Increased Insulin-Stimulated Expression of Arterial Angiotensinogen and Angiotensin Type 1 Receptor in Patients With Type 2 Diabetes Mellitus and Atheroma

Wassim Hodroj, Liliana Legedz, Nabil Foudi, Catherine Cerutti, Marie-Claude Bourdillon, Patrick Feugier, Michel Beylot, Jacques Randon, Giampiero Bricca

Objective—Because inhibition of the renin–angiotensin system (RAS) reduces the onset of type 2 diabetes (T2D) and prevents atherosclerosis, we investigated the expression of RAS in the arterial wall of T2D and nondiabetic (CTR) patients.

Methods and Results—mRNA and protein levels of angiotensinogen (AGT), angiotensin-converting enzyme (ACE) and AT1 receptor (AT1R) were determined in carotid atheroma plaque, nearby macroscopically intact tissue (MIT), and in vascular smooth muscle cells (VSMCs) before and after insulin stimulation from 21 T2D and 22 CTR patients. AGT and ACE mRNA and their protein levels were 2- to 3-fold higher in atheroma and in MIT of T2D patients. VSMCs from T2D patients had respectively 2.5- and 5-fold higher AGT and AT1R mRNA and protein contents. Insulin induced an increase in AGT and AT1R mRNA with similar ED50. These responses were blocked by PD98059, an inhibitor of MAP-kinase in the two groups whereas wortmannin, an inhibitor of PI3-kinase, partially prevented the response in CTR patients. Phosphorylated ERK1–2 was 4-fold higher in MIT from T2D than from CTR patients.

Conclusions—The arterial RAS is upregulated in T2D patients, which can be partly explained by an hyperactivation of the ERK1–2 pathway by insulin. (Arterioscler Thromb Vasc Biol. 2007;27:525-531.)

Key Words: renin ■ carotid atheroma ■ type 2 diabetes mellitus ■ MAP-kinase ■ P85-PI3-kinase

Type 2 diabetes mellitus (T2D) is an important risk factor of atherosclerosis, and nearly 80% of diabetic patients die of cardiovascular disease.1 The inhibition of the renin–angiotensin system (RAS) may be achieved with angiotensin converting enzyme (ACE) inhibitors or angiotensin type 1 receptor (AT1R) antagonists. These drugs demonstrated a decrease in the mortality and frequency of cardiovascular events, in primary prevention of cardiovascular diseases in high risk patients (Heart Outcomes Prevention Evaluation study [HOPE], Losartan Investigation For Endpoints reduction in hypertension study [LIFE]) and in secondary prevention after myocardial infarction (Gruppo Italiano per lo Studio della Soppravvivenza nell’Infarto miocardico [GISSI3], International Study of Infarct Survival-4 [ISIS4]).2–4 In atherosclerotic regions of arteries, the amounts of immunoreactive ACE and angiotensin II (Ang II) are significantly increased.5–8 We previously showed that ACE and other enzymes such as cathepsin G and tissue kallikrein that are able to cleave Ang II from AGT mRNA were also increased in carotid atheroma with a concomitant decrease in AT1R mRNA.9,10

This suggests that RAS is involved in the atherogenic process. Inhibition of RAS in T2D patients has been shown to be more beneficial than in nondiabetic subjects in terms of reduction of cardiovascular events.1,2 Moreover, RAS blockers reduced the development of diabetes and insulin resistance in hypertensive patients.2,11 Thus, RAS is likely to be involved in the development of both insulin resistance and atheroma.

Insulin has been shown to activate, via the MAP kinase ERK1–2 pathway, the expression of AGT and AT1R in vascular smooth muscle cells (VSMCs),12,13 whereas insulin-induced glucose uptake is mediated via the PI3kinase and altered in T2D patients.14,15 However, the mechanisms involved in the diabetic atheroma formation and in the higher sensitivity of T2D patients to the beneficial effects of RAS inhibitors are still poorly understood.

In this work, we have tested the hypothesis that the increased vascular risk and greater benefit of RAS blockade in T2D patients result from a higher activity of RAS in the arterial wall. In a previous study, we did not detect renin nor AT2 receptor mRNA in patients with or without T2D.9 Consequently, we investigated the mRNA and protein levels of RAS in atheroma plaques and nearby macroscopically intact tissues (MIT) of the carotid wall from patients with or without T2D. In addition, cultured VSMCs from similar

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TABLE 1. Clinical Parameters From 43 Patients Included in this Study

