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Nitric Oxide-Induced Apoptosis in RAW 264.7 Macrophages Is Antagonized by Protein Kinase C- and Protein Kinase A-Activating Compounds

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SUMMARY

Endogenously generated or exogenously applied nitric oxide (NO) redox species induce apoptotic cell death in murine RAW 264.7 macrophages. Activation of the inducible NO synthase by incubation of cells with a combination of lipopolysaccharide and interferon-γ produced internucleosomal DNA fragmentation and morphological alterations, i.e., chromatin condensation, indicative of apoptotic cell death. These alterations, reflecting the production of NO, were prevented by an inhibitor of NO synthase, N^G-monomethyl-L-arginine. Moreover, NO derived from endogenous or exogenous sources caused accumulation of the tumor suppressor gene *p53*. Proposing a link between NO generation and DNA fragmentation, we investigated interfering biochemical signaling pathways. Therefore, we tested the ability of four NO-releasing compounds [sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1), *S*-nitroso-*N*-acetylpenicillamine (SNAP), and *S*-nitrosoglutathione (GSNO)] to cause specific DNA fragmentation. All NO donors induced DNA fragmentation in a time- and concentration-dependent manner. However, substance-specific differences became obvious. After an 8-hr incubation period, GSNO proved to be the strongest apoptotic inducer, whereas SIN-1 was much less active. Apoptosis was rapid with GSNO and SNP, yielding specific DNA fragments after 4 hr and 5 hr, respectively. In contrast, SNAP and SIN-1 produced DNA fragmentation after considerable lag times of 9 hr and 14 hr, respectively. Furthermore, an inhibitory effect of protein kinase C (PKC) and cAMP-dependent protein kinase became apparent. 12-*O*-Tetradecanoylphorbol-13-acetate, an activator of PKC, inhibited DNA fragmentation by all four NO donors, whereas PKC inhibitors such as staurosporine and calphostin C sensitized macrophages to apoptosis induced by SNP and GSNO. Lipophilic cAMP analogues suppressed SNP-, SIN-1-, and SNAP-induced DNA fragmentation. Thus, our study suggests the existence of specific down-modulatory mechanisms related to NO-induced apoptotic DNA fragmentation.

Free radical formation, such as the generation of NO or superoxide anion (O_2^-), is among the various cellular signals produced in response to diverse agonists. NO exerts a number of patho-physiological activities (see Ref. 1 for review) and is synthesized from molecular oxygen and the guanidino group of L-arginine by several isoforms of NOS (EC 1.14.13.39) (2, 3). The constitutive isoforms of NOS release small amounts of NO, accounting for endothelium-derived relaxing factor activity mediated through soluble guanylyl cyclase activation (4), followed by cGMP generation, causing downstream protein phosphorylation events. The production of large quantities of NO is coupled to macrophage activation by bacterial endotoxins like LPS and lymphokines such as IFN-γ (5). Under these conditions, NO redox species contribute to the cytostatic and cytotoxic activities of these cells during the nonspecific defense against pathogens (see Ref. 1 and references cited therein). To date, the exact mechanisms by which NO exerts its cytostatic/cytotoxic properties are largely unknown. Inhibition of iron-sulfur proteins, including the Krebs cycle aconitase, complexes I and II of the mitochondrial respiratory chain, and ribonucleotide reductase, NAD^+-dependent covalent modification of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (6), DNA-deaminating reactions (7), induction of DNA damage (8) and DNA strand breaks (9), and interactions with targets via redox and additive chemistry might be involved.

Recently, a link between NO formation and apoptosis was

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ABBREVIATIONS: NO, nitric oxide; GSNO, *S*-nitrosoglutathione; IFN-γ, interferon-γ; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NMMA, N^G-monomethyl-L-arginine; NOS, nitric oxide synthase; PKC, protein kinase C; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TBS, Tris-buffered saline; BSA, bovine serum albumin; PKA, protein kinase A; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid.

proposed (10, 11). Apoptosis, or programmed cell death, is distinguished from necrosis by several morphological characteristics. Necrosis is regarded as "accidental" cell death (see Ref. 12 and references cited therein), with early signs of cell swelling, membrane rupture, randomly digested DNA, and cell dissolution (13). In contrast, apoptosis is more selective, eliminating cells programmed for death during embryogenesis and development (14, 15). Removal of apoptotic cells or apoptotic bodies is achieved by macrophages. However, macrophages themselves are sensitive to apoptotic cell death as a result of UV irradiation, mild hyperthermia (16), gliotoxin treatment (17), and NO intoxication (10, 11). The apoptotic death process is characterized by morphological changes, i.e., nuclear and cytoplasmatic condensation, nuclear fragmentation, and apoptotic body formation (18), as well as biochemical markers, i.e., internucleosomal DNA fragmentation, known as "DNA laddering."

Expression of wild-type *p53*, a tumor suppressor gene, seems to be closely linked to several forms of apoptosis caused by DNA-damaging agents (19, 20). Accumulated wild-type *p53* acts as a checkpoint control in the cell cycle to permit the repair of damaged DNA or, in the case of severe damage, to cause apoptosis. Recently, the *p53* gene product has been shown to participate directly in the death program (21).

Although NO is known to kill macrophages by an apoptotic mechanism (10, 11), the signal transduction mechanisms, the involvement of *p53*, and the opposing intracellular pathways have not been defined. These aspects have been addressed in our study. Generation of NO causes p53 accumulation and DNA fragmentation. Initiation of apoptosis was different for several NO-releasing compounds and was negatively regulated by PKC and PKA.

