A small molecule C5a receptor antagonist protects kidneys from ischemia/reperfusion injury in rats

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Background. C5a has been implicated in numerous pathophysiological conditions, including ischemia/reperfusion (I/R) injury of the kidney. We examined whether a novel and specific C5a receptor antagonist, the cyclic compound AcF-[OPdChaWR] could moderate I/R-induced renal injury in rats.

Methods. Female Wistar rats were subjected to renal ischemia (60 min) and reperfusion (5 h). Rats were treated with either 1 mg/kg IV in 5% ethanol/saline or 10 mg/kg PO in 25% ethanol/saline prior to ischemia. I/R injury was characterized by significant tissue hemorrhage with increased microvascular permeability, elevated renal tissue levels of tumor necrosis factor-α (TNF-α) and myeloperoxidase (MPO), increased serum levels of creatinine and aspartate aminotransferase (AST) and hematuria.

Results. Pre-ischemic treatment with the C5a receptor (C5aR) antagonist (1 mg/kg IV or 10 mg/kg PO) substantially inhibited or prevented I/R-induced hematuria, vascular leakage, tissue levels of TNF-α and MPO, and serum levels of AST and creatinine. Histological examination of kidneys from antagonist pretreated I/R animals showed a marked reduction in tissue damage compared to drug-free I/R rats. This antagonist, however, did not inhibit complement-mediated lysis of red blood cells, suggesting unimpaired formation of the membrane attack complex (MAC).

Conclusions. The results demonstrate for the first time that a selective antagonist of both human and rat C5a receptors, given either intravenously or orally, significantly protects the kidney from I/R injury in the rat. We conclude that C5a is an important pathogenic agent in renal I/R injury, and that C5a receptor antagonists may be useful therapeutic agents for the pretreatment of anticipated renal reperfusion injury in humans.

Renal ischemia/reperfusion (I/R) injury is a significant complication of vascular surgery of the aorta and kidney. It also commonly occurs when renal perfusion is reduced during shock, and I/R-induced renal failure remains a major cause of morbidity and mortality in hospitalized patients [1]. There is currently no effective therapy to prevent cellular injury resulting from renal I/R [2]. Although there is continuing debate about the nature of the mediators of renal I/R, numerous studies have identified the following inflammatory mediators as likely being involved: oxygen-derived reactive species [3], tumor necrosis factor-α (TNF-α) [1, 4], phospholipase A2 (PLA2) [5], leukotriene B4 (LTB4) [6], intercellular adhesion molecule-1 (ICAM-1) [7, 8], P-selectin [9], polymorphonuclear leukocytes (PMNs) [10] and platelet-activating factor (PAF) [11].

Polymorphonuclear leukocytes play an important role in mediating the tissue injury associated with I/R injuries, accumulating in tissues during ischemia, and this is exacerbated by reperfusion [12]. PMN activation has been demonstrated in renal I/R [13, 14], which is seen as adherence of leukocytes to vascular endothelium, retention of PMNs in the affected tissue, and release of myeloperoxidase (MPO). ICAM-1 and P-selectin, which facilitate the adhesion of PMNs to the vascular endothelium, are important in the pathology of renal I/R. ICAM-1 deficiency [7], treatment with antibodies against ICAM-1 [8], or P-selectin blockade [9] reduce renal I/R injury.

Some experimental studies have shown that I/R injury results in local tissue production of the complement factor 5a (C5a) [15, 16]. C5a involvement in I/R injury has been demonstrated by the use of a variety of agents that blocked the action of C5a and reduced I/R-induced injury [17–20]. However, the effects of a specific C5a receptor (C5aR) antagonist have not been determined in renal I/R injury to date. Activation of C5a receptors on PMNs leads to chemotaxis, expression of cellular adhesion receptors, and release of pro-inflammatory mediators [21, 22]. The involvement of activated PMNs in local tissue damage is well established for reperfusion injury, and agents that can

Key words: acute renal ischemia-reperfusion, C5a receptor antagonist, AcF-[OPdChaWR], polymorphonuclear leukocytes, transplantation, organ storage.

