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Mitochondrial-generated ROS down regulates insulin signaling via activation of the p38MAPK stress response pathway

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ABSTRACT

Impairment of insulin signaling and hepatic insulin resistance has been attributed to ROS-mediated activation of p38MAPK stress response signaling. Our research focused on whether (a) ROS generated by mitochondrial electron transport chain complex I (ETC-CI) dysfunction, via the use of Rotenone, inactivates insulin signaling; and (b) the p38MAPK pathway is involved in the ROS-induced impairment of insulin signaling. Our results show that in primary mouse hepatocytes the CI inhibitor, Rotenone, (a) induces IRS-1 Ser^{307} phosphorylation that is blocked by the anti-oxidant NAC or by the p38MAPK inhibitors, SB203580 and SB202190; (b) inhibits insulin-stimulated AKT-Ser^{473} and GSK3β-Ser^{9} phosphorylations, in a manner that is not responsive to reversal by the anti-oxidant NAC or by the p38MAPK inhibitors, SB203580 and SB202190. We conclude that rotenone-induced insulin resistance involves a p38MAPK-dependent mechanism for the inhibition of the proximal end of insulin signaling (IRS1), and a p38MAPK-independent mechanism for the inhibition of the distal end (AKT and GSK3β). Our study suggests that ROS generated by inhibition of ETC CI, promotes hepatic insulin resistance partly via activation of the p38MAPK stress-response pathway.

1. Introduction

Increased levels of oxidative stress (ROS) generated by mitochondrial dysfunction or ROS-associated inflammation are associated with the onset, progression, and development of Type 2 diabetes (T2D) as well as development of its late complications such as chronic insulin resistance, cardiovascular disease, and metabolic syndrome (Henriksen et al., 2011; Pomytkin, 2012; Zhai et al., 2011; Zhang et al., 2013). Mitochondrial dysfunction and more specifically, dysfunctional electron transport chain (ETC) activity, mainly at complex I (CI) and complex III (CIII), is a major source of ROS and may play a key role in promoting diseases of oxidative stress such as T2D (Bloch-Damti and Bashan, 2005; Evans et al., 2005, 2003; Fridlyand and Philipson, 2006; Rolo and Palmeira, 2006). Furthermore, the common stress signaling kinases, p38MAPK, IKKβ, JNK and ERK, which are induced by ROS-associated oxidative stress and inflammation, promote the development of T2D characteristics that include impaired insulin signaling and insulin resistance (Henriksen et al., 2011; Pomytkin, 2012).

The mechanism of ROS-mediated inhibition of insulin signaling has been shown to involve the oxidative stress-mediated inactivation of IRS-1/IRS-2 by serine phosphorylation and proteasomal-independent degradation (Alexander et al., 2003; Goldstein et al., 2005; Potashnik et al., 2003). The fact that mitochondria are a major source of endogenous ROS emphasizes the importance of understanding the mechanism by which mitochondrial-ROS induced stress signaling contributes to the impairment of the insulin signaling processes. The consequences of oxidative stress and chronic inflammation involve the activation of multiple serine kinase cascades (Copps and White, 2012) some of which target the insulin signaling pathway by phosphorylation of specific serine/threonine residues of IRS1and IRS2 (Birnbaum, 2001; Sykiotis and Papavassiliou, 2001; Zick, 2001). These serine/threonine phosphorylations attenuate the association of IRS proteins with the β-subunit of the insulin receptor (IR), as well as association with their downstream target proteins such as PI3K (Aguirre et al., 2000; Griffin et al., 1999; Henriksen et al., 2011; Hers et al., 2002; Kellerer et al., 1998; Li et al., 1999; Luo et al., 2005; Paz et al., 1997; Qiao et al., 1999; Styskal et al., 2012).

Furthermore, it has been shown that the major stress response

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kinases, p38MAPK, IKKβ, NFκB and ERK that are induced by dysfunctional mitochondrial-generated ROS and inflammation, inhibit insulin-stimulated IRS-1 tyrosine phosphorylation as well as its interaction with PI3K resulting in a decrease of the downstream signaling activities and eventually attenuation of insulin action (Dhand et al., 1994; Gao et al., 2010; Henriksen et al., 2011; Luo et al., 2005; Taniguchi et al., 2007; Yu et al., 1998).

Past studies have shown that oxidative stress generated by H_2O_2 (in vitro) and by physiological conditions such as hyperglycemia, inhibit insulin stimulated glucose transport (Pomytkin, 2012; Rudich et al., 1999; Tirosh et al., 1999; Wu et al., 2005) and that this response is blocked by antioxidant treatment (Evans et al., 2002). Furthermore, in L6 muscle cells activation of p38MAPK by H_2O_2 is linked to the oxidative stress-mediated inhibition of insulin signaling (glucose transport) (Blair et al., 1999; Carlson and Rondinone, 2005; Henriksen et al., 2011; Styskal et al., 2012) and is reversed by inhibition of p38MAPK (Konrad et al., 2001; Somwar et al., 2002; Somwar et al., 2000). Our studies have shown that oxidative stress generated by inhibition of ETC-CI (rotenone), CII (3-NPA); CIII (antimycin) activate the ASK1 → p38MAPK stress response pathway (Hsieh and Papaconstantinou, 2006). We thus hypothesized that ETC-generated ROS-mediated activation of p38MAPK would link oxidative stress generated by mitochondrial dysfunction to the inhibition of insulin signaling via serine phosphorylation. We propose that the pathway of development of T2D and insulin resistance in response to mitochondrial generated ROS involves the phosphorylation/dephosphorylation of certain serine residues of the insulin signaling pathway, *e.g.*, IRS-1, AKT (PKB) and GSK3β, and that treatment with antioxidants would protect against oxidative stress-mediated inhibition of the pathway (Henriksen et al., 2011).

