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Original Article

A METHOD FOR INDUCTION OF CHRONIC RENAL FAILURE IN RATS

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Chronic Renal Disease (CRD) is a major health burden, which has recieved increased attention in recent times and has thus become one major focus of intensive research. All is agreed that the complex interplay of major pathophysiological factors that are characteristic of CRD and end stage renal failure (ESRF) is of multifactorial aetiology. However, commonly used animal models for CRD are bedeviled by methodologically induced complexities, which make the procedures not only laborious but also make interpretation of results less explicit. More often than not, some of these procedures present in addition, pathological parameters that may not universally reflect the settings of clinical forms of CRD. We have therefore characterized a simple and reproducible method for inducing chronic renal failure (CRF) in rats; in which the pathological parameters better reflect the usual findings in clinical situations. This approach has methodological and experimental advantages with respect to commonly used procedures for inducing CRF in rats, which may involve extensive renal surgery in which the renin-angiotensin system is often markedly stimulated. This later complication is at variance with clinical CRD in which low to normal renin activity is more often the rule rather than the exception. The simplicity and reproducibility of this model, coupled with a better correlation with the known features of CRF makes it a useful rat model not only for research purposes but also for testing of therapeutic maneuvers commonly used in the clinical setting.

Key words: chronic renal failure-hypertension-acid-base balance-kallikrein

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INTRODUCTION

Chronic renal disease (CRD) has become a major burden to mankind in recent times (United States Renal Data System, 1990 Annual data report). A majority of patients with CRD suffer from hypertension, irrespective of the cause of their renal disease. In these groups of patients, uncontrolled hypertension, as a result of increased cardiovascular risk factors, contributes to the high morbidity and mortality associated with the disease. Although fluid overload plays a significant role in the sustenance of hypertension in CRD, euvolaemic patients are also known to have significantly higher blood pressures compared to controls, thereby suggesting the operation of a persistent hypertensive stimulus other than volume overload (Schmidt & Baylis, 2000).

Most commonly used models of hypertension require nephron reduction to the level of nephrectomy to effect significant features of end-stage renal disease (ESRD). Thus the classical "DOCA salt model of hypertension" requires considerable nephron reduction to achieve significant hypertensive renal disease (Selye *et al*., 1943). More over, the remnant kidney model of progressive renal disease, is associated with arterial hypertension in which, nephrectomy is combined with partial infarction (Ibrahim & Hostetter, 2000). The effects of renal infarction presents on its own, a unique spectrum of pathological manifestations which may add on to the effects of hypertension on renal function; thereby making data interpretation less explicit. For instance, the known suppressive effect of potassium loading on plasma renin activity (PRA) is inhibited by the scar-derived renin secretion in the remnant kidney model (Linas, 1981).

Other commonly used models of CRD besides ablation of renal mass and

ureteral obstruction (Reyes *et al*., 1994), includes cyclosporine induced renal damage (Andoh *et al*., 1997) and chronic Nitric Oxide Synthase (NOS) inhibition (Zatz & Baylis, 1998) which for obvious reasons also have predictable short-comings. Generally, CRF is often associated with low to normal plasma renin activity (Ibrahim *et al*., 1997). This observation is in direct conflict with the fact that most commonly employed models of chronic renal disease are of the high renin variety (Ibrahim & Hostetter , 2000).

We have therefore characterized a method for inducing hypertensive renal disease where renin angiotensin activation is not marked and where extracellular volume expansion plays a significant role in the established stage of hypertension, a setting similar to what obtains in CRD; in order to provide a rat model, not only for research purposes but also for testing of therapeutic maneuvers that are relevant to real clinical situations.

MATERIALS AND METHODS

Animal preparation:

Male Sprague-Dawley rats weighing 230.4±2.3 g were used for the experiment. The rats were allowed free access to normal rat chaw containing 0.4% sodium (Na^+), 1.4% potassium (K^+) and 0.5% chloride (Cl^-) and tap water *ad libitum* up to the day of operation and throughout the observation period. The operation for the induction of hypertensive renal disease was performed in two stages under ether anaesthesia: During the first operation, a 0.2mm "U" shaped silver clip (internal diameter) was placed on the left renal artery via a left-flank incision. The control rats were sham-operated. After recovery from the first operation, usually 36 to 48 hours, the experimental and control rats were subjected to a right nephrectomy under ether anaesthesia. The rats were kept under observation for one day in the laboratory. They were thereafter returned to the animal-care house which had a room temperature of 28 ^oC, dark / light cycle of 12 hours and relative humidity of 60%, all regulated by electronic devices. The rats received no antibiotics and infection was not observed in any of them.

