

Variable number tandem repeats in the promoter region of prostacyclin synthase gene in choline deficient rats

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ABSTRACT: Weanling Sprague-Dawley rats were fed on a choline-deficient diet with hydrogenated vegetable oil and corn oil as lipids develop acute renal failure. Pathogenesis of the latter is controversial and an ischemic mechanism has been proposed. Arachidonic acid derivatives are involved in the regulation of vascular tonus. Vasospasm could be due to an increase in thromboxane A₂-mediated vasoconstriction or to a decrease in prostacyclin-induced vasodilatation. Enzymes involved in the synthesis of both compounds are thromboxane A₂- and prostacyclin-synthase respectively. The aim of this study was to identify the variable number tandem repeats (VNTR) in the promoter region of prostacyclin synthase gene and verify if there exists a relationship between the occurrence of VNTR in those choline-deficient rats which die because of acute renal failure and those which do not. We verified the presence of the VNTR in the prostacyclin synthase rat gene, but we did not find any difference in the molecular weight of the alleles between experimental and control rats. Renal reparation of the acute kidney injury due to choline deficiency in some rats is not related with differences in VNTR in the promoter region of the prostacyclin synthase gene.

Introduction

Choline is crucial for sustaining life. It modulates basic signaling processes within the cell, it is a structural element in membranes and it is vital during critical periods in brain development. Since choline is widely distributed in food, it has been difficult to identify a human deficiency syndrome in healthy humans. However; vulnerable populations may become choline deficient, including growing infants, pregnant and lactat-

ing woman (Zeisel and Blusztajn, 1994). Choline requirements are modified by individual growth rate and by complex interrelationships between choline and methionine, folic acid, and vitamin B₁₂. This quaternary amine is present in tissues predominantly as a constituent of phosphatidylcholine and sphingomyelin.

It is known that female rats are more resistant to ischemic acute kidney injury (acute renal failure) than male rats (Takayama *et al.*, 2007). We have observed that weanling male rats fed on a choline-deficient diet (with hydrogenated vegetable oil and corn oil as lipids) develop acute kidney injury with morphological alterations that vary from focal tubular necrosis to massive cortical necrosis, but may either die as a consequence of acute kidney failure (Montes de Oca *et al.*, 1980) or may resolve for unknown reasons.

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The pathogenesis of acute kidney injury due to choline deficiency is controversial, and both an ischemic mechanism and primary tubular damage have been proposed (Montes de Oca *et al.*, 1980). The quality as well as the quantity of diet lipids modulates the renal lesion due to choline deficiency (O'Neal *et al.*, 1961). Menhaden oil has a protective effect in this experimental model. This oil is rich in eicosapentanoic (20:5) and docosahexaenoic acids (22:6) and it may influence renal fatty acid composition and arachidonic acid metabolism which plays an important role in renal physiopathology (Courrèges *et al.*, 2002). This effect could be due to modifications in the vascular tonus as well as susceptibility of each particular rat.

Prostaglandins and thromboxans (=prostanoids) are cyclic eicosanoids have potent vasodilatory and vasoconstrictory activities, respectively. Prostaglandin H2 synthase catalyzes the conversion of arachidonic acid to prostaglandin G2, and of the latter to prostaglandin H2, and prostacyclin synthase catalyzes the rearrangement of prostaglandin H2 to prostacycline. Prostacycline increases levels of intracellular cAMP inducing vasodilatation and suppressing platelet aggregation (Mayes, 1992) and may have direct cytoprotective actions (Vane, 1983). Thromboxane A2 is also produced from prostaglandin H2, but induces both vasoconstriction and platelet aggregation (Mayes, 1992). Hence, when the action of prostacyclin synthase is hindered, diseases such as hypertension, thrombosis and embolism may result, and thus the prostacyclin synthase gene may represent a disease susceptibility gene. Prostacyclin synthase is widely distributed in the organism. It is a membrane-associated P450-like enzyme with a molecular mass of 52 kDa. Like many others P450 molecules, prostacyclin synthase binds not only with its substrate, prostaglandin H2, but also with heme molecules (Nelson *et al.*, 1996). Miyata *et al.* (1994) reported the DNA copy sequence of human prostacyclin synthase, and Nakayama *et al.* (1996) clarified the genomic structure. The genetic locus of prostacyclin synthase is 20q13.11-13 (NCBI Gene ID:5740). A single copy gene contains 10 exons and is approximately 70 kbp in length. The size of the exons ranges from 74 bp (ATG-exon 1) to 182 bp (exons 6 and 8) and the size of the introns ranges from approximately 1 kb (intron 7) to 19 kb (intron 1). In the core promoter region, 9 bp repeated sequences exist just upstream of the initiation codon, and transcription factors bind in tandem, as variable number tandem repeats (VNTR). A VNTR is a short nucleotide sequence ranging from 14 to 100 nucleotides in length. It is or-

