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A Preliminary Study on T-786C Endothelial Nitric Oxide Synthase Gene and Renal Hemodynamic and Blood Pressure Responses to Dietary Sodium

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Summary

The purpose of the present study was to examine the role of the T-786C endothelial nitric oxide synthase (eNOS) gene polymorphism on changes in renal hemodynamics and blood pressure due to Na^+ loading. Twenty-eight older (63±1 years), moderately obese (39±2 % fat) hypertensives had their glomerular filtration rate (GFR), renal plasma flow (RPF), blood pressure (BP) and plasma nitric oxide (NO_x) levels determined after eight days of low (20 mEq) and high (200 mEq) Na^+ diets. The two Na^+ diets were separated by a 1-week washout period. Subjects were genotyped for the eNOS-786 site and were grouped on whether they were homozygous or heterozygous for the C allele (TC+CC, n=13) or only homozygous for the T allele (TT, n=15). The TC+CC genotype group had a significantly greater increase in diastolic (P=0.021) and mean arterial (P=0.018) BP and a significant decline in both RPF (P=0.007) and GFR (P=0.029) compared to the TT genotype group with Na^+ loading. Furthermore, Na^+ loading resulted in a significant (P=0.036) increase in plasma NO_x in the TT, but not in the TC+CC genotype group as well as a trend (P=0.051) for an increase in urine NO_x in TC+CC, but not in the TT genotype group. The increase in BP during Na^+ loading in older hypertensives was associated with the eNOS genotype and may be related to changes in renal hemodynamics due to changes in NO metabolism.

Key words
Aging • Glomerular filtration rate • Renal plasma flow

Introduction

A number of endogenous substances have been shown to produce changes in renal hemodynamics. Many of these substances exert their vasodilatory action through endothelial-derived nitric oxide (NO_X). It has been

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hypothesized that impairments in endothelial NO generation may be influenced by gene polymorphisms, which ultimately may result in impaired renal hemodynamics (Noiri *et al.* 2002). The endothelial nitric oxide synthase (eNOS) gene consists of 26 exons and 25 introns located on the long arm of chromosome 7 at 7q35→36 (Marsden *et al.* 1993). In the promoter (5′ flanking region), a single nucleotide polymorphism with a T to C substitution occurring at nucleotide position -786 (T-786C) has been identified (Karantzoulis-Fegaras *et al.* 1999, Wang and Wang 2000). Recently, *in vitro* studies have shown the C allele to have significantly lower promoter activity compared to the T allele in a luciferase-based transcription analysis (Nakayama *et al.* 1999). The C allele influences eNOS transcription, which is consistent with reduced NO production. Therefore, it is possible that the eNOS gene is involved in the regulation of NO in the kidney and may play a role in the variable response of blood pressure and renal hemodynamics to sodium (Na^+) intake in humans.

In humans, there is a great variation in both blood pressure and renal hemodynamic responses to changes in dietary Na^+ intake, even when the dietary Na^+ intake is standardized (Cooperative Research Group 1988). Previously, we reported that Na^+-sensitive hypertensive individuals have an increase in glomerular filtration rate (GFR) with an increase in dietary Na^+ intake. However, in Na^+-resistant individuals, an increase in dietary Na^+ did not result in any changes in renal hemodynamics (Weir *et al.* 1995). We undertook the present study to determine if the variability in renal hemodynamics induced by dietary Na^+ in older hypertensive individuals was related to the eNOS T-786C gene polymorphism. Given that the C allele has been associated with lower transcription rates, and presumably with a reduction in NO production, we hypothesized that Na^+ loading would result in a greater increase of blood pressure in these individuals. In addition, we hypothesized that renal hemodynamics might contribute to the differential blood pressure response to Na^+ loading.

Methods

Study population

Twenty-eight older (63±1 years) subjects (10 males and 18 females) with mild hypertension were recruited for this study. Subjects were recruited through a newspaper advertisement, from the University of Michigan Turner Geriatric Clinic, and from the Human Subjects Core of the University of Michigan Geriatrics Center. All subjects were community dwelling and in good health apart from their hypertension.

