

Angiotensin II Type 1 Receptor Gene Polymorphism and the Response to Hyperglycemia in Early Type 1 Diabetes

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Recent studies suggest that there is an association between the A¹¹⁶⁶→C polymorphism of the angiotensin II type 1 receptor (AGT₁R), glycemic control, and the risk of diabetic nephropathy in subjects with type 1 diabetes. Because hypertension and renal hemodynamic function are also related to the risk of diabetic nephropathy and because hyperglycemia can activate the renin angiotensin system, we sought to determine if there is an association between the AGT₁R polymorphism, baseline renal and peripheral hemodynamic function, and pressor response to high glucose in subjects with early uncomplicated type 1 diabetes. There were 39 diabetic subjects genotyped for the AGT₁R polymorphism by polymerase chain reaction and segregated into 2 groups: those with and those without the C1166 allele (AA and AC/CC). The average age was 27 ± 1 years, and the mean duration of diabetes was 3.5 ± 0.6 years. HbA_{1c} values were <10% in all subjects and were similar in the 2 groups (8.2 ± 0.3 vs. 9.1 ± 0.4%). After a 7-day controlled diet (150 mmol sodium, 1.5–2.0 g · kg⁻¹ · day⁻¹ protein), renal hemodynamic function was assessed by inulin and para-aminohippurate clearance during clamped euglycemic conditions (4–6 mmol/l). Mean values for glomerular filtration rates did not differ between groups during euglycemia. In contrast, mean values for renal plasma flow and renal blood flow were significantly greater in the AC/CC group compared with the AA group. Values for mean arterial pressure were similar in the 2 groups, whereas renal vascular resistance was significantly reduced in the AC/CC group. In 20 subjects (10 from each genotype subgroup), hemodynamic function was assessed on a second occasion during controlled clamped hyperglycemia (9–11 mmol/l) after a similar preparatory period. In response to high glucose, plasma renin activity increased in both genotype groups to the same extent, but a pressor response was noted only in subjects with the C1166 allele. Mean arterial pressure increased significantly in the AC/CC subgroup and remained unchanged in the AA subgroup. We conclude that there is an association between the AGT₁R A¹¹⁶⁶→C polymor-

phism and renal hemodynamic function in early type 1 diabetes. But more importantly, the pressor response to hyperglycemia is augmented in those diabetic patients with the C1166 allele and may represent a factor that predisposes them to renal injury during periods of inadequate glucose control. *Diabetes* 49:1585–1589, 2000

Studies of pharmacological blockade of the renin angiotensin system (RAS) have clearly implicated angiotensin (ANG) II in the progression of diabetic nephropathy (1), at least in part through its action on transforming growth factor-β₁ (2). Most of the known actions of ANG II are mediated by the angiotensin II type 1 receptor (AGT₁R), which is expressed on a variety of cell types, including the vascular smooth muscle cells of the afferent and efferent arterioles of the kidney, glomerular mesangial cells, proximal tubule cells of the kidney, and adrenal glomerulosa cells (3,4). A polymorphism of the AGT₁R gene (AGT₁R A¹¹⁶⁶→C) has recently been described in which there is either an adenine (A) or cytosine (C) base at position 1166 in the 3' untranslated region of the gene (5). This polymorphism has been shown to be associated with diabetic nephropathy in some (6) but not all (7) studies. Recently, Doria et al. (6) reported that there is an association between the C1166 allele, glycemic control, and the risk of developing diabetic nephropathy in patients with type 1 diabetes. Although this has not been a universal finding (8), we hypothesized that there may be an association between renal hemodynamic function, the pressor response to hyperglycemia, and the AGT₁R polymorphism in subjects with early type 1 diabetes.

The rationale for these studies was 2-fold. First, renal hemodynamic function is related to the risk of diabetic nephropathy (9–11), and second, diabetic subjects exhibit a renal and systemic pressor response to hyperglycemia, which we have shown to be ANG II-dependent (12,13). Therefore, we first compared renal hemodynamic function under clamped euglycemic conditions in subjects with type 1 diabetes segregated on the basis of the AGT₁R polymorphism. We next studied renal and peripheral hemodynamic function during clamped hyperglycemic conditions (12,13).

