Computational Insights on the Competing Effects of Nitric Oxide in Regulating Apoptosis

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Abstract

Despite the establishment of the important role of nitric oxide (NO) on apoptosis, a molecular-level understanding of the origin of its dichotomous pro- and anti-apoptotic effects has been elusive. We propose a new mathematical model for simulating the effects of nitric oxide (NO) on apoptosis. The new model integrates mitochondria-dependent apoptotic pathways with NO-related reactions, to gain insights into the regulatory effect of the reactive NO species N2O3, non-heme iron nitrosyl species (FeLNO), and peroxynitrite (ONOO⁻). The biochemical pathways of apoptosis coupled with NO-related reactions are described by ordinary differential equations using mass-action kinetics. In the absence of NO, the model predicts either cell survival or apoptosis (a bistable behavior) with shifts in the onset time of apoptotic response depending on the strength of extracellular stimuli. Computations demonstrate that the relative concentrations of anti- and pro-apoptotic reactive NO species, and their interplay with glutathione, determine the net anti- or pro-apoptotic effects at long time points. Interestingly, transient effects on apoptosis are also observed in these simulations, the duration of which may reach up to hours, despite the eventual convergence to an anti-apoptotic state. Our computations point to the importance of precise timing of NO production and external stimulation in determining the eventual pro- or anti-apoptotic role of NO.

Introduction

The survival of an organism depends on homeostatic mechanisms that establish a balance between cell proliferation and cell death. Apoptosis, a form of programmed cell death, assists in regulating cell proliferation. This process stands in contrast to necrosis, which is thought to be uncontrolled. Dysregulation of apoptosis has been implicated in various disease processes in which the cells apoptose to a higher or lower extent compared to those in healthy tissues [1]. When cells undergo apoptosis, a series of morphological and biochemical changes occur, the mechanisms of which are current topics of broad interest [2].

Apoptosis may be induced by various events, such as binding of extracellular (EC) death signaling ligands to host cell receptors, the lack of pro-survival signals, and genetic damage. These events are usually followed by the activation of caspases, cysteine-dependent aspartate-specific proteases, which initiate and execute apoptosis. Caspases are activated through two major pathways: (a) ligand-dependent or receptor-induced activation (extrinsic pathway), involving death receptors such as Fas or the members of tumor necrosis factor (TNF) receptor superfamily, and (b) mitochondria-dependent activation (intrinsic pathway) via cytochrome c (cyt c) release from mitochondria, triggered by stress, irradiation or inflammation [3,4].

Binding of death ligands such as Fas ligand (FasL), TNF, or tumor necrosis-related apoptosis-inducing ligand (TRAIL) usually induces the oligomerization of associated receptors, followed by binding of adaptor proteins, e.g., Fas-Associated Death Domain proteins (FADD), to the cytoplasmic domains of the receptors [5]. The resulting Death Inducing Signaling Complex (DISC) recruits multiple procaspase-8 molecules that mutually cleave and activate one another into caspases-8 (casp8). In Type I cells, large quantities of casp8 activate other caspases including the executioner caspase-3 (casp3) molecules that ultimately lead to apoptosis. In Type II cells, on the other hand, the amount of casp8 activated at the DISC is small, such that the activation of casp8 does not propagate directly to casp3, but instead is amplified via the mitochondria.

Nitric oxide has opposite, competing effects in regulating apoptosis: it exerts an anti-apoptotic effect on hepatocytes [6–8], endothelial cells [9–13] and keratinocytes [14], whereas it is pro-apoptotic in the case of macrophages [15–18]. The variability and complexity of the effects of NO on ultimate cellular fate may arise from this molecule’s ability to react with oxygen, reactive oxygen species, metal ions, small thiol-containing molecules, and proteins. The resulting reactive NO species can either trigger or suppress apoptosis through various mechanisms. Chief among them is the S-nitrosative suppression of caspase activation, subsequent to the generation of FeLNO or other species capable of carrying out S-nitrosation reactions (see below) [7,19]. Differences in the levels of NO and its reaction products may also arise from diverse inflammatory settings in which the expression of nitric oxide

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Effects of NO on Apoptosis

Figure 1. (A) Mitochondria-dependent apoptotic pathways in Model I. The dotted box includes the interactions considered in the model. Solid arrows indicate chemical reactions or upregulation; those terminated by a bar indicate inhibition or downregulation; and dashed arrows indicate subcellular translocation. The components of the model are procaspase-8 (pro8), procaspase-3 (pro3), procaspase-9 (pro9), caspase-8 (casp8), caspase-9 (casp9), caspase-3 (casp3), IAP (inhibitor of apoptosis), cytochrome c (cyt c), Apaf-1, the heptameric apoptosome complex (apop), the mitochondrial permeability transition pore complex (PTPC), p53, Bcl-2, Bax, Bid, truncated Bid (tBid). The reader is referred to our previous work [28] for more details. Three compounds (N2O3, FeLnNO and ONOO−) not included in the original Model I [28] are highlighted. These compounds establish the connection with the nitric oxide pathways delineated in panel B. (B) Nitric oxide (NO)-related reactions to GSNO, which is then converted to GSSG and finally back to GSH. Those compounds and interactions are shown in blue. See Table 1 for the complete list of reactions and rate constants. doi:10.1371/journal.pone.0002249.g001

Results

First, we illustrate how different strengths of EC pro-apoptotic signals may result in opposite qualitative responses or different quantitative (time-dependent) responses in the same type of cells [37], using our recently introduced bistable model [28] (illustrated in Figure 1A). Then, we examine the differences in the bistable response of diverse NO producing cells, e.g. cells with different concentrations of GSH and FeLn, in different settings, i.e., with or without production of superoxide.

