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Neutrophils Accentuate Renal Cold Ischemia-Reperfusion Injury. Dose-Dependent Protective Effect of a Platelet-Activating Factor Receptor Antagonist[1]

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ABSTRACT

This study was undertaken to evaluate whether the renal damage induced by cold ischemia-reperfusion was worsened by neutrophils (PMN), and if blockade of platelet-activating factor (PAF) could effectively decrease this injury. After flushing with EuroCollins, 85 kidneys from Sprague-Dawley rats underwent either no cold ischemia or a 4-h cold ischemia, and then were reperfused for 75 min at 37°C and 100 mm Hg in an isolated perfusion circuit. Reperfusion was performed with a Krebs-Henseleit solution containing 4.5% albumin, with and without human PMN (7.5×10^5 cells/ml) and with and without addition of a PAF receptor antagonist (BN 52021). Hemodynamic and functional parameters were continuously assessed during reperfusion. At end of the study, PAF production was evaluated. Presence of PMN during reperfusion of nonischemic kidneys produced no alteration of functional parameters or PAF production. After 4-h cold ischemia, the presence of PMN during reperfusion produced a significant worsening of plasma flow rate, glomerular filtration rate and sodium reabsorption in comparison with kidneys reperfused without PMN. Also, higher production of PAF was observed in the kidneys reperfused with PMN than in the kidneys reperfused without PMN. After 4-h cold ischemia, addition of BN 52021 during reperfusion in the presence of PMN significantly increased the plasma flow rate, glomerular filtration rate and sodium reabsorption in comparison with kidneys reperfused without this PAF antagonist. This effect was dose dependent. After 4-h cold ischemia, addition of BN 52021 during reperfusion in the absence of PMN produced no significant effect on functional parameters in comparison with kidneys reperfused without this PAF antagonist. These results indicate that PMN contribute to renal cold ischemia-reperfusion injury evaluated in the isolated perfused kidney. Treatment with a PAF receptor antagonist attenuated this injury in a dose-dependent manner, which suggests that it is mediated by PAF.

Posttransplant ischemic renal failure influences short and long term prognosis of renal transplant (Moreso *et al.*, 1995). There is a growing body of evidence which indicates that reperfusion of ischemic tissues leads to an acute inflammatory response in which neutrophils (PMN) are involved (Hansen, 1995; Marzi *et al.*, 1991; Suzuki *et al.*, 1993; Bienvenu and Granger, 1993). In normal conditions, PMN do not adhere to the endothelium. However, when stimulated, they become more adherent to the endothelial cells. This adhesion is mediated by Platelet Activating Factor (PAF), by a group of intercellular adhesion molecules expressed, constitutively or not, on the endothelial surface (intercellular adhesion molecule-1, E-selectin, P-selectin), and by a group of surface glycoproteins on the PMN surface called leukocyte cell adhesion molecules or integrins from the CD11/CD18 family (Arnould *et al.*, 1993; Zimmerman *et al.*, 1992; Adams and Shaw, 1994). Endothelial cells may be activated by different stimuli, and it is well known that a time-dependent expression of signalling and tethering molecules by activated endothelial cells exists (Zimmerman *et al.*, 1992). Thrombin or leukotriene C_4 are able to stimulate endothelial cells leading to increased PMN adherence. This adherence is optimal within minutes and involves P-selectin overexpression and platelet activating factor (PAF) synthesis (Zimmerman *et al.*, 1992). Interleukin 1 or tumor necrosis factor induces the expression

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ABBREVIATIONS: EC, Euro-Collins solution; EDTA, ethylenediaminetetraacetic acid; FF, filtration fraction; FRNa, fractional sodium reabsorption; GFR, glomerular filtration rate; PAF, platelet-activating factor; PMN, polymorphonuclear cells; PFR, plasma flow rate; QO_2, oxygen consumption; RVR, renal vascular resistance; TNa, net sodium reabsortion; RIA, radioimmunoassay.

of E-selectin and interleukin-8. Both take hours, require "*de novo*" protein synthesis, and are involved in PMN adherence. Recently, *in vitro* studies have shown that hypoxia can activate endothelial cells by itself and this activation can account for the increased PMN adherence observed in ischemic tissues (Arnould *et al.*, 1993; Milhoan *et al.*, 1992). As a result, PMN induce damage to endothelial cells. In these studies, the role of PAF and of adhesion molecules has also been demonstrated (Nishiyama *et al.*, 1993; Taylor *et al.*, 1993).

The aim of the present study was to know the role of PMN and PAF in the pathophysiology of renal cold ischemia and reperfusion damage. Because long-term storage of rat kidneys in EC solution may result in severe renal injury, as we reported (Herrero *et al.*, 1995), short preservation time (4 h) was used to obtain renal functional damage capable of being worsened by other experimental conditions. So, in this study, we evaluated whether the renal damage induced by cold ischemia-reperfusion was worsened by PMN the behavior of PAF in cold ischemia-reperfusion, and whether the PAF blockade could effectively decrease this injury.

Material and Methods

Animals and surgical technique. Kidneys were obtained from male Sprague-Dawley rats (250–300 g b.wt.). Animals had free access to commercial chow and tap water, and they did not fast before the experiment. Anesthesia was induced and maintained by intramuscular injection of a mixture of ketamine (75 mg/kg b.wt.), diazepam (5 mg/kg b.wt.) and atropine (0.5 mg/kg b.wt.). Surgery was performed according to Schurek and Alt (1981). The abdominal cavity was opened, and the left ureter was cannulated for the collection of urine with a short polyethylene tubing (PE-10 tubing, 5 mm) connected to a larger polyethylene catheter (PE-50, 100 mm) to prevent ureteral back-pressure. Aorta, cava and renal vessels were dissected carefully. A double-barreled cannula was introduced in the aorta and progressed to the origin of the left renal artery. The aorta above the renal artery was clamped, and the kidney was flushed immediately *in situ* with 20 ml of cold EC (4°C) (table 1) at a maximum pressure of 100 mm Hg monitored through the inner part of the cannula. Renal vein was cannulated with a short polyethylene catheter (PE-80, 10 mm). After excision, the kidney was placed in a beaker containing preservation solution at 4°C.

