Propionyl-L-carnitine prevents early graft dysfunction in allogeneic rat kidney transplantation

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Ischemia-reperfusion injury is an important cause of graft failure. Because carnitine regulates substrate flux and energy balance across membranes which may be deranged in ischemia we determined whether its use was effective in preventing kidney injury in an allogeneic transplant model. Brown Norway rats received a Lewis rat kidney transplant and were then treated with cyclosporine A to avoid rejection. The grafts were stored in Belzer solution supplemented with propionyl-L-carnitine during the cold ischemia period. Compared to rats receiving untreated kidneys but with equal cold ischemia times, the post-transplant serum creatinine values of the carnitine-treated transplants were significantly lower. Histological evaluation 16 h after transplant showed that propionyl-L-carnitine significantly inhibited tubular necrosis and neutrophil infiltration of the allografts and improved the 3 month graft survival. Treated transplants also had decreased lipid peroxidation, inducible nitric oxide synthase expression and protein nitration compared to the untreated grafts. Post-transplant serum creatinine levels were significantly reduced and graft survival was slightly prolonged in rats not receiving cyclosporine A treatment and transplanted with a kidney treated with propionyl-L-carnitine. The efficacy of propionyl-L-carnitine to modulate ischemia-reperfusion injury during transplantation suggests that its use in human transplantation is worth testing.

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Worldwide 20–40% of patients who are given a cadaveric renal graft suffer from impaired or delayed graft function in the first few weeks after transplantation which mostly reflects acute tubular necrosis due to peritransplant ischemia-reperfusion (I/R) injury.[1] Patients transplanted with organs undergoing a longer cold I/R time have a higher risk of early post-transplant complications including acute rejection, and their long-term graft outcome is considerably less favorable.[2–4] Thus every effort to achieve immediate diuresis and function after transplantation is of great clinical relevance.

Oxidative damage is recognized to be a key process in tissue damage that follows an ischemic insult.[5,6] In hypoxic cells, mitochondrial dysfunction leads to calcium accumulation and activation of proteases that augment xanthine oxidase production, which in turn, forms oxygen free radicals. Endothelial cells are induced by ischemic damage to express high levels of surface adhesion molecules and to produce cytokines and chemokines capable to attract neutrophils and macrophages, then promoting a nonspecific host inflammatory response.[7] Vasoactive mediators, lysosomal enzymes, and superoxide anions released by activated granulocytes concur to amplify the injury.[8] Indirect evidence in support to the above sequence of events rests on the protective effect of free radical scavengers in experimental models of I/R injury.[9–13]

Despite significant advances in understanding its pathophysiology, the prevention and treatment of postischemic injury remain one of the most difficult areas of kidney transplant medicine with very modest achievements after years of trials.

Carnitine is an essential cofactor required for the translocation of activated long-chain fatty acids from extramitochondrial coenzyme A into the inner mitochondrial matrix and then to intramitochondrial coenzyme A.[14] This transport is essential to allow mitochondrial β-oxidation of long-chain fatty acids and thus to provide energy supply to the cells. Therefore, carnitine is an important factor in regulating substrate flux and energy balance across cell membranes, which might become critical in the context of I/R, possibly preventing cell injury. Support for such possibility is provided by findings that L-carnitine has protective properties in heart I/R injury in dogs.[15]

Propionyl-L-carnitine (PC), a short-chain acyl derivative of L-carnitine, has the potential not only to restore tissue carnitine stores but also to replenish key mitochondrial tricarboxylic acid intermediates as documented by studies in skeletal muscle mitochondria[16] and in ischemic heart tissues.[17] In human subjects with preexisting ventricular dysfunction, PC administration improved myocardial contractility and function.[18,19] PC also protected ischemic myocardium and stimulated contractile recovery during reperfusion in animal models.[20–22] Intravenous administration of PC to hemodialysis patients with peripheral arterial disease improved both the hemodynamic flow and the oxidative profile.[23]

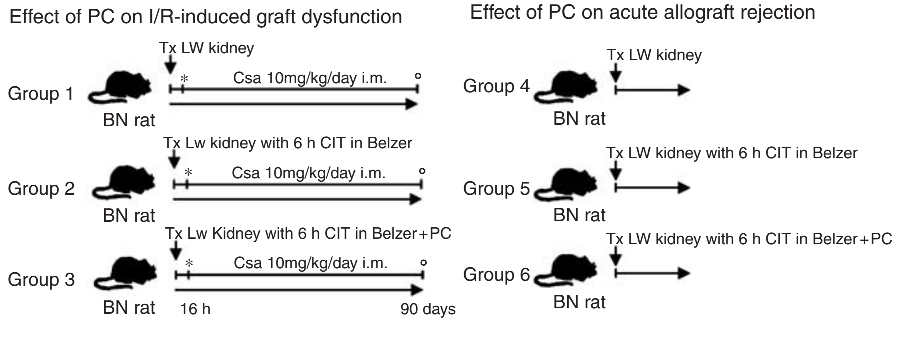
We previously documented that PC prevented renal function deterioration and structural injury induced by I/R in a rat model of isolated perfused kidney and *in vivo* in a model of syngeneic kidney transplantation.[24]

This study was aimed to investigate whether PC were effective in reducing I/R injury in an allogeneic kidney transplant setting and to study the biochemical mechanisms underlying the protective effect. For this purpose we set up a model of kidney graft I/R injury in the fully mismatched Lewis (LW) to Brown Norway (BN) rat strain combination, in which recipient rats were daily treated with cyclosporine A (CsA) to avoid acute rejection. It has been suggested that the tissue injury caused in the graft by I/R might be important in amplifying the response of T cells to alloantigens, thus facilitating allograft rejection.[25] Thus, a further aim of this study was to examine whether cold ischemia damage could accelerate allograft rejection in LW-BN rat model when no immunosuppression was given, and whether this phenomenon could be prevented by addition of PC during cold storage.

