Downregulation of Renal Endothelin-Converting Enzyme 2 Expression in Early Autoimmune Diabetes

JANA ORTMANN,*,[1] PHILIPP C. NETT,*‡,[1] JENNIFER CELEIRO,* REGINA HOFMANN-LEHMANN,† LUIGI TORNILLO,§ LUIGI M. TERRACCIANO,§ AND MATTHIAS BARTON*,[2]

Medical Policlinic, Department of Internal Medicine, University Hospital Zürich, and †Clinical Laboratory, Vetsuisse-Faculty, University of Zürich, CH-8091 Zürich, Switzerland; ‡Department of Visceral and Transplant Surgery, University Hospital Bern, CH-3010 Bern, Switzerland; and §Institute of Pathology, University Hospital Basel, CH-4031 Basel, Switzerland

To determine whether renal expression of endothelin-converting enzymes (ECEs) and endothelin (ET) is affected in the early stages of autoimmune diabetes mellitus and whether ET_A receptors are involved, prediabetic nonobese diabetic (NOD) and control mice were treated with the ET_A receptor antagonist BSF461314 (a follow-up compound of darusentan) or with placebo. Blood samples were analyzed for glucose levels, and renal gene expression of ECE-1, ECE-2, and prepro-ET-1 was determined using real-time polymerase chain reaction. Renal morphology was assessed using standard histologic techniques. ECE-1, ECE-2, and prepro-ET-1 mRNA was detected in the kidneys of NOD and control mice. Despite normal renal histology, expression of ECE-1 and prepro-ET-1 was reduced in NOD mice by approximately 50% compared with controls ($P < 0.01$); ECE-2 was markedly decreased by almost 90% compared with controls ($P < 0.001$). Treatment with BSF461314 for 6 weeks delayed the onset of diabetes ($P < 0.05$) and increased expression of all three genes ($P < 0.05$) in NOD mice only. Hyperglycemia at an early stage of autoimmune diabetes is associated with transcriptional downregulation of ECE-1, ECE-2, and prepro-ET-1 in the kidney. Blockade of ET_A receptors inhibits diabetes-associated gene regulation and delays the onset of diabetes, suggesting its therapeutic potential for the treatment of autoimmune forms of diabetes. Exp Biol Med 231:1030–1033, 2006

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[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed at Medizinische Poliklinik, Departement für Innere Medizin, Universitätsspital Zürich, Rämistrasse 100, CH-8091 Zürich, Switzerland. E-mail: barton@usz.ch

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Introduction

Diabetic nephropathy currently affects about 20% of patients with type 1 diabetes mellitus and 35% of patients with type 2 diabetes mellitus (1). Several vasoactive factors are involved in the initiation and progression of glomerulosclerosis, resulting in diabetic nephropathy (2). Endothelin (ET), a potent vasoconstrictor and mitogenic peptide initially isolated from endothelial cells (3), has been implicated in the pathogenesis of glomerulosclerosis because of its action on chemotaxis, extracellular matrix accumulation, and mesangial cell proliferation (4–6). ET exerts its biologic effects by activating ET_A and ET_B receptors (5). Mature ET-1 peptide is generated via a specific two-step processing pathway, which involves the cleavage from the physiologically inactive precursor peptide prepro-ET to big ET by endopeptidases and conversion to bioactive ET by endothelin-converting enzymes (ECEs) (3, 5).

Under hyperglycemic conditions, expression of prepro-ET-1 and ECE-1 increases in the kidney (7). Although blockade with ET_A receptor antagonists reduces proteinuria and normalizes renal matrix protein expression in type 2 diabetic rats (8) and in patients with diabetes (9), there is no information (to our knowledge) about how autoimmune type 1 diabetes affects gene expression of renal ECEs and ET-1. Herein, we investigated to what extent transcriptional regulation of renal ECE-1, ECE-2, and prepro-ET-1 is affected during the early stages of autoimmune diabetes and whether renal ET_A receptors contribute to gene regulation and the development of early diabetes.