Experimental Procedures

Materials. The mouse macrophage-like cell line RAW 264.7 was provided by Prof. A. Wendel, Faculty of Biology, University of Konstanz (Konstanz, Germany). SNAP was kindly provided by Dr. M. Feelisch, Schwarz Pharma AG (Monheim, Germany), whereas SIN-1 was donated by Cassella-Pharma (Frankfurt, Germany). LPS (*Escherichia coli* serotype 0127:B8), Hoechst dye 33258, NMMA, TPA, Protein A-Sepharose, cycloheximide, dibutyryl-cGMP, diphenylamine, sulfanilamide, *N*-naphthylethylenediamine dihydrochloride, and SNP were purchased from Sigma (Deisenhofen, Germany), whereas ^{125}I-Protein A (10 mCi/mg) was purchased from DuPont-New England Nuclear (Dreieich, Germany). Dibutyryl-cAMP, 8-(4-chlorophenylthio)-cAMP, recombinant murine IFN-γ, and RNase A from bovine pancreas were from Boehringer Mannheim (Mannheim, Germany). Calphostin C was purchased from Biomol (Hamburg, Germany). Kaiser's glycerol gelatin was purchased from Merck (Darmstadt, Germany). RPMI 1640 medium supplemented with 0.532 g/liter *N*-acetyl-L-alanyl-L-glutamine was obtained from Biochrom (Berlin, Germany). Cell culture supplements, fetal calf serum, and agarose were from GIBCO (Berlin, Germany). All other chemicals were of the highest grade of purity commercially available.

Cell culture. The mouse monocyte/macrophage cell line RAW 264.7 was maintained in RPMI 1640 medium (with 0.532 g/liter *N*-acetyl-L-alanyl-L-glutamine) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (complete RPMI 1640 medium). All experiments were performed using complete RPMI 1640 medium. To investigate DNA fragmentation, cells were cultured in six-well culture plates (10^6 cells/ml, 4 ml/well), allowed to adhere overnight, and stimulated with the indicated inducers or inhibitors after the medium was changed. Except for TPA and the cAMP analogues, which were applied 30 min before the NO donors, all combinations were applied simultaneously. Stimulation of endogenous NO production was achieved with a combination of LPS (10 μg/ml) and IFN-γ (100 units/ml) applied for 24 hr, using 2×10^7 cells/assay (5×10^5 cells/ml), followed by nitrite determination and DNA agarose gel electrophoresis. The NOS inhibitor NMMA (1 mM) was added simultaneously with LPS/IFN-γ.

Morphological investigations. Cells (4×10^5) were grown on glass coverslips (15-mm diameter) in 12-well plates. After adhesion, cells were stimulated for 24 hr, followed by fixation with 3% paraformaldehyde for 5 min. Samples were washed with phosphate-buffered saline, stained with Hoechst dye 33258 (8 μg/ml) for 5 min, washed with distilled water, and mounted in Kaiser's glycerol gelatin. Nuclei were visualized using a Axiovert 35 microscope (Carl Zeiss, Oberkochen, Germany) and images were obtained with a charge-coupled device camera (Kappa Messtechnik GmbH, Gleichen, Germany), with a resolution of 768×512 pixels.

GSNO synthesis. GSNO was freshly synthesized before use, as described previously (22). Glutathione was dissolved in 0.625 N HCl at 0°, to a final concentration of 625 mM. $NaNO_2$ was added at an equimolar concentration and the mixture was stirred at 0° for 40 min. After the addition of 2.5 volumes of acetone, stirring was continued for another 20 min, followed by filtration of the precipitate. GSNO was washed once with 80% acetone, two times with 100% acetone, and finally three times with diethyl ether and was then dried under vacuum. GSNO was characterized by high performance liquid chromatographic analysis and UV spectroscopy.

Quantitation of DNA fragmentation. DNA fragmentation was mainly assayed as reported previously (23). After the appropriate incubation time, cells were scraped off the culture plates using a rubber policeman, centrifuged at 1500 rpm for 10 min, resuspended in 250 μl of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE buffer), and incubated with an additional 1 volume of lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 4°. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at $13,000 \times g$. Pellets were resuspended in 500 μl of TE buffer, and samples were precipitated by addition of 500 μl of 10% trichloroacetic acid at 4°. Samples were pelleted at 4000 rpm for 10 min and the supernatant was removed. After addition of 300 μl of 5% trichloroacetic acid, samples were boiled for 15 min. DNA contents were quantitated using the diphenylamine reagent (24). The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

DNA agarose gel electrophoresis. For the preparation of DNA for agarose gel electrophoresis, cells were harvested, lysed, and centrifuged as described above, to separate DNA fragments from intact chromatin. Supernatants were precipitated with 1 ml of ice-cold ethanol and 50 μl of 5 M NaCl at $-20°$ and centrifuged again at $13,000 \times g$ for 15 min, and each pellet was incubated for 30 min at 37° in 500 μl of TE buffer supplemented with 100 μg/ml RNase A. Samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once again with an equal volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated overnight with 1 ml of ethanol and 50 μl of 5 M NaCl at $-20°$. DNA pellets were recovered by centrifugation ($13,000 \times g$ for 15 min), air dried, resuspended in 10 μl of TE buffer supplemented with 2 μl of sample buffer (0.25% bromphenol blue, 30% glyceric acid), and electrophoretically separated on a 1% agarose gel containing 1 μg/ml ethidium bromide, for 2.5 hr at 100 V. Pictures were obtained by UV transillumination.

Nitrite determination. Nitrite, a stable NO oxidation product, was determined using the Griess reaction (25). Cell-free culture supernatants were collected (200 μl), adjusted to 4°, and mixed with 20 μl of sulfanilamide (dissolved in 1.2 M HCl) and 20 μl of *N*-naphthylethylenediamine dihydrochloride. After 5 min at room temperature, the absorbance was measured at 560 nm with a reference

wavelength of 690 nm. Nitrite concentrations were calculated using a $NaNO_2$ standard.

