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Measurement of the vasoconstrictive substances endothelin, angiotensin II, and thromboxane B_2 in cold storage solution can reveal previous renal ischemic insults

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Abstract In a rat model, the left kidney was subjected to 60 min of normothermic ischemia followed by 15 min of reperfusion, whereas the right kidney, serving as a paired control, was not rendered ischemic. Both kidneys were then perfused in situ with either Euro-Collins (EC) solution ($n = 12$) or University of Wisconsin (UW) solution ($n = 6$) for 10 min. Each kidney was then harvested and stored at 4 °C in its respective solution. After 24 and 48 h of cold storage, the following vasoactive substances were measured in the preservation media: endothelin (ET), angiotensin II (A-II), thromboxane (B_2) (TxB_2), and prostaglandin I_2 (PGI_2). After 24 h in EC solution, left kidneys uniformly produced significantly higher concentrations of each vasoactive substance than right kidneys: ET 1.64 ± 0.3 pg/ml vs 0.82 ± 0.1 pg/ml ($P \leq 0.009$); A-II 20.8 ± 6.2 pg/ml vs 7.75 + 2.3 pg/ml ($P \leq 0.007$); TxB_2 100.8 ± 17.7 pg/ml vs 40.1 ± 11.7 pg/ml ($P \leq 0.04$); PGI_2 638.3 ± 41.1 pg/ml vs 318.3 ± 36.4 pg/ml ($P \leq 0.001$), respectively. At 48 h, a similar pattern of results was obtained as the kidney continued to produce TxB_2 and prostacyclins during the 24–48 h period. In the UW solution, basal levels of ET and A-II were lower than those in EC solution, but similarly increased after initial ischemia. At 24 h, the concentrations produced by the left and right kidneys were as follows: ET 0.66 ± 0.1 pg/ml vs 0.48 ± 0.1 pg/ml ($P \leq 0.14$); A-II 10.36 ± 3.7 pg/ml vs 2.14 ± 0.7 pg/ml ($P \leq 0.006$); TxB_2 178 ± 53 pg/ml vs 52 ± 23.1 pg/ml ($P \leq 0.001$); and PGI_2 448.3 ± 49 pg/ml vs 323 ± 44.3 pg/ml ($P \leq 0.01$), respectively. After 48 h, the range of concentrations of each substance was similar to that obtained after 24 h. In further studies, the concentrations of ET and A-II were measured in solution previously used to preserve human kidneys ($n = 7$). The mean concentration of ET and A-II in these samples was 3.82 ± 1.14 pg/ml and 21.3 ± 9.2 pg/ml, respectively, whereas in control media both substances were below the limits of detection. These results demonstrate that vasoconstrictive substances can be measured in the preservation media after a kidney has been stored cold and that higher concentrations are found when the organ has been subjected to prior normothermic ischemia. The measurement of these vasoactive substances before transplantation may reveal that the kidney has been subjected to previous ischemic events. Moreover, these vasoactive substances could be involved in the early recovery of renal function after kidney transplantation.

Key words Renal transplantation, preservation · Vasoactive substances, renal preservation Preservation, vasoactive substances

Introduction

Acute renal failure is still a frequent complication in the immediate post-transplant period [2, 13]. This event often results from ischemic injury. Both warm and cold ischemia are well-known factors influencing post-transplant renal failure. It is, nevertheless, difficult to assess the renal damage produced by those ischemic insults and especially difficult to predict the consequences on early graft function.

Because the pathophysiology of acute renal failure during the initiation phase involves the production of different vasoactive substances, i.e., the renin-angiotensin system axis, the arachidonic acid metabolite thromboxane A_2, we thought that their concentration in the surrounding media might reflect the ischemic insults undergone by the kidney. This study measured the concentrations of several vasoactive substances in media that are used to preserve kidneys, with the aim of assessing whether it is possible to establish a relationship between ischemic injury and the concentration level of these substances. The substances we measured included angiotensin II (A-II), thromboxane B_2 [TxB_2; an inactive end-product of the vasoactive thromboxane (A_2)] and endothelin (ET). Endothelin is a newly discovered peptide said to be the most potent endothelium-derived vasoconstrictive agent [5].

Our results suggest, that there is a leakage/release of these vasoactive substances from the kidney into the preservation media during cold ischemia. If the kidney has been subjected to warm ischemia previously, significantly greater concentrations of those substances are released into the media. These results suggest that the measurement of these products would be useful in estimating the degree of renal injury induced by both warm and cold ischemia before transplantation.

