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Presentation of Nitric Oxide Regulates Monocyte Survival through Effects on Caspase-9 and Caspase-3 Activation*

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In the absence of survival factors, blood monocytes undergo spontaneous apoptosis, which involves the activation of caspase-3. Although nitric oxide can block caspase-3 activation and promote cell survival, it can also induce apoptosis. We hypothesized that nitrosothiols that promote protein *S*-nitrosylation would reduce caspase-3 activation and cell survival, whereas nitric oxide donors (such as 1-propamine 3-(2-hydroxy-2-nitroso-1-propylhydrazine (PAPA) NONOate and diethylamine (DEA) NONOate) that do not target thiol residues would not. Using human monocytes as a model, we observed that nitrosothiol donors *S*-nitrosoglutathione and *S*-nitroso-*N*-acetylpenicillamine suppressed caspase-9 and caspase-3 activity and DNA fragmentation. In contrast, PAPA or DEA NONOate did not promote monocyte survival events and appeared to inhibit monocyte survival induced by macrophage colony-stimulating factor. The caspase-3-selective inhibitor DEVD-fluoromethyl ketone reversed DNA fragmentation events, and the caspase-9 inhibitor LEHD-fluoromethyl ketone reversed caspase-3 activity in monocytes treated with PAPA or DEA NONOate in the presence of macrophage colony-stimulating factor. These results were not caused by differences in glutathione levels or the kinetics of nitric oxide release. Moreover, *S*-nitrosoglutathione and *S*-nitroso-*N*-acetylpenicillamine directly blocked the activity of recombinant caspase-3, which was reversed by the reducing agent dithiothreitol, whereas PAPA or DEA NONOate did not block the enzymatic activity of caspase-3. These data support the hypothesis that nitrosylation of protein thiol residues by nitric oxide is critical for promoting the survival of human monocytes.

In the absence of specific survival factors, human monocytes spontaneously undergo apoptosis in 24–48 h (1, 2). In contrast to cellular necrosis, apoptosis is an energy-requiring, non-inflammatory mechanism of programmed cell death. The apoptotic pathway can be extrinsic or intrinsic. The extrinsic pathway is regulated by cell receptors such as Fas, which is in the tumor necrosis factor receptor family. Once clustered by Fas ligand, Fas activation leads to the formation of the death-inducing signaling pathway (DISC). This complex contains the adapter protein Fas-associated death domain protein and cysteinyl-aspartic acid protease (caspase)-8 and caspase-10, which can activate cellular apoptosis. In type I cells, caspase-8 activation directly activates downstream caspases, resulting in cellular apoptosis. In contrast, type II cells rely on an amplification loop where caspase-8 cleaves and activates the pro-apoptotic protein Bid, inducing the release of cytochrome *c* and Diablo from the mitochondria. These events lead to caspase-3 activation, which induces the execution of cellular apoptosis. Moreover, active caspase-3 activates caspase-8, further amplifying this feedback loop.

In contrast to the Fas receptor system, the intrinsic apoptotic pathway is initiated by the release of cytochrome *c* from the mitochondria. Cytochrome *c* interacts with procaspase-9, APAF-1, and ATP to liberate active caspase-9. Active caspase-9 cleaves and activates caspase-3, leading to cellular apoptosis. As a family, these caspases exist as inactive procaspase zymogens that require activation by proteolytic or autocatalytic cleavage. Processing and activation of upstream caspases such as caspase-8 and/or caspase-9 lead to activation of executioner caspase-3, -6, and -7, which are responsible for the characteristic changes associated with apoptosis such as membrane blebbing, nuclear condensation, and DNA fragmentation. Caspase-3 is a primary executioner caspase and inactivates ICAD (inhibitor caspase-activated deoxyribonuclease) to induce the cleavage of DNA into oligonucleosomal fragments (5). These cysteine proteases are described as mammalian homologs to *Caenorhabditis elegans* CED-3 (*C. elegans* death gene 3), originally discovered to be involved in the regulation of apoptotic cell death (3, 4).

Nitric oxide promotes cell survival by blocking caspase-3 activation by *S*-nitrosylating the cysteine residue in the catalytic site of caspase-3 (6). In fact, it has been suggested that the binding of Fas to Fas ligand induces apoptosis by denitrosylating and activating caspase-3, providing a causal link between nitric oxide and cell survival (6). Consistent with a key role for nitric oxide effects on caspase-3 function, *S*-nitrosylation of Cys-163 by nitric oxide specifically inhibits caspase-3 enzymatic activity (7). Thus, *S*-nitrosylation is widely believed to be the primary mechanism by which nitric oxide prevents caspase-3 cleavage. Because certain nitric oxide donors such as *S*-nitrosoglutathione (GSNO)[1] and *S*-nitroso-*N*-acetylpenicillamine (SNAP) promote *S*-nitrosylation by transferring nitrosyl groups to protein thiol residues (8), we speculated that these

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[1] The abbreviations used are: GSNO, *S*-nitrosoglutathione; SNAP, *S*-nitroso-*N*-acetylpenicillamine; M-CSF, macrophage colony-stimulating factor; PAPA, 1-propamine 3-(2-hydroxy-2-nitroso-1-propylhydrazine; DEA, diethylamine; FMK, fluoromethyl ketone; AFC, aminotrifluoromethylcoumarin; PIPES, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; HPLC, high performance liquid chromatography.