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prevent the activation and extravasation of PMNs may be therapeutic for the prevention or treatment of I/R.

The purpose of the present study, therefore, was to determine the effects of a new, potent and selective C5aR antagonist, AcF-OPdChaWR, against renal I/R injury in rats. This cyclic antagonist is orally active [18, 24, 25], an effective inhibitor of gut I/R injury [18], endotoxin-induced hypotension and neutropenia [26], and the reverse-passive Arthus reaction [24, 27]. In the present study, we demonstrate that either intravenous or oral administration of the antagonist is effective in inhibiting both biochemical and tissue changes that occur in a rat model of renal I/R injury, suggesting that C5a is a major pathogenic mediator of this condition in rats.

METHODS

Antagonist preparation

The C5aR antagonist, the cyclic molecule AcF-OPdChaWR, was synthesized as previously described [23, 28]. The compound, as the acetate salt, was purified by reversed-phase high-pressure liquid chromatography (HPLC) and fully characterized by mass spectrometry and proton nuclear magnetic resonance (NMR) spectroscopy as reported [23, 28].

Red blood cell lysis assay

To determine whether AcF-OPdChaWR inhibited activation of the complement cascade and formation of the terminal membrane attack complex (MAC), a red blood cell lysis assay was used as previously described [29]. Sheep erythrocytes were sensitized with hemolysin and incubated with dilutions of human serum. A serum dilution (1/100) causing approximately 70% hemolysis (determined spectrophotometrically at 415 nmol/L) was used to assay inhibitory activity of AcF-OPdChaWR and a control inhibitor, rosmarinic acid [29]. AcF-OPdChaWR or rosmarinic acid were preincubated with serum for 10 minutes at 37°C prior to addition of sensitized erythrocytes. Results were expressed as a percentage of the maximal red blood cell lysis that occurred in the presence of distilled water. Data are shown as the mean ± SEM maximum lysis for each experiment.

Model of renal ischemia/reperfusion injury

Female Wistar rats (250 to 300 g) were fasted and given water only for 12 hours prior to all experiments. Animals were anesthetized by intraperitoneal injection of zolazepam (25 mg/kg), tiletamine (25 mg/kg) and xylazine (10 mg kg⁻¹), and anesthesia was maintained by administration of a single dose of 80 mg/kg ketamine and 10 mg/kg xylazine during the procedure. During the procedure rats were placed on a heating pad to maintain normal body temperature. A polyethylene catheter (0.5 mm O.D.) was inserted into the femoral vein to allow the infusion of either 1 mg/kg AcF-OPdChaWR (1 mg/mL stock solution; 5% ethanol in endotoxin-free saline) over a two-minute period 15 minutes prior to occlusion of the renal blood vessels. Oral dosing of AcF-OPdChaWR (10 mg/kg) was achieved by gavage (10 mg/mL stock solution in 25% ethanol/saline) 60 minutes prior to occlusion. Rats in the sham-operated and I/R injury groups were infused with 0.2 mL of 5% ethanol in saline or gavaged with 0.2 mL of 25% ethanol in saline at the same rate as drug treated groups. The left renal pedicle was exposed through a midline abdominal incision and a polyethylene catheter (0.8 mm O.D.) was inserted into the left ureter to collect urine from the occluded kidney. Renal ischemia was induced by clamping the pedicle with a non-traumatic occlusive device (Yasergil aneurysm clamp; Jarit Instruments, Sydney, Australia). After 60 minutes of occlusion the clamp was removed and the animal was monitored for a further five hours of reperfusion. We used a similar surgical and experimental procedure as previously reported [4]. Sham-operated animals underwent an identical surgical procedure, including isolation of the renal pedicle, but occlusion of the pedicle was not performed. At the end of the five-hour reperfusion period, whole blood was collected into heparinized Eppendorf tubes and plasma samples collected and stored at −20°C for later measurement of plasma aspartate aminotransferase (AST) and creatinine. The animals were then euthanized with an overdose of pentobarbital. The left and right kidneys were removed and immediately fixed in 10% buffered formaldehyde-saline for histological studies.