Measurement of LDH release. The integrity of the plasma membrane was determined by measuring the LDH activity released into the culture medium. LDH activity was monitored by following the oxidation of NADH as the decrease in absorbance at 334 nm. Reactions were carried out in a potassium phosphate buffer (40 mM K_2HPO_4, 10 mM KH_2PO_4), pH 7.5, containing 0.24 mM NADH and 0.62 mM pyruvate. The percentage of LDH released was defined as the ratio of LDH activity in the supernatant to the sum of the LDH amount released plus the activity measured in the cell lysate.

p53 quantification. The amount of p53 was quantified by immunoprecipitation followed by Western blot analysis. For each assay, 2 $\times 10^7$ cells were incubated in 10-cm Petri dishes for the times indicated, scraped off with a rubber policeman, and lysed for 20 min in 700 μl of lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, pH 8.0). Lysed cells were sonicated for 10 sec using a Branson sonifier (duty cycle, 100%; output control, 1). After centrifugation for 5 min at 13,000 × *g*, nonspecific adsorbents were removed from the resulting supernatant by incubation with 40 μl of 50% (v/v) Protein A-Sepharose for 10 min at 4°, followed by centrifugation for 15 min at 13,000 × *g*. p53 was immunoprecipitated overnight at 4° by addition of 200 μl of hybridoma supernatant (clone PAb122) and 50 μl of 50% Protein A-Sepharose. Immune complexes were centrifuged at 13,000 × *g* for 60 sec and washed three times with 500 μl of SNNTE (5% sucrose, 1% Nonidet P-40, 0.5 M NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4) and one time with 1 ml of SNNTE. Finally, samples were resuspended in 40 μl of sample buffer (125 mM Tris, 2% sodium dodecyl sulfate, 10% glycerin, 1 mM dithiothreitol, 0.002% bromphenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels and blotted onto nitrocellulose sheets, using the Pharmacia semi-dry blot system (0.8 mA/cm^2, for 1.25 hr, with 25 mM Tris/192 mM glycine as the buffer system). The sheets were washed twice with TBS (140 mM NaCl, 50 mM Tris, pH 7.2) containing 0.1% Tween-20 before blocking of nonspecific binding with TBS/2% BSA for 1 hr at 20°. The p53-specific antibody (clone PAb122 hybridoma supernatant, diluted 1/6 in TBS/0.2% BSA) was added and incubated overnight at 4°. Nitrocellulose sheets were washed five times and nonspecific binding was blocked as described. For detection, blots were incubated with ^{125}I-Protein A (2 ng/ml Protein A, 1 μCi in TBS/0.06% Tween-20/0.1% BSA) for 2 hr, followed by quantitative determination of radioactivity using the PhosphorImager system (Molecular Dynamics) (26).

Statistical analyses. Statistical analyses were performed using paired Student's *t* tests.

Results

Induction of apoptotic cell death by endogenous NO generation. A combination of LPS and IFN-γ caused the expression of the inducible NOS in RAW 264.7 macrophages, as determined by nitrite accumulation in the cell supernatant. Whereas unstimulated cells produced <0.1 nmol of $NO_2^-/10^6$ cells in 24 hr (mean ± standard deviation, five experiments), nitrite accumulation in LPS/IFN-γ-treated samples amounted to 63.6 ± 3.9 nmol of $NO_2^-/10^6$ cells × 24 hr (mean ± standard deviation, five experiments). The NOS inhibitor NMMA, in the presence of LPS/IFN-γ, reduced nitrite production significantly (16.3 ± 4.2 nmol of $NO_2^-/10^6$ cells × 24 hr, mean ± standard deviation, five experiments). Internucleosomal DNA fragmentation, leading to an apoptotic DNA ladder, was detectable in cells releasing large amounts of NO (Fig. 1, *right*, *lane 2*). Suppression of the inducible NOS completely blocked DNA fragmentation, whereas NO_2^- accumulation in the cell supernatant was significantly reduced.

Investigation of cell morphology using the Hoechst dye 33258 gave a clear indication of chromatin condensation in stimulated versus control macrophages (Fig. 1, *left*, A versus B). As demonstrated for DNA laddering, addition of the NOS inhibitor NMMA completely prevented chromatin condensation (Fig. 1, *left*, C).

Induction of apoptotic DNA fragmentation by NO donors. Next, four commonly used NO donors were used to induce apoptotic cell death in RAW 264.7 cells, with internucleosomal cleavage of nuclear DNA as a reliable marker. Incubation of RAW 264.7 cells with increasing concentrations of GSNO, SNP, SNAP, or SIN-1 for 8 hr led to concentration-dependent DNA fragmentation (Fig. 2A). GSNO proved to be the strongest inducer, whereas SIN-1 was much less active. GSNO at a concentration of 250 μM induced roughly 22% DNA fragmentation, whereas 1 mM SNP, 2.5 mM SNAP, and 5 mM SIN-1 were needed to produce comparable fragmentation. GSNO was maximally active at approximately 1 mM and SNP showed most intensive fragmentation at 2 mM, whereas for SNAP and SIN-1 there was a strict concentration-dependent effect up to 5 mM concentrations of the individual NO donors. Interestingly, with higher concentrations (≥2 mM) of SNP DNA fragmentation started to decline. As shown in Fig. 2B, the pattern of DNA fragmentation elicited by the various NO donors generated the characteristic DNA ladder, confirming the results obtained with the diphenylamine reaction. Control incubations lacked accumulation of significant DNA fragments in the supernatant.

In parallel with DNA fragmentation, cell morphology was studied. All NO donors caused extensive chromatin condensation, indicative of apoptotic cell death, after an 8-hr incubation period (data not shown). Of course, morphological alterations were absent in control cells. In apoptosis, in contrast to necrosis, DNA fragmentation, nuclear condensation, and cell shrinkage precede the destruction of the plasma membrane. To confirm apoptotic cell death induced by various NO-releasing compounds, LDH activity released into the culture medium was determined. The absence of significant LDH release after an 8-hr incubation period with the NO donors (Table 1) indicates that membrane integrity was preserved. Importantly, DNA fragmentation was clearly visible under these conditions, i.e., 8-hr incubation period. As expected, at longer times the NO donors elicited significant LDH leakage. For example, SNP at 1 mM caused 49.0 ± 7.5% LDH release (mean ± standard deviation, nine experiments) after 24 hr, whereas control cells exhibited 2.9 ± 1.2% LDH release.