Delay in apoptosis induction (Model I)

Tyas et al. [37] showed that cells of the same type simultaneously subjected to EC stimuli initiate their apoptotic reactions/pathways through these compounds. GSH modulates their concentrations by reacting with them. GSH is converted by these reactions to GSSG, which is then converted to GSSG and finally back to GSH. Those compounds and interactions are shown in blue. See Table 1 for the complete list of reactions and rate constants.

syntheses (NOS) is affected. For example, quiescent endothelial cells express constitutive NOS (eNOS) that directly produce NO molecules and mediate so-called “direct” effects [20]. Some inflammatory stimuli, on the other hand, lead to inducible NOS (iNOS) expression that subsequently generates reactive NO species, which in turn mediate “indirect” effects of NO. The simultaneous presence of oxygen radicals can generate other reactive NO species that mediate further indirect effects of NO [20]. As another example, hepatocytes and macrophages have different amounts of non-heme iron complexes, which affect the levels of iron-nitrosyl species when NO is produced [21]. Finally, different intracellular levels of glutathione (GSH) can also modulate the time evolution of NO-related compounds [22].

Computational approaches have been used previously to help unravel the complex biology of NO. Biotransport of NO was first modeled by Lancaster [23,24] followed by other groups, among them Zhang and Edwards [25] (reviewed by Buerk [26]). Recently, Hu and coworkers focused on detailed reaction mechanism of NO [22]. These models have shed light into the biotransport of NO and the types of chemical reactions that involve NO and related reactive species. Additionally, a number of mathematical models have been proposed for understanding the mechanisms of apoptosis [27–35], including in particular the work of Eissing et al., which demonstrated the importance of IAP inhibition for imparting bistability in type I cells [30], and that of Rehm et al. [33] and Legewie et al. [32] that showed the same effect in type II cells. These studies have improved our understanding of the robustness of switch mechanisms for regulating apoptosis, but none of them has addressed the dichotomous effects of NO [27–35].

Herein, we propose a mathematical model that may shed light on the pro- and anti-apoptotic effects of NO in specific contexts. The model we propose couples the apoptotic cascade [28] to an extended model of NO reaction pathways initially proposed by Hu et al. [22]. First, we illustrate how identical cells can undergo apoptosis at different time points after being exposed to apoptotic stimuli, in accord with experimental data collected on single cells [36,37]. Then, we examine the apoptotic behavior in response to changes in N2O3, FeLnNO, ONOO−, and GSH levels in the presence of NO production by iNOS. Our simulations provide insights into the origin of the dichotomous effects of NO on apoptosis observed in experiments.

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response at different times. Figure 2 panels A–C illustrate the theoretical time evolutions of casp3 in three identical cells subjected to different strengths of EC apoptotic stimuli (represented here by the initial concentration of casp8) in the absence of NO. For these simulations, we used Model I with three different values of $[\text{casp8}]_0$; 10$^{-5}$ $\mu$M, 10$^{-4}$ $\mu$M, and 1.5$ \times $10$^{-4}$ $\mu$M in the respective panels A–C, while $[\text{casp3}]_0$ was 10$^{-5}$ $\mu$M in all three cases. Panel A shows that low $[\text{casp8}]_0$ leads to the depletion of $[\text{casp3}]$, while $[\text{casp8}]_0$ above a certain threshold (8.35$ \times $10$^{-5}$ $\mu$M) (panels B and C) lead to increase in $[\text{casp3}]$ and thereby onset of cell death. Furthermore, comparison of panels B and C shows that a relatively lower $[\text{casp8}]_0$ (or weaker EC apoptotic signal) results in a time-delayed initiation of apoptosis, in agreement with the single cell experiments done by Tyas et al. [37]. The sharp increase in $[\text{casp3}]$ to its equilibrium level indeed starts about 30 minutes later in panel B, compared to panel C.

Next, we examined how this onset time varies with $[\text{casp8}]_0$. Figure 2D displays the results. An increase in onset time is predicted with decreasing $[\text{casp8}]_0$ up to $[\text{casp8}]_0 = 8.35 \times 10^{-5}$ $\mu$M, after which no apoptotic effect is observed. The time delay is found to obey a logarithmic decay with increasing $D[\text{casp8}]_0$; $[\text{casp8}]_0$–8.35$ \times $10$^{-5}$ $\mu$M, as indicated by the best fitting curve. This analysis shows that cells of the same type may undergo apoptosis at different times due to their different EC microenvironments. Hence, the difference in the onset times among cells of the same type in a given cell culture may be explained without recourse to alterations in the underlying network of biochemical reactions [28].