ganized into clusters of tandem repeats, usually repeated in the range of between 4 and 40 times per occurrence. Clusters of such repeats are scattered on many chromosomes. Each variant is an allele and they are inherited codominantly. The number of times that a sequence is repeated varies between different individuals and between maternal and paternal loci of an individual. The likelihood of two individuals having the same band pattern is extremely improbable. The number of repeats will determine the length of the fragment of DNA. The repeat sequence itself can be used as a probe, or if the repeat is not long enough, a sequence from the upstream or downstream side can be used. There are two principal families of VNTR: mini- and microsatellites. The former are sequences of 11-16 bp repeated 1000 times. They are important because they are highly repetitive and dispersed into the genome. The other members of the VNTR family are the microsatellites or short tandem repeats. They are represented by short sequences of 100-200 bp given by the repetition of 1-6 bp sequences (Claros *et al.*, 2004).

Expression of prostacyclin synthase is high in heart, lung, smooth muscle, kidney and ovary, moderate in brain, pancreas and prostate, and low in placenta, spleen and leukocytes (Jeremy *et al.*, 1985). A previous study, using prostacyclin synthase knockout mice, found that the complete loss of prostacyclin synthase (-/- type) caused hypertension, fibrosis and vascular injury in atrophic kidneys, and increased levels of blood urea, nitrogen and creatinine (Yokoyama *et al.*, 2002). The locus of *Rattus norvegicus* prostacyclin synthase is 3q42 (NCBI Gene ID:25527). Genetic mutations that bring about changes in amino acid residues could therefore affect enzymatic activity. Poole *et al.* (2007) suggested that individuals with fewer prostacyclin synthase repeats would be at increased risk of colorectal polyps. Small number repeats allele was found more frequently associated to cerebral infarction and transcriptional activity increases with increasing numbers of repeats (Nakayama *et al.*, 2000; Nakayama, 2005). On the other hand, this polymorphism did not seem to be associated with the development of chronic thromboembolic pulmonary hypertension and essential hypertension (Amano *et al.*, 2004; Nakayama *et al.*, 2001).

The aim of this study was to identify the VNTR in the promoter region of prostacyclin synthase gene and verify if there exists a relationship between the VNTR and those rats fed on the choline deficient diet which die as a consequence of acute renal failure and those which do not.

Material and Methods

Animals

This study was performed on ten buffered formalin fixed, paraffin embedded renal tissue samples from a previous study (experiment 1, group 1; Courrèges *et al.*, 2002). They were from Sprague-Dawley weanling male rats (21-23 days) which were fed *ad libitum* on a choline-deficient diet with hydrogenated vegetable oil and corn oil as lipids (Table 1). Some rats were kept on this diet until they died from acute renal failure (group 1, $n=6$) or were sacrificed on day 21 of choline-deficient diet feeding (group 2, $n=4$). Both body and kidneys weights were recorded for each animal (Courrèges *et al.*, 2002). Tissue samples were fixed in buffered formalin and embedded in paraffin.

TABLE 1.

Choline-deficient diet composition (g/100 g)

Components	Diet (%)
Soybean protein ¹	20.0
Hydrogenated vegetable oil ²	14.3
Corn oil ³	5.7
Sucrose	49.5
Cellulose ⁴	4.0
Vitamin mixture (without choline) ⁵	4.0
Salts ⁶	2.0
L-cystine ⁷	0.5
Choline chloride	0.00

¹Grade II, Biochemical Corporation, U.S.; ²Flora Dánica, Buenos Aires, Argentina; ³≈Mazola, Refinerías de Maíz, Buenos Aires, Argentina; ⁴Celufil, non-nutritive bulk, Biochemical Corporation, U.S.; ⁵Vitamin Diet Fortification Mixture, Biochemical Corporation, U.S.; ⁶Salt Mixture, Wesson Modification, Biochemical Corporation, U.S.; ⁷Biochemical Corporation, U.S.