Blood pressure was measured in conscious rats twice weekly using rat-tail plethysmography. Based on previous pilot studies, the rats were used for standard clearance experiments 4 weeks after operation. Only rats whose blood pressures were clearly above 140 mmHg were used for clearance experiment.

Clearance experiment:

On the day of experiment, the rats were anaesthesized with inactin^R (sodium salt of 5-ethyl-5-(1'-methyl-propyl)-2-thiobarbituric acid; Byk Gulden, Konstanz Germany) at a dose of about 75-80 mg/Kg body weight. This dose of inactin is about 75% to 80% of the usual dose of inactin, in order to eliminate anaesthesia induced alterations in acid-base balance studies as previously reported (Odigie & Marin-Grez, 2000). A tracheostomy was performed to guarantee spontaneous breathing. The rectal temperature was maintained at 37.0 $\pm$ 0.5^oC using a heated rat-operating table coupled to a rectal thermometer. A PP50 polyethylene catheter was placed in the carotid artery for blood pressure measurement (strain gauge transducer, Tekman Electronic GmbH, Germany) and for blood samples. A PP50 femoral catheter was inserted for continuous infusion of physiological saline solution (PSS) containing 150 mmol sodium chloride and 6% polyfructosan (inutest^R Laevosan GmbH, Linz) at the rate of 1ml /hr i.v. A PP100 polyethylene catheter filled with PSS containing 50 U/ml heparin was placed in the lower abdominal aorta for venous pressure measurement. The ureter was canulated via a mid-abdominal incision and the ureter catheter was fixed with a ligature. After 120 min equilibration period, urine flow was controlled by collecting 30 min samples of ureter urine.

It was assumed that the rat was stable when constant urine samples were obtained. Urine samples were then collected in pre-weighed vials (eppendorf) for two 30 min periods.

Blood samples were collected anaerobically in heparinized capillary tubes and were immediately subjected to complete acid-base-balance analysis on an Automatic Blood-Gas Analyzer (AVL 990, AVL Biochemical Instruments, Graz, Austria) as previously reported (Odigie & Marin-Grez, 2000). Blood was also collected in heparinized capillary tubes for haematocrit determination. Blood plasma was obtained by centrifugation of blood samples at 3000 r.p.m. for 10 min (Eppendorf Centrifuge, Hamburg). Aliquots of plasma samples were stored in a deep freezer at -30 ^{o}C until the day of measurement. Plasma from first blood sample was used for aldosterone measurement. Plasma creatinine and plasma electrolytes were measured within 24 hours. At the end of the experiment, the kidney was decapsulated in-vivo and together with the heart were removed and weighed (Sartorious Digital Balance). The hearts were examined optically and the kidneys were sliced using a series of blades mounted on a holder. A piece of renal cortex was immediately shock-frozen in liquid nitrogen and stored at -30 ^{o}C and later used for renal kallikrein measurement.

Analytical Methods:

Urine was determined gravimetrically without correcting for specific gravity. Kallikrein excretion in urine and renal tissue kallikrein was measured using a chromogenic substrate as previously described (Klein *et al*., 1989). Plasma aldosterone concentration was determined using a computer assisted specific Aldosterone radioimmunoassay. Creatinine was measured using the Jaffe's reaction. Glomerular filtration rate (GFR) was calculated using the clearance of polyfructosan (Fuhr *et al*., 1955). Plasma and urinary electrolyte concentration was measured using a flame photometer (Instrumentation Laboratory IL943, IL Fisher Science, U.S.A). Chloride concentration was determined with an automatic chloridometer (Buchler-Cotlove Automatic Chloride Titrator; Buchler Instruments Inc. Fort Lee N. J. USA). Urinary bicarbonate concentration was calculated from the measured values of urinary PCO_2 and pH after taking the ionic strength of the urine into consideration as previously reported (Odigie & Marin-Grez, 2000). Haemoglobin concentration was measured using the method of Van Kampten and Zilstra, which involves the conversion of haemoblobin to haemoglobin cyanide. Crystallized rat haemoglobin was used as standard (Sigma Chemie GmbH, Diesenhofen, Germany). The determination of renal blood flow using p-amino-hippuric acid (PAH) was avoided because this substance interferes with acid base balance determination (Silbernagl , 1986).

