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HIPPOCAMPAL HEAT SHOCK PROTEIN 25 EXPRESSION IN STREPTOZOTOCIN-INDUCED DIABETIC MICE

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Abstract—Hippocampal abnormalities are believed to increase the risk of cognitive decline in diabetic patients. The underlying mechanism is unknown, but both hyperglycemia and oxidative stress have been implicated. Cellular stresses induce the expression of heat shock protein 25 (HSP25) and this results in cytoprotection. Our aim was to assess hippocampal expression of HSP25 in experimental diabetes. Mice were rendered diabetic by streptozotocin injection. Ten weeks after diabetes onset hippocampal HSP25 expression was studied by immunoblotting and immunohistochemistry (IHC). Expression of glial fibrillary acidic protein, nitrotyrosine, iNOS, HSP72, HSP90, and Cu/Zn superoxide dismutase (SOD) was assessed by either IHC or immunoblotting, Cu/Zn-SOD activity by enzymatic assay, and malondialdehyde (MDA) content by colorimetric assay. Hippocampal HSP25 was significantly increased in diabetic as compared to non-diabetic animals and localized predominantly within the pyramidal neurons layer of the CA1 area. This was paralleled by overexpression of nitrotyrosine, iNOS, SOD expression/activity, and enhanced MDA content. In experimental diabetes, HSP25 is overexpressed in the CA1 pyramidal neurons in parallel with markers of oxidative stress.

Key words: HSP25, Oxidative stress, experimental diabetes, hippocampus.

INTRODUCTION

Multiple organ systems are adversely affected by diabetes, including the brain, which undergoes changes that may increase the risk of cognitive decline (Ryan et al., 1993; Ott et al., 1999; Ristow, 2004). Studies in experimental diabetes have demonstrated progressive astrogliosis, pyramidal neuron apoptosis, altered synaptic plasticity, and reduced dendritic complexity within the hippocampus (Magariños and McEwen, 2000; Saravia et al., 2002; Valastro et al., 2002). The underlying mechanisms of these abnormalities are unknown; however, both hyperglycemia and oxidative stress have been implicated (Mastrocola et al., 2005; Nishikawa et al., 2000).

Heat shock proteins (HSPs) are ubiquitous, highly evolutionary conserved intracellular proteins (Ellis and van der Vies, 1991). Thermal, oxidative, hemodynamic, osmotic, and hypoxic stresses induce HSPs expression, and this stress response results in cytoprotection (Ellis and van der Vies, 1991; Feder and Hofmann, 1999). Specifically, HSPs prevent non-specific protein assembly, assist in denatured protein refolding, and interfere with pro-apoptotic pathways (Yenari et al., 2005).

Studies on the expression of HSPs in the diabetic hippocampus have shown that HSP60, a mitochondrial-specific molecular chaperon, is overexpressed in the hippocampal CA1 region and that HSP60 expression strongly correlates with that of superoxide dismutase (SOD) (Yuan et al., 2006), a marker of mitochondrial oxidative stress. By contrast, no abnormalities in HSP70 expression were observed (Guven et al., 2009).

HSP25 is a chaperone, binds to F-actin, and protects cells from both oxidative stress and apoptosis (Mehlen et al., 1997; Sanz et al., 2001). In addition, HSP25 interacts with HSP90 and synergizes with HSP72 in preventing neural cell death (Patel et al., 2005). HSP25 is weakly expressed in the normal hippocampus, but strongly induced in hippocampal neurons exposed to thermal stress (Kirbach and Golenhofen, 2011). Recent studies have shown that HSP25 expression is enhanced in the retina and the kidney (Barutta et al., 2008; Pinach et al., 2011) from mice made diabetic with streptozotocin (STZ), an antibiotic that causes pancreatic β-cell destruction, resulting in a state of insulin-dependent type 1 diabetes mellitus. Whether HSP25 is also altered in the brain, another important target organ of diabetes-induced injury, remains unknown.

The aim of the present study was to assess HSP25 expression in the hippocampus of STZ-induced diabetic mice in parallel with markers of oxidative stress.

EXPERIMENTAL PROCEDURES

Materials

All materials were purchased from Sigma (St. Louis, MO) unless otherwise stated.

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Abbreviations: DM, diabetic; GFAP, glial fibrillary acidic protein; HSP25, heat shock protein 25; HSPs, heat shock proteins; IHC, immunohistochemistry; MDA, malondialdehyde; ND, non-diabetic; SOD, superoxide dismutase; STZ, streptozotocin.