Isolation of human neutrophils. PMN were purified from human blood anticoagulated with sodium citrate. The buffy coat from voluntary blood donors from our blood bank was used. Buffy coat (5 ml) was layered onto 4 ml of Polymorphprep (Nycomed Pharma, Oslo, Norway) and centrifuged for 35 min at 3,000 rpm. PMN were aspirated from their layer, and thereafter a hypotonic lysis of erythrocytes was performed with 0.75% NaCl and centrifuged at 1,000 rpm for 1 min. After a saline wash, cells were resuspended with Krebs-Henseleit solution and incubated at 37°C in a sterile atmosphere of 95% O_2 and 5% CO_2 until their use. Final preparations contained 94 to 96% PMN (May Grünwald-Giemsa) with 99% of cell viability (trypan blue). Contaminations included eosinophils, basophils and a few lymphocytes. Platelet contamination was scarce.

TABLE 1
Electrolytic composition of EC for kidney preservation

	mM
Sodium	10
Potassium	115
Chloride	15
Bicarbonate	10
Phosphate	50
D-Glucose	195
Osmolality (mOsm/kg)	355
pH	7.0

Isolated kidney perfusion system. The basic perfusion medium (200–250 ml) consisted of a modified Krebs-Henseleit solution (37.5°C) containing 4.5 g/100 ml of dialyzed bovine serum albumin (fraction V; Sigma Chemical Co., Madrid, Spain) and (in mM): sodium, 140.0; potassium, 4.9; chloride, 123.0; calcium, 2.2; ionic calcium, 1.2; magnesium, 1.2; bicarbonate, 25.0; inorganic phosphates, 1.2; sulfates, 1.2; EDTA, 0.04; urea, 6.0; creatinine, 0.13; malic acid, 1.0; pyruvate, 0.3; lactate, 2.1; α-ketoglutarate, 1.0; and D-glucose, 5.0. Basic salts contained were (in mM): NaCl, 115; KCl, 3.7; $CaCl_2{\cdot}2H_2O$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4{\cdot}7H_2O$, 1.2). Streptomycin (10 mg/l) and penicillin G (100,000 I.U./l) were used for antibiotic prophylaxis. To improve the stability of the tubular function, a mixture of 22 L-amino acids in concentrations between 0.05 and 2.3 mM was added with a commercial solution (Amyloplasmal L-12.5, B. Braun Medical, Rubi, Spain) supplemented with tyrosine, lysine, glycine, cysteine and glutamine (amino acids in mM: leucine, 0.4; phenylalanine, 0.32; methionine, 0.33; lysine, 1.0; valine, 0.33; histidine, 0.24; threonine, 0.24; tryptophan, 0.07; alanine, 2.0; glycine, 2.3; arginine, 0.5; tyrosine, 0.2; cysteine, 0.5; aspartate, 0.2; glutamate, 0.5; asparagine, 0.2; glutamine, 2.0; serine, 1.0; proline, 0.31; isoleucine, 0.3; N-acetyltyrosine, 0.05; ornithine, 0.16). Insulin (4 I.U./l) and thyroid hormone (1.5 μg/l) were also added. Polyfructosan (1 g/l) (Laevosan, Linz, Austria) was added to determine the GFR. This solution was filtered through a 0.22-μm filter (Millipore, Barcelona, Spain). All ingredients used were purchased from Sigma in Spain.

Perfusion was performed with a Watson Marlow model 502S roller pump at a flow rate that maintained an effective perfusion pressure of 100 mm Hg and was monitored by an electronic pressure transducer (Nihon Kohden Co., Madrid, Spain). The perfusate was recirculated by draining back the venous effluent into the reservoir and oxygenated with a gas mixture (95% O_2-5% CO_2) by a neonatal membrane oxygenator (VPCML plus, Cobe), which brought the pH to 7.4. In studies with PMN, 7.5×10^5 cells/ml were added to the reperfusion circuit at the beginning of the perfusion. When BN 52021 was used, it was also added to the circuit at the desired concentration at the beginning of the perfusion. Vehicle for BN 52021 is mannitol and NaCl (100 mM mannitol, 22.4 mM NaCl).

Estimations. The study protocol is detailed in figure 1. After 15 min of equilibration, the following parameters were evaluated every 10 min over a 60-min period: PFR (ml/min/g) was measured by collecting perfusate in a sterile graded pipette for a known interval; RVR (mm Hg/ml/min/g) was calculated from the formula RVR = arterial pressure/PFR; urine was collected in preweighted tubes and

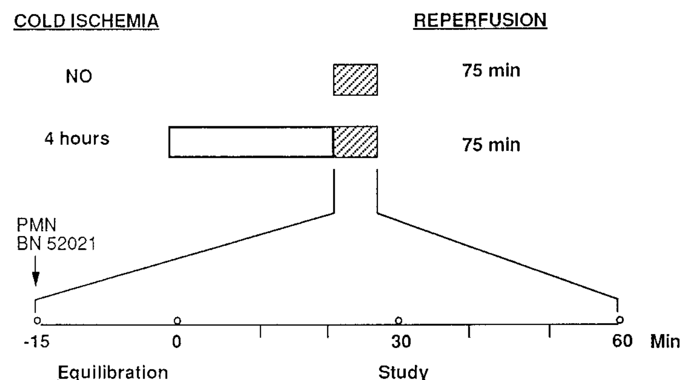

Fig. 1. Schematic diagram of the study protocol. After flushing with EC, kidneys from Sprague-Dawley rats underwent either no cold ischemia or a 4-h cold ischemia, and then were reperfused for 75 min at 37°C and 100 mm Hg in an isolated perfusion circuit. Reperfusion was performed with a Krebs-Henseleit solution containing 4.5% albumin, with and without human PMN (7.5×10^5 cells/ml) and with and without addition of a PAF receptor antagonist (BN 52021)

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urine output (μl/min/g) was determined, assuming a urine specific gravity of 1.000; GFR (μl/min/g) was evaluated from inulin clearance by the formula, GFR = urine output × urine inulin/perfusate inulin; FF was calculated from the formula (GFR/PFR) × 100 (%); TNa and FRNa were calculated from the formulas: TNa = (perfusate Na × GFR − urine Na × urine output)/1000, and FRNa = 100 × (1 − urine Na/perfusate Na × perfusate inulin/urine inulin).

Kidney QO_2 (μmol/min/g) was calculated every 30 min from the arteriovenous pO_2 difference of physically dissolved oxygen and the perfusion flow rate. Concentrations of dissolved oxygen were calculated from the arterial and venous pO_2 values with the absorption coefficient for oxygen ($\alpha\ O_2 = 0.0227$) in physiological saline at 37°C. All results are expressed for 1 g kidney wet weight, and the right kidney was used as a weight basis for calculations.