Histopathology

Both kidneys were removed and weighed; they were fixed in buffered-formalin and embedded in paraffin. Four to five μm sections were stained with hematoxylin and eosin to analyze histopathological al-

terations, which were classified according to Monserrat *et al.* (1981) into (grade 0), no necrosis; (grades 1 to 4), tubular necrosis; and (grades 5 to 8), cortical necrosis. Tissue repair was also observed.

Urea measurement

Blood was drawn from the abdominal aorta under ether anesthesia. Urea was measured in serum by the urease method (Wiener Lab, Buenos Aires, Argentina).

VNTR detection in purified DNA from 7 μm sections of paraffin-embedded kidneys.

Sections were cleansed with 100% xylene-ethanol (Carlo Erba, Italy) and digested with proteinase K (100mM Tris-HCl, pH=8, 25mM EDTA, 0.5% SDS, 0.01% proteinase K) at 42°C overnight. Lipoprotein fractions were removed with phenol-chloroform-isoamyllic acid (Carlo Erba, Italy). Purified DNA was precipitated with NaCl/isopropanol and reimmersed in $T_{10}E_1$ (Tris-EDTA). The presence of the VNTR in the promoter region of prostacyclin synthase gene was analyzed with a semi-quantitative PCR technique. DNA purity and yield were measured by spectrophotometry, and 200 ng of DNA were used for PCR. Reaction volume was 25 μL . The PCR mixture contained 50 pmol of each primer, and a final concentration of 100 μM of each deoxyribonucleoside triphosphate, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM MgCl_2 , and 1 unit of Taq DNA Polymerase (Promega Corporation, Madison, WI, USA). PCR was performed in a Triothermoblock cyler (Biometra, Göttingen, Germany) under the following conditions: 2 min at 95°C, for 35 cycles: 30 s at 95°C, 60 s at 68°C, 60 s at 72°C, and a final 10-min extension at 72°C. A new pair of primers homologous to the region flanking possible VNTR in the promoter prostacyclin synthase rat gene was designed with a computational primer design tool (Primer3 Output). These primers amplified a 220-320 bp fragment. The oligonucleotide sequence was PGIS F (5'-CGCCTCCCCAATCCCCTCTC-3') y PGIS R (5'-CGGCTCAGCAGCAGCAGCAA-3'). PCR products were analyzed by electrophoresis on a 9% polyacrylamide gel in Tris-boric-EDTA 1X buffer, visualized with ethidium bromide under ultraviolet light and photographed. A 100 bp ladder (Promega Corporation) was used as a size reference marker.

Results

Initial body weight was 41.9 ± 1.0 g ($n=10$, mean \pm SEM). By the fifth day of feeding on the deficient diet body weight was 57.3 ± 0.7 g ($n=10$). Final body weight was 49.3 ± 2.1 g ($n=6$) in group 1 (those which died) and 98.3 ± 4.0 g ($n=4$) in group 2 (surviving animals). Both renal absolute weight and renal weight relative to body weight, as well as serum urea concentrations (only from group 2) are shown in Table 2. Urea levels in serum were markedly high in the animals which survived (group 2). Relative renal weight was significantly higher in group 1 than in group 2 ($P < 0.05$, Student's *t* test) but absolute weights were similar in both groups (Table 2). Survival of rats in group 1 was 9.2 ± 0.2 days.

TABLE 2.

Absolute (g) and relative renal weight (g/100 g body weight) and serum urea concentration (mg/dL) in rats which developed acute renal failure and died (group 1) and those which do not (and were sacrificed on day 21 of choline-deficient feeding; group 2).

	<i>n</i>	Absolute renal weight	Relative renal weight	Urea
Group 1	6	1.981 ± 0.156	4.004 ± 0.232	*
Group 2	4	1.910 ± 0.088	1.957 ± 0.145	99 ± 11

Results are expressed as mean \pm SEM. *Serum urea could not be determined in animals which died.