<table>
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<tr>
<th>Clinical Parameters</th>
<th>CTR Patients</th>
<th>T2D Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year</td>
<td>67.7±1.6</td>
<td>69.7±1.8</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>150±3</td>
<td>149.5±3</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>83.1±1.9</td>
<td>80.4±1.7</td>
</tr>
<tr>
<td>Plasma glucose, mmol/L</td>
<td>5.4±0.1</td>
<td>11.1±0.3</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.3±0.2</td>
<td>5.1±0.2</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>3.1±0.1</td>
<td>3±0.1</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.2±0.1</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>1.5±0.1</td>
<td>1.5±0.1</td>
</tr>
</tbody>
</table>

Results are the mean±SEM of 22 CTR and 21 T2D patients. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein. *P<0.05 T2D vs CTR.

patients were used to verify whether the alterations of tissue RAS in T2D patients are an intrinsic property of the cells or a result of extracellular factors associated with T2D (insulin, glucose, oral-antidiabetic drugs). To go deeper into the cellular mechanisms, the insulin signaling via the MAP-kinase ERK1–2 and P85–PI3-kinase pathways was investigated in VSMCs and MIT.

Materials and Methods

Patients and Tissue Sampling

The investigation conforms to the principles outlined in the declaration of Helsinki16; all procedures were approved by the local ethical committee and the patients gave informed consent. Forty-three hypertensive patients who underwent carotid endarterectomy at the University Hospital of Lyon (Hôpital Edouard Herriot) were included in the study (Table 1). The endarterectomy tissues of 31 patients served for the carotid wall study: 15 T2D (mean age 69.2±9.9; 8 M) and 16 CTR (mean age 68.2±8.5; 10 M). The carotid samples of 24 patients were used for primary VSMC culture experiments: 12 T2D (mean age 67.8±8.9; 6 M) and 12 CTR (mean age 67.4±6.3; 8 M). Patients were under various therapies and T2D was defined as the use of an antidiabetic treatment (Table 1).

Histological Analysis

Fragments from endarterectomy tissue were fixed in 4% paraffin-embedded in phosphate buffer saline and subsequently paraffin-embedded. Tissue samples were numbered and given blind to the pathologist for conventional processing and analysis to determine the stage of the lesion.

Cell Culture

VSMCs were obtained from carotid sample with the explant technique. In brief, a strip of MIT was cut into small fragments which were placed in 25 cm² culture dishes in human VSMC culture medium (Promocell–Heidelberg) maintained at 37°C and 5% CO₂. Experiments on cells were performed from passages 2 to 4. The smooth muscle phenotype was controlled with anti–α-smooth muscle actin (DAKO) immuno-staining. For the experiments, 150,000 cells were seeded in 6-well plates in their usual medium for at least one day. Twenty-four hours before the experiments, culture medium was replaced by a basal VSMC medium without fetal calf serum. On the day of the experiments, culture medium was replaced and the test substances were added at appropriate concentrations (insulin: 0 to 100 μU/mL; Life Sciences; glucose: 0 to 50 mmol/L; glibenclamide: 0 to 100 μmol/L; metformine: 0 to 100 μmol/L, Sigma) for another 24 hours. PD98059 (10 μmol/L) or Wortmannin (1 μmol/L; Calbiochem), was applied 30 minutes before insulin addition. Medium was then collected for AGT measurements, and cells were immediately treated for RNA and/or protein extraction. All experimental points were performed in duplicate.

RNA Purification and RT-PCR

RNA purification and reverse transcription were performed from duplicate cell samples as described in Legedz et al.10 Real-time PCR was performed using 5 μL of the RT reaction mix (qSYBR Green Supermix, Biorad), 2.5U Platinum TaqDNA Polymerase (Invitrogen), and the appropriate set of primers (Table 2) in a Biorad MyIQ thermal cycler. All samples were run in duplicate along with dilutions of known amounts of target sequence for absolute quantitation of initial cDNA copies. Data are expressed as absolute values or in ratio to no treatment.

Protein Extraction, Immunoprecipitation, and Western Blot

For atheroma plaque and MIT, proteins were extracted using TriZol Reagent (Invitrogen). VSMCs were lysed with 150 mmol/L NaCl, 10 mmol/L EDTA, 1% Triton X-100, 50 mmol/L HEPES (pH 7.5), 100 mmol/L NaF, 10 mmol/L Na₃P₂O₅, and 2 mmol/L sodium orthovanadate supplemented with a protease inhibitor cocktail (Complete mini, Roche). Insoluble material was removed by centrifugation, and the protein concentrations of supernatants were measured with the Lowry method. Protein extract (50 μg) was immunoprecipitated with either anti–ERK1–2 (Cell Signaling Technologies) or anti-P85 α-subunit of the PI3-kinase (Clinciscience) antibodies and recovered with protein G sepharose. Immunoprecipitated proteins or total proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4°C with primary antibodies: anti-AGT (kindly provided by Dr E. Clauser, INSERM, U36, Paris, France), anti-ACE (Santa Cruz Biotechnology), anti-AT1R and anti–phospho-ERK1–2Thr202/Tyr204 (Cell Signaling Technology), and anti–phospho-P85 (Clinciscience). After washing and incubation with the peroxidase-linked secondary antibody for 1 hour, immunoreactive proteins were visualized by ECL reagent (Amer sham). The blots were normalized with either α-actin for angiotensinogen, AT1R, ACE, or the nonphosphorylated form of the protein investigated.