Nitric oxide-associated network (Model II) (Figure 1B)

The results from our calculations using Model II are shown in Figure 3. Here, we focused on the time evolution of four compounds, GSH, $\text{N}_2\text{O}_3$, Fe$\text{LnNO}$ and $\text{ONOO}^-$, displayed in respective panels A–D. The NO species $\text{N}_2\text{O}_3$, Fe$\text{LnNO}$ and $\text{ONOO}^-$ have been proposed to carry out various indirect effects of NO on cellular pathways, including apoptosis [20].

GSH is an anti-oxidant reduced to GSSG by reacting with nitrosative $\text{N}_2\text{O}_3$ and Fe$\text{LnNO}$, and with oxidative $\text{ONOO}^-$ (Table 1). GSH is depleted to low levels in a switch-like manner due to those reactions (panel A). The depletion of GSH is accompanied by increases in $\text{N}_2\text{O}_3$ and Fe$\text{LnNO}$ concentrations (panels B–C). On the other hand, this switch-like behavior is not that pronounced in $[\text{ONOO}^-]$ time dependence (panel D). Simulations performed with different initial GSH concentrations (three different curves in each panel) change the steady-state concentrations of all three NO-related compounds that interfere with apoptotic pathways (panels B–D). The switch-like increase in $[\text{N}_2\text{O}_3]$ and non-switch-like increase in $[\text{ONOO}^-]$ is in agreement with the results of Hu et al. [22].

Anti-apoptotic and pro-apoptotic effects of NO (Model III)

We analyze here the dynamics of the reduced mitochondria-dependent apoptosis model coupled to anti- and pro-apoptotic response at different times. Figure 2 panels A–C illustrate the shift in the onset time of apoptosis depending on $[\text{casp8}]_0$. Panels B and C illustrate the shift in the onset time of apoptosis (evidenced by increase in $[\text{casp3}]$, see panels B–C). The threshold concentration of $[\text{casp8}]_0$ required for the switch from anti-apoptotic to pro-apoptotic response is calculated to be 8.35$ \times $10$^{-5}$ $\mu$M. Panels B and C illustrate the shift in the onset time of apoptosis depending on $[\text{casp8}]_0$. D) Dependence of apoptotic response time on the initial caspase-8 concentration. The ordinate is the onset time of caspase-3 activation, and the abscissa is the initial concentration of caspase-8 in excess of the threshold concentration required for the initiation of apoptosis (evidenced by increase in $[\text{casp3}]$, see panels B–C). The onset time of caspase-3 activation exhibits a logarithmic decrease with $\Delta[\text{casp8}]_0 ([\text{casp8}]_0–8.35 \times 10^{-5} \mu$M).
pathways associated with NO; see Materials and Methods for the list of reactions/interactions/steps that come into play in this model (III). As mentioned above, NO-related pathways are coupled to apoptotic pathways through \( \text{N}_2\text{O}_3 \), \( \text{FeLnNO} \), and \( \text{ONOO}^- \) that are produced by the reaction of NO with \( \text{O}_2 \), \( \text{FeLn} \), and \( \text{O}_2^- \), respectively. For simplicity, those effects of NO mediated by cGMP \([38,39]\) are not included in this initial mathematical model.

Modulating roles of \( \text{N}_2\text{O}_3 \) and GSH in apoptosis. We initially excluded non-heme iron compounds in order to assess the effect of \( \text{N}_2\text{O}_3 \) exclusively. The production rate of superoxide was likewise assumed to be zero. \( \text{N}_2\text{O}_3 \) is produced by reactions \((\text{xii})\) and \((\text{xiii})\) in Table 1. NO production and EC stimulation were initiated simultaneously. Figures 4A–C are the counterparts of Figures 2A–C, respectively (same initial conditions, except for the interference of NO pathways through \( \text{N}_2\text{O}_3 \)), where the time-dependence of \([\text{casp}3]\) (solid curve) and \([\text{GSH}]\) are shown. The bistable response to apoptotic stimuli, dependent on \([\text{casp}8]_0\), is shown to be maintained despite the interference of NO pathways through \( \text{N}_2\text{O}_3 \). The three columns refer to different initial concentrations of GSH, decreasing from \([\text{GSH}]_0 = 10^4 \) (Panels A–C), to \([\text{GSH}]_0 = 10^3 \) (panels D–F) and \([\text{GSH}]_0 = 0 \) (panels G–I). The threshold \([\text{casp}8]_0\) value for \( \text{casp}3 \) activation was \( 8.35 \times 10^{-2} \) \( \mu \text{M} \) in Figure 2, where NO was not produced at all. This value remains the same for both \([\text{GSH}]_0 = 10^4 \) \( \mu \text{M} \) (not shown) and \( 10^3 \) \( \mu \text{M} \) (panels A–C) in the presence of NO, but increases to \( 9.9 \times 10^{-2} \) \( \mu \text{M} \) when \([\text{GSH}]_0 = 10^4 \) \( \mu \text{M} \) (panels D–F) and to \( 1.26 \times 10^{-2} \) \( \mu \text{M} \) when \([\text{GSH}]_0 \) is zero (panels G–I), hence the different (pro-apoptotic) behavior observed in panel H. These results suggest that \( \text{N}_2\text{O}_3 \) does not affect the bistable character of the response to EC stimuli, except for modifying the threshold for onset of apoptosis, which is shifted to higher \([\text{casp}8]_0\) (i.e. rendered more difficult) with decreasing \([\text{GSH}]_0\). However, high initial concentrations of GSH restore the threshold back to \( 8.35 \times 10^{-2} \) \( \mu \text{M} \). Therefore, \( \text{N}_2\text{O}_3 \) can serve as an effective modulator of apoptosis provided that the level of GSH in the system is sufficiently low.

