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NADPH Oxidase-Dependent Reactive Oxygen Species are Important to the Early Stage of CD95 Engagement in Hepatocytes

Reinehr R, Becker S, Eberle A, Grether-Beck S, Häussinger D. Involvement of NADPH Oxidase Isoforms and Src Family Kinases in CD95-dependent Hepatocyte Apoptosis. J Bio Chem 2005;280:27179-27194. (Reprinted with permission from the American Society for Biochemistry and Molecular Biology.)

Abstract

CD95 ligand (CD95L) triggers a rapid formation of reactive oxygen species (ROS) as an upstream event of CD95 activation and apoptosis induction in rat hepatocytes. This ROS response was sensitive to inhibition by diphenyleneiodonium, apocynin, and neopterin, suggestive of an involvement of NADPH oxidases. In line with this, hepatocytes expressed mRNAs not only of the phagocyte gp91^{phox} (Nox 2), but also of the homologs Nox 1 and 4 and Duox 1 and 2, as well as the regulatory subunit p47^{phox}. gp91^{phox} (Nox 2) and p47^{phox} were also identified at the protein level in rat hepatocytes. CD95L induced within 1 min ceramide formation and serine phosphorylation of p47^{phox}, which was sensitive to inhibitors of sphingomyelinase and protein kinase Cζ (PKCζ). These inhibitors and p47^{phox} protein knockdown inhibited the early CD95L-induced ROS response, suggesting that ceramide and PKCζ are upstream events of the CD95Linduced Nox/Duox activation. CD95L also induced rapid activation of the Src family kinase Yes, being followed by activation of c-Src, Fyn, and c-Jun-N-terminal kinases (JNK). Only Yes and JNK activation were sensitive to *N*-acetylcysteine, inhibitors of NADPH oxidase, PKCζ, or sphingomyelinase, indicating that the CD95L-induced ROS response is upstream of Yes and JNK but not of Fyn and c-Src activation. Activated Yes rapidly associated with the epidermal growth factor receptor (EGFR), which became phosphorylated at Tyr^{845} and Tyr^{1173} but not at Tyr^{1045}. Activated EGFR then triggered an AG1478-sensitive CD95-tyrosine phosphorylation, which was a signal for membrane targeting of the EGFR/CD95 complex, subsequent recruitment of Fas-associated death domain and caspase 8, and apoptosis induction. All of these events were significantly blunted by inhibitors of sphingomyelinase, PKCζ, NADPH oxidases, Yes, or EGFR-tyrosine kinase activity and after protein knockdown of either p47^{phox}, Yes, or EGFR. The data suggest that CD95L-induced apoptosis involves a sphingomyelinase- and PKCζ-dependent activation of NADPH oxidase isoforms, which is required for Yes/EGFR/CD95 interactions as upstream events of CD95 activation. (HEPATOLOGY 2005;42: 956-958.)

Comments

Hepatocytes are susceptible to many death stimuli, such as the activation of death receptors, ischemia/reperfusion insults, and toxic chemicals. Almost invariably, reactive oxygen species (ROS) are among the most serious culprits that lead to cell death and tissue injury.[1,2] It is thus important to define what species of ROS are generated, how they are generated, and what their roles are in cell death.

One of the best-studied cases of ROS in cell death is perhaps conducted with tumor necrosis factor α (TNFα). Most of the studies conducted in nonhepatocyte systems indicate that mitochondria are the major source of the ROS.[3] This may not be too surprising, as mitochondria are the single largest source of ROS in normal cells, due to the activities of the respiratory chain. Early studies showed that TNFα could cause mitochondrial respiration disturbance and therefore the leakage of superoxide into the cytosol. Suppression of mitochondrial respiration could reduce TNFα-induced ROS generation.[3] ROS generation in CD95-activated cells could be also mediated by the mitochondria.[4] Studies conducted on hepatocytes indicated that mitochondrial dysfunction is caused by the pro-death Bcl-2 family proteins, such as Bid.[4] Bid acts on Bax and/or Bak and induces cytochrome *c* release, mitochondrial depolarization, and the collapse of electron transport along the respiratory chain. The last two events could be further exacerbated by the activated caspases, which inactivate one of the complex I components (p75 NDUSF1).[5]

Mitochondria are certainly not the only source of ROS, even in death receptor-mediated cell death. In nonhepatocytes, ROS generation following death receptor engagement had been found to be derived from phospholipase A2-arachidonic acid-5-lipoxygenase[6,7] and NADPH oxidase,[8,9] respectively. Cytochrome P450 enzymes had also been implicated in ROS generation, particularly during the catabolism of ethanol or certain drugs in the hepatocyte.[2]

