Research Article

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Sab (Sh3bp5) dependence of JNK mediated inhibition of mitochondrial respiration in palmitic acid induced hepatocyte lipotoxicity

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Background & Aims: Sustained c-Jun N-terminal kinase (JNK) activation by saturated fatty acids plays a role in lipotoxicity and the pathogenesis of non-alcoholic steatohepatitis (NASH). We have reported that the interaction of JNK with mitochondrial Sab leads to inhibition of respiration, increased reactive oxygen species (ROS), cell death and hepatotoxicity. We tested whether this pathway underlies palmitic acid (PA)-induced lipotoxicity in hepatocytes.

Methods: Primary mouse hepatocytes (PMH) from adeno-shlacZ or adeno-shSab treated mice and HuH7 cells were used.

Results: In PMH, PA dose-dependently up to 1 mM stimulated oxygen consumption rate (OCR) due to mitochondrial β-oxidation. At ⩾1.5 mM, PA gradually reduced OCR, followed by cell death. Inhibition of JNK, caspases or treatment with antioxidant butylated hydroxyanisole (BHA) protected PMH against cell death. Sab knockdown or a membrane permeable Sab blocking peptide prevented PA-induced mitochondrial impairment, but inhibited only the late phase of both JNK activation (beyond 4 h) and cell death. In PMH, PA increased p-PERK and its downstream target CHOP, but failed to activate the IRE-1α arm of the UPR. However, Sab silencing did not affect PA-induced PERK activation. Conversely, specific inhibition of PERK prevented JNK activation and cell death, indicating a major role upstream of JNK activation.

Conclusions: The effect of p-JNK on mitochondria plays a key role in PA-mediated lipotoxicity. The interplay of p-JNK with mitochondrial Sab leads to impaired respiration, ROS production, sustained JNK activation, and apoptosis.

Keywords: Palmitic acid; Reactive oxygen species; Apoptosis; Mitochondria; Hepatocytes.

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Abbreviations: ASK1, mitogen-activated protein kinase kinase kinase 5; ATP, Adenosine 5′-triphosphate; Bcl2, B-cell lymphoma 2; BHA, butylated hydroxyanisole; CCCP, Carbonyl cyanide 3-chlorophenylhydrazone; CHOP, DNA-damage-inducible transcript 3; ECAR, extracellular acidification rate; ER, endoplasmic reticulum; IRE1α, Inositol-requiring enzyme-1α; JNK, c-Jun N-terminal kinase; JNKi, JNK inhibitor SP600125; MAP kinase, mitogen-activated protein kinase; MOMP, mitochondrial outer membrane permeabilization; MPT, membrane permeable transition; NASH, non-alcoholic steatohepatitis; $O2^{\cdot-}$, superoxide; OCR, oxygen consumption rate; Ox-Phos, oxidative phosphorylation; OTC, ornithine carbamoyltransferase; PA, palmitic acid; PERK, eukaryotic translation initiation factor 2-alpha kinase; PKR, protein kinase R; PMH, primary mouse hepatocyte; RCR, respiratory-control-ratio; Reserve capacity, reserve oxidative capacity; ROS, reactive oxygen species; Sab, SH3 homology associated BTK binding protein; sXBP1, spliced X-box binding protein 1; XBP1, X-box binding protein 1.

Introduction

Non-alcoholic steatohepatitis (NASH), mainly related to obesity and type II diabetes, is a significant cause of cirrhosis in Western countries. This disease represents a progression from fatty liver to cirrhosis with hepatocellular death believed to be a pivotal factor in promoting inflammation and fibrosis [1–3]. The hepatocellular death is mainly induced by free fatty acids with saturated fatty acids such as palmitic acid (PA) being much more toxic than unsaturated fatty acids [4,5]. This phenomenon is referred to as lipotoxicity or lipoapoptosis [6–9]. The mechanism for PA-induced lipotoxicity has been the subject of considerable investigation and has revealed a pivotal role for c-Jun N-terminal kinase (JNK) in mediating the toxicity in hepatocytes [10–16]. The pathways for saturated fatty acid-induced JNK activation have been extensively studied and evidence supports a role for Src dependent activation of the MAP3K, MLK3 [17–20]. Recently, autophagy-mediated degradation of KEAP-1 has been demonstrated to be upstream of JNK in PA-induced apoptosis, possibly upstream of MLK3 [21]. The role of ER stress in activating ASK-1 has also been suggested [22] but recent evidence indicates that ER stress is somehow linked to MLK3 activation [11,20,23]. On the other hand, the effector cell death pathway which mediates the action of JNK in PA toxicity, has been linked to induction and activation of PUMA and Bim [13,21], pro-apoptotic Bcl2 family members, which mediate mitochondrial permeabilization.