RESULTS

Effect of PC on I/R-induced graft dysfunction

To evaluate the effect of PC on I/R-induced graft dysfunction in allogeneic transplantation, LW kidneys were transplanted into BN recipients who were treated chronically with CsA (10 mg/kg/day i.m., starting from the day of transplant; Figure 1, left panel). In BN rats receiving a LW kidney allograft not preexposed to cold ischemia (group 1), a slight but statistically significant elevation ($P<0.05$) of serum creatinine levels was observed at 16 and 24 h as compared to pretransplant values (basal), likely due to warm ischemia damage (Figure 2). However in all animals in this group, serum creatinine values returned to basal levels within 2 days, and remained normal until the end of the 90 days observation period. Accordingly, in this group animal survival was 100% (Figure 3). In animals receiving kidneys preexposed to 6 h cold ischemia time (CIT) in Belzer solution alone (group 2), serum creatinine increased soon after surgery reaching values that, at 16 h, 24 h, and 2 days post transplantation, were significantly higher ($P<0.01$) than those observed in the control group 1 (Figure 2). In three out of five animals of group 2, I/R-induced renal dysfunction was irreversible and the animals died of renal failure at days 12, 18, and 24 post transplantation (Figure 3). In the other two animals, serum creatinine levels progressively decreased thereafter, reaching values of group 1 at day 15 post transplantation. In animals receiving allogeneic kidneys stored in Belzer solution added with 1.2 mg/ml PC during



*n=5 animals for each group killed at 16 h post-transplant
°killing at 90 days post-transplant

Figure 1 | Experimental design. Left panel: to evaluate the effect of PC in I/R injury in allotransplantation, the following experimental groups of animals were considered: group 1 ($n=8$), rat recipients of a kidney allograft not exposed to cold ischemia time (CIT); group 2 ($n=10$), rat recipients of a kidney allograft exposed to 6 h CIT in Belzer UW solution; group 3 ($n=10$), rat recipients of a kidney allograft exposed to 6 h CIT in Belzer UW solution containing PC (1.2 mg/ml). To prevent acute rejection, all rats received CsA (10 mg/kg/day i.m.) starting from the day of transplant until the end of follow-up. Five animals in each group were killed 16 h after transplant for graft histology, intragraft infiltration, energy charge, and oxidative damage evaluation. The other animals were followed for 90 days, to evaluate the effect of PC on graft and animal survival and on chronic renal function preservation. Right panel: to investigate whether the addition of PC to the preservation solution might delay allograft rejection, the following experimental groups of animals have been considered, to which no immunosuppression was given: group 4 ($n=4$), rat recipients of a kidney allograft not exposed to CIT; group 5 ($n=8$), rat recipients of a kidney allograft exposed to 6 h CIT in Belzer UW solution; group 6 ($n=10$), rat recipients of a kidney allograft exposed to 6 h CIT in Belzer UW solution containing PC (1.2 mg/ml). Animals were followed until rejection.

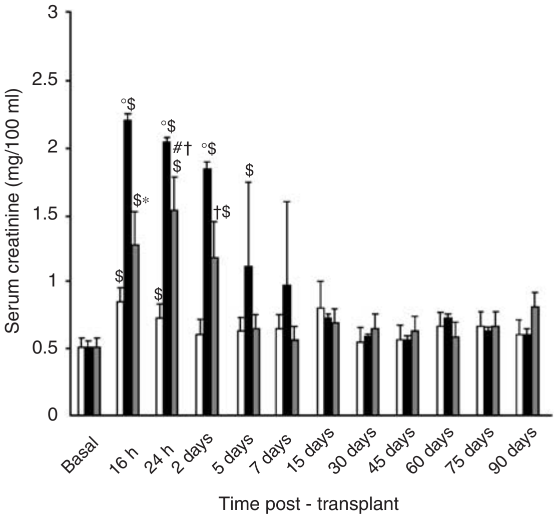


Figure 2 | Effect of PC on I/R induced graft dysfunction. Serum creatinine levels in BN rats treated with CsA and receiving a Lewis kidney allograft not preexposed to CIT (group 1, white bars, $n=8$ till 16 h, $n=3$ thereafter) or preexposed to 6 h CIT in Belzer solution (group 2, black bars, $n=10$ till 16 h, $n=5$ till 12 days, $n=4$ till 18 days, $n=3$ till 24 days, $n=2$ thereafter) or in Belzer solution added with PC (group 3, gray bars, $n=10$ till 16 h, $n=5$ thereafter). Five animals in each group were killed 16 h after transplant; the other animals were followed for 3 months. In group 2, three animals died of renal failure at days 12, 18, and 24 post transplant. Values are mean ± s.d. $^{\circ}P<0.01$ vs group 1, $^{\#}P<0.05$ vs group 1, $^{*}P<0.01$ vs group 2, $^{\dagger}P<0.05$ vs group 2, $^{\$}P<0.05$ vs basal.

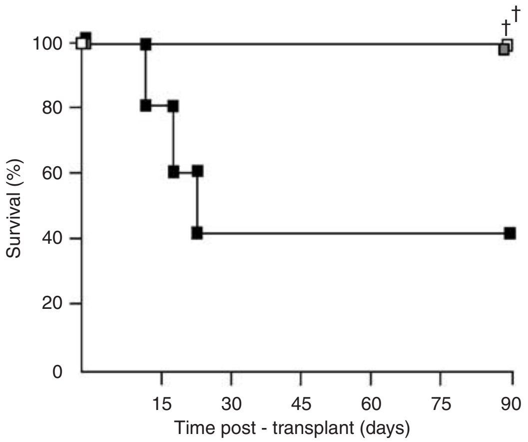


Figure 3 | Effect of PC on graft survival. Graft survival of BN rats treated with CsA and receiving a Lewis kidney allograft not preexposed to CIT (group 1, white squares; $n=3$) or preexposed to 6 h CIT in Belzer solution (group 2, black squares, $n=5$) or in Belzer solution added with PC (group 3, gray squares, $n=5$). $^{\dagger}P<0.05$ vs group 2.