Effect of \( \text{N}_2\text{O}_3 \) on the threshold degradation rates of Bax for transition from bistable to monostable behavior. In our previous computational study of apoptotic pathways, we observed a bistable behavior (selecting between cell death and survival) for degradation rates of Bax \( (\mu_{\text{Bax}}) \) lower than a threshold value \( (0.11 \text{ s}^{-1}) \), while monostable cell survival was predicted when \( \mu_{\text{Bax}} > 0.11 \text{ s}^{-1} \) (Figure 4A in Ref. [28]). This critical value of
Effects of NO on Apoptosis

Roles of non-heme iron complexes and GSH in apoptotic response. One of the important anti-apoptotic effects of NO is presumed to occur via its ability to react with non-heme iron complexes (FeLn) to form FeLnNO. These species inhibit caspases by S-nitrosating the catalytic cysteine in the active site of these enzymes [19,40,41].

The results are presented in Figure 3, panels A-F, organized similarly to Figure 4 (i.e. using different [casp8]0 in each row, and different [GSH]0 in the two columns). Our calculations suggest that when the FeLn concentration is higher than 0.03 μM, there are no longer two stable steady-states at long times: caspase-3 levels always decrease to zero, even though their time evolutions depend on [casp8]0 and [GSH]0. Yet, depending on the level of GSH, both apoptosis and cell survival may be possible. Panels A–C correspond to relatively high [GSH]0. In panel A, [casp3] decreases to 10^{-7} μM that is less than 1 molecule per cell, hence zero, from 10^{-3} μM within the first two hours. However, in panels B and C, [casp3] increases to nanomolar values and remains at those levels for more than three hours. Caspase-3 may cause enough damage to kill the cell before it is depleted at longer times. We note that lower [GSH]0 (e.g. [GSH]0 = 10^{-3} μM, panels D–F and [GSH]0 < 10^{-5} μM, data not shown) do not permit the casp3 concentration to reach such pro-apoptotic levels and monostable cell survival is observed irrespective of [casp8]0.

Various cell types subject to different intracellular microenvironments, or even the same cells under different settings (e.g. healthy state vs. inflammation or oxidative stress), may produce or experience different reactive NO intermediates [7,20,42]. For example, more FeLnNO may be produced in hepatocytes than in RAW264.7 macrophage-like cells due to the high level of non-heme iron complexes in hepatocytes [21]. In our previous study, RAW264.7 cells underwent apoptosis in the presence of NO; conversely, no casp3 activation was observed in either hepatocytes or iron loaded RAW264.7 cells [21]. The results (Figure 4 and data not shown) suggest that in cells with iron concentrations lower than 0.03 μM (e.g. RAW264.7 cells), both cell survival and apoptosis are possible depending on the strength of apoptotic stimuli (in agreement with our experimental results) [21]. However, a change in the intracellular environment of the same cell can change the response. Figure 5D–F shows that casp3 is not activated in the presence of non-heme iron ([FeLn]0 = 0.05 μM) when [GSH]0 = 10 μM and [GSH]0 < 10^{-5} μM (data not shown). We also checked if casp3 is activated when [casp8]0 is as high as 0.0007 μM (data not shown) do not permit the casp3 concentration to reach such pro-apoptotic levels and monostable cell survival is observed irrespective of [casp8]0.
apoptotic effects is not well established [43]. In the present study, we assume that the pro-apoptotic effect of NO occurs via formation of ONOO$^-$, as has been suggested from a large number of experimental studies both in vitro and in vivo [44,45]. Experimental studies suggest that ONOO$^-$ may induce the opening of mitochondrial permeability transition pores (MPTPs) and subsequent cyt$c$ release from mitochondria [38].

