Inflammatory Modulation of Hepatocyte Apoptosis by Nitric Oxide: *In Vivo*, *In Vitro*, and *In Silico* Studies

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Abstract: Nitric oxide (NO•) and its reaction products are key players in the physiology and pathophysiology of inflammatory settings such as sepsis and shock. The consequences of the expression of inducible NO• synthase (iNOS, NOS-2) can be either protective or damaging to the liver. We have delineated two distinct hepatoprotective actions of NO•: the stimulation of cyclic guanosine monophosphate and the inhibition of caspases by S-nitrosation. In contrast, iNOS/NO• promotes hepatocyte death under conditions of severe redox stress, such as hemorrhagic shock or ischemia/reperfusion. Redox stress activates an unknown molecular switch that transforms NO•, which is hepatoprotective under resting conditions, into an agent that induces hepatocyte death. We hypothesize that the magnitude of the redox stress is a major determinant for the effects of NO• on cell survival by controlling the chemical fate of NO•. To address this hypothesis, we have carried out studies in relevant *in vivo* and *in vitro* settings. Moreover, we have constructed an initial mathematical model of caspase activation and coupled it to a model describing some of the reactions of NO• in hepatocytes. Our studies suggest that modulation of iron, oxygen, and superoxide may dictate whether NO• is hepatoprotective or hepatotoxic.

INTRODUCTION

The L-arginine → NO• pathway was first fully elucidated in 1987 [1-3]. The magnitude of this discovery was recognized by *Science* Magazine in 1992 when NO• was named molecule of the year [4]. In 1998, Furchgott, Ignarro, and Murad received the Nobel Prize in Medicine for their discoveries on the role of the NO• → cyclic guanosine monophosphate (cGMP) system in the cardiovascular system. Nitric oxide is produced enzymatically by one of three NO• synthases (NOS) [5-7]. These enzymes are products of distinct genes that share approximately 50% sequence homology. Their nomenclature is defined by the cellular expression patterns that led to their discovery and the order in which the cDNAs were first cloned: neuronal nNOS (NOS-1), inducible or inflammatory iNOS (NOS-2), and endothelial eNOS (NOS-3). Neuronal NOS and eNOS are typically referred to as constitutive because these isoforms are present in tissues under resting conditions. Regulation of these enzymes is predominantly post-translational. A dominant mechanism for control involves the Ca^{2+}-dependent binding of calmodulin to eNOS or nNOS that then transiently activates the enzyme to produce small bursts of NO•. Numerous other regulatory mechanisms have been identified, and both constitutive isoforms are now known to have a wide range of tissue expression [8,9].

The focus of our research is iNOS. This isoform is not typically expressed in the cellular resting state (exceptions include intestinal and bronchial epithelial cells, and renal tubular epithelial cells [6,7]. Instead, gene transcription is activated under acute inflammatory or stress conditions, and because calmodulin binds to iNOS in the absence of calcium elevations, [10] the enzyme produces NO in large quantities in a sustained manner. Pro-inflammatory cytokines, microbial products, and oxidative stress are all particularly effective at stimulating iNOS transcription [5-7,11]. Not surprisingly, transcription factors activated by these stimuli (*e.g.* NF-κB, STATs, AP-1, HNF4-α) are all known to be involved in iNOS gene activation [5-7]. Although iNOS expression was first identified in murine macrophages, it is now known that iNOS can be expressed by virtually every cell type, if appropriately stimulated, in all mammalian species.

Some of the earliest effects ascribed to iNOS involved cell viability and apoptosis: Hibbs *et al.* [1] first proposed a cytotoxic role for macrophage-derived NO in 1987, and the Albina laboratory first showed that NO• could increase apoptosis in 1993 [12]. Stamler and coworkers [13] first revealed in 1994 the capacity of NO• to prevent apoptosis. As is the case in many other physiological and pathological processes, NO• plays a dual role vis-à-vis cell death in hepatocytes. Under conditions that

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induce apoptosis, NO• is protective. In contrast, under conditions of redox stress, NO• potentiates cell death. Below we summarize extensive studies, both from our laboratory of those of others, designed to address this paradox. These studies have been carried out *in vitro*, using hepatocyte cell culture; *in vivo*, using relevant rodent models; and, more recently in our laboratory, *in silico*, using an initial mathematical model that combines the likely chemistry of NO• in hepatocytes with the apoptotic pathway.

NITRIC OXIDE SYNTHESIS AND THE LIVER

Many surgical diseases and the surgical procedures used as therapy for these diseases result in acute stress and inflammation that can lead to end-organ dysfunction or damage. Common insults experienced by surgical patients include exposure to septic stimuli, shock resulting from numerous etiologies, and ischemia/reperfusion injury. The inflammation that ensues from these injuries results in the activation of inflammatory cascades, including the up-regulation of iNOS [14]. This manuscript is focused on the hypothesis that it is not only the magnitude of iNOS up-regulation that dictates the consequences of induced NO•, but just as important to the homeostatic balance is the cellular context in terms of the local redox state of the tissue.