Measurement of biochemical parameters

Plasma concentrations of creatinine were measured as indicators of impaired glomerular function [30]. Plasma concentrations of AST, an enzyme that occurs in the proximal tubule cells, were measured as an indicator of renal parenchymal cell injury [31, 32]. AST and creatinine concentrations were measured within 48 hours of collecting plasma. Concentrations were derived from calibration curves. AST results are expressed as Sigma-Franke (SF) U/mL. Creatinine results are expressed as μmol/L.

Measurement of leukocytes in the urine

Urine samples from the ureters of occluded kidneys and sham-operated kidneys were collected at the end of the reperfusion period and leukocytes counts were performed using a hemocytometer. Leukocytes counts were expressed as cells/μL urine.

Determination of renal microvascular permeability

In a separate series of identical experiments, renal vascular permeability was assessed by measuring the extravasation of Evans blue (EB) dye into the renal tissues. EB (1% solution of EB in saline injected at 2.5 mL/kg) was
injected into the femoral vein catheter 60 minutes prior to the end of reperfusion period. At the end of the experiment anesthetized animals were killed by exsanguination from the thoracic aorta. The kidneys were excised, dried at 80°C for 12 hours and weighed. The kidneys were incubated in 2 mL formamide for 24 hours and the concentration of EB dye in the formamide extract determined by spectrophotometry at a wavelength of 620 nm. Tissue concentrations of EB were determined from a standard curve. The EB dye concentration for each sample was expressed as μg/g kidney tissue.

**Measurement of renal levels of TNF-α**

In another set of experiments, kidneys were removed at the end of the reperfusion period, blotted dry and weighed, then homogenized in 10 mmol/L phosphate buffer (pH 7.4) containing 0.05% (wt/vol) of sodium azide at 4°C. Homogenates were then sonicated for 20 seconds and centrifuged (10,000 × g for 10 min). The supernatants were stored at −20°C until analysis. Renal concentrations of TNF-α were measured using an enzyme-linked immunosorbent assay (ELISA; OptEIA; Pharmingen, San Diego, CA, USA), according to the manufacturer’s instructions. Concentrations of TNF-α in supernatant were determined by linear regression analysis from the standard curve.

**Determination of renal MPO activity**

The technique for measuring renal MPO activity was a modification of the technique previously described [33]. At the end of the reperfusion period, the kidneys were removed, weighed, homogenized with 10 mmol/L phosphate buffer (pH 7.4) and then sonicated for 20 seconds. After centrifugation (10,000 × g for 10 min), 5 μL of supernatant was added to 195 μL of 10 mmol/L phosphate buffer (pH 6.0). Substrate solution containing 2.85 mg/mL o-dianisidine and 0.025% hydrogen peroxide was added to the diluted supernatant. After five minutes, the absorbance at 460 nm was measured with a spectrophotometer. MPO activity of the supernatant was expressed as the optical density/g kidney tissue.

**Renal histopathology**

The fixed kidneys were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin (H&E). Sections were examined in a blinded fashion and the degree of damage was scored between 0 and 4. A score of 0 was assigned to tissues with no visible damage. Kidneys showing lesions of mild hemorrhage as the only pathological change were scored 1. A score of 2 was given to kidneys that showed hemorrhage and mild changes, like protein casts, in the renal tubules. Kidneys with moderate hemorrhage and more severe tubular pathology were scored 3, while a score of 4 was reserved for kidneys with severe hemorrhage, marked peritubular edema and visible evidence of renal tubular necrosis.