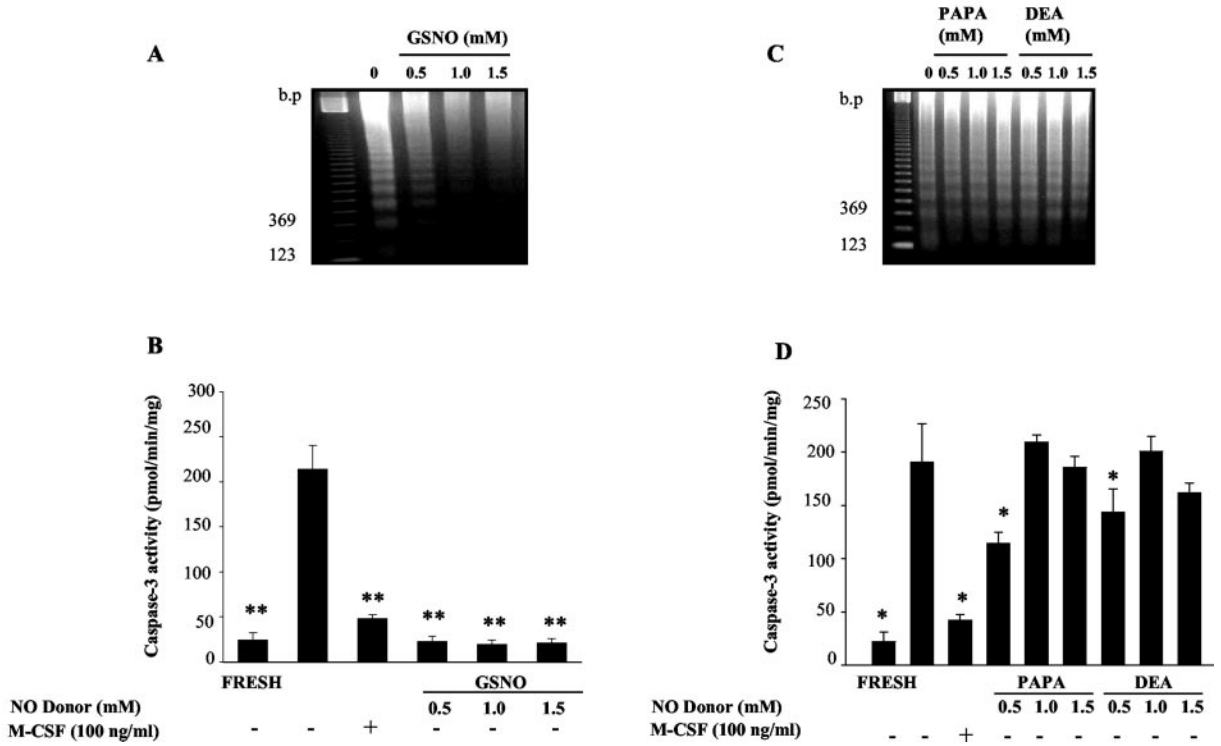

FIG. 1. **Nitric oxide presentation influences survival and caspase-3 activation in primary human monocytes.** Human monocytes (5×10^6/condition) were left non-stimulated (0, −) or were incubated with the nitrosothiol donor GSNO or the nitric oxide donor PAPA NONOate or DEA NONOate at 0.5, 1.0, and 1.5 mM as indicated for 24 h. M-CSF (+) was used as a control. *A*, cytosolic extracts from monocytes incubated with GSNO were assayed for DNA fragmentation as described under "Experimental Procedures." *B*, monocytes were lysed immediately after isolation (*FRESH*) or lysed after 24 h of incubation with GSNO and assayed for caspase-3-like activity using the fluorogenic substrate DEVD-AFC. Data are expressed as means ± S.E. for four independent experiments. GSNO at 0.5, 1.0, and 1.5 mM suppressed caspase-3 activation compared with non-stimulated control cells incubated for 24 h. **, $p < 0.001$. *C*, cytosolic extracts from monocytes incubated with PAPA NONOate or DEA NONOate were assayed for DNA fragmentation. *D*, lysates from monocytes treated with PAPA NONOate or DEA NONOate were assayed for caspase-3 activity as described above. Data are means ± S.E. for four independent experiments, with *, $p < 0.05$ reduction in caspase-3 activity by PAPA NONOate and DEA NONOate compared with the non-stimulated 24-h control.

nitrosothiol donors would reduce caspase-3 activation and promote survival of human monocytes.

Paradoxically, nitric oxide can also induce apoptosis in certain cells (9–11). The specific mechanism for this effect is not clear; however, nitric oxide reacts rapidly with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) (12–14). Peroxynitrite can nitrate tyrosine residues and inhibit tyrosine phosphorylation-mediated activation events. The monocyte survival factor macrophage colony-stimulating factor (M-CSF) induces reactive oxygen species production (15) and relies on the phosphorylation of tyrosine residues in the cytoplasmic domain of the receptor to activate signaling pathways. We speculated that nitric oxide donors (such as 1-propamine 3-(2-hydroxy-2-nitroso-1-propylhydrazine) (PAPA) NONOate and diethylamine (DEA) NONOate) that do not target thiol residues would interrupt phosphorylation of these tyrosine residues and reduce monocyte survival induced by M-CSF.

Consistent with these hypotheses, we found that the nitrosothiol donors GSNO and SNAP inhibited caspase-3 activity and reduced DNA fragmentation in human monocytes, whereas the nitric oxide donors PAPA NONOate and DEA NONOate did not consistently reduce caspase-3 activation or DNA fragmentation. Homeostasis of blood monocytes by these donors appeared to involve the activation state of caspase-9 and caspase-3 and correlated to modification of thiol residues by nitric oxide.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium and Dulbecco's modified Eagle's medium were purchased from BioWhittaker, Inc. (Walkersville, MD). Fetal calf serum was obtained from Hyclone Laboratories (Logan, UT). GSNO and SNAP was obtained from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). PAPA NONOate was purchased from Alexis Biochemicals (San Diego, CA). DEA NONOate was obtained from Calbiochem. Recombinant human M-CSF was purchased from R&D Systems (Minneapolis, MN). The anti-phosphotyrosine antibody used is a 30:30:1 ratio of clonal antibodies PY72, PY20, and 4G10 obtained from Dr. Bart Sefton (Transduction Laboratories, San Diego, CA) and Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-caspase-3 antibody was purchased from Upstate Biotechnology, Inc. The caspase inhibitors DEVD-fluoromethyl ketone (FMK) and LEHD-FMK and the fluorogenic substrates DEVD-aminotrifluoromethylcoumarin (AFC) and LEHD-AFC were obtained from Enzyme Systems Products (Livermore, CA). The active recombinant human caspase-3 enzyme was purchased from BioVision (Mountain View, CA). All other reagents were purchased from Sigma unless indicated otherwise.

Purification of Peripheral Blood Monocytes—Monocytes were isolated from buffy coats obtained from the American Red Cross according to a method described previously (16). For DNA fragmentation analysis, nitric oxide measurement, and caspase 3-like activity measurement, monocytes (5×10^6/sample) were treated in the indicated conditions immediately after monocyte isolation. In signaling experiments for tyrosine-phosphorylated proteins, monocytes were resuspended at 10×10^6 cells/ml of RPMI 1640 medium, 10% fetal bovine serum, 10 μg/ml polymyxin B, and 20 ng/ml recombinant human M-CSF. After 24 h in culture, the cells were serum-starved for 2 h, treated for 1 h with the indicated nitric oxide donors, and stimulated for 2 min with 100 ng/ml M-CSF.

Cytosolic DNA Fragmentation Analysis—Apoptotic DNA fragments were purified using DNA isolation kits (Suicide-Track DNA isolation kit, Oncogene Research Products, Cambridge, MA). DNA fragments were resolved by 1.6% agarose gel electrophoresis. DNA bands were visualized by staining with Syber Green (Molecular Probes, Inc., Eugene, OR). The DNA fragments were analyzed on a digital gel documentation system (Gel-Doc 1000, Bio-Rad).

Quantification of Apoptosis—Apoptosis was measured by using an annexin V-FITC apoptosis dectection kit according to the manufactur-