RESEARCH DESIGN AND METHODS

Subjects. Thirty-nine normal men ($n = 22$) and women ($n = 17$) with type 1 diabetes were recruited to participate in the study. Their mean age was 27 ± 1 years. Each subject underwent a detailed history, physical, and laboratory examination. All were insulin-dependent and were studied within 5 years of diagnosis (mean 3.5 ± 0.6 years). They were otherwise healthy nonsmokers

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AGT₁R, angiotensin II type 1 receptor; ANG, angiotensin; ERPF, effective renal plasma flow; FF, filtration fraction; GFR, glomerular filtration rate; MAP, mean arterial pressure; PAH, para-aminohippurate; PRA, plasma renin activity; RAS, renin angiotensin system; RBF, renal blood flow; RVR, renal vascular resistance.

who were normotensive and nonobese, with a normal BMI (mean $23 \pm 0.7 \text{ kg/m}^2$), on no medications except for insulin. None of the women used oral contraceptive medications. No subject had evidence of retinopathy, microalbuminuria (as measured in a 24-h sample by radioimmunoassay), a decrease in creatinine clearance, or an orthostatic decline in blood pressure, as determined by a qualified internist. HbA_{1c} was $<10\%$ in all cases (normal range 4–6%, optimal target $<7\%$). For these experiments, subjects were subdivided into 2 groups on the basis of the $\text{AGT}_1\text{R A}^{1166} \rightarrow \text{C}$ polymorphism: those without the C1166 allele (AA [$n = 20$]) and those with the C1166 allele (AC/CC [$n = 19$]). In a previous study, in 100 normal volunteers, we found the AGT_1R gene frequencies to be AA = 56 (56%), AC = 37 (37%), and CC = 7 (7%) (14). From the 39 subjects, 10 were chosen randomly from each genotype subgroup for an examination of renal and peripheral hemodynamic responses to clamped hyperglycemia. Their characteristics did not differ significantly from the entire sample. Repeat physical examination, urine albumin excretion, and HbA_{1c} did not differ significantly from values obtained during the euglycemic phase of the experiment. The study was performed with the approval of the University of Toronto Human Subjects Review Committee and with the informed written consent of each subject.

All subjects were counseled to adhere to a diet that maintained normal caloric intake, sodium intake of 150–200 mmol/day, and protein intake of $1.5\text{--}2.0 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 7 days before each study day. To assess compliance with the controlled diet, a 24-h urine was obtained 1 day before each study for measurement of sodium and urea excretion. Subjects were considered properly prepared for study if the excretion of sodium was 140–220 mmol in 24 h and urea excretion was 3–6 mmol/kg. No subjects were excluded on this basis. All subjects refrained from caffeine intake for 48 h before each study day. Subjects were admitted to the Clinical Investigation Unit of the Toronto Hospital the evening before the study day. All studies were conducted at 0830 after an overnight fast, with the subjects lying supine in a warm quiet room.

Study protocol. On the evening prior during the prestudy period, an 18-gauge peripheral venous cannula was inserted into an antecubital vein for infusion of insulin, and a second 19-gauge sampling line was inserted in the contralateral arm for blood sampling. Insulin was infused at an average rate of $0.9 \pm 0.01 \text{ U/h}$ during the night and continued throughout the study. During the prestudy period, blood glucose levels were measured every hour (AccuCheck), and euglycemia (blood glucose 4.0–6.0 mmol/l) was maintained by varying the insulin infusion rate (12,13,15,16). Subjects who were also studied while hyperglycemic were admitted to the Clinical Investigation Unit on a separate occasion, and a similar protocol was followed, but blood glucose levels were maintained at 9–11 mmol/l—a plasma level chosen to avoid glucosuria and activation of the RAS due to volume contraction secondary to osmotic diuresis. For this portion of the study, insulin was infused at an average rate of $0.7 \pm 0.02 \text{ U/h}$.