All animal handling and experimentation were in accordance with the guidelines laid down by the American Physiological Society for animal care and experimentation.

Statistical analysis:

Values are expressed as means ± SEM (Standard Error of Mean). Unpaired "t" test was used for hypothesis testing between hypertensive rats and controls. A difference was considered statistically significant when $P < 0.05$

RESULTS

Application of a 0.2mm clip on the renal arteries of rats with body weight of 230.4 ± 2.3 g led to a rapid progression of hypertension, resulting in CRF. A significant reduction in blood PCO_2, BE, BEecf, Blood HCO_3^- and standard HCO_3^- was found in Rats with CRF (all $P<0.001$). The clipped rats developed a metabolic acidosis (Table 1). Rats with CRF had a significantly reduced body weight ($P<0.001$) and an increased respiratory rate ($P<0.05$). A significant elevation in arterial blood pressure ($P<0.001$) and venous pressure ($P<0.05$) was observed. There was no significant difference in the weight of the kidneys of controls compared to the clipped rats (Table 2).

Table 1:
Acid-base balance parameters of rats with chronic renal failure (CRF) compared to controls. H^+ = proton concentration; PCO_2 = partial pressure of carbon dioxide in arterial blood; BE=base excess; BEecf=Base excess of extracellular fluid; BB= Buffer base; O_2.Sat.=arterial oxygen saturation; St.HCO_3^- = standard bicarbonate.

	CONTROL (n = 8)	CRF (n = 5)	P-VALUES
$[H^+]$ (nmol/L)	40.2 ± 0.7	38.1 ± 2.0	NS
PCO_2 (mmHg)	45.7 ± 1.2	30.7 ± 2.6	P< 0.001
BE (mmol/L)	2.7 ± 0.5	- 3.5 ± 1.1	P< 0.001
BE_{ecf} (mmol/L)	3.2 ± 0.5	- 4.4 ± 1.2	P< 0.001
BB (mmol/L)	46.9 ± 3.8	43.5 ± 1.0	NS
$[HCO_3^-]$ (mmol/L)	27.7 ± 0.5	19.2 ± 1.2	P< 0.001
(PO_2) (mmHg)	76.3 ± 2.4	87.8 ± 5.7	NS
O_2.Sat (%)	94.9 ± 0.4	96.4 ± 0.7	NS
[St.HCO_3^-] (mmol/L)	26.1 ± 0.4	21.0 ± 0.8	P< 0.001

Table 2: Arterial blood pressure (BP) and venous blood pressure (VP), Body weight (BW), Kidney weight (Kid.Wt), Heart weight (Hrt.Wt) and Respiratory rate (RR) in rats with CRF and Controls.

	CONTROL (n = 8)	CRF (n = 5)	P-VALUES
BP (mmHg)	125.0 ± 3.8	154.0 ± 2.4	P < 0.01
V.P. (mmH_2O)	5.6 ± 0.13	6.9 ± 0.42	P < 0.05
B. W. (g)	390.4 ± 8.4	345.2 ± 3.30	P <0.001
Kid. Wt (g)	2.17 ± 0.12	1.89 ± 0.10	NS
Hrt. Wt (g)	0.74 ± 0.01	0.99 ± 0.05	P < 0.05
Resp. rate (Rate/min)	88.0 ± 0.8	109.4 ± 3.4	P < 0.05

Plasma potassium concentration (P<0.005) and plasma chloride concentration (P<0.005) were significantly elevated. The Anion-Gap was comparable in the two groups. Plasma creatinine on the other hand was significantly elevated P<0.001) in rats with CRF. The haematocrit values were significantly lower than controls (P<0.001, Table 3).