Oxidative stress and proinflammatory cytokines are associated with activation of the apoptosis signal regulating kinase (ASK1), a MAPK kinase, which phosphorylates and activates the MAPK kinases 3/6, or 4/7 (Seong et al., 2010). These upstream kinases are responsible for p38MAPK and JNK phosphorylation and activation (Aikin et al., 2004; Hsieh and Papaconstantinou, 2006; Johnson and Nakamura, 2007). Phosphorylation of Thr^{180}/Tyr^{182} in the activation loop of p38MAPKs result in their activation. The involvement of p38MAPK in inhibiting insulin signaling is indicated by a) its up regulation of PTEN expression through its activating transcription factor-2 (ATF-2) that binds to the PTEN promoter region, leading to increased PTEN transcription (Shen et al., 2006) and b) the activation of p38MAPK by free fatty acids (FFA)-induced insulin resistance in cells (Gao et al., 2010; Liu et al., 2007; Ragheb et al., 2009). Furthermore, p38MAPK has been shown to be involved in the distal end of the insulin signaling by increasing or decreasing glucose uptake in both skeletal muscle and adipose cells (Carlson and Rondinone, 2005; Styskal et al., 2012). However, the exact mechanisms of p38MAPK involvement in insulin signaling and the hepatic molecular mechanism that links oxidative stress and insulin resistance are not well understood (Rother et al., 1998). To address this, we examined the role of rotenone, an inhibitor-ROS generator at ETC CI, in the down regulation of insulin signaling and the involvement of p38MAPK in this down regulation.

2. Materials and methods

2.1. Animals

Young (3–4 months old) C57BL/6 male mice were obtained from the National Institute on Aging (NIH). Mice were housed in groups of 1–6 per cage, maintained on 12-h light/dark cycle, with free access to food and water for at least one week to allow them to acclimatize to their new environment before performing the perfusion experiments described below. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

2.2. Primary hepatocyte isolation, and culture

Mouse primary hepatocytes were isolated by collagenase perfusion. Briefly, mice were anesthetized by an intraperitoneal injection of pentobarbital (Ovation Pharmaceuticals, Inc., Deerfield, IL). The inferior vena cava was cannulated with a 24-gauge catheter, and heparin (APP Pharmaceuticals, LLC, Schaumburg, IL) was injected. The liver was blanched with Ca^{++}/Mg^{++}-free Hanks balanced salt solution (Cellgro, Manassas, VA) at 37 °C for 5–10 min through a peristaltic pump, then perfused with 0.05% collagenase Type 4 (Worthington Biochemical, Lakewood, NJ) perfusion solution in Hanks balanced salt solution (with Ca^{++} and Mg^{++}) at 37 °C until they were adequately perfused and starting to release hepatocytes from the extracellular matrix. The digested liver was excised and the cells were shaken from their sac in diluted collagenase solution (0.023%). Isolated cells were rotated at 37 °C and 125 rpm for 5 min. Cells were filtered through a 70 μm nylon cell strainer (BD Biosciences, Bedford, MA) into a 50-ml Falcon tube. The cell suspension was centrifuged at 700 rpm for 3 min. The cell pellet was washed 3 times and resuspended in Williams' E medium (Sigma–Aldrich, St. Louis, MO) containing 5% fetal bovine serum (FBS) (Serum Source International, Inc., Charlotte, NC), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco, Grand Island, NY). Cells were counted using a hematocytometer and plated on 100 mm culture dishes using Williams' E medium containing 5% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and insulin (0.6 μg/ml) (Gibco, Grand Island, NY) and incubated overnight (37 °C, 5% CO_2).

2.3. Primary hepatocyte treatment, and harvesting

Following an overnight incubation, cells were treated with either 20 μM ROT (Sigma–Aldrich, St. Louis, MO) or 100 nM insulin (Sigma–Aldrich) for the time points specified in each figure, or they were pretreated with ROT then treated with insulin. In other experiments, cells were pretreated with 20 μM SB203580 (Sigma–Aldrich), or 20 μM SB202190 (Sigma–Aldrich), or 20 mM N-acetylcysteine (NAC) (Sigma–Aldrich) for 30 min and then treated with ROT or ROT and insulin. Control treatments had only media as untreated control or dimethylsulfoxide (DMSO) (Sigma–Aldrich) as a vehicle control for ROT, and the kinase inhibitors SB203580 and SB202190.

Treated cells were harvested at various time points by washing and scraping the cells in ice-cold Tris-buffered saline (TBS) (25 mM Tris, pH 7.4, 5 mM KCl, 0.8% NaCl). Cells were pelleted by centrifugation at 2000 rpm and 4 °C for 5 min. Cell pellets were resuspended and lysed in ice-cold protein lysis buffer [10 mM HEPES, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride (NaF), 10 mM sodium pyrophosphate (NaPP), 1 mM sodium orthovanadate, 10 μM sodium molybdate, 20 mM β-glycerophosphate, 1 μg/ml each of the protease inhibitors Antipain, Chymostatin, Leupeptin, and Pepstatin] for 30 min on ice. Whole cell lysates were vortexed for 30 s and centrifuged at 20,000 rpm for 30 s. Supernatants were collected as the cytosolic fractions.

2.4. ROS quantification

Primary hepatocytes were plated on 24-well plates and incubated overnight (37 °C, 5% CO_2). Intracellular ROS levels were determined the next day using the fluorogenic probe 2′,7′-