Additional experiments established a time dependence of NO-induced apoptotic cell death, comparing individual NO donors on a molar basis. As shown in Fig. 3, apoptosis was rapid with GSNO and SNP; specific DNA fragments were observed after 4 and 5 hr, with no further increase in fragmentation after 8 and 15 hr, respectively.

In contrast, SNAP and SIN-1 produced DNA fragmentation after considerable lag times of 9 and 14 hr, respectively. Although SNAP revealed a significant lag period, it then induced DNA fragmentation comparable to that produced by GSNO and SNP. SIN-1 at a concentration of 1 mM only slightly increased DNA fragmentation. For all NO donors, detailed kinetic studies revealed no strict correleation be-

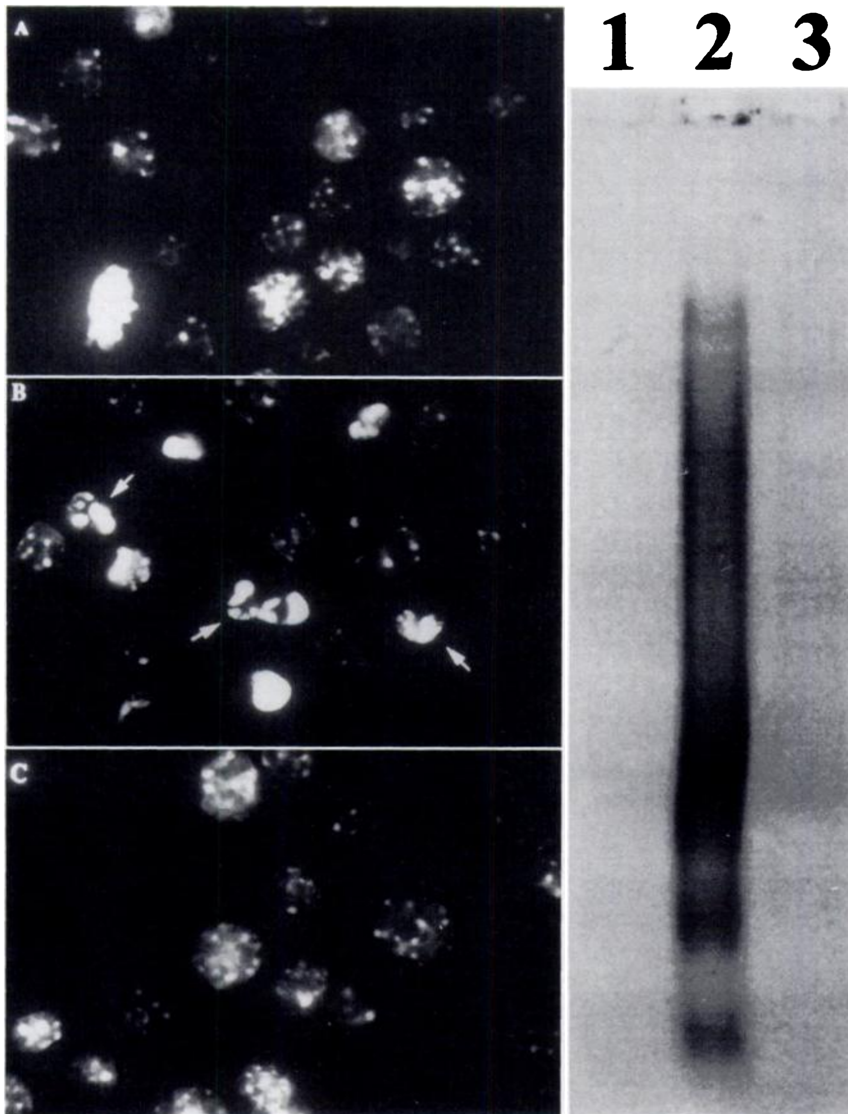

Fig. 1. Chromatin condensation and DNA fragmentation in RAW 264.7 macrophages, induced by endogenous NO production. *Left*, cells were cultured, incubated for 24 hr without further addition (A), with 10 μg/ml LPS and 100 units/ml IFN-γ (B), or with 10 μg/ml LPS, 100 units/ml IFN-γ, and 1 mM NMMA (C), fixed, and stained using the DNA-specific fluorochrome Hoechst dye 33258, as outlined in Experimental Procedures. A and C, Control cells and stimulated cells incubated with the NOS inhibitor NMMA exhibited a normal nuclear morphology characterized by a diffuse chromatin structure and, therefore, only weak fluorescent staining. Chromatin condensation present in the control incubation indicates cells in the mitotic phase of the cell cycle. B, About 50% of the stimulated cells showed chromatin condensation and nuclear fragmentation; some characteristic cells are marked (*arrows*). *Right*, cells (2 × 10^7) were cultured for 24 hr as described in Experimental Procedures and incubated without further addition (*lane 1*), with 10 μg/ml LPS and 100 units/ml IFN-γ (*lane 2*), or with 10 μg/ml LPS, 100 units/ml IFN-γ, and 1 mM NMMA (*lane 3*). An ethidium bromide-stained agarose gel is shown. Results are representative of three similar experiments.

tween NO_2^- generation and donor ability to induce DNA fragmentation.

SIN-1 simultanously forms O_2^- and NO during decomposition (27), thereby producing peroxynitrite ($OONO^-$) (28). Peroxynitrite by itself is thought to account for several biological effects of NO. Therefore, we probed for O_2^- generation from SIN-1 by incubating RAW 264.7 macrophages in the presence of up to 2000 units/ml superoxide dismutase and 500 units/ml catalase. There was no alteration of the amount of DNA fragmentation induced by SIN-1 under these conditions (data not shown).

p53 accumulation in response to NO. Incubation of RAW 264.7 macrophages with a combination of LPS and IFN-γ for 15 hr resulted in a dramatic accumulation of the tumor suppresssor p53, as detected by immunoprecipitation of the protein followed by Western blot analysis (Fig. 4). In control cells, little p53 was detectable. Accumulation of p53 in response to LPS/IFN-γ and concomitant NO generation were suppressed by NMMA. Similarly to endogenously generated NO, exogenously applied NO caused massive p53 accumulation in response to 1 mM GSNO and 1 mM SNP, compared with controls (Fig. 4). The response was less pronounced with SNAP (measured after 4, 6, and 8 hr) and was virtually absent with SIN-1 (data not shown).