Analytical methods. Glucose was measured enzymatically by the hexokinase/glucose-6-phosphate dehydrogenase method (Boehringer Mannheim, Barcelona, Spain), and polyfructosan was measured after acid hydrolysis by adding a glucose-6-phosphate isomerase into the assay (Schmidt, 1961). Sodium level was measured by flame photometry (Ciba Corning, Barcelona, Spain). Oxygen partial pressure was measured by a gas analyzer (288 blood gas system, Ciba Corning).

Measurement of PAF production. At the end of the experiment, 10 ml of venous effluent were collected in polypropylene tubes, mixed with equal volume of 20% acetic acid in water (v/v) to stop degradation of PAF to lyso-PAF by acetylhydrolase and immediately frozen at −80°C. For PAF extraction, samples were thawed and partially purified with reverse SEP-PACK columns (SEP-PACK C18 Waters) previously equilibrated by step elution with the following solvents: methanol, 5 ml; chloroform, 5 ml; hexane, 2 ml; chloroform, 2 ml; methanol, 3 ml; water, 3 ml. Afterward, 2 ml of the acidified PAF-containing samples were layered on the columns that were sequentially eluted with 2 ml distilled water, 5 ml ethyl acetate and 8 ml methanol, with fractions collected in polypropylene tubes. PAF was contained in the methanol fraction. With radiolabeled PAF, an average recovery yield of 75% was calculated. Methanol was evaporated with a nitrogen stream, the dry residue was resuspended in 0.7 ml of the radioimmunoassay solution provided by the supplier (PAF RIA kits, Dupont, Les Ulis) and the samples were stored until the assay. PAF assay was performed according to the supplier specifications. The results are expressed as picograms per milliliter and were corrected for the recovery yield.

Experimental groups. Eighty-five kidneys were studied and divided into nine groups: NonISC group, no cold ischemia, reperfusion with basic solution without PMN (n = 11); BN 1600 NonISC group, no cold ischemia, reperfusion with basic solution without PMN plus BN 52021, 1600 ng/ml (n = 4); NonISC-PMN group, no cold ischemia, reperfusion with basic solution plus 7.5×10^5 PMN/ml (n = 12); ISC group, EC flushing, 4 h of cold ischemia, reperfusion with basic solution without PMN (n = 11); ISC-PMN group, EC flushing, 4 h of cold ischemia, reperfusion with basic solution plus 7.5×10^5 PMN/ml (n = 12); BN 1600 group, EC flushing, 4 h of cold ischemia, reperfusion with basic solution plus BN 52021, 1600 ng/ml (n = 11); BN 400-PMN group, EC flushing, 4 h of cold ischemia, reperfusion with basic solution plus 7.5×10^5 PMN/ml and BN 52021, 400 ng/ml (n = 8); BN 800-PMN group, EC flushing, 4 h of cold ischemia, reperfusion with basic solution plus 7.5×10^5 PMN/ml and BN 52021, 800 ng/ml (n = 8); BN 1600-PMN group, EC flushing, 4 h of cold ischemia, reperfusion with basic solution plus 7.5×10^5 PMN/ml and BN 52021, 1600 ng/ml (n = 8).

Statistical analysis. To compare more than two groups throughout the reperfusion, statistical analysis was performed by two-way analysis of variance to factor in time. On the other hand, at 20 min of reperfusion, and when it was needed at any time point, comparison of more than two groups was performed by one-way analysis of variance followed by Fisher's procedure for multiple pairwise comparisons. When a nonparametric test was needed, the Kruskall-Wallis analysis was used. All P values were two tailed, and a P value of $< .05$ was considered statistically significant. Data are presented as mean ± standard error of the mean.

Results

Effect of PMN on kidneys after cold ischemia. Figures 2 and 3 summarize the PFR and GFR profile. GFR was not statistically different between nonischemic kidneys, regardless of the presence of PMN. These nonischemic kidneys showed a slight reduction in PFR during reperfusion in the presence of PMN compared with that obtained without PMN, although this was not statistically significant. Addition of BN 52021 at 1600 ng/ml during reperfusion without PMN in nonischemic kidneys produced no variation in PFR or GFR (data not shown). Urine output, FF, FRNa (fig. 4), TNa and QO_2 were not significantly different between these two groups (table 2)

In 4-h cold ischemia kidneys, GFR decreased significantly as expected (fig. 3). When kidneys were reperfused in the presence of PMN, a significantly greater decrease in GFR was observed. Concerning the PFR in these 4-h ischemic groups (fig. 2), kidneys reperfused without PMN showed slightly but not significantly higher values than nonischemic kidneys. However, when reperfusion was performed in the

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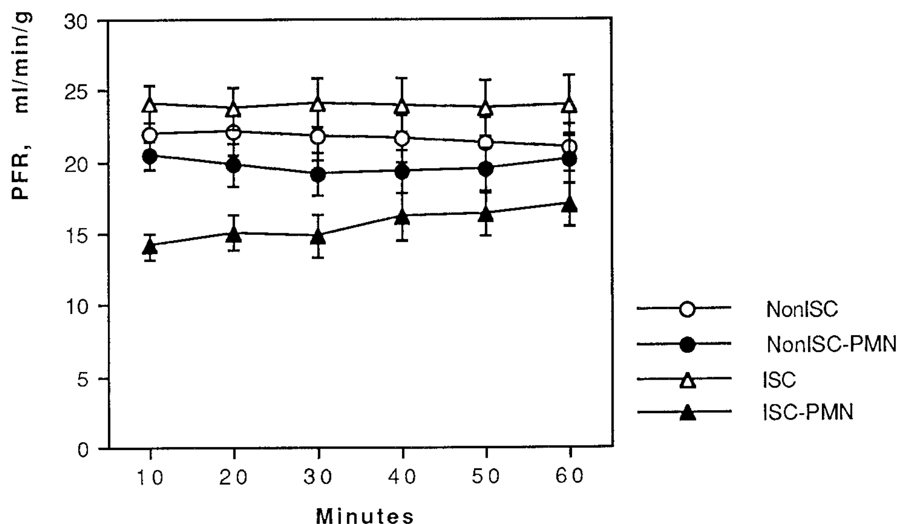

Fig. 2. Effect of addition of neutrophils on PFR of nonischemic and 4-h ischemic isolated perfused kidneys. NonISC, nonischemic kidneys, reperfusion without neutrophils; NonISC-PMN, nonischemic kidneys, reperfusion with neutrophils; ISC, 4-h ischemic kidneys, reperfusion without neutrophils; ISC-PMN, 4-h ischemic kidneys, reperfusion with neutrophils. Statistical analysis was performed by two-way analysis of variance to factor in time. P < .05 NonISC, NonISC-PMN and ISC *vs*. ISC-PMN.