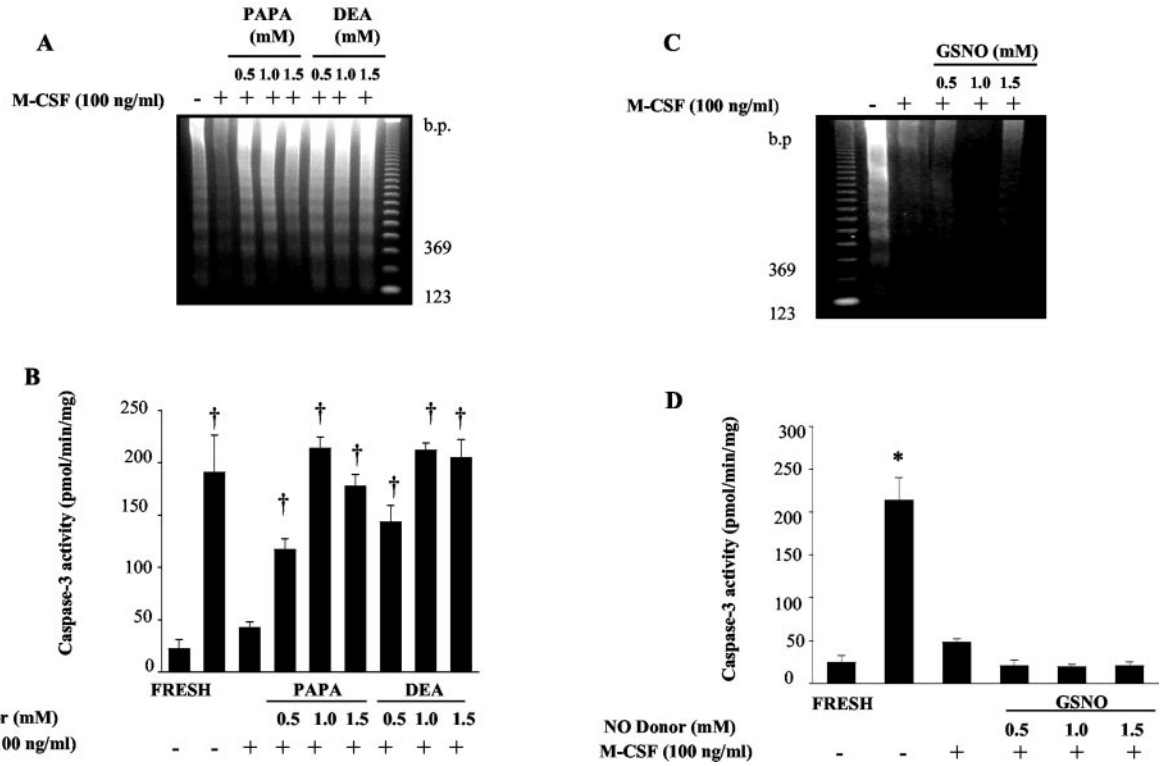

FIG. 2. **PAPA NONOate and DEA NONOate inhibit M-CSF-induced monocyte survival and activate caspase-3.** Monocytes (5×10^6/condition) were left non-stimulated or were incubated with the nitric oxide donor PAPA NONOate, DEA NONOate, or GSNO (0.5, 1.0, and 1.5 mM) as indicated for 30 min and then incubated without (−) or with (+) M-CSF (100 ng/ml) for 24 h. *A*, cytosolic extracts from non-stimulated or M-CSF-stimulated monocytes treated with the indicated concentrations of PAPA NONOate or DEA NONOate were assayed for DNA fragmentation. *B*, lysates from monocytes prepared as described above were assayed for caspase-3-like activity using the fluorogenic substrate DEVD-AFC. Data are expressed as means ± S.E. for four experiments. Cells left non-stimulated (−) for 24 h and cells treated with PAPA NONOate or DEA NONOate in the presence of M-CSF had no caspase-3 activation *versus* cells treated with M-CSF alone or freshly isolated (*FRESH*). †, $p < 0.01$. *C*, cytosolic DNA fragmentation was assayed in extracts of monocytes left non-stimulated or stimulated with M-CSF alone or with the indicated concentrations of GSNO. *D*, lysates from monocytes as cultured above were assayed for caspase-3-like activity. Data are means ± S.E. from four experiments. Only non-stimulated cells for 24 h had more caspase-3 activity compared with cells incubated with M-CSF with or without GSNO. *, $p < 0.05$.

er's protocol (Pharmingen). Samples were analyzed by a flow cytometer (FACSCalibur, BD Biosciences).

Nitric Oxide Measurement by Chemiluminescence—Monocytes were cultured for 24 h in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with the following NO donors at 2 μM to 2 mM concentrations: GSNO, SNAP, DEA NONOate, and PAPA NONOate. Nitrate and nitrite in the cultured supernatants were injected into a collection chamber and reduced to nitric oxide by VCl_3 in 1 N HCl. The nitric oxide reacted with ozone, producing a chemiluminescent signal that was measured by a nitric oxide analyzer (Sievers NOA 280).

Caspase Enzymatic Activity Measured with AFC—Monocytes were collected by centrifugation and washed with KPM buffer (50 mM KCl, 50 mM PIPES, 10 mM EGTA, 1.92 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml cytochalasin B, and a 2 μg/ml concentration of each the following protease inhibitors: chymostatin, pepstatin, leupeptin, and antipain). The cell suspension was lysed by four cycles of freezing and thawing. The cytosolic protein was obtained after centrifugation at 12,000 × *g* for 20 min at 4 °C. The extracts were incubated with the fluorogenic substrate DEVD-AFC for caspase-3-like activity or with LEHD-AFC for caspase-9 activity in a cyto-buffer (10% glycerol, 50 mM PIPES, pH 7, and 1 mM EDTA) containing 1 mM DTT and 20 μM DEVD-AFC as previously described (16). For experiments using the active recombinant human caspase-3 enzyme, the NO donors were incubated with the protein at one-seventh of their half-lives in an attempt to control for the varying half-lives of the different nitric oxide donors, except GSNO. Because of its extremely long half-life, GSNO was incubated with recombinant caspase-3 at ½₄₂ of its half-life. DTT was omitted from KPM buffer to prevent denitrosylation of recombinant human caspase-3. DTT was added to the indicated samples for 15 min prior to measurement of caspase-3 activity to reverse *S*-NO formation. All conditions were assayed for caspase-3 activity by measuring the release of free AFC as determined with a Cytofluor 4000 fluorometer (filters, excitation at 400 nm and emission at 505 nm; PerSeptive Diagnostics, Ramingham, MA).

Immunoprecipitation and Immunoblotting—Stimulated samples were left on ice for 15 min in lysis buffer containing 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10 mM NaF, 0.5% deoxycholate, 10 mM EDTA, 0.1% SDS, and 1% Nonidet P-40. Lysates were centrifuged at 12,000 × *g* for 10 min at 4 °C. Equivalent amounts of protein were used for each immunoprecipitation or whole cell lysate as determined by the Bradford protein assay (Bio-Rad). Immunoprecipitated samples were incubated with the mouse anti-phosphotyrosine antibody mixture overnight at 4 °C. After incubation with protein G-agarose beads (Invitrogen), the immune complexes attached to the beads were washed three times with lysis buffer. Laemmli sample buffer containing 2-mercaptoethanol was added to the agarose beads and whole cell lysates and incubated for 5 min at 95 °C. The samples were run on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin in 1× Tris-buffered saline and 1% Tween and probed with the indicated primary antibodies, followed by incubation with the appropriate secondary antibody linked to horseradish peroxidase. The membrane was evaluated by enhanced chemiluminescence detection (Amersham Biosciences).

High Performance Liquid Chromatography (HPLC) Electrochemical Measurement of GSH—Samples were prepared as described (17). Glutathione was separated using a C_{18} column and detected using an HPLC colorimetric electrode array detector (Coularray Detector Model 5600 with 12 channels, ESA Inc., Chelmsford, MA). The mobile phase consisted of 50 mM sodium dihydrogen phosphate, 0.5 mM octanosulfonic acid, and 3% acetonitrile at pH 2.7 (18).

Statistical Analysis—All data are expressed as means ± S.E. Statistical significance was defined as $p < 0.05$ using analysis of variance with *post hoc* testing (Minitab, College Park, PA).

RESULTS

Nitric Oxide Supports Survival by a Caspase-3-dependent Mechanism—The spontaneous apoptosis of human monocytes

