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Editorial
EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Who pulls the trigger: JNK activation in liver lipotoxicity?

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Nonalcoholic fatty liver disease (NAFLD) has emerged as the most common cause of liver disease in the developed countries [1]. NAFLD is strongly associated with obesity, insulin resistance, and increased serum levels of saturated free fatty acids (SFA)s [2]. SFAs are directly hepatotoxic, perhaps by mediating an endoplasmic reticulum (ER) stress response, and inducing hepatocyte apoptosis, referred to as lipoapoptosis or lipotoxicity.

Several studies have implicated ER stress in lipotoxicicity [3,4]. There are three resident trans-membrane-ER proteins, that sense and transduce the ER stress response, and their downstream mediators serve as ER stress markers. Biosensors of ER stress include: (1) protein kinase RNA-like ER kinase (PERK); (2) inositol-requiring protein-1α (IRE-1α); and (3) activating transcription factor 6 (ATF6). These three resident trans-membrane-ER proteins are held inactive by the chaperone glucose-regulated protein (GRP) 78; however, an excess of unfolded proteins relieves GRP78 inhibition resulting in activation of the ER stress transducers. ATF6 and PERK activation drive the expression of the proapoptotic transcription factor C/EBP-homologous protein (CHOP). IRE-1α activation creates a spliced form of *XBP-1* mRNA, a transcription factor that promotes degradation of misfolded ER glycoproteins. IRE-1α also leads to C-Jun N-terminal kinase (JNK) activation [5,6]. ER stress is a homeostatic process to restore protein folding in the ER by decreasing protein load, increasing the abundance of protein chaperones, and enhancing protein degradation. However, sustained or unrelenting ER stress induces cell death by CHOP induction and JNK activation, the role of CHOP in lipotoxicity is controversial, as animals genetically deficient in CHOP display accentuated lipotoxicity [7]. JNK activation, on the other hand, has been more strongly implicated in FFA mediated apoptosis *in vivo* and *in vitro* [8–10].

JNK activation is fundamental in both the metabolic syndrome accompanying NAFLD and cellular apoptosis by SFA. For example, JNK activation has been well recognized in both rodent and human non alcoholic steatohepatitis (NASH) [8–10]. However, the precise cellular and molecular mechanisms resulting in JNK activation have not been fully revealed, and mechanistic insight into this process may identify therapeutic targets to treat NASH. The concomitant occurrence of both ER stress markers and JNK activation has been inferred to indicate JNK activation is downstream of ER stress. However, a smoking gun does not always infer causing and effect. Nature often camouflages the true identity of the assassin, in this instance JNK activation. For example, several MAP3K have been implicated in JNK activation independent of ER stress, including (apoptosis signal-regulating kinase 1) ASK1 [11] and mixed lineage kinases (MLK) [12]. Recently, the double-stranded RNA-dependent protein kinase (PKR) has also shown to be a required component of JNK activation in response to lipids, inflammatory stimuli, and ER stress [13]. Thus, there are multiple pathways converging on JNK activation.

JNK activation in NASH has been presumed to be activated through ER stress by IRE-1α/ASK pathway. The article by Sharma *et al.* in this issue of the *Journal of Hepatology* challenges this concept and reveals a new mechanism of SFA-induced JNK activation. In this remarkable study, the authors suggest JNK activation is mediated by small GTP binding protein Cdc42 (cell division cycle protein) and Rac1 (Ras-related C3 botulinum toxin substrate), independent of IRE-1α and ASK1. Cdc42 and Rac1 have been established as critical regulators of JNK in response to oncogenic growth factors and inflammatory cytokines [14], but their role in SFA-induced JNK activation had not yet been explored. The authors used siRNA-mediated knockdown of MLK3, ASK1, IRE-1α in Hepa1c1c7, AML-12 cell lines, and primary mouse hepatocytes. Silencing *MLK3* reduced SFA-induced JNK activation, without affecting p38 MAPK phosphorylation, suggesting an important role of MLK3 in SFA induced JNK activation. Interestingly, silencing *MLK3* did not reduce SFA-induced ER stress markers (i.e., CHOP and sXBP1), suggesting MLK3 activation was either downstream or independent of ER stress. As an

Keywords: ER stress; GSK; NAFLD; Small GTPase; Saturated free fatty acid (SFA).
☆ DOI of original article: 10.1016/j.jhep.2011.03.019.
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Abbreviations: ASK1, apoptosis regulating kinase 1; CHOP, C/EBP-homologous protein; Cdc42, cell division cycle protein; ER, endoplasmic reticulum; SFA, saturated free fatty acids; GRP, glucose-regulated protein; GSK, glycogen synthase kinase; IRE-1α, inositol-requiring protein-1α; JNK, c-Jun-N-terminal kinase; MAP3K, mitogen activated protein kinase; MLK, mixed lineage kinase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PERK, protein kinase RNA-like ER kinase; PP2A, protein phosphatase 2A; PKR, double-stranded RNA-dependent protein kinase; Rac1, ras-related C3 botulinum toxin substrate; SFA, saturated free fatty acid, TG, thapsigargin..

