Comparative Effects of Aprotinin and Human Recombinant R24K KD1 on Temporal Renal Function in Long-Evans Rats

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ABSTRACT

Bovine aprotinin, a reversible inhibitor of plasmin and kallikrein, has been clinically approved for over two decades to prevent perioperative blood loss during cardiac surgery. However, because of postoperative renal dysfunction in thousands of these patients, aprotinin was voluntarily withdrawn from the market. Our earlier studies indicated that a R24K mutant of the first Kunitz-type domain of human tissue factor pathway inhibitor-2 (R24K KD1) exhibited plasmin inhibitory activity equivalent to aprotinin *in vitro*. In this study, we compared the effects on renal function after infusion of aprotinin and recombinant R24K KD1 in chronically instrumented, conscious rats. Aprotinin-infused rats exhibited statistically significant decreases in glomerular filtration rate and effective renal plasma flow relative to rats infused with phosphate-buffered saline (PBS) or R24K KD1 dissolved in PBS. In addition,

aprotinin-treated rats exhibited marked increases in serum creatinine, blood urea nitrogen, urinary protein, and effective renal vascular resistance, whereas these renal parameters remained essentially unchanged in vehicle and R24K KD1-treated rats for a one-week period. Moreover, with use of a highly sensitive apoptosis detection assay, a significant increase in the rate of early and late apoptotic events in renal tubule cells occurred in aprotinin-treated rats relative to R24K KD1-treated rats. In addition, histological examination of the rat kidney revealed markedly higher levels of protein reabsorption droplets in the aprotinin-infused rats. Our data collectively provide suggestive evidence that R24K KD1 does not induce the renal dysfunction associated with aprotinin, and may be an effective clinical alternative to aprotinin as an antifibrinolytic agent in cardiac surgery.

Aprotinin, also known as bovine pancreatic trypsin inhibitor and Trasylol, is the prototypical member of the Kunitz-type family of serine proteinase inhibitors. Aprotinin reversibly inhibits trypsin, plasmin, and kallikrein through its reactive site lysine residue at position 15 acting as a pseudosubstrate for serine proteinases. Given its strong antifibrinolytic activity, aprotinin was used perioperatively in millions of cardiac surgeries to effectively reduce inflammation and excessive bleeding after cardiopulmonary bypass (Ray and O'Brien, 2001). However, recent large-scale observational studies (Mangano et al., 2006; Wagener et al., 2008) have concluded that the use of aprotinin in cardiac surgery was associated with a significantly increased risk of renal failure postoperatively, necessitating dialysis in thousands of patients. As a result of numerous adverse renal complica-

tions associated with its use in surgery, aprotinin was voluntarily withdrawn from the market by the manufacturer in 2007 and replaced with antifibrinolytic agents such as *e*-aminocaproic acid and tranexamic acid in these patients. The mechanism whereby aprotinin induces renal dysfunction in these patients is not known with certainty, but presumably involves uptake of aprotinin by proximal tubule cells (Vio et al., 1998), where it has been shown to inhibit tubule kallikrein secretion, prostaglandin and renin synthesis, and bradykinin release (Seto et al., 1983).

Our laboratory has previously reported that an R24K mutant of the first Kunitz-type domain of human tissue factor pathway inhibitor-2, designated R24K KD1, exhibits plasmin inhibitory activity comparable with aprotinin (Chand et al., 2004), suggesting its potential as an alternative to aprotinin in cardiac surgery. Whether R24K KD1 exhibits the same nephrotoxic effects as aprotinin in an animal model has not been investigated. In this study, we report that infusion of aprotinin into chronically instrumented rats resulted in statistically significant decreases in glomerular filtration rates and effective renal plasma flow relative to rats treated with either vehicle (phosphate-buffered saline, or PBS) or equiv-

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ABBREVIATIONS: R24K KD1, the first Kunitz-type domain of tissue factor pathway inhibitor-2-mutated at position 24; GFR, glomerular filtration rate; ERPF, effective renal plasma flow; ERVR, effective renal vascular resistance; IN, inulin; PAH, *p*-aminohippurate; BUN, blood urea nitrogen; BW, body weight.