Subjects then presented to the Renal Physiology Laboratory at 0800 the next morning. They voided and then drank 800 ml water in the first 45 min to induce a water diuresis. To maintain an adequate urine output for collection of spontaneously voided samples, 200 ml was ingested in each hour of the protocol. A third venous catheter was inserted for infusion of inulin and paraaminohippurate (PAH). While the hemodynamic measures were being made, plasma glucose levels were measured every 30 min by the glucose oxidase method (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, CA), and minor adjustments were made in the insulin infusion rate, which maintained plasma glucose in the desired range. Blood for plasma renin activity (PRA) was collected. Hemodynamic parameters (mean arterial pressure [MAP] and heart rate) were measured every 5 min throughout the study by an automated sphygmomanometer (Dinamapp), and the mean result for each period was recorded once in each half-hour of the protocol.

Renal hemodynamics were measured using inulin and PAH clearance techniques, as previously described (12,13,16,17). In brief, a priming infusion containing 25% inulin (60 mg/kg) and 20% PAH (8 mg/kg) was administered; then inulin and PAH were infused at a rate calculated to maintain their respective concentrations constant at 20 and 1.5 mg/dl. After a 90-min equilibration period, 3 timed urine collections of 20 min duration were obtained by spontaneous voiding. Blood samples were collected for inulin and PAH determinations. **Sample collection and analytical methods.** Blood samples collected for inulin and PAH determinations were immediately centrifuged at 3,000 rpm for 10 min at 4°C . Plasma was separated, placed on ice, and then stored at -70°C before the assay. Urine samples were all tested for the presence of glucose, and data were discarded if glucosuria was present. Although no subject developed glucosuria during the study, urine samples collected for inulin and PAH were promptly alkalinized by the addition of 23 μl of 4 mol/l NaOH to 4 ml urine to prevent the possible formation of an adduct between PAH and glucose (18). Inulin concentrations in plasma and urine were measured by a modified method of Walser et al. (19), and PAH concentration was measured by a spectrophotometric method according to Brun (20). The mean of the final 2 clearance periods represent glomerular filtration rate (GFR) and effective renal plasma

flow (ERPF), expressed per 1.73 m^2 . Filtration fraction (FF) represented the ratio of GFR to ERPF. Renal blood flow (RBF) was calculated by dividing the ERPF by $(1 - \text{hematocrit})$. Renal vascular resistance (RVR) was derived by dividing MAP by RBF. PRA was determined by the quantitation of ANG I generation by radioimmunoassay using the New England Nuclear Kit. Urine sodium was measured by a flame photometry method.

Polymerase chain reaction. Genomic DNA was extracted from peripheral blood leukocytes as previously described (21,22). To determine the AGT_1R genotype of the subjects, 0.1 μg genomic DNA was subjected to polymerase chain reaction amplification (14) using the following 2 primers: 5'-GCA CCA TGT TTT GAG GTT-3' and 5'-CGA CTA CTG CTT AGC ATA-3'. The 546-bp amplicon was then subjected to overnight incubation with *DdeI* at 37°C , and the digestion products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide. The C allele contains a recognition site for *DdeI* so that the digestion yields a 435- and 111-bp fragment. The A allele does not contain the restriction site so that the amplicon remains unaltered by incubation with *DdeI*.

Statistical analysis. Subjects were segregated into subgroups on the basis of the presence of the C1166 allele (AA vs. AC/CC). Data are presented as means \pm SE. Within-subject and between-group comparisons of all parameters at baseline and of the renal and peripheral hemodynamic response to hyperglycemia were made using nonparametric methods (Wilcoxon's rank-sum test). All statistical analyses were performed using the SAS statistical package (SAS Institute, Cary, NC).

RESULTS

Subject characteristics. The average age of the 39 patients with type 1 diabetes was 27 ± 1 years, and the average duration of diabetes was 3.5 ± 0.6 years. The mean HbA_{1c} was $8.7 \pm 0.6\%$. The MAP was 89 ± 3 mmHg, the albumin excretion rate was $9.1 \pm 2 \mu\text{g}/\text{min}$, and the 24-h urine sodium excretion was 154 ± 3 mmol/day.