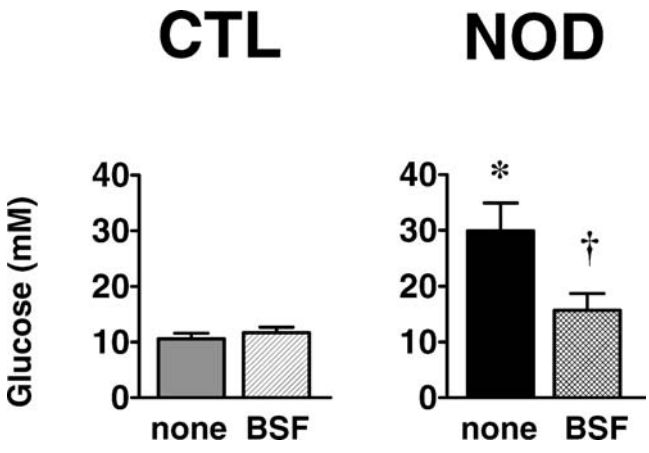

Figure 1. Blood glucose levels of control (left) and NOD (right) mice (n = 7–9 mice/group) at the age of 22 weeks. Treatment with BSF461314 for 6 weeks inhibited the increase of blood glucose levels in NOD mice ($P < 0.05$ vs. untreated). None indicates untreated animals; and BSF, BSF461314 treated. Data are means ± SEM and are expressed in m*M*. *$P < 0.01$ vs. control. †$P < 0.05$ vs. untreated NOD.

Materials and Methods

Animal Experiments and Tissue Sampling. Prediabetic 16-week-old female nonobese diabetic/LtJ (NOD) and sex-matched SWR/LtJ control mice (10) were obtained from the Jackson Laboratory (Bar Harbor, ME) and were randomly allocated (n = 7–9/group) to receive placebo or an orally active ET_A receptor–selective antagonist (BSF461314, 30 mg/kg/day; Knoll AG, Ludwigshafen, Germany) administered in the drinking water as previously described (11). BSF461314 is a follow-up compound of darusentan, with a selectivity toward ET_A receptors of 800-fold vs. ET_B receptors (Dr. K. Münter, personal communication). After 6 weeks of treatment, animals were humanely killed and the kidneys excised, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Tissue of each kidney was embedded in paraffin for histologic analysis. Blood glucose was assayed using automated analysis (Cobas Mira; Hoffmann-La Roche AG, Basel, Switzerland) as previously described (11). The study design was in accord with the principles set out by the National Health and Medical Research Council of Switzerland and the local authorities for Animal Research (Kommission für Tierversuche des Kantons Zürich, Switzerland).

Renal Gene Expression. Quantitative RT-PCR was used to determine the expression of genes encoding for murine ECE-1, ECE-2, and prepro-ET-1, calculated using the $\Delta\Delta_{CT}$ method as previously described (11, 12). Primers were designed to allow detection of all ECE isoforms (Table 1) (13, 14).

Histologic Analysis. Hematoxylin-eosin–stained sections of the kidneys of the study animals were evaluated blindly by a pathologist (L.T.) using standard histologic methods as previously described (11).

Statistical Analysis. Data were analyzed by means of analysis of variance with or without repeated measures using the Statview SE program (SAS Institute Inc., Cary, NC). Comparisons of group means were performed using the Fisher least significant difference test or the Mann-Whitney U test. Data are given as means ± SEM, with n indicating the number of animals used in the experiment. $P < 0.05$ was considered significant.

Results

Metabolic Parameters and Renal Histology. Blood glucose levels of all animals were within the normal range at the beginning of the study (data not shown). At the age of 22 weeks, blood glucose levels had increased in NOD mice (29.9 ± 4.8 m*M*), which was 2.8-fold higher than those in controls (10.6 ± 1.1 m*M*; $P < 0.05$; Fig. 1). Treatment with BSF461314 for 6 weeks largely prevented the increase of blood glucose levels in NOD mice (15.7 ± 2.7 m*M*; $P < 0.05$ vs. untreated). Histologic analysis of kidneys of NOD and control mice revealed no pathologic changes (data not shown).

Diabetes and Gene Expression. Gene expression of ECE-1, ECE-2, and prepro-ET-1 was detected in the kidneys of all animals (Fig. 2), with mRNA levels of ECE-2 being approximately 400-fold lower than those of ECE-1 (Fig. 2A and B). Compared with control mice, transcriptional levels of the ECE-1 and prepro-ET-1 genes in NOD mice were reduced by approximately 50% ($P < 0.01$; Fig.

