Sustained Nitric Oxide Delivery Delays Nitric Oxide-Dependent Apoptosis in Macrophages: Contribution to the Physiological Function of Activated Macrophages

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Treatment of the macrophage cell line RAW 264.7 with the short-lived NO donor S-nitrosoglutathione triggers apoptosis through the release of mitochondrial mediators. However, continuous supply of NO by long-lived NO donors protected cells from apoptosis through mechanisms that involved the maintenance or an increase in the levels of the inhibitor of apoptosis proteins (IAPs) cIAP-1, cIAP-2, and XIAP and decreases in the accumulation of p53 and in the levels and targeting of Bax to the mitochondria. As a result of these changes, the activation of caspases 9 and 3 was notably delayed, expanding the time of viability of the macrophages. Moreover, inhibition of NO synthase 2 activity after 8 h of stimulation of RAW 264.7 cells with LPS and IFN-γ accelerated apoptosis via an increase in the processing and activation of caspases. These data suggest that NO exerts an important role in the autoregulation of apoptosis in macrophages. The Journal of Immunology, 2003, 171: 6059–6064.

Macrophage activation plays a central role in innate and acquired immunity, and this process is subjected to a fine regulation to respond and adapt to the specific necessities of the inflamed area (1, 2). Among other functions, macrophages participate actively in host defense against pathogen infection through phagocytosis and pathogen killing and in inflammatory reactions. In the course of activation by pathogens and proinflammatory cytokines, macrophages express a series of genes involved in host defense through the release of proinflammatory mediators such as bioactive lipids (i.e., prostanoids and other arachidonic acid-derived metabolites), reactive oxygen and nitrogen species, and matrix metalloproteinases to accomplish tissue remodeling (1, 3). Elevated NO synthesis after NO synthase-2 (NOS-2)3 expression by activated macrophages is one of the main cytostatic, cytotoxic, and proapoptotic mechanisms participating in the innate response, at least in rodents (4–7). The proapoptotic activity of NO has been well documented (4–6); it involves the release of cytochrome c from the mitochondria (7), the rise in the tumor suppressor p53 (8, 9), and the accumulation and targeting of proapoptotic members of the Bcl-2 family, in particular Bax (10). However, most physiological macrophage functions, such as phagocytosis, require preservation of cell integrity, in particular an attenuation of the autoapoptotic response. Indeed, it has been reported that NO plays a dual role, favoring cell viability or inducing apoptotic death depending on the cell type and amount of NO produced (11–15). For example, moderate NO synthesis inhibits basal apoptosis in B lymphocytes (16, 17) or the TGF-β-induced apoptosis in hepatocytes (18).

Regarding the protective mechanisms mediated by NO, several targets have been identified, including the regulation of caspase activation (19, 20) and the expression of antiapoptotic genes of the inhibitor of apoptosis protein (IAP) family (21, 22). Caspases are a family of proteases that catalyze specific protein cleavage through a cysteine/histidine catalytic mechanism, but require intermolecular processing to be active (23, 24). Because the active site of the enzyme and the procaspase contain a cysteine residue susceptible to S-nitrosylation by NO, it has been proposed that this could be a physiological mechanism of apoptotic regulation (25–28).

In studies of apoptosis induced by treatment of macrophages with an NO donor, such as S-nitrosoglutathione (GSNO), we realized that the appearance of characteristic hallmarks of apoptosis was very rapid compared with those observed with molecules that release NO in a sustained way or after NOS-2 expression via LPS and IFN-γ challenge of the cells. In addition to this, a clear cell-specific susceptibility to NO-dependent apoptosis has been observed, and in general, macrophages appear to be more resistant than other cells, such as neurons or T and B lymphocytes. In view of these observations we analyzed the possibility that high output NO concentrations would contribute to delay the occurrence of apoptosis. Using NO donors with different half-lives of NO generation and pharmacological inhibitors of NOS-2 activity, we characterized a dual role for NO in macrophage viability; although NO rapidly triggers the release of early mitochondrial mediators of apoptosis (cytochrome c), sustained NO levels paradoxically prevent caspase processing and activity and favor the accumulation of IAPs, delaying the action of the downstream apoptotic pathway and contributing to cell viability and integrity.