Subjects were screened prior to participation with a medical history and physical examination, a complete blood count, and routine blood chemistries, and a urinalysis. Individuals were excluded from the study if they had clinically significant concomitant medical illness such as cardiac, renal (serum creatinine greater than 135 mmol/l), hepatic or gastrointestinal disease, or required medications that might affect glucose metabolism or renal function. Individuals with a recent history of smoking or drug or alcohol abuse, or clinically relevant mental disorders were also excluded. Absence of diabetes mellitus was confirmed in all subjects by a 2-hour 75 g oral glucose tolerance test (American Diabetes Association 1997). Hypertension was defined as a seated systolic blood pressure $\geq$140 mm Hg and/or a seated diastolic blood pressure $\geq$90 mm Hg (Chobanian *et al.* 2003).

General study protocol

At the initial screening visit to determine their eligibility for participation as described above, subjects signed an informed consent form. All studies were performed according to the *Declaration of Helsinki*, and had been approved by University of Michigan Institutional Review Board. Hypertensive subjects who were being treated with antihypertensive medications were tapered off their medications and were studied following a 4-week period during which no anti-hypertensive medications were taken. During the tapering period, subjects were given a blood pressure unit to monitor their blood pressure on a daily basis. In addition, patients had their blood pressure taken weekly, by the research staff.

Subjects were randomized in a double-blind design to begin either a 20 or 200 mmol/l/day Na^+ diet, which they consumed over an 8-day period. All meals during the 8-day Na^+ diet period were prepared by the General Clinical Research Center Metabolic Kitchen at the University of Michigan. The two diets were identical in composition except for the Na^+ content and consisted of 50-55 % calories as carbohydrate, 30-35 % as fat, 15-20 % as protein and 300 to 350 mg per day of cholesterol. After completion of the first 8-day Na^+ diet and the associated metabolic and renal tests, the subjects consumed their own diet for a one week washout period and then were switched to the alternative Na^+ diet, which they consumed for a second 8-day period. Compliance with the diet was monitored by 24 h urine collections for Na^+.

Measurement of body composition

The waist-to-hip circumference ratio (WHR) was calculated as the ratio of the minimal circumference of the abdomen to the circumference of the buttocks at the maximal gluteal protuberance. Body fat, lean body mass (LBM) and percent body fat were determined by dual energy x-ray absorptiometry (DXA, Model DPX-IQ Lunar Radiation Corporation, Madison, WI).

Measurement of blood pressure

On the eighth day of each Na^+ diet, blood pressure measurements were made while the subject rested in the seated position, following a 20 min resting period. Systolic, diastolic and mean arterial blood pressure (MABP) were continuously monitored for a 30-min period using Ohmeda 2300 Finapress blood pressure monitor.

Measurement of renal hemodynamics

Glomerular filtration rate (GFR) and renal plasma flow (RPF) were measured by the clearance of ^{99m}Tc-DTPA (200 μCi) and ^{131}I-hippuran (60 μCi), respectively. Briefly, upon arrival at the General Clinical Research Center patients consumed 950 ml of water to establish brisk urine flow after which the subjects were asked to void and resting urine and blood sample were obtained. Following the collection of the resting urine and blood sample an intravenous bolus injection of 100 μCi of ^{99m}Tc-DTPA and 60 μCi of ^{131}I-hippuran was then given and after 60 min, the patient's bladder was emptied, blood samples were withdrawn, and three timed sequential 1-hour urine collections were obtained, after which additional blood samples were withdrawn (Klassen *et al.* 1992). The ^{99m}Tc-DTPA and ^{131}I-hippuran activity in the samples was determined by liquid scintillation counting. Urinary clearances of ^{99m}Tc-DTPA were calculated for each 1-hour collection period as urine activity times urine flow rate divided by average plasma activity. Average plasma activity was calculated as the mean of the plasma values over the interval from the beginning to the end of each urinary collection. The GFR was expressed as the average of the three 1-hour collection values (Klassen *et al.* 1992). RPF was determined by measuring the disappearance from the serum of 60 μCi of ^{131}I-hippuran at precisely 44 min after injection as previously described (Tauxe *et al.* 1971). The filtration fraction was calculated by dividing GFR by RPF.