The possible mechanisms of cyt$c$ release from mitochondria are diverse and controversial [46,47]. In our model, we assume that cyt$c$ release is mediated by activation of MPTPs, independent of Bax channel formation on mitochondria. The complex that forms the MPTPs is called mitochondrial permeability transition pore complex (PTPC). The complex consists of peripheral benzodiazepine receptor, cyclophilin D, adenine nucleotide translocator (ANT), voltage-dependent anion channel (VDAC), and other proteins [48]. ANT is proposed to be converted from a specific transporter to a non-specific pore which then releases cyt$c$ into the cytoplasm and subsequently induces apoptosis. It has been suggested that ONOO$^-$ acts on PTPC, specifically on ANT, to convert it to a non-specific pore (PTPC$_{act}$) [49]. We represent this process as:

$$\text{ONOO}^- + \text{PTPC} \rightarrow \text{PTPC}_{act} + \text{products}$$

Cytochrome $c$ is then released from the pore formed by PTPC$_{act}$

$$\text{cyt } c_{mito} + \text{PTPC}_{act} \rightarrow \text{cyt } c + \text{PTPC}_{act}$$

The results are shown in Figure 6. The initial concentration of PTPC is assumed to be high (0.01 $\mu$M). At that value, Model I predicts the response to apoptotic stimuli to be monostable apoptosis (Figure 6 in Ref. [28]). We see a similar response in Figure 6A; a low initial value of casp8 (10$^{-5}$ $\mu$M) results in an increase of [casp3] to nanomolar levels. Casp3 activation was observed with even lower values of [casp8]. However, casp3 does not reach nanomolar concentrations when [GSH]$_0$ = 10$^5$ $\mu$M (Figures 6D–F) and [GSH]$_0$ $\leq$ 10$^4$ $\mu$M (data not shown). Initial concentrations [casp8]$_0$ higher than 1.5 $\times$ 10$^{-4}$ $\mu$M did not change this prediction.

These results suggest that in cells with large numbers of MPTPs (probably with high numbers of mitochondria), there are two possible outcomes in the presence of NO and O$_2^-$ production:
pathological cell death when GSH level is high ($10^4$ μM) and solely cell survival when GSH level is low ([GSH] $\approx 10^3$ μM) in the presence of O$_2$ and FeLn. This result stands in contrast with studies in which GSH protects against oxidative stress (high concentrations of O$_2^\cdot$ and ONOO$^-\cdot$) that can cause apoptosis. The reason for this paradoxical prediction is that GSH has both protective and pro-apoptotic effects in our simulations: it exerts apoptotic effects via its reaction with anti-apoptotic N$_2$O$_3$ and FeLn, and protective effects due to its reaction with pro-apoptotic O$_2^\cdot$ and ONOO$^-\cdot$. Simulations (Figure 6) suggest that the pro-apoptotic effect of GSH is stronger than its protective effect using the interactions and parameters adopted in current simulations.

To examine the possibility of an alternative response, we repeated the computations depicted in Figure 6 in the absence of O$_2$ (so that N$_2$O$_3$ is not produced) and FeLn. We also used initial PTPC concentration of 0.0001 μM, at which Model I predicts bistability (Figure 6 in ref [28]). As seen in Figure 7, both cell survival and apoptosis are possible under these conditions, depending on [casp8]. Higher [GSH]$_0$ ($10^4$ μM) results in cell survival (Figure 7B) in contrast to lower [GSH]$_0$ resulting in apoptosis (Figure 7E) under the same amount of EC stimulus ([casp8]$_0$ = $7 \times 10^{-5}$ μM). The present analysis thus shows that the protection by GSH against oxidative stress is possible provided that O$_2$ and FeLn levels are sufficiently low.

**Discussion**

We present here the results from simulations that incorporate the main chemical interactions of NO with components of the apoptotic interactions network, with the goal of shedding light on the dichotomous effects of NO on apoptosis. Based on previously published studies, we considered N$_2$O$_3$ and Fe$_{Ln}$NO to be anti-apoptotic and ONOO$^-\cdot$ pro-apoptotic. The results predict that cell survival or apoptosis is determined by a complex interplay among these reactive NO species and GSH. We observed that relative concentrations of anti-apoptotic and pro-apoptotic species determine the ultimate cell fate at late time points. Interestingly, transient apoptotic effects were observed under specific conditions (e.g. Figure 5 panels B–C). These intriguing findings point to the importance of the timing of NO production and apoptotic stimuli in determining the actual anti- or pro-apoptotic effect, even if steady state conditions favor cell survival, in agreement with our previous observations [50–52]. Another interesting effect we observed in our simulations was the time shift/delay in the onset of apoptosis in the presence of weak EC stimulus (panel B–D in Figure 2), consistent with the experiments of Tyas et al. [37].

Our simulations suggest that N$_2$O$_3$ and non-heme iron nitrosyl form in a switch-like manner after depletion of GSH. ONOO$^-\cdot$ formation, on the other hand, hardly shows any switch-like
behavior. We further found that N2O3 does not eliminate the bistability between cell survival and apoptosis, but rather increases the threshold \([casp8]_0\) for onset of apoptosis. However, high initial concentrations of GSH restore the threshold back to its original value. Therefore, we would predict, non-intuitively, that N2O3 does not influence cell survival when \([GSH]_0\) level is high.