TABLE 2. Primer Sequences Used for Quantitative Real Time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Left Primer</th>
<th>Right Primers</th>
<th>Size (bp)</th>
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<tr>
<td>18S rRNA</td>
<td>TGGAGCCATGATTTAAGGAGG</td>
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<tr>
<td>ACE I</td>
<td>TGGAGGTTATTATTAGGAGAG</td>
<td>GTAAGGCAGCTAGTGGAGAA</td>
<td>156</td>
</tr>
<tr>
<td>AT1R</td>
<td>GGAAAACGACTTGTTGCGAT</td>
<td>CCAAGGATATCTGACGTC</td>
<td>150</td>
</tr>
<tr>
<td>AGT</td>
<td>CCATGGACACACTTTAGACC</td>
<td>GCGAGCACAGATAAACACC</td>
<td>128</td>
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</tbody>
</table>

ACE-1 indicates angiotensin converting enzyme 1; AT1R, angiotensin II receptor type 1; AGT, angiotensinogen.
ACE Activity

Tissue ACE activity was determined with the method of Faure-Delanoe17 in duplicate with the synthetic specific substrate furylacyloyl-phenylalanlyl-glycyl-glycine (FAPGG). The final concentration of FAPGG was 0.8 mmol/L. Nonspecific ACE I activity was measured in the presence of 10 µmol/L captopril (Sigma) and was subtracted from each determination. One unit (U) of ACE activity is the amount of enzyme that hydrolyzes 1 µmol of substrate per minute.

Statistical Analysis

Results are expressed as mean±SEM. Two-way analysis of variance considering T2D and the stage of lesion (atheroma or MIT) as factors was performed after normal distribution of data were verified. Subsequent pair-wise comparisons were performed with the Tukey test. The effect of each class of drug taken by the patients was verified with t test without taking into account the presence of T2D. The amounts of mRNA and protein in cells and cell culture media were compared between CTR and T2D patients with t test. Dose–response curves were analyzed with SigmaPlot (SPSS Inc), and fitted to a three-parameter logistic regression for calculation of ED50. A probability value lower than 0.05 was considered significant.

Results

Endarterectomy Samples

Histological Analysis

Histological analysis confirmed that the endarterectomy samples contained only media and intima. The fragment considered as atheroma plaque consisted mostly of stage IV and/or V lesions according to the AHA classification. MIT was almost exclusively composed of stage I and II lesions, with some restricted stage III lesions in a few patients.18–19

Gene Expression

No effect from the patients’ treatment was detected on AGT, ACE, and AT1R mRNA, probably because of the low number of patients treated with a single drug of each class and the unavailability of untreated patients.

In both groups of patients, ACE mRNA content was 3 times higher in atheroma compared with MIT, whereas AT1R mRNA content was about 2 times lower. AGT mRNA was not significantly modified (Figure 1A). In T2D patients, AGT and ACE mRNA contents, both in atheroma and MIT, were about 2-fold higher than in CTR patients. The difference in AT1R mRNA did not reach statistical significance (P=0.11).

Protein quantification of AGT, AT1R, and ACE in MIT was performed with Western blot. (Figure 1B). Carotid tissue samples from T2D compared with CTR patients showed a 3-fold increase in AGT and 2-fold increase in ACE (P<0.001).

Tissue ACE activity in MIT was increased from 11±3 U/L in CTR to 23±4 U/L in T2D patients. After normalization to the ACE protein content of each patient, ACE activity was similar in 3 T2D (28.0±0.6) and 3 CTR (28.8±1.7) patients.

VSMC Culture

Basal characteristics of VSMCs

In basal conditions without fetal calf serum and in the presence of physiological concentrations of insulin and glucose, all three mRNA for AGT, AT1R, and ACE were expressed in human VSMCs. ACE mRNA was about 10 times less abundant than AT1R and AGT mRNAs. Both AGT and AT1R mRNA and protein contents were about 5- and 2.5-fold higher in cells from T2D patients, whereas ACE mRNA levels were similar in both groups (Figure 2). AGT secreted during 24 hours in the culture medium measured with quantitative dot blot reached levels nearly 8 times higher levels in T2D patients (5.8±1.8 versus 42.7±6.0 µg/mg protein).