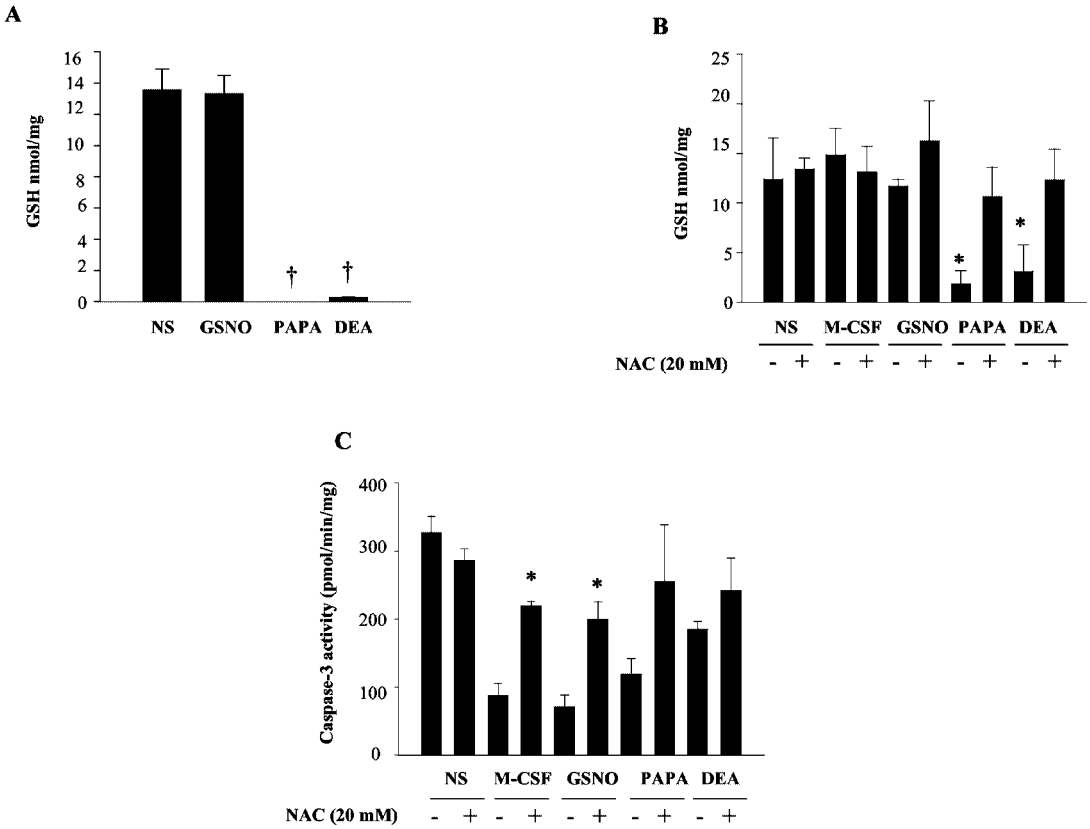

FIG. 3. **Intracellular glutathione levels are not responsible for the effects of PAPA NONOate and DEA NONOate.** *A*, monocytes (5×10^6/condition) were left non-stimulated (*NS*) or were stimulated with the nitric oxide donor GSNO, PAPA NONOate, or DEA NONOate (0.5 mM) for 24 h and measured for GSH. PAPA NONOate or DEA NONOate reduced intracellular glutathione compared with cells treated with GSNO and the non-stimulated control. †, $p < 0.01$. *B*, monocytes (5×10^6/condition) were treated as described above without (−) or with (+) exogenous *N*-acetylcysteine (*NAC*; 20 mM) for 24 h, and glutathione was measured. Only in glutathione levels did significant differences exist in cells treated with PAPA NONOate or DEA NONOate in the absence of *N*-acetylcysteine compared with the non-stimulated + *N*-acetylcysteine control. *, $p < 0.05$. *C*, lysates from monocytes (5×10^6/condition) treated as described for *B* were assayed for caspase-3-like activity. These data represent three independent studies and demonstrate that the addition of *N*-acetylcysteine increased caspase-3 activity compared with samples treated with M-CSF and GSNO alone. *, $p < 0.05$.

appears to be partly regulated by the activation of caspase-3 (1). GSNO has been shown to *S*-nitrosylate the critical cysteine residue in the catalytic domain of caspase-3 to inhibit caspase-3 activation and to facilitate cell survival (6). Thus, we speculated that nitrosothiol donors such as GSNO would suppress caspase-3 activation and facilitate cell survival. The nitrosothiol donor GSNO suppressed DNA fragmentation events (Fig. 1*A*) and inhibited the activation of native caspase-3 (Fig. 1*B*). Low concentrations of DEA NONOate and PAPA NONOate also reduced caspase-3 activation in human monocytes (Fig. 1, *C* and *D*), but did not appear to reduce DNA fragmentation. Higher concentrations of DEA NONOate and PAPA NONOate resulted in caspase-3 activation, whereas similar concentrations of GSNO did not.

Compared with non-stimulated cells staining positive for annexin V/propidium iodide, GSNO and SNAP reduced annexin V/propidium iodide staining ($p < 0.01$ non-stimulated *versus* GSNO or SNAP; non-stimulated, 84.8 ± 2.3%; GSNO, 39.8 ± 10.1%; SNAP, 53.2 ± 0.76%), whereas PAPA NONOate and DEA NONOate did not ($p > 0.4$ non-stimulated *versus* PAPA NONOate or DEA NONOate; non-stimulated, 84.3 ± 2.3%; PAPA NONOate, 71.52 ± 5.6%; DEA NONOate, 80.46 ± 6.6%).

PAPA NONOate and DEA NONOate Block M-CSF-induced Monocyte Survival through Caspase-3 Activation—Previously, we demonstrated that M-CSF promotes the survival of human monocytes (15). We next investigated whether PAPA NONOate or DEA NONOate would reverse the suppression of caspase-3 and DNA fragmentation promoted by M-CSF in human monocytes. We found that PAPA NONOate or DEA NONOate independently induced DNA fragmentation and caspase-3 activation in M-CSF-stimulated human monocytes (Fig. 2, *A* and *B*). In contrast to the effects of PAPA NONOate or DEA NONOate, GSNO did not promote DNA fragmentation or the activation of native caspase-3 in parallel samples of M-CSF-stimulated monocytes (Fig. 2, *C* and *D*).

Glutathione Levels and Cumulative Nitric Oxide Production over 24 h Are Not Responsible for the Differing Effects on DNA Fragmentation and Caspase-3 Activation in Human Monocytes—Because GSNO is an *S*-nitrosoglutathione, we first examined the possibility that enhanced glutathione stores resulting from treatment with GSNO in monocytes were responsible for differences seen between GSNO and NONOates. We found that intracellular glutathione levels were decreased in monocytes treated with PAPA NONOate or DEA NONOate in the absence of M-CSF (Fig. 3*A*). However, replenishing intracellular glutathione with *N*-acetylcysteine (Fig. 3*B*) did not inhibit caspase-3 activation by these nitric oxide donors and even appeared to enhance caspase-3 activity in monocytes treated with M-CSF or GSNO (Fig. 3*C*). To ensure that the protective effect of GSNO was not related solely to increased glutathione levels donated by GSNO, we also used the nitrosothiol donor SNAP, which does not generate glutathione. Similar to GSNO, SNAP reduced DNA fragmentation over a 24-h period and reduced the activation of native caspase-3 in human monocytes (Fig. 4, *A* and *B*).

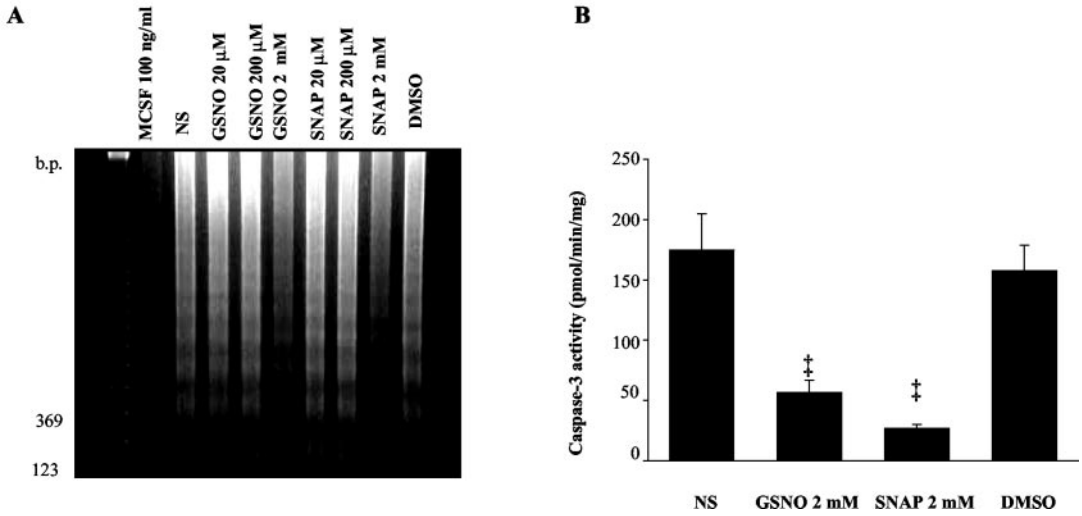

FIG. 4. **The nitrosothiol SNAP also blocks DNA fragmentation and caspase-3 activity in human monocytes.** *A*, monocytes (5×10^6) were left non-stimulated (*NS*) or were treated with the Me_2SO (*DMSO*) solvent control and assayed for DNA fragmentation as described under "Experimental Procedures." Cells were treated with the nitric oxide donors SNAP and GSNO (20 and 200 μM and 2 mM). *B*, monocytes were incubated with the above controls and with 2 mM SNAP or GSNO. Lysates were assayed for caspase-3 activity by measuring the caspase-3 substrate DEVD-AFC. These data are representative of two separate experiments. ‡, $p < 0.02$ *versus* non-stimulated and Me_2SO controls.

