Targeting signal transduction pathways which regulate necrosis in acetaminophen hepatotoxicity

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Acetaminophen (APAP) is a widely used medication which has been associated with dose-dependent liver injury and is the leading cause of acute liver failure in the U.S. and most of Europe [1]. Because of its highly reproducible effects in rodents, it has served as a model hepatotoxin for more than forty years. A great deal of information about the mechanism of its toxic effect has been gained over this period, although research continues on the details of several aspects of the cell death and tissue injury process. To summarize some of the generally accepted facts on the mechanism of injury, the drug is mainly metabolized to non-toxic sulfate and glucuronide conjugates but a lesser fraction is converted to an electrophilic reactive metabolite, NAPQI, mainly mediated by Cyp2e1. NAPQI is preferentially detoxified by GSH; when the GSH in cytoplasm and mitochondria is depleted, the NAPQI covalently binds to protein-thiols. The mitochondria are the key locus of dysfunction and an oxidative stress is induced which ultimately leads to MPT-mediated necrosis [2]. ER stress induced by covalent binding in the ER may also contribute [3], but is likely by the amplifying toxic effects on mitochondria. The ultimate development of MPT, however, depends on the participation of signal transduction pathways in which the initial oxidative stress activates kinases leading to JNK activation. Activated JNK binds to its target, SH3BP5 (Sab) on the cytoplasmic face of the mitochondria outer membrane. This leads to a self-sustaining mechanism which further impairs mitochondria function and amplifies ROS production which has two consequences: sustaining JNK activation and promoting MPT [4,5]. Knockdown of Sab or both JNK 1 and/or 2 prevents acetaminophen toxicity without affecting GSH depletion or covalent binding [4,6]. Therefore, one may view this as a form of regulated necrosis.

In the current issue Kim *et al.* have explored the hepatoprotective properties of the commonly used drug, metformin, in the mouse APAP model [7]. Indeed, metformin, pretreatment 30 min before APAP was markedly protective. Importantly an effect of metformin on APAP metabolism was excluded. The protection was accompanied by inhibition of JNK activation as well as its upstream activator MKK4, but not MKK7. As previously described [8], APAP caused a rapid decline in p-AMPK, which could not be reversed by metformin, making it unlikely that metformin was protective through its known effect to activate AMPK. The authors then looked for upstream modulators of MAPK activation which might account for metformin's inhibition of JNK activation, focusing their attention on Gadd45β, a stress response gene, known to downregulate JNK [9]. Gadd45β expression was rapidly induced by APAP and was greatly amplified by metformin. The enhanced Gadd45β expression was associated with decreased JNK activation and toxicity. Although Gadd45β was only examined at the mRNA level and no data were provided on protein levels, strong support for a causal relationship between Gadd45β and its protective properties was provided by administering APAP to Gadd45β knockout (KO) mice which revealed markedly enhanced toxicity. The effect was cell autonomous, i.e. hepatocytes from these KO mice were much more susceptible to APAP-induced JNK activation and to death. Most importantly, Gadd45β KO abrogated the metformin protective effect. Furthermore, metformin protected wild-type mice even when given one hour after APAP, offering some promise as a therapeutic approach. However, the doses of metformin required to exert such an effect may not be achievable in humans (Fig. 1).

This very well executed study provides convincing evidence that metformin protects against APAP toxicity upstream of p-JNK in a Gadd45β dependent fashion. JNK activation in APAP toxicity involves at least two MAP3 kinases with the early participation of MLK3 [10] and the subsequent participation of ASK1 [11]. Mitochondrial ROS are believed to play an important role in activation of these MAP3K, either by oxidizing cytoplasmic tethers or activation of upstream signaling (e.g. GSK3β or Src) [11,12]. Gadd45β induction appears to be a compensatory stress response which dampens toxicity. Although it is conceivable that it is acting to directly inhibit MKK4, as there is experimental support it binds and inhibits MKK7 in other contexts [13], other possibilities should be considered such as direct inhibitory effects on the upstream signaling kinases or indirect effects somehow limiting mitochondrial ROS production.

