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Renal protection by low dose irbesartan in diabetic nephropathy is paralleled by a reduction of inflammation, not of endoplasmic reticulum stress

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ABSTRACT

Diabetes can disrupt endoplasmic reticulum (ER) homeostasis which leads to ER stress. ER stress-induced renal apoptosis seems to be involved in the development of diabetic nephropathy. The present study was designed to investigate the contribution of reduced ER stress to the beneficial effects of an angiotensin receptor blocker. Insulin-dependent diabetes mellitus was induced by streptozotocin injections to hypertensive mRen2-transgenic rats. After 2 weeks animals were treated with 0.7 mg/kg/day irbesartan. Blood glucose, blood pressure and protein excretion were assessed. Expression of ER stress markers was measured by real-time PCR. Immunohistochemistry was performed to detect markers of ER stress, renal damage and infiltrating cells. Glomerulosclerosis and apoptosis were evaluated. Diabetic mRen2-transgenic rats developed renal injury with proteinuria, tubulointerstitial cell proliferation as well as glomerulosclerosis and podocyte injury. Moreover, an increase in inflammation, podocyte ER stress and apoptosis was detected. Irbesartan somewhat lowered blood pressure and reduced proteinuria, tubulointerstitial cell proliferation and glomerulosclerosis. Podocyte damage was ameliorated but markers of ER stress (calnexin, grp78) and apoptosis were not reduced by irbesartan. On the other hand, inflammatory cell infiltration in the tubulointerstitium and the glomerulus was significantly attenuated. We conclude that irbesartan reduced renal damage even in a very low dose. The beneficial effects of low dose irbesartan were paralleled by a reduction of blood pressure and inflammation but not by a reduction of ER stress and apoptosis. Thus, sustained endoplasmic reticulum stress in the kidney does not necessarily lead to increased inflammation and tubulointerstitial or glomerular injury.

1. Introduction

End stage renal failure is often a consequence of nephropathy developing in patients with diabetes mellitus [1]. Endoplasmic reticulum stress is a typical feature of diabetic kidney disease [2]. The endoplasmic reticulum is responsible for proper folding of secretory and membrane proteins, which can be disturbed in conditions of nutrient deprivation, oxidative stress and abnormal calcium levels in the endoplasmic reticulum [2]. Accumulation of misfolded proteins leads to the unfolded protein response, which includes an increased expression of endoplasmic chaperone proteins like glucose-regulated protein 78 (grp78) [3]. On the one hand, the endoplasmic reticulum stress-induced unfolding protein response can help to restore endoplasmic reticulum homeostasis and proper protein folding, while on the other hand sustained endoplasmic reticulum stress results in increased apoptosis, which may contribute to organ damage [2,4].

In vitro studies in tubular epithelial cells revealed that albumin as well as high glucose both induce endoplasmic reticulum stress and thus might be causative for diabetic endoplasmic reticulum stress in the diabetic kidney [5]. In addition to tubular cells, endoplasmic reticulum stress was described also in glomerular cells of diabetic kidneys [6,7]. Inhibition of the renin–angiotensin system with an ACE inhibitor or the angiotensin receptor antagonist (AT1RA) olmesartan ameliorated renal tubular apoptosis and endoplasmic reticulum stress in normotensive rats with experimental diabetes [8,9].

Diabetic nephropathy is frequently associated with hypertension in humans [1]. Therefore, animal models were developed to mimic human diabetic nephropathy in combining hyperglycemia and hypertension. One of these models is the streptozotocin diabetic, mRen-2 transgenic hypertensive rat [10]. This model has the advantage of leading to marked damage to kidney tissue although the pathogenesis of this injury is complex, with high blood pressure per se playing an important role [11,12]. On the other hand, a considerable part of the

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glomerular lesions detected in the model is diabetes-induced [11]. Administration of angiotensin receptor antagonists (AT1RA) proved to be beneficial to attenuate diabetic nephropathy in hypertensive as well as normotensive models of diabetes mellitus [13–17]. The AT1RA irbesartan was used in doses from 5 to 65 mg/kg/day to ameliorate murine diabetic renal injury [17–19]. We aimed to attenuate diabetic nephropathy by suppressing diabetes-induced endoplasmic reticulum stress and renal apoptosis using a very low, non-hypotensive dose of irbesartan (0.7 mg/kg/day).