Materials and Methods

Chemicals

Reagents were obtained from Roche (Mannheim, Germany), Calbiochem (Darmstadt, Germany), Bachem (Bubendorf, Switzerland), and Sigma-Aldrich (St. Louis, MO). Fluorescent probes and Abs were purchased from BD PharMingen (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA). Culture media were obtained from BioWhittaker (Verviers, Belgium).
Cell culture conditions

The murine macrophage cell line RAW 264.7 was maintained in RPMI 1640 medium supplemented with 10% FCS, l-glutamine, and antibiotics, as previously described (5, 6).

Flow cytometric analysis of apoptosis

Propidium iodide (PI) staining was performed after incubation of the cells with 0.005% PI, following a previously described protocol (6, 7, 16). Cells were carefully resuspended and run in a FACScan cytometer (BD Biosciences, San Jose, CA) equipped with a 25-mW argon laser. Quantification of the percentage of apoptotic cells was performed using a dot plot of the forward scatter against the PI fluorescence. Apoptotic and viable cells were sorted, and the integrity of the DNA was analyzed in agarose gels to confirm the criteria of gating (6) and by TUNEL (29).

NO measurement

Real-time NO levels were determined in the culture medium using an NO-selective amperometric sensor (World Precision Instruments, Sarasota, FL). The electrode was calibrated following the decomposition of S-nitroso-N-acetyl-penicillamine using Cu²⁺ as catalyst, following the instructions of the supplier.

Preparation of cytosolic and total protein extracts

RAW 264.7 cells were washed twice with ice-cold buffer A (10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, 2 µg/ml aprotinin, 10 µg/ml leupeptin, 2 µg/ml N-tosyllys-chloromethyl ketone, 5 mM NaN₃, 1 mM NaVO₄, and 10 mM Na₂MoO₄) containing 120 mM NaCl and scraped off the plate. Cells were lysed at 4°C with 0.2 ml of buffer A supplemented with 0.5% Nonidet P-40 and under continuous shaking. After centrifugation of the cell lysate, the supernatant was stored at −80°C (cytosolic extract), and protein content was assayed using the Bio-Rad protein reagent. The DEVDase (corresponding mainly to caspases 3 and 7) and caspase 9 activities were determined in cell lysates using N-acetyl-DEVD-7-amino-4-trifluoromethylcoumarin and N-acetyl-LEHD-7-amino-4-trifluoromethylcoumarin as fluorogenic substrates and following the instructions of the supplier (Calbiochem, La Jolla, CA). The corresponding peptide aldehyde and Z-VAD.fmk were used to inhibit caspase activity in vivo and to ensure the specificity of the reaction in the in vitro assay. The caspase activities were linear over a 30-min reaction period. In vivo activation of caspase 3 was measured by confocal microscopy after labeling the cells with PhyPhyLux-G1D2 (a derivative of GDEVDGDI that yields a fluorescent peptide (green) after caspase excision; OncolImmunin, Gaithersburg, MD) following the instructions of the supplier. Plates were visualized using an MRC-1024 confocal microscope (Bio-Rad), and the fluorescence was acquired and electronically evaluated. Staining with 40 nM chloromethyl X-rosamine was also performed to label the mitochondria (red).

Western blot analysis of proteins

Protein extracts were size-separated in 10–12% SDS-PAGE. The gels were blotted onto a Hybond-P membrane (Amersham Pharmacia Biotech, Arlington Heights, IL) and incubated with the following Abs: anti-IAPs (R&D Systems, Minneapolis, MN); anti-p53, anti-Bax, an anti-caspases 3 and 9 (Santa Cruz Biotechnology; sc-6243, sc-7148, and sc526, respectively); and anti-cytochrome c Ab (BD Biosciences). The blots were submitted to sequential reprobing with Abs after treatment with 100 mM 2-ME and 2% SDS in TBS and heated at 60°C for 30 min. The blots were revealed by ECL following the manufacturer’s instructions (Amersham Pharmacia Biotech). For in vitro caspase assays, cell extracts were prepared in 10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 120 mM NaCl, 1 mM DTT, 0.5 mM PMSF, 2 µg/ml aprotinin, 10 µg/ml leupeptin, 2 µg/ml N-tosyl-lys-chloromethyl ketone, 5 mM NaN₃, 1 mM NaVO₄, 10 mM Na₂MoO₄, and 0.5% Nonidet P-40. After centrifugation of the cell lysate the supernatant was stored at −80°C (cytosolic extract), and protein content was assayed using the Bio-Rad protein reagent. The DEVDase (corresponding mainly to caspases 3 and 7) and caspase 9 activities were determined in cell lysates using N-acetyl-DEVD-7-amino-4-trifluoromethylcoumarin and N-acetyl-LEHD-7-amino-4-trifluoromethylcoumarin as fluorogenic substrates and following the instructions of the supplier (Calbiochem, La Jolla, CA). The corresponding peptide aldehyde and Z-VAD.fmk were used to inhibit caspase activity in vivo and to ensure the specificity of the reaction in the in vitro assay. The caspase activities were linear over a 30-min reaction period. In vivo activation of caspase 3 was measured by confocal microscopy after labeling the cells with PhyPhyLux-G1D2 (a derivative of GDEVDGDI that yields a fluorescent peptide (green) after caspase excision; OncolImmunin, Gaithersburg, MD) following the instructions of the supplier. Plates were visualized using an MRC-1024 confocal microscope (Bio-Rad), and the fluorescence was acquired and electronically evaluated. Staining with 40 nM chloromethyl X-rosamine was also performed to label the mitochondria (red).