NADPH oxidase, or Nox, was initially defined in phagocytes and is responsible for the microbial-killing activity of these cells.[10] Nox is a multi-component enzyme, composed of the core membrane-bound heterodimer of gp91^{phox} and p22^{phox} (flavocytochrome *b558*), which is responsible for the electron transport from NADPH to molecular oxygen to form O_2^-, and several cytoplasmic regulatory elements, including p67^{phox} (NoxA2), p47^{phox} (NoxO2), p40^{phox}, and the small GTPase Rac2. Independent activation of p47^{phox},

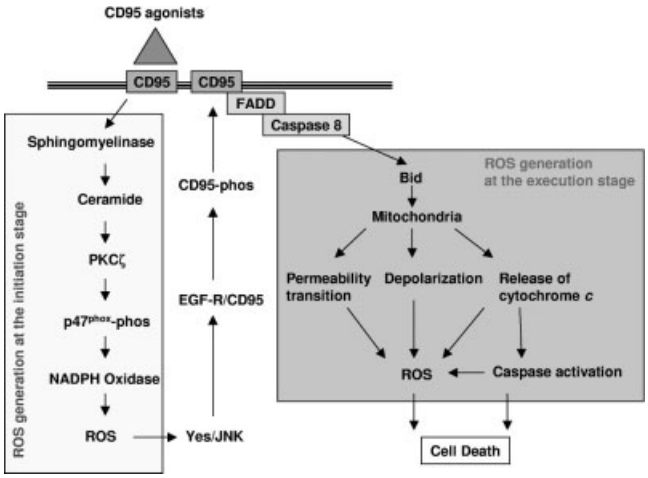

Fig. 1. A schematic representation of CD95 activation induced hepatocyte apoptosis and the role of ROS. Ligation of CD95 by the agonists (CD95L or anti-CD95) rapidly activates sphingomyelinase and the generation of ceramide, which activates PKCζ. The latter induces the phosphorylation of $p47^{phox}$, the regulatory element of the NADPH oxidase. The phosphorylated $p47^{phox}$ ($p47^{phox}$-phos) translocates to the membranes and activates the NADPH oxidase to generate the first wave of reactive oxygen species (ROS). This early stage of ROS generation seems to be important for the subsequent DISC formation via the effects of Yes, JNK, EGF-receptor (EGF-R), and CD95 phosphorylation (CD95-phos). See the text for further details. Once caspase 8 is activated, Bid can be cleaved, and truncated Bid translocates to the mitochondria to induce a series of mitochondrial dysfunctions, culminating in the generation of a second wave of ROS, which might be more involved in the direct damage of cell structure and function, leading to cell death.

via serine phosphorylation, and Rac2, via dissociation from its GDI (guanine nucleotide dissociation inhibitor), by the upstream signaling events, leads to their translocation to the membranes where they stabilize the interaction of $p67^{phox}$ with $gp91^{phox}$ and activate the latter.[11]

A number of $gp91^{phox}$ proteins have been discovered, including Nox1, Nox3, Nox4, Nox5, DUOX1 and DUOX2, with Nox2 being the prototype $gp91^{phox}$.[10,11] Homologs to $p47^{phox}$ and $p67^{phox}$ have also been described and termed as NoxO1 and NoxA1, respectively. Tissue distribution of these homologs varies significantly. Neither their activation nor their functions are clearly understood. On the other hand, Rac1, a Rac2 homolog, is expressed ubiquitously and can functionally substitute Rac2, which is only expressed in leukocytes.

Although Nox is classically known to be responsible for the superoxide generation in the phagocytes following phagocytosis, Nox-dependent ROS generation has been widely reported in other types of cells as well, *e.g.,* in the lymphocytes[9] and in the vascular cells,[11] due to the presence of the various Nox members. The stimulation signals could be very diverse, including the activation of death receptor by CD95 agonists[8,9] or by TNFα.[11] However, the significance of Nox in the hepatocyte was not known until the work of Reinehr et al.[12]

This work reveals several important features of an early generation of ROS and their roles in mediating hepatocyte apoptosis induced by CD95 ligation[12] This is the first report examining the expression of NADPH oxidase component in the liver system. Thus, rat hepatocytes express mRNAs not only of $gp91^{phox}$, but also of the homologs Nox1, Nox4, Duox1, and Duox2, as well as the regulatory subunit $p47^{phox}$. $gp91^{phox}$ and $p47^{phox}$ were also identified at the protein level, although it seems that rat $gp91^{phox}$ was present as a nonglycosylated form, whereas human $gp91^{phox}$ was heavily glycosylated. Interestingly, the Kupffer cells and the stellate cells also expressed the same set of Nox molecules. Expression of NADPH oxidase components in the mouse liver or human liver is not known yet, but could be detected as well.