On the other hand, our simulations suggest that there are no longer two stable steady states (cell survival and apoptosis) in the presence of non-heme iron at a level higher than a threshold value. Caspase-3 levels always decrease to zero even though its time evolution may depend on \([casp8]_0\) and \([GSH]_0\). Yet, despite the steady state conditions that favor cell survival, executioner caspase concentrations can reach and retain apoptotic levels for several hours before they level off, when \([GSH]_0\) is high. When \([GSH]_0\) is low, on the other hand, our simulations predict resistance to apoptosis, in agreement with experimental observation [21].

In cells with high numbers of MPTPs (probably cells that contain high numbers of mitochondria), our simulations suggest two possibilities in the presence of simultaneous NO and O2\(^\cdot\) production and sufficiently high \([FeLn]_0\): pathological cell death when \([GSH]_0\) is high \((10^4 \text{ M})\) or solely cell survival when \([GSH]_0\) level is low \((10^3 \text{ M})\). On the other hand, GSH is protective against oxidative stress when \(O_2\) and \(FeLn\) levels are low in cells with low numbers of MPTPs.

Tiedge et al. [53] have shown that pancreatic beta cells have low anti-oxidant levels (notably, GSH) and that the number of mitochondria is a determining factor in survival. They have also shown that transfection of the cells with a peroxide-inactivating enzyme, catalase, can protect against high-glucose induced apoptosis. An interesting experiment would be to correlate the number of mitochondria in the transfected cells with their survival. Oyadomari et al. [54] have shown that the endoplasmic reticulum (ER) plays a crucial role in the fate of NO-sensitive beta cells via calcium signaling. A natural next step in the present model would be to include these effects via a model which incorporates the effects of NO on the ER.

Our results are subject to several limitations. While we have adopted values for kinetic parameters and concentrations in accord with experimental data whenever available (Tables 1 and 2), many of the true intracellular rate constants for the reactions in our simulations are unknown. Given that the observed apoptotic responses are so sensitive to model parameters, detailed knowledge of reaction mechanisms and accurate values of rate constants are needed in modeling reaction networks as complicated as the ones

Figure 6. Time evolutions of \([GSH]\) and \([casp3]\) predicted by Model III in the presence of N2O3, FeLnNO and ONOO\(^\cdot\). The initial concentration of PTPC is 0.01 \(\mu\text{M}\). Each column is a counterpart of Figure 2A–C and has a different initial concentration for GSH. A–C) \([GSH]_0 = 10^4 \text{ M};\) D–F) \([GSH]_0 = 10^3 \text{ M}.\) Solid line is for time evolution of \([casp3]\) and dashed line is for time evolution of \([GSH]\). Caspase-3 concentrations at long times are \(2.4 \times 10^{-4} \text{ M}\) and \(2.5 \times 10^{-5} \text{ M}\) for panels A–C and D–F, respectively.

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presented here. Due to an extensive literature basis, we have posited that the pro-apoptotic NO species is ONOO$^-_{2}$; however, other species may in fact exert this effect. Additionally, the hypotheses raised by our simulations remain to be tested by further experiments. Some of the predictions could be tested by iron chelation and/or treatment with superoxide donors in a cell-free system or in single-cell studies, though each of these manipulations may have additional, artifactual effects. The hypothesis of bistability with regards to the apoptotic response can be tested as suggested by Legewie et al. [32], either in cell free-systems by adding caspase-3 or in single living cells by microinjecting caspase-3. The time evolution of caspase-3 can be monitored by fluorescent caspase-3 substrates. The time needed for caspase-3 activation will increase abruptly as caspase-3 concentration added will approach threshold value in a bistable system (Figure 2D). Such combined experimental and computational studies may potentially help us understand and design therapeutics for diseases associated with apoptosis dysregulation.

**Materials and Methods**

**Models**

Three models are considered in this study. **Model I**, proposed in our earlier work [28], focuses on the pathways involved in mitochondria-dependent apoptosis (Figure 1A). **Model II** is an extension of the kinetic model of NO-associated reactions recently proposed by Hu et al. [22] (Figure 1B). Finally, **Model III** is the integration of Models I and II, proposed in the present study, to examine the pro-apoptotic and anti-apoptotic effects of NO.

| Equilibrium concentrations | References |
|----------------------------|------------|
| [SOD]$^-_{2}$ = 10 $\mu$M | [74]       |
| [GPX]$^-_{2}$ = 5.8 $\mu$M | [67]       |
| [CO2]$^-_{2}$ = 10$^{3}$ $\mu$M | [61]       |
| [O$_2$]$^-_{2}$ = 35 $\mu$M | [67]       |
| [cyt c]$^-_{2}$ = 400 $\mu$M | [75]       |

| Initial concentrations | References |
|------------------------|------------|
| [CeOx]$^-_{2}$ = 0.1 $\mu$M | [76]       |
| [Fe]$^-_{2}$ = 0.05 $\mu$M | [77]       |
| [GSH]$^-_{2}$ = 10$^{3}$ $\mu$M (or otherwise specified) | [22]       |

