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Modulation of glutathione peroxidase activity by age-dependent carbonylation in glomeruli of diabetic mice

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ABSTRACT

Aims: Low levels of reactive oxygen species and resulting oxidative protein modifications may play a beneficial role in cellular function under stress conditions. Here we studied the influence of age-dependent protein carbonylation on expression and activity of the anti-oxidative selenoenzyme glutathione peroxidase (GPx) in insulin-deficient *Ins2*^{Akita} mice and type 2 diabetic obese db/db mice in context of diabetic nephropathy.
Methods: Protein carbonylation, GPx expression and activity were examined in kidney tissue and lysates by common histological and protein biochemical methods.
Results: In kidneys of *Ins2*^{Akita} mice, carbonylated proteins, GPx-1 and GPx-4 expression were mainly detected in podocytes and mesangial cells. GPx activity was increased in kidney cortex homogenates of these mice. Remarkably, young animals did not show a concomitant increase in GPx expression but enhanced GPx carbonylation. No carbonylation-dependent modification of GPx activity was detected in db/db mice. In cultured podocytes hyperglycemia induced an increase in GPx expression but had no effect on activity or carbonylation. In kidney tissue sections of type 1 or type 2 diabetes patients, GPx-1 and GPx-4 expression but not overall protein carbonylation was significantly decreased.
Conclusions: These results indicate the existence of a threshold for beneficial carbonylation-dependent redox signaling during the progression of diabetic nephropathy.

1. Introduction

Diabetic nephropathy is the main reason for end stage renal disease in Western societies.[1] It is characterized by glomerular hyperfiltration, albuminuria, mesangial expansion, and loss of podocytes.[2] In addition to reactive glucose metabolites, increased levels of oxidative stress in diabetic individuals are considered as one of the driving forces for the development of diabetic nephropathy.[3] The enhanced formation of reactive oxygen species (ROS) can cause detrimental oxidation of DNA, lipids or proteins. One of the most frequent types of oxidative protein modification is the introduction of a carbonyl group into certain amino acids.[4] They appear most often on lysine, proline, arginine and threonine residues and can lead to a conformational change of the affected protein.[5] Elevated levels of protein carbonyls can be found in a variety of diseases including diabetes, Alzheimer's disease and multiple sclerosis. On the other hand, ROS are also an essential component of intracellular signaling pathways. ROS are produced in response to certain cellular stimuli and can modulate gene expression by activation of transcription factors.[6] In this context, protein carbonylation is also known for its role in oxidative signaling pathways.[7] However, the threshold beyond which ROS no longer act as signal transducing molecules but cause oxidative damage to cell constituents has yet to be determined.

Cellular elimination of ROS is chiefly mediated by anti-oxidative enzymes. Among these, the glutathione peroxidases (GPx) are of specific relevance for the detoxification of hydrogen peroxide (H_2O_2). The

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cytosolic GPx-1 is expressed ubiquitously in nearly all mammalian cells and forms tetramers whereas GPx-4 functions as a monomer.[8] In contrast to other GPx isoforms, GPx-4 is therefore able to reduce large fatty acid hydroperoxides such as phospholipid or cholesterol hydroperoxides. As a result, GPx-4 plays an important role in the protection of cellular membranes against oxidative stress.

Because expression of anti-oxidative enzymes can be enhanced by intense oxidative stress,[9] an oxidizing environment may also influence their activity. Thus, we could observe a carbonylation-dependent increase in activity of human recombinant GPx-1 and hypothesize that GPx carbonylation might be a novel mechanism to quickly adapt GPx activity to changing levels of oxidative stress below a certain threshold. Hence, this study investigates the carbonylation and activity of the anti-oxidative enzymes GPx-1 and GPx-4 in comparison to the overall amounts of carbonylated proteins during the progression of diabetic nephropathy. To this end, *Ins2^{Akita}* developing an insulin-dependent diabetes, including hyperglycemia and hypoinsulinemia, and the non-insulin-dependent type 2 diabetic db/db mice as well as podocytes cultured under normo- or hyperglycemic conditions were studied.

2. Material and methods

2.1. Animals

All animal experiments were performed with permission of the Regional Council Karlsruhe and conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 2011). Diabetic heterozygous Ins2Akita$^{+/-}$ (*Ins2^{Akita}*) mice, purchased from Jackson Laboratory (Charles River Laboratories, Germany), were bred at the animal facility of the University Hospital Mannheim, Heidelberg University. Age-matched non-diabetic homozygous Ins2Akita$^{-/-}$ littermates served as control. Kidneys of 1, 3, 6, and 8 months old male *Ins2^{Akita}* and control mice were used. Inbred male db/db (B6.BKS(D)-Lepr^{db}/J) and gender-matched control litter mates (10, 18 and 26 weeks old) were housed in the Interfaculty Biomedical Research Facility (IBF) of Heidelberg University, Germany. After killing approximately 0.5 ml blood was drawn by cardiac puncture, mixed with EDTA, stored at −20 °C, and used for determination of the relative HbA1c content by high performance liquid chromatography. Blood glucose levels were measured with the Accu-check Aviva glucometer (Roche, Germany). Body weight, HbA1c values and blood glucose levels of the mice used in this study can be found in Suppl. Fig. 1.

