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Research paper

Redox imbalance and mitochondrial abnormalities in the diabetic lung

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ABSTRACT

Although the lung is one of the least studied organs in diabetes, increasing evidence indicates that it is an inevitable target of diabetic complications. Nevertheless, the underlying biochemical mechanisms of lung injury in diabetes remain largely unexplored. Given that redox imbalance, oxidative stress, and mitochondrial dysfunction have been implicated in diabetic tissue injury, we set out to investigate mechanisms of lung injury in diabetes. The objective of this study was to evaluate NADH/NAD^+ redox status, oxidative stress, and mitochondrial abnormalities in the diabetic lung. Using STZ induced diabetes in rat as a model, we measured redox-imbalance related parameters including aldose reductase activity, level of poly ADP ribose polymerase (PAPR-1), NAD^+ content, NADPH content, reduced form of glutathione (GSH), and glucose 6-phophate dehydrogenase (G6PD) activity. For assessment of mitochondrial abnormalities in the diabetic lung, we measured the activities of mitochondrial electron transport chain complexes I to IV and complex V as well as dihydrolipoamide dehydrogenase (DLDH) content and activity. We also measured the protein content of NAD^+ dependent enzymes such as sirtuin3 (sirt3) and NAD(P)H: quinone oxidoreductase 1 (NQO1). Our results demonstrate that NADH/NAD^+ redox imbalance occurs in the diabetic lung. This redox imbalance upregulates the activities of complexes I to IV, but not complex V; and this upregulation is likely the source of increased mitochondrial ROS production, oxidative stress, and cell death in the diabetic lung. These results, together with the findings that the protein contents of DLDH, sirt3, and NQO1 all are decreased in the diabetic lung, demonstrate that redox imbalance, mitochondrial abnormality, and oxidative stress contribute to lung injury in diabetes.

1. Introduction

Diabetes is a problem of glucose metabolism and diabetes complications is the outcome of glucose toxicity, which is often manifested by increased protein glycation, activation of the polyol pathway and poly ADP ribose polymerase (PARP), and protein kinase C activation [1–5]. Mechanistically, all these hyperglycemia upregulated pathways can eventually lead to production of reactive oxygen species (ROS) that then induce oxidative stress, mitochondrial dysfunction, and cell death [6,7]. Although the lung is one of the least studied organs in diabetes complications, increasing evidence has indicated that the lung is a target of diabetic injury [8–11]. Nevertheless, the underlying mechanisms remain largely unknown.

As glucose is one of the major sources of NADH, its excess can often lead to excess NADH production and NAD^+ deficiency, thereby causing NADH/NAD^+ redox imbalance [12]. The major source of this redox imbalance is thought to come from the activation of the polyol pathway and poly ADP ribose polymerase (PARP) [13–16]. On one hand, the polyol pathway converts NADPH to NADH when it transforms glucose to fructose via a two–reaction mechanism [17], resulting in NADH overproduction at the consumption of glucose [18,19]. On the other hand, as PARP uses NAD^+ as its substrate and is usually over-activated by DNA oxidative damage in diabetes [20], cellular NAD^+ could be potentially depleted [21–23]. Therefore, the overall outcome of the two activated pathways is NADH/NAD^+ redox imbalance with diminished levels of NAD^+ and increased levels of NADH, leading to reductive stress that gradually progresses to oxidative stress [24].

Oxidative stress occurs when cellular antioxidative system is defeated by ROS that are overproduced under a variety of disease conditions including diabetes [25]. As mitochondrion is a major source of ROS and a target of ROS [26,27], its abnormalities have been thought to contribute to diabetic pathogenesis [28]. However, whether mitochondrial abnormalities also occur in the diabetic lung remains to be evaluated. In the present study, using STZ induced diabetes in rat as a model; we characterized pulmonary redox imbalance and its associated pathways. Specifically, we measured the activities of mitochon-

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drial membrane complexes I to V. We also measured the enzyme activities of mitochondrial dihydrolipoamide dehydrogenase (DLDH) and its possible modifications by protein acetylation. Additionally, lung mitochondrial ROS production and overall protein oxidative damage were quantified. NAD(P)H: quinone oxidoreductase 1 (NQO1) protein content and activity and sirtuin 3 (sirt3) protein content were also evaluated in the context of redox imbalance and mitochondrial abnormalities in the diabetic lung.

2. Materials and methods

2.1. Chemicals

Biotin-linked aldehyde reactive probe ARP) for protein carbonyl assay was from Cayman Chemical (Ann Arbor, MI). Dihydrolipoamide was synthesized from lipoamide in our own laboratory using sodium borohydride as previously described [29,30]. ε-amino-*N*-caproic acid was obtained from MP Biochemicals. Acrylamide/bisacrylamide, ammonium persulfate, Bradford protein assay solution, coomassie brilliant blue (CBB) R-250, immunoblotting membrane, and an ECL immunochemical detection kit were from Bio-Rad laboratories (Richmond, CA, USA). NADH, BSA, lipoamide, EDTA, ATP, and NBT chloride tablets were obtained from Sigma (St. Louis, MO, USA). Serva Blue G was purchased from Serva (Heidelberg, Germany). Anti-PARP antibody was purchased from Trevigen (Gaithers burg, MD). Anti-NQO1 antibodies were from Sigma. Rabbit anti-DLDH polyclonal antibodies (IgG) and goat anti-rabbit IgG conjugated with horseradish peroxidase were purchased from US Biological (Swampscott, MA, USA) and Invitrogen (San Diego, CA, USA), respectively. Other antibodies were from Abcam (Cambridge, MA).

