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Review

The anti-apoptotic actions of nitric oxide in hepatocytes

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Abstract

Nitric oxide (NO) has anti-apoptotic actions in hepatocytes *in vitro* and *in vivo*. The protection is mediated via the S-nitrosylation of procaspases as well as the active caspase enzymes. NO stimulation of the cGMP/protein kinase G pathway also appears to contribute to the protective effect of NO. Here we review the evidence for the direct inhibition of NO with the apoptotic signaling pathways in hepatocytes.

Keywords: caspases; S-nitrosylation; apoptosis; inducible NO synthase

Abbreviations: ActD, actinomycin D; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; TNFα, tumor necrosis factor α; TGFβ, transforming growth factor β; NIL, L-N^G-iminoethyl-lysine; DTT, dithiothreitol

It is well established that liver produces either small amounts of nitric oxide (NO) constitutively or large amounts of NO under a number of inflammatory conditions.[1] Both parenchymal and nonparenchymal liver cells have been shown to generate NO. Hepatocytes constitute the majority cells of the liver (over 70%) and play important roles in metabolism, detoxification and production of plasma proteins. In the absence of exogenous stimulation hepatocytes probably produce little if any NO. However, following exposure to cytokines such as interleukin 1 or tumor necrosis factor alpha (TNFα), hepatocytes express the inducible NO synthase (iNOS) and produce high levels of NO for prolonged periods. Other stimuli such as bacterial lipopolysaccharide, interferon gamma and redox stress can also augment hepatocyte iNOS expression. The low levels of NO produced by constitutive endothelial NO synthase (eNOS) maintain hepatic perfusion and protect the liver under inflammatory conditions. The higher amounts of NO produced by iNOS participate in immune-defense against invading pathogens. However, recent studies indicate that the inducible NO production also plays important roles in modulating hepatocellular cell death and tissue injury. Depending on the type and extent of insults, iNOS expression can either protect the liver from damage or promote hepatocellular injury. For example, we[2,3] and others[4] have shown that pharmacological doses of NO donor conferred protection against TNFα-induced liver injury in D-galactosamine-sensitized animals and that administration of iNOS inhibitors resulted in increased apoptosis in the liver of animals treated with endotoxin.[2] Similarly, iNOS expression limited hepatocyte apoptosis during liver regeneration.[5] In contrast, iNOS expression in response to hemorrhagic shock actually promoted liver damage.[6] We have postulated that one of the determinants of iNOS toxicity in liver is the degree of associated redox stress.[7]

The potent anti-apoptotic properties of NO in hepatocytes have now been established in *in vitro* and *in vivo* studies. Hepatocytes are rather resistant to NO-mediated toxicity. Millimolar concentrations of NO donors, much higher than levels known to induce apoptotic cell death in many other cell types,[8] actually cause hepatocyte necrosis but not apoptosis (unpublished observations). However, cultured hepatocytes readily undergo apoptotic cell death upon exposure to Fas ligand, TNFα plus the transcriptional inhibitor actinomycin D, transforming growth factor β (TGFβ) or growth factor depletion.[9–13] We have found that NO suppresses not only Fas- or TNFα-mediated apoptosis, but also non-receptor-mediated apoptosis such as spontaneous and hydrogen peroxide-induced apoptotic cell death in hepatocytes.[10,14] Both the exogenously provided NO via various NO donors and endogenous NO derived from induction of iNOS with cytokines prevented hepatic apoptosis.[10] Adenoviral gene transfer of human iNOS into hepatocytes also effectively inhibited TNFα+ActD-induced apoptosis, a response completely reversed by blocking NO generation with iNOS inhibitor L-N^G-iminoethyl-lysine (NIL).[15,16] Moreover, NO exhibits a similar potency for suppressing apoptosis in the liver *in vivo*. Using a NO donor that concentrates NO release in hepatocytes, we have shown that NO almost completely prevented the massive and rapid apoptotic liver destruction induced by the administration of TNFα and D-galactosamine.[2]

Our laboratory has begun to examine the mechanism by which NO inhibits hepatic apoptosis with a focus on how NO interacts with the apoptotic signaling cascade in hepatocytes. Initial studies examined the effect of NO pretreatment on hepatocyte susceptibility to TNFα-induced cell death. We found that pretreatment with a NO donor or pre-induction of iNOS stimulated the expression of the inducible heat shock protein 70 (Hsp70) in hepatocytes and inhibited subsequent TNFα+ActD-induced hepatocyte apoptosis.[17] The protective effect of NO pretreatment on TNFα+ActD-induced apoptosis correlated with the expression level of Hsp70, and this protection was lost when