Statistical analysis

The data shown are the mean ± SEM (n = 3–4). Statistical significance was estimated with Student’s t test for unpaired observations. A value of p < 0.05 was considered significant. In studies of Western blot analysis, a linear correlation between increasing amounts of input protein and signal intensity was observed (correlation coefficients, >0.84).

Results

Sustained NO synthesis delays apoptosis in macrophages

Treatment of macrophages with the short-lived NO donor GSNO induced apoptosis, followed by the appearance of hypodiploid DNA 6 h after addition of this molecule. However, when macrophages were incubated simultaneously with GSNO and a sustained NO donor such as diethylenetriamine NONOate (DETA-NO), the percentage of cells exhibiting hypodiploid DNA was significantly
reduced, and only after 18 h in culture was a significant increase in the percentage of apoptotic cells evidenced. To explain this distinct behavior when sustained NO release occurs, the kinetics of NO concentration in the culture medium were determined using an amperometric NO electrode. As Fig. 1B shows, the release of NO in cells treated with GSNO showed a rapid decrease, with a half-life of ~40 min. However, DETA-NO maintained sustained levels of NO for at least 5 h. In addition to this, an evaluation of NO levels in vivo was deduced by measuring the nitrosylation pattern of proteins using the Jaffrey method of selective oxidation of S-nitrosylated groups (31). One of the most intense bands corresponded to a protein of 14 kDa, which was identified as cytochrome c. This band was evidenced at short times, whereas it disappeared after longer periods of time (4 h). Moreover, the sustained release of NO influenced the profile of proteins controlling apoptosis. As Fig. 1D shows, the expression levels of the anti-apoptotic proteins cIAP-1, cIAP-2, and xIAP correlated with the severity of the induced apoptotic response. When the accumulation of cytochrome c in the cytoplasm was determined by Western blot as a marker of the release of mitochondrial-dependent apoptotic mediators, the levels were notably lower in the presence of DETA-NO than in the GSNO-only counterparts.

In view of these data we compared the levels of proteins related to apoptosis in macrophages treated for 4 and 8 h with GSNO or GSNO plus DETA-NO. As Fig. 2 shows, the levels of IAPs decreased notably after GSNO treatment; however, these changes did not occur in the presence of DETA-NO. Moreover, the tumor suppressor p53 was up-regulated at 4 and mainly at 8 h in response to GSNO treatment, whereas reduced levels were observed when DETA-NO was present. Regarding proapoptotic proteins of the Bcl-2 family, the amount of Bax increased after GSNO treatment, and interestingly, the protein translocated to the mitochondria was enhanced after GSNO treatment, but this association remained much lower when DETA-NO was present. In agreement with these data, when DNA laddering was investigated at 8 h as a hallmark of apoptosis, a fragmentation pattern was evidenced in GSNO-treated cells, but not in the GSNO plus DETA-NO condition, which reflects the delay in the kinetics of apoptotic changes in the presence of DETA-NO.