Measurement of plasma and urinary values

Prior to the determination of renal hemodynamics on each Na^+ diet blood sample were collected into chilled glass tubes containing heparin sodium, stored on ice, and separated immediately after each study. Plasma and urine samples for aldosterone, renin, creatinine, urea, potassium, protein, chloride, and sodium were measured in the University of Michigan Medical Center Laboratory. Blood samples for measuring NO_X were drawn directly into a vacuum tubes containing 0.01 % EDTA anticoagulant. Immediately after blood collection, these blood samples were stored temporarily on ice and then centrifuged at 3000 rpm for 20 min at 4 °C. Plasma isolated from these samples for the NO_X assay was placed into 1 ml microtubes and frozen at -80 °C until analyzed. Before assaying, plasma samples were ultrafiltered to remove protein with single-use filters (Ultrafree-MC Centrifugal Filter, Millipore, Bedford, MA). To eliminate inter-assay variation, the NO_X assay of the baseline and final plasma samples were performed in the same assay at the end of the study. Since *in vivo* NO is rapidly oxidized to the stable end products, $NO_2^- + NO_3^-$, reported values will represent the total amount of stable plasma NO_X as measured by a colormetric assay based on the Griess reaction as previously described (Brown *et al.* 2000).

eNOS genotyping

Genomic DNA was extracted from leukocytes of the blood sample utilizing a PureGene kit (Gentra Systems, Minneapolis, MN). Subjects were genotyped for the eNOS –786 site using polymerase chain reaction amplification with flanking primers F: 5′- CACCCAGGC CCACCCCAACT-3′ and R: 5′-GCCGCAGGTCGAC AGAGA GACT-3′. DNA was denatured for 5 min at 95 °C followed by 35 cycles of denaturation (30 s, 95 °C), annealing (15 s, 54 °C) and extension (30 s, 72 °C). The amplicon was digested overnight at 37 °C using 5 units of MspI followed by electrophoresis for 4 h in a gel composed of 2 % agarose + 1 % Nusieve (FMC, Inc.). The T allele yields one fragment of 415 bp, and the C allele yields two fragments of 370 bp and 45 bp. Subjects were classified into two different groups based upon whether they were homozygous or heterozygous for the C allele (TC+CC, n=13) or only homozygous for the T allele (TT, n=15).

Table 1. Physical characteristics of older hypertensives by T-786C polymorphism (TT, TC+CC) in the 5′-flanking region of the eNOS gene

	TC+CC	TT	P value
Number	13	15	
Gender (M/F)	4/9	6/9	0.611
Ethnicity	2/11	3/12	0.750
Age (years)	63.7±2.0	62.5±2.0	0.676
Height (cm)	165.1±3.2	170.0±1.9	0.187
Weight (kg)	80.2±4.5	82.0±3.7	0.752
Body fat (%)	39.2±2.3	38.2±2.3	0.751
Waist:hip ratio	0.86±0.02	0.87±0.02	0.704

Data are mean ± S.E.M. Ethnicity (African-American/Caucasian)

Statistical analysis

Data were analyzed using Statview (Abacus Concepts, Inc., Berkeley, CA). An alpha level of 0.05 was accepted for statistical significance. Comparisons of the physical characteristics of the two eNOS groups were made using analysis of variance (ANOVA). A two-way repeated measures ANOVA with eNOS genotype group (TT and TC+CC) as one variable and diet (low Na^+ and high Na^+) as the other was utilized to examine within and between group differences. All data are reported as means ± S.E.M.

Results

Twenty-eight older (63.0±1.4 years), moderately overweight (38.7±1.6 % fat) subjects with essential hypertension were studied (Table 1). When these subjects were divided into groups based upon the T-786C polymorphism (TT, TC, CC), 15 individuals were TT and 12 individuals were TC. One subject (3 %) was a CC homozygote, which agrees with the reported frequency of the CC genotype (Yoshimura *et al.* 2000). Based on available data, the frequency of the CC genotype in the population is between 0.02 and 0.10. Thus, we did not have adequate statistical power to include a separate CC genotype group. Furthermore, the C allele is considered to have deleterious cardiovascular effects, with CC and TC genotype group demonstrating similar responses that are different from the group with the TT genotype (Nakayama *et al.* 1999, Zanchi *et al.* 2000, Rossi *et al.* 2003). Thus, it appears appropriate from a mechanistic perspective to group the C allele carriers into a combined TC+CC genotype group. Therefore, in the present study, subjects were categorized into a combined TC+CC genotype group and compared to the TT genotype group. The distribution of eNOS genotypes in this group of older hypertensive individuals was 54 % TT, and 46 % TC+CC, which is similar to the distribution in the general population (Zanchi *et al.* 2000, Rossi *et al.* 2003). There were no statistically significant differences in age, weight, body mass index or percentage body fat between the two eNOS genotype groups (Table 1).