**RESULTS**

**Plasma markers**

Animals incurring renal I/R injury exhibited significant increase in the plasma concentrations of creatinine and AST compared to uninjured, sham-operated animals (Fig. 1). After five hours of reperfusion, the plasma level of creatinine (Fig. 1A) had increased from 41.8 ± 1.5 μmol/L (sham-operated) to 129.6 ± 16.5 μmol/L (I/R). In animals pre-treated with the C5aR antagonist, the elevation in plasma creatinine after reperfusion was significantly less than I/R animals. Pre-treat-
low levels, but did not completely abolish urinary leukocytes or leukocytes. Pre-treatment with 1 mg/kg IV C5aR antagonist only did not affect plasma AST or creatinine levels. Renal tissue MPO and TNF-α activity

Administration of the C5aR antagonist significantly inhibited the I/R-induced increases in renal tissue levels of TNF-α and MPO. The concentration of TNF-α in kidneys from rats subjected to renal I/R increased from 206 ± 12 pg/g tissue in sham-operated animals to 618 ± 134 pg/g tissue (N = 6, P < 0.001; Fig. 4). Renal tissue levels of TNF-α were significantly lower in rats pretreated with 1 mg/kg IV C5aR antagonist (171 ± 14 pg/g tissue; N = 4, P < 0.001) or 10 mg/kg PO (130 ± 7 pg/g tissue; N = 5, P < 0.001).

Renal tissue MPO also increased from 1.17 ± 0.2 OD/g tissue in sham-operated animals to 4.6 ± 0.9 OD/g tissue after reperfusion (N = 10, P < 0.001; Fig. 5). The I/R-induced increases in renal levels of MPO optical density were significantly lower in rats pretreated with the C5aR antagonist at 1 mg/kg IV (2.2 ± 0.2 OD/g tissue; N = 8, P < 0.001) and 10 mg/kg PO (2.1 ± 0.3 OD/g tissue; N = 5, P < 0.001). Pretreatment of sham-operated rats with 1 mg/kg IV C5aR antagonist only did not affect tissue TNF-α or MPO levels (Figs. 4 and 5).
Fig. 4. Renal I/R induced tissue TNF-α. Renal I/R resulted in significant elevation in tissue TNF-α compared with sham-operated animals. Rats pretreated with the C5aR antagonist AcF-[OPdChaWR] at either 1 mg/kg IV or 10 mg/kg PO showed inhibition of the elevation of tissue TNF-α levels compared with I/R animals. Data are shown as means ± SEM (N = 4 to 6 in each group). Symbols are: (□) Sham; (⚫) Sham + 1 mg/kg IV AcF-[OPdChaWR]; (●) I/R injury; (▲) I/R + 1 mg/kg IV AcF-[OPdChaWR]; (●) I/R + 10 mg/kg PO AcF-[OPdChaWR]. *P < 0.001 vs. sham-operated animals, +P < 0.001 vs. I/R animals.

Histopathology

Sham-operated animals and drug-treated sham animals had no detectable histological changes in the kidneys (Figs. 6A and 7). In animals subjected to renal I/R, the most common lesion was hemorrhage in the renal parenchyma. This appeared to be most severe in the zone between the cortex and medulla (Fig. 6B). Other detectable changes included peritubular edema of the distal tubules and collecting ducts, some tubular casts and in a few cases there was also varying degrees of damage to proximal tubule epithelial cells. The tubule cells had loss of nuclei, with the most severely affected cells being in the distal tubules. Animals that were pretreated with antagonist had less severe lesions (Figs. 6 C, D and 7).

Unimpaired MAC activity

AcF-[OPdChaWR] did not have any affect on hemolysin-induced lysis of sheep red blood cells in concentrations up to 100 μmol/L. This standard hemolytic assay for complement activation requires C5b-mediated formation of the MAC [29]. In the same assay, a reported C3/C5 convertase inhibitor, rosmarinic acid, inhibited red blood cell lysis by approximately 70% at 100 μmol/L (Fig. 8). We conclude that the selective C5aR antagonist does not impair the formation of the MAC.

DISCUSSION

The complement system is activated during I/R injury in a number of organs, including the gut [16, 34], kidney [35], heart [36], liver [37] and central nervous system [38].
The present study shows that a new C5aR antagonist inhibits a rat model of renal I/R injury. The drug was effective when administered either by intravenous or oral routes. We have recently reported that this antagonist also is effective in treating I/R injury of the gastrointestinal tract when administered either orally or intravenously to rats [18].