alent amounts of pure recombinant human R24K KD1 dissolved in PBS. Moreover, aprotinin-treated rats exhibited marked increases in serum creatinine, blood urea nitrogen, urinary protein and effective renal vascular resistance, whereas these renal parameters remained essentially unchanged in vehicle and R24K KD1-treated rats over a 7-day period. By use of a highly sensitive apoptosis detection assay, we also demonstrate a statistically significant increase in the rate of early and late apoptotic events in renal tubule cells of aprotinin-treated rats at 24, 48, and 168 h after infusion relative to R24K KD1-treated rats. Collectively, our data provide suggestive evidence that R24K KD1 does not induce the nephrotoxic effects associated with aprotinin use, and may be an effective alternative to aprotinin as an antifibrinolytic agent in cardiac surgery.

Materials and Methods

Materials. Sterile PBS was obtained from Invitrogen (Carlsbad, CA). Recombinant human R24K KD1 was expressed in *Escherichia coli* and prepared as described previously (Schmidt et al., 2005). Bovine lung aprotinin (lot 095K7008), Pyrogallol Red, and inulin were obtained from Sigma-Aldrich (St Louis, MO). Para-aminohippurate was purchased from Merck Research Labs (West Point, PA). The ApopTag ISOL Dual Fluorescence Apoptosis Kit was a product of Millipore Bioscience Research Reagents (Temecula, CA). All other reagents were of the highest quality commercially available.

Animal Preparation. Eighteen Long-Evans female rats, 9 to 12 weeks old and weighing 250 to 275 g, were purchased from Harlan Laboratories (Indianapolis, IN) and provided Techlad Rodent Irradiated Diet 2018 containing 0.23% sodium (Harlan-Techlad Feed, Madison, WI) and water ad libitum. The rats were maintained on a 12-h light/dark cycle in an Institutional Animal Care and Use Committee-approved animal resource facility at the University of New Mexico. In preparation for experimentation, the rats were trained for several hours in an appropriate-size Plexiglas restraining cage (Bain-tree Scientific Co., Braintree, MA) on at least five different occasions, progressing in length of time, before chronic instrumentation.

Details of the surgical procedures have been described previously (Danielson et al., 1999). In brief, with use of halothane/oxygen gas for anesthetic purposes and aseptic technique, Tygon vascular catheters were implanted in the abdominal aorta and inferior vena cava via the femoral artery and vein, respectively. A silastic-covered stainless steel cannula was then sewn into the urinary bladder with a purse-string suture and exteriorized through the ventral abdominal wall. All vascular catheters were tunneled subcutaneously and exteriorized between the scapulae. The bladder catheter was plugged with a silastic-covered obturator to allow the rat to urinate normally through the urethra when in her home cage. The abdominal muscle incision for the bladder catheter was sutured, and both the femoral vascular and abdominal skin was closed with sterile Autoclips. All of these procedures were performed by use of sterile techniques, and were approved by the University of New Mexico Health Sciences Center Laboratory Animal Care and Use Committee.

Analytical Techniques. Plasma and urine inulin (IN) and *p*-aminohippurate (PAH) were assayed by standard procedures (Danielson et al., 1999). Blood urea nitrogen (BUN) and creatinine assays were performed on a Vitros System DT slide system (Ortho-Clinical Diagnostics, Rochester, NY), which uses dry-slide technology for measurement. Urine protein was determined by a dye-binding protein assay with use of Pyrogallol Red. To eliminate interfering protein from either food or feces, the urine was centrifuged before analysis. Resected kidneys were sliced on the long axis of each organ to obtain 1- to 2-mm slices of cortex and medulla. These tissue samples were fixed in 10% neutral formalin solution for >6 h at 4°C. The tissues were then dehydrated in an ascending series of alcohol/

water mixtures, cleared with toluene, and subsequently embedded in paraffin. The paraffin-embedded tissue was sliced into 7- μ m sections, mounted on slides, and subsequently stained with either periodic acid/Schiff reagent or Jones' methenamine silver stains for examination by light microscopy, or processed for apoptosis labeling. For each section examined histologically, the number of tubular cytoplasmic protein reabsorption droplets were quantified in five high-power fields (100 $\times$; under oil).