Intracellular signaling pathways involved in NO-induced DNA fragmentation. Probing for the involvement of cGMP in NO-induced apoptosis, our experiments were negative. SNP (1 mM) produced 22.3 ± 3.4% DNA fragmentation (mean ± standard deviation, three experiments, 8-hr incubation), whereas in combination with 2.5 mM dibutyryl-cGMP similar results were observed (20.5 ± 8.2% DNA fragmentation, mean ± standard deviation, three experiments, 8-hr incubation). Moreover, cell-permeable cGMP analogues produced no apoptosis.

Additional experiments characterized signaling components possibly involved in DNA fragmentation induced by chemically generated NO. We examined the effect of cycloheximide, an inhibitor of protein biosynthesis, on SNP-induced DNA fragmentation. At low cycloheximide concentrations (1 μM), SNP (1 mM)-induced DNA fragmentation was not affected (23.2 ± 2.7% DNA fragmentation, mean ± standard deviation, three experiments). Whereas cycloheximide alone at concentrations of 1 μM induced only minor DNA fragmentation (7.7 ± 2.8% DNA fragmentation, mean ±

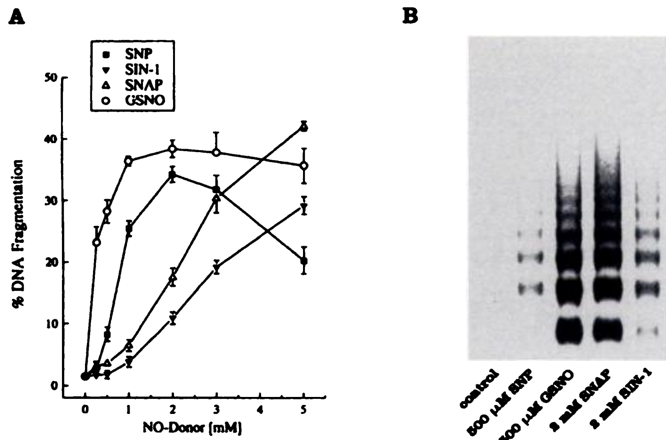

Fig. 2. Dose-dependent DNA fragmentation in RAW 264.7 macrophages, induced by various NO donors. Cells [4 × 10^6 cells (A) or 8 × 10^6 cells (B)] were cultured as outlined in Experimental Procedures and incubated for 8 hr with the NO donors indicated, followed by DNA extraction as described. A, Cells were incubated with different concentrations of SNP, SIN-1, SNAP, or GSNO, and DNA fragmentation was quantitated using the diphenylamine reaction. Values are means ± standard errors of four individual experiments. B, An ethidium bromide-stained agarose gel shows internucleosomal DNA fragmentation in cells incubated without (control) and with the NO donors indicated. The gel is typical of three independent experiments.

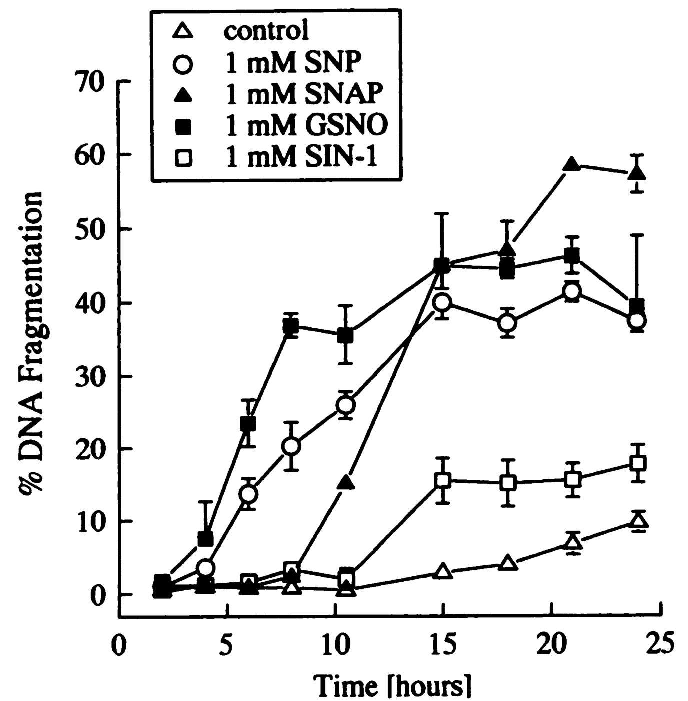

Fig. 3. Time-dependent DNA fragmentation in RAW 264.7 macrophages, induced by NO-releasing compounds. Cells were cultured as described in Experimental Procedures and incubated without a NO donor (control) or with SNP, SIN-1, SNAP, or GSNO, as indicated. DNA fragmentation was determined using the diphenylamine reaction. Values are means ± standard errors of four individual experiments.

standard deviation, four experiments), DNA breakdown reached significantly higher values (21.5 ± 1.8% DNA fragmentation, mean ± standard deviation, three experiments, 8-hr incubation) with elevated concentrations of 10 µM cycloheximide. Interestingly, actinomycin D at concentrations of 2 µg/ml also led to the onset of apoptosis (data not shown). Furthermore, the role of Ca^{2+} as a prerequisite for NO-induced DNA fragmentation was determined. Depletion of intracellular Ca^{2+} by incubation of macrophages with 20 µM BAPTA/acetoxymethyl ester or chelation of extracellular Ca^{2+} by addition of 5 mM EGTA did not produce any inhibitory effect on SNP-induced DNA fragmentation. Unexpectedly, BAPTA/acetoxymethyl ester and EGTA by themselves induced significant apoptotic DNA fragmentation (data not shown). Because it is known that Zn^{2+} inhibits the endonuclease responsible for internucleosomal DNA fragmentation in some cells (29), we investigated the inhibitory effects of Zn^{2+}. Zn^{2+} at 500 µM negatively interfered with the SNP (1 mM)-induced apoptotic process in RAW 264.7 macrophages (Fig. 5D). The chosen concentrations of $ZnCl_2$ were nontoxic to RAW 264.7 macrophages, as established by the absence of LDH release and judged by cell morphology (trypan blue exclusion assay) (data not shown).