The role of C5α is undergoing increasing scrutiny in renal inflammatory diseases. C5α has been shown to be present in thrombotic glomerulonephritis [39], while enhanced expression of C5αR mRNA has been demonstrated in the human kidney in membranous nephropathy and mesangial proliferative glomerulonephritis [40]. C5αRs have been identified on human renal proximal tubular cells [41] and in leukocytes in rat renal tissue [42]. Despite these findings, it is still not clear what role complement in general, and C5α in particular, play (if any) in renal I/R injury, and the present study was carried out with a specific C5α receptor antagonist for these reasons. The present study demonstrates that the small molecule C5αR antagonist AcF-[OPdChaWR], has impressive tissue-protective effects against renal I/R-induced cellular injury and renal dysfunction in the rat. Pretreatment with this C5αR antagonist also reduced I/R-increased plasma levels of creatinine and AST, hematuria, renal vascular permeability, and markedly inhibited elevated renal tissue TNF-α concentrations and renal tissue MPO activity caused by I/R.

In other organs, C5α involvement in mediating leukocyte activation during certain I/R injuries has been previously demonstrated with C5α-induced sequestration of PMN in the coronary vascular bed [43] and in focal cerebral ischemia [44]. Neutrophil infiltration into the kidney parenchyma in response to renal I/R injury is a well-known phenomenon and activation of these neutrophils releases cytokines, reactive oxygen species, MPO and other enzymes [10, 45]. Reperfusion of microvessels results in the adherence of leukocytes to the endothelium, increased migration of leukocytes to the affected site, and increased microvascular permeability resulting in tissue edema [46]. Several studies have demonstrated that inhibition of neutrophil activation prevented or reduced renal I/R mediated injury [7–9]. In rats, leukopenia also significantly inhibited the increase in renal vascular permeability as well as helped to maintain renal tissue blood flow during renal I/R [33]. In the present study, neutrophils were not seen prominently in the damaged parenchyma of mild-to-moderately damaged kidneys. The relatively low numbers of neutrophils seen may be due in part to the relatively short time course of the experiments. Tissue MPO activity was significantly increased, however, which may reflect some contribution by adhering resident macrophages [47], as it has been reported that there is a lack of correlation between renal MPO levels and tissue neutrophils after I/R [47].

The appearance of blood in the urine suggests the degree of renal damage resulted in tissue hemorrhage and escape of blood into the urine. We attempted to quantify the escape of blood-borne cells from the plasma to the renal tubule and urine, so leukocyte counts were performed because there was almost complete hemolysis of erythrocytes in urine. There was extensive hemorrhage in the kidney parenchyma following I/R. The C5α antagonist blocked both the appearance of urinary leukocytes and hemolyzed red blood cells in the urine, as well as the hemorrhage caused by I/R. Renal I/R activates the TNF-α transcription factor nuclear factor-κB and increases TNF-α activity in the kidney [1, 4]. Our recent in vivo studies showed that pretreatment with C5αR antagonists blocked any rise in plasma TNF-α levels to either small intestinal I/R injury [18], immune complex-mediated peritoneal [27] or dermal inflammation [24], or acute administration of lipopolysaccharide [26]. These findings, along with those from the present study, suggest that renal I/R-induced increase in MPO and TNF-α may be due to activation of PMNs, monocytes and macrophages by C5α following activation of complement. Inhibition of the activation of these inflammatory cells by the C5αR antagonist likely reduces I/R-induced renal injury through inhibiting production of proinflammatory cytokines as well as leukocyte trafficking. Inhibition of these inflammatory cells also would result in the blockade of both vascular leakage and leukocyte egress into the urine and interstitium.