Table 1 lists the clinical characteristics of the 2 groups of patients after genotyping for the $\text{AGT}_1\text{R A}^{1166} \rightarrow \text{C}$ polymorphism. Of the subjects, 20 displayed the AA genotype, 17 displayed the AC genotype, and 2 displayed the CC genotype; therefore, subjects were segregated into 2 groups: AA ($n = 20$) and AC/CC ($n = 19$). Mean values for HbA_{1c} were similar in these 2 groups, averaging $8.2 \pm 0.3\%$ in the AA group and $9.1 \pm 0.4\%$ in the AC/CC group. There was no difference between groups in urinary albumin and sodium excretion rates. PRA levels were not different between groups.

Baseline renal hemodynamic function. Table 2 shows the mean values for renal hemodynamic function in the 2 groups of diabetic subjects segregated on the basis of the $\text{AGT}_1\text{R A}^{1166} \rightarrow \text{C}$ polymorphism during euglycemic clamping conditions, wherein the blood glucose levels were main-

TABLE 1
Clinical characteristics of diabetic patients segregated by genotype

Parameter	AA	AC/CC
Age (years)	27 ± 1	27 ± 2
Male/female	12/8	10/9
Duration of type 1 diabetes (years)	3.6 ± 0.3	3.2 ± 0.4
HbA_{1c} (%)	8.2 ± 0.3	9.1 ± 0.4
Albumin excretion rate ($\mu\text{g}/\text{min}$)	9.1 ± 2.2	9.3 ± 2.2
24-h Urine sodium excretion (mmol/day)	156 ± 2	152 ± 4
Plasma renin activity ($\text{ng ANG I} \cdot \text{ml}^{-1} \cdot \text{s}^{-1}$)	1.4 ± 0.02	1.3 ± 0.01

Data are n or means \pm SE.

TABLE 2
Renal and peripheral hemodynamic function in diabetic patients segregated by genotype

Parameter	AA	AC/CC
MAP (mmHg)	89 ± 2	89 ± 4
GFR (ml/min)	127 ± 4	125 ± 3.4
ERPF (ml/min)	624 ± 29	794 ± 50*
FF	0.21 ± 0.007	0.16 ± 0.005*
RBF (ml/min)	1,066 ± 54	1,342 ± 86*
RVR (mmHg · l ⁻¹ · min ⁻¹)	88 ± 5	69 ± 3*

Data are means ± SE. **P* < 0.05 vs. the AA genotype subgroup.

tained between 4.0 and 6.0 mmol/l. There was no difference in the average values for heart rate, MAP, or GFR in the 2 groups. However, under euglycemic clamp, the AC/CC group exhibited significantly greater ERPF and RBF values than the AA group. Because values for MAP were similar, higher values for ERPF and RBF in the AC/CC group reflected significantly lower values for RVR compared with the AA group. In addition, mean values for FF were significantly lower in the AC/CC subjects.

Response to high glucose. There were 10 subjects chosen who displayed the AA genotype and 10 who displayed the AC genotype. Mean age, duration of diabetes, MAP, HbA_{1c}, urine albumin excretion, and urine sodium excretion were not statistically significantly different between groups and were not different from the cohort as a whole. Both genotype subgroups responded to the increased plasma glucose level with an increase in PRA, the AA group value being 1.5 ± 0.004 ng ANG I · l⁻¹ · s⁻¹ while euglycemic and 2.8 ± 0.002 while hyperglycemic (*P* = 0.06 vs. euglycemia) and the AC/CC group value being 1.23 ng ANG I · l⁻¹ · s⁻¹ while euglycemic and 3.5 ± 0.01 while hyperglycemic (*P* = 0.04 vs. euglycemia; NS vs. response of AA group). As shown in Table 3, the renal hemodynamic changes in response to hyperglycemia did not differ strikingly between groups. There was a numerical increase in FF that was statistically significant in the AC/CC genotype subgroup, but the response was not significantly different between groups (*P* = 0.07). The difference between groups in the MAP response was striking, as shown in Fig. 1 and Table 3, with the AA group remaining stable and the AC/CC group increasing significantly.