2. Material and methods

2.1. Rat model of diabetic nephropathy

Rats were housed in a room maintained at 22 ± 2 °C, exposed to a 12 hour dark/light cycle. The animals were allowed unlimited access to chow (#1320, Altromin, Lage, Germany) and tap water. EU Directive 2010/63/EU for animal experiments was followed. All procedures performed on animals were done in accordance with guidelines of the American Physiological Society and were approved by the local government authorities (Regierung von Mittelfranken, AZ # 621-2531.31-19/96). 20 male rats heterozygous for the mouse ren-2 transgene (TGR) with angiotensin II dependent hypertension [15] at an average body weight of 250 g were used for induction of diabetes by intraperitoneal injection of streptozotocin (STZ) (Sigma, Deisenhofen, Germany) (70 mg per kg of body weight) dissolved in 0.1 M sodium citrate buffer (pH 4.5) at the age of 12 weeks. 6 age-matched Sprague–Dawley–Hannover (SD) served as controls (Möllegaard, Eijby, Denmark). Two days later, blood was obtained from the tail vein and diabetes was confirmed by measurement of blood glucose using a reflectance meter (Glucometer Elite II, Bayer, Leverkusen, Germany). Only rats with a consistent blood glucose > 250 mg/dL were included (16 TGR and 6 SD). In 8 TGR-STZ, a low dose of irbesartan (0.7 mg/kg body weight/day; Sanofi-Aventis, Frankfurt, Germany) was administered 1 week after STZ injection for 4 weeks. The animals received osmotic minipumps (Alzet model 2004; Alza Scientific Products, Palo Alto, CA, USA) intraperitoneally, which delivered 0.25 μL/h for 28 days. All groups were followed for 5 weeks. Blood glucose and systolic blood pressure (as measured by tail-cuff plethysmography under light ether anesthesia) were monitored weekly (at 8 a.m.). At the end of the experiment, the rats were kept in metabolic cages to collect urine for 24 h. Subsequently, the rats were equipped with a femoral catheter under light isoflurane anesthesia and arterial blood pressure was measured in conscious rats via transducers (Grass Instruments, Quincy, USA) connected to a polygraph (Hellige, Freiburg, Germany) 4 h after termination of anesthesia. Urinary protein and serum glucose were analyzed with the automatic analyzer Integra 800 (Roche Diagnostics, Mannheim, Germany). Rats were sacrificed and kidneys were weighed and decapsulated. Renal tissue was fixed in methyl-Carnoy solution (60% methanol, 30% chloroform and 10% glacial acetic acid) for histology and immunohistochemistry. Cortical tissue was snap frozen in liquid nitrogen and processed for RNA analysis.

2.2. Real-time RT-PCR

Renal tissue was homogenized in 500 μL of RLT buffer reagent (Qiagen, Hilden, Germany) with an ULTRA-TURRAX for 30 s and total RNA was extracted from homogenates with RNeasy Mini columns (Qiagen) according to the standard protocol. First-strand cDNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Darmstadt, Germany) using random hexamers as primers. Final RNA concentration in the reaction mixture was adjusted to 0.1 ng/μL. Reactions without Multiscribe reverse transcriptase were used as negative controls for genomic DNA contamination. RT-products were diluted 1:1 with dH_2O before PCR procedure. PCR was performed with an ABI PRISM 7000 Sequence Detector System and SYBR Green or TaqMan reagents (Applied Biosystems) according to the manufacturers' instructions. All samples were run in triplicate. Specific mRNA levels were calculated and normalized to 18S rRNA as a housekeeping gene with the ΔΔCT method as specified by the manufacturer (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). Primer Express software (PerkinElmer, Foster City, CA) was used to design primer pairs. For detection of Bcl-2, the forward primer was 5′-GATGACTGAGTACCTGAACC-3′; the reverse primer was 5′-CCATATAGTTCCACAAAGGCAT-3′. For the detection of the housekeeping gene 18S, the forward primer was 5′-TTGA TTAAGTCCCTGCCCTTTGT-3′; the reverse primer was 5′-CGATCCGAGG GCCTCACTA-3′.