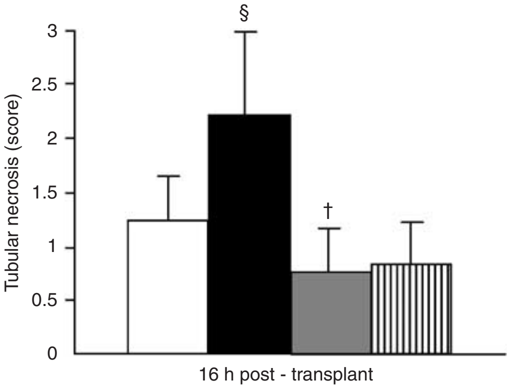


Figure 4 | Histological examination of tissue damage. Tubular necrosis (score) at 16 h post transplant in kidneys of BN rats treated with CsA and receiving a Lewis kidney allograft not preexposed to CIT (group 1, white bars, $n=5$) or preexposed to 6 h CIT in Belzer solution (group 2, black bars, $n=5$) or in Belzer solution added with PC (group 3, gray bars, $n=5$) or in native tissue kidneys (hatched bars, $n=3$). Values are mean ± s.d. $^{\dagger}P<0.05$ vs group 2, $^{\S}P<0.05$ vs native kidneys.

6 h CIT (group 3), serum creatinine values at 16 h, 24 h, and 2 days post transplantation were significantly lower than values observed in animals receiving an untreated ischemic allogeneic kidney (16 h: $P<0.01$, 24 h and 2 days: $P<0.05$). In addition, in group 3 serum creatinine levels returned to control values at day 5 post transplantation. PC treatment resulted in improved graft survival, indeed all the animals in group 3 were alive at the end of the 90 days observation period, so that the percent survival in animals of group 3 was significantly higher than that of animals receiving untreated kidneys exposed to CIT (100 vs 40%, $P<0.05$; Figure 3).

Effect of PC on I/R-induced graft histology changes and intragraft cell infiltration

Histological examination of sections from allogeneic kidneys was undertaken in a subgroup of animals killed 16 h post transplantation (Figure 4). A mild–to-moderate tubular necrosis was found in the outer medulla of grafts not exposed to cold ischemia (group 1). Kidneys preexposed to CIT (group 2) showed moderate–to-severe tubular necrosis manifested by swollen, vacuolated proximal tubular cells with pycnotic nuclei and denudation of tubular basement membrane, mainly in the outer medulla. When PC was added to the Belzer solution during CIT (group 3), a significantly ($P<0.05$) lower degree of tubular necrosis at 16 h post transplantation was observed as compared with group 2 grafts.

Figure 5a summarizes the results of quantitative evaluation of granulocyte numbers by immunoflorescence. In groups 1 and 2 allografts, higher numbers of granulocytes were found in the interstitium at 16 h post transplantation, as compared with those found in nontransplanted kidneys. Interstitial granulocyte numbers were comparable in kidneys from groups 1 and 2, indicating that warm ischemia induced granulocyte recruitment in the graft and that cold ischemia did not have an additive effect on this phenomenon. Addition of PC to the storage solution completely prevented granulocyte infiltration in the interstitial area of allogeneic kidneys ($P<0.01$ vs groups 1 and 2; Figure 5a). Low numbers of granulocytes were found in intra and periglomerular areas and in perivascular area in all groups.

Some $CD4^{+}$ lymphocytes and MHC II^{+} cells were found in the interstitium of groups 1 and 2 kidney allografts

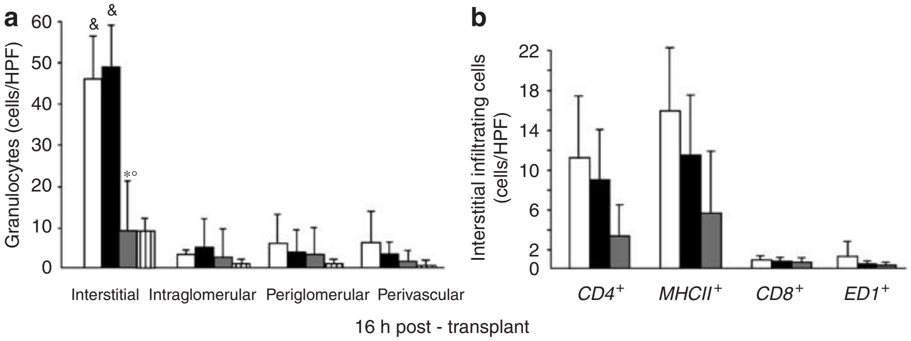


Figure 5 | Analysis of graft infiltrating cells. Immunohistochemical staining at 16 h post transplant in kidneys from animals treated with CsA and receiving a Lewis kidney allograft not preexposed to CIT (group 1, white bars, $n = 5$) or preexposed to 6 h CIT in Belzer solution (group 2, black bars, $n = 5$) or in Belzer solution added with PC (group 3, gray bars, $n = 5$) or in native tissue kidneys (hatched bars, $n = 3$). (**a**) Granulocyte counts in the interstitial area, in the glomeruli (intra and periglomerular area) and in the perivascular area; (**b**) immunostaining of $CD4^+$ lymphocytes, MHC II^+ cells, $CD8^+$ lymphocytes and $ED1^+$ cells. Values are mean ± s.d., in at least 10 randomly selected high power field (HPF). $^{\circ}P<0.01$ vs group 1, $^*P<0.01$ vs group 2, $^{\&}P<0.01$ vs native kidneys.

(Figure 5b). In PC-treated kidneys (group 3), lower numbers of infiltrating $CD4^+$ lymphocytes and MHC^+ cells were found, although the difference among groups did not reach statistical significance. Very few $CD8^+$ lymphocytes and $ED1^+$ monocytes/macrophages were found in the interstitium of kidney allografts, with no difference among groups.

Effect of PC on I/R-induced oxidative tissue damage

To evaluate whether the I/R damage in kidney grafts was associated with increased oxidative stress and whether the protective effect of PC was related to an antioxidant effect, analysis of lipid peroxidation products was undertaken in renal tissues 16 h after transplantation. The amount and distribution of 4-hydroxynonenal (4-HNE)-modified proteins, formed by a major aldehydic product of lipid peroxidation (4-HNE) and the lysine residues of structural proteins,[26] were assessed using a specific antibody. 4-HNE-lysine staining was very faint in tubuli, glomeruli, and vessels (both arterioles and venules) of nontransplanted kidneys, whereas signals were intense in kidney sections from both group 1 and group 2 animals (Figure 6). In PC-treated grafts (group 3), 4-HNE-lysine staining scores in all areas examined were significantly lower then those in groups 1 and 2. Of note, in tubuli and vessels of group 3 kidneys, the amount of lipid peroxidation was not different from that found in nontransplanted kidneys.