Material and methods

Experimental study in rats

Male Wistar rats weighing 250–300 g were used in this study. All animals were fed standard rat chow and libitum and housed at 24 °C, with an alternating cycle of light and dark for at least 3 days prior to the experiment. Anesthesia was induced by ether inhalation and followed by an intraperitoneal injection of pentobarbital sodium (25 mg/kg). Through a midline incision, the left renal artery was dissected and occluded with a smooth vascular clamp for 60 min. After 60 min, the clamp was removed and perfusion was restored to the left kidney for 15 min. This ischemia/reperfusion procedure was intended to mimic a clinical situation in which a donor arrests (warm ischemia) but is successfully resuscitated. The right kidney was not manipulated and thus served as a paired control. During reflow, the suprarenal aorta and the supra- and infrarenal vena cavae were located. A 16 g silicon thin wall needle (Venothin, Labomed, Cremasca, Calif., USA) was introduced into the infrarenal aorta and secured with a suture. At the conclusion of the 15-min reperfusion, the ligature was tightened and in situ cold perfusion (4 °C) administered at a rate of 2 ml/min for 10 min.

Either of two types of cold preservation media was infused: Euro-Collins (EC) solution (n = 12) or University of Wisconsin (UW) solution (n = 6; Dupont Pharmaceuticals, Wilmington, Del., USA.

After in situ cold perfusion, a bilateral nephrectomy was performed. The kidneys were weighed and then placed into a flask containing 40 ml of the chosen cold perfusate at 4 °C. After 24 h of preservation, 20 ml of each solution was withdrawn and frozen at − 35 °C. The remaining 20 ml of solution was removed and stored in a similar fashion after 48 h. In all samples, the following vasoactive substances were assessed: endothelin 3 (ET-3, rat endothelin), angiotensin II (A-II), thromboxane B_2 (TxB_2), and prostacyclin (6-keto-$PGF_{1\alpha}$). The techniques for extraction and radioimmunoassay for the two latter substances have been previously described [7]. ET-3 and A-II were assessed as follows:

Sample extraction

Twenty milliliters of each sample was added to 4 g of guanidine HCL, vortexed and applied to Sep Pack C_{18} cartidges previously activated with 81 acetonitrile containing 0.1 % trifluoroacetic acid (B solution). After washing the cartridges with 8 ml of 0.1 % trifluoroacetic acid in water (A solution), adsorbed material was eluted with 3 ml of 60 % B in A, dried in a Speec Vac concentrator, redissolved in 0.5 ml of RIA buffer (0.1 M PBS, pH 7.4, containing 0.1 % HSA and 0.1 % Triton X-100), and submitted to RIA for ET-1 and A-II. When 20 ml of preservation media was spiked with 50 pg of synthetic peptides 89 % ET-1 and 97 % A II were recovered by this extraction method.

RIA

Rabbit antisera for ET-1 and A-II were from Peninsula Laboratories (Belmont, Calif., USA). ET-1 antiserum displays 7 %, 7 %, and 35 % crossreactivity with ET-2, ET-3, and big ET-1, respectively. A-II antiserum crossreacts 100 % with A-II but 0 % with renin substrate, A-I, or arginine-vasopressin.

Tracers were synthetic ET-1 or A-II (Peninsula Laboratories), labelled with iodine-125 by the lactoperoxydase method and subsequently purified by RP-HPLC. The standard (synthetic ET-1 or A-II) and experimental samples were incubated with antibodies at room temperature for 16 h. After the addition of tracers and 5 h of further incubation, bound ligands were separated by precipitation with 1 % goat anti-rabbit antiserum (in 6 % PEG 6000 solution in RIA buffer) and centrifugation (2800 g for 30 min). The limits of detection of the assays, expressed as 10 % tracer displacement, were 1 pg of ET-1 and 0.9 pg of A-II per tube (0.025 pg of ET-1 and 0.022 pg of A-II per ml of starting sample). ET-1 or A-II levels were measured in all samples in the same RIA. The intra-assay CV was 9 % for ET-1 and 5 % for A-II. ET-1 and A-II immunoreactivities in control media were below the limits of detection of the assays.

