L-GLUTAMINE METABOLISM IS NOT A MAJOR SOURCE OF INCREASED FREE RADICAL GENERATION IN GOLDBLATT RENOVASCULAR HYPERTENSION

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Running title: Glutamine and Renovascular Hypertension

Summary: Glutamine has been implicated in the generation of free radicals and free radicals-induced impairment of vascular responses to nitrovasodilators may underlie the pathogenesis of vasospasm in 2K-1C hypertension. Plasma glutamine levels were therefore determined in 2K-1C and 1K-1C hypertensive rats in order to ascertain the direction of change of this amino acid in this model of renovascular hypertension. Hypertension was induced in male SD rats (99±2.3 g) by subjecting them to left renal artery clamping using a 0.2mm silver clip (2K-1C, n=7) under ether anesthesia. Control rats (n=7) were sham-operated (Sh-Op). Rats with 1K-1C hypertension (n=8) and uni-nephrectomized controls (1K:, n=8), additionally underwent right nephrectomy. 10 weeks (2K-1C) and 4 weeks (1K-1C) respectively after renal artery clamping, clipped rats exhibited elevated blood pressures (P<0.001), which was sustained under anaesthesia. No significant difference in plasma glutamine levels were found in hypertensive rats compared to controls (11.3±1.3 mg/l in 2K-1C vs. 12.3±2.1 mg/l in Sh-Op, n=7 and 9.0±1.4 mg/l in 1K-1C vs. 9.6±1.4 mg/l in 1K:, n=8). Serum creatinine and. serum electrolyte concentrations were not significantly different in clipped rats compared to their respective controls. This study shows that plasma glutamine level is not altered in the established stages of Goldblatt renovascular hypertension. The significance of this observation is unclear. Nevertheless, these findings suggest that glutamine is not in the main stream of free radicals generation and is therefore not a reliable index of oxidative stress in this model of renovascular hypertension.

Key Words: Glutamine, Glutamate, Superoxide Radicals, Experimental Renovascular Hypertension.

Introduction

The most abundant free amino acid in the body is glutamine (Newsholme et al, 1988). This amino acid, which is derived primarily from skeletal muscle, is rapidly metabolized by a wide variety of cells under a wide range of physiological and pathological conditions. The leucocytes are a subset of cells amongst a variety of others, which metabolize glutamine at high rates (Newsholme, 2001). Activated neutrophils actively metabolize glucose and glutamine (Pithon-Curi et al, 1997) such that a

marked reduction in the plasma concentration of glutamine characterizes certain severe infections and oxidative stress in man (Novak et al, 2002; Houdijk et al, 1998; Goeters et al, 2002). Thus reduced plasma glutamine level is associated with impairment in immunological defense systems (Calder & Yaqoob, 1999). Glutamine supplementation is beneficial in elective surgery and accidental injury (Wilmore, 2001). Interestingly, changes in serum glutamine levels in human and in laboratory animals have been identified in situations of severe acute injury, with the re-establishment of normal values as the stress situation resolves (De-Souza & Greene, 1998). Glutamine also has a lot of nutritional advantages, some of which have been highlighted recently (Calder, 2000).

The importance of Glutamine as a substrate in the rapid metabolism in leucocytes lies in the fact that glutamine is important for the production of $0_2^{\cdot}$ (Superoxide radicals) by these cells (Garcia et al, 1999; Pithon-Curi et al, 2002). Even though the precise mechanisms involved are yet to be fully worked out, many studies have shown that glutamine is clearly involved in the respiratory burst in immunocompetent cells (Furukawa et al, 2000 (a)). Interestingly, glutamine is involved in the production of free radicals in post-operative patients (Furukawa et al, 2000 (b)). In addition, glutamine has the ability to regulate the expression

and production of cytokines (Fukatsu et al, 2001; Yassad et al, 2000), which are involved in free radicals production.

In an ischaemic-reperfusion model in the rat, an event known to be associated with increased free radicals production, renal uptake of glutamine and citrulline increased after ischaemic-reperfusion (Prins et al, 2002). The kidneys normally synthesize arginine, which is the sole precursor amino acid in the generation of vasodilatory agent nitric oxide (Palmer et al, 1988), from circulatory citrulline (Dhanakoti et al, 1992).