To evaluate whether the expression of inducible nitric oxide enzyme (iNOS) was upregulated following ischemic injury in kidney grafts, analysis of iNOS mRNA levels in the grafts was performed at the same time post transplantation. The expression of iNOS was only slightly enhanced in group 1 kidneys in respect to native kidneys (Figure 7a); by contrast iNOS expression was significantly higher ($P<0.05$) in group 2 kidneys exposed to CIT in respect to native and group 1 kidneys. PC completely blocked iNOS expression upregulation (group 3 $P<0.01$ vs group 2) so that in group 3 grafts iNOS levels were comparable to those recorded in native nontransplanted kidneys (Figure 7a).

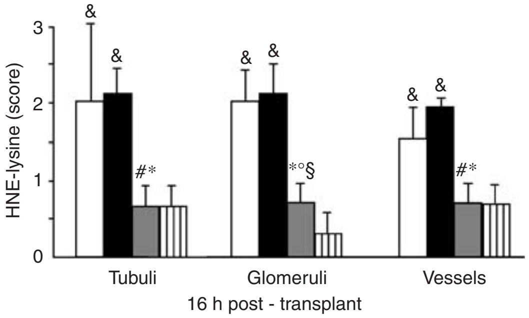


Figure 6 | Immunocytochemistry of 4-hydroxynonenal protein adducts. 4-HNE lysine immunoperoxidase staining mean score in kidney tissues from rats treated with CsA and receiving a kidney allograft not preexposed to CIT (group 1, white bars, $n = 5$) or preexposed to 6 h CIT in Belzer solution (group 2, black bars, $n = 5$) or in Belzer solution added with PC (group 3, gray bars, $n = 5$) or in native tissue kidneys (hatched bars, $n = 3$). Values are mean ± s.d. $^{\circ}P<0.01$ vs group 1, $^{\#}P<0.05$ vs group 1, $^*P<0.01$ vs group 2, $^{\&}P<0.01$ vs native kidneys, $^{\S}P<0.05$ vs native kidneys.

High amounts of free oxygen radicals in the presence of nitric oxide could generate toxic products such as peroxinitrite, whose formation can be detected by measuring the extent of protein nitration by nitrotyrosine staining.[27] This was done in kidneys from rats killed 16 h post transplantation. As shown in Figure 7b, kidney grafts undergoing cold ischemia in Belzer solution alone were more markedly stained by an antinitrotyrosine antibody in tubuli, glomeruli, and vessels (both arterioles and venules) than native kidneys and kidney grafts not exposed to CIT. Staining for nitrotyrosine in kidneys from group 3 treated with PC was significantly lower in tubuli ($P<0.01$), glomeruli ($P<0.01$), and vessels ($P<0.01$) than in kidneys from group 2 and not different from that found in native kidneys. Altogether the data indicate that PC added to Belzer solution during CIT prevented lipid peroxidation, iNOS upregulation and peroxinitrite generation.

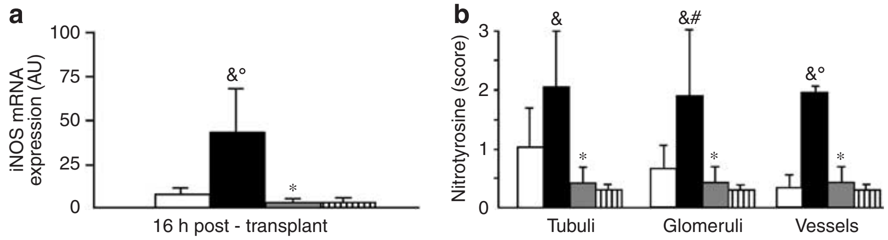


Figure 7 | Expression of inducible nitric oxide enzyme and immunocytochemistry of nitrotyrosine. (**a**) iNOS mRNA expression (AU) by real-time PCR and (**b**) nitrotyrosine staining mean score in kidney tissues from rats treated with CsA and receiving a Lewis kidney allograft not preexposed to CIT (group 1, white bars, $n=5$) or preexposed to 6 h CIT in Belzer solution (group 2, black bars, $n=5$) or in Belzer solution added with PC (group 3, gray bars, $n=5$) or in native kidney tissues (hatched bars, $n=3$). Values are mean ± s.e. (**a**) or s.d. (**b**). $^{\circ}P<0.01$ vs group 1, $^{\#}P<0.05$ vs group 1, $^{*}P<0.01$ vs group 2, $^{\&}P<0.01$ vs native kidneys.

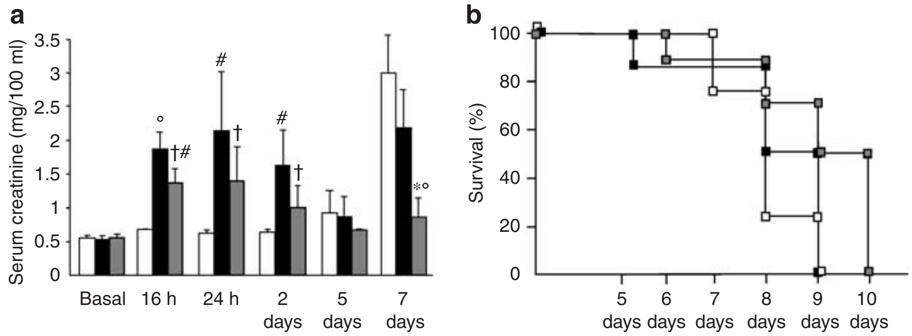


Figure 8 | Effect of PC on acute allograft rejection. (**a**) Serum creatinine levels (mean ± s.d.) and (**b**) graft survival (%) of BN rats receiving a Lewis kidney allograft not preexposed to CIT (group 4, white squares, $n=4$) or preexposed to 6 h CIT in Belzer solution (group 5, black squares, $n=8$) or in Belzer solution added with PC (group 6, gray squares, $n=10$). $^{\circ}P<0.01$ vs group 4, $^{\#}P<0.05$ vs group 4, $^{*}P<0.01$ vs group 5, $^{\dagger}P<0.05$ vs group 5.

Effect of PC on acute allograft rejection

We then investigated whether I/R damage had an impact on the severity of acute allograft rejection.[25] For this purpose BN rats received an allogeneic LW kidney transplant preexposed or not to 6 h CIT, but were not given CsA immunosuppression (Figure 1, right panel).