Inhibition of DNA fragmentation by PKC and PKA. Modulation of apoptosis by PKC and PKA was studied in the following experiments. TPA, a commonly used PKC-activating agent, suppressed DNA fragmentation induced by SIN-1, SNP, GSNO, or SNAP (Figs. 5A and 6). Prolonged incubation of cells with TPA or high concentrations of other phorbol esters is known to down-regulate certain PKC isoenzymes. Therefore, it was of interest to determine whether the inhibitory effect of TPA at 100 nM is based on PKC activation or, alternatively, on PKC down-regulation. Therefore, we preincubated RAW 264.7 macrophages for 24 hr with 500 nM TPA; this was followed by another 8-hr stimulation with TPA in the presence of NO-releasing compounds. Under these conditions, we observed only a marginal inhibitory effect of TPA

TABLE 1

LDH release by RAW 264.7 macrophages incubated for 8 hr with different NO donors

Cells were cultured and LDH activity was determined as outlined in Experimental Procedures. Values are means ± standard deviations of three individual experiments, performed in triplicate. Values are not statistically different ($p > 0.05$).

	LDH release			
	SNP	SIN-1	SNAP	GSNO
	%			
Control	1.72 ± 1.11			
250 µM	2.40 ± 1.96	4.36 ± 3.31	4.04 ± 3.59	3.69 ± 1.57
500 µM	3.38 ± 3.24	3.27 ± 1.26	1.82 ± 1.20	3.19 ± 1.48
1 mM	3.89 ± 2.24	4.55 ± 3.90	1.29 ± 0.76	5.07 ± 2.52
2 mM	3.38 ± 1.54	0.48 ± 0.73	1.07 ± 1.10	3.77 ± 2.53
3 mM	5.43 ± 3.34	0.94 ± 1.41	1.25 ± 1.43	6.27 ± 5.52
5 mM	6.12 ± 3.40	2.01 ± 2.28	1.89 ± 4.14	8.13 ± 8.39

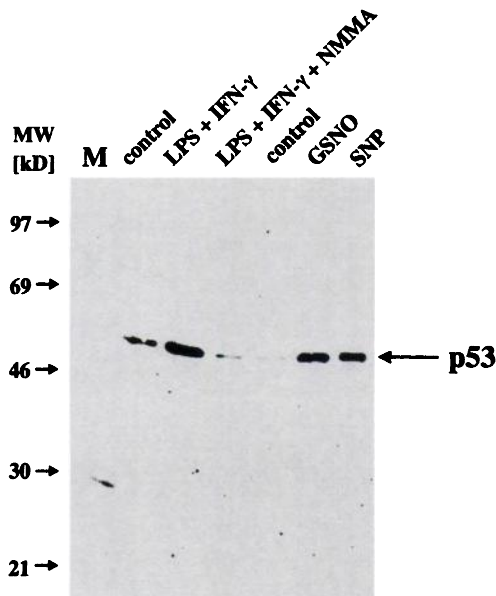

Fig. 4. NO-induced p53 accumulation in RAW 264.7 macrophages. Cells (2×10^7) either were incubated for 15 hr with 10 μg/ml LPS and 100 units/ml IFN-γ or with LPS/IFN-γ in the presence of 1 mM NMMA or remained as controls. Incubations with 1 mM GSNO and 1 mM SNP were for 4 hr. p53 was immunoprecipitated using the monoclonal antibody PAb122, followed by Western blot analysis as described in Experimental Procedures. *M*, ^{14}C-labeled molecular weight markers. The blot is representative of three similar experiments.

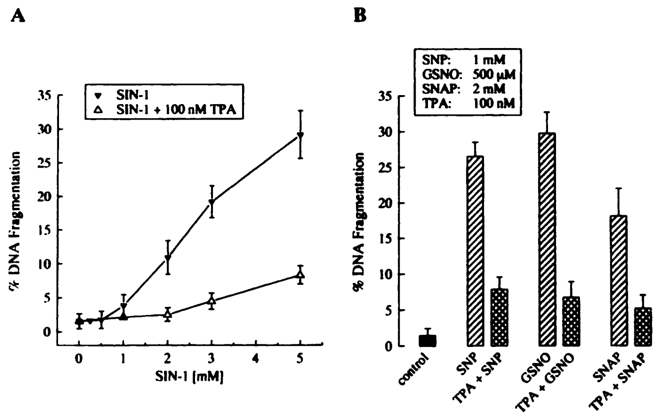

Fig. 6. Antagonism by the PKC activator TPA of DNA fragmentation in RAW 264.7 cells. RAW 264.7 cells were cultured as described in Experimental Procedures and incubated for 8 hr with different NO-releasing compounds. TPA (100 nM) was added 30 min before the addition of the NO donors. The values are means ± standard deviations of three individual experiments.

(data not shown), suggesting that TPA mainly activates PKC, rather than producing down-regulation. In line with these experiments, PKC inhibitors such as staurosporine and calphostin C sensitized macrophages to NO-induced apoptosis. As presented in Fig. 7, staurosporine alone, at a concentration of 40 nM, induced some DNA fragmentation, compared with controls. Coincubation of the cells with staurosporine and SNP or GSNO resulted in increased DNA fragmentation in each case. Because staurosporine is considered a nonspecific PKC inhibitor, we also used calphostin C, a more specific PKC inhibitor (IC_{50} of 0.05 μM, compared with >50 μM for PKA and >20 μM for protein kinase G) (30). Fig. 5B indicates that 0.25 μM calphostin C sensitized macrophages to SNP-induced DNA fragmentation in a manner similar to that of staurosporine. Furthermore, we focused on cAMP as a potential modulator of NO-mediated signaling pathways. Figs. 5C and 8 address the question of whether lipophilic cAMP analogues interfere with SNP-induced DNA fragmentation. Two membrane-permeable cAMP analogues, i.e., 8-(4-chlorophenylthio)-cAMP and dibutyryl-cAMP, dose-dependently decreased NO-induced DNA cleavage.

