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High-mobility group box 1 enhances the inflammatory process in diabetic lung

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ABSTRACT

Diabetes mellitus generates metabolic changes associated with inflammatory events that may eventually affect all body tissues. Both high-mobility group box 1 (HMGB1) and β-catenin are active players in inflammation. The study aimed to determine whether HMGB1 modulates the β-catenin activity in supporting inflammation, using an experimental type 1 diabetes mouse model. The protein and gene expression of HMGB1 were significantly increased (2-fold) in the diabetic lung compared to control and were positively correlated with the HMGB1 levels detected in serum. Co-immunoprecipitation of HMGB1 with RAGE co-exists with activation of PI3K/AKT1 and NF-kB signaling pathways. At the same time β-catenin was increased in nuclear fraction (3.5 fold) while it was down-regulated in diabetic plasma membrane (2-fold). There was no difference of β-catenin gene expression between the control and diabetic mice. β-Catenin phosphorylation at Ser552 was higher in diabetic nuclear fraction, suggesting that AKT1 activation promotes β-catenin nuclear translocation. In addition, c-Jun directly binds β-catenin indicating the transcriptional activity of β-catenin in diabetes, sustained by significantly COX2 increase by 6-fold in the cytosolic extract of diabetic lung compared to control. Taken together, the data support the new concept that HMGB1 maintains the inflammation through RAGE/AKT1/β-catenin pathway in the diabetic lung.

1. Introduction

Diabetes mellitus, a disorder characterized by chronic hyperglycemia and impaired insulin signaling, generates metabolic changes and an inflammatory status that will eventually affect all body tissues. The presence of biochemical modifications accompanied by alteration of lung structure in diabetes has been reported by numerous experimental studies over the years. Therefore, elevated activity of angiotensin-converting enzyme [1], diminished level of insulin-like growth factor-I [2], suppression of aniline p-hydroxylase activity [3] and increased susceptibility to glycation of lung proteins [4] are only few deregulated proteins described. The thickening of the alveolar basement membranes of the diabetic lung in human or rats with streptozotocin-induced diabetes [5,6], the drastic morphological alterations of the lung structure in experimental diabetes associated with hyperlipidaemia [7] or the reduced strength of respiratory muscle observed in patients with diabetes mellitus [8] are events that signal the functional disturbance of pulmonary system. The lung dysfunction in diabetes can also be the consequence of the systemic inflammation and its abnormal regulation. Therefore, the inflammation is now thought to be an important link between diabetes and impaired lung function [9].

High-mobility group box 1 (HMGB1) is a nuclear DNA-binding protein with alarmin activity, that has been shown to activate pro-inflammatory responses after being released by necrotic cells into the extracellular environment [10] or through its active secretion from living inflammatory cells [11]. Therefore, studies in both patients and animal models have established the involvement of HMGB1 in the pathogenesis of several inflammatory conditions including autoimmunity, cancer, rheumatic diseases, trauma, hemorrhagic shock, and ischemia-reperfusion injury [12,13]. Extracellular HMGB1 interacts with a variety of receptors, including the receptor for advanced glycation end products (RAGE) [13]. HMGB1 signaling via RAGE triggers intracellular pathways that promote cytokines production through nuclear factor-kappa B (NF-kB) activation [14]. Phosphatidylinositol 3-kinase (PI3K)/AKT is one of the signaling pathways that can be activated by HMGB1-RAGE

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interaction and is able to regulate the multifunctional protein β-catenin [15]. Several studies have shown that nuclear β-catenin interacts with the transcription factors, T-cell factor (TCF) and lymphoid enhancer factor (LEF) to form a complex that is involved in the transcriptional regulation of inflammatory molecules as cyclooxygenase 2 (COX2) and matrix metalloproteinases (MMPs) [15–17].

Based on these reported data, the study aims to investigate whether HMGB1 signaling cascade was activated in the diabetic lung tissue and to search a possible link between HMGB1 and β-catenin pathway. The results will be important for understanding the role of HMGB1 in the inflammatory mechanisms underlying lung dysfunction associated with diabetes.

2. Materials and methods

2.1. Reagents and consumables

The chemicals and solvents used were of analytical grade, liquid chromatography or mass spectrometry (MS) grade (Sigma, St. Louis, MO, USA). The protein concentration of the samples was measured with Precision Red Protein Assay Reagent (Cytoskeleton, Denver, USA) or Amido Black protein assay (J.T. Baker Chemical Co, Phillipsburg, USA). The columns, the equipment and software used in MS analysis were from Thermo Fisher Scientific (San Jose, CA, USA). All primary antibodies were purchased from Abcam (Cambridge, GB), except antibodies for GSK3β, phospho- GSK3β (Ser9), phospho- β-catenin (Ser552) and COX2 that were acquired from Thermo Fisher Scientific (IL, USA). Secondary antibodies linked to horseradish peroxidase (HPR) were purchased from Sigma (MO, USA), Enhanced chemiluminescence (ECL) Western Blotting Substrate kit was supplied by Thermo Fisher Scientific (IL, USA) and HMGB1 ELISA kit was from IBL International GMBH (Hamburg, DE). RNeasy Mini Kit was purchased from Qiagen (Hilden, DE). Transcriptor First Strand cDNA Synthesis Kit, LightCycler 480 SYBR Green I Master mix and protease inhibitor mixture were obtained from Roche (Mannheim, DE). Protein A/G plus-agarose was from Santa Cruz Biotechnology (Dallas, USA) and trypsin was purchased from Promega (WI, USA).