Clinical study

We also measured the concentration of endothelin 1 (ET-1, human endothelin) and A-II in the preservation media (EC solution n = 5 or UW solution n = 2) of seven kidneys (six cadaveric and one living related) destined for human recipients. Some hours prior to transplantation, we withdrew 20 cc of the perfusion medium (4 °C), which had been preserving the kidney for a time ranging from 3 to 35 h (mean 23.8 ± 10 h). The kidney was flushed thereafter with fresh

Table 1 Vasoactive substances in EC solution

	Control ($n = 12$)	Ischemia ($n = 12$)	P
After 24 h of cold preservation			
Endothelin 3 (pg/ml)	0.82 ± 0.1	1.64 ± 0.3	0.009
Angiotensin II (pg/ml)	7.75 ± 2.3	20.80 ± 6.2	0.007
Thromboxane B_2 (pg/ml)	40.10 ± 11.7	100.80 ± 17.7	0.04
6-keto-$PGF_{1\alpha}$ (pg/ml)	318.30 ± 36.4	638.30 ± 41.1	0.001
After 48 h of cold preservation			
Endothelin 3 (pg/ml)	0.79 ± 0.1	1.58 ± 0.3	0.008
Angiotensin II (pg/ml)	8.10 ± 2.3	22.10 ± 6.3	0.007
Thromboxane B_2 (pg/ml)	94.10 ± 18.7	255.00 ± 73.0	0.06
6-keto-$PGF_{1\alpha}$ (pg/ml)	863.30 ± 125	1706.0 ± 383	0.06

Table 2 Vasoactive substances in UW solution

	Control ($n = 6$)	Ischemia ($n = 6$)	P
After 24 h of cold preservation			
Endothelin 3 (pg/ml)	0.48 ± 0.1	0.66 ± 0.1	0.07
Angiotensin II (pg/ml)	2.14 ± 0.7	10.36 ± 3.7	0.04
Thromboxane B_2 (pg/ml)	52 ± 23.1	178 ± 53	0.04
6-keto-$PGF_{1\alpha}$ (pg/ml)	323.30 ± 44.3	448.30 ± 49.0	0.02
After 48 h of cold preservation			
Endothelin 3 (pg/ml)	0.46 ± 0.04	0.60 ± 0.1	0.14
Angiotensin II (pg/ml)	1.41 ± 0.2	6.59 ± 1.1	0.006
Thromboxane B_2 (pg/ml)	56.60 ± 12.6	242 ± 36.0	0.001
6-keto-$PGF_{1\alpha}$ (pg/ml)	481.60 ± 59.7	773.30 ± 104.0	0.01

preservation media. The sample was centrifuged and frozen at −35°C until the aforementioned assays for ET-1 and A-II extractions could be performed.

All values are expressed as mean ± SEM. Statistical analysis included Student's unpaired or paired t-test. Correlation between ET-1 concentration and the duration of conservation time (hours) or correlation between ET-1 production and $PGF_1\alpha$ or A-II release was assessed by an adequate linear regression. Statistical significance was defined as a P value lower than or equal to 0.05.

Results

Experimental study in rats

All vasopeptides were always undetectable in the preservation media before their use for preservation. Table 1 shows that ET-3, A-II, TxB_2, and 6-keto-PGF_1 were already found at significant concentration levels in the EC solution in which the right kidney (cold ischemia alone) had been preserved for 24 or 48 h. Moreover, 60 min of normothermic acute renal ischemia prior to preservation produced a 2–2.7-fold increase in the concentrations of ET-3, A-II, TxB_2 and 6-keto-$PGF_1\alpha$ in comparison with the right kidney that had not been rendered ischemic previously. Furthermore, after 24 h of preservation, both the left and right kidneys continued to produce TxB_2 and 6-keto-$PGF_1\alpha$, but not ET or A-II during the period 24–48 h in EC solution.

Comparable results were obtained in the experimental group that used UW solution (Table 2). However, concentrations of ET-3 and A-II in the left and right kidneys were lower than those in the EC-preserved group after 24 and 48 h of cold preservation. After 60 min of warm ischemia, the increase in the ET-3 concentration was significant after 24 h of cold ischemia ($P \leq 0.07$) but not after 48 h ($P = 0.14$); the 6-keto-$PGF_1\alpha$ concentration increased less after cold storage in UW solution than in EC solution. In contrast, A-II and TxB_2 concentrations after ischemia rose more in the UW group (4.8 and 3.4-fold, respectively) than in the EC group (2.7 and 2.5-fold, respectively), despite a lower basal level. In both the EC and UW groups, a positive and significant correlation between the production of ET-1 and the release of PGI_2 and A-II was found: as the concentration of ET rose, so PGI_2 and A-II ($r = 0.76$ and $P \leq 0.05$ for PGI_2; $r = 0.89$ and $P \leq 0.01$ for A-II).