Effect of Vehicle, Aprotinin, and R24K KD1 on Renal Function in Conscious Rats. After a 5- to 7-day recovery period from anesthesia and surgical procedures, the rats were randomly assigned to three groups of six rats. Renal function parameters and mean arterial pressures were initially measured in six age-matched, chronically instrumented conscious rats, for a 90-min baseline period. Before the infusion of any fluids, blood (300 μ l) was drawn for baseline plasma analyses on each rat for BUN and creatinine. Mean arterial pressure/heart rate were measured by a Gould P231D pressure transducer and Gilson ICT-2H Duograph. To obtain estimates of glomerular filtration rates (GFR) and effective renal plasma flow (ERPF), the silastic covered pin in the bladder catheter was removed in each rat and the cannula was extended with a short piece of silastic tubing for the collection of urine. A bolus of inulin (100 μ l of a 50% stock solution/100 g BW) and *p*-aminohippurate (50 μ l of a 4% working solution/100 g BW) was administered via the venous catheter followed by constant infusion (15 μ l/min) of the two reagents for 1 h at 0.4 and 0.1 mg/min/100 g BW, respectively. After the 1-h infusion, three baseline urine and blood collections were obtained at 30-min intervals to measure the renal clearances of IN and PAH. After centrifugation and removal of plasma, all red cells were gently resuspended in Ringer's solution and returned to the animal to ensure euvolemia.

After these baseline measurements were completed, each group of six rats were intravenously administered either vehicle (PBS), R24K KD1 or aprotinin, both dissolved in sterile PBS and further sterilized by filtration (0.22 μ m membrane). Through a separate intravenous line, each rat was concurrently administered IN and PAH (0.4 and 0.1 mg/min/100 g BW, respectively). To simulate the dosing of aprotinin administration with that employed for cardiopulmonary bypass surgery in humans (Nuttall et al., 2002; Ray et al., 2005), each rat in the aprotinin group was infused with a bolus of aprotinin (~1 mg) for 10 min, followed by continuous infusion of aprotinin (0.28 mg/ml) for an additional 230 min at a rate of 15 μ l/min (0.25 mg of aprotinin/h/rat). A second group of six rats were similarly administered a bolus of R24K KD1 (~1 mg) during the first 10 min of infusion, followed by continuous infusion of R24K KD1 (0.28 mg/ml) for the remaining 230 min. The third group of six rats was continuously infused with sterile PBS for 240 min. After the first hour of infusion, blood and urine samples were collected from each rat at hourly intervals for estimation of GFR, ERPF, and effective renal vascular resistance (ERVR). At 24 h after infusion, two rats from each of the three groups were anesthetized, killed by exsanguination, and kidney sections collected for histology and apoptosis assessment. A second set of two rats from each group were sacrificed at 48 h, and the third remaining set of two rats from each group were sacrificed at 7 days. In addition, 24-h urine samples were collected for total urine protein analyses on day 6 before their sacrifice on day 7.