Inducible NOS can be expressed in all cell types in the liver, but hepatocyte expression still appears to be the most prominent and consistent across species. *In vitro*, interleukin-1 (IL-1), tumor necrosis factor (TNF), interferon-γ (IFN-γ), and bacterial lipopolysaccharide (LPS) act synergistically to induce iNOS expression maximally in hepatocytes [15]. Interleukin-1 is the most potent single inducer of iNOS,[16] while redox stress further activates iNOS gene transcription [11].

In vivo, hepatocyte iNOS expression has been identified in response to endotoxemia, [17] hemorrhagic shock, [18,19] hepatic and intestinal ischemia/reperfusion, [20] and during hepatic regeneration [21] or hepatitis [22,23]. Whereas acute insults result in a rapid but transient increase in iNOS expression, massive and sustained hepatocyte iNOS expression is observed following injection of killed *Corynebacterium parvum* [24,25]. This exuberant hepatic iNOS expression lasts for days with little evidence of hepatic injury [24]. Evidence for up-regulation of iNOS in humans under conditions similar to the animal models came first from a study in septic patients, demonstrating a four-fold increase in circulating levels of reactive nitrogen species [26]. Similarly, iNOS mRNA is present in human liver following trauma [19].

Thus, ample evidence exists that iNOS expression is part of the pathophysiology of sepsis and shock. However, unraveling the actions of iNOS *in vivo* has been much more challenging. Much has been learned using nonselective pharmacological NOS inhibitors, selective iNOS inhibitors, and iNOS- and eNOS- null mice to assess hepatic NOS function. It appears from several reports that inhibition of eNOS enhances hepatic injury in animal models of sepsis, hemorrhagic shock and ischemia/reperfusion [25,27]. Endothelial NOS appears to be critical for preserving tissue perfusion and endothelial cell viability [20,28]. In contrast, iNOS has opposing actions depending on the specific redox milieu. From studies employing both iNOS-selective inhibitors and iNOS knockout mice, it has been suggested that iNOS expression increases hepatocellular injury in hemorrhagic shock [18] and ischemia/reperfusion [20]. This damage is evident within three to six hours of either resuscitation or reperfusion and hence occurs prior to significant polymorphonuclear neutrophil (PMN) infiltration. In hemorrhagic shock, iNOS expression is also associated with enhanced IL-6 production and NF-κB activation, while iNOS expression in ischemia/reperfusion appears to enhance the efficiency of reperfusion. However, in both models the temporal sequence of events support direct hepatocellular injury in the presence of iNOS. Expression of iNOS has also been shown to contribute to toxicity in models of alcohol-induced cirrhosis [23] and hepatitis induced by concanavalin A or acetaminophen [22,29,30].

These observations stand in contrast to studies in animal models of endotoxemia, [31,32] lipopolysaccharide/D-galactosamine (LPS/D-gal) administration, [33] hepatic regeneration [21] and cold ischemia/reperfusion following transplantation [34]. In each of these settings, either the absence or the inhibition of iNOS resulted in enhanced hepatocyte apoptosis. The anti-apoptotic capacity of NO• in the liver was most evident in the TNF/D-gal model of massive hepatic apoptosis. Using a liver selective NO• donor, it was demonstrated that NO• almost completely inhibited the widespread cell death seen in this model [33]. Although the anti-apoptotic actions are profound, other iNOS-mediated protective effects include the inhibition of PMN influx [35] and improved liver perfusion [36].

