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Original Contribution

AT_1-receptor blockade by telmisartan upregulates GTP-cyclohydrolase I and protects eNOS in diabetic rats

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ABSTRACT

Several enzymatic sources of reactive oxygen species (ROS) were described as potential reasons of eNOS uncoupling in diabetes mellitus. In the present study, we investigated the effects of AT_1-receptor blockade with chronic telmisartan (25 mg/kg/day, 6.5 weeks) therapy on expression of the BH_4-synthesizing enzyme GTP-cyclohydrolase I (GCH-I), eNOS uncoupling, and endothelial dysfunction in streptozotocin (STZ, 60 mg/kg iv, 7 weeks)-induced diabetes mellitus (type I). Telmisartan therapy did not modify blood glucose and body weight. Aortas from diabetic animals had vascular dysfunction as revealed by isometric tension studies (acetylcholine and nitroglycerin potency). Vascular and cardiac ROS produced by NADPH oxidase, mitochondria, eNOS, and xanthine oxidase were increased in the diabetic group as was the expression of NADPH oxidase subunits at the protein level. The expression of GCH-I and the phosphorylation of eNOS at Ser1177 was decreased by STZ treatment. Therapy with telmisartan normalized these parameters. The present study demonstrates for the first time that AT_1-receptor blockade by telmisartan prevents downregulation of the BH_4 synthase GCH-I and thereby eNOS uncoupling in experimental diabetes. In addition, telmisartan inhibits activation of superoxide sources like NADPH oxidase, mitochondria, and xanthine oxidase. These effects may explain the beneficial effects of telmisartan on endothelial dysfunction in diabetes.

Introduction

Diabetes mellitus is a major risk factor for the development of cardiovascular disease. Endothelial dysfunction is encountered early during the development of vascular damage [1]. Animal and human studies have demonstrated that increased oxidative stress largely accounts for this phenomenon since vitamin C was able to correct endothelial dysfunction in patients with diabetes mellitus type 1 and 2 [2,3]. As predominant sources of superoxide, the vascular NADPH oxidase [4–6], an uncoupled endothelial nitric oxide synthase (eNOS) [5,7,8], xanthine oxidase [9], and mitochondria [10] have been identified. The uncoupled eNOS has gained growing attention, since in almost all animal models with endothelial dysfunction, eNOS expression was up- rather than downregulated [5,11], but also dysfunctional, thereby shifting the superoxide ($O_2^{\cdot-}$)/nitric oxide ($^{\bullet}NO$) equilibrium toward $O_2^{\bullet-}$, but can also be a source of $O_2^{\bullet-}$ itself by transferring electrons to molecular oxygen in the uncoupled state [12]. The uncoupling reaction of eNOS is triggered largely by a peroxynitrite ($ONOO^-$) mediated oxidation of the eNOS cofactor tetrahydrobiopterin (BH_4) leading to the formation of the BH_3 radical and subsequently to dihydrobiopterin (BH_2) [8]. Intracellular depletion of BH_4 is counteracted mainly by the activity of the BH_4-synthesizing enzyme GTP cyclohydrolase I (GCH-I) and the BH_2 reducing enzyme dihydrofolate reductase (DHFR). There is good experimental evidence that GCH-I is downregulated or its activity is decreased in diabetic animals [13–16] and genetic overexpression represents a rescue mechanism to prevent endothelial dysfunction in diabetes [17,18]. Our group provided the first evidence that long-term AT_1-receptor blockade retards plaque formation [19], recouples eNOS, and simultaneously improves endothelial dysfunction in hypercholesterolemic Watanabe rabbits

Abbreviations: ACh, acetylcholine, DHE, dihydroethidine, DHFR, dihydrofolate reductase, DPI, diphenyleneiodonium, ECL, enhanced chemiluminescence, eNOS, endothelial $^{\cdot}NO$ synthase (type 3), GCH-I, GTP-cyclohydrolase I, GTN, glyceryl trinitrate (nitroglycerin), L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2*H*,3*H*) dione sodium salt, ROS, reactive oxygen species, STZ, streptozotocin, VASP, vasodilator-stimulated phosphoprotein.