However, what determines the duration of sustained JNK activation required for toxicity is not fully understood.

We have been investigating the mechanism of JNK-mediated cell death in models of hepatotoxicity due to acetaminophen, TNF/galactosamine, and severe ER stress due to tunicamycin [24,25]. In all three models we have identified a key role for SH3BP5 (SH3 homology associated BTK binding protein, Sab), an outer membrane mitochondrial protein which is a binding target and substrate of JNK. When JNK phosphorylates Sab, mitochondrial respiration becomes impaired and reactive oxygen species (ROS) release is enhanced. This both sustains JNK activation, as ROS activate the MAPK pathways, and further impairs mitochondrial function. Thus, the interaction of JNK with mitochondria sustains JNK activation and ROS production, which can promote membrane permeable transition (MPT) in APAP necrosis or MOMP via modulation of Bcl2 proteins in TNF and ER stress-induced apoptosis. In all these models, knockdown of Sab *in vitro* or *in vivo* largely abrogates sustained JNK activation and thereby inhibits toxicity.

Since sustained JNK activation plays an important role in lipotoxicity, our goal in the present study was to determine if PA-induced JNK activation induces impaired mitochondrial function in a Sab-dependent fashion and if this contributes to cell death.

Materials and methods

Animals and reagents

Male C57BL/6NHsd mice (6–8 weeks of age) were obtained from Harlan Bioproducts for Science Inc. (Indianapolis, IN). Antisera to p-JNK, PERK, p-PERK, CHOP (Cell Signaling Technology), total JNK (JNK 1/2/3) (Santa Cruz Biotechnology), GAPDH and β-actin (Sigma Aldrich) and Sab (Proteintech, Abnova) were used. The p-JNK antiserum does not distinguish p-JNK 1 and 2. PA, butylated hydroxyanisole (BHA), TUDCA, 4-PBA, tunicamycin, oligomycin, CCCP, rotenone, etomoxir, necrostatin-1 were from Sigma. JNK Inhibitor II (SP600125), PP2, Src inhibitor 1, PERK inhibitor 1 (GSK2606414) (EMD-Millipore) were dissolved as described by manufacturer. Organic solvent free PA-BSA (20 mM stock) was prepared as follows. Equal volume of sodium palmitate (40 mM) dissolved in 150 mM NaCl at 70 °C and 45% BSA (99% fat free, Roche) dissolved in 150 mM NaCl at 37 °C were mix gradually to generate PA-BSA stock (20 mM PA/3.4 mM BSA; ratio of 6:1) and stored in −80 °C which remains stable for months. BSA control stock (3.4 mM, 99% fat free) was prepared in 150 mM NaCl at 37 °C. PA-BSA or BSA stock was thawed at 37 °C and resuspended thoroughly.

Primary mouse hepatocytes isolation, culture, treatment and protein extraction

Primary mouse hepatocytes (PMH) from wild-type or adenoviral shRNA (shlacZ or shSab) or ASO for JNK1 and 2 pretreated C57BL/6N mice were isolated and cultured as described previously (see Supplementary Methods) for adenoviral [24,26] and ASO preparation [27]. Briefly, 3 h after plating of isolated hepatocytes in seeding medium (DMEM/F12 supplemented with HEPES, L-methionine, L-glutamine, penicillin, streptomycin, $NaHCO_3$, insulin, hydrocortisol, FBS), medium was changed to serum free DMEM/F-12 culture medium and cells were rested overnight. Into the resting medium PA-BSA or relevant control reagents were added and incubated for 1, 4, 8, 16, 24 h. At indicated times, hepatocytes were washed twice in DPBS and protein was extracted in RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Whole cell lysate was centrifuged at 20,000 g at 4 °C for 15 min and supernatant was collected and stored in −80 °C.