2.3. Renal histology and immunohistochemistry

After overnight fixation in methyl-Carnoy solution, tissues were dehydrated by bathing in increasing concentrations of methanol, followed by 100% iso-propanol. After embedding in paraffin, 3 μm sections were cut with a Leitz SM 2000 R microtome (Leica Instruments, Nussloch, Germany). After deparaffinization, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 20 min at room temperature. A mouse monoclonal antibody detecting proliferating cells (PCNA) was purchased from Santa Cruz Biotechnologies (Heidelberg, Germany) and used at a dilution of 1:50. The mouse monoclonal antibody against desmin was purchased from DAKO (Hamburg, Germany) and used at a dilution of 1:50. The mouse monoclonal antibody against synaptopodin was from Progen (Heidelberg, Germany) and used at a dilution of 1:100. The rabbit polyclonal antibody to WT-1 was from NeoMarkers (Fremont, CA, USA) and used at a dilution of 1:50. The rabbit polyclonal antibody to phosphorylated SMAD2/3 was from Santa Cruz Biotechnologies and used at a dilution of 1:5000. The rabbit polyclonal antibodies against calnexin and grp78 both were from Abcam (Cambridge, UK) and used at a dilution of 1:100. The mouse monoclonal antibody against the macrophage marker ED-1 was from Serotec (Biozol, Eching, Germany) and used at a dilution of 1:250. The mouse monoclonal antibody against the pan leukocyte marker CD45 was from BD Pharmingen (Heidelberg, Germany) and used at a dilution of 1:100. The rabbit polyclonal antibody against osteopontin was used as described before [20].

Intraglomerular ED-1 and WT-1 positive cells were counted in every third glomerulus of a given kidney section (no selection) and expressed as cells per glomerular section. Glomerular pSMAD2/3 positive cells were evaluated as described [21]. Interstitial PCNA, CD45 or ED-1 positive cells were counted in 30 medium-power (magnification 250×) cortical views per section. Counting was started in a random cortical field and in consecutive non-overlapping cortical fields to the right of the previous view without selection; if necessary, counting was continued at the opposite (left) edge of the section. Glomerular desmin, synaptopodin, calnexin and grp78 stainings were measured by Metaview software (Visitron Systems, Puchheim, Germany) in every third glomerulus per cross section, and the stained area was expressed as percentage of the total area of the glomerular tuft. Tubulointerstitial calnexin and osteopontin stainings were measured by Metaview software in 30 medium-power (magnification 250×) cortical views per section, and the stained area was expressed as percentage of the total area of the cortical view.

The kit to detect apoptotic cells (TACS 2 TdT Blue Label in situ Apoptosis Detection Kit) was from Trevigen (Biozol, Eching, Germany) and used according to the manufacturer's instructions. For analysis of glomerulosclerosis, 2 μm thick deparaffinized sections were stained with periodic acid-Schiff (PAS) and then evaluated on 50 consecutive glomeruli using a ×40 objective. A score from 0 (no sclerosis) to 4 (>75% sclerosis) was applied. Tubulointerstitial damage was assessed in 10 non-overlapping medium power randomly sampled views per PAS-stained kidney section using a tubulointerstitial damage score grading tubular atrophy, tubular dilatation, interstitial fibrosis and