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Animals and induction of diabetes

Eight-week-old male C57BL6 mice from Jackson Laboratories (Bar Harbor, ME) were used in the present study. All animal procedures were in accordance with the Italian law (D.L.116/1992) and animals were maintained on a normal diet under standard animal house conditions. Diabetes was induced by intraperitoneal injections of STZ in citrate buffer, pH 4.5 (55 mg/kg body wt/day), delivered in five consecutive daily doses. Mice sham injected with sodium citrate buffer were used as controls. Diabetes onset was confirmed by blood glucose levels > 250 mg/dl 4 weeks after the first dose of STZ. After 10 weeks of experimental diabetes, mice were euthanized by decapitation. Brain hemispheres were fixed in 4% neutral-buffered formaldehyde and then paraffin embedded for light microscopy. The hippocampus was isolated, frozen in N_2, and stored at −80 °C for protein analysis.

Metabolic and physiological parameters

Before euthanasia blood samples were taken via saphenous vein puncture on alert 4-h-fasted animals, and glucose levels measured using a glucometer (Accu-chek; Roche Applied Science). Glycated hemoglobin was measured in whole blood samples obtained at the time of killing by quantitative immunoturbidimetric latex determination (Sentinel Diagnostic, Milan, Italy).

Immunohistochemistry

Immunohistochemical staining was performed on 4-μm paraffin sections of fixed tissue. Briefly, sections were dewaxed, rehydrated, and immersed in 0.01 M citrate buffer pH 6.0 at 100 °C for antigen retrieval. Endogenous peroxidase activity was quenched by incubation with 3% H_2O_2. Endogenous avidin-binding activity was inhibited by sequential treatment with avidin–biotin, and non-specific binding sites blocked with 3% BSA. For immunodetection, sections were incubated overnight with mouse anti-glial fibrillary acidic protein (GFAP, Calbiochem, Darstadt, Germany), rabbit anti-HSP25 (Stressgen, Ann Arbor, MI, USA), or rabbit anti-nitrotyrosine (anti-N-Tyr Abcam, Cambridge, UK) antibodies, then the specific staining was detected using the high-sensitive-Labeled StreptAvidin Biotin (LSAB) + system-HRP (Dako, Glostrup, Denmark) that uses a refined avidin–biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. Sections were counterstained with Mayer's hemallume and visualized with an epifluorescence microscope (Olympus-Bx4I) connected by a photographic attachment (Carl Zeiss, Oberkochen, Germany). For each antibody, a negative control was included in which the primary antibody was replaced with a non-immune isotypic control antibody. In semiquantitative immunohistochemical analysis, the cellular area of nucleated-positive cells with a staining intensity over an established threshold was calculated by a computer-aided image-analysis system (Axiovision 4.7; Carl Zeiss) on digital images of hippocampal sections, as previously described (Saravia et al., 2002). Results were then expressed as percentage increase over non-diabetic controls. In addition, GFAP staining was also calculated as the number of GFAP-positive cells per area (65×10^3 μm^2). Evaluations were performed by two independent investigators in a blinded fashion.

Cu/Zn-SOD activity

Cu/Zn-SOD activity was evaluated in cytosolic hippocampal extracts by a kinetic enzymatic commercial assay kit (Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer's instructions.

Malondialdehyde measurement

Malondialdehyde (MDA), a marker of lipid peroxidation, was measured by a commercial colorimetric assay kit (BioVision, Milpitas, USA) following manufacturer's instructions. MDA levels in hippocampal sample homogenates were expressed as nanomoles of MDA per milligram of protein.

Immunofluorescence

Expression of Cu/Zn SOD was assessed by indirect immunofluorescence using a sheep anti-mouse primary antibody (Calbiochem-Merck, Milan, Italy) and a FITC-conjugated donkey anti-sheep secondary antibody (Dako). Sections were examined using an Olympus epifluorescence microscope (Olympus Bx4I) and digitized with a high-resolution camera (Carl Zeiss).

Double immunofluorescence

Double immunofluorescence was performed for HSP25 and either Cu/Zn-SOD or GFAP on hippocampal sections. After blocking, sections were incubated with either an anti-Cu/Zn-SOD antibody or an anti-GFAP antibody for 1 h, followed by a labeled secondary antibody (Cu/Zn-SOD:FITC-conjugated donkey-anti-sheep antibody; GFAP:TRITC-conjugated goat-anti-mouse antibody). After washing, sections were incubated with an anti-HSP25 antibody for 18 h at 4 °C, followed by 1-h incubation with a biotinylated swine anti-rabbit IgG (Dako) and then with FITC/Alexa 555-conjugated streptavidin (Invitrogen, Milan, Italy). Sections were examined using an Olympus epifluorescence microscope (Olympus Bx4 I) with photographic attachment (Carl Zeiss). The images were color-combined and assembled into photomontages by using Adobe Photoshop (Universal Imaging, West Chester, PA).

Protein extraction

For total protein extraction isolated hippocampal tissue was homogenized in a modified RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 μg/mL leupeptin, 1 μg/ml aprotinin, 1 mM PMSF).