In the Goldblatt two-kidney, one-clip hypertensive rat (2K-1C), enhanced production and systemic delivery of Angiotensin II, forms the basic endocrine defect (Ploth & Fitzgibbon, 1994). 2K-1C renovascular hypertension is associated with increased vascular O_2^- (superoxide) production, which leads to impaired vascular response to endogenous and exogenous nitrovasodilators (Heitzer et al, 1999). This pathological process which results in markedly elevated blood pressure, occurs via NO (nitric oxide) scavenging by highly reactive free oxygen radicals generated by endothelial NAD(P)H oxidase systems which mediate superoxide production via a protein kinase C dependent pathway (Heitzer et al, 1999). Experimental evidence reveals that endothelial cells as well as smooth muscle cells possesses free radicals generating systems (Mohazzab et al, 1994) and angiotensin II stimulates these oxidase systems by as yet unidentified mechanism (Heitzer et al, 1999). These observations allow the postulate that increased production of free radicals of such a grave magnitude to significantly affect endothelial performance might manifest as alteration in plasma levels of glutamine since this amino acid has been reported to be involved in free radicals production (Pithon-Curi et al, 2002).

Plasma glutamine levels were therefore measured in rats with established 2K-1C and one kidney-one clip (1K-1C) hypertension in order to determine to what extent plasma levels of this amino acid reflect its possible involvement in increased free radicals production.

Materials and Methods

Animals Preparation:

Male Sprague Dawley (SD) rats were used for the experiment. After admission, the rats were allowed to adapt to the laboratory environment (room temperature of 28±2 °C and humidity of 60 %) for one week. The animals received normal rat chow (containing 0.4% Na^+, 1.4% K^+ and 0.5% Cl^-) and tap water ad libitum and were subjected to a light-dark cycle of 12 hours.

Induction Of 2K-1C And 1K-1C Hypertension:

Under ether anesthesia, male SD rats (body weight, 99±2.3 g) were subjected to left renal artery clamping using a 0.2mm "U" shaped silver clip (inner diameter) via a left flank incision while the right kidney was left untouched (2K-1C, n=7). Control rats (n=7) were sham-operated (Sh-Op). Muscle and skin were sutured separately. For the induction of 1K-1C renovascular hypertension, the rats were first subjected to left renal artery clamping under ether anaesthesia as above. 48 hours after left renal artery clamping, both control (1K:, n=8) and clipped rats (1K-1C, n=8) underwent right nephrectomy under ether anaesthesia.

The blood pressure of the clipped (2K-1C and 1K-1C) and control rats (Sh-Op and 1K:) was measured sphygmomanometrically in the rat tail weekly using a piezoelectric pulse detector and an occluding tail-cuff in conscious animals as previously reported (Odigie et al, 2000 (a)).

Clearance Experiment:

At the end of the observation period (10 weeks in 2K-1C and 4 weeks in 1K-1C), the rats were anesthetized with 100mg/kg of inactin^R (Na^+-5-ethyl-(1`-methyl-propyl)-2-thiobarbituric acid; Byk Gulden, Konstanz Germany) intraperitonially. The trachea was intubated with a small piece of polyethylene tubing (PE-240; Fisher Scientific, Springfield NJ USA) to guarantee spontaneous breathing. Through a mid-abdominal incision, the right and left ureter of 2K-1C and Sh-Op and the remaining left ureter of 1K-1C and 1K: were catheterized for direct ureter-urine collection. The left carotid artery and the right femoral vein were cannulated using a PE-50 polyethylene catheter. The carotid artery catheter was connected to a strain gauge blood pressure transducer (P23D Statham, Hato Rey, Puerto Rico) and the blood pressure was recorded on a Grass Polygraph Model 7D (Grass Instruments Co., Quincy Mass. USA). The carotid catheter was also used for the purpose of withdrawal of blood samples. Through the femoral vein catheter (PE-50), the rats received sterile intravenous fluid (0.9% saline in distilled water, 150mM) at the rate of 2ml/hr (2K-1C and Sh-Op) and 1ml/hr (1K-1C and 1K:) respectively. The rectal temperature of the rat was maintained at 37 °C ± 0.5°C.

After 120 min equilibration period, blood pressures were recorded and urine volume was determined by collecting urine into pre-weighed vials. Terminal blood samples were collected into heparinized tubes and blood plasma was separated immediately by centrifugation (3000 r.p.m. for 15mins). The plasma so obtained was stored in a deep freezer at –30 °C until required. At the end of the experiment, the rats were killed with an overdose of pentobarbital sodium (100 mg / kg) administered intra-arterially.