Even at a rather low dose (100 μM), 8-(4-chlorophenylthio)-cAMP reduced SNP-induced DNA fragmentation to values below 50%, compared with controls. Similar results were obtained with SNAP or SIN-1 and PKA-activating compounds. These results show an inhibitory effect of cAMP-elevating agents on NO-induced DNA fragmentation in RAW 264.7 macrophages. Lipophilic cAMP analogues alone produced no apoptotic response.

Discussion

NO-stimulated apoptosis. NO causes peritoneal macrophage cell death (10, 11). We established a similar scenario for RAW 264.7 macrophages stimulated with LPS/IFN-γ. In our system, morphological parameters, i.e., chromatin condensation, and biochemical markers, i.e., DNA ladder forma-

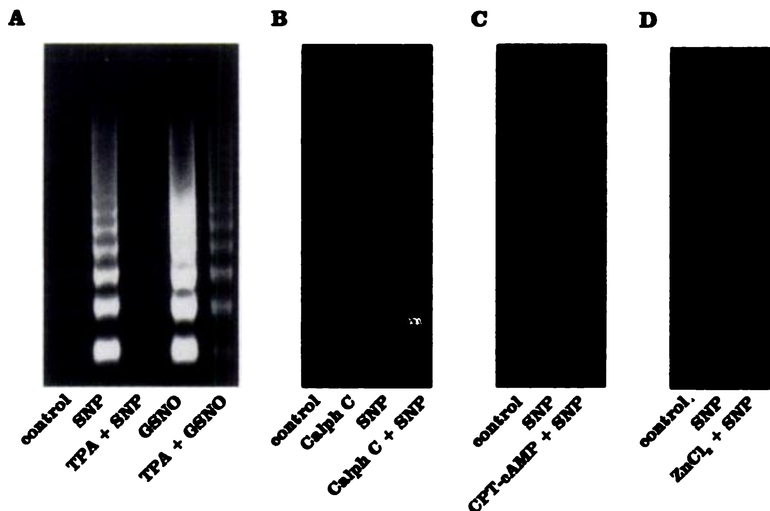

Fig. 5. Modulation of NO-induced DNA fragmentation in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated for 8 hr with 1 mM SNP, 500 μM GSNO, and/or 100 nM TPA (A), 0.25 μM calphostin C (*Calph C*) and/or 400 μM SNP (B), 1 mM SNP and 500 μM 8-(4-chlorophenylthio)-cAMP (*CPT-cAMP*) (C), 1 mM SNP and 500 μM $ZnCl_2$ (D), or no additions (control). TPA and 8-(4-chlorophenylthio)-cAMP were added 30 min before the NO donor. All other combinations were added simultaneously. Preparation of DNA and agarose gel electrophoresis were performed as described in Experimental Procedures. The gels are representative of three individual experiments.

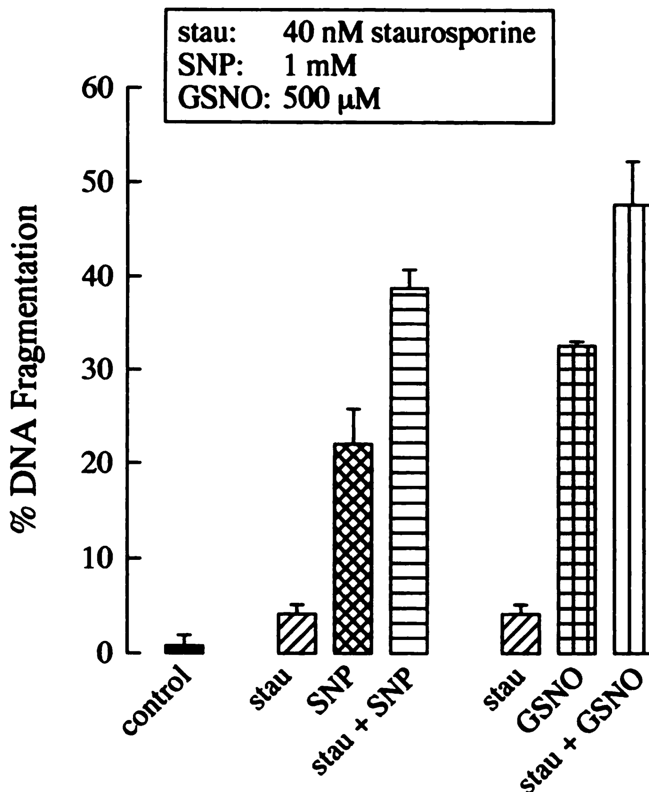

Fig. 7. Effects of staurosporine on SNP- and GSNO-induced DNA fragmentation in macrophages. RAW 264.7 cells were cultured as outlined in Experimental Procedures and incubated for 8 hr without any addition (control) or with 1 mM SNP, 500 μM GSNO, and/or 40 nM staurosporine. DNA fragmentation was quantitated using the diphenylamine reaction. Values are means ± standard deviations of four individual experiments.