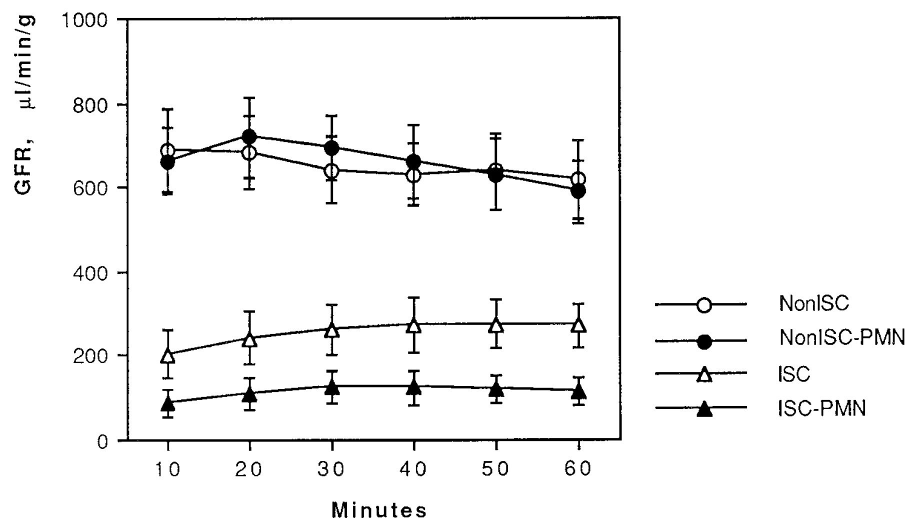

Fig. 3. Effect of addition of neutrophils on GFR of nonischemic and 4-h ischemic isolated perfused kidneys. P not significant, NonISC *vs.* NonISC-PMN; P < .05, ISC *vs.* ISC-PMN; P < .05, NonISC *vs.* ISC and NonISC-PMN *vs.* ISC-PMN.

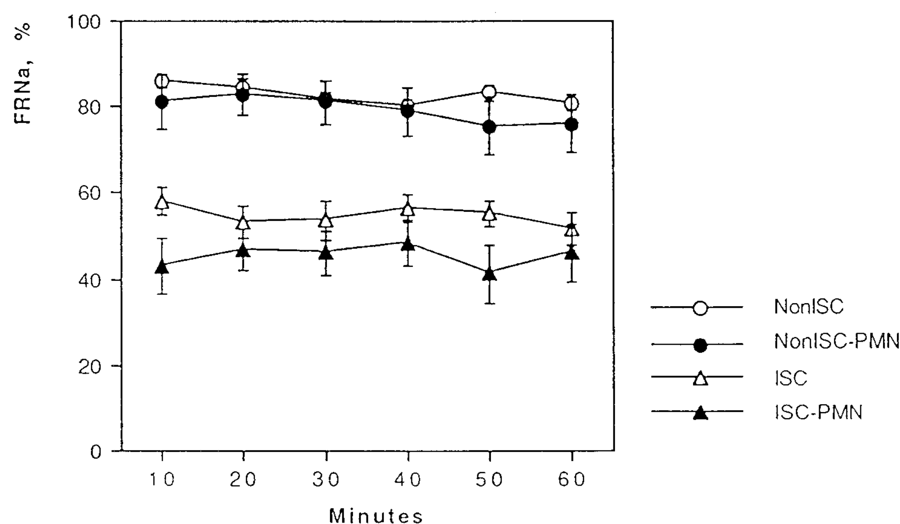

Fig. 4. Effect of addition of neutrophils on fractional reabsorption of sodium of nonischemic and 4-h ischemic isolated perfused kidneys. P not significant, NonISC *vs.* NonISC-PMN; P < .05, ISC *vs.* ISC-PMN; P < .05, NonISC *vs.* ISC and NonISC-PMN *vs.* ISC-PMN.

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TABLE 2
Functional parameters evaluated at 20 min of the reperfusion period in all study groups[a]

	NonISC	NonISC-PMN	ISC	ISC-PMN	BN 400-PMN	BN 800-PMN	BN 1600-PMN
PFR (ml/min/g)	22 ± 2	20 ± 1	24 ± 1	14 ± 1	17 ± 1	21 ± 2	24 ± 2
RVR (mm Hg/ml/min/g)	4.6 ± 0.5	5.0 ± 0.2	4.3 ± 0.3	7.4 ± 0.5	6.0 ± 0.5	4.9 ± 0.4	4.4 ± 0.4
Urine output (µl/min/g)	136 ± 24	136 ± 35	89 ± 21	83 ± 31	104 ± 41	126 ± 37	155 ± 26
GFR (µl/min/g)	641 ± 78	694 ± 75	262 ± 60	126 ± 37	261 ± 53	348 ± 70	638 ± 71
FF (%)	3.4 ± 0.4	3.6 ± 0.4	1.0 ± 0.3	0.7 ± 0.3	1.4 ± 0.3	1.6 ± 0.3	2.6 ± 0.4
FRNa (%)	84 ± 2	83 ± 2	60 ± 5	47 ± 4	52 ± 5	57 ± 7	74 ± 3
TNa (µmol/min/g)	68 ± 9	82 ± 12	20 ± 6	12 ± 5	16 ± 5	34 ± 11	65 ± 12
QO_2 (µmol/min/g)	7.9 ± 0.5	7.7 ± 0.5	7.3 ± 0.5	5.5 ± 0.3	5.9 ± 0.8	7.1 ± 0.7	7.1 ± 0.5

[a] Statistical analysis was performed by one-way analysis of variance. See text for differences.

presence of PMN, a significant decrease of PFR was observed. When we evaluated each time point (one-way analysis of variance), we observed that this decrease was clearly different from the other groups during the first 30 min of the experiment; nevertheless, throughout the rest of the study, it was not significantly different because the PFR gradually increased. RVR followed an profile inverse to PFR (table 2). FF was similar between both 4-h ischemic groups and significantly lower than in nonischemic groups. FRNa (fig. 4) was significantly lower in kidneys reperfused with PMN than in those reperfused without these cells. TNa and QO_2 were significantly lower in the 4-h ischemic group reperfused with PMN than in the ischemic group reperfused without PMN (table 2).