In animals receiving untreated kidneys exposed to CIT (group 5), serum creatinine increased soon after surgery reaching values that, at 16, 24 h and 2 days post transplantation, were significantly higher ($P<0.01$, $P<0.05$, $P<0.05$, respectively) than those observed in a control group of animals receiving a nonischemic allogeneic kidney transplant (group 4; Figure 8a). In animals of group 5, serum creatinine decreased in the following days and within 5 days reached mean values comparable to those recorded in rats of group 4. Serum creatinine increased thereafter in both groups due to acute rejection that occurred from days 5 to 9. Graft survival (Figure 8b) did not differ between animals of groups 4 and 5 (mean survival 8 and 8.1 days, respectively).

In animals receiving allogeneic kidneys stored in Belzer solution added with PC (1.2 mg/ml) during the 6 h CIT (group 6), serum creatinine values at 16 h, 24 h, and 2 days post transplant were significantly lower than values observed in those of group 5 animals ($P<0.05$; Figure 8a). In addition, in this group, rise in mean serum creatinine levels at day 7 post transplant, due to acute rejection, was significantly lower than in rats of groups 4 and 5 ($P<0.01$). The survival time was slightly prolonged in group 6 animals (mean survival 9 days) as compared with animals receiving an untreated allograft, however the difference did not reach statistical significance (Figure 8b).

DISCUSSION

In a previous study in a rat syngeneic kidney transplantation model,[24] we documented a protective effect of PC against I/R injury. This study confirms the protective properties of PC in a fully allogeneic transplant model, a setting that more closely mimics the clinical condition. Furthermore, we found that immunosuppression with CsA did not hamper the action of PC to protect the graft against I/R damage, which makes our results even more clinically relevant.

Renal function preservation by PC was documented by lower serum creatinine levels at early times after transplant in rats whose graft were treated with PC compared to animals receiving untreated grafts exposed to CIT. By histological analysis, we documented a structural preservation of kidneys stored in Belzer solution added with PC, in particular renal tubular damage, the most typical feature of I/R injury,[8] was clearly prevented by PC.

Another main finding of this study is that the exposure of kidneys to cold ischemia had a strong impact on graft survival. This is in line with clinical observations that, in kidney transplant recipients, CIT is a negative risk factor for long term renal allograft survival.[2,3] On the other hand, when kidneys underwent CIT in the presence of PC, 100% graft survival was achieved at the end of the observation period, suggesting that the addition of this drug to preservation solution might result in improved graft success in human kidney transplantation.

We then tried to gain insight into the mechanisms involved in PC protection from I/R damage. Leukocyte infiltration in response to I/R injury is a well known phenomenon.[28] As in acute inflammation, neutrophils infiltrate down a concentration gradient of chemotactic factors such as interleukin-8 and macrophage inflammatory protein-2, after adherence to endothelial cells through adhesion molecules.[29] In turn, neutrophils release lysosomal enzymes and superoxide anions, which concur to amplify the tissue injury.[30] In several models of I/R injury, prevention of granulocyte infiltration resulted in a beneficial effect on postischemic organ function.[31–38] We then analyzed the degree of granulocyte infiltration and found an increased number of neutrophils in interstitial areas of all untreated transplanted kidneys, irrespectively of whether they were or not exposed to CIT. We speculate that in our model warm ischemia during surgery was sufficient to trigger signals that recruited neutrophils and sustained their graft infiltration, as already observed in experimental models of renal I/R injury induced by arterial clamping.[34,38–42] The slight increase in tubular damage and the significant raise in serum creatinine that we observed following warm ischemia, were probably attributable to the contribution of neutrophil infiltration to graft dysfunction. PC addition to the Belzer solution completely prevented intragraft infiltration of neutrophils as well as $CD4^+$, $CD8^+$, $ED1^+$, and MHC II^+ cells. The effect of PC on leukocytes was probably secondary to a protective effect of this drug on renal tissue, so that inflammatory stimuli such as adhesion molecules and chemokines triggered by I/R damage were reduced in PC treated grafts.

The property of PC to modify the molecular dynamics of the membrane bilayer[43] and to stabilize plasma membrane during ischemia[44] could contribute to the protection observed at tubular cell level. The possibility exists that PC may protect ischemic tissue by a metabolic effect, related to an improvement in mitochondrial respiration, as already described in models of cardiac[22] and forebrain ischemia.[45] However, other studies in rat isolated perfused kidneys[24] and hearts[46] did not find an effect of PC on ATP and energy charge levels in ischemic tissues. Further studies are needed to investigate a possible metabolic effect of PC in our experimental model.

Cytoprotection could also be attributed to direct antioxidant properties of PC as previously reported *in vitro*[47] and in models of rat forebrain ischemia-induced neuronal injury,[45] in liver and heart of spontaneous hypertensive rats,[48] in rat renal[24] and cardiac[49,50] I/R injury. According to this hypothesis, we found that allogeneic grafts treated with PC were protected from oxidative stress injury that was instead present in untreated ischemic kidney grafts. Notably, as observed for neutrophil infiltration, oxidative stress injury was also present in kidney grafts not exposed to CIT, indicating that warm ischemia was sufficient to cause increased lipid peroxidation and that apparently cold ischemia did not worsen oxidative stress. Although granulocyte infiltration and HNE production were both ameliorated by PC, they were not different in the cold and warm ischemic kidneys, so they cannot simply account for the injury observed.

In this study, we also showed a pro-oxidant pathway activated by cold but not by warm ischemia in kidney grafts. Peroxinitrite generation, a phenomenon reflecting the increased tissue concentration of reactive oxygen species and nitric oxide,[27] occurred specifically in kidneys undergoing CIT, but not in kidneys only exposed to warm ischemia as demonstrated by nitrotyrosine staining. Accordingly, mRNA for iNOS—the inducible NO-synthase isoform expressed in inflammatory cells[51] and in renal tissue[52,53]—was upregulated in grafts exposed to CIT but not in grafts only exposed to warm ischemia. We did not investigate the cell localization of iNOS, however we found nitrotyrosine staining signals both in resident cells and in inflammatory infiltrates (data not shown), which suggests that both PMN and renal cells could contribute to the generation of peroxinitrite. Altogether these data indicate that cold ischemia was associated to an enhancement of iNOS expression and NO release in the graft that combining with reactive oxygen species released by PMN and by renal tissue upon warm ischemia could result in the production of peroxinitrite and tissue damage. We found that PC treatment inhibited the upregulation of iNOS mRNA in kidney grafts exposed to cold ischemia and consequently the production of peroxinitrite. However, we were unable to demonstrate if there was a direct antioxidant effect of PC or if its various primary effects in ameliorating the cellular insult could have contributed to limit the oxidative injury.