Analytical Methods:

Urine volume was determined gravimetrically without correcting for specific gravity. Serum creatinine was measured using the Jaffe's reaction according to Popper et al (Popper et al, 1937). Plasma electrolytes were determined using flame photometry (Instrumentation Laboratories, IL 943, IL Fisher Science USA). Chloride was determined electrometrically (Buchler-Cotlove Automatic Chloride Titrator; Buchler Instruments Inc Fort Lee, NJ, USA). Glutamine level in plasma was determined using the method of Beutler and Michal (Beutler and Michal, 1974; Beutler, 1985). Briefly, the principle of measurement is based on the oxidative deamination of L-glutamate by the enzyme glutamate dehydrogenase in the presence of Nicotine-Adenine-Dinucleotide (NAD). The reduced Nicotine-Adenine-Dinucleotide (NADH) produced in this reaction is used by the enzyme diaphorase to convert iodonitro tetrazolium chloride to formazan. The amount of formazan produced is proportional to the L-glutamate concentration in the samples and was determined spectrophotometrically at 492 nm. L-Asparaginase, which shows a glutaminase side activity, was first added to the samples in order to convert L-glutamine to L-glutamate. This enzyme is judged suitable for the determination of L-glutamine in assay mixtures (Biochemica 1988, Boehringer Mannheim GmbH, Germany).

For the measurement of glutamine in plasma, samples were first deproteinated using perchloric acid (1.0 mmol/l) followed by neutralization using tertiary potassium phosphate solution (1.93 mmol/l) for 10mins in ice-bath (0 °C). After centrifugation at 3,000 r.p.m. for 5 mins, the supernatant was used for the determination of glutamine levels. Iodonitro tetrazolium chloride is sensitive to light. Therefore, the lighting system in the laboratory was subdued in order to prevent interference with measured parameters.

All reagents and materials used for L-glutamine / L-glutamate determination were obtained as combination kit from Boehringer Manheim (Boehringer Biochemica GmbH, Manheim Germany). For the preparation of L-glutamic acid standard solution, L-glutamic acid (biochemical grade) was obtained from Merck (Merck, Darmstadt, Germany). The American Physiological Society's guidelines for experimental animal research were adhered to in all the experiments.

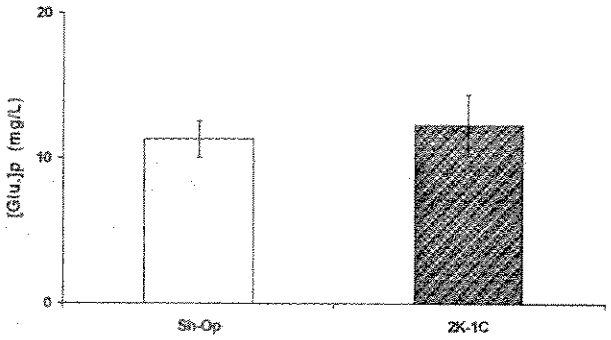
Statistical Analysis

Results are given as means ± SEM. The significance of differences in mean values was analyzed (SPSS 7.5 for windows) using the two-tailed independent Student's "t" test. The level of statistical significance was set at $P<0.05$.

Results

Conscious 2K-1C rats had elevated blood pressure 10 weeks after renal artery clamping (175.4±5.4 mmHg vs. 121.7±2.7 mmHg, n=7, $P<0.001$) and under anesthesia (156.0±5.4 mmHg vs. 108.0±4.0 mmHg, n=7, $P<0.001$). Similarly, the blood pressure of 1K-1C was elevated above those of controls in awake rats, 4 weeks after renal artery clamping (192.3±7.0 mmHg vs. 121.5±4.2 mmHg,

Figure 1.
Plasma Glutamine levels in 2K-1C (n=7) and Sham-Operated controls (Sh-Op, n=10).
$[Glu.]_p$ = plasma glutamine concentration. Blood samples were collected 10 weeks after renal artery clamping.



n=8, $P<0.001$) and under anaesthesia (180.9±6.9 mmHg vs. 112.8±3.8 mmHg, n=8, $P<0.001$). Serum creatinine concentration was not significantly different in the clipped rats compared to controls (1.22±0.10 mg/dl in 2K-1C vs. 1.04±0.22 mg/dl in Sh-Op, n=7 and 1.15±0.11 mg/dl in 1K-1C vs. 1.21±0.08 mg/dl in 1K: n=8).

Serum electrolyte concentrations were comparable in the clipped rats (2K-1C & 1K-1C) and their respective controls (Sh-Op & 1K:) and are shown in Table 1.