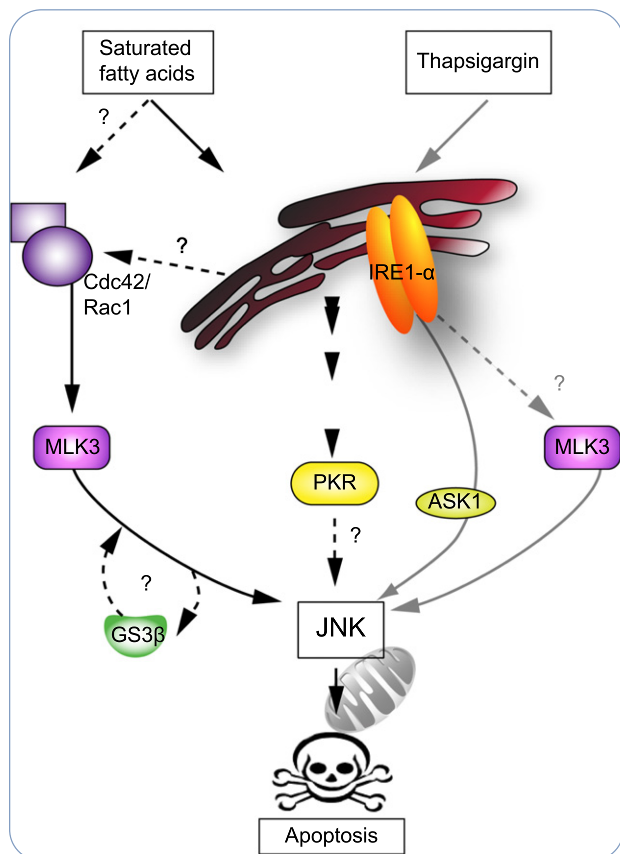

Fig. 1. JNK activation by thapsigargin versus saturated free fatty acid (SFA). Thapsigargin (TG) induced ER stress activates the transmembrane ER protein IRE-1α. IRE-1α subsequently activates ASK1 and JNK leading to cell death. MLK-3 contributes to TG induced JNK activation, most probably, downstream of ER stress. Saturated fatty acids (SFA) induce an ER stress response, which may activates double-stranded RNA-dependent protein kinase (PKR) leading to JNK activation. SFA also activates the small GTP binding protein Cdc42 (cell division cycle protein) and Rac1 (Ras-related C3 botulinum toxin substrate). Direct interaction between Cdc42 and MLK3 (downstream or parallel to the ER stress) may then cause SFA-induced JNK activation. Finally, sustained JNK activation may also potentially be mediated by direct interaction between MLK-3 and GSK-3β, leading to a feed forward mechanism for this process. By whatever mechanism, JNK activation is a key mediator of apoptosis.

important control, silencing *MLK3* reduced JNK activation associated with thapsigargin (TG; a protein glycosylation inhibitor which is classically used to induce ER stress), suggesting MLK3/JNK activation may be downstream of ER stress. Although, ASK1 and IRE-1 α have a major role in JNK activation through ER stress induced by TG, they were dispensable for saturated FFA-associated JNK activation. These data dispel the myth that SFA-induced JNK activation is due to IRE-1α/ASK axis. This study also demonstrates that silencing both *Cdc42* and *Rac1* concomitantly strongly inhibits SFA-induced JNK activation. The exact mechanism of SFA-induced Cdc42 and Rac1 activation was not addressed in this study. However, direct interaction between Cdc42 and MLK3 was required for SFA-induced JNK activation. Finally, the authors demonstrate that hepatocytes depleted of Cdc42/Rac1 or MLK 3, but not IRE1α, are protected against SFA-induced lipoapoptosis. Altogether, these results reveal that the small GTPases Cdc42 and Rac1 are major components of the SFA stimulated signaling pathway that regulates MLK3-dependent JNK activation in hepatocytes, independent of the ER stress transducer IRE-1α.

A recent study by us demonstrated that inhibition of glycogen synthase kinase, (GSK)-3α and β, serine/threonine kinases, by either pharmacological inhibitors or shRNA technology, also attenuates SFA-induced JNK activation, without affecting other markers of SFA-induced ER stress response [15]. Several studies have suggested that GSK-3β is involved in JNK activation through interaction with upstream MAP3Ks such as MLK-3 or MEKK1 in neuronal and kidney cell lines [16,17]. MLK-3 may also be regulated by JNK-mediated phosphorylation. These observations suggest the existence of a feed forward loop where JNK activates GSK-3 β which in turn activates MLK-3 further enhancing JNK activating phosphorylation [18]. Thus, emerging data indicate a unique, yet incompletely understood complex process for SFA-mediated JNK activation.

The integrative analyses of our data with the results of Sharma *et al.*, point to a novel pathway of SFA-induced JNK activation (Fig. 1). SFA-induced JNK activation is mediated by direct interaction between CdC42/Rac1 and MLK-3 (downstream or parallel to the ER stress). A key question remains, is ER stress still responsible of JNK activation independent of IRE1α/ASK or is ER stress an epiphenomenon in this process? GSK-3 may participate in this process by directly phosphorylating MLK3 in feed forward loop involving JNK as described above and depicted in Fig. 1. Future direction for research in the field should include exploration of the molecular pathways mediating JNK activation including a role for PKR, the exact mechanism of SFA-mediated Cdc42/Rac1 activation, and a potential interaction between GSK-3 and MLK-3.

Two JNK isoforms exist in the liver, many investigators have implicated JNK 1 in lipotoxicity [19,20]. However, not all investigators agree that these isoforms mediate different cellular process (Roger Davis, University of Massachusetts, personal communication); whether JNK1 and JNK2 execute redundant pathways or isoform specific cytoxic cascades, was not examined in the current study and will require further definition.

Because indiscriminate pharmacologic JNK inhibition may not be advisable, unraveling and elucidating the intricacies of SFA-induced JNK activation are important. Inhibiting a potentially specific or unique SFA-induced pathway could ultimately be therapeutically beneficial in NASH. We await such studies with anticipation; our patients are still in need of therapy!

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Financial support

This work was supported by NIH Grants DK41876 to GJG, and the Mayo Foundation.

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