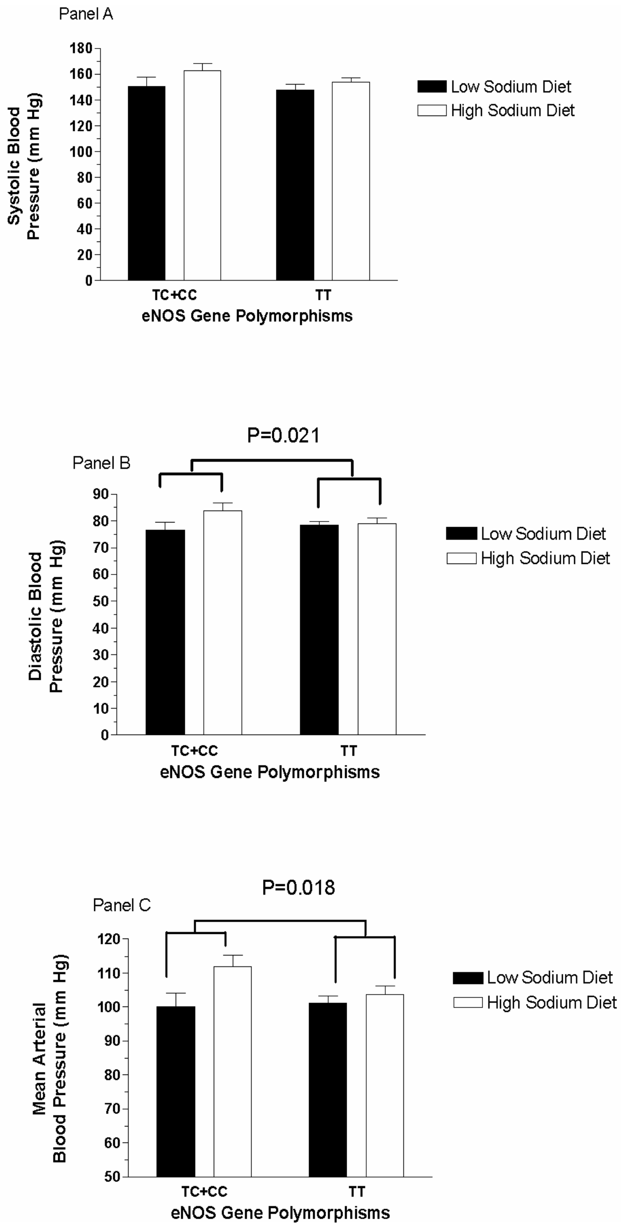

Fig. 1. Mean arterial (Panel A), systolic (Panel B) and diastolic (Panel C) blood pressure on low (20 mEq/day) (open bar) and high (200 mEq/day) (solid bar) sodium (Na^+) intake in TC+CC (n=13) and TT (n=15) eNOS gene polymorphisms.