No significant differences were found in serum glutamine levels in the 2K-1C compared to Sh-Op (11.3±1.3 mg/L vs. 12.3±2.1 mg/L; n=7; Fig. 1). Similarly, the serum glutamine levels were comparable in 1K-1C and 1K: (9.0±1.4 mg/L vs. 9.6±1.4 mg/L; n=8; Fig. 2). Urine flow rate obtained by direct ureter catheterization was comparable in 1K-1C and 1K: (7.1±1.3 □l/min vs. 8.2±1.9 □l/min; n=8; P=NS). The urine flow rate of the right kidney (Ureter catheterization) was elevated above that of Sh-Op (12.0±2.5 □l/min in 2K-1C and 8.3±1.6 □l/min in Sh-Op; n=7; $P<0.05$). The urine flow rate of the left kidney of 2K-1C was markedly reduced compared to that of the Sh-Op (4.4±0.6 □l/min in 2K-1C and 7.3±1.4 □l/min in Sh-Op; n=7; $P<0.001$).

The differences in body and organ weights in the clipped and control rats were as expected and are

shown in (Table 2). Hypertensive rats had cardiomegaly ($P<0.05$ in 2K-1C and $p<0.001$ in 1K-1C vs. their respective controls; Table 2). The weight of the left kidney of 2K-1C was significantly lower than that of Sh-Op ($P<0.001$; Table 2). A significant reduction in body weight; consistent with the metabolic stress of chronic severe hypertension, was observed in 2K-1C rats ($P<0.001$, Table 2).

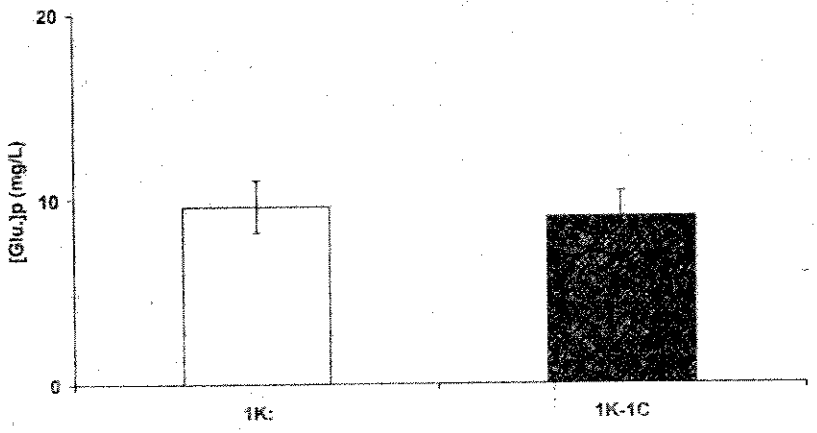
Table 1: Plasma electrolyte concentrations in 2K-1C (n=7) and 1K-1C (n=8) hypertensive rats and controls. Sh-Op=sham-operated controls (n=7); 1K: uni-nephrectomized control (n=8). [Na^+], [K^+] and [Cl^-] represent plasma sodium, potassium and chloride concentrations respectively.

	Sh-Op	2K-1C	1K:	1K-1C
[Na^+] (mmol/l)	143.9± 1.7	140.4± 3.6	146.0± 1.7	148.6± 1.3
[K^+] (mmol/l)	4.3± 0.1	4.7± 0.2	4.2± 0.1	4.4± 0.1
[Cl^-] (mmol/l)	101.6± 2.0	100.2± 1.3	100.3± 1.3	105.0± 1.8

*Table 2: Body and organ weights of 2K-1C (n=7, 10 weeks) and 1K-1C (n=8, 4 weeks) and controls. Sh-Op=sham-operated controls (n=7); 1K:, Uni-nephrectomized control. (n=8) * = P<0.05, *** =P<0.001, hypertensive rats compared to their respective controls.*

	Sh-Op	2K-1C	1K:	1K-1C
Body weight (g)	430.6±1.3	*** 407.1±4.6	294.0±8.8	281.4±13.1
Rt. Kidney wt. (g)	1.83±0.06	2.04±0.39	-	-
Lt. Kidney wt (g)	1.65±0.03	*** 1.27±0.37	1.42±0.12	1.24±0.15
Heart wt. (g)	1.06±0.03	* 1.27±0.37	0.74±0.01	*** 0.99±0.05

Figure 2
Plasma Glutamine levels in 1K-1C (n=8) and Uni-nephrectomized rats (1K:, n=8).
$[Glu.]_p$ = plasma glutamine concentration. Blood samples were collected 4 weeks after renal artery clamping.