It has been hypothesized that ischemic renal injury increases the risk of acute graft rejection because stimulation of the innate immunity in response to injury amplifies adaptive immune response to the allograft.[25] Indeed, after I/R, increased expression of MHC class I and II molecules in the kidney have been reported, which can elicit a strong immune response via either the direct or indirect pathway of allorecognition.[54] Tubular cells mainly upregulate MHC class I antigens, whereas in infiltrating interstitial cells MHC class II expression increases in response to I/R.[55] Ischemic injury also upregulates intragraft expression of costimulatory molecules, resulting in increased immunogenicity of transplanted organs.[35] The possibility that ischemic injury is an initiating factor in allograft rejection is supported by several clinical studies that reported an higher incidence of acute rejection in human kidney allografts with post-transplant acute tubular necrosis and delayed graft function compared with allografts with immediate function.[56–58] In the fully mismatched rat kidney transplantation model here reported, graft survival was not worsened by cold ischemia, suggesting that I/R damage did not accelerate acute allograft rejection. However, in the group of animals receiving PC-treated ischemic grafts, mean serum creatinine levels due to acute rejection were significantly lower at 7 days post transplantation and the graft survival time was slightly prolonged as compared to rats receiving untreated kidneys either exposed or not to CIT. These data would suggest that the protective properties of PC on pathogenetic mechanisms unchained by both warm and cold ischemia had an effect in reducing the graft capacity to stimulate T-cell alloreactivity, although this protection was not enough to block the reaction of rejection in this fully mismatched model, in the absence of any immunosuppression.

In conclusion, our results indicate that addition of PC to the storage Belzer solution during cold ischemia led to structural and functional graft preservation toward I/R damage in rat kidney allotransplantation. This benefit was associated with inhibition of leukocyte recruitment and reduction of oxidative stress. These results support the clinical applicability of PC addition to organ storage solution, which might be useful for the prevention of I/R injury-induced delayed graft function in kidney transplant patients.

MATERIALS AND METHODS

Animals

Inbred adult male BN rats ($RT1^n$; Charles River Italia Spa, Calco, Italy) were used as recipients and LW ($RT1^l$) as donors.

Animal care and treatment were conducted in accordance with institutional guidelines in compliance with national and international laws and policies (European Economic Community Council Directive 86/609). All animals were allowed free access to standard rat chow and tap water.

Kidney transplantation and graft function evaluation

Kidney transplantation was performed as described previously.[24] The donor kidney was flushed with Belzer UW solution containing 1000 U/ml heparin and, according to experimental design, placed or not in an iced Belzer UW solution for 6 h (CIT) with or without addition of PC. After ischemia time had elapsed, recipient rats underwent removal of the native left kidney. Donor kidney grafts were then washed with saline solution and transplanted. An anastomosis was created between the donor and recipient renal artery as well as renal vein with end-to-end anastomosis. Vascular clamps were released after 30 min (warm ischemia). Donor and recipient ureters were attached end-to-end. The native right kidney was then removed. Rejection was defined as animal death or killing because of moribund state. Serum creatinine levels were measured by Reflovet (Roche, Mannheim, Germany).[24,59]

Histological examination

Kidney specimens fixed in Dubosq-Brazil were dehydrated in alcohol. After paraffin embedding, 3-μm sections of the blocks were cut and stained with periodic acid-Schiff, Masson's trichrome, and hematoxylin eosin (H and E). Tubular damage—defined as necrosis—was assessed and graded according to a semiquantitative scale (0–3).[24]

Immunohistochemistry

To evaluate leukocyte infiltration, indirect immunofluorescence was performed on frozen sections, with the following antibodies: mouse anti-rat granulocyte (MOM/3F12/F2; Walter Occhiena, Turin, Italy); mouse anti-rat CD4 (W3/25; Serotec, Oxford, UK); mouse anti-rat MHC class II (0X6; Walter Occhiena); mouse anti-rat CD8 (OX8; Pharmingen, San Diego, CA); mouse anti-rat ED1 (Chemicon, Temecula, CA). As secondary antibodies, Cy3-conjugated donkey anti-mouse immunoglobulin G (Jackson ImmunoResearch, West Grove, PA) were used.

Oxidative damage in renal tissue was localized by specific mouse monoclonal antibodies against nitrotyrosine (Upstate Biotechnology Inc., Lake Placid, NY) and 4-HNE-lysine adduct (NA59, kindly provided by Dr Witzum, The Scripps Research Institute, La Jolla, CA). Briefly, 3-μm paraffin sections previously fixed with 10% formalin were incubated with the primary antibody and developed with diaminobenzidine-nickel. The sections were counterstained with Harris hematoxylin. Negative controls were incubated with a nonimmune antibody. Each section was scored for intensity of immunostaining (absent, faint, moderate, intense: 0–3). At least 8–10 fields per section were examined. The final score *per se*ction was calculated as a weighted mean.

Real-time quantitative PCR

Total RNA was obtained by homogenization of renal tissue followed by Trizol extraction (Invitrogen, Carlsbad, CA, USA). RNA was treated with DNase and reverse transcribed to cDNA by Supescript II (Invitrogen). Quantitative real-time PCR was performed on a TaqMan ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA USA) with Power Syber Green Master Mix as previously described.[60] The $\Delta\Delta$Ct equation was used to compare the iNOS gene expression in each sample with the expression in a pool of three native kidneys, taken as calibrator (iNOS expression set to 1 arbitrary unit).

Statistical analysis

Results are expressed as mean ± s.d. or s.e. as specified in the figure legends. Data were analyzed using log-rank test, ANOVA-factorial, or ANOVA-repeated measures for multiple comparisons as appropriate. Statistical significance was defined as $P<0.05$.