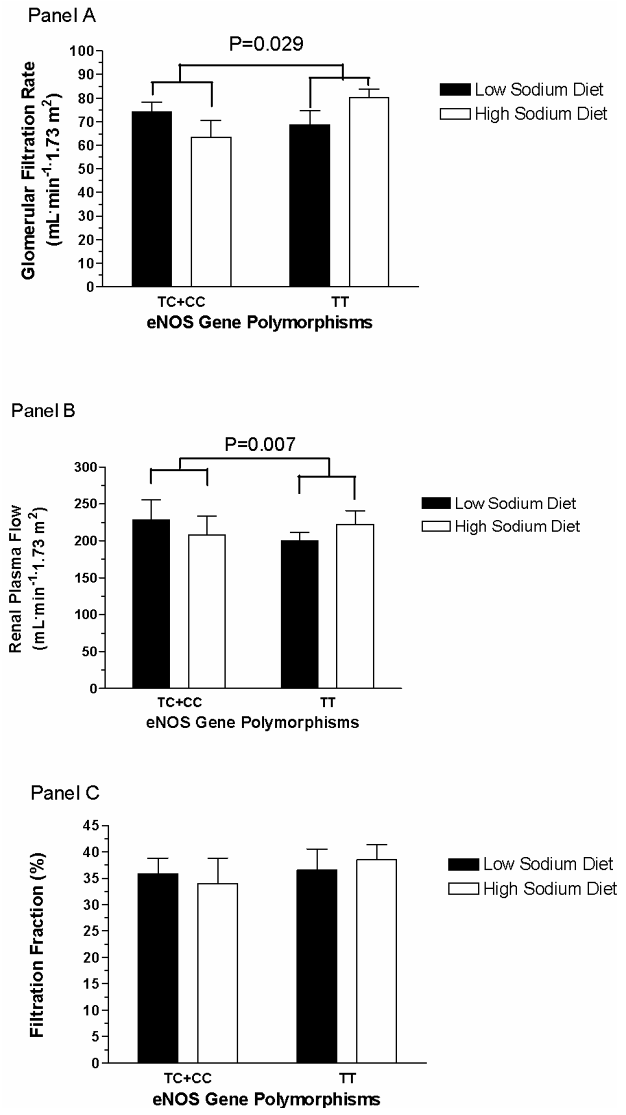

Fig. 2. Glomerular filtration rate (Panel A), renal plasma flow (Panel B) and filtration fraction (Panel C) in individuals with the TC+CC (n=13) and TT (n=15) eNOS gene polymorphisms on low (20 mEq/day) (open bar) and high (200 mEq/day) (solid bar) sodium (Na^+) intake.

Blood pressure

Although there was no significant (P=0.411) interaction of the eNOS gene polymorphism and dietary Na^+ on systolic blood pressure (Figure 1, Panel A) there was a significant (P=0.021) interaction of the eNOS gene polymorphism and dietary Na^+ on diastolic blood pressure (Figure 1, Panel B), indicating that TC+CC genotype individuals increased their diastolic blood pressure in response to Na^+ loading, while the TT genotype individuals did not. Similarly, there was a significant (P=0.018) interaction of the eNOS gene polymorphism and dietary Na^+ on mean arterial blood pressure (Figure 1, Panel C), indicating that the TC+CC genotype individuals increased their mean arterial blood pressure in response to Na^+ loading, while the TT genotype individuals did not.

Renal hemodynamics

There was a significant interaction between eNOS gene polymorphism and dietary Na^+ on the glomerular filtration rate (P=0.029) (Fig. 2A), demonstrating an increase in GFR with Na^+ loading in TT genotype individuals, while GFR decreased with Na^+ loading in TC+CC genotype individuals. Similarly, there was a significant (P=0.007) interaction between dietary Na^+ and eNOS polymorphism on renal plasma flow indicating that the TC+CC genotype individuals reduced their RPF in response to Na^+ loading, while the TT genotype individuals increased their RPF in response to Na^+ loading (Fig. 2B). There was no interaction between dietary Na^+ and eNOS gene polymorphism on filtration fraction (P=0.493) (Fig. 2C). Although Na^+ clearance was not significantly different between TT and TC+CC genotype groups, as expected there was a significant (P<0.0001) increase in both groups in response to Na^+ loading (Table 2).

Plasma and urine values

The mean plasma Na^+ levels were significantly (P=0.027) higher during the high Na^+ diet in both groups (Table 2). In addition, the increase in dietary Na^+ resulted in a significant decrease in plasma levels of urea, creatinine and aldosterone in both groups (Table 2). There was a trend (P=0.072) for the TC+CC genotype group to have higher plasma renin levels than the TT genotype group on both high and low Na^+ diets (Table 2).