TABLE 3
Hemodynamic responses to hyperglycemia in patients segregated by genotype

Parameter	AA		AC/CC	
	Euglycemia	Hyperglycemia	Euglycemia	Hyperglycemia
MAP (mmHg)	86 ± 2	87.5 ± 3	85 ± 1	93 ± 1†‡
GFR (ml/min)	124 ± 5	123 ± 4	125 ± 4	127 ± 3
ERPF (ml/min)	613 ± 27	592 ± 24	784 ± 47*	650 ± 42
FF	0.19 ± 0.01	0.21 ± 0.009	0.17 ± 0.005	0.21 ± 0.01†
RBF (ml/min)	1,096 ± 77	964 ± 53	1,203 ± 22*	1,178 ± 56
RVR (mmHg · l ⁻¹ · min ⁻¹)	80 ± 6	89 ± 3	71 ± 3*	80 ± 3†

Data are means ± SE. **P* < 0.05 vs. baseline AA genotype subgroup values; †*P* < 0.05 vs. baseline values; ‡*P* < 0.05 vs. response of the AA genotype subgroup.

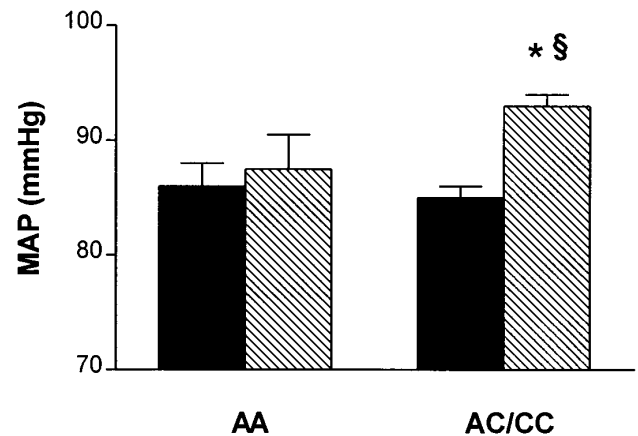


FIG. 1. MAP response during euglycemia (■) and hyperglycemia (▨) in subjects with type 1 diabetes segregated on the basis of the presence of the C1166 allele. **P* < 0.05 compared with MAP during euglycemia; §*P* < 0.05 compared with the response of the AA genotype subgroup.

DISCUSSION

The Diabetes Control and Complications Trial demonstrated that hyperglycemia is an important factor in the initiation and progression of diabetic nephropathy (23). This landmark study also showed that high glucose cannot be the only factor involved in the pathogenesis of this complication. It is clear that although poor glucose control puts many diabetic patients at risk, others appear to be protected. It is highly probable that the physiological response to high glucose determines which patients will develop nephropathy rather than the hyperglycemic state itself. The mechanism linking hyperglycemia and the initiation and progression of diabetic renal disease is unknown, but it has been demonstrated that high glucose levels activate the RAS in patients with early uncomplicated type 1 diabetes (12,13) and in normal nondiabetic subjects (24) and that RAS activation is an important factor in the pathogenesis of renal disease in naturally occurring (1) and experimental diabetes (25).

In a recent important study, Doria et al. (6) reported evidence of an interaction between glycemic control, the AGT₁R A¹¹⁶⁶→C polymorphism, and the risk of developing diabetic nephropathy in patients with type 1 diabetes. In that study, the C1166 allele was more frequent among nephropathy cases, but the result was not significant until glycemic control was factored into the analysis. The authors concluded that the C1166

allele may have modified the harmful effects of hyperglycemia on the kidney, but the mechanism was obscure.

Our present study was designed to further examine the interaction between renal hemodynamic function, hyperglycemia, and the AGT₁R A¹¹⁶⁶→C polymorphism. Based on the results of the study by Doria et al., we hypothesized that the renal and peripheral pressor response to high glucose would differ between groups of subjects with and without the C1166 allele. To test our hypothesis, we genotyped subjects for the AGT₁R A¹¹⁶⁶→C polymorphism and then divided the subjects into 2 groups based on the presence of the C1166 allele (AA and AC/CC). We then compared baseline renal and peripheral hemodynamic function between the groups while they were clamped in a euglycemic state. Because Du et al. (26) reported that renal AGT₁R mRNA levels are increased in rats maintained on a low-sodium diet and because protein intake can influence activity of the RAS (27) as well as increase GFR (28), subjects ingested a 150–200 mmol sodium diet (12,13,16,17) containing 1.5–2.0 g protein per kilogram body weight. Urinary sodium and urea excretion rates were measured and found to be similar between the 2 groups.