Hsp70 expression was blocked with antisense oligonucleotides. Although Hsp70 has also been shown to suppress apoptosis in a number of other cell systems, how Hsp70 prevents cells from TNFα-mediated cell death remains unknown. Recent evidence suggests that Hsp70 acts downstream of caspase activation in tumor cell lines.[18] In addition, NO also induces heme oxygenase-1 expression in hepatocytes, and this enzyme produces the potent antioxidant biliverdin to prevent H_2O_2-induced toxicity.[14]

Simultaneous exposure to NO donor not only suppressed TNFα-induced apoptosis but also Fas and nutrient deprivation-induced apoptosis.[10,16] These findings suggested a direct interaction between NO and the apoptotic signaling cascade. Based on this observation we assessed the effects of NO on caspase activity and activation. Several of the 14 identified mammalian caspases are known to participate in apoptotic signaling.[19] These enzymes exist in cells as zymogens and require proteolytic cleavage into the enzymatically active form. Initiator caspases, such as caspase-8, can propagate the apoptotic signal by activating downstream effector caspases, such as caspase-3, which serve as executioners in the terminal phase of apoptosis by cleaving key death substrates (such as PARP, DNA-dependent kinase, and the inhibitor of the caspase-activated deoxyribonuclease). Activated upstream caspases can also cleave certain Bcl2 family members, leading to the release of cytochrome *c* from mitochondria. Released cytochrome *c* then forms a complex with apoptotic protease activating factor 1 (Apaf-1), dATP and procaspase-9, resulting in activation of procaspase-9 that in turn activates procaspase-3. Growing evidence has demonstrated the essential involvement of caspase-3-like proteases, but not caspase-1, in hepatocyte apoptosis induced by TNFα, Fas, TGFβ, ischemia/reperfusion and toxic bile salts.[10,16,20–26] We have confirmed that caspase activation takes place in hepatocytes stimulated to undergo apoptosis.[10,16] Numerous caspases are activated in hepatocytes and pan-caspase inhibitors and inhibitor more specific for caspase-3-like proteases prevent hepatocyte apoptosis.[16] Hepatocytes exposed to NO via donors or through the expression of iNOS display much lower levels of caspase activity coinciding with enhanced cell survival.[10,16] Similar observations take place *in vivo* in TNFα and D-galactosamine treated rats infused with a liver selective NO donor.[3] Under all circumstances caspase-3-like activity could be increased if lysates from the cells or whole liver were treated with the strong reducing agent dithiothreitol (DTT),[10] implicating S-nitrosylation as a mechanism for the NO-dependent inhibition of caspase activity and hence protection from cell death.[22,27–31] This is consistent with similar observations made in endothelial cells,[27] neurons,[28] eosinophils,[32] lymphocytes[33,34] and several tumor cell lines.[35] Indeed, when seven recombinant active caspases were exposed to NO, they were all inhibited by NO in a manner reversible by DTT.[22]

Besides direct inactivation of active caspases through S-nitrosylation, NO also inhibits the activation process of procaspases. To determine if NO prevents the proteolytic processing of procaspases we first identified the total number of caspases activated in apoptotic hepatocytes. Affinity labeling of caspases activated in TNFα+ActD-treated hepatocytes with biotin-tagged the pan-caspase inhibitor VAD-fmk revealed that at least four different active caspase species were present in hepatocytes undergoing apoptosis.[16] NO blocked the detection and appearance of all four active caspase species. Western blotting analysis for the detection of the proteolytic fragment of caspases confirmed that NO treatment prevented the proteolytic processing of both procaspase-3 and -8.[16] These findings support the idea that NO can act at the earliest steps of the apoptosis signal cascade by blocking the apical procaspase-8 proteolytic activation. This is consistent with the observation that NO inhibits Bcl2 cleavage, cytochrome *c* release, loss of mitochondrial transmembrane potential and activation of caspase-3, all events distal to the proteolytic activation of caspase-8.[16,36]

Although it is currently unknown whether NO has any effect on the assembling and recruitment of procaspase-8 to the TNFα-receptor 1 (death signaling complex), the fact that NO directly S-nitrosylates caspase-8 and inhibits caspase-8 activity *in vitro* suggests that a plausible mechanism is the S-nitrosylation of procaspase-8.[22] This could prevent autoactivation of procaspase-8 that is thought to occur when the zymogen is bound to FADD. However, this proposed mechanism poses a significant problem. If NO inhibits apoptosis by blocking caspase processing and catalytic activity, how is it that NO initiates caspase-dependent apoptosis in some cell types (see parallel review by Brune in this issue).[37] The answer is probably that cells differ in their capacity to carry out S-nitrosylation reactions. The efficiency of S-nitrosylation in cells is dependent on the chemical fate of NO in a particular environment. For NO to nitrosylate a target, it must first give up one electron to form NO^+ or its equivalent. Potential electron acceptors include oxygen molecule and transition metals such as iron (also see the review by Lipton in this issue).[38] The abundant iron content in hepatocytes may explain the efficiency of S-nitrosylation of protein targets including caspases in these cells. We have found that it is necessary to pretreat MCF-7 cells[36] and RAW264.7 cells with iron to observe NO-dependent inhibition of caspase activity and prevention of apoptosis. Other pathways to S-nitrosylation undoubtedly exist and it is likely that the redox status of a cell will determine the capacity of NO to either protect or injure a cell. This is supported by data indicating that iNOS expression, typically well tolerated by hepatocytes, becomes injurious in states of severe redox stress such as ischemia/reperfusion or hemorrhagic shock.[6] The finding that endogenous NO nitrosylates endogenous caspase-3 in intact cells reinforced the concept that NO suppresses apoptosis via inhibiting caspases through S-nitrosylation.[39,40]