Effect of cold ischemia and PMN on kidney PAF production. In kidneys with neither cold ischemia nor PMN, the PAF levels were below the lower limit of detection of the RIA kit. Kidneys with no cold ischemia and reperfused with PMN showed slightly but not statistically significant higher levels of PAF than the group without PMN (fig. 5). It is interesting to note that four kidneys reperfused with PMN showed levels of PAF below the lower detection limit of the kit, and the five remaining kidneys showed appreciable but very low levels.

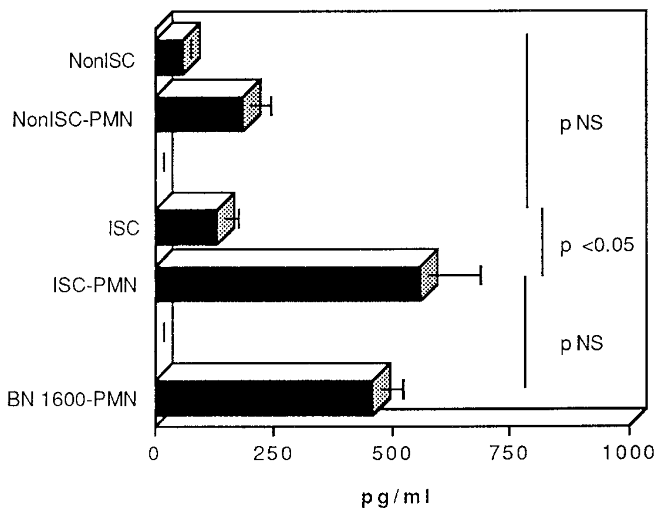

Fig. 5. PAF production by nonischemic and 4-h cold ischemic kidneys and reperfused with or without neutrophils. Effect of addition of the highest concentration of BN 52021. PAF production was assayed by a specific commercial RIA kit. Statistical analysis was performed by one-way analysis of variance.

After 4-h cold ischemia, in kidneys reperfused in the absence of PMN, low levels of PAF were observed, which are probably derived from ischemic endothelial and mesangial cells. Nevertheless, these PAF levels were not significantly different from levels in nonischemic kidneys. After 4-h cold ischemia, the presence of PMN during reperfusion induced significantly higher levels of PAF than in the former groups.

Among 4-h cold ischemic kidneys reperfused in the presence of PMN and with the addition of BN 52021, we only measured the levels of PAF in the group with the highest drug concentration (1600 ng/ml). The PAF levels in this group were high and similar to the group without the drug and with PMN.

Effect of PAF receptor antagonist on cold ischemia-injured kidneys reperfused with PMN. To investigate the role of PAF produced by these cold ischemic kidneys reperfused in the presence of PMN, experiments were performed using three doses of PAF antagonist in 4-h ischemic kidneys reperfused with PMN (figs. 6 and 7). We observed a highly significant and dose-dependent protective effect on GFR. Kidneys reperfused with the lowest concentration of BN 52021 (400 ng/ml) showed slight but not significantly higher GFR than 4-h ischemic kidneys reperfused without the drug. Kidneys reperfused with the medium concentration of BN 52021 (800 ng/ml) showed significantly higher GFR than 4-h ischemic kidneys. Finally, kidneys reperfused with the highest concentration of BN 52021 (1600 ng/ml) showed a GFR similar to that in nonischemic kidneys. When we evaluated each time point (one-way analysis of variance) in these latter two groups, we observed significantly higher GFR in nonischemic kidneys in the first 10 min of the study. Nevertheless, as the GFR gradually increased throughout the experiment in kidneys reperfused with the drug, GFR became similar to that in nonischemic kidneys.

Concerning PFR and RVR, we also observed a similar dose-dependent protective effect of BN 52021. FRNa in kidneys reperfused with the lowest concentration of BN 52021 was significantly higher than in ischemic kidneys reperfused without the drug (fig. 8). FRNa gradually ameliorated as concentration of BN 52021 was progressively increased, but it was not significantly different. Finally, FRNa in kidneys reperfused with the highest concentration of BN 52021 was significantly lower than in nonischemic kidneys. FF, TNa and QO_2 gradually ameliorated as BN 52021 concentration increased (table 2).

Effect of PAF receptor antagonist on cold ischemia-injured kidneys reperfused without PMN. To evaluate the effect of BN 52021 on cold ischemic kidneys without the participation of PMN, we studied 4-h ischemic kidneys reperfused without PMN with only the highest BN 52021 concentration (1600 ng/ml). We observed no effect in PFR (fig. 9). GFR (fig. 10) was slightly higher in this group than in 4-h ischemic kidneys reperfused without either PMN or the addition of BN 52021. This difference was not statistically significant, however. RVR, FF, FRNa, TNa and QO_2 were similar between both ischemic groups (data not shown).

Discussion

The present study shows that PMN contribute to renal cold ischemia-reperfusion injury evaluated in the isolated perfused kidney, and that treatment with the PAF receptor antagonist attenuated this injury, which suggests that it is mediated by

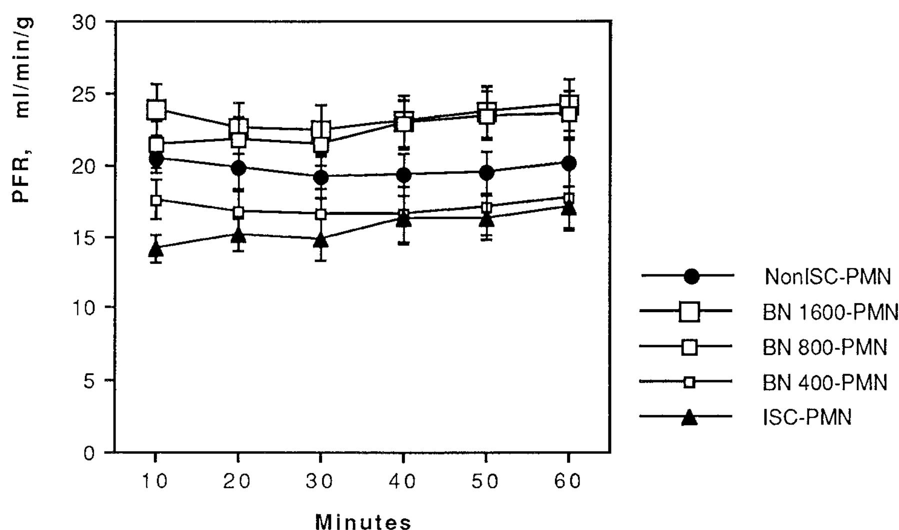

Fig. 6. Effect of no addition and addition of BN 52021, at increasing concentration, on PFR in kidneys made cold ischemic for 4 h and reperfused with neutrophils. BN 1600-PMN, 4-h ischemic kidneys, reperfusion with neutrophils and BN 52021, 1600 ng/ml; BN 800-PMN, 4-h ischemic kidneys, reperfusion with neutrophils and BN 52021, 800 ng/ml; BN 400-PMN, 4-h ischemic kidneys, reperfusion with neutrophils and BN 52021, 400 ng/ml. Statistical analysis was performed by two-way analysis of variance to factor in time. P not significant, NonISC-PMN *vs.* BN 800-PMN and BN 1600-PMN; $P < .05$, BN 400-PMN *vs.* BN 800-PMN and BN 1600-PMN; P not significant, ISC-PMN *vs.* BN 400-PMN.