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[20]. Recently, we were able to show that HMG-CoA-reductase inhibition with atorvastatin treatment recouples the dysfunctional eNOS in diabetic rats by increasing the expression of GCH-I and normalizing vascular BH_4 levels [13].

Antioxidative pleiotropic effects have been reported for the AT_1-receptor blocker (ARB) telmisartan in a mouse model of atherosclerosis by suppression of systemic oxidative stress and regression of atherosclerotic plaques [21], in patients with essential hypertension by suppression of RAGE (receptor for advanced glycation end products) [22] and in hemodialysis patients by decreasing the oxidative status of serum albumin [23]. In obese diabetic patients, telmisartan seemed to be superior to valsartan in terms of blood pressure lowering effects [24]. Moreover, telmisartan seems to work as effective as the antioxidant vitamin C in improving endothelial dysfunction in type I diabetic patients after normalization of glycemia [25]. More recently, the group of Bauersachs has reported on normalization of vascular oxidative stress and vascular dysfunction by telmisartan therapy of diabetic rats without providing detailed mechanistic insights in the process of eNOS un- and recoupling [26].

It remains to be established , whether in vivo treatment with AT_1-receptor blockers is able to upregulate downregulated expression of the GCH-I and whether sartan treatment is thereby able to prevent harmful events downstream of eNOS uncoupling mediated by decreased $^{\bullet}NO$ and increased $O_2^{\bullet-}$ formation.

Material and methods

Materials

For isometric tension studies, GTN was used from a Nitrolingual infusion solution (1 mg/ml) from G.Pohl-Boskamp (Hohenlockstedt, Germany). For induction of *diabetes* we used streptozotocin from Fluka (Steinheim, Germany). L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2*H*,3*H*)dione sodium salt) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich, Fluka, or Merck.

Animals and in vivo treatment

Male Wistar rats (6 weeks old, 250 g, Charles River Laboratories, Sulzfeld, Germany) were devided into 4 treatment groups: untreated controls (Ctr) versus telmisartan (Telmi) treatment (10 or 25 mg/day/kg body weight,) versus streptozotocin-induced diabetes mellitus type 1 (STZ) versus STZ/Telmi. For induction of diabetes mellitus type 1, rats were injected with a single dose of STZ into the vena dorsalis penis (60 mg/kg body weight, in 5 mM citrate buffer, pH 4.5). Animals from the other study arms were injected with the solvent. Telmisartan treatment was started 1 week after STZ injection and continued for 7 weeks. Diabetes was diagnosed by measuring glucose levels in whole blood (for STZ-treated rats it was diluted 1:5 with NaCl solution) using the ACCU-CHEK Sensor system from Roche Diagnostics GmbH (Mannheim, Germany).

Isometric tension studies

Vasodilator responses to ACh and GTN were assessed with endothelium-intact isolated rat aortic rings mounted for isometric tension recordings in organ chambers, as described previously [27,28]. The rat aorta was preconstricted with phenylephrine.

Detection of oxidative stress in serum, mitochondria, membrane fractions, cardiac tissue, and isolated aorta