Table 1. Genbank Accession Numbers and PCR Primers of Mouse ECE-1, ECE-2, and Prepro-ET-1 Genes

Genbank	Primer	Sequence
ECE-1	Sense	CTC GCT CTC CAA CTC CAA GGA
XM_131743	Antisense	CTT ACC AGA CTT CGC ACT TGT GAT
	Probe	FAM-AGA ACA CTT TCG CTG CCC GCC TGG-TAMRA
ECE-2	Sense	GCT TTG CTC TGG GTT CAC TC
AF396699	Antisense	CGG GTC TTC TCA TCC ATC CA
	Probe	FAM-ACC GAC AAA GCA AGG AAA TCG CC-TAMRA
Prepro-ET-1	Sense	TGT GTG GCT TCT ACA GTT TCT TGT
U35233	Antisense	CTC AGC CTT TCT TGG AAT GTT TGG
	Probe	FAM-CAG ACG GGC AGA GGA CCA GCA TCC-TAMRA

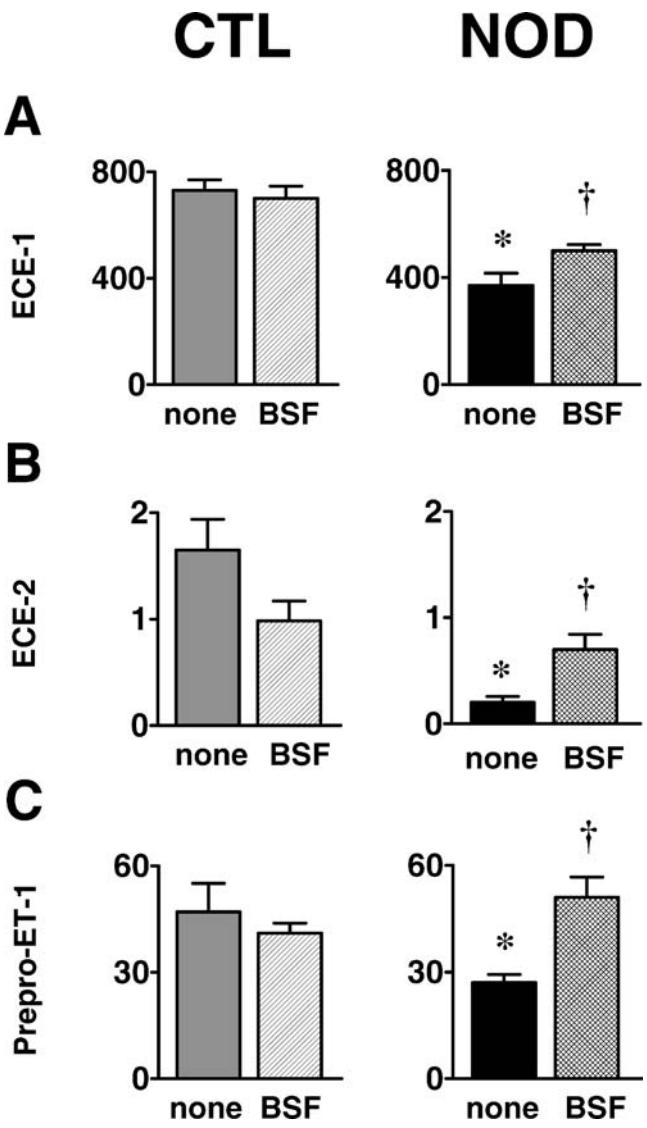

Figure 2. Gene expression of ECE-1 (A), ECE-2 (B), and prepro-ET-1 (C) in the kidneys of control (left panels) and NOD (right panels) mice ($n = 7$–9 mice/group). Expression of ECE-2 in both groups was approximately 400-fold lower than ECE-1 expression ($P < 0.001$ for all groups of NOD or control mice). Treatment with BSF461314 for 6 weeks increased gene expression of ECE-1, ECE-2, and prepro-ET-1 in the kidneys of NOD mice only. None indicates untreated animals; and BSF, BSF461314 treated. Data are means ± SEM and are expressed in arbitrary units (AU) = $\Delta\Delta_{CT}$ of gene of interest and housekeeping gene. *$P < 0.05$ vs. control. †$P < 0.05$ vs. untreated NOD.

2A and C). The decrease of ECE-2 mRNA in NOD mice was approximately 90% ($P < 0.001$; Fig. 2B).

