidogrel and may interact with Bax to antagonize its death-promoting activity. In our model, acute renal failure results from hypoperfusion and hypoxia. Hypoxia activates the heterodimeric transcription factor hypoxia-inducible factor (HIF), leading to changes in gene expression that promote tissue adaptation and survival [36]. It has been recently established that *Bcl-xL* is a target gene regulated by HIF-α through binding with ischaemia-responsive element [37]. This explains why the expression of Bcl-xL is higher in IR groups. Moreover, it has been discovered (WO/2006/094 292) that thienopyridine compounds like clopidogrel could stabilize the alpha subunit of HIF-1α and then increase its activity. This would explain why IR and clopidogrel together synergized to increase Bcl-xL expression.

Thus, clopidogrel prevents the apoptosis of renal cells through inhibition of the apoptotic cascade at the mitochondrial level and at the levels of caspase-8 and -3. Kumar *et al* [38] observed the same phenomenon in rats submitted to renal IRI, where a single dose of dexamethasone prevented acute kidney injury, up-regulated Bcl-xL, down-regulated Bax, and inhibited caspase-9 and caspase-3 activation. As described in several studies [38–41], the Bcl-xL induction observed in our model could induce profound effects favouring cell survival.

Since the overproduction of ROS following tissue reperfusion has long been recognized as one of the pivotal mechanisms implicated in the initiation of cellular apoptosis [42], we investigated the effects of clopidogrel on ROS metabolism. The enzymatic activities of SOD, CAT, and GSH in kidney tissues were not significantly modified by the pretreatment with clopidogrel. However, the pretreatment by clopidogrel increased the total antioxidant capacity (TAC) of the kidney submitted to ischaemia. The TAC encompasses the cumulative action of all the antioxidant molecules present in body fluids and tissues, thus providing an integrated parameter of measurable antioxidants [43]. This capacity refers to a full spectrum of antioxidant activities against various reactive oxygen/nitrogen radicals, but we were not able to demonstrate which type of antioxidant system is impacted by clopidogrel. This property of clopidogrel could explain its beneficial effect on IRI. Indeed, elevated ROS levels induced by ischaemia lead to the peroxidation of DNA, proteins, and lipids, and result in damage of the biological membranes. A number of processes are involved in the pathogenesis of cell injury induced by oxygen deprivation [44]. In rat kidney ischaemia, re-oxygenation causes an increase in nitrosative and oxidative molecules [45]. Conversely, the protective effects of antioxidants described in IRI of the heart, liver, intestine, and kidney suggest an important role for ROS [46,47]. For example, pretreatment of

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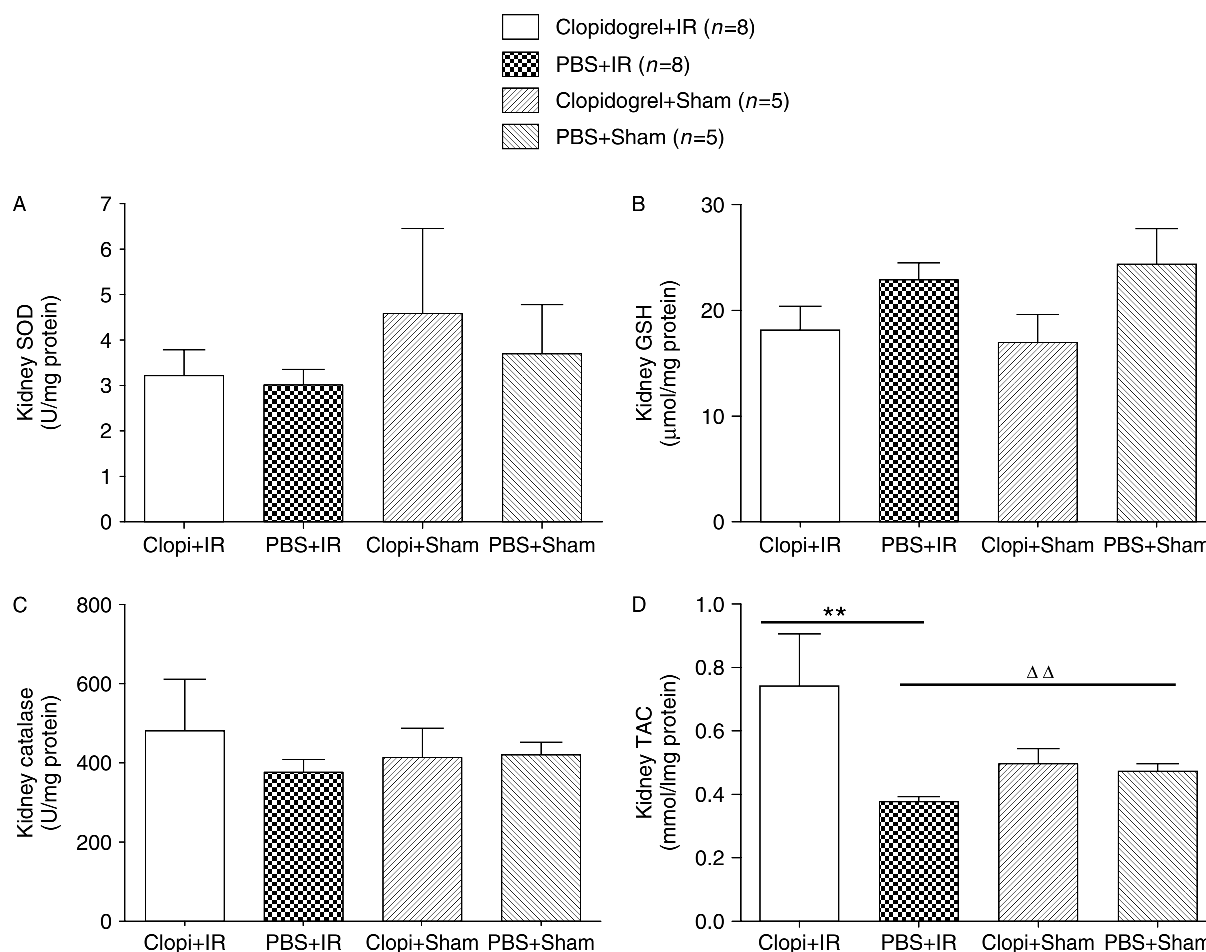