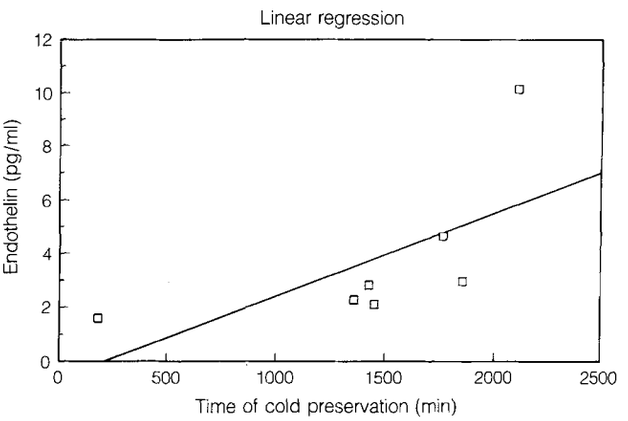

Fig. 1 Relationship between the ET-1 concentration in the surrounding medium and the cold ischemia time in seven human kidneys stored in EC ($n = 5$) or UW ($n = 2$) solution. The linear regression is significant at $P < 0.01$

Clinical results

In the clinical study, a correlation existed ($P \leq 0.01$; Fig. 1) between the concentration of ET-1 and the length of preservation in either EC or UW solution. A longer preservation time produced higher concentrations of ET-1 (range 1.6–10.2 pg/ml; mean 3.82 ± 1.14 pg/ml). No correlation with the duration of the preservation time was found with A-II, though concentrations averaged 21 ± 9.2 pg/ml (range 2.5–58 pg/ml). The relationship between these concentrations in the medium and early graft function is, nonetheless, obsolete in our experience

because we always reperfuse the kidney with fresh, matched preservation media some hours before the kidneys transplanted.

Discussion

Our experimental results demonstrate that abundant concentrations of various vasoactive substances are measured in the preservation media in which a kidney has been cold preserved for 24 or 48 h. The release of these agents is significantly higher when the kidney has sustained prior warm ischemic injury. The three potent vasoconstrictive substances identified in the preservation media were ET, A-II, and TxB_2.

ET is a recently described 21-amino acid peptide initially isolated from supernatants of cultured porcine aortic endothelial cells [17]. Three isopeptides have been identified: ET-1 in humans and swine; ET-2 in humans, containing two amino acid substitutions; and ET-3 in rats, containing six amino acid substitutions [16]. These three peptides are structurally similar to a snake venom (*Atractaspis engaddensis*), sarafotoxin 6b [9]. Synthesis of ET is not limited to vascular endothelial cells, but no other cell has been found to produce as large quantities. Cytoplasmic storage granules of ET do not exist as they do for other peptides [17].

The kidney is a preferential target of ET and the renal arteries are particularly sensitive to this peptide. The specific high-affinity binding sites are located in the glomeruli, the inner medulla, and the renal vessels [6]. In experimental studies, ET has been shown to decrease cardiac output, reduce renal blood flow, increase renal artery resistance, slow glomerular filtration, and reduce urinary sodium excretion [11].

Recent evidence suggests that ET is involved in the pathophysiology of acute renal failure. Shibouta et al. demonstrated that plasma levels of rats rose from 1.6 pg/ml to 18 pg/ml after 45 min of renal artery occlusion [14]. However, acute injury resulting from postischemic vasoconstriction could be ameliorated by infusion of an anti-ET monoclonal antibody into the renal artery [14]. This specific antibody has been shown to improve renal function recovery and to protect the kidney from renal tubular necrosis. The fact that some Ca^{++} channel blockers have recently been reported to inhibit ET release may provide a possible explanation for the ability of the calcium antagonists to protect against acute renal ischemia [11].

In our rat study, the concentration of ET-3 in EC or UW solution preserving the right kidney reached a level of 0.46–0.82 pg/ml after 24 or 48 h, whereas ET was not detected in the preservation media alone. In the solutions that contained a left kidney subjected to 60 min of warm ischemia and reperfusion, significantly higher levels were obtained: 0.66–1.64 pg/ml. The total amount of ET found in the 40 ml of medium in which the left kidneys were stored ranged from 24 to 65 pg. In comparison, Shibouta et al. [14] demonstrated that the concentration in kidney tissue in similar warm ischemia conditions reached 1000 pg/g, but they did not wash the blood from the kidney at the time of harvesting. Because one of the most probable mechanisms of ET clearance includes receptor binding followed by internalization, we can expect a high tissue concentration in the kidney, liver, and lungs [9]. While our study leaves no doubt that the quantity of ET detected in the surrounding media was released by the kidney, our measurement is indirect and certainly an underestimation of the total amount of ET produced in the kidney.