Effect of ET_A Receptor Blockade on Gene Expression. Treatment of NOD mice with BSF461314 for 6 weeks was associated with an upregulation of gene expression of renal ECE-1, ECE-2, and prepro-ET-1 ($P < 0.05$ vs. untreated NOD; Fig. 2). Treatment had no significant effect on gene expression in control mice (Fig. 2, left panels).

Discussion

This study demonstrates that hyperglycemia at an early stage of autoimmune diabetes is associated with transcriptional regulation of renal ECE-1, ECE-2, and prepro-ET-1 expression in the absence of structural injury of the kidney. Treatment of prediabetic NOD mice with the ET_A receptor antagonist BSF461314 for 6 weeks prevented the onset of diabetes and resulted in upregulation of ECE-1, ECE-2 and prepro-ET-1. To the best of our knowledge, this is the first report investigating the role of ET in autoimmune diabetes and demonstrating effects on ECE-2 expression. We also show in this model that ET_A receptors contribute to transcriptional regulation of ECE-2 *in vivo*.

The NOD mouse provides a suitable animal model for studying the early metabolic and expressional changes of autoimmune diabetes (10). Female NOD mice generally develop spontaneous insulitis beginning on average at 4 weeks of age. Insulitis leads to progressive destruction of insulin-producing pancreatic beta cells, and animals become overtly diabetic around 18–20 weeks of age (10). Expression of ECEs and prepro-ET-1 genes is largely confined to the glomerular and vascular endothelium in the non-diseased kidney (15). Formation of bioactive ET-1 protein depends on the activity of ECEs and the production of the inactive precursor prepro-ET-1 (5), and the conversion of big ET-1 to ET-1 by ECEs determines ET-1 production by renal mesangial and endothelial cells (5, 15). The results of the present study suggest that ECE-1 is important for renal ET-1 formation, because gene expression of ECE-1 was about 400-fold higher compared with ECE-2 gene expression in all animals. Previous studies reported that hyperglycemia induces gene expression of ECE-1 and ET-1 in endothelial cells (16) and mesangial cells *in vitro* (17, 18) and *in vivo* (19, 20). In the present study, however, we unexpectedly found that, in the kidney of a model of autoimmune type 1 diabetes, hyperglycemia is associated with a decrease rather than an increase in ECE-1, ECE-2, and prepro-ET-1 gene expression. Moreover, it appears that this decrease is specific for and restricted to the kidney, because upregulation of these genes occurs in the aorta of the NOD animals of the same age (11). The mechanism underlying this differential regulation is unclear but may involve negative feedback regulation of gene expression by elevated ET-1 protein levels (5). Such a concept would be supported by the increase of ET-1 plasma levels found at the early stages of diabetes in NOD mice (11), indicating an activated ET system (5). Previous studies investigating ET in the setting of experimental type 1 diabetes exclusively used the streptozotocin-induced toxicity model, leading to islet destruction (19, 20); these animals develop hyperglycemia and severe renal injury that may be partly caused by streptozotocin toxicity (Dr. K. Amann, personal communication). Renal injury was absent in our animals, which makes the results even more remarkable.

Our findings that glucose levels were lower in

BSF461314-treated NOD mice suggest that inhibition of ET_A receptors could at least partially contribute to the development of autoimmune diabetes. Indeed, ET-1 regulates pancreatic beta-cell function and thus glucose activity (11). Alternatively, regulation of immune-mediated processes by ET or its receptors (21) could contribute to the development of type 1 diabetes. This notion arguing against a hyperglycemia-dependent regulation of the renal genes investigated is further supported by the results of experiments started in 10-week-old prediabetic NOD mice that show upregulation of genes after 6 weeks of BSF461314 treatment (authors' unpublished observation, 2005). An immune-mediated mechanism is further suggested by the results of a recent study showing that treatment of NOD mice with a sphingosine-1-phosphate receptor agonist (FTY720) prevented the initiation of type 1 diabetes (22).

In conclusion, our data demonstrate that an unexpected decrease in renal gene expression of ECE-1 and ECE-2 occurs in the early stages of autoimmune diabetes in the absence of any structural injury and that ET_A receptor–dependent mechanisms control blood glucose levels and gene expression in the early stages of experimental type 1 diabetes. Further studies are needed to investigate the potential immunomodulatory role of ET receptors in autoimmune models of diabetes and, possibly, in patients.

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