We next wanted to determine whether the dichotomous effects of the nitric oxide donors could be explained by quantitative differences in nitric oxide release by the donors. We found that, although GSNO blocked the activation of caspase-3, it produced less nitric oxide than DEA NONOate and PAPA NONOate measured at 24 h (Table I). To ensure that the higher amounts of nitric oxide delivered by DEA NONOate or PAPA NONOate did not independently injure the cells, lower concentrations of PAPA NONOate and DEA NONOate were measured at 24 h (Table II), and they did not consistently prevent the activation of caspase-3 (Fig. 5). Of note, using these lower concentrations of nitric oxide donors, only SNAP significantly suppressed caspase-3 activity.

Nitric Oxide Donors Suppress Tyrosine Phosphorylation Events Induced by M-CSF-stimulated Human Monocytes—We next sought to explain the mechanism of DEA NONOate and PAPA NONOate suppression of M-CSF-induced monocyte survival. Because we have shown that M-CSF induces reactive oxygen species production in monocytes (15), we speculated that nitric oxide combined with reactive oxygen species forms peroxynitrite and suppresses tyrosine phosphorylation events in M-CSF-stimulated monocytes. As predicted, we found that each of the three nitric oxide donors reduced tyrosine phosphorylation events in M-CSF-stimulated monocytes (Fig. 6).

GSNO and SNAP Prevent the Proteolytic Processing of Caspase-3 to Promote Monocyte Survival—Because GSNO and DEA NONOate or PAPA NONOate reduced cellular tyrosine phosphorylation events in M-CSF-stimulated monocytes, but only GSNO promoted monocyte survival events, we concluded that GSNO promoted cell survival by acting downstream of the receptor. In keeping with this hypothesis, only GSNO and SNAP blocked the cleavage of caspase-3 to its active 17-kDa subunit at 24 h of incubation, whereas cells incubated with DEA NONOate or PAPA NONOate or incubated alone did not (Fig. 7*A*). The 17-kDa subunit was also measured by densitometry (Fig. 7*B*).

GSNO and SNAP Inhibit the Activity of Active Recombinant Caspase-3—In addition to reducing the activation of caspase-3, we next wanted to determine whether the nitrosothiol donors GSNO and SNAP could also interrupt the biological activity of active caspase-3. This prediction was based on the finding that the active cysteine site of caspase-3, which can be modified via *S*-nitrosylation, influences the enzymatic activity of caspase-3 (7). To test this hypothesis, we first determined the kinetics of active recombinant caspase-3 and found that this enzyme spontaneously lost biological activity quickly after 15 min, but stabilized between 30 and 90 min of incubation (Fig. 8*A*). Using a time point of 40 min, we then added the nitric oxide donors SNAP, PAPA NONOate, and DEA NONOate at one-seventh of their respective half-lives and GSNO at 1/242 of its half-life. GSNO and SNAP reduced the enzymatic activity of active recombinant caspase-3, whereas DEA and PAPA NONOate did not (Fig. 8*B*). To determine whether the direct inhibition of caspase-3 by GSNO and SNAP was due to modification of the sulfhydryl group, the reducing agent dithiothreitol was added to remove NO modification of thiol residues by nitric oxide. In the presence of GSNO or SNAP, DTT restored caspase-3 enzymatic activity, suggesting that GSNO and SNAP modify active recombinant caspase-3 by *S*-nitrosylation of the active caspase (Fig. 8*C*).

Blocking Caspase-3 and Caspase-9 Activation Reverses Apoptosis Induced by PAPA NONOate or DEA NONOate in

TABLE I
Nitric oxide production in human monocytes

Monocytes (5×10^6) were incubated for 24 h with the indicated nitric oxide donors in millimolar concentrations, with the exception of M-CSF. The Culture medium was measured for nitric oxide production by ozone chemiluminescence as described under "Experimental Procedures." Data are expressed as means ± SE for four independent experiments.

NO donor	Nitric oxide production
	μM
Non-stimulated	15.53 ± 4.43
M-CSF, 100 ng/ml	23.56 ± 0.59
GSNO[a]	
0.5 mM	234.85 ± 17.50
1.0 mM	512.12 ± 35.12
1.5 mM	914.53 ± 73.00
PAPA NONOate[a,b]	
0.5 mM	677.3 ± 100.70
1.0 mM	1294.44 ± 155.90
1.5 mM	1988.68 ± 238.80
DEA NONOate[a,b]	
0.5 mM	830.60 ± 122.38
1.0 mM	1099.912 ± 101.08
1.5 mM	1369.71 ± 143.61

[a] $p < 0.05$; differences among GSNO, PAPA NONOate, and DEA NONOate compared with non-stimulated and M-CSF controls.
[b] $p < 0.05$; differences in DEA NONOate and PAPA NONOate compared with GSNO at equal concentrations of nitric oxide donor (0.5–1.5 mM).

TABLE II
Nitric oxide production at lower doses of NO donors in human monocytes

Monocytes (5×10^6) were incubated for 24 h with the indicated nitric oxide donors in micromolar concentrations. The culture medium was measured for nitric oxide production by ozone chemiluminescence as described under "Experimental Procedures." Data are expressed as means ± S.E. for two independent experiments.

No donor	Nitric oxide production
	μM
Non-stimulated	43.99 ± 0.26
GSNO	
2 μM	39.94 ± 6.46
20 μM	52.75 ± 0.75
200 μM	114.28 ± 11.31
SNAP	
2 μM	55.77 ± 16.94
20 μM	64.20 ± 20.76
200 μM	107.75 ± 21.12
PAPA NONOate	
2 μM	62.62 ± 18.16
20 μM	74.87 ± 17.47
200 μM	225.06 ± 6.28
DEA NONOate	
2 μM	48.75 ± 2.00
20 μM	64.74 ± 20.15
200 μM	208.22 ± 2.99

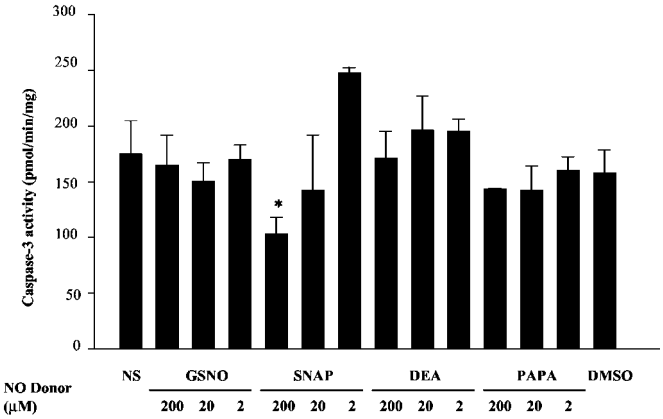

FIG. 5. **DEA NONOate and PAPA NONOate do not reduce caspase-3 activation at lower nitric oxide concentrations.** Monocytes (5×10^6) were treated with GSNO, SNAP, DEA-NONOate, and PAPA-NONOate (2–200 μM, as indicated). Monocytes were also left non-stimulated (*NS*) or were treated with the vehicle control Me_2SO (*DMSO*). The fluorogenic substrate DEVD-AFC released from these lysates was measured as described under "Experimental Procedures." Data are means ± S.E. from two independent experiments. *, $p < 0.05$ for SNAP compared with the non-stimulated control.