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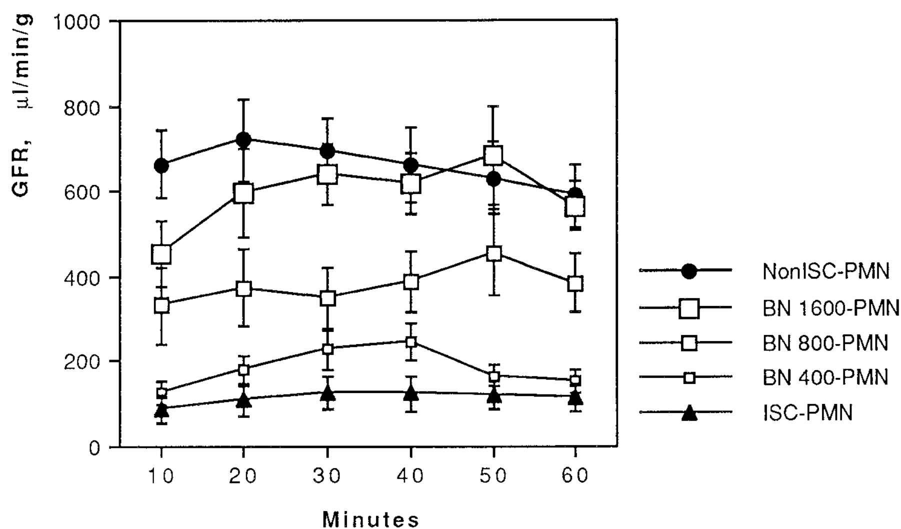

Fig. 7. Effect of no addition and addition of BN 52021, at increasing concentration, on GFR in kidneys made cold ischemic for 4 h and reperfused with neutrophils. P not significant, BN 1600-PMN *vs.* NonISC-PMN; P < .05, BN 400-PMN *vs.* BN 800-PMN *vs.* BN 1600-PMN; P not significant, BN 400-PMN *vs.* ISC-PMN.

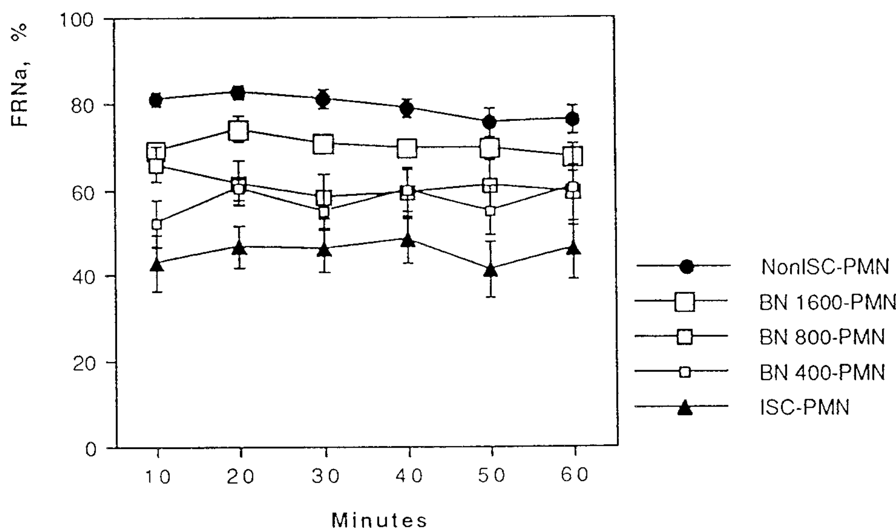

Fig. 8. Effect of no addition and addition of BN 52021, at increasing concentration, on fractional reabsorption of sodium in kidneys made cold ischemic for 4 h and reperfused with neutrophils. P < .05, BN 1600-PMN *vs.* NonISC-PMN; P not significant, BN 400-PMN *vs.* BN 800-PMN *vs.* BN 1600-PMN; P < .05, BN 400-PMN *vs.* ISC-PMN.

PAF. These findings are supported by some observations: First, addition of PMN during reperfusion induced hemodynamic alterations and worsened functional injury of previously hypothermically ischemic kidneys, whereas reperfusion with PMN did not cause either functional or hemodynamic injury in nonischemic kidneys. Second, previously hypothermically ischemic kidneys reperfused in the presence of PMN produced higher amounts of PAF than those kidneys reperfused in the absence of PMN. Third, the addition of the PAF receptor antagonist during reperfusion produced an amelioration in the kidney hemodynamics and functional injury induced by PMN in a concentration-dependent manner.

As we have reported, the isolated perfused rat kidney has proved to be an excellent model for the study of kidney function after preservation (Herrero *et al.*, 1995). This *ex vivo* preparation is optimal because renal cold ischemia could be evaluated separately without the confounding effects of changes in systemic hemodynamics or in renal nerve function. Furthermore, the specific effects of PMN on kidney function could be determined in the absence of other blood cells, circulating factors or vasoactive hormones coming from systemic sources which are present *in vivo* after transplantation. The utilization of human PMN in the isolated perfused kidney model has already been reported by Linas *et al.* (1988, 1992) in kidneys subjected to warm ischemia. These authors have shown how inactive (Linas *et al.*, 1987), primed (Linas *et al.*, 1992) or activated (Linas *et al.*, 1987, 1988) PMN contribute to acute renal failure produced by variable degrees of warm ischemia *in vitro*. In our work, the deterioration of renal function and the higher production of PAF observed in cold ischemic kidneys reperfused in the presence of PMN suggests that PMN interacted with endothelial cells to contribute to further renal damage. Recently, with use of isolated guinea pig heart, a similar exacerbation in reperfusion injury by PMN after brief ischemia has been reported (Raschke and Becker, 1995). This report reinforces the results of our study.