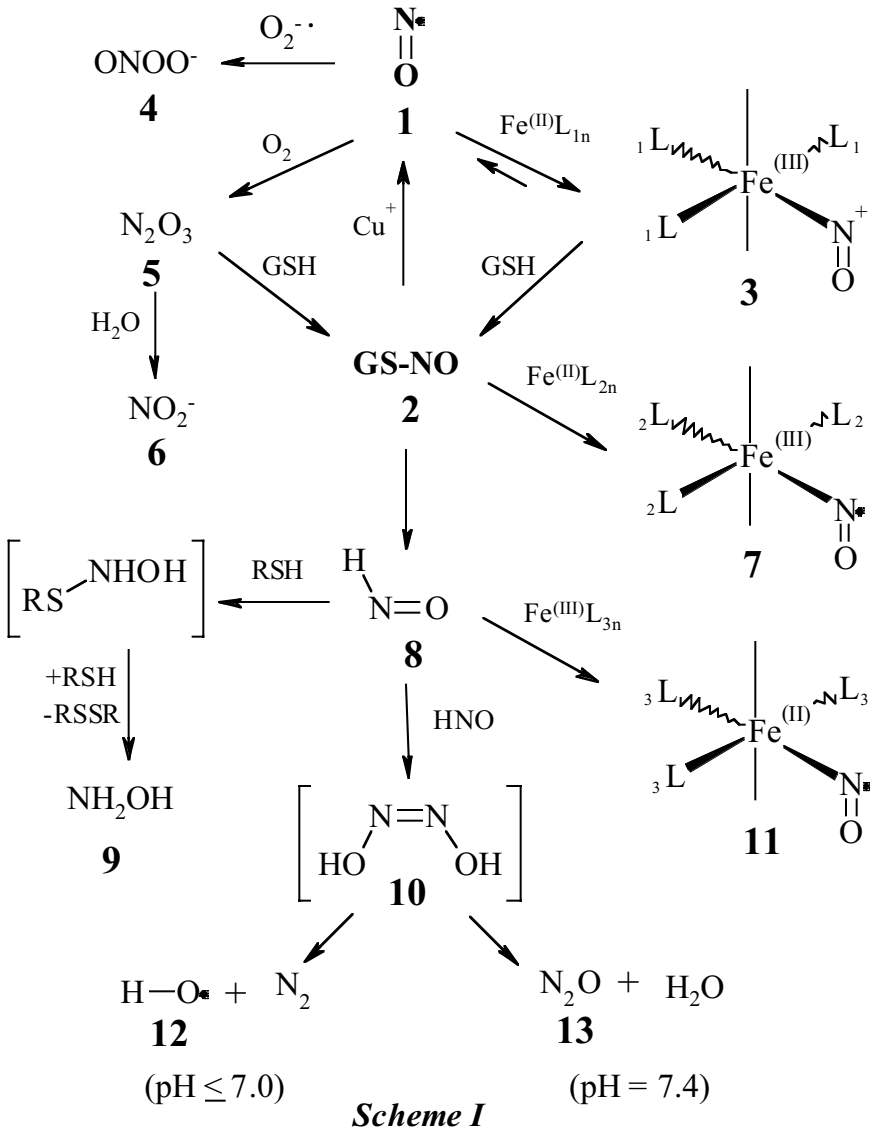
THE CHEMICAL FATE OF NO• IN BIOLOGICAL SYSTEMS

These *in vivo* observations provide the basis for our hypothesis that the chemical fate of NO• dictates whether iNOS expression contributes to damage or inhibits cell death. In endotoxemia, hepatic regeneration, or TNF administration, hepatocytes are exposed to pro-apoptotic signals (e.g. death receptor ligands, TNF, or bile salts) in the setting of preserved antioxidant defenses. Recent studies suggest that the NO•-mediated protection involves the S-nitrosation of caspases and the formation of cGMP, [37] leading to the hypothesis that the pathways to S-nitrosation and cGMP production are preserved in hepatocytes protected by NO•. On the

other hand, hemorrhagic shock and warm ischemia/reperfusion deplete antioxidant defenses and result in severe redox stress. In this setting, iNOS may contribute to cell damage, possibly through the formation of oxidizing species.

If the chemical fate of NO• ultimately dictates the consequence of NO• synthesis on cell survival, it is necessary to understand the chemistry of NO• in cells. The chemical fate of NO• in cells remains the topic of considerable debate due, in large part, to the complexities associated with measuring the abundance of short-lived radicals. It is reasonably well accepted that NO•, with its one unpaired electron, will react avidly with oxygen, superoxide anion radical (O_2^-), and transition metals. These reactions can lead to the modification of proteins, resulting in the activation or inactivation of enzymes, or lead to cellular toxicity through various other means. It is also safe to conclude that the chemistry resulting from these interactions can be separated into nitrosation or oxidation [38,39]. The challenge in the field has been in defining the pathways to nitrosation or oxidation in intact tissue. The chemical fate of NO (i.e., nitrosation vs. oxidation) is likely to depend on the redox status of the cell. This process will depend not only on cell type, but also environmental factors such as ischemia.

Figure 1 depicts the main reaction pathways of NO• in biological systems. In a resting cell, the interaction of NO• with oxygen (1→5) and iron complexes (1→3) leads to the formation of S-nitrosoglutathione (GSNO, 2) via the intermediate formation of N_2O_3 and iron-nitrosyl complexes ($L_nFe(NO)_m$). In turn, N_2O_3, ($L_nFe(NO)_m$) and GSNO



Scheme I

Figure 1. Proposed scheme for the biochemical reactions of NO• in hepatocytes exposed to redox stress. Nitric oxide can possibly react with oxygen (O_2), superoxide (O_2^-), and thiol groups to generate various products, including nitroxyl anion (HNO) and peroxynitrite ($ONOO^-$). In turn, HNO can react to form hydroxyl radical (•OH) under acidic conditions.

can S-nitrosate thiols on proteins including caspases [37,40,41]. One consequence of GSH depletion, as seen during redox stress, would be enhanced peroxynitrite formation resulting from the interaction of NO• and O_2^- (1 → 4). Glutathione may also be involved in the detoxification of peroxynitrite. Hence, excessive reactions of nitrosation may lead to "nitrosative stress" via depletion of low molecular weight and protein thiols.