**Sustained NO release impairs caspase 9 activation**

NO-dependent apoptosis involves the release of mitochondrial cofactors (7, 29). Therefore, to evaluate the effect of continuous supply of NO by DETA-NO on proteins involved in the apoptotic pathway, the time course of activation of procaspases 9 and 3, p53 accumulation, and release of cytochrome c from mitochondria to cytosol were measured. As Fig. 3 shows, caspase activity using LEHD as substrate (a preferred caspase 9 and 7 substrate) increased after 4 h of treatment of cells with GSNO, whereas in the presence of DETA-NO this activity was notably impaired. Similar results were obtained with DEVD as substrate. Moreover, when cells were loaded with PhyPhyLux, a permeable fluorescent substrate of caspase 3, the percentage of positive cells was ~70% in macrophages treated for 8 h with GSNO, but only 20% when cells were maintained under continuous NO supply. In addition to these

**FIGURE 2.** Sustained NO synthesis impairs Bax processing and DNA degradation. RAW 264.7 macrophages were treated as indicated in Fig. 1, and at 0, 4, and 8 h samples were obtained to determine the protein levels of IAPs and p53 as well as those of Bax in different subcellular localizations. Analysis of DNA laddering was conducted in agarose gels. Results show a representative experiment of four performed.

**FIGURE 3.** Caspase activation is delayed by continuous NO supply. Macrophages were treated with 0.5 mM GSNO in the absence or the presence of 0.1 mM DETA-NO, and the LEHDase and DEVDase activities were measured in cell extracts prepared at the indicated times. When cells were labeled with the permeable caspase 3 fluorescent substrate PhyPhyLux (green) and the mitotracker chloromethyl X-rosamine (red), the percentage of caspase 3-positive cells was determined and plotted as a function of time in culture. A representative picture corresponding to the distribution of fluorescence at 6 h is shown. The levels of p53, the release of cytochrome c to the cytosol, and the processing of caspases 9 and 3 were analyzed by Western blot. The results show the mean of three experiments (left panels) and a representative blot of three performed.
parameters, the amount of p53 and the release of cytochrome c to the cytoplasm were notably attenuated when DETA-NO was present. Analysis of the processing of procaspase 9 and 3 showed that in the presence of DETA-NO, caspase processing was significantly inhibited.

To further evaluate the contribution of sustained NO synthesis to a subsequent high output GSNO burst, cells were treated for the indicated periods of time with DETA-NO and after the change in culture medium (time zero), they were challenged with GSNO for 18 h and the percentage of apoptotic cells and the levels of IAPs and mitochondrial-associated Bax were determined. As Fig. 4 shows, treatment of macrophages for >4 h with DETA-NO significantly protected macrophages against GSNO-dependent apoptosis. With regard to IAPs, the amount of cIAP-1 correlated with the protection from apoptosis after treatment with DETA-NO, whereas the amounts of cIAP-2 and xIAP remained preserved. However, after treatment with DETA-NO for at least 6 h, translocation of Bax to the mitochondria was almost completely inhibited.

**Inhibition of NO synthesis accelerates apoptosis in LPS- and IFN-γ-activated macrophages**

To evaluate the contribution of NO to the appearance of the apoptotic phenotype in activated macrophages, cells were treated with LPS and IFN-γ, and at various times the selective NOS-2 inhibitor N-(3-aminomethylbenzyl)acetamidine (1400W) was added to the culture medium. As Fig. 5 shows, when 1400W was added before NOS-2 expression, the percentage of apoptotic cells determined after 18 h of incubation was significantly reduced with respect to that of NO-synthesizing cells. However, when 1400W was added 8 h after activation, the percentage of apoptotic cells determined at 18 h was notably increased, indicating that endogenous NO contributes to regulation of the kinetics of apoptosis. Indeed, when caspase activity was measured using LEHD and DEVD as substrates, the inhibition of NOS-2 at short times, when the expression levels of the enzyme are negligible, resulted in a low caspase activity compared with LPS- and IFN-γ-treated cells. However, the inhibition of NOS-2 at 8 h resulted in a marked increase in caspase 3 activity.

**Discussion**

We investigated in this work the contribution of NO to the autoregulation of apoptosis in macrophages. Previous observations with short-lived NO donors, such as GSNO, indicated that macrophages respond rapidly to initiate apoptosis, as deduced by the prompt release of cytochrome c, activation of caspases, and appearance of DNA fragmentation as soon as 5–6 h after GSNO challenge (7, 29). However, these responses are markedly delayed when macrophages are expressing NOS-2 in response to proinflammatory stimulation or when NO is continuously supplied, as shown by the present data, which suggests that sustained NO synthesis contributes to the improvement of cell viability in the macrophage. In the model investigated, the optimal range of DETA-NO to mediate these protective effects was ~50–150 μM.