In the preliminary human results, the concentration of ET-1 in the perfusion media ranged between 1.6 and 10.2 pg/ml. If the total amount of media used to preserve each human kidney is approximately 300 ml, then the quantity of ET-1 released by the kidney in an average of 24 h was 0.5–3.6 ng. By comparison, the daily urinary production of ET in renal graft recipients has been measured to be 4 ± 1.9 ng/24 h [1]. Shibouta et al. [14] demonstrated that the renal tissue concentration of ET can be very high after warm ischemia and, therefore, if ET enters the recipient circulation upon reperfusion of the allograft, the resulting effects could be detrimental to the kidney. This hypothesis is supported by the results of Berbinschi et al. [1], who found higher concentrations of ET in transplant recipients just after unclamping [plasma levels in these patients ($n = 8$) reached before unclamping averaged 22.7 ± 7 pg/ml (unpublished data)]. After unclamping, the plasma ET concentration rose to 25 ± 4 pg/ml and 31 ± 8 pg/ml after 4 and 8 h, respectively, and then finally decreased to 18 ± 3 pg/ml after 24 h. These higher post-reperfusion levels may reflect locally produced ET washed into the peripheral circulation, but the effect of the surgical procedure cannot be excluded as a possible cause. When compared to healthy volunteers, patients with acute renal failure, cyclosporin induced nephrotoxicity and the postrenal transplantation were found to have higher plasma levels of ET [3, 7, 8, 15].

Paradoxically, ET has been found to stimulate the release of prostacyclin, atrial natriuretic factor, and endothelium derived relaxing factor (EDRF), agents that counteract vasoconstriction [11]. Our data found a close relationship between the concentrations of ET and PGI_2 in the preservation media.

Prior to the discovery of ET, A-II was the most potent renal vasoconstrictive substance known. Even now, the site of A-II formation remains controversial. Among the hypotheses are those that suggest that A-II is generated either in the peripheral blood or in the extravascular space of the kidney [10]. By virtue of A-II's presence at high concentrations in the preservation media, our results clearly support the theory that the kidney can produce very high concentrations of this vasoconstrictive substance. A-II may act as a local hormone, producing con-

striction of the afferent glomerular artery, reducing glomerular filtration and renal blood flow, and thus precipitating acute renal failure.

The arachidonic acid metabolic thromboxane A_2 (TxA_2) is produced by aggregated or ischemically injured platelets, mesangial cells, and monocytes. TxA_2, a potent vasoconstrictive substance, is counteracted by prostaglandin I_2 (PGI_2), a vasodilator secreted by endothelial cells subjected to ischemia. The ratio of TxA_2 to PGI_2 may be the most relevant parameter is assessing the status of a postischemic organ [4].

Since we did not compare the concentration of vasoactive substances with ischemia intervals of varying duration, it is not possible to know if the release of those substances, rather than only a diffusion time effect, corresponds to the severity of graft injury. It is likely that at 4 °C, the measurable concentrations of vasopeptides represent a progressive release and leakage, rather than a continuous production or synthesis. Nonetheless, the high concentrations of vasoative substances released by a kidney subjected to warm ischemia clearly reflect the resulting injury.

Recently, Parrot and coworkers [12] advocated additional perfusion with fresh preservation media just prior to transplantation, which reportedly decreases the incidence of primary nonfunction (30.8% vs 57.5% in patients without late perfusion). These interesting results suggest that late perfusion removes any vasoactive compounds from the organ and prevents these substances from being washed into the circulation when transplantation is completed. Our experimental and preliminary clinical results suggest that the presumed vasoconstrictive substances could be ET-1, A-II, and TxB_2. Late perfusion did not entirely eliminate primary nonfunction, indicating that this condition is the result of multiple factors.

Our experimental results demonstrate a relationship between high concentrations of various vasoactive substances in the surrounding medium after cold storage and previous acute ischemic injury. Although we have yet to determine whether vasopeptides are continuously produced during cold storage, we can at least suggest that increased concentrations of those substances in the surrounding medium reflect a previous ischemic injury.

Extended human studies must be performed in order to establish the relationship between ischemic time and vasoactive peptide concentration. It is worth mentioning that the only recipient who exhibited an acute tubular necrosis had a very high concentration of A-II (53 pg/ml) and a significant level of ET (2.13 pg/ml) in the surrounding medium. This kidney had been preserved in EC solution. Moreover, the donor arrested 10 min prior to kidney harvesting.

The evaluation of these substances before transplantation may have prognostic value, indicating whether additional treatment should be performed by the transplant surgeon. Reperfusion with fresh media before transplantation, as proposed by Parrot et al. [12], should be considered. However, the relevance of vasoactive substances in the recovery of early graft function remains hypothetical.

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