Metformin is a known AMPK activator and AMPK activation promotes autophagy, a compensatory protective pathway which removes damaged mitochondria. Enhanced autophagy/mitophagy is known to decrease APAP toxicity [14]. Kim *et al.* were unable to demonstrate reversal of APAP-induced inhibition of
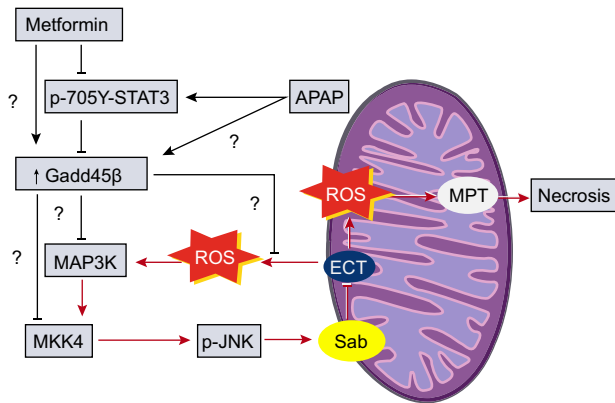
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Editorial

Fig. 1. Signal transduction in APAP toxicity. JNK plays a key role in APAP toxicity through its interaction with mitochondrial Sab which promotes ROS release, sustaining JNK activation and ROS production, ultimately leading to MPT-induced necrosis. Gadd45β is induced by either APAP or metformin alone and to a greater extent by both. p-Stat3 is a repressor of Gadd45β expression which is activated by APAP and suppressed by metformin. The exact mechanism of Gadd45β induction by APAP and/or metformin is at present uncertain. However, Gadd45β KO abrogates the protective effect of metformin and enhances APAP-induced JNK activation and toxicity.

AMPK by metformin. Nevertheless, they also excluded an effect of metformin on autophagy to account for protection.

The possible mechanism of induction of Gadd45β was considered in the Supplementary data. A role for transcription factors, CAR and NF-κB, was excluded. Another known transcriptional regulator (repressor) of Gadd45β is Stat-3 which was activated after APAP, while metformin blocked this activation, suggesting that activated Stat-3 may repress Gadd45β. However, no direct evidence for the role of Stat-3 in APAP toxicity is provided and this will require more extensive exploration. Furthermore, under basal conditions the authors show that metformin induces Gadd45β without an effect on Stat-3. Therefore, metformin induces Gadd45β before APAP, independent of Stat-3, although an effect on Stat-3 after APAP may amplify the induction by 'lifting the break' but does not fully explain induction. Moreover, APAP itself leads to submaximal Gadd45β induction and it is not clear if APAP and metformin induce Gadd45β by the same or independent mechanisms, nor if their effects are simply additive or potentiated.

Metformin exerts its effects through AMPK dependent and independent mechanisms at pharmacologic and super-pharmacologic doses [15]. The latter appears to be accounted for by electrophoretic accumulation of positively-charged drug into the mitochondrial matrix where it modestly inhibits Complex1 which then restrains hepatic gluconeogenesis and increases accumulation of lactic acid [16,17]. Metformin has been shown to inhibit MPT and this effect is likely dependent on inhibiting Complex1 at the initiation of the electron transport chain [18]. Complex1 inhibition by metformin can inhibit ROS production [19]. How this factors into the regulation of Gadd45β induction before or after APAP administration is not certain.

A number of uncertainties and controversies remain to be clarified in the field of APAP toxicity, including exactly how the interaction of JNK and Sab exert effects on mitochondrial function, the contribution of ER stress, the role of mitochondrial fission, the contribution of necroptosis, and the role of DAMP release from necrotic cells in inducing secondary inflammation and collateral damage. These issues are under investigation in many laboratories. However, the current work is an important reminder that injury promoting mechanisms of cell death simultaneously are countered by adaptive measures to dampen the progression of injury, and further enhancement of these adaptive responses (e.g. enhanced mitophagy or Gadd45β) offer promising therapeutic targets.

In summary, the work of Kim *et al.* reveals a protective effect of metformin against APAP toxicity by inducing Gadd45β and downregulating JNK. Further work will be needed to determine the precise locus of action of Gadd45β and the mechanism of its induction by metformin and APAP.

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Conflict of interest

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

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