The pathophysiological effects of nitroxyl (HNO), the one-electron reduction product of NO•, have attracted much interest in recent years. The biosynthesis of HNO has been suggested to proceed via reduction of NO• by SOD, [42] ferrocytochrome c, [43] and ubiquinol, [44] and/or reduction of GSNO by GSH and the alcohol dehydrogenase system [45-47]. Several groups have reported that HNO, per se a strong reductant, [48] can trigger reactions of oxidation. Mechanistically, these reactions were suggested to occur with the intermediate formation of either •OH [49-53] or (an isomer of) [54] peroxynitrite; [53,55] direct detection of the corresponding oxidizing species, however, has proven to be difficult. Irrespectively of the exact mechanism of these reaction(s), overproduction of HNO in tissues subjected to acidosis may lead to oxidative stress inasmuch as the ability of HNO to act as a pro-oxidant dramatically increases in acidic solutions (ref.[51]; D. Stoyanovsky, personal communications). It could be generalized that metabolic hyperactivity or limited oxygen supply can cause a decrease of tissue pH. Acidosis is characteristic for such disease states as sepsis, arthritis, ischemia, and cancer [56-60].

Given the myriad possible reaction products of NO• in hepatocytes, unraveling the effects of NO• in the process of apoptosis is a daunting task. One approach by which to begin this process, recognizing that the specific chemical fate of NO• may affect its pro- or anti-apoptotic properties, is to create a mathematical model that encompasses the key reactions described above. Such a mathematical model, and the *in silico* experiments it allows, may allow for the better understanding of how NO• carries out diverse effects. The transport and kinetics of NO• in several cellular systems have recently been modeled (reviewed in [61]). Mathematical simulations of NO• production and diffusion in endothelial cells have suggested that NO• diffuses to neighboring cells and may therefore modulate targets there [62]. We incorporated this information into a mathematical model describing the likely reactions of NO• in hepatocytes, a model that unifies the rate constants of the preponderant intracellular reactions of NO• (Figure 1). It should be noted, however, that some of the rate constants reported in the literature for the reactions of NO• vary considerably with small modifications of the experimental settings. Hence, a major effort in such an undertaking involves experimental validation, as well as the adjustment of the mathematical model, in order to unify the thermodynamic data on NO• in predictive algorithms. For example, in order to determine whether NO• can be converted to HNO in hepatocytes, the signature for HNO formation, hydroxylamine (reaction scheme I; 8 → 9), was measured in cells treated with the chemical NO• donor S-nitroso-N-acetylpenicillamine (SNAP; Figure 2A; D. Stoyanovsky, personal communications). A concentration-dependent increase in hydroxylamine formation was seen in the NO•-treated cells, confirming that HNO is formed from NO• in hepatocytes (Figure 2A). Our mathematical model verifies this result: when iNOS concentration is increased to high levels, the virtual concentration of NH_2OH increases linearly (Figure 2B).

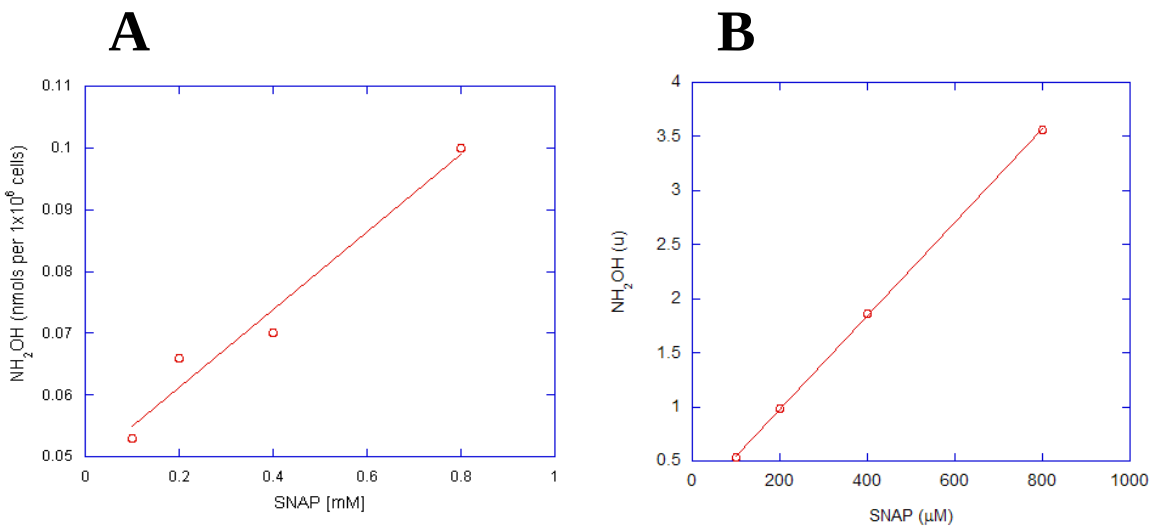


Figure 2. Nitroxyl radicals can be detected in hepatocytes exposed to NO• under normoxia: *in vitro* and in silico studies. A) HPLC-EC-monitored formation of NH_2OH (the stable reaction product of nitroxyl anion) in PBS (pH 7.4) containing hepatocytes ($5x10^6$ per mL) exposed to SNAP (0.4 mM; t_{inc} = 20 min at 37 °C). B) Mathematical simulation of the chemistry of NO• in hepatocytes, showing generation of NH_2OH with increasing amount of iNOS.