NO-mediated protection against apoptosis in some cell types, such as motor and sympathetic neurons[41,42] and splenocytes,[43] occurs via a cGMP-dependent mechanism. Hepatocytes are also partially protected by cyclic nucleotides[10] (and unpublished data), and NO has been previously shown to increase cGMP levels in hepatocytes.[44] NO-mediated inhibition of apoptotic cell death in cultured hepatocytes can be partially mimicked with a cGMP analog.[10] In fact, 8-Br-cGMP at concentration of

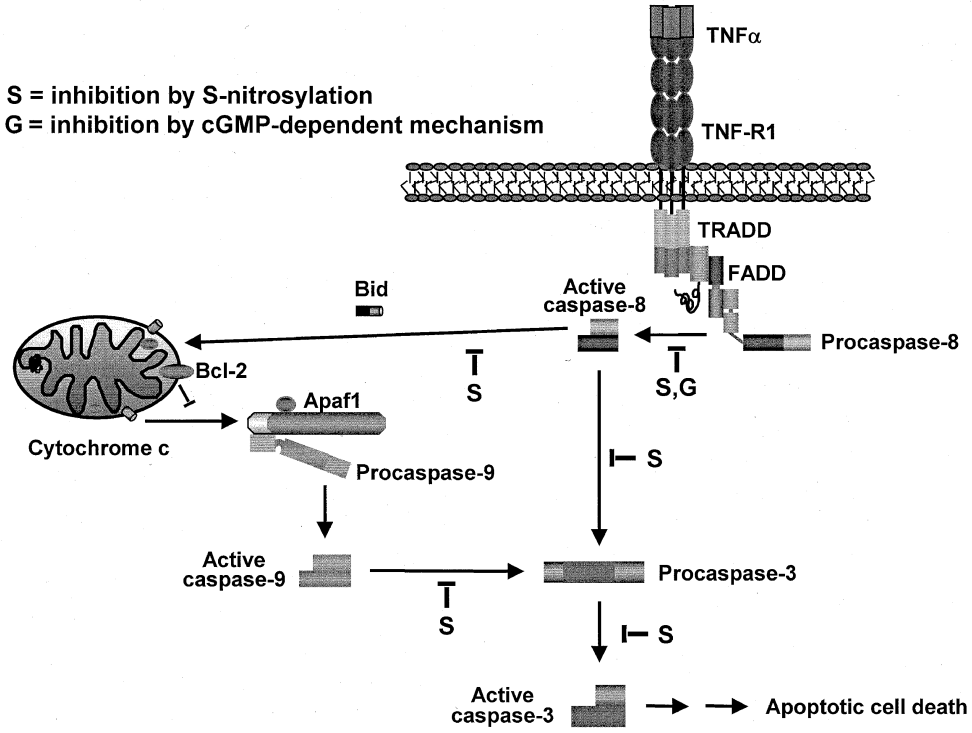

Figure 1 The proposed sites for NO in the inhibition of TNFα+ActD-induced apoptosis in hepatocytes

800 μM blocks the proteolytic activatioan of caspase-3, the release of cytochrome *c*, and the loss of mitochondrial transmembrane potential in cells challenged with TNFα and ActD (unpublished data). This cGMP effect appears to be mediated through the activation of protein kinase G, but may also depend on the activation of protein kinase A. Inhibition of soluble guanylyl cyclase with ODQ, partially prevented the anti-apoptotic effect of NO,[10] suggesting that the protective actions of NO in hepatocytes are mediated in part via cGMP. The mechanism by which cGMP suppresses caspase activation is not clear, but probably involves the interaction of the cGMP-dependent protein kinase pathway with the apoptotic signaling pathway.

In summary, the dominant protective action of NO in hepatocytes appears to be direct inhibition of caspases through S-nitrosylation with cGMP playing a minor role. Nonetheless, the dual mechanisms for protection by NO are unique to hepatocytes and the proposed sites for NO-mediated inhibition of apoptotic signaling in hepatocytes are depicted in Figure 1. As our understanding of the mechanisms by which NO inhibits apoptosis in hepatocytes advances and methods to deliver NO to liver improve, it may be possible to devise therapies to limit hepatic injury.

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