There was a marked reduction in urine flow rate (P<0.005) compared to controls and GFR was significantly reduced (P<0.001). A marked reduction in the ability of the clipped kidney to excrete electrolytes was noted. Thus the sodium (P<0.05), potassium (P<0.001), chloride (P<0.001) and bicarbonate (P<0.05) excretion rates were all reduced (Table 4).

Table 3:
Serum electrolytes, serum creatinine and haematocrit values of rats with CRF compared to controls. Na^+ p=plasma sodium concentration; K^+ p=plasma potassium concentration; Cl^- p=plasma chloride concentration; AN-GP=anion-gap; creat.p=serum creatinine concentration; Hct=haematocrit of arterial blood.

	CONTROL (n = 8)	CRF (n = 5)	P-VALUES
$[Na^+]_p$ (mmol/L)	148.5 ± 0.8	146.7 ± 0.5	NS
$[K^+]_p$ (mmol/L)	5.10 ± 0.90	6.20 ± 0.30	P< 0.005
$[Cl^-]_p$ (mmol/L)	102.7 ± 1.2	107.8 ± 0.3	P< 0.005
An- Gp (mmol/L)	18.1 ± 1.5	19.7 ± 1.4	NS
(Creat.) p(mg/100ml)	1.02 ± 0.03	1.82 ± 0.08	P< 0.001
Hct. (%)	49.6 ± 0.5	40.0 ± 3.2	P< 0.001

Table 4: Urine flow rate (V), Glomerular filtration rate (GFR), and electrolyte excretion in rats with CRF compared to controls. $[Na^+]u.V$=sodium excretion rate; $[K^+]u.V$=potassium excretion rate; $[Cl^-]u.V$=chloride excretion rate; $[HCO_3^-]u.V$=bicarbonate excretion rate

	Controls (N = 8)	Crf (N = 5)	P-Values
V (µl/min.)	7.9 ± 0.5	3.9 ± 0.8	P< 0.005
GFR (ml/min)	1.68± 0.11	0.36± 0.08	P< 0.001
$[Na^+]$.V(µmol/min	0.30± 0.06	0.09± 0.05	P < 0.05
$[K^+]_u$.V(µmol/min	2.52± 0.21	0.28 ± 0.11	P< 0.001
$[Cl^-]_u$.V(µmol/min	1.16± 0.19	0.13 ± 0.06	P< 0.001
$[HCO_3^-]_u$.V(µmol/min	17.7 ± 6.9	1.30 ± 0.40	P < 0.05

Rats with CRF had reduced fractional excretion of sodium (P<0.05), potassium (P<0.001), and bicarbonate (P<0.001). The fractional excretion of chloride was not significantly different from controls. Rats with CRF had a significant reduction in urinary kallikrein excretion (P<0.001, Table 5)

Renal tissue kallikrein content was significantly reduced in the clipped rats

(112.5 ± 6.3 vs 87.5 ± 4.2 mU/g kidney, n=12, P<0.001). The haemoglogin concentration in rats with CRF and controls showed no significant difference (16.7 ± 0.4 g/dl vs 17.3 ± 0.3 g/dl). Plasma aldosterone concentration was markedly elevated in rats with CRF (3.14 ± 0.41 vs 0.63 ± 0.16nmol/L, n=13, P<0.001)

Table 5: Urinary kallikrein excretion and fractional electrolyte excretion in rats with CRF compared to controls. Ukalli=urinary kallikrein excretion rate; FE-Na^+ =fractional excretion of sodium; FE-K^+ =fractional excretion of potassium; FE-Cl- =fractional excretion of chloride; FE-HCO_3^- =fractional excretion of bicarbonate

	CONTROL (n = 8)	CRF (n = 5)	P-VALUES
Ukalli(mU/min.	1.59 ± 0.12	0.46 ± 0.03	P < 0.001
FE-Na^+ (%)	0.12 ± 0.02	0.07 ± 0.03	P < 0.05
FE-K^+ (%)	29.5 ± 1.50	11.9 ± 3.50	P < 0.001
FE-Cl^- (%)	0.65 ± 0.11	0.45 ± 0.30	NS
FE-HCO_3^- (%)	0.08 ± 0.04	0.02 ± 0.01	P < 0.001