Effect of Glucose, Oral Antidiabetics, and Insulin

Glucose (2.5 mmol/L to 50 mmol/L) did not modify the mRNA amount of the 3 genes in the 2 groups of patients. Metformin, up to 300 µmol/L, did not affect on all 3 genes, but doses of glibenclamide, up to 100 µmol/L, dependently reduced AGT and AT1 mRNA of CTR and T2D patients, with a maximal decrease of 90% at 50 µmol/L.

A concentration–response curve for insulin was obtained for AGT and for AT1R mRNA. Insulin induced much higher mRNA responses for AGT and AT1R in cells from T2D
patients with very similar ED50 (30 to 35 \( \mu U/mL \)) in both groups of patients (Figure 3A and 3B). Protein levels as well were increased by insulin: more than 3-fold for AGT and close to 2-fold for AT1R in both groups of patients (Figure 3E and 3F). ACE mRNA level was not modified at any concentration of insulin (data not shown).

In both groups of patients, the effects of insulin on AGT, AT1R mRNA, and proteins were prevented by PD98059 (Figure 3C through 3F). The PI3 kinase inhibitor wortmannin attenuated the effect of insulin on AGT mRNA only in CTR subjects, whereas its attenuating effects on AT1R mRNA and protein were similar in both group of patients. (Figure 3C through 3F).

**Insulin Induced Phosphorylation of P85-PI3-Kinase and ERK1–2**

Insulin stimulated the phosphorylation of P85-PI3 kinase and ERK1–2 in VSMCs from CTR patients, which were blocked as expected respectively with wortmannin and PD98059 (Figure 4A). In T2D patients, a higher insulin-induced phosphorylation of ERK1–2 was observed while the phosphorylation of P85-PI3 kinase was more than 2-fold reduced.

Results obtained in MIT (Figure 4B) indicate that the proportion of phosphorylated ERK1–2 was almost 4 times higher in T2D patients than in CTR, whereas similar proportions of phosphorylated P85-PI3 kinase were found.

**Discussion**

Beneficial effects of RAS inhibition in preventing atheroma complications (stroke, myocardial infarction) and renal alterations in T2D patients strongly suggest an overactivation of RAS in T2D. This study demonstrates, for the first time, an overexpression of RAS genes in the atherosclerotic arterial wall of T2D compared with CTR patients. Indeed, we have shown an increase in AGT and ACE, as well as a marked trend for a higher AT1R mRNA and protein levels in atheroma plaques and in the adjacent macroscopically intact tissues of human carotid arteries in type 2 diabetes patients. Furthermore, the investigation of cellular mechanisms revealed an increase of the phosphorylation of ERK1–2 in situ whereas insulin effect on RAS gene expression in VSMCs was associated with a decreased PI3 kinase transduction and an increase MAP-kinase pathway stimulation.

Different factors (hyperglycemia, cellular glucopenia, hyperinsulinemia, and oral antidiabetic drugs) may contribute to the increased expression of AGT and ACE in the arterial wall of T2D patients. In this study, glucose and metformin had no effect, whereas the hyperglycemic sulfamide glibenclamide decreased AGT and AT1R mRNA in VSMCs. Thus, in vascular tissue in vivo, a direct effect of glibenclamide on VSMCs would be expected to reduce the overexpression of RAS observed in T2D patients, whereas its insulin secreting effect would counterbalance this alteration. Glucose has been reported to increase AGT gene expression in rat kidney tubular cells via the generation of reactive oxygen species. In liver and fat, glucose has been reported to differentially affect AGT expression in lean or obese rats via the generation of UDP-Nacetyl glucosamine. In contrast, our in vitro study suggests that the involvement of hyperglycemia in atheroma formation is most likely not mediated by direct RAS expression stimulation in VSMCs.

The expression of ACE mRNA was higher in the arterial wall from T2D patients but not in the cultured VSMCs, suggesting that either the culture conditions have altered ACE expression responsiveness or these cells are not responsible for the in situ overexpression of ACE. Indeed, immunohistochemical studies in different arteries with or without atheroma have shown that ACE was mostly found in endothelial cells of the arterial wall, in the neocapillaries of the atheroma plaque, as well as in macrophages.