Table 2. Equilibrium levels and initial concentrations used in Model II
Model II—Generation of NO-related oxidative and nitrosative species ONOO⁻, N₂O₃, and Fe₄NO

We extended the network originally proposed by Hu and coworkers [22] by introducing additional reactions involving NO, as well as additional compounds such as the NO-related species Fe₄NO (L denotes ligands that do not contain heme), N₂O₃, and cytochrome c oxidase (CcOx). Figure 1B illustrates the extended network of interactions. Table 1 lists the corresponding reactions and rate constants. The reactions (xxii) and (xxiii) break down the production of N₂O₃ from NO and O₂ into two steps that replace the corresponding reaction (with rate constant k₁₂) used in the model of Hu et al. [22]. Reactions (xxiv)–(xxv) are introduced in the present study. The identity of the products are not written when these compounds do not serve as reactants in any of the reactions listed in Table 1. Table 3 lists the rate laws for these reactions (the first 20 rows), which are used in the differential rate equations (rows 21–29) that control the time evolution of the concentration of the individual compounds. Model II contains 16 components. Eleven of them reach steady-state concentrations.

Table 3. Rate equations for Model II (*)

| Rate laws (Eqs. 1–20) and differential rate equations (Eqs. 21–29) | Equation numbers |
|---------------------------------------------------------------|------------------|
| f₁NO = k₁NO                                                 | (1)              |
| f₂NO = k₂NO                                                 | (2)              |
| f₃NO = k₃NO                                                 | (3)              |
| f₄NO = k₄NO[O₂⁻]                                             | (4)              |
| f₅NO = k₅NO[SO₂]                                            | (5)              |
| f₆NO = k₆NO[ONO]                                            | (6)              |
| f₇NO = k₇NO[ONO]                                            | (7)              |
| f₈NO = k₈NO[ONO][CO₂]                                       | (8)              |
| f₉NO = k₉NO[ONO][cyt c]                                      | (9)              |
| f₁₀NO = k₁₀NO[GSNO][O₂⁻]                                    | (10)             |
| f₁₁NO = k₁₁NO[N₂O₃][GSH]                                    | (11)             |
| f₁₂aNO = k₁₂aNO[NO][O₂]                                      | (12)             |
| f₁₂bNO = k₁₂bNO[NO][NO]                                      | (13)             |
| f₁₂cNO = k₁₂cNO[N₂O₃]                                        | (14)             |
| f₁₃NO = k₁₃NO[NO][O₂]                                       | (15)             |
| f₁₄NO = k₁₄NO[GSNO]                                         | (16)             |
| f₁₅NO = k₁₅NO[CcOx][NO]                                     | (17)             |
| f₁₆NO = k₁₆NO[Fe₄NO][NO]                                     | (18)             |
| f₁₇NO = k₁₇NO[Fe₄NO][GSH]                                   | (19)             |
| f₁₈NO = f₁₉NO[Fe₄NO][O₂]                                    | (20)             |
| d[N₂O₃]/dt = f₁₈NO[Fe₄NO][O₂]                               | (21)             |
| d[Fe₄NO]/dt = f₁₉NO[Fe₄NO][O₂]                              | (22)             |
| d[ONOO⁻]/dt = f₂₀NO[Fe₄NO][Fe₄NO][O₂]                      | (23)             |
| d[N₂O₃]/dt = f₂₁NO[Fe₄NO][O₂]                               | (24)             |
| d[Ono]/dt = f₂₂NO[Fe₄NO][O₂]                                | (25)             |
| d[GSNO]/dt = f₂₃NO[Fe₄NO][O₂]                               | (26)             |
| d[CcOx]/dt = f₂₄NO[Fe₄NO][O₂]                               | (27)             |
| d[Fe₄NO]/dt = f₂₅NO[Fe₄NO][O₂]                              | (28)             |

All interactions (chemical or physical; single step or multiple steps) are modeled using mass action kinetics theory and methods. The simulations are performed using XPPAUT software (http://www.math.pitt.edu/~bard/xpp/xpp.html) [55].

Table 5. The modified equations from either Model I or II (*)

| Rate laws (Eqs. 30–34) and differential rate equations (Eqs. 35–43) | Equation numbers |
|---------------------------------------------------------------------|------------------|
| f₁NO = k₁NO[ONO]                                                   | (30)             |
| f₂NO = k₂NO[N₂O₃][GSH]                                            | (31)             |
| f₃NO = k₃NO[Fe₄NO][Fe₄NO][O₂]                                      | (32)             |
| f₄NO = [Fe₄NO][Fe₄NO][O₂]                                         | (33)             |
| f₅NO = k₅NO[Fe₄NO][Fe₄NO][O₂]                                      | (34)             |
| d[ONOO⁻]/dt = f₁₆NO[Fe₄NO][O₂]*f₂₂NO                                 | (35)             |
| d[PTPC]/dt = f₁₉NO                                                 | (36)             |
| d[N₂O₃]/dt = f₁₉NO[Fe₄NO][O₂]                                      | (37)             |
| d[casp8]/dt = f₁₆NO[Fe₄NO][Fe₄NO][O₂]                              | (38)             |
| d[Fe₄NO]/dt = f₁₆NO[Fe₄NO][Fe₄NO][O₂]                              | (39)             |
| d[Fe₄NO]/dt = f₁₆NO[Fe₄NO][Fe₄NO][O₂]                              | (40)             |
| d[casp3]/dt = f₁₆NO[Fe₄NO][Fe₄NO][O₂]                              | (41)             |
| d[casp9]/dt = f₁₆NO[Fe₄NO][Fe₄NO][O₂]                              | (42)             |
| r₁₇NO = k₁₇NO[PTPCact][cyt c]                                       | (43)             |