Apoptosis Detection by the Dual Fluorescence Labeling and Confocal Microscopy. To assess the extent of renal tubular cell damage caused by the infusion of either human R24K KD1 or bovine aprotinin, we used the ApopTag ISOL Dual Fluorescence Apoptosis Detection Kit. This kit allows for the detection of both early, caspase-dependent (DNase I) specific cuts and late, caspase-independent (DNase II) specific cuts in apoptotic cells. The kit also facilitates the differentiation of apoptotic cells from necrotic or transiently damaged cells, and reduces false-positive signals and high background (Arends et al., 1990; Mukae et al., 1998; Counis and Torriglia, 2006). In this procedure, the kidneys were removed by dissection from each rat after sacrifice and fixed in 10% neutral

Fig. 1. Effect of PBS, R24K KD1, and aprotinin on renal function. Renal function and arterial pressure in conscious rats were determined during a 4-h infusion period with either PBS, R24K KD1, or aprotinin. A, glomerular filtration rate; B, effective renal plasma flow; C, effective renal vascular resistance; and D, mean arterial pressure before (baseline) and during infusion in chronically instrumented rats. *, $P < 0.05$ for aprotinin-infused group versus PBS/R24K KD1/baseline by Student's paired t test. Baseline, $n = 3$; PBS, $n = 6$; R24K KD1, $n = 6$; aprotinin, $n = 6$.

formalin solution and embedded in paraffin wax as described above. The glass slides with 7- μ m-thick paraffin sections were rehydrated and processed for labeling by use of the ApopTag ISOL Dual Fluorescence Apoptosis kit according to the instructions provided by the manufacturer. After labeling, the slides were washed extensively with several changes of deionized water for approximately 1 h in the dark. The slides were further treated with a drop of mounting media containing 4,6-diamidino-2-phenylindole, and covered with a glass coverslip in preparation for confocal microscopy. Images from five different fields in each treatment group were used to determine the average number of apoptotic cells at each time point after infusion.

Results

Influence of PBS, Aprotinin, and R24K KD1 Infusion on Renal Function in Conscious Rats. To determine the effects of R24K KD1 and aprotinin on renal function, several renal parameters were monitored after the infusion of these two proteins in chronically instrumented, conscious rats. Infusion of PBS in control rats served as a reference point for comparison. In our chronically instrumented rats, GFR, ERPV, and ERVR remained unchanged during the infusion of either PBS or R24K KD1 (Fig. 1, A–C). In contrast, infusion of aprotinin significantly decreased GFR and ERPF with a concomitant increase in ERVR (Fig. 1, A–C) relative to baseline values ($P < 0.05$ by Student's paired t test). Mean arterial pressure was not significantly affected in all rats during this time period (Fig. 1D). Plasma creatinine/urea ratios revealed a significant increase in the aprotinin-treated rats at 24 h, 48 h, and 7 days (168 h) compared with either baseline values, PBS-treated, or R24K KD1-treated rats (Table 1). In addition, total 24-h urine protein at day 6 increased approximately 20-fold in the aprotinin-treated rats (654 ± 22 mg/24

TABLE 1

Ratios of creatinine and BUN in rats infused with vehicle, R24K KD1, and aprotinin

The rats were infused with either PBS alone, R24K KD1, or aprotinin, and creatinine and BUN levels were measured as described in *Materials and Methods*. Aprotinin-infused rats showed a significant increase in creatinine/BUN compared with the PBS and R24K KD1 groups.

Treatment	Creatinine/BUN			
	Baseline	24 h	48 h	1 wk
	mg/dl			
PBS	0.3/12	0.3/12	0.3/12	0.4/12
R24K KD1	0.3/12	0.3/10	0.3/12	0.3/11
Aprotinin	0.3/12	0.3/13	0.6/15	0.8/18*

* $P < 0.01$.

h) compared with PBS-treated (31 ± 10 mg/24 h) and R24K KD1-treated (29 ± 12 mg/24 h) rats. Overall, these results strongly suggest that aprotinin significantly alters renal function compared with PBS and R24K KD1.