Discussion:

The main finding in the present study is an unchanged plasma level of glutamine. This observation suggests that plasma glutamine levels are not significantly depleted in the established stages of Goldblatt renovascular hypertension. One possible implication of this conclusion is that free radicals production in the 2K-1C model of renovascular hypertension may not be primarily glutamine dependent since elevated levels of free radicals are known to underlie the pathophysiology of 2K-1C hypertension (Heitzer et al, 1999). Even though intracellular levels of glutamine were not determined in this study, there are indications to suggest that plasma levels of glutamine reflect intracellular levels (Novak et al, 2002; Houdijk et al, 1998; Goeters et al, 2002).

In neutrophils, glutamine metabolism is important for superoxide (O_2.-) production (Garcia et al, 1999; Pithon-Curi et al, 2002; Furukawa et al, 2000 (a)). Ever. though endogenous glutamine is known to be involved in the respiratory burst in neutrophils, the pathway for glutamine metabolism in vascular endothelial cells and vascular smooth muscle cells (VSMC) has not been fully worked out. There are indications to show that intracellular levels of this amino acid are higher than plasma levels in neutrophils. This is consistent with the observation that neutrophils normally have a high content of glutamine (Pithon-Curi et al, 1997; Curi et al, 1999). The data presented herein does not allow for postulations concerning the role of glutamine in 2K-1C hypertension, which is still controversial. In addition, it is not known with certainty what role glutamine plays in free radicals generation in endothelial cells in the rat. The possibility of a species variation in glutamine metabolism in endothelial cell in the rat must be entertained and cannot be completely ignored. Nevertheless, it must be determined whether like in the neutrophils, vascular endothelial cells rely heavily on glutamine for the generation of superoxide radicals. Studies on intracellular levels of glutamine in VSMC should clarify this possibility.

In the neutrophils, the pathway for glutamine metabolism involves its conversion into glutamate and oxoglutarate, which are subsequently oxidized via the citric acid cycle (Pithon-Curi et al, 1997; Curi et al, 1999) for ATP production and protein synthesis. Recent reports that glutamine is able to regulate the expression of the components of NADPH oxidase (Pithon-Curi et al, 2002) allows the postulate that glutamine may also play an important role in the production of superoxide radicals in vascular endothelium. Interestingly, the pathway for superoxide production by NAD(P)H oxidase in vascular endothelial and smooth muscle cells (Heitzer et al, 1999) follows closely that which occurs in neutrophils (Kobayashi et al, 2001). Thus superoxide radicals generated by endothelial NAD(P)H oxidase systems is a center point in the production of highly reactive oxidants and potent free radicals, which scavenge endothelial nitric oxide and induce endothelial dysfunction with resultant vascular hypertonia that characterize 2K-1C hypertension (Heitzer et al, 1999). The role of glutamine in this scenario remains to be determined against the background of other key players of free radicals generation in this model of renovascular hypertension. For instance, aldosterone, which has been implicated in endothelial dysfunction in humans (Farquharson et al, 2002), is known to increase the production of cytokines and induce oxidative stress, which then breaks down formed nitric oxide (Rocha and Stier, 2001). Plasma levels of aldosterone are significantly elevated in Goldblatt renovascular hypertension (Odigie and Marin-Grez, 2000 (b)). This steroid hormone also inhibits inducible nitric oxide release in response to cytokines stimulation (Ikeda et al, 1995). In addition, high levels of angiotensin II associated with 2K-1C hypertension stimulates endothelial NAD(P)H oxidase systems leading to release of free radicals (Heitzer et al, 1999). The observation that aldosterone amplifies the response of angiotensin II in-vitro (Sun and Weber, 1993; Ullian et al, 1992) suggest a more significant contribution of these systems, compared to glutamine, in the generation and release of free radicals in 2K-1C hypertension.

The lack of a significant difference in plasma creatinine level in the hypertensive rats compared to their respective controls speak against renal failure as a possible underlying cause of elevated blood pressure. The preservation of serum electrolyte levels observed in the established stages of Goldblatt renovascular hypertension in this study is consistent with previous reports (Odigie and Marin-Grez, 2000 (c)).

In conclusion, this study suggests that in this model of renovascular hypertension, plasma glutamine level is unchanged and is therefore not a reliable index of oxidative stress.

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