Our ultimate goal is to understand how NO• and/or its reaction products impact hepatocyte viability. As discussed below, the mathematical model of the chemistry of NO• in hepatocytes has been extended to address apoptotic pathways. For clarity, we first describe the apoptotic process and the known effects of NO• on this process.

MODELING APOPTOSIS

In order to understand how NO• impacts hepatocyte apoptosis, it is first necessary to understand the apoptotic process. Ligand-dependent apoptosis in hepatocytes takes place via activation of the type II or mitochondrial-dependent pathway [63]. The binding of pro-death ligands (e.g. FasL, TNF, or TRAIL) to specific receptors can induce oligomerization of the receptors, followed by the recruitment of adapter proteins to the cytoplasmic portions of the receptor [64]. Common to all death receptors is the binding of FADD (Fas-Associated Death Domain protein) [65,66]. The TNF signal transduction pathway is more complex, in that signaling through the p55 receptor activates inflammatory pathways distinct from apoptotic pathways [67]. The p55 TNF receptor also recruits TRADD (TNF Receptor-Associated Death Domain) as well as RIP (Receptor Interacting Protein) and RAIDD (RIP-associated Protein with a Death Domain) [68]. Recruitment and binding of large pro-domain initiator

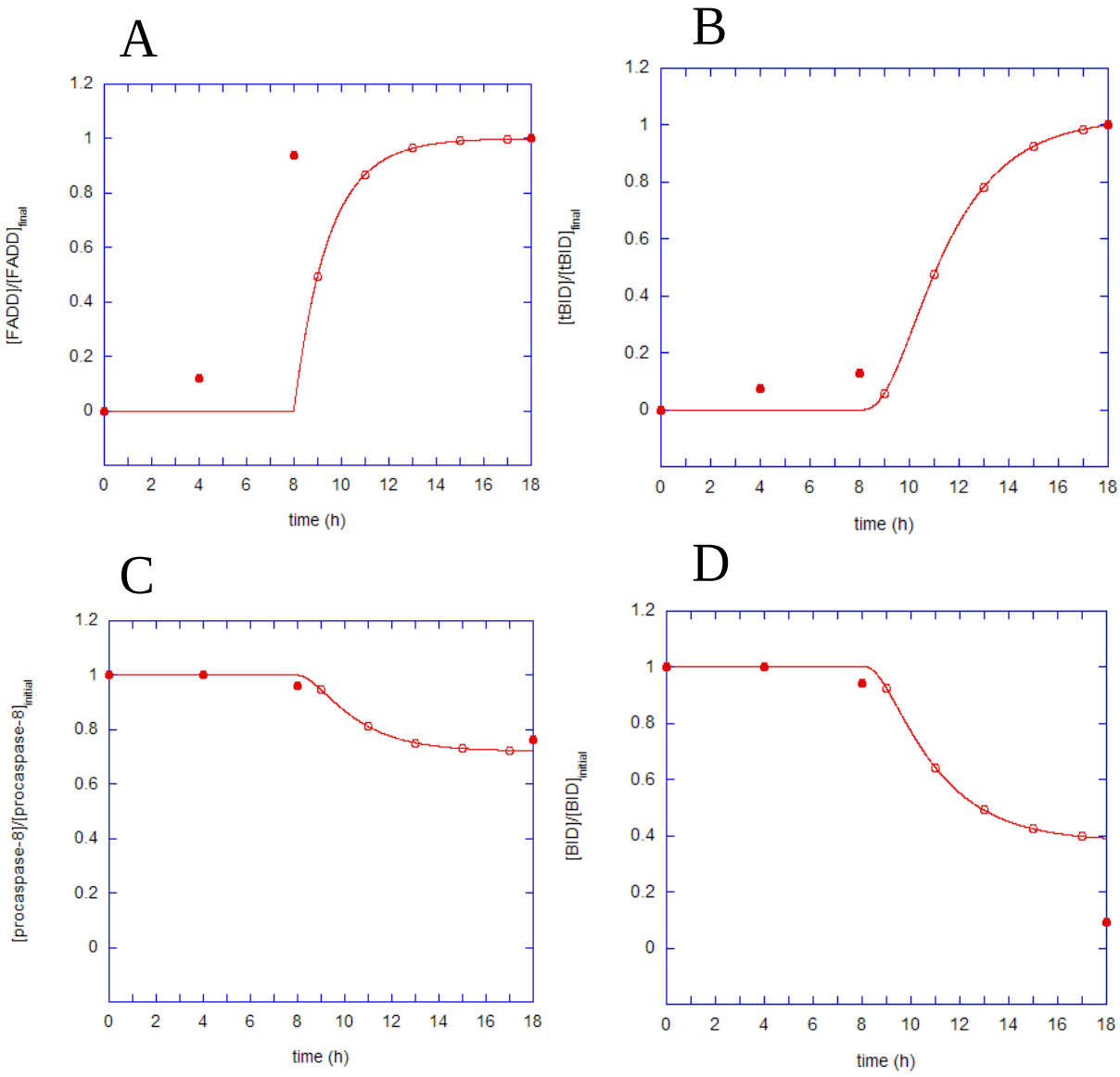


Figure 3. Mathematical simulation of the effects of FADD over-expression on levels of procaspase-8, cleaved caspase-8, BID, and tBID. A) FADD and B) tBID levels increase whereas C) procaspase-8 and D) BID decrease in stimulated rat hepatocytes in which FADD levels are increased, simulating the effects of transduction with an adenovirus expressing FADD.[82] The dark symbols show the experimental values obtained by quantifying western blots with Scion Image software (http://www.scioncorp.com). The curves with blank dots show simulation results. The concentrations are shown relative to the initial or final values in both experimental and simulation results.