2.2. Animals

Ins-HA transgenic mice express the influenza virus A/PR8/34 hemagglutinin (HA) protein specifically in pancreatic β cells under the rat insulin promoter. TCR-HA/Ins-HA mice are double transgenic for expressing specific T cell receptor (TCR) for immunodominant peptide 110–120 of influenza virus A/PR8/34 HA presented by I-E^d MHC molecule and the HA of the same virus in the pancreatic beta-cells [18]. TCR-HA/Ins-HA (D) mice which develop spontaneous diabetes and non-diabetic Ins-HA (C) mice were used as experimental models. The two groups of male mice ($n = 8$ each) were maintained in standard housing conditions and had free access to standard diet and fresh water. TCR-HA/Ins-HA mice were considered diabetic when blood glucose exceeded 250 mg/dl on two successive blood assays. At 3 months of age, the blood was collected than the animals were killed and the lungs were harvested and kept frozen until use. All animal experiments were performed in accordance with the EU Directive 2010/63/EU, regulations of the ethic Committee of ICBP N. Simionescu and Romanian Law no. 43/2014.

2.3. Protein extraction

Fractions enriched in nuclear, cytoplasmic and membrane proteins were isolated through a slightly modified sequential extraction method [19]. Briefly, equally weighted tissue fragments were homogenized in buffer containing 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% Triton X-100 and 10 μg/ml protease inhibitor mixture, followed by centrifugation at 4500× *g* for 5 min. The collected supernatants were considered the cytosolic fractions. The pellets were suspended and kept at 4 °C for 1 h in 20 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 20% glycerol, 0.1% Triton X-100 buffer and protease inhibitor mixture, with occasional agitation. After centrifugation at 14,000× *g* for 15 min at 4 °C, the supernatants were collected as nuclear fractions. The remaining pellets were further re-suspended in 2× concentrated Laemmli buffer and incubated over night at 4 °C. After a vigorous vortex, the membrane extracts were recovered by centrifugation at 14,000× *g* for 30 min at 4 °C.

2.4. Mass spectrometry analysis

The samples were cleaned-up for lipid and salt removal by precipitation with acetone for 60 min at −20 °C. The reduction of cysteine residues of the precipitated proteins was performed with a denaturant buffer containing 8 M urea, 0.1 M Tris–HCl (pH 8.8), 0.1 mM EDTA and 20 mM dithiothreitol, for 1 h, under agitation at room temperature. This step was followed by alkylation using 80 mM iodacetamide in 0.1 M Tris–HCl and 0.1 mM EDTA buffer, for 90 min, under agitation at room temperature, in the dark. Samples were subjected to trypsin digestion according to manufacturer's instructions and the desalting step was conducted using Waters Sep Pek C18 columns (Waters, MA, USA). LC-MS/MS experiments were performed using the EASY −nLC II system coupled to the LTQ-Orbitrap Velos Pro mass spectrometer. For each analysis, the sample was loaded into C18 EASY trap column (2 cm × 100 μm internal diameter, 5 μm, 120 Å) connected to the C18 EASY analytical column (10 cm × 75 μm internal diameter, 3 μm, 120 Å). Peptides were eluted at a flow rate of 300 nl/min during 90 min of chromatographic separation using 2–35% gradient generated from solvent B (acetonitrile with 0.1% formic acid) over A (0.1% formic acid in water). For ionization, 2 kV on-emitter voltage and 275 °C capillary temperature were used. The analyses were carried out with CID fragmentation mode in a top 10 data-dependent configuration at 60 k resolving power for a full scan, with monoisotopic precursor selection enabled and mass lock correction across the 350–2000 m/z domain.

2.5. Protein identification and data mining

Protein identification was performed using Proteome Discoverer 1.4. The search was achieved with the Mascot algorithm 2.4.1 (Matrix Science, London, GB) against the mouse UniProtKB/SwissProt fasta database, build 04.2013. A maximum of 2 missed cleavage sites was allowed. A mass tolerance for the precursor was set on 10 ppm and for the fragment on 0.8 Da. Oxidation of methionine and deamidation of asparagine and glutamine were considered as dynamic modifications while carbamidomethylation of cysteine was treated as fixed modification. The search workflow contained also a Percolator validation node [20] using a decoy database search with a target FDR lower than 0.05. Proteome Discoverer Daemon 1.4 was utilized for performing Multidimensional Protein Identification Technology (*MudPIT*) as well as batch searches based on 3 technical replicates for each biological condition. General protein annotation and classification based on *Cellular Component*, *Molecular Function* and *Biological Process* were performed using the Gene Ontology (slim version) database accession characteristic within the Protein Center software v.3.12.