As expected, the mean 24 h urinary Na^+ excretion was significantly (P<0.0001) higher during the high Na^+ diet in both groups (Table 2). There was also a significant (P<0.0001) increase in urinary volume in both genotype groups with the increase in dietary Na^+ (Table 2). There was a significant interaction between eNOS genotype and dietary Na^+ intake in those individuals with the TC+CC genotype increasing their urinary output to a greater degree than those individuals with the TT genotype. There was no change in urinary creatinine or protein levels with the change in dietary Na^+ (Table 2). There was no main effect of eNOS genotype or dietary Na^+ on plasma or urinary creatinine levels (Table 2).

There was a significant interaction for dietary Na^+ and the eNOS gene polymorphism on plasma NO_X levels (Table 2). Following the high Na^+ diet plasma,

Table 2. Plasma and urinary values in older hypertensives on low and high sodium (Na$^+$) diets by T-786C polymorphism (TT, TC+CC) in the 5′-flanking region of the eNOS gene.

	TC+CC (n=13)		**TT (n=15)**		**P-Value**		
	Low Na$^+$	**High Na$^+$**	**Low Na$^+$**	**High Na$^+$**	**Group Effect**	**Diet Effect**	**Interaction Effect**
Plasma Variables							
Sodium (mmol/l)	138.0±0.5	138.5±0.3	138.5±0.4	139.5±0.5	0.205	0.027	0.422
Urea (mmol/l)	5.3±0.2	4.5±0.2	5.6±0.4	4.5±0.2	0.838	<0.0001	0.551
Creatinine (µmol/l)	84.9±3.5	79.6±4.4	83.1±4.4	79.6±4.4	0.877	0.001	0.423
Aldosterone (pmol/l)	543.7±91.5	174.8±38.8	502.1±83.2	202.5±36.0	0.927	<0.0001	0.614
Renin (ng/s)	1.16±0.22	0.39±0.28	2.71±0.08	0.20±0.03	0.072	<0.0001	0.425
Plasma NO$_x$ (µmol/l)	18.5±2.8	17.2±3.5	17.7±3.6	23.6±4.7	0.578	0.158	0.036
Urine Variables							
Volume (ml/day)	1785±158	2262±180	1888±166	2038±124	0.779	<0.0001	0.014
Sodium (mmol/day)	30.5±3.3	197.0±14.3	42.3±11.6	185.5±6.8	0.988	<0.0001	0.215
Creatinine (mmol/day)	12.0±0.9	12.1±0.9	11.3±0.7	11.7±0.7	0.605	0.593	0.717
Protein (g/l)	0.05±0.01	0.05±0.01	0.03±0.01	0.04±0.01	0.418	0.570	0.417
Urine NO$_x$ (µmol/l)	20.4±4.2	28.6±5.3	27.6±5.6	24.5±7.0	0.838	0.359	0.051

Values are means ± S.E.M.

NO$_X$ levels significantly (P=0.036) increased in the TT genotype group compared with the TC+CC genotype group (5.0±2.3 vs. –1.3±2.1 µmol/l, respectively). There was a significant interaction between dietary Na$^+$ and the eNOS gene polymorphism on urine NO$_X$ levels (Table 2). There was a trend (P=0.051) for the TC+CC genotype group to increase their urine NO$_X$ levels in response to Na$^+$ loading while the TT genotype group maintained urine NO$_X$ levels during Na$^+$ loading (Table 2).

Discussion

The present study demonstrates an association between the T-786C polymorphism in the eNOS gene promoter and changes in renal hemodynamics and mean arterial blood pressure in response to dietary Na$^+$ loading in older hypertensives. Individuals homozygous for the T allele had no significant increase in their MABP in response to the increase in dietary Na$^+$; however, individuals with the C allele had a significant increase in MABP with Na$^+$ loading. One possible explanation for the elevation of MABP with the increase in dietary Na$^+$ in individuals with the C allele may be the abnormal response in GFR with Na$^+$ loading. In individuals homozygous for the T allele, the increase of dietary Na$^+$ resulted in an enhanced GFR. However, in individuals with the C allele, the increase in dietary Na^+ resulted in a decline in GFR. Typically, higher dietary Na^+ results in an increase in GFR (Laragh and Sealey 1992). This change in GFR may be mediated in part by changes in renal renin secretion and thus angiotensin II formation (Laragh and Sealey 1992). Normally an increase in dietary Na^+ intake depresses renal renin secretion while a reduction in dietary Na^+ intake increases renal renin secretion (Sealey *et al.* 1972). Although individuals homozygous for the T allele and individuals with the C allele in the present study both demonstrated a reduction in plasma renin levels with Na^+ loading. However, there was a trend for those individuals homozygous for the T allele to have higher renin levels during the low Na^+ diet and lower renin levels during Na^+ loading than those with the C allele. Therefore, those individuals homozygous for the T allele demonstrated a greater overall change in renal renin secretion with the change in dietary Na^+, which may explain the ability of this group to preserve blood pressure in response to an increase in dietary Na^+ intake.