Apoptosis Detection by Dual Fluorescence Labeling and Confocal Microscopy. To assess the extent of renal damage caused by the infusion of human R24K KD1 and aprotinin, we used a highly sensitive apoptosis detection kit that detects both early (type I DNase-specific cuts) and late (type II DNase-specific cuts) in cells undergoing apoptosis. Consistent with the results of the renal function tests (Fig. 1) and blood analyses (Table 1), we observed a progressive increase in early and late apoptosis in aprotinin-infused rat kidney sections from 24 to 168 h. In contrast, significantly smaller numbers of apoptotic cells were observed in PBS and R24K KD1-infused rat kidney sections in this same time period (Fig. 2). In addition, by dual fluorescence labeling, it was possible to observe augmented type I and type II DNase

Fig. 2. Analyses of apoptosis in paraffin-embedded rat kidney tissue sections. For immunofluorescent detection of apoptotic cells by use of confocal microscopy, paraffin sections from the 48-h treatment time point were labeled with the apoptosis detection kit as described in *Materials and Methods*. The images were obtained by confocal microscopy using a 63 $\times$ oil-immersion objective lens. A, representative images from each treatment group consisting of vehicle-infused rat (a), R24K KD1-infused rat (b), and aprotinin-infused rat (c). The red/CR590 label represents DNase type I DNA cleavages, whereas green/FAM-labeled oligonucleotide fluorescence represents DNase type II DNA cleavages. B, bar diagrams that depict the mean number of type I DNase and type II DNase-induced apoptotic cells derived from a total count of five fields in each experiment. Error bars represent the standard deviation, and *P* values are shown above the bars.

activity with time in aprotinin-infused rat kidney sections (Fig. 2). These data collectively indicate that aprotinin specifically induces high levels of apoptosis in rat kidney tubule cells, whereas the level of apoptosis induction in R24K KD1 kidney sections was approximately 2-fold greater than that observed in PBS-infused rats. Furthermore, aprotinin seems to activate both caspase-dependent (type I DNase) and caspase-independent (type II DNase) apoptotic pathways (Arends et al., 1990; Mukae et al., 1998).

Histological Analysis of Rat Kidney Sections by Light Microscopy. To further assess potential histological changes that occur after infusion of PBS, R24K KD1, and aprotinin, kidney sections were stained with either periodic acid/Schiff reagent or Jones' methenamine silver stain. By light microscopic examination, a small number of proximal tubule cytoplasmic protein reabsorption droplets were observed in PBS-treated rat kidney sections at all time points (Fig. 3A). The number of cytoplasmic protein droplets was slightly increased in R24K KD1-treated rats compared with PBS-treated rats (Fig. 3B), and markedly increased in the aprotinin-treated animals (Fig. 3, C and D). In contrast to the

results of the dual fluorescence labeling, definitive tubular apoptotic bodies were not observed histologically in the aprotinin-treated rat kidney sections, and the interstitial compartment, vessels, and glomeruli seemed normal by this technique.

Discussion

Bovine aprotinin, clinically known as Trasylol, is a potent plasmin and kallikrein inhibitor that was widely used in cardiopulmonary bypass surgery as an antifibrinolytic agent until its withdrawal from the market due to a large number of patients presenting with postoperative renal dysfunction. Aprotinin has largely been replaced clinically by either amino-caproic acid or tranexamic acid, two competitive inhibitors of plasminogen activation, but, in most cases, the use of these inhibitors has resulted in greater blood loss, transfusion requirements, and reoperation for bleeding in relation to patients treated with aprotinin (Later et al., 2009; Wang et al., 2009). Thus, the need for a safer antifibrinolytic agent with plasmin-inhibitory properties similar to aprotinin but without the deleterious sequelae would be valuable clinically.

Fig. 3. Histological analysis of rat kidney. For the histological examination of rat kidney, 7- μ m sections of formalin-fixed, paraffin-embedded rat kidney were stained with Jones' methenamine silver stain as described in *Materials and Methods*. Representative images of renal cortex taken at 100 $\times$ magnification are shown: A, vehicle-infused rat; B, R24K KD1-infused rat; and C, aprotinin-infused rat. The arrows indicate tubular cytoplasmic protein reabsorption droplets. D, bar graph diagram showing the mean number of protein reabsorption droplets per five high-power fields (100 $\times$) for each experiment. Error bars represent standard deviation, and *P* values of <0.05 were obtained by Student's paired *t* test for both the aprotinin-infused group versus the PBS group and between the PBS and R24K KD1 groups.