Our first major observation was that baseline values for renal hemodynamic parameters differed in the 2 groups during euglycemic conditions. Specifically, subjects with the C1166 allele exhibited significantly higher mean values for ERPF and RBF compared with subjects homozygous for the A1166 allele. Values for GFR were similar. MAP did not differ between the groups; therefore, the differences in ERPF and RBF reflected significantly lower values for RVR in the AC/CC group compared with the AA group. Mean values for FF were lower in the AC/CC group compared with the AA group. It is important to note that these data were obtained from patients in the resting euglycemic sodium-replete state, where one would expect the RAS to be suppressed; therefore, these results did not suggest reduced responsiveness to ANG II.

We then went on to test the pressor response to hyperglycemia in 20 patients, 10 from each genotype subgroup. Our rationale reflected the fact that hyperglycemia increases arterial pressure in diabetic subjects because of activation of the RAS (12,13) and that the AGT₁R polymorphism in part predicts the response to the AGT₁R blockade (29). Our second major observation was that subjects with the C1166 allele exhibited a significantly augmented systemic pressor response to high glucose. In contrast, values for MAP were maintained close to baseline in those homozygous for the A1166 allele. Pressor responses to hyperglycemia have also been observed in studies of experimental type 1 diabetes. Brands and Hopkins (30) studied blood pressure changes in response to hyperglycemia in the streptozotocin-induced diabetic rat and observed that hyperglycemia resulted in elevations in arterial pressure in spite of a natriuresis. The hypertensive and natriuretic effects of poor glycemic control were completely reversible with restoration of insulin therapy and normalization of blood glucose (30). In a recent study from our laboratory, we demonstrated that hyperglycemia increases arterial pressure and FF, probably secondary to activation of the RAS, because the pressor effect was abolished with the ANG II receptor blockade (13). The possibility exists that in the present study, hyperglycemia activated the RAS, resulting in the release of ANG II. The fact

that both groups experienced an increase in PRA but only those with the C1166 allele experienced an augmented pressor response may indicate that the C1166 allele predicts enhanced ANG II responsiveness.

The mechanism(s) responsible for the associations between the AGT₁R A¹¹⁶⁶→C polymorphism, renal hemodynamic function, and poor glucose control in subjects with early type 1 diabetes must remain speculative. However, it is clear that hypertension is an important risk factor for progression of renal disease (31–33), and it is tempting to speculate that the observed association between the AGT₁R polymorphism and the enhanced pressor response to glucose is responsible, at least in part, for the association reported by Doria et al. (6). Finally, although the rationale for our study was based on the role of the RAS and hyperglycemia in diabetic nephropathy, the association between the pressor response to glucose and the AGT₁R A¹¹⁶⁶→C polymorphism in our diabetic subjects may be unrelated to the AGT₁R gene. It is possible that the AGT₁R A¹¹⁶⁶→C polymorphism is in linkage disequilibrium with a functional polymorphism in a nearby gene on chromosome 3q that is responsible for our association (34).

In summary, the current study establishes that there is an association between the AGT₁R A¹¹⁶⁶→C polymorphism and renal hemodynamic function in subjects with early type 1 diabetes. Subjects with the C1166 allele exhibit higher values for ERPF and RBF than subjects homozygous for the A1166 allele and exhibit significantly lower values for RVR under clamped euglycemic conditions. More importantly, these subjects exhibit an enhanced pressor response to high glucose. This high pressure state may contribute to glomerular injury and may explain the relationship between the C1166 allele and diabetic nephropathy in those patients with poor glucose control (6). Further studies will be necessary to determine the mechanism(s) responsible for this association.

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