Reactive Nitrogen Species in Acetaminophen-Induced Mitochondrial Damage and Toxicity in Mouse Hepatocytes: A Cautionary Note on the Impact of Cell Culture Conditions

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To the Editor: We read with great interest the recent paper by Burke et al. (1). However, on the basis of findings published after the submission of this manuscript we think that the experimental conditions may have significantly influenced the results obtained in these cell culture experiments.

First, the authors appear to be able to quite rapidly trigger severe injury with short-term exposure to 1 mM acetaminophen (APAP) (1), which is different from what has been reported by a number of other groups where continuous exposure to 5–25 mM APAP was used to get a much more delayed hepatocellular injury (2–5). One likely explanation for these unexpected results could be the fact that the authors used hepatocytes in suspension (1). The authors observed a very steep increase in enzyme release as early as 3–4 h after a 1 mM APAP exposure in hepatocytes in suspension (approximately 20-fold increase over baseline at 4 h) (1). In contrast, a similar time course of enzyme release in adherent cells after exposure to 5 mM APAP did not result in any significant enzyme release at 3 h and showed only a 2-fold increase at 6 h (2). In fact, when we directly compared APAP toxicity in adherent versus suspended cells we observed 4–5-fold higher cell death in suspended cells independent of the dose of APAP used (Ramachandran, A., Yan, H. M., Jaeschke, H., unpublished work). Thus, cells in suspension are generally more susceptible to APAP exposure than adherent cells. Another caveat of the authors’ experiments is the use of extremely high oxygen concentrations, i.e., incubation of cells under 95% oxygen (1). Hepatocytes in the intact liver in vivo experience oxygen concentrations in sinusoids between 3–9% (6). In a recent study, we demonstrated that even culturing cells under room air (21% oxygen) leads to an accelerated and higher mitochondrial oxidant stress and peroxynitrite formation causing accelerated and higher cell death compared to cells cultured under 10% oxygen (7). Interestingly, the differences in mitochondrial oxidant stress and peroxynitrite formation do not appear to exist under baseline conditions but only after APAP exposure (7). Furthermore, these differences contribute to the fact that lipophilic antioxidants such as vitamin E partially protect only when cells are cultured under 21% oxygen but not when cells are cultured under more physiological oxygen levels of 10% or lower (7). These in vitro observations are consistent with in vivo experiments where vitamin E did not protect against APAP overdose (8). Thus, our data suggest that exposing cells to APAP when cultured under 21% oxygen enhances the mitochondrial oxidant stress and peroxynitrite formation. Together, these more recent findings may explain the observations reported by Burke et al. (1). In these in vitro studies, the severe stress induced by keeping the cells in suspension and the initial mitochondrial dysfunction triggered by APAP is further exaggerated by the hyperoxic conditions (95% oxygen) used throughout these experiments. Thus, the apparently high susceptibility of these hepatocytes to low concentrations of APAP is actually the result of a triple hit, i.e., drug toxicity, hyperoxia, and cellular stress by preventing adherence.

Second, the authors conclude that “the data are consistent with the hypothesis that reactive nitrogen species (RNS) cause mitochondrial damage and that mitochondrial damage induces additional RNS...” (1). However, the results in Table 1 of their work show that the mitochondrial membrane permeability transition (MPT) inhibitor cyclosporine A eliminates dichlorodihydrofluorescein fluorescence and nitrotyrosine protein adducts (1). Thus, one would conclude that virtually all of the oxidant stress and peroxynitrite formation under these experimental conditions occurred after the MPT pore opening. This would be inconsistent with the assumption that peroxynitrite causes the MPT. However, the observations in this experimental system do not agree with a number of different in vitro and in vivo findings. First, the oxidant stress after APAP overdose starts right after GSH depletion but well before cell death (2), which correlates with the MPT (3). A number of in vivo studies showed that scavenging peroxynitrite effectively prevented cell necrosis (9–13). Most importantly, a recent study demonstrated that APAP-induced liver injury and DNA damage was completely prevented in cyclophilin D-deficient mice but that there was only a partial reduction of the oxidant stress and peroxynitrite formation (14). Thus, in vivo data clearly support the conclusion that the formation of reactive oxygen species (ROS) and peroxynitrite occurs prior to the MPT pore opening and are at least in part involved in triggering the MPT (14).

Third, the authors conclude that in isolated hepatocytes NO for peroxynitrite is generated mainly by the neuronal nitric oxide synthase (nNOS) and not by the inducible NOS (iNOS) (1). These data are consistent with some (8, 15, 16) but not all in vivo data (17–20). The involvement of iNOS may be more prominent when the generation of anti-inflammatory cytokines such as interleukin-10 is reduced (21). In addition, a role for endothelial NOS (eNOS) has been suggested (20). Thus, all three isoforms of NOS appear to be able to contribute to NO formation in vivo. In addition to hepatocytes, NOS enzymes are located in endothelial cells and Kupffer cells. Consequently, the source of NO may vary depending on the experimental conditions and may therefore not be the most relevant therapeutic target for preventing APAP-induced cell death. Scavenging ROS and peroxynitrite (9–13), supporting mitochondrial energy metabolism (12), and preventing the MPT directly (1, 14, 22) or indirectly by c-jun N-terminal kinase inhibition (16, 23) appear to be more effective strategies.

Taken together, on the basis of these newly available findings one would conclude that the APAP-induced oxidant stress and peroxynitrite formation observed by the authors in their studies is certainly favored by the experimental conditions of using cells in suspension and exposure to 95% oxygen. How much hyperoxia affects NO formation by nNOS remains to be investigated. Preventing adherence of the cells reduces viability, and hyperoxic conditions tend to amplify mitochondrial ROS and peroxynitrite formation and accelerate cell death. However, this note of caution does not only apply to the conditions used by Burke et al. (1) but also to all cell culture experiments where adherent cells are incubated in room air (21% oxygen) (7). Consequently, any extrapolation of findings obtained under experimental conditions not closely mimicking the in vivo environment to the intact animal or humans should be done very cautiously.
References


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