M-CSF-stimulated Monocytes—Because GSNO and SNAP appeared to reduce DNA fragmentation and activation of caspase-3 in human monocytes, we next wanted to determine whether caspase-3 activation was involved in promoting DNA fragmentation in PAPA NONOate- and DEA NONOate-treated monocytes. The caspase-3 inhibitor DEVD-FMK reduced oligonucleosomal DNA fragmentation in M-CSF-stimulated monocytes treated with DEA NONOate or PAPA NONOate (Fig. 9). To evaluate the role of caspase-9 in this pathway, we found that GSNO and SNAP also suppressed the activation of caspase-9 in human monocytes (Fig. 10*A*). Moreover, the caspase-9-selective inhibitor LEHD-FMK inhibited caspase-3 activation in monocytes incubated with PAPA NONOate or DEA NONOate (Fig. 10*B*), suggesting that caspase-3 is regulated by caspase-9 in this pathway.

DISCUSSION

This study was designed to test the hypothesis that the presentation of nitric oxide determines whether nitric oxide

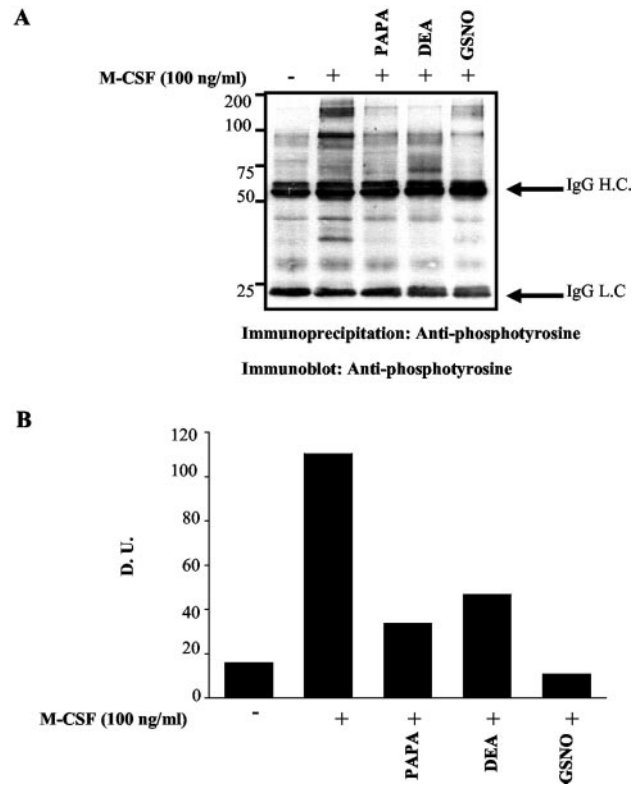

FIG. 6. **Nitric oxide donors suppress tyrosine phosphorylation events induced by M-CSF-stimulated human monocytes.** *A*, monocytes (10×10^6/condition) were left non-stimulated (−) or were pretreated with the nitric oxide donors GSNO, PAPA NONOate, and DEA NONOate (0.5 mM) for 1 h prior to stimulating these samples with M-CSF (100 ng/ml; +) for 2 min. The cells were lysed, immunoprecipitated, and blotted for tyrosine-phosphorylated proteins with anti-phosphotyrosine antibodies as described under "Experimental Procedures." IgG heavy chain (*H.C.*) and light chain (*L.C.*) are labeled. Data are representative of three independent studies. B, shown are densitometric units (*D.U.*) of bands representing tyrosine-phosphorylated proteins of the blot in *A*.

functions to promote cell life or cell death. Incubating human monocytes with the nitrosothiol donors GSNO and SNAP suppressed the activation of native caspase-3, reduced the enzymatic activity of active recombinant caspase-3, and reduced DNA fragmentation in monocytes incubated with or without M-CSF. In contrast, incubating parallel samples of these monocytes with the nitric oxide donor PAPA NONOate and DEA NONOate, which do not target thiol groups, did not consistently suppress the activation of native caspase-3 or reduce the activity of active recombinant caspase-3. Furthermore, in the presence of M-CSF, PAPA NONOate and DEA NONOate induced the activation of native caspase-3 and also induced cytosolic oligonucleosomal DNA fragmentation. Thus, it appears that the ability to donate nitric oxide to protein thiol residues is important in reducing caspase-3 activation and in promoting the survival of human monocytes.

To support the hypothesis that the interruption of caspase-3 is important in monocyte survival, we found that the caspase-3 inhibitor DEVD-FMK blocked DNA fragmentation in monocytes treated with PAPA NONOate or DEA NONOate in the presence of M-CSF, suggesting that caspase-3 activation regulates these apoptotic events. Moreover, the caspase-9-selective inhibitor LEHD-FMK suppressed caspase-3 activation in monocytes treated with PAPA NONOate or DEA NONOate, suggesting that caspase-9 activation is involved as the upstream activator of caspase-3 in human monocytes. To confirm that caspase-9 is involved in GSNO- or SNAP-induced mono-

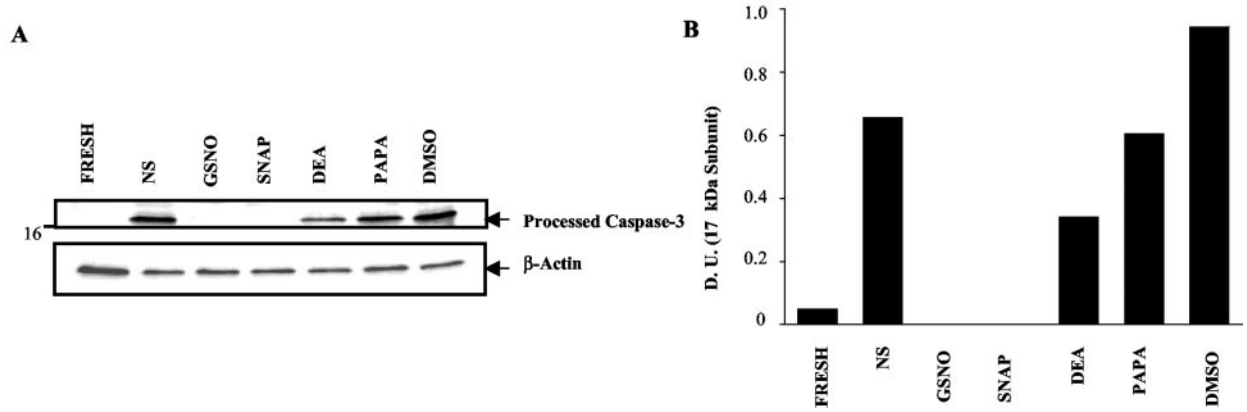

FIG. 7. **The nitrosothiols GSNO and SNAP can prevent the formation of caspase-3 cleavage products.** *A*, monocytes (5×10^6) were freshly isolated (*FRESH*) from buffy coats or were isolated and left non-stimulated (*NS*) or were treated with 0.5 mM GSNO, SNAP, DEA-NONOate, or PAPA-NONOate for a 24-h incubation period. Processed 17-kDa products of caspase-3 were detected using anti-caspase-3 antibodies according to the immunoblotting procedure described under "Experimental Procedures." Anti-β-actin antibody was used as an indicator to show equal protein loading. *B*, a ratio of the 17-kDa caspase-3 cleavage product to total β-actin protein is represented in densitometric units (*D.U.*). *DMSO*, Me_2SO.