caspases, such as caspase-8, through binding of its pro-domain with the death effector domain of FADD, is characteristic of the formation of the DISC (Death Inducing Signaling Complex) [69]. This is thought to result in auto-cleavage and activation of caspase-8 [70,71]. In hepatocytes, caspase-8 then cleaves BID (BCL-2 Interacting Domain), a pro-death Bcl-2 family member [63]. The BID cleavage product interacts with the mitochondria, leading to loss of membrane potential and release of pro-death signaling proteins including cytochrome *c*. Cytochrome *c* supports the formation of the apoptosome, which is comprised of Apaf-1 (Apoptotic Protease Activating Factor-1), caspase-9, and adenosine triphosphate (ATP) in addition to cytochrome *c*. This complex activates downstream effector caspases, such as caspase-3 and -7 [72]. Enzymatic cleavage of downstream targets of these effector caspases results in the morphological and structural changes characteristic of apoptosis. Hepatocytes are representative of Type II cells in that ligand-dependent apoptosis depends on BID cleavage.[63] In contrast, cells utilizing the Type I pathway bypass the mitochondria, and the proximal caspases directly cleave and activate the downstream effector caspases [73].

A mathematical model of the caspase activation cascade has been reported recently, [74] though this model does not account for the possibly central role of S-nitrosation of caspases in the initiation of this process [75]. Thus, an *in silico* approach to understanding the chemistry of NO• may aid in the understanding of the effects of this free radical in biological systems. Accordingly, we sought to combine mathematical models of apoptosis and of the chemistry of NO• in order to begin to understand how NO• affects apoptosis in hepatocytes (Bagci *et al*, manuscript in preparation). We introduced a number of changes to the model of Fussenegger *et al*, [74] aimed at increasing the physical realism of the model. Also some additions were made to their apoptosis model, such as making the function of p53 explicit [76-79] and including the pro-apoptotic protein Bid [80] in the pathways. This mathematical model also accounts for the release of cytochrome *c* more accurately, secondary to cleavage of BID to truncated BID (tBID), [81] consistent with previous experimental observations.

The predictions of this model were compared to a previous experimental study in which apoptosis was induced in hepatocytes using an adenovirus expressing mouse FADD [82]. That study showed that FADD, cleaved caspase-8, and tBid levels increase, whereas procaspase-8 and Bid decrease [82]. This result was verified by the model as shown in Figure 3. Kim *et al* showed that cell viability decreases with increase in multiplicity of infection with FADD [82]. The mathematical model conforms to this observation because increase in FADD expression (achieved by increase in the rate of formation of FADD) increases the levels of caspase-3 that is a key enzyme for apoptosis (Figure 3).

NITRIC OXIDE APOPTOSIS: S-NITROSATION, CGMP PRODUCTION, AND MODULATION OF APOPTOSIS-RELATED GENES

In macrophages, pancreatic islet cells, neurons, enterocytes, thymocytes, cardiac myocytes, endothelial cells, and fibroblasts, even low level NO• lead to apoptosis. In contrast, B lymphocytes, natural killer cells, eosinophils, embryonic motor neurons, pheochromocytomas, ovarian follicles, and hepatocytes can be protected by NO• against apoptosis induced in various ways [81]. Hepatocytes are unique in that not only are these cells protected by NO•, but also necrotic death is not seen until the cells are exposed to supraphysiologic concentrations of NO• donors in the millimolar range [83,84]. This response changes dramatically when the cells are subjected to redox stress. Under these circumstances, NO• induces hepatocyte necrosis even at low to moderate concentrations. In NO• -sensitive cell types, the pro-apoptotic actions of NO• involve DNA damage and the activation of p53, [85] the up-regulation of Fas and Fas ligand (FasL), [86-89] and direct effects on mitochondria. The mitochondria may be a particularly important target in susceptible cells and hepatocytes exposed to redox stress. Targets for NO• in the mitochondria include cytochrome *c* oxidase, mitochondrial complexes I, II, III, IV, and V, aconitase, creatine kinase, the mitochondrial membrane, mitochondrial DNA, and superoxide dismutase [90,91,91].

Most studies suggest that the dominant anti-apoptotic effect of NO• in hepatocytes involves the direct modification of caspases by S-nitrosation [28,37,40,75,92]. Caspases are cysteine proteases, and all possess active-site cysteine residues essential for their activity. This cysteine is susceptible to S-nitrosation that blocks enzyme activity. At least 5 of the 14 known caspases are activated in hepatocytes stimulated to undergo apoptosis [41]. For S-nitrosation to take place, NO• must react with an electron acceptor (e.g. oxygen, transition metals). This process is very efficient in hepatocytes in the absence of redox stress, and the direct inhibition of caspases seems to be the dominant mechanism for the inhibition of apoptosis in hepatocytes.