DISCUSSION

The pathogenesis of elevated blood pressure in patients with chronic renal failure is being debated on many platforms. Among the factors currently implicated for elevated blood pressure is Nitric Oxide (NO). This physiologically important endogenous vasodilator has been reported to be deficient in CRD (Schmidt & Baylis, 2000; Ketteler & Ritz, 2000; Blum *et al*, 1998) and in ESRF (Vallence *et al*., 1992; Reyes *et al*., 1994). The reasons advanced for reduction of NO production includes NO substrate deficiency as a result of reduced renal mass (Morris Jr., 1992); accumulation of NO inhibitors in progressive renal disease (Vaziri *et al*., 1998) and endothelial dysfunction due to increased inactivation of endothelial NO as a result of oxidative stress (Vaziri *et al*., 1998). In renovascular hypertension, at least in the 2K-1C model, increased free radicals generation leading to impaired vascular response to endogenous and exogenous nitrovasodilators has been reported (Heitzer *et al*., 1999). Besides, NO influences blood flow distribution in renovascular hypertension (Sigmon & Beierwaltes, 1994) so that its deficiency readily sets the pace for the abnormal renal haemodynamics that characterize CRD. The strength of this assertion is exemplified by the fact that chronic arginine supplementation in the diet protects against progressive deterioration of renal function in a wide range of CRD models (Andoh *et al*., 1997; Reyes *et al*., 1994). However, this is not always the case as shown by the fact that a randomized double-blind, placebo-controlled study of supplementation of L-argininie in patients with moderate CRF did not improve renal function (Denicola *et al*., 1999); an observation that calls for further studies. The role of NO in CRD remains controversial as some workers have reported increased exhaled NO in CRF rather than a reduction (Matsumoto *et al*., 1999). Since exhaled NO in mainly generated in the lungs, it may not necessarily reflect systemic or renal events. The role played by other vasoactive substances in CRD remains to be completely elucidated.

In the rat model of CRF characterized above, widespread electrolyte and acid-base balance disturbances were observed. The markedly elevated serum aldosterone levels and reduced fractional excretion of electrolytes as well as reduced urinary excretion speak for the possibility of fluid overload in the pathogenesis of hypertension in this model of CRD. Renal Kallikrein excretion and renal tissue content of the enzyme were markedly reduced as expected. Significantly reduced haematocrit levels reveal evidence of anaemia. Serum creatinine was significantly elevated and the reduced body weight of rats with CRF reveals a negative nitrogen balance, as is characteristic of the disease.

In the setting of CRD and hypertension, the kidney could be the victim or the culprit or both. Hypertension is a major risk factor that determines not only the rate of progression of renal disease but also generally aggravates the development of glomerular sclerosis.

One way it could do this is as a result of increase in glomerular capillary pressure and the resulting pressure damage ("barotrauma") then leads to glomerular sclerosis (Brenner, 1985). These effects are generally more severe in the face of glomerular hypertrophy (Miller *et al*., 1991) as obtains in progressive glomerular loss of CRD. On the other hand, the intrinsic vascular damage of chronic hypertension by causing reduction in arterial lumen may lead to glomerular hypoperfusion and chronic ischaemic renal damage (Greco & Breyer, 1997). The role played by angiotensin II in this regard is significant since angiotensin converting enzyme inhibitors attenuates glomerular sclerosis independent of glomerular capillary pressure (Fogo *et al*., 1988). It follows therefore, that glomerular hyperperfusion and hypertension are not essential for the development of glomerular sclerosis. However, a significant difference between human and rat kidney is the increased glomerular sclerosis with normal aging present in the rat (Bolton & Sturgil, 1980). These factors should be taken into consideration in the interpretation of rodent results, as these results cannot necessarily be substituted for clinical observations.

Non-haemodynamic factors mediating glomerular damage and interstitial fibrosis include angiotensin II as well as various growth factors and cytokines (Chung & Chevalier, 1996). The effects of angiotensin II on glomerular damage, in particular, are known to be independent of hypertension (Yoo *et al*., 1998). The pathophysiological effects of these additional factors can only be exhaustively investigated in a suitable animal model of CRD.

In summary, it is clear to see that although isolated breakthroughs have been made in the management of CRD, we certainly need more information on the pathophysiology involved before meaningful interventions can be made and the model, presented here serves as a simple, reproducible and uncomplicated model for such interventions.

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