The mechanisms that account for the ischemic-reperfusion injury are partially known, but links between them remain undefined (Lefer and Lefer, 1993). The majority of evidence comes from basic experimental studies on inflammation (Zimmerman *et al.*, 1992). Recent observations have confirmed that PMN kinetics and margination in hypoxic damage are regulated by the same mediators as in inflammatory damage (Arnould *et al.*, 1993; Milhoan *et al.*, 1992; Kubes *et al.*, 1990). The production of oxygen free radicals is the initial event, peaks in the first 5 min, is likely produced endogenously by the endothelium (Ko *et al.*, 1993) and is prolonged

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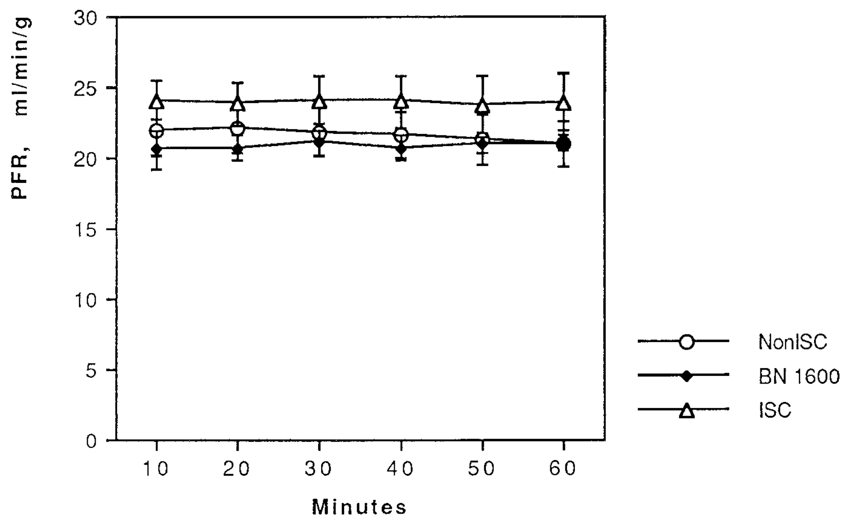

Fig. 9. Effect of no addition and addition of BN 52021, at 1600 ng/ml, on PFR in kidneys made cold ischemic for 4 h and reperfused in the absence of neutrophils. Statistical analysis was performed by two-way analysis of variance to factor in time. P not significant, NonISC *vs.* BN 1600-PMN *vs.* ISC.

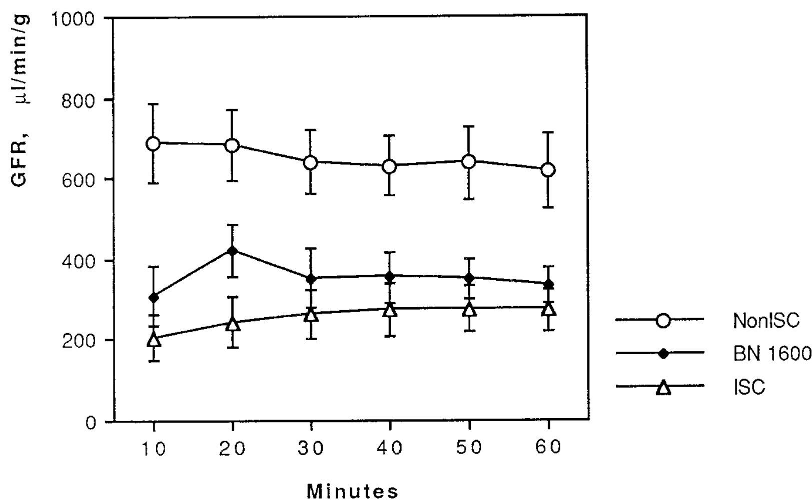

Fig. 10. Effect of no addition and addition of BN 52021, at 1600 ng/ml, on GFR in kidneys made cold ischemic for 4 h and reperfused in the absence of neutrophils. GFR was slightly but not significantly (P NS) higher in the group reperfused with the PAF antagonist than in the ischemic group reperfused without the addition of the drug.

for several hours. These oxygen free radicals produce a transmembrane calcium flux which presumably would activate phospholipase A_2 from endothelial cells (Arnould *et al.*, 1993; Zimmerman *et al.*, 1992) or PMN (Hansen, 1995; Bednar *et al.*, 1987; Lotner *et al.*, 1980). Phospholipase A_2 activation mobilizes membrane lipids, especially arachidonic acid, and results in generation of 5′-lypoxygenase products (*e.g.*, leukotriene B_4), cyclooxygenase products (*e.g.*, 6-keto prostaglandin F1a, thromboxane B_2) and the phospholipid PAF (Imatzumi *et al.*, 1995). Furthermore, activated PMN can release phospholipase A_2 into the external environment (Hansen, 1995), thereby enhancing production of PAF by other cells. Support for the enhancement of PAF release by oxygen free radicals is provided by the observation that hydrogen peroxide stimulates cultured endothelial cells to produce PAF and consequently promotes the adhesion of PMN to endothelial cell monolayers (Lewis *et al.*, 1988), and by the report of Alloati *et al.* (1994) which shows high intracoronary production of PAF after infusion of a potent oxygen radical. Some other studies have provided evidence that PMN adhere to endothelial cells during hypoxia/ischemia (Bienvenu and Granger, 1993; Arnould *et al.*, 1993; Milhoan *et al.*, 1992; Ma *et al.*, 1992). After adhesion, PMN amplifies the ischemia-reperfusion injury-generating factors, including oxygen-derived free radicals, cytokines, proteases and lipid mediators (Lefer and Lefer, 1993). Moreover, adhesion molecules on the surface of the PMN, along with their ligands on the endothelial cell membrane, are expressed. These interactions lead to neutrophil adherence to the endothelium, PMN migration into the underlying tissues and subsequent tissue injury.