In the NO•-sensitive macrophage-like cell line RAW 264.7, exposure to SNAP at concentrations $\geq$ 0.5 mM led to cell death marked by DNA fragmentation and caspase activation [83]. In clear distinction to hepatocytes, caspase inhibitors prevented this death. It was suggested that caspases were efficiently S-nitrosated in hepatocytes but not macrophages due to the high non-heme iron content in hepatocytes. Iron is important because NO• can react with Fe^{2+} to form iron-nitrosyl complexes, and formation of these complexes would be an efficient pathway for caspase S-nitrosation. Iron-nitrosyl complexes can S-nitrosate caspases, and iron supplementation increased the formation of iron-nitrosyl complexes in NO•-treated RAW 246.7 cells. The formation of these complexes in the RAW

264.7 cells was associated with higher cellular S-nitrosothiol content as well as an inhibition of NO•-mediated toxicity. Thus, diverting the chemical fate of NO• toward S-nitrosation reduced the toxicity of NO• in at least one cell type. It may be hypothesized that redox stress will further modulate the fate of NO• in hepatocytes, such that S-nitrosothiols that are formed will be metabolized to toxic •OH during conditions of redox stress.

However, not all of the protection afforded to hepatocytes by NO• is due to S-nitrosation. One downstream effector of NO• is cGMP, a cyclic nucleotide that is produced by the soluble guanylyl cyclase [93,94]. In some cells, the pro-death signaling is initiated by cGMP, [95,96] while in others (including hepatocytes) cGMP inhibits apoptosis [37,97,98]. Similar to NO•, cell permeable cGMP analogs suppressed caspase-8 activation, loss of mitochondrial membrane potential, cytochrome *c* release, and caspase-3 activation. Non-hydrolyzable analogs of cyclic adenosine monophosphate (cAMP) were even more potent in the inhibition of hepatocyte cell death. The protective effects of both cGMP and cAMP appear to be dependent, in part, on protein kinase A (PKA). Furthermore, NO•, cGMP, and cAMP all activated the Akt/protein kinase B pathway in cultured hepatocytes [97]. Activation of the protein kinase Akt is protective in many cell types, through the phosphorylation and inhibition of BAD (Bcl-Associated Death Promoter) and caspase-9 [99]. However, inhibition of Akt activation minimally altered the protective effects of either NO• or the cyclic nucleotides in hepatocytes [97]. Hepatocytes may therefore be unique in that NO• protects both through the efficient S-nitrosation of caspases and through the activation of soluble guanylyl cyclase, and that both mechanisms suppress the activation of the proximal caspases. Taken together, these observations indicate that the capacity of NO• to limit cell death in hepatocytes should be correlated primarily with the efficiency of S-nitrosation and to a lesser extent on the production of cGMP. These events should, in turn, be dictated by the chemical fate of NO•.

Other anti-apoptotic actions of NO• include the up-regulation of anti-apoptotic Bcl-2 family proteins,[100,101] the down-regulation of pro-death genes [102,103] and proteins (e.g. the E1B 19K/Bcl-2-binding protein Nip3 [BNIP3]), [103] The decrease in BNIP3 was shown to depend on cGMP [103]. At higher levels, NO• can up-regulate heat shock protein 70 (Hsp70), which in turn can interfere with the proximal signaling steps in apoptosis [104]. The effect of NO• on Hsp70, as well as other apoptosis-related genes, was also observed in DNA microarray studies [102].

This large series of pro- and anti-apoptotic mechanisms induced by NO• and its reaction products makes prediction of the effect of NO• under any given setting difficult. To begin to address this complexity, we combined our mathematical model of caspase activation in apoptosis and the model of the chemistry of NO• (described above) in order to begin to model the mechanisms by which NO• may inhibit apoptosis in hepatocytes. The chemical pathways of NO• were coupled to the apoptotic pathways directly through S-nitrosation of caspases and the