2.2. Diabetes induction in rats

Young adult male Sprague Dawley rats obtained from Charles River were used in this study. Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg body weight) into rats weighing 220–250 g after overnight fasting as previously described [31]. STZ solutions were made fresh by dissolving in 0.1 M sodium citrate buffer (pH 4.5) and control animals received sodium citrate buffer only. Blood glucose concentration was monitored once a week using blood glucose test strips (FreeStyle lite from Abbott Diabetes Care Inc., Alameda, California). Rats with blood glucose contents exceeding 200 mg/dl were deemed diabetic. Four weeks post STZ injections, rats were sacrificed and tissues were collected. All animal studies procedures were approved by the UNTHSC committee for research.

2.3. Isolation of lung mitochondria

Mitochondria from the lung were isolated according to a previously described method [32] with slight modifications. Essentially, lung tissues were homogenized (1g/10 ml isolation buffer) in mitochondrial isolation buffer containing 15 mM MOPS (pH 7.2), 70 mM sucrose, 230 mM mannitol, and 1 mM K^+-EDTA. The homogenates were centrifuged at 800g for 10 min at 4 °C. The resulting supernatant was further centrifuged at 8,000g for 10 min also at 4 °C. The resulting pellet containing crude mitochondria was washed with 10 ml of the isolation buffer followed by centrifugation under the same conditions. The obtained mitochondrial pellet was either used immediately or frozen at −80 °C until use.

2.4. Measurement of H_2O_2 and protein carbonyls

Lung tissue homogenate H_2O_2 was measured by the Amplex Red method [33] using a kit purchased from Invitrogen (catalog number A22188). Protein carbonyls of whole mitochondrial preparation were measured by derivatization with biotin-linked aldehyde reactive probe (ARP) [34] followed by SDS-PAGE resolution of the carbonylated proteins and Western blot assay and densitometric quantification of each gel lane.

2.5. Measurement of NAD^+/NADH ratio, NADPH, and ATP

Lung tissue homogenate NAD^+/NADH ratio was measured spectrophotometrically by following the changes at 340 nm using a kit from BioAssay (Hayward, CA). NADPH content was measured by a kit from BioVision (Milpitas, CA, Catalog number: K347-100) according to the manufacturer's instructions. ATP content was determined colorimetrically by the ATP Colorimetric/Fluorometric Assay kit that is also from BioVision (Milpitas, CA, catalog number K354-100). This method quantifies phosphorylated glycerol that can be easily monitored at 570 nm.

2.6. Measurement of enzyme activities

Aldose reductase activity was measured spectrophotometrically by following the decrease of NADPH's absorption at 340 nm as previously described [35]. DLDH dehydrogenase activity was measured in the forward reaction as previously described [36,37]. Measurement of mitochondrial complexes I, IV and V activities was also conducted as previously described using in-gel based assays or spectrophotometric assays [38]. Activities for complexes II and III were measured spectrophotometrically as previously described [39,40]. NQO1 activity was measured according to the method of Lind et al. [41] and G6PD activity was measured by monitoring NADPH production at 340 nm as previously described [42]. Caspase-3 activity was measured using a kit also from BioAssay (Hayward, CA). Mitochondrial membrane potential was measured by a kit purchased from BioVision (Milpitas, CA) according to the manufacturer's instruction manual.

2.7. Polyacrylamide gel electrophoresis and Western blot analysis

SDS-PAGE (typically 10% resolving gel) was performed according to standard procedures [43]. One of the resulting gels was stained with Coomassie colloid blue [38], and the other gel was subjected to electrophoretic transfer to membrane for immunoblotting [44]. Signals on the immunoblotting membrane were visualized with an enhanced chemiluminescence kit. Nongradient blue native gel electrophoresis (BN-PAGE) was performed as previously described [36]. All images were scanned by an EPSON PERFECTION 1670 scanner. All densitometric quantifications of gel images were analyzed by AlphaEaseFC software.

2.8. Data analysis

Where appropriate, all values were presented as mean ± SEM. Statistical data analysis was performed using GraphPad's 2-tailed unpaired *t*-test (GraphPad, San Diego, CA). A p value less than 0.05 ($p < 0.05$) was deemed statistically significant.

3. Results

3.1. Redox imbalance in the diabetic lung

In many diabetic tissues that have been well studied, redox imbalance between NADH and NAD^+ is the primary driving force for ROS production and oxidative stress [12,13]. This redox imbalance is believed to mainly originate from two enzyme systems activated by persistent hyperglycemia. One reaction is the polyol pathway including aldose reductase and sorbitol dehydrogenase [45]. This pathway converts glucose to fructose and NADPH to NADH, resulting in overproduction of NADH [46]. Another pathway is poly ADP ribose polymerase (PARP) that uses NAD^+ as its substrate [47]. This enzyme