Attempts to prevent or diminish ischemic injury associated with organ procurement and preservation have involved the utilization of several pharmacological agents (Lefer *et al.*, 1993; Ma *et al.*, 1991). The PAF antagonists have been shown to have a protective effect on postischemic organ function after renal warm ischemic injury (Lopez-Farre *et al.*, 1989; Torras *et al*, 1993), after liver cold and warm ischemic injury (Ontell *et al.*, 1987, 1988), after experimental lung transplantation (Conte *et al.*, 1991) and after human renal transplantation (Grinyo *et al.*, 1994). PAF is a glycerophospholipid that is an inflammatory mediator. In addition to its effects on platelets, PAF is a potent stimulator of PMN chemotaxis, adhesion to endothelial cells (Zimmerman *et al.*, 1990) and its oxidative metabolism and degranulation (Zimmerman *et al.*, 1992; Hogg, 1992), and it may serve to amplify PMN-mediated tissue injury and vascular permeability (Wedmore and Williams, 1981). Moreover, PAF is a potent vasoactive substance and increases vascular permeability (Stahl *et al.*, 1988), thus enhancing other proinflammatory actions (Handley and Saunders, 1986). Effects of PAF on kidney

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hemodynamics have been controversial. A decrease of renal plasma flow after PAF infusion *in vivo* (Hebert *et al.*, 1989), a potent and reproducible vasodilator effect in isolated rat kidney (Gerkens, 1990) and a receptor-mediated biphasic effect in the isolated microperfused afferent arterioles of the rabbit kidney, dilating them at low concentrations although constricting them at higher concentrations (Juncos *et al.*, 1993), have been reported. Besides these vasoactive and cellular effects, it has been established that PAF has glomeruli and tubular actions. In tubules, it decreases the sodium chloride transport rate in the medullary thick ascending limb of Henle's loop (Bailly *et al.*, 1992). Because it has been said that this decrease can prevent the damage induced by hypoxia in the epithelium, it has been suggested that PAF may play a role in the preservation of cell integrity during renal injury (Bailly *et al.*, 1992).

Reasons for the beneficial effect of BN 52021 on cold ischemic injury observed in our study are unclear. There is a vasoactive effect that is probably induced by the high amounts of PAF delivered during reperfusion of ischemic kidneys in the presence of PMN. This vasoactive action has been counteracted by BN 52021, because the reduction of PFR and the increase in RVR has been overcome with the addition of the drug. Nevertheless, an effect on PMN may have been produced. Although we have not demonstrated it, there are several lines of evidence: 1) reperfusion of our cold ischemic kidneys in the presence of PMN is associated with increased serum levels of PAF; 2) exposition of PMN to PAF induces PMN priming, adhesion to unstimulated endothelial cells and CD 11b receptor expression (Read *et al.*, 1993); 3) it is known that endothelial cells under hypoxia increase their adherence to PMN (Arnould *et al.*, 1993; Milhoan *et al.*, 1992), and mesenteric warm ischemia reperfusion increases the adherence and extravasation of PMN to mesenteric venular endothelium (Kubes *et al.*, 1990); 4) PAF receptor antagonists attenuate the adhesion of PMN and endothelium in cultured cells under hypoxia (Arnould *et al.*, 1993; Milhoan *et al.*, 1992) and in mesenteric ischemia (Kubes *et al.*, 1990). Unfortunately, in our study we have insufficient data to judge whether BN 52021 lowered intrarenal neutrophil retention. Finally, it is easy to discard a platelet-dependent mechanism because our experimental model is almost platelet-free because platelets have been washed out during the process of blood fractionating before the buffy coat is obtained. Our results suggest that the antagonism of the PAF receptor blocks the actions of PAF, but there is no down-regulation of the production of PAF, because we did not observe a decrease in the mean levels of PAF when the highest concentration of BN 52021 was used.

Apart from its specific PAF antagonistic property, BN 52021 has been described as a nonspecific inhibitor of proteases (Deby-Dupont *et al.*, 1986) related to the presence of a lactone ring in its molecule. It is well known that proteases constitute mediators of ischemia-reperfusion because they facilitate the generation of oxygen free radicals by activation of xanthine oxidase (Clavien *et al.*, 1992). This effect could also explain, at least in part, the beneficial effect of BN 52021 in our study.

Concentrations of BN 52021 used in our study (400, 800 and 1600 ng/ml) correspond approximately to mean plasma levels obtained after three different intravenous doses (2.5, 5 and 10 mg/kg b.wt.) in healthy volunteers (Henri Beaufour Institute, 1993). These are the doses usually reported in experimental studies (Torras *et al.*, 1993; Kubes *et al.*, 1990) and their geometrical gradation in our study allows us to evaluate a dose-dependent effect.

Reasons for the lack of effect of the highest concentration of BN 52021 (1600 ng/ml) in cold ischemic kidneys reperfused without PMN are not clear. Nevertheless, studies from the literature with other organs have given some evidence. It has been reported that isolated rabbit heart is responsive to PAF only if blood (Kenzora *et al.*, 1984), or platelets are present in the coronary vessels (Montrucchio *et al.*, 1989; Salinas *et al.*, 1995). On the other hand, a lack of enhancement of PAF release during heart ischemia in the absence of blood cellular components has been described (Montrucchio *et al.*, 1989). This has been related to the inability of myocardial cells to produce PAF (Sugiura and Waku, 1987). Glomeruli, specially mesangial cells, and medulla are known to be the major sources of PAF production in kidney (Pirotzki *et al.*, 1984). Isolated glomeruli produce PAF after ischemia (Lopez Farre et al.,1988), but there are no data about global renal PAF production after ischemia in isolated kidney in the absence of blood cells. Furthermore, it is well known that PAF is not released into the blood stream by endothelial cells and it is mainly expressed on the endothelium surface in a juxtacrine way (Zimmerman *et al.*, 1990). So, quantification of perfusate PAF levels may undervalue the real PAF amount produced by the system. However, there is not any method to quantify this PAF attached to the cell.

Recently, Salinas *et al.* (1995) have shown a lack of response to BN 52021 after warm ischemia-reperfusion in the isolated interventricular septum of rabbit heart reperfused without blood cells. The infusion of exogenous PAF aggravated the effects of ischemia, and the PAF receptor blocking with BN 52021 antagonized this effect. So, in our study, cold ischemic kidneys reperfused with PMN produced a bulk of PAF which may have acted as exogenous PAF, as in Salinas' study, aggravating ischemic damage. Therefore, we suggest that in ischemic kidneys reperfused without PMN there may be a small participation of PAF on the pathophysiology of ischemia, and other mediators may produce the ischemic-reperfusion injury observed. On the contrary, in kidneys reperfused with PMN, the action of PAF may be the most prominent, and perhaps the other mediators are much less actives and therefore the BN 52021 produces this beneficial effect.

In summary, the results of this study provide objective data to substantiate that PMN and PAF play an important role in renal failure induced by reperfusion of cold ischemic kidneys. In addition, PAF antagonists may constitute valuable drugs in preventing cold renal ischemia-reperfusion injury and in renal preservation. Results of further experimental and clinical studies will confirm the participation of PAF in these situations and whether the PAF antagonist currently in use, or another more potent one, can offer extensive clinical potential.

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