The increase in plasma creatinine after renal I/R may indicate impaired glomerular function [30]. Increased levels of AST in serum or plasma usually denote liver damage, but this enzyme is not only specific to liver, it also is found in some other organs such as kidney and smooth muscle. Plasma AST is regarded also as a marker of renal
tubular damage [32] and plasma levels of this enzyme are elevated after renal reperfusion injury [31]. Pre-treatment with the C5aR antagonist AcF-[OPdChaWR] reduced plasma levels of creatinine and completely blocked the I/R-induced rise in AST, suggesting that C5a is a mediator of injury to the glomerular and tubular cells. The mechanism by which the C5aR antagonist reduces tubular and glomerular damage is not yet known. Recent studies have shown that both glomerular and tubular cells express C5aR [40, 41, 48] and glomerular mesangial cells are an important source of TNF-α [49]. As C5a has been shown to produce TNF-α in vivo [27], the C5aR antagonist may be involved in inhibition of TNF-α production within glomerular and tubular cells.

In certain models of I/R injury in other species such as the mouse, the MAC has been suggested to be a more important pathogenic factor than C5a [50–52]. One study reported that neither C5a-mediated neutrophil infiltration, nor the classical complement pathway in which C4 participates, appears to contribute to renal I/R injury in this model [52]. Other studies in C3-deficient mice reported only partial protection from renal I/R-induced injury, and the use of a rodent C3a convertase inhibitor (Crry-Ig) was ineffective, suggesting that other pro-inflammatory mediators operate in parallel with complement-mediated lysis of red blood cells, indicating that the drug does not inhibit the formation of the MAC. The C5a antagonist did not completely block the appearance of histopathology, suggesting that other factors also might be involved. In this light, the role of the MAC in promoting tissue injury in I/R awaits clarification, at least in the species [53]. In the rat, in intestinal [18] and now renal I/R injury, we have found that a specific C5a receptor antagonist effectively blocks the expression of multiple markers of cellular injury and is quite effective in reducing pathology. It is not yet known if species differences alone can account for these discrepancies between our results and those previously published in mice [54]. In mice, the C5a antagonist used in the present study binds poorly to circulating PMNs [54].

AcF-[OPdChaWR] did not inhibit complement-mediated lysis of red blood cells, indicating that the drug does not inhibit the formation of the MAC. The C5a antagonist did not completely block the appearance of histopathology, suggesting that other factors also might be involved. In this light, the role of the MAC in promoting tissue injury in I/R awaits clarification, at least in the rat. The use of the large, proteinaceous complement blockers, such as soluble complement receptor type-1 [55], or an antibody against C5 [56], which block the formation of both C5a and the MAC, may help to elucidate this issue. Although oral administration of the C5a antagonist appeared to provide greater tissue protection than the intravenous route, the relatively crude quantitative measure of the scoring system does not allow any conclusions regarding drug efficacy and differing routes of administration at this stage.

The C5aR antagonist used in the present study is a potent inhibitor of the actions of C5a on human PMNs and tissue macrophages [23, 28, 57] and behaves as an insurmountable antagonist at both kinds of C5aRs [57]. The drug binds to rat PMNs with similar affinity to human PMNs [54]. In rats, the plasma half-life for the drug following oral or intravenous administration is approximately 70 minutes [24], but the effect of the drug in vivo is much longer than the blood levels would suggest because of the insurmountable nature of its antagonism at C5aRs [57]. For example, inhibition of C5a-induced neutropenia in rats, following a single intravenous or oral dose, lasts for up to 24 hours after administration, when the drug is undetectable in the circulation [24]. We attribute the long duration of drug action in vivo to a slow off-rate from the receptor, so that drug levels in the circulation are not necessarily relevant to therapeutic effect or duration.

Renal I/R injury remains a significant cause of patient morbidity and mortality. Our study shows, to our knowledge for the first time, that the first reported, orally active small molecule C5aR antagonist is highly effective in reducing the renal dysfunction and tissue damage caused by I/R in the rat. The results suggest an important role for C5a in I/R in the kidney, and a potential role for C5aR antagonists in the treatment or prevention of human I/R kidney injury.

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