Isolation of mitochondria from PMH

PMH was rinsed and scrapped in 1 ml of ice-cold homogenizing buffer (H-medium) with protease and phosphatase inhibitor cocktails, and collected into homogenizer in ice. Mitochondria from PMH 30×10^6 cells for each time point were pelleted by differential centrifugation as described before [24], and resuspended in RIPA lysis buffer.

Measurements of respiration by Seahorse XF24 analyzer

PMH were plated in seeding medium for 5 h in collagen coated 24 well XF24 cell culture microplates [25,28–30]. Cells were then rested overnight in serum free, phenol red free DMEM/F12 medium. The resting medium was then removed and cells were washed twice with DMEM running medium (XF assay modified DMEM supplemented with 2.5 mM glucose, pH 7.4) and incubated at 37 °C without CO_2 for 1 h to allow cells to pre-equilibrate with the assay medium. PA or reagents of interest were diluted in running buffer, loaded into port-A, and injected into XF24 extracellular flux assay plate. Oligomycin, CCCP or rotenone (Sigma) diluted in DMEM running medium were loaded into port-B, port-C or port-D, respectively. Final concentrations in XF24 cell culture microplates were 1 μg/ml oligomycin, 20 μM CCCP and 20 μM rotenone. The sequence of measurements was as follow unless otherwise described. Basal level of oxygen consumption rate (OCR) was measured 3 times, and then port-A was injected and measured 7 times for 1, 4 or 6 h. The medium was mixed every 10 min without data acquisition if intervals of OCR measurements were longer than 10 min. At the end of continuous real-time measurement of cellular OCR, port-B, port-C and port-D were injected sequentially and OCR was measured two times after each injection to determine mitochondrial or non-mitochondrial contribution of OCR. All measurements were normalized to average three measurements of the basal (starting) level of cellular OCR of each well. Each sample was measured in 3–5 wells. Experiments were repeated 3–5 times with different cell preps. Mitochondrial oxidative phosphorylation was determined by OCR before oligomycin injection minus OCR after oligomycin injection. Mitochondrial proton leak was determined by OCR after oligomycin injection minus OCR after rotenone injection. Non-mitochondrial OCR was determined by OCR after rotenone injection minus OCR of wells without cells. Mitochondrial reserve oxidative capacity was determined by OCR after CCCP injection minus OCR before oligomycin injection. Data was analyzed in groups of wells of each sample cell prep and statistical analysis was by ANOVA and *t* test.

Biological replicates and statistical analysis

A minimum of three biological replicates were considered for all cell-based studies. Statistical analyses were performed using the Student's *t* test. $p < 0.05$ was defined as statistically significant.

The methods for assessment of cell death, caspase 3 activation, immunoblotting, and peptide preparation are as we have previously reported [25,31] and details can be found in the Supplementary Material.

Results

Decreased mitochondrial respiration in PA-induced hepatocyte lipotoxicity and cell death

To determine the threshold of β-oxidation of PMH, we measured oxygen consumption induced by PA without supplementing mitochondrial respiratory substrates other than PA with low concentration glucose (2.5 mM) using Seahorse analyzer (Fig. 1A and B). Mitochondrial carnitine palmitoyltransferase (CPT)-1 inhibitor (Etomoxir) [32] prevented PA-induced increases in OCR, but did not prevent increases in OCR in response to pyruvate 1 mM with high concentration glucose (25 mM) (Fig. 1C). Thus, increased OCR represented PA-induced mitochondrial β-oxidation. Cellular oxygen consumption of PMHs in response to PA steadily increased with dose up to 4 h. At 4 h after PA treatment, both ATP-producing (oligomycin inhibitable) and non-ATP-producing (oligomycin uninhibitable proton leak) mitochondrial OCR increased dose dependently (180% of basal OCR with 0.5 mM PA) (Fig. 1A). However, higher dose of PA (1.5 mM) for 1 h markedly decreased reserve capacity, and by 4 h mitochondrial respiration (ATP-producing and proton leak) decreased 60% compared to 0.5 mM (Fig. 1B). Ultrapure fatty acid free BSA alone (up to 1.7% BSA used to complex with PA 1.5 mM) slightly (<20% of basal) increased cellular OCR, but proton leak