2.2. Cell culture

The podocyte cell line E11 (Cell Line Services, Germany) was cultivated at 33 °C with 10 U/ml interferon-γ. When a confluency of 75% was reached, cells were placed at 37 °C for 14 days to induce differentiation. Cells were cultured in RPMI medium (w/o glucose, Thermo Fisher Scientific, Germany, 11879-020) supplemented with 10% FCS, 1% antibiotics, 5 mmol/l glucose and 35 nmol/l Na_2SeO_3. Stimulation was performed with different glucose concentrations or mannitol as osmotic control for 24 h and 7 days, respectively, or with methylglyoxal (Sigma-Aldrich, Germany, M0252) for 24 h. To reduce GPx-1 protein levels in podocytes, cells were transfected using an siRNA transfection reagent (Santa Cruz Biotechnology, Germany, sc-29528).

2.3. Detection of intracellular reactive oxygen species

The fluorescent indicator dichlorodihydrofluorescein diacetate (DCFH-DA, Thermo Fisher Scientific, D-399, dissolved in DMSO) was used for the measurement of ROS. Cells were cultured in a 12-well-plate prior to the assay and pre-incubated for 30 min with 5 μmol/l DCFH-DA. 20 mmol/l glucose or mannitol was added for 1 h. Cells were carefully washed and light emission at 538 nm after excitation with 485 nm was measured (Fluoroskan Ascent, Thermo Fisher Scientific). A blank value of fluorescence derived from unstained cells was subtracted from the readings.

2.4. siRNA-based gene silencing

For the knock down of GPx-1 undifferentiated podocytes were cultured in a 6-well-plate at a density of 5×10^4 cells per well and cultivated at 37 °C. After 72 hrs cells were transfected with a GPx-1 targeting siRNA (Sigma-Aldrich, SASI_Mm02_00313265) or a non-targeting control siRNA (Dharmacon, USA, D-001206-13-05). Eight μl transfection reagent (Santa Cruz Biotechnology, sc-29528) was used with 1 μg siRNA. To increase transfection efficiency cells were transfected again 48 h after the first transfection and the effects of the knock down were analyzed 48 h later.

2.5. Protein detection

Protein detection by Western Blot and immunohistochemistry was done according to standard protocols. The following primary antibodies were used: GPx-1 (GeneTex, Irvine, CA, USA, GTX116040, 1:1,000 dilution), GPx-4 (abcam, Cambridge, UK, ab125066, 1:2,000 dilution), Dinitrophenolhydrazone (DNP) (invitrogen, Germany, A6430, 1:2,500 dilution), and β-actin (abcam, ab6276, 1:5,000 dilution) for Western Blot and α-Actinin-4 (Adipogen, Switzerland, AG-25T-0107, 1:300 dilution), CD31 (Santa Cruz Biotechnology, sc-18916, 1:50 dilution), DNP (1:100 dilution), GPx-1 (1:100 dilution), GPx-4 (1:100 dilution), PDGFRB (LifeSpan BioSciences, Seattle, WA, USA, LS-C106587, 1:50 dilution), and synaptopodin (Santa Cruz, sc-21537, 1:50 dilution) for immunohistochemistry.

For the detection of carbonylated proteins 10 μg protein from kidney or podocyte homogenates was treated with 2,4-dinitrophenolhydrazine (DNPH) solution (OxyBlot protein detection kit, Merck, Germany, S7150) according to the manufacturer's instructions and the reaction stopped by the addition of neutralization solution. To neutralize the pH of the samples for loading on an SDS gel, proteins were precipitated by adding four times the volume of ice-cold ethanol. After incubation at 0–4 °C for 15 min samples were centrifuged at 20,800 ×*g* and 4 °C for 15 min. The supernatant was removed and the protein pellet air-dried before resuspending it in 10 μl of 2× SDS sample buffer and detection of carbonylated proteins by Western Blot with an anti-DNP-antibody.

2.6. Western Blot

For the preparation of cell homogenates, cells were washed once and resuspended in homogenization buffer (25 mmol/l Tris-HCl, 0.55% KCl, 1 mmol/l EDTA, 2.5 mmol/l glucose) supplemented with protease and phosphatase inhibitors. Protein extraction was achieved by three times treating with ultrasound for 30 s (UP50H ultrasound processor, Hielscher Ultrasonics, Germany). Kidney homogenates were prepared by pestling 20 mg kidney tissue in a small mortar in the presence of 190 μl homogenization buffer. Cell or kidney homogenates were centrifuged at 1100 ×*g* and 4 °C for 5 min and the supernatants were stored at −80 °C. For Western Blot analysis 10–15 μg protein extracts were mixed with 4× sample buffer and boiled (95 °C for 5 min). Samples were then separated by denaturing 10 or 12% SDS-polyacrylamide gel electrophoresis according to standard protocols and subsequently transferred to a polyvinylidene fluoride transfer membrane. The membrane was blocked in 5% (w/v) BSA in TBS-T for 1 hr and then probed with primary antibodies at 4 °C overnight. The secondary, horse radish peroxidase (HRP)-labeled antibody was added for 1 h at ambient temperature. Proteins were detected using a chemiluminescence imaging system (ImageQuant LAS 4000 mini, GE Healthcare, Germany).

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