(*) J refers to fluxes of components, for details see ref [28]. PTPCact refers to the non-specific pore at the mitochondria that releases cyt c. Note that [PTPCact] = [PTPC]−[PTPC].

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We extended the network originally proposed by Hu and coworkers [22] by introducing additional reactions involving NO, as well as additional compounds such as the NO-related species Fe₄NO (L denotes ligands that do not contain heme), N₂O₃, and cytochrome c oxidase (CcOx). Figure 1B illustrates the extended network of interactions. Table 1 lists the corresponding reactions and rate constants. The reactions (xxii) and (xxiii) break down the production of N₂O₃ from NO and O₂ into two steps that replace the corresponding reaction (with rate constant k₁₂) used in the model of Hu et al. [22]. Reactions (xxiv)–(xxv) are introduced in the present study. The identity of the products are not written when these compounds do not serve as reactants in any of the reactions listed in Table 1. Table 3 lists the rate laws for these reactions (the first 20 rows), which are used in the differential rate equations (rows 21–29) that control the time evolution of the concentration of the individual compounds. Model II contains 16 components. Eleven of them reach steady-state concentrations.

Table 4. Reactions bridging between Models I to II (*)

| Reaction                          | Rate constant | Reference | Reaction index |
|-----------------------------------|---------------|-----------|----------------|
| ONOO⁻+PTPC=PTPCact+products       | k₁₈NO         | accounts for ONOO⁻ induced formation of non-specific pore associated with mitochondrial permeability transition (49) |
| N₂O₃+casp8→casp8:NO+Fe₄NO         | k₁₉NO         | [78]      | (xxii)         |
| Fe₄NO+casp8→casp8:NO+Fe₄NO       | k₂₀NO         | [38]      | (xxiii)        |
| Fe₄NO+casp9→casp9:NO+Fe₄NO        | k₃₈NO         | [38]      | (xxiv)         |
| Fe₄NO+GSH→casp3→casp3+NO+Fe₄NO   | k₃₂NO         | [38]      | (xxv)          |

(*) The parameters used in the present study are k₁₂NO = 1 μM⁻¹ s⁻¹ (varying the value between 0.01 μM⁻¹ s⁻¹ and 100 μM⁻¹ s⁻¹ does not affect the results), k₁₈NO = 10 μM⁻¹ s⁻¹ [78], k₂₀NO = k₂₁NO = k₂₂NO = 66 μM⁻¹ s⁻¹ (the same value as k₁₃NO).

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within a short time interval (~20 minutes) after initiation of the simulations for \([\text{GSH}]_0 \leq 10^3 \mu M\) and within four and half hours for \([\text{GSSH}]_0 \leq 10^4 \mu M\), whereas five compounds (superoxide dismutase (SOD), glutathione peroxidase (GPX), CO\(_2\), O\(_2\), and cytochrome c) retain their equilibrium concentrations. Table 2 lists the initial and equilibrium concentrations different from zero, adopted in Model II, and the corresponding references.

Model III—Effects of NO-related reactions on apoptotic pathways

Model III combines Models I and II upon inclusion of the additional reactions presented in Table 4. See the highlighted compounds in Figure 1, which point to the species that couple the apoptotic and NO pathways. We note that ONOO\(^-\) has a pro-apoptotic effect, while \(\text{NO}_2\) and \(\text{Fe}^{\text{II}}\text{NO}\) (reactions labeled (xxii)–(xxv)) deactivate the caspases, thus inducing anti-apoptotic effects. The associated rate constants and references are given in Table 4. Table 5 provides the rate expressions (rows 30–34) and differential rate equations (rows 35–43) for these reactions and involved compounds, respectively.

The steady-state concentrations \([\text{H}^+]_0\) in reaction (v), \([\text{H}_2\text{O}]_0\) in reactions (x) and (xiv), \([\text{NADPH}]_0\) and \([\text{H}^+]_0\) in reaction (xv), \([\text{Cu}^2+]_0\) in reaction (xvi) are incorporated into the corresponding rate constants.

**Author Contributions**

Conceived and designed the experiments: TB YY IB BE. Performed the experiments: EB. Analyzed the data: EB. Contributed reagents/materials/analysis tools: YY BE. Wrote the paper: EB.
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