Our results confirm previous observations showing that insulin and insulin-like growth factor stimulate AGT production and AT1R expression. For both AGT and AT1R mRNAs, the similarity of insulin ED_{50} in all patients indicates that in T2D patients the response to insulin is amplified at a postreceptor level. Thus, basal and insulin-stimulated overexpression of angiotensin system genes in VSMCs may contribute to the higher levels observed in carotid arteries.

The insulin signaling pathway was investigated at the 2 different levels of PI3 kinase and ERK-MAP kinase. Using a specific inhibitor for each of these pathways, our results show that the effect of insulin on AGT and AT1 receptor mRNA is mediated via both the MAP kinase and PI3 kinase pathways in CTR patients, whereas only the MAP kinase pathway is involved in the AGT increase observed in T2D patients,
possibly the result of an imbalance of insulin receptor signaling between MAP kinase and PI3 kinase pathways in VSMCs from T2D patients. In contrast, only the ERK1 MAP kinase pathway is involved in the AGT increase observed in T2D patients, which may result from an imbalance of insulin receptor signaling between ERK Map Kinase and PI3 kinase pathways in VSMCs from T2D patients. This observation is in accordance with the results of Cusi et al and Bouzakri et al showing a lack of PI3 kinase pathway stimulation by insulin in human skeletal muscle of T2D patients while insulin stimulation of the MAP kinase pathway was normal.15,23

Similar results were found by Jiang et al in small arteries of obese insulin resistant Zucker rats.14 Interestingly, in vivo, the obvious imbalance in favor of the ERK1–2 pathway argues in favor of parallel variations of insulinemia and expression of AGT and AT1R expression in arterial wall. A specific study testing the correlation between insulinemia or insulin resistance index and AGT and AT1R expression in the arterial wall is necessary to verify this hypothesis.

Because ACE, cathepsins D and G, and tissue kallikrein are present, increased levels of AGT in arterial wall would result in increased Ang II formation. This may occur in intact tissue and contribute to the initiation and accelerated development of atheroma in T2D patients. Ang II inhibits insulin signaling in VSMCs at different levels, particularly by inhibiting the insulin-stimulated PI3 kinase pathway.24–25 This may help in understanding the UKPDS study in which the expected benefit in terms of macroangiopathy, with aggressive treatment (by insulin and/or oral antidiabetic agents) to lower blood glucose, was not observed.26

The observation of higher AGT and AT1R expression in VSMCs from T2D patients (found even in the absence of insulin stimulation) could be useful for future research on insulin resistance. Indeed, smooth muscle cells originating from carotid wall, in culture for at least 1 month after numerous medium changes and cell divisions, maintained decreased insulin mediated PI3-kinase transduction, which is a feature of insulin resistance phenotype, as has been already
observed in striated muscle cells. The overexpression of angiotensin system in vascular wall cells of T2D patients may be either a genetic or an epigenetic property. Cortisol is a potent activator of AGT and AT1R gene expression and abnormal cortisol metabolism and/or tissue sensitivity to cortisol were frequently found in patients with T2D, glucose intolerance, and increased cardiovascular risk. It would be interesting to study this mechanism in VSMCs from T2D patients.

In conclusion, there is overexpression of arterial RAS components: AGT, ACE, and AT1R in T2D patients. These components could be considered as new biomarkers of diabetic atherosclerotic complications.

This hyperactivity may be attributable to increased basal and amplified insulin-stimulated RAS gene expression in VSMCs. Amplified response to insulin in insulin-resistant subjects seems to be paradoxical. It may be attributable to an imbalance between altered PI3-kinase pathway and normal MAP-kinase signaling. Paracrine Ang II overproduction may, in turn, contribute to inhibition of PI3-kinase signaling, to increase in insulin resistance, and to activation of the MAP kinase signaling. It may also partly explain the higher basal and amplified responsiveness to insulin.

Figure 4. Phosphorylation of ERK1–2 and P85. Western blot analysis of Phospho-ERK1–2 and phospho-P85-Pi3kinase in ERK1–2 and P85 immuno precipitated proteins from: A, VSMCs were treated for 30 minutes with insulin in the presence or not of PD 98058 or Wortmannin and B, in MIT tissue from carotid endarterectomy samples. Typical results from 12 CTR and 12 T2D patients and quantitation in 6 T2D and 6 CTR patients. *P<0.05 vs control without insulin treatment. B, Typical Western blot macroscopically intact tissue from 2 T2D and 2 CTR patients (MIT) and quantification from 6 T2D and 6 CTR patients. Results are the mean ± SEM; in A, †P<0.01 T2D vs CTR.
cardiovascular risk of T2D patients as well as the clinical benefits of RAS blockade.

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Disclosures
None.

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