DISCLOSURE

All the authors declare no conflict of interest.

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REFERENCES

1. Daly PJ, Power RE, Healy DA *et al.* Delayed graft function: a dilemma in renal transplantation. *BJU Int* 2005; **96**: 498–501.
2. Perico N, Cattaneo D, Sayegh MH *et al.* Delayed graft function in kidney transplantation. *Lancet* 2004; **364**: 1814–1827.
3. Quiroga I, McShane P, Koo DD *et al.* Major effects of delayed graft function and cold ischaemia time on renal allograft survival. *Nephrol Dial Transplant* 2006; **21**: 1689–1696.

4. Shoskes DA, Halloran PF. Delayed graft function in renal transplantation: etiology, management and long-term significance. *J Urol* 1996; **155**: 1831–1840.
5. Johnson KJ, Weinberg JM. Postischemic renal injury due to oxygen radicals. *Curr Opin Nephrol Hypertens* 1993; **2**: 625–635.
6. Burke TJ, Schrier RW. Pathophysiology of cell ischemia.In: Schrier RW, Gottschalk CW (eds). *Diseases of the Kidney*. Little Brown and Co: Boston, 1997, pp 1013.
7. Sutton TA, Fisher CJ, Molitoris BA. Microvascular endothelial injury and dysfunction during ischemic acute renal failure. *Kidney Int* 2002; **62**: 1539–1549.
8. Bonventre JV, Weinberg JM. Recent advances in the pathophysiology of ischemic acute renal failure. *J Am Soc Nephrol* 2003; **14**: 2199–2210.
9. Toledo-Pereyra LH, Lopez-Neblina F, Toledo AH. Reactive oxygen species and molecular biology of ischemia/reperfusion. *Ann Transplant* 2004; **9**: 81–83.
10. Tahara M, Nakayama M, Jin MB *et al.* A radical scavenger, edaravone, protects canine kidneys from ischemia-reperfusion injury after 72 h of cold preservation and autotransplantation. *Transplantation* 2005; **80**: 213–221.
11. Zahmatkesh M, Kadkhodaee M, Moosavi SM *et al.* Beneficial effects of MnTBAP, a broad-spectrum reactive species scavenger, in rat renal ischemia/reperfusion injury. *Clin Exp Nephrol* 2005; **9**: 212–218.
12. Hines IN, Harada H, Wolf R *et al.* Superoxide and post-ischemic liver injury: potential therapeutic target for liver transplantation. *Curr Med Chem* 2003; **10**: 2661–2667.
13. Simpson PJ, Mickelson JK, Lucchesi BR. Free radical scavengers in myocardial ischemia. *Fed Proc* 1987; **46**: 2413–2421.
14. Bremer J. Carnitine—metabolism and functions. *Physiol Rev* 1983; **63**: 1420–1480.
15. Suzuki Y, Kamikawa T, Kobayashi A *et al.* Effects of L-carnitine on tissue levels of acyl carnitine, acyl coenzyme A and high energy phosphate in ischemic dog hearts. *Jpn Circ J* 1981; **45**: 687–694.
16. Davis EJ, Spydevold O, Bremer J. Pyruvate carboxylase and propionyl-CoA carboxylase as anaplerotic enzymes in skeletal muscle mitochondria. *Eur J Biochem* 1980; **110**: 255–262.
17. Tassani V, Cattapan F, Magnanimi L *et al.* Anaplerotic effect of propionyl carnitine in rat heart mitochondria. *Biochem Biophys Res Commun* 1994; **199**: 949–953.
18. Ferrari R, DeGiuli F. The propionyl-L-carnitine hypothesis: an alternative approach to treating heart failure. *J Card Fail* 1997; **3**: 217–224.
19. Caponnetto S, Canale C, Masperone MA *et al.* Efficacy of L-propionylcarnitine treatment in patients with left ventricular dysfunction. *Eur Heart J* 1994; **15**: 1267–1273.
20. Paulson DJ, Traxler J, Schmidt M *et al.* Protection of the ischaemic myocardium by L-propionylcarnitine: effects on the recovery of cardiac output after ischaemia and reperfusion, carnitine transport, and fatty acid oxidation. *Cardiovasc Res* 1986; **20**: 536–541.
21. Shug A, Paulson D, Subramanian R *et al.* Protective effects of propionyl-L-carnitine during ischemia and reperfusion. *Cardiovasc Drugs Ther* 1991; **5**(Suppl 1): 77–83.
22. Ferrari R, Ceconi C, Cargnoni A *et al.* The effect of propionyl-L-carnitine on the ischemic and reperfused intact myocardium and on their derived mitochondria. *Cardiovasc Drugs Ther* 1991; **5**(Suppl 1): 57–65.
23. Signorelli SS, Fatuzzo P, Rapisarda F *et al.* A randomised, controlled clinical trial evaluating changes in therapeutic efficacy and oxidative parameters after treatment with propionyl L-carnitine in patients with peripheral arterial disease requiring haemodialysis. *Drugs Aging* 2006; **23**: 263–270.
24. Mister M, Noris M, Szymczuk J *et al.* Propionyl-L-carnitine prevents renal function deterioration due to ischemia/reperfusion. *Kidney Int* 2002; **61**: 1064–1078.
25. Lu CY, Penfield JG, Kielar ML *et al.* Hypothesis: is renal allograft rejection initiated by the response to injury sustained during the transplant process? *Kidney Int* 1999; **55**: 2157–2168.
26. Esterbauer H, Cheeseman KH, Dianzani MU *et al.* Separation and characterization of the aldehydic products of lipid peroxidation stimulated by ADP-Fe2+ in rat liver microsomes. *Biochem J* 1982; **208**: 129–140.
27. Radi R. Nitric oxide, oxidants, and protein tyrosine nitration. *Proc Natl Acad Sci USA* 2004; **101**: 4003–4008.
28. De Greef KE, Ysebaert DK, Ghielli M *et al.* Neutrophils and acute ischemia-reperfusion injury. *J Nephrol* 1998; **11**: 110–122.
29. Mackay CR. Chemokines: immunology's high impact factors. *Nat Immunol* 2001; **2**: 95–101.
30. Welbourn CR, Goldman G, Paterson IS *et al.* Pathophysiology of ischaemia reperfusion injury: central role of the neutrophil. *Br J Surg* 1991; **78**: 651–655.
31. Cugini D, Azzollini N, Gagliardini E *et al.* Inhibition of the chemokine receptor CXCR2 prevents kidney graft function deterioration due to ischemia/reperfusion. *Kidney Int* 2005; **67**: 1753–1761.
32. Sekido N, Mukaida N, Harada A *et al.* Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8. *Nature* 1993; **365**: 654–657.
33. Hellberg PO, Kallskog TO. Neutrophil-mediated post-ischemic tubular leakage in the rat kidney. *Kidney Int* 1989; **36**: 555–561.
34. Kelly KJ, Williams Jr WW, Colvin RB *et al.* Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *J Clin Invest* 1996; **97**: 1056–1063.
35. Takada M, Nadeau KC, Shaw GD *et al.* The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. *J Clin Invest* 1997; **99**: 2682–2690.
36. Souza DG, Cara DC, Cassali GD *et al.* Effects of the PAF receptor antagonist UK74505 on local and remote reperfusion injuries following ischaemia of the superior mesenteric artery in the rat. *Br J Pharmacol* 2000; **131**: 1800–1808.
37. Souza DG, Coutinho SF, Silveira MR *et al.* Effects of a BLT receptor antagonist on local and remote reperfusion injuries after transient ischemia of the superior mesenteric artery in rats. *Eur J Pharmacol* 2000; **403**: 121–128.
38. Rouschop KM, Roelofs JJ, Claessen N *et al.* Protection against renal ischemia reperfusion injury by CD44 disruption. *J Am Soc Nephrol* 2005; **16**: 2034–2043.
39. Linas SL, Whittenburg D, Parsons PE *et al.* Ischemia increases neutrophil retention and worsens acute renal failure: role of oxygen metabolites and ICAM 1. *Kidney Int* 1995; **48**: 1584–1591.
40. Willinger CC, Schramek H, Pfaller K *et al.* Tissue distribution of neutrophils in postischemic acute renal failure. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1992; **62**: 237–243.
41. Paller MS. Effect of neutrophil depletion on ischemic renal injury in the rat. *J Lab Clin Med* 1989; **113**: 379–386.
42. Thornton MA, Winn R, Alpers CE *et al.* An evaluation of the neutrophil as a mediator of *in vivo* renal ischemic-reperfusion injury. *Am J Pathol* 1989; **135**: 509–515.
43. Arduini A, Gorbunov N, Arrigoni-Martelli E *et al.* Effects of L-carnitine and its acetate and propionate esters on the molecular dynamics of human erythrocyte membrane. *Biochim Biophys Acta* 1993; **1146**: 229–235.
44. Hulsmann WC. Biochemical profile of propionyl-L-carnitine. *Cardiovasc Drugs Ther* 1991; **5**(Suppl 1): 7–9.
45. Al-Majed AA, Sayed-Ahmed MM, Al-Omar FA *et al.* Carnitine esters prevent oxidative stress damage and energy depletion following transient forebrain ischaemia in the rat hippocampus. *Clin Exp Pharmacol Physiol* 2006; **33**: 725–733.
46. Di Lisa F, Menabo R, Barbato R *et al.* Contrasting effects of propionate and propionyl-L-carnitine on energy-linked processes in ischemic hearts. *Am J Physiol* 1994; **267**: H455–H461.
47. Calo LA, Pagnin E, Davis PA *et al.* Antioxidant effect of L-carnitine and its short chain esters: relevance for the protection from oxidative stress related cardiovascular damage. *Int J Cardiol* 2006; **107**: 54–60.
48. Gomez-Amores L, Mate A, Revilla E *et al.* Antioxidant activity of propionyl-L-carnitine in liver and heart of spontaneously hypertensive rats. *Life Sci* 2006; **78**: 1945–1952.
49. Ronca G, Ronca F, Yu G *et al.* Protection of isolated perfused working rat heart from oxidative stress by exogenous L-propionyl carnitine. *Drugs Exp Clin Res* 1992; **18**: 475–480.
50. Packer L, Valenza M, Serbinova E *et al.* Free radical scavenging is involved in the protective effect of L-propionyl-carnitine against ischemia-reperfusion injury of the heart. *Arch Biochem Biophys* 1991; **288**: 533–537.
51. Dedon PC, Tannenbaum SR. Reactive nitrogen species in the chemical biology of inflammation. *Arch Biochem Biophys* 2004; **423**: 12–22.
52. Bachmann S, Mundel P. Nitric oxide in the kidney: synthesis, localization, and function. *Am J Kidney Dis* 1994; **24**: 112–129.
53. Morrissey JJ, McCracken R, Kaneto H *et al.* Location of an inducible nitric oxide synthase mRNA in the normal kidney. *Kidney Int* 1994; **45**: 998–1005.
54. Sayegh MH. Why do we reject a graft? Role of indirect allorecognition in graft rejection. *Kidney Int* 1999; **56**: 1967–1979.