NO is a short-lived mild oxidant molecule that acts as an intracellular messenger mediating several physiologic and pathologic processes, including the regulation of cell viability (i.e., through the inhibition of stimulus-dependent apoptosis), apoptosis, and necrotic death (1, 4, 5, 7, 32). Furthermore, high output NO synthesis, such as occurs after the expression of NOS-2, contributes to the induction of apoptosis in inflammatory cells and therefore to the resolution of inflammation (5). In addition to the effects observed with elevated concentrations of NO, it has been described in neurons, which are very susceptible to NO-dependent apoptosis, that low, but prolonged, NO supply via NOS-1 activation through excitotoxicity mechanisms is sufficient to promote apoptotic death in these cells (33, 34). The pathways involved in NO-dependent apoptosis have been described in some detail, and there are specific...
patterns dependent on the cell type (6). In macrophages, for example, NO induces apoptosis in the absence of apoptotic volume decrease and promotes the release of mitochondrial mediators, such as cytochrome c and apoptosis-inducing factor, in the absence of a decrease in the mitochondrial inner membrane potential (7). NO also promotes a rapid up-regulation of the levels of p53 preceding cytochrome c release (8), followed by an activation of caspases and changes in the expression of proteins of the Bcl-2 family. In this way, monocytes express relatively high levels of the caspases and changes in the expression of proteins of the Bcl-2 family. In contrast to these proapoptotic effects of NO, this molecule can contribute to maintain cell viability, and this capacity appears to be dependent on the cell type considered, the amount of NO released, and the activation status of the cell (20, 35–37). Indeed, proapoptotic effects of NO have been described in many cell types, such as macrophages, mesangial cells, hepatocytes, endothelial cells, and B and T lymphocytes (16–18, 21, 27, 38). In macrophages, several mechanisms have been described to protect against activation-dependent apoptosis. For example, chaperones of the heat shock protein 70 family facilitate efficient apoptosis (39, 40), and signaling through the membrane receptor Ron, a Met family member tyrosine kinase receptor that is activated by macrophage-stimulating protein and negatively regulates the activation process in response to LPS, inhibits apoptosis not only in response to LPS, but also after incubation of macrophages with GSNO (41).

In addition to these mechanisms, NO appears to be very effective in inhibiting the process of caspase activation (19, 28, 37), probably through S-nitrosylation modifications. The incorporation of up to three molecules of NO in the p17 and p12 subunits of caspase 3 has been observed, but not when the protein was mutated in the cysteine residue present in the active site of the enzyme (26). In addition to NO, other molecules, such as the H2O2 released by activated macrophages, might also contribute in vitro to the reversible inhibition of caspases 3 and 8 (42). Moreover, it has been recently described that NO donors reduce the efficiency of formation of the apoptosis protease-activating factor 1 (caspase-9) Apoptosome through a deficient assembly of the subunits, which impairs later steps of apoptosis activation through the caspase-receptor domain motifs of Apaf 1 and procaspase 9 (20). Furthermore, the effects of modifications of cytochrome c, via nitrosylation of tyrosine residues, on the assembly and function of the apoptosis cannot be excluded (7).

In addition to the effects of NO on procaspase processing and activity, our data show that IAPs are up-regulated under conditions of continuous supply of NO. IAPs were identified in insect cells infected with baculovirus and then were described in mammalian cells, showing a high degree of conservation among species in two domains: the baculovirus inhibitory repeat and the C-terminal, zinc-binding motif. Several IAPs have been described, including cIAP-1, cIAP-2, and XIAP (43–45). These proteins bind to and potently inhibit caspases 9, 3, and 7 (with affinity constants in the nanomolar concentration range). Moreover, the IAPs can be recruited to the complexes activated in response to proinflammatory signaling, such as TNF-α, redirecting signaling toward the activation of NF-kB and the generation of additional survival signals through this pathway (22).

In summary, our data describe a mechanism by which NO delays apoptosis in the macrophage, allowing the cell to maintain its viability for longer periods of time, but primed to accomplish apoptosis as soon as the synthesis of NO decreases, a characteristic of the resolution step of inflammation, or when the balance favoring the accumulation of proapoptotic factors (mainly Bax and p53) and activation of caspases dominates.

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References