Rats in group 1 showed tubular ($n=2$) or cortical ($n=4$) necrosis, while rats in group 2 showed reparative changes ($n=4$).

The PCR-amplified product showed three different weight bands in a 9% polyacrilamide gel, which run between 220 and 320 bp standards. Both alleles were paired in differently in each rat. We did not find differences in the molecular weight of the alleles between groups 1 and 2. Control results were as expected.

Discussion

We investigated the possible association of a VNTR polymorphism of the prostacyclin synthase gene with the development of acute kidney injury. We found that

the overall distribution of allele frequencies was not significantly different between animals which died due to acute kidney failure and those which did not.

Research priorities in critical care are increasingly focusing on long-term outcomes and prognosis for survivors of critical illness. Few studies have described the long-term outcomes after acute renal failure. The long-term survival after acute kidney injury is poor, variable and range from 46 to 74%, 55 to 73%, 57 to 65% and 65 to 70% at 90 days, 6 months, 1 year and 5 years, respectively. Further research is needed to explore the relationship between survival, markers of morbidity and costs after acute kidney injury (Bagshaw, 2006). The treatment of critically ill patients with acute kidney injury has changed dramatically over the last 30 years. As treatment has increased in both sophistication and efficacy, so has the severity of the condition (Bellomo, 2006). Despite technical progress in the management of acute kidney injury over the last 50 years, mortality rates seem to have remained unchanged at around 50% (Ympa *et al.*, 2005). Acute kidney injury is present in 1 to 5 percent of patients at hospital admission and affects up to 20 percent of patients in intensive care units (Needham, 2005). All the data previously mentioned emphasizes the necessity of new models of acute kidney injury.

The pathogenesis of the acute kidney injury due to choline deficiency is controversial. As it has been mentioned above, the ischemic mechanism as well as a primary tubular damage has been proposed. Common genetic polymorphisms are associated with changes in the susceptibility to choline deficiency (Da Costa *et al.*, 2006). The rat is a useful experimental model for human acute kidney injury (Hammerman, 2000).

Several recent reports documented that VNTR polymorphisms located in the promoter region influence the transcriptional activity, resulting in phenotypes with functional differences (Tovar *et al.*, 2003; Chevalier *et al.*, 2001). We described that the VNTR repeats upstream of the insulin gene is a susceptibility locus for latent autoimmune diabetes in adults (Cerrone *et al.*, 2001). Although the biological mechanism(s) behind these polymorphisms are poorly understood, a defined length for the regulatory region seems to be important for optimal gene transcription (Sabol *et al.*, 1998). We considered that acute kidney injury due to choline deficiency was an adequate experimental model to study its pathophysiology.

As arachidonic acid derivatives are involved in the regulation of vascular tonus and play an important role in renal physiology and pathology, we hypothesized that it would exist a possible relationship between this bio-

logical phenomenon and the genetic structure of one of the molecular candidates in terms of differential expression levels of some proteins in the animals which died of acute renal failure and those which recovered.

There are many studies that described a relationship between the number of tandem repetitions in the promoter region of human prostacyclin synthase gene and different pathologies. There is no evidence of the VNTR region in the promoter region in the prostacyclin synthase rat gene, although there is a great homology between human and rat. The coding region of the rat prostacyclin synthase DNA copy shows 82.5% homology with the human DNA copy (Miyata *et al.*, 1994). Besides the VNTR of prostacyclin synthase promoter gene was hard to describe in human pathologies, and the high homology that exists between rat and human, we could not use the human primers described in the literature. Then, we had to design the primers for the prostacyclin synthase gene promoter region to be used in the rat, flanking the possible VNTR sequence, and we then analysed a cohort of rats by using PCR with primers which encompass the VNTR region. Genotyping of 10 unrelated rats revealed three PCR products that differed in size.

In conclusion, the existence of VNTR sequence was established for the rat prostacyclin synthase gene promoter region, but the reparation of the acute kidney injury which occurs in some choline deficient rats is not associated with differences in VNTR in the promoter region of this gene.

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