Xanthine oxidase activity was measured in serum, which was diluted 1:1 with cytochrome *c* (100 μM) in PBS containing either hypoxanthine (1 mM) or allopurinol (1 mM). The reduction of cytochrome *c* at 550 nm was measured as the difference between hypoxanthine and allopurinol containing buffer. Superoxide formation rates were calculated using ε_{550}=19500 $mM^{-1}cm^{-1}$ for reduced cytochrome *c*. Isolated mitochondria were prepared from rat hearts according to a previously published protocol and ROS formation was detected by L-012 (100 μM) ECL as recently described [28–30]. Mitochondrial suspensions were diluted to a final protein concentration of 0.1 mg/ml in 0.5 ml of PBS buffer containing L-012 (100 μM). ROS production was detected after stimulation with succinate (5 mM final concentration). The CL was registered at intervals of 30 s over 5 min with a Lumat chemiluminometer (Berthold Techn., Bad Wildbad, Germany) and the signal was expressed as counts per minute at 5 min. Vascular superoxide production in intact aortic rings (5 mm) was determined in the presence or absence of L-NAME (500 μM) using lucigenin (5 μM)-enhanced chemiluminescence without any cofactor stimulation as described [31]. The CL was registered at intervals of 60 s over 20 min with a Lumat chemiluminometer and was normalized for the dry weight of aortic tissue. Vascular ROS formation was also determined using dihydroethidine (DHE, 1 μM)-dependent fluorescence in aortic cryo-sections as reported elsewhere [32]. Membrane fractions were prepared and NADPH oxidase activity was measured by lucigenin (5 μM) ECL in the presence of NADPH (200 μM) according to a published protocol [32,33]. Oxidative stress and superoxide were also measured by a modified HPLC-based method to quantify ethidium and 2-hydroxyethidium levels as previously described [34]. Briefly, heart tissue was incubated with 50 μM DHE for 30 min at 37 °C in PBS buffer. Heart pieces were snap-fozen and stored at − 80 °C until they were homogenized in 50% acetonitrile/50% PBS and centrifuged and 50 μl of the supernatant was subjected to HPLC analysis. The system consisted of a control unit, two pumps, mixer, detectors, column oven, degasser, and an autosampler (AS-2057 plus) from Jasco (Groß-Umstadt, Germany) and a C_{18}-Nucleosil 100-3 (125×4) column from Macherey & Nagel (Düren, Germany). A high pressure gradient was employed with acetonitrile and 25 mM citrate buffer, pH 2.2, as mobile phases with the following percentages of the organic solvent: 0 min, 36%; 7 min, 40%; 8–12 min, 95%; 13 min, 36%. The flow was 1 ml/min and DHE was detected by its absorption at 355 nm whereas 2-hydroxyethidium and ethidium were detected by fluorescence (Ex. 480 nm/Em. 580 nm). The signal was normalized on wet weight of the heart tissue.

Western blot analysis

Isolated aortic tissue was frozen and homogenized in liquid nitrogen. Proteins were separated by SDS-Page and blotted onto nitrocellulose membranes. After blocking, immunoblotting was performed with antibodies against α-aktinine (100 kDa) or actin (42 kDa) (1:2500, Sigma-Aldrich) as controls for loading and transfer, Nox1 (1:100, Santa Cruz Biotechnologies, USA) and Nox2 ($gp91^{phox}$, 1:1000, BD Biosciences, USA), Phospho-VASP (Ser239) (P-VASP: 1.5 μg/ml, Calbiochem, USA), eNOS (1:1000, BD Biosciences) and Phospho-eNOS (Ser1177) (1:1000, Cell Signaling Tech., USA), GTP-cyclohydrolase 1 (GCH-1: 1 μg/ml, Abnova Corp., Germany), dihydrofolate reductase (DHFR: 1 μg/ml, RDI Div. of Fitzgerald Ind., USA), $p67^{phox}$ and Rac1 (1:500 and 1:1000, BD Biosciences) and $p47^{phox}$ (1:500, Upstate, USA). To investigate the membrane association of soluble NADPH-oxidase subunits $p67^{phox}$, $p47^{phox}$, and Rac1 aortic lysates were separated into cytosolic and membrane fractions by ultracentrifugation (100,000 *g* for 1 h at 4 °C). Detection was performed by ECL with peroxidase-conjugated anti–rabbit/mouse (1:10000, Vector Lab., Burlingame, CA) and anti-goat (1:5000, Santa Cruz Biotechnologies) secondary antibodies. The antibody-specific bands were quantified by densitometry.

Statistical analysis

Results are expressed as mean ± SE. One-way ANOVA (with Bonferroni's or Dunn's correction for comparison of multiple means)