Ligation of CD95 led to a rapid induction of ROS, accompanied with the phosphorylation of $p47^{phox}$. Activation of $p47^{phox}$ is important to the assembly and therefore the activation of the Nox complex. Consistently, ROS response was sensitive to inhibition by various Nox inhibitors, and most importantly by the knockdown of $p47^{phox}$. Thus, it seems that the hepatic Nox system is functional and can generate ROS following CD95 ligation. The next important question then became how the hepatic Nox was activated following CD95 ligation and how important this Nox-dependent ROS generation was in CD95 mediated killing.

Reinehr et al.[12] thus found that it was the serial effects of sphingomyelinase, ceramide and PKCζ that led to the $p47^{phox}$ activation in the rat hepatocyte stimulated by CD95 ligand. Acid sphingomyelinase (ASMase) and ceramide are no strangers in CD95-mediated events. Earlier studies had shown that they were important to CD95-mediated apoptosis. Mice deficient in ASMase were relatively resistant to anti-CD95-induced liver injury.[13] It was found that ASMase was important for the CD95-dependent production of ceramide, which then promoted CD95 capping.[14] It seemed that this event could be important for the efficient activation of the death inducing signaling complex (DISC).[14]

Now if the Nox activation was the result of ceramide action, one might wonder whether Nox-generated ROS had anything to do with the receptor capping. Reinehr et al. did not report any finding on this aspect, but they reported that inhibition of this Nox-mediated ROS generation did affect DISC formation. Further investigation revealed an interesting signaling pathway from ROS to the activation of JNK and Yes, to the formation of Yes/EGF-receptor complex and then the EGF-receptor/CD95 complex. The latter seemed to be required for the efficient translocation of CD95 from the cytosol to the membrane, where the DISC was formed. The study com-

bined the use of pharmacological agents, gene knockdown, traditional biochemical analysis, and the advanced FRET technology. The data shown looked quite convincing for most of the studies. The only puzzling finding was about the formation and dissociation of the intermediate complex, Yes/EGF-receptor, and the role of JNK in this process. It seemed that EGF receptor was phosphorylated through this process and became competent to bind to and phosphorylate CD95. However, what is puzzling is that knockdown of Yes did not affect the formation of EGF-receptor/CD95 complex, although it did affect the subsequent CD95 phosphorylation, translocation and DISC formation. Further studies will be required to address this apparent paradox.

Although the bottom line of this study was that early ROS generation through hepatic Nox system was important to DISC formation and therefore CD95-mediated cell death, there was a plethora of additional information contained in this complicated study. From the point of ROS generation, one may wonder how the hepatic Nox system is assembled, whether the nonglycosylated $gp91^{phox}$ is equally functional, and whether other Nox homologs might be more important. The relationship of the Nox-mediated ROS generation and the mitochondria-mediated ROS generation is also fascinating. Kinetically, the Nox-mediated ROS generation might come significantly earlier, although the level of ROS might be lower. The significance of extra-mitochondrial ROS and mitochondrial ROS could thus be quite different (Fig. 1). While the former might be involved in the early initiation step, the latter is more likely involved at the execution step of direct disruption of cellular structure and functions. The implication would be that suppression of both types of ROS could be necessary for the optimal protection and that antioxidants directed to scavenging superoxide might be more effective death repressors.[4,15]

On the other hand, the role of ROS in CD95 receptor translocation, reorganization, and DISC formation seems to be much more intriguing. While the current findings were exciting, many more questions arise as well. Limited by the space, one cannot possibly discuss every single issue here, but it is worth mentioning just a few of them for further thinking. For example, where would the normal CD95 receptors be in hepatocytes and how are they organized? Why would (additional?) CD95 receptors be translocated to the cytoplasmic membranes following the initial ligation of CD95, which presumably occurs on the cytoplasmic membrane? Why would this translocation have a significant impact on DISC formation? CD95 translocation from intracellular locations, such as the Golgi complex, to the cytoplasmic membranes had been reported to be important for p53-mediated killing.[16] Furthermore, it is not known whether the complexity of CD95 activation may reflect the different types of cells being studied. Clearly, a lot more mechanistic details have yet to be worked out for the death receptor activation-induced apoptosis.

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