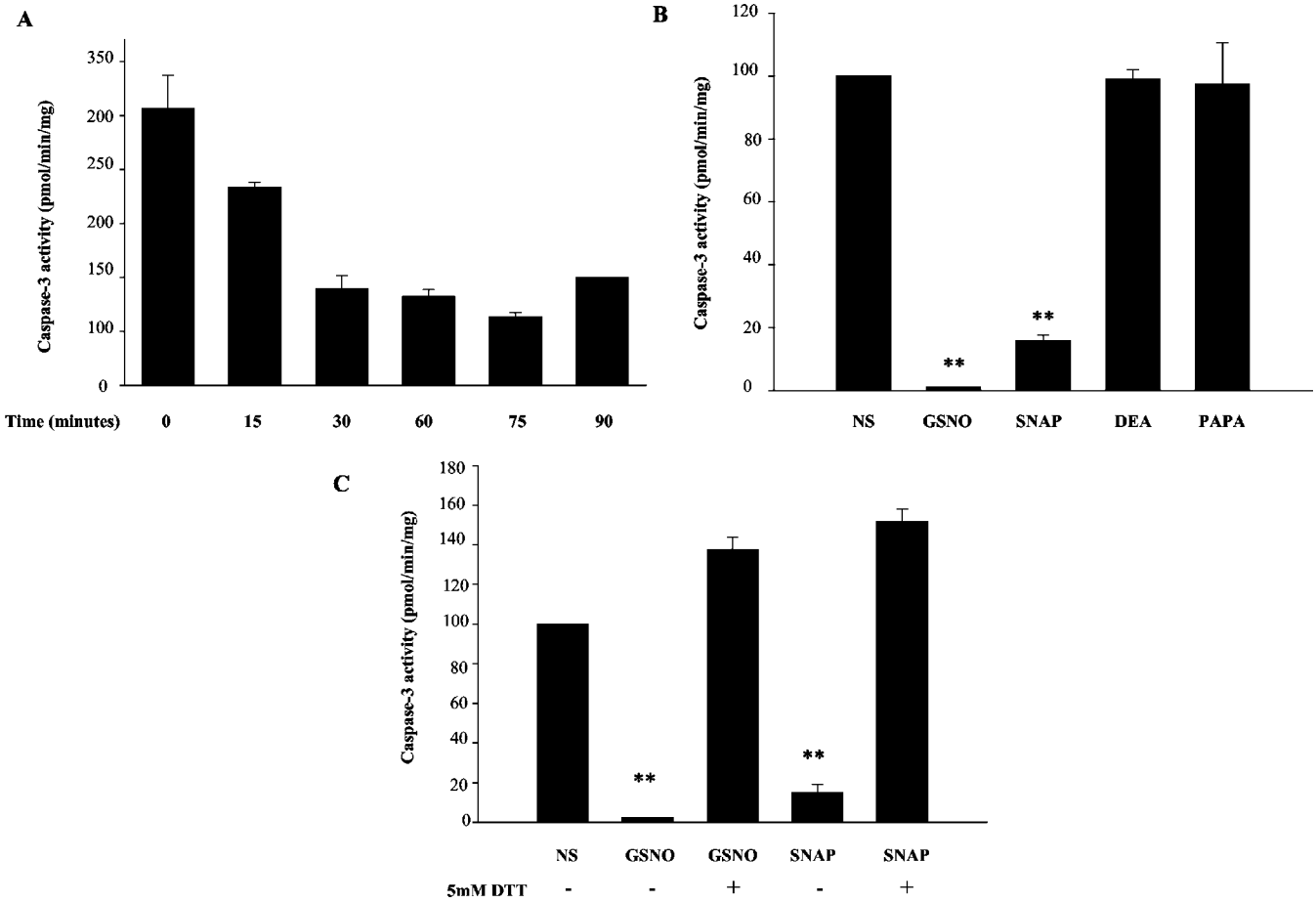

FIG. 8. **GSNO and SNAP directly inhibit active recombinant human caspase-3, which is reversible by DTT.** *A*, the active recombinant human caspase-3 enzyme was incubated at 37 °C for 0, 15, 30, 60, 75, and 90 min. The spontaneous loss of enzymatic activity stabilized between 30 and 90 min. *B*, the nitric oxide donors SNAP, DEA NONOate, and PAPA NONOate were added at one-seventh of their half-lives, or GSNO was added at ½₄₂ of its half-life and incubated with active recombinant human caspase-3. Caspase-3 activity was determined as described under "Experimental Procedures." Results are means ± S.D. for three independent experiments. **, $p < 0.001$ *versus* non-stimulated recombinant caspase-3 enzyme. *C*, because only GSNO and SNAP reduced caspase-3 activity, we next added dithiothreitol (+) for 15 min to samples of active recombinant caspase-3 that were incubated with GSNO or SNAP. The non-stimulated (*NS*) samples are taken as 100% control. Data are means ± S.D. for three independent experiments. **, $p < 0.001$ *versus* GSNO + DTT, SNAP + DTT, or non-stimulated.

cyte survival, we found that treating monocytes with the nitrosothiol donors GSNO and SNAP also suppressed native caspase-9 activity in the absence of M-CSF, suggesting that nitrosothiol donors interrupt the activation of caspase-9 upstream of caspase-3. These data suggest that modulating the activity of caspases is a critical determinant of monocyte survival by the nitrosothiol donors.

We considered the possibility that intracellular glutathione depletion by PAPA NONOate and DEA NONOate may lead to caspase activation, as glutathione depletion can induce the

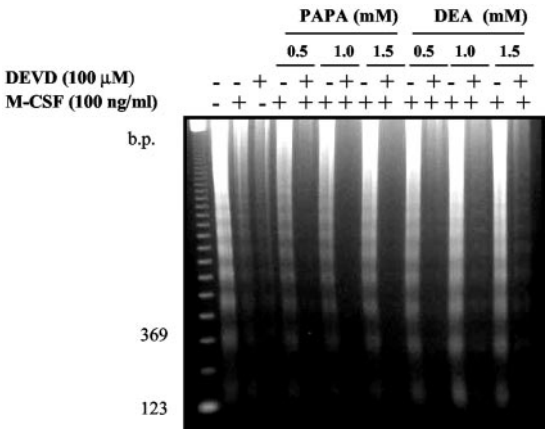

FIG. 9. **Blocking caspase-3 activation with DEVD-FMK reverses DNA fragmentation induced by PAPA NONOate or DEA NONOate in M-CSF-stimulated monocytes.** Monocytes (5×10^6/condition) were left non-stimulated (−) or were stimulated with M-CSF alone (+) or with the indicated concentrations of PAPA NONOate or DEA NONOate in the presence (+) or absence (−) of the caspase-3-selective peptide benzyloxycarbonyl-DEVD-FMK (100 μM) or an equal concentration of Me_2SO for 24 h. Cytosolic extracts were assayed for DNA fragments. Data are representative of three independent studies.