55. Shoskes DA, Parfrey NA, Halloran PF. Increased major histocompatibility complex antigen expression in unilateral ischemic acute tubular necrosis in the mouse. *Transplantation* 1990; **49**: 201–207.
56. Halloran PF, Hunsicker LG. Delayed graft function: state of the art, November 10–11, 2000. Summit meeting, Scottsdale, Arizona, USA. *Am J Transplant* 2001; **1**: 115–120.
57. Yokoyama I, Uchida K, Kobayashi T *et al*. Effect of prolonged delayed graft function on long-term graft outcome in cadaveric kidney transplantation. *Clin Transplant* 1994; **8**: 101–106.
58. Ojo AO, Wolfe RA, Held PJ *et al*. Delayed graft function: risk factors and implications for renal allograft survival. *Transplantation* 1997; **63**: 968–974.
59. Dols JL, Sanders GT. Evaluation of the creatinine assay on the Reflotron analyser under centralized and non-centralized conditions. *Eur J Clin Chem Clin Biochem* 1993; **31**: 359–366.
60. Aiello S, Cassis P, Cassis L *et al*. DnIKK2-transfected dendritic cells induce a novel population of inducible nitric oxide synthase-expressing CD4+CD25- cells with tolerogenic properties. *Transplantation* 2007; **83**: 474–484.