To this end, we have performed studies in a well-established rat kidney model to directly compare temporal changes in renal parameters after the administration of either aprotinin or a functional recombinant analog to aprotinin designated as human R24K KD1. In several previous studies, we established that R24K KD1, the first Kunitz domain of human tissue factor pathway inhibitor-2 mutated at the reactive site, exhibited strong inhibitory activity toward human plasmin with a K_i of 0.85 nM (Chand et al., 2004). By comparison, aprotinin inhibits human plasmin with a K_i of 0.75 nM (Chand et al., 2004). In the present study, aprotinin-infused rats exhibited statistically significant decreases in glomerular filtration rate and effective renal plasma flow relative to rats infused with either PBS or R24K KD1 dissolved in PBS. Moreover, aprotinin-infused rats exhibited marked increases in serum creatinine, blood urea nitrogen, urinary protein, and effective renal vascular resistance. In comparison, these latter renal parameters remained essentially unchanged in vehicle- and R24K KD1-treated rats over a one-week period. It is perhaps important to point out that it is unknown as to whether or not the deleterious changes in renal parameters experienced by the aprotinin-infused rats over a one-week period are irreversible, and that, over time, these parameters may return to baseline values. This question is currently being addressed in ongoing studies in our laboratory.

Having demonstrated that aprotinin adversely affects renal function, we next examined the effects of aprotinin infu-

sion at the cellular level using a highly sensitive, dual fluorescence apoptosis detection assay that distinguishes early and late apoptotic events. By this assay, a significant increase in the rate of early and late apoptotic events in renal tubule cells was evident in aprotinin-treated rats relative to R24K KD1-treated rats. In addition, examination of the kidney sections by light microscopy revealed markedly higher levels of protein reabsorption droplets, often seen in nephrotic syndrome (Kretchmer and Bernstein, 1974), in the aprotinin-infused animals. It is important to mention that no evidence for apoptosis was observed by light microscopy, which may reflect the vast differences in sensitivity between this technique and the highly sensitive ApopTag detection assay.

Our results confirm and significantly extend data on the negative effects of aprotinin on rat kidney function. Precisely why aprotinin adversely affects kidney function has been a matter of speculation for years, but many investigators agree it is most probably related to aprotinin's ability to inhibit renal kallikrein after its specific uptake by the kidney (Vio et al., 1998). Although aprotinin clearly is a strong inhibitor of plasmin and kallikrein, it is widely believed that its anti-fibrinolytic activity plays a dominant role during surgery to limit intraoperative blood loss. In this regard, R24K KD1 is distinct from aprotinin in that it exhibits plasmin inhibitory activity equivalent to aprotinin with essentially no inhibitory activity toward glandular kallikrein (Petersen et al., 1996).

Thus, it is conceivable that the benign effect of R24K KD1 on rat kidney function and the rate of apoptosis is related to its inability to inhibit kidney kallikrein. It is also conceivable that aprotinin not only inhibits kallikrein, but other kidney proteinase(s) that regulate intracellular events. In this connection, others have shown that aprotinin reduces the urinary excretion of epidermal growth factor and may be responsible for inhibiting the proteinase involved in the renal processing of the epidermal growth factor precursor (Jørgensen et al., 1990). In light of its benign effect on renal function and apoptosis, its strong antifibrinolytic activity, and its human origin, R24K KD1 may be a promising replacement for aprotinin in cardiac surgery.

In summary, we show that infusion of bovine aprotinin into chronically instrumented, conscious Long-Evans rats adversely affected renal function and induced apoptosis in renal tubule cells. In contrast, infusion of PBS or R24K KD1 had either no effect on renal function and apoptosis or minimal effect on these parameters, respectively. Future studies will focus on whether the effects seen with aprotinin are either permanent or reversible. Overall, our results suggest that R24K KD1 may be a suitable replacement for aprotinin during cardiac surgery.

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