Figure 7. Effects of clopidogrel on renal SOD, GSH, CAT, and TAC activities. The levels of SOD (A), GSH (B), CAT (C), and TAC (D) in kidneys were measured 24 h after reperfusion. Results are presented as means ± SE. $^{**}p < 0.01$; $^{\Delta\Delta}p < 0.01$.

animals by catechin, a bioflavonoid antioxidant, attenuates renal dysfunction and morphological alterations. Catechin probably exerts its protective effects through its radical scavenging and antioxidant activities. Similarly, the devastating IRI associated with a deficiency in ROS scavenging in a genetic (SOD1)-deficient mouse model shows the preventive role of ROS detoxification.

In addition to its apoptotic effects, clopidogrel could prevent IRI through its anti-inflammatory properties. Indeed, following the activation that occurs during reperfusion, platelets generate ROS and release pro-apoptotic and pro-inflammatory mediators as well as growth factors. Clopidogrel and its active metabolites have been described, respectively, to inhibit platelet–PMN adhesion and platelet-dependent generation of ROS *ex vivo* in the mouse and *in vitro* in human cells [23]. In patients with coronary artery disease, platelet ADP receptor blockade by clopidogrel exerts a beneficial effect that improves systemic endothelial function and reduces the oxidative stress and inflammatory response [48].

The overproduction of ROS following tissue reperfusion can also participate in the inflammatory process associated with IRI. The anti-inflammatory properties of clopidogrel have already been demonstrated under ischaemic conditions [49]. By reducing platelet-dependent up-regulation of inflammatory and pro-atherothrombotic functions in leukocytes, clopidogrel can reduce inflammation [23,50]. Clopidogrel increases tissue perfusion by decreasing platelet and leukocyte adhesion and reducing blood viscosity. Furthermore, clopidogrel prevents early progression of renal injury following five-sixths nephrectomy by inhibiting the activation of platelets, the expression of inflammatory mediators and growth factors, and the infiltration of monocytes/macrophages [51]. The major decrease of GR-1-bearing cells observed in the clopidogrel-treated mice, compared with the untreated mice, is in agreement with the hypothesis of the anti-inflammatory effect of clopidogrel. The lower expression of CD41 in clopidogrel-pretreated mice confirms the inhibition of the activation and aggregation of platelets in animals and may contribute to improve the outcome, as observed in those animals.

In conclusion, clopidogrel is endowed with anti-apoptotic, antioxidant, and anti-inflammatory properties. This compound provides significant functional protection in our renal murine model of IRI. In this study, the effects of clopidogrel were studied in a pre-treatment design but the molecule is also endowed with

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a curative action. Thus, this molecule offers a helpful tool to interfere with the complex physiopathology of IRI.

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Abbreviations

ARF acute renal failure
BUN blood urea nitrogen
CAT catalase
DAB 3,3′-diaminobenzidine tetrahydrochloride
GSH glutathione
IR ischaemia reperfusion
IRI ischaemia–reperfusion injury
SOD superoxide dismutase
TAC total antioxidant capacity

Author contribution statement

The authors contributed in the following way: HH, CC, CG, and DB: the acquisition, analysis, and interpretation of data; and drafting the paper. FB and CN: study conception and design; the analysis and interpretation of data; and drafting the paper. ATDX and BW: study conception and design; and drafting the paper.

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