The mechanism of this abnormal response in RPF and GFR is not known, but it is possible that endothelial-derived NO plays a role. Nitric oxide exerts a powerful influence on the regulation of RPF and Na^+ excretion. NO regulates the glomerular microcirculation by modulating afferent blood vessels (Wang and Wang 2000). It relaxes mesangial cells and contributes to the regulation of renal Na^+ excretion and the release of renin (Wang and Wang 2000). Of all the tissues that are sensitive to the effects of NO, the renal vasculature appears to be the most sensitive (Majid *et al.* 1999, Zou and Cowley 1997, Zuckerman *et al.* 1997). In the present study, individuals with the T allele had a significant increase in plasma levels of NO_X with the increase in dietary Na^+, while those individuals with the C allele had little or no change in plasma NO_X levels. The elevated plasma NO_X level in individuals with the T allele may be indicative of an increase in systemic and/or renal NO_X production. This increased NO_X production could result in vasodilation of the vascular beds resulting in the increase of RPF and GFR and an overall maintenance of blood pressure during Na^+ loading. Alteration in the NO_X system may lead to impairment in renal hemodynamics and reduced Na^+ excretion during elevation in dietary Na^+ resulting in an elevation in blood pressure. Shultz and Tolins (1993) demonstrated that the NO system directly modulates renal hemodynamics and Na^+ excretion in Sprague-Dawley rats. These investigators measured GFR, RPF, and urinary Na^+ excretion during NO synthase inhibition. In a dose-dependent fashion, NO synthase inhibition resulted in renal vasoconstriction, and reduced GFR and RPF. Shultz and Tolins (1993) found that Na^+ loading significantly increased total serum and urinary NO_X excretion suggesting that Na^+ loading increases NO_X production. Other studies have shown a direct relationship between changes in renal arterial pressure and renal NO_X production and that these changes paralleled those in urinary Na^+ excretion (Hu and Manning 1995, Majid *et al.* 1993, Majid and Navar 1997). Recently, Majid *et al.* (1999) found that dose-dependent changes in renal arterial pressure were correlated with changes in renal cortex NO_X production and in urinary excretion of Na^+ and total nitrates.

Tracer studies in humans have demonstrated that 50 % of systemic nitrates originate from the NO_X synthesis substrate, L-arginine (Castillo *et al.* 1993, Rhodes *et al.* 1995) and that NO_X synthase inhibition induces a pronounced reduction of urinary nitrates (Boger *et al.* 1996). Although it is clear that nitrate is synthesized by mammalian endothelial cells as a result of eNOS activity, increasing dietary nitrate intake also increases urinary nitrate excretion (Granger *et al.* 1991). However, in the present study the participants were on a stable diet for 8 days before plasma NO_X samples were drawn. Baylis *et al.* (1998) have demonstrated that if samples are collected after a stable period of fasting (>10 h) and during a controlled nitrate diet, plasma and urine levels of nitrates provide an estimate of total body NO_X generation, but not an estimation of biologically active NO_X.

Although this is a preliminary study with a small number, it may be that the increase in MABP during Na^+ loading in the TC+CC group may be related to impaired NO production and a fall in RPF and GFR. It is more than likely that multiple genes influence the renal hemodynamics response to dietary Na^+. Thus, the influence of any single gene on renal hemodynamic responses during alterations in dietary Na^+ is likely to be small. However, the eNOS T-786C gene polymorphism is a particularly attractive candidate because it has been shown to alter gene promoter and transcriptional activities. Future studies will be needed to examine the mechanisms that contribute to the observed interaction between changes in dietary Na^+ induced alterations in renal hemodynamics and the eNOS genotype.

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