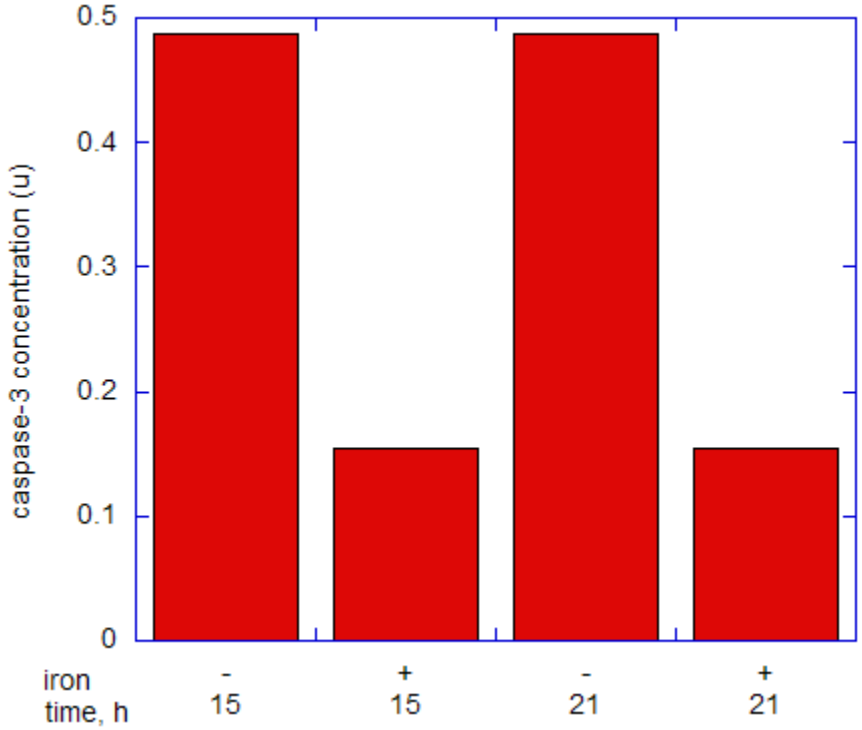


Figure 4. Effects of iron on NO-induced cell death. Mathematical simulation of caspase-3 concentration in the absence and presence of iron, in agreement with previous results.[83]

generation of cGMP, and indirectly through the DNA-damage induced production of p53 and activation of protein kinase G (PKG) that in turn inhibits mitochondrial cytochrome *c* release.

The model was first used to examine how the modulation of intracellular iron affects the anti-apoptotic role of NO•. Cellular non-heme iron modulates effects of NO• [83]. Iron levels are low in the macrophage-like RAW264.7 cells and their caspase-3-like activity is high. When iron is introduced to those cells in levels comparable to those in hepatocytes, caspase-3-like activity is lowered in the presence of high levels of NO (maintained via exposure to SNAP) [83]. The mathematical model reproduces this result (Figure 4). Furthermore, the model shows that the anti-apoptotic effects of NO• mediated via cGMP (through the activation of PKG and inhibition of cytochrome *c* release [105,106]) are not as effective as the iron pathway. In the absence of iron, the concentration of caspase-3 is still high despite the presence of cGMP. While still requiring additional experimental validation and calibration, this mathematical model may prove to be of benefit to investigators struggling with the complexity of the chemistry of NO• in biological systems, and may serve as a template for integrating the chemistry of NO• with pathways other than caspase activation.

CONCLUSIONS

Nitric oxide exerts both pro-necrotic and anti-apoptotic effects on hepatocytes in settings of inflammation and tissue damage, apparently depending on the level of concomitant redox stress. In settings of inflammation that do not involve redox stress, NO• and its reaction products inhibit apoptosis by S-nitrosation of caspases at their catalytic site cysteine residue, triggering of the cGMP pathway, modulation of heat shock proteins and possibly other apoptosis-related gene products, and prevention of mitochondrial dysfunction. In settings of redox stress, NO• potentiates cell death induced by oxygen free radicals. For the first time, some of these complex effects can be examined by traditional *in vitro* and *in vivo* methods, as well as by complex systems analysis that allows for *in silico* experiments. This combination of approaches may yield novel insights into the role that the chemistry of NO• plays in modulating apoptosis and necrosis.

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ABBREVIATIONS

AP-1 = Activator Protein-1

Apaf-1 = Apoptotic Protease Activating Factor-1

ATP = adenosine triphosphate

BAD = Bcl-Associated Death Promoter

BID = BCL-2 Interacting Domain

tBID = truncated BID

BNIP3 = the E1B 19K/Bcl-2-binding protein Nip3

BSO = buthione sulfoximine

cAMP = cyclic adenosine monophosphate

cGMP = cyclic guanosine monophosphate

DCF = dichlorofluoroscein

DEM = dimethyl maleate

DISC = Death Inducing Signaling Complex

DTT = dithiothreitol

eNOS, FADD = Fas-Associated Death Domain protein

GSH = glutathione

GSNO = S-nitrosoglutathione

GSSG = oxidized GSH

HNF4-α = Hepatocyte Nuclear Factor 4

inducible NO• synthase

iNOS = inducible NO• synthase

IFN-γ = interferon-γ

IL- = interleukin

LPS = bacterial lipopolysaccharide

LPS/D-gal = lipopolysaccharide/D-galactosamine

NO• = nitric oxide

NOS = NO• synthase NF-κB, Nuclear Factor κB

PKA = protein kinase A

PMN = polymorphonuclear neutrophil

RAIDD = RIP-associated Protein with a Death Domain

RIP = Receptor Interacting Protein

RSNO = S-nitrosothiols

SNAP = S-nitroso-N-acetylpenicillamine

STAT = Signal Transducer and Activator of Transcription

TNF = Tumor Necrosis Factor-α

TRADD = TNF Receptor-Associated Death Domain

TRAIL = TNF-related Apoptosis Inducing Ligand

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