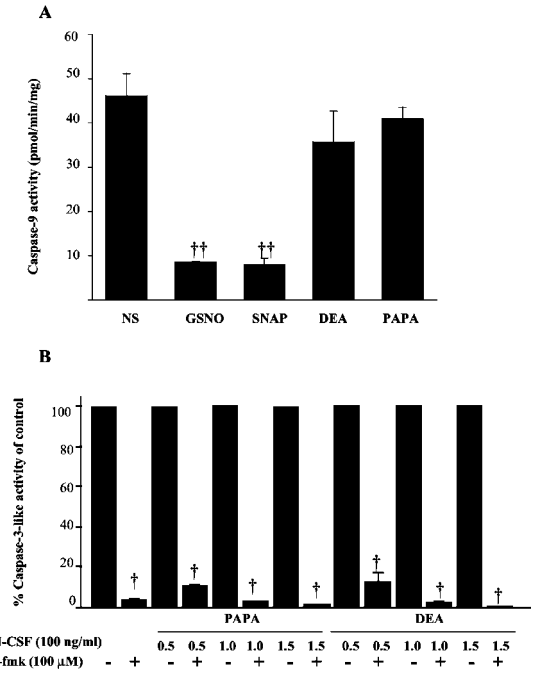

FIG. 10. **GSNO and SNAP reduce caspase-9 activation, and blocking caspase-9 activation inhibits caspase-3 activity in monocytes treated with PAPA NONOate or DEA NONOate.** *A*, monocytes (5×10^6/condition) were incubated with 1.0 mM GSNO, 1.0 mM SNAP, 0.5 mM PAPA NONOate, or 0.5 mM DEA NONOate for 24 h. These concentrations of NO donors gave more equivalent concentrations of NO at 24 h. Caspase-9-like activity was assayed and is expressed as means ± S.E. for two studies. ††, $p = 0.004$ *versus* the non-stimulated (*NS*) control. *B*, monocytes (5×10^6/condition) were left non-stimulated (−) or were stimulated with M-CSF treated with PAPA NONOate or DEA NONOate in the presence (+) or absence (−) of the caspase-9-selective inhibitor LEHD-FMK (100 μM) for 24 h and measured for caspase-3-like activity. Me_2SO was added to all conditions as the solvent control for LEHD-FMK. LEHD-FMK reduced caspase-3-like activity in monocytes treated with M-CSF and either PAPA or DEA NONOate. Results are expressed as means ± S.E. for four independent studies. †, $p < 0.01$ *versus* the non-stimulated control.

release of cytochrome *c*, leading to caspase-9 and caspase-3 activation (19). Other reports suggest that caspase-3 function may be impaired upon depletion of glutathione (20). Consistent with the possibility that glutathione depletion by PAPA NONOate and DEA NONOate promotes caspase-3 activity, monocytes treated with PAPA NONOate or DEA NONOate had lower concentrations of intracellular glutathione than monocytes treated with GSNO. However, replenishing glutathione stores using *N*-acetylcysteine did not reduce caspase-3 activation in monocytes stimulated with PAPA NONOate or DEA NONOate. Moreover, the addition of *N*-acetylcysteine increased the activation of caspase-3 in GSNO-treated monocytes, suggesting that replenishment of glutathione is not responsible for the observed differences in cell survival mediated by the different nitric oxide donors. To confirm that glutathione is not directly responsible for monocyte survival or apoptosis, we incubated monocytes with the nitrosothiol donor SNAP, which can *S*-nitrosylate thiol residues without donating glutathione. SNAP also promoted monocyte survival and blocked the activation of caspase-3.

We then considered the possibility that quantitative differences in nitric oxide delivered by the different donors determine cell fate and caspase-3 and caspase-9 inhibition. We found that the quantity of nitric oxide produced by GSNO, SNAP, and PAPA NONOate or DEA NONOate did not correlate with the inhibition of caspase-9 or caspase-3. To determine the mechanism of caspase-3 inhibition by GSNO or SNAP, we found that these donors blocked the production of the 17-kDa caspase-3 cleavage product in human monocytes. Because these donors also blocked caspase-9 activation in human monocytes, we speculated that nitrosothiol donors inhibit the upstream activation of caspase-3 to block monocyte apoptosis. Interestingly, using active recombinant caspase-3, we found that GSNO and SNAP also blocked enzymatically active caspase-3. We next sought to understand how these nitrosothiol donors are able to block caspase-3 activity and found that the reducing agent DTT, which can regenerate disulfide bonds in modified thiol residues, reversed the effects of these nitrosothiol donors and restored caspase-3 activity. In composite, these data argue that GSNO and SNAP reduce caspase-3 activity by both blocking the upstream activation of caspase-3 by caspase-9 and blocking the biological activity of enzymatically active caspase-3, likely through *S*-nitrosylation.

We next wanted to understand why PAPA NONOate and DEA NONOate did not promote monocyte survival, even though these donors augment the *N*-nitrosylation of proteins. We previously described that M-CSF stimulation augments the production of reactive oxygen species by human monocytes (15). We speculated that PAPA NONOate and DEA NONOate promote caspase-9 and caspase-3 activation by reducing tyrosine phosphorylation events in monocytes stimulated with M-CSF. Interestingly, we found that PAPA NONOate, DEA NONOate, and GSNO each reduced tyrosine phosphorylation events in M-CSF-stimulated monocytes, suggesting that the protective effects of GSNO are downstream of the receptor. We speculated that it is the ability of GSNO and SNAP to directly suppress caspase-3 activation that differentiates GSNO and SNAP from DEA NONOate or PAPA NONOate in promoting monocyte survival. However, it is also possible that GSNO and SNAP influence other signaling events that result in the suppression of caspase-9 or caspase-3 activation after M-CSF stimulation, and this is being actively investigated in our laboratory.

It is interesting to note that, in the absence of M-CSF, PAPA NONOate and DEA NONOate were intermittently able to reduce the activation of caspase-3. These data are very interesting and suggest either that there is some targeting of thiol residues by PAPA NONOate or DEA NONOate or that, perhaps more likely, *N*-nitrosylation events may play some role in caspase activation. We are investigating the mechanism of this observation.

These findings may have relevance to inflammatory human diseases in which nitric oxide may be produced in a localized milieu. In the presence of superoxide, nitric oxide may combine to form peroxynitrite and block tyrosine phosphorylation of proteins. In the absence of being able to suppress caspase activation, nitric oxide may lead to cellular apoptosis (21). This pathway may be important in the loss of left ventricular heart function after acute myocardial infarction, as inducible nitric-oxide synthase-deficient mice appear to be protected from cell death more than control animals (22–24). Similarly, nitric oxide has been implicated in immune destruction of islet cells in the pancreas, leading to diabetes mellitus (25–27). In contrast, nitric oxide may also directly nitrosylate protein thiol residues regulating cell survival through suppressing caspase activation, favoring cell survival (6, 28). The resulting accumulation of inflammatory cells may create a burden for the involved organ and precipitate injury. Interestingly, correlating with the important *in vitro* effects of GSNO, a GSNO reductase that is conserved from yeast to man has been identified recently (29), suggesting that GSNO likely has important biological functions *in vivo*. Thus, regulation of GSNO in mammalian cells appears to be of importance.

In summary, the data generated in this study support the hypothesis that the presentation of nitric oxide plays an important role in determining cell fate. When SNAP or GSNO was used, each of which can *S*-nitrosylate protein residues, nitric oxide inhibited the activation of caspase-9 and caspase-3 and DNA fragmentation in human monocytes. Moreover, these donors also suppressed the activity of active recombinant caspase-3. These data suggest that GSNO and SNAP can both inhibit the activation of casapse-3 and interfere with the biological activity of enzymatically active recombinant caspase-3, likely via protein *S*-nitrosylation. In contrast, the addition of PAPA NONOate or DEA NONOate, which primarily leads to *N*-nitrosylation of protein tyrosine residues in human monocytes, does not consistently reduce caspase-9 or caspase-3 activation or DNA fragmentation. These data begin to unravel the molecular details of how nitric oxide may be able to induce both cell survival and cell death. Our data suggest that the presentation of nitric oxide and the subsequent suppression of caspase-9 and caspase-3 activation determine whether nitric oxide promotes cell survival or cell death in human monocytes.

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Presentation of Nitric Oxide Regulates Monocyte Survival through Effects on Caspase-9 and Caspase-3 Activation

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