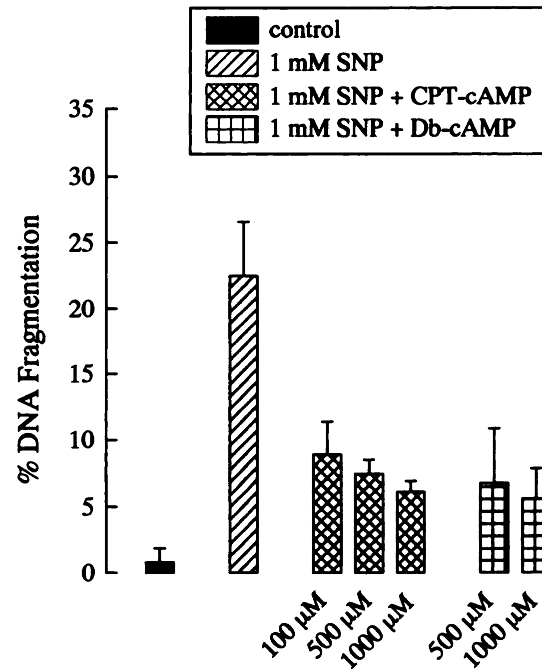

Fig. 8. Prevention by cAMP analogues of SNP-induced DNA fragmentation in RAW 264.7 macrophages. Cells were incubated for 8 hr with 1 mM SNP, 8-(4-chlorophenylthio)-cAMP (*CPT-cAMP*), and/or N^6,2′-*O*-dibutyryl-cAMP (*Db-cAMP*) at the indicated concentrations. cAMP analogues were added 30 min before SNP. Controls were without any addition. Culture conditions and quantitation of DNA fragmentation were as in Fig. 1. Values are means ± standard deviations of three or four separate experiments.

tion, demonstrated the characteristics of an apoptotic process. Stimulated macrophages express a high level of inducible NOS and produce NO from L-arginine, which is detected as nitrite. Because macrophages underwent internucleosomal DNA fragmentation, endogenous NO production is clearly sufficient to induce this type of cell death. Apoptotic alterations typified by DNA degradation and cell morphology were absent when NOS activity was blocked through the addition of NMMA, establishing a definite link between endogenously produced NO and cell destruction.

Thus, the role of modulation of the apoptotic response during NO-induced cell death was a central issue of our experiments, because intracellular pathways promoting or antagonizing NO-mediated cell killing have not been defined. To exclude possible interference with NOS induction in these experiments, we examined the ability of four different NO-generating compounds, i.e., GSNO, SNP, SNAP, and SIN-1, to induce apoptotic RAW 264.7 cell death (Fig. 2). The induction of apoptosis versus necrosis was supported by the absence of significant LDH release but the occurrence of morphological and biochemical apoptotic markers. Interestingly, we observed differences in the concentrations needed and the time required for NO donors to induce apoptotis (Fig. 2B). Among the NO donors used, SIN-1 alone generates significant amounts of oxygen free radicals (27) and NO, resulting in a diffusion-controlled generation of peroxynitrite (28). As shown previously, incubation of rat cerebrocortical cultures with SIN-1 induces neuronal cell death through the formation of peroxynitrite, i.e., cells were completely protected by exogenously added superoxide dismutase (31). Although the potential for peroxynitrite to kill cells exists, this seems not to be the case in our system. Firstly, SIN-1, producing peroxynitrite, is a weak apoptotic inducer. Secondly, in agreement with reported experiments (31), addition of exogenous superoxide dismutase and catalase did not alter SIN-1-mediated killing. Following the suggested argumentation, i.e., prevention of peroxynitrite formation by exogenous superoxide dismutase, our results would rule out any participation of the primary reaction product formed by the combination of O_2^- and NO.

The reactivity of NO in cellular systems is not fully understood, and several factors can be proposed to explain variations in the potency of NO donors. Under physiological conditions NO can be interconverted among different redox forms with distinct chemistries (32). Individual reactions of nitrogen monoxide (NO·), nitrosonium ion (NO^+), and nitroxyl anion (NO^-) in relation to their relative abundance could explain varied biological responses related to the generation of one or more reactive and potentially toxic nitrogen oxides (NO_x). The primary products of the reaction of cellular constituents with the intermediates of the NO/O_2 reactions are *S*-nitrosothiol adducts (33). Considering GSNO and SNP, the most potent apoptotic inducers, as potential NO^+-delivering compounds, one might assume a role of nitrosonium ion and probably *S*-nitrosylation reactions in apoptosis. One even can consider SIN-1 as a weak NO^+ donor in the presence of thiols (34), an observation in line with its weak DNA-damaging ability. It has been demonstrated that cys-

teine residues have an affinity for the NO_x species that is 3 orders of magnitute greater than that of the nonsulfhydryl amino acids and $>10^6$ times greater than that of the exocyclic amino groups of the DNA bases (33). Therefore, one might predict formation of *S*-nitrosothiol adducts of thiol-containing enzymes that are especially vulnerable to NO_x species being formed during apoptosis. Rates of decomposition of exogenously applied NO donors, donor transport, *S*-nitrosothiol formation, donor and *S*-nitrosothiol stability, and decrease of the critical, intracellular, steady state concentration of *S*-nitrosothiols via breakdown may determine the apoptosis-inducing ability of the NO donors. Not surprisingly, there was no direct correlation between NO_2^- accumulation and the death-eliciting potency. Nitrite, a final NO oxidation product, cannot be correlated with NO·, NO_x, or *S*-nitrosothiol formation possibly involved in apoptotic signaling.

Investigation of the potency of various NO donors in another well established assay system, i.e., activation of soluble guanylyl cyclase associated with inhibition of platelet aggregation or smooth muscle relaxation, revealed similar variations (35). In those studies, all NO-releasing compounds showed considerable variations in their chemical stabilities, abilities to release an NO-related redox species, and potencies to elicit soluble guanylyl cyclase activation. Even the rank order of potency for a single NO donor to stimulate cGMP accumulation differed among various cells or tissues examined.

The ability of NO to influence various signal-transducing mechanisms has recently been demonstrated for individual enzymes (36, 37). However, because lipophilic cGMP analogues did not affect DNA fragmentation, soluble guanylyl cyclase and the cGMP cascade seem not involved. The challenging question will be to evaluate apoptotic signaling pathways used by individual NO donors and various NO redox species.

Our observation that generation of NO causes accumulation of p53 may indicate a relevant signaling step promoting apoptosis in RAW 264.7 cells. Apparently, NO can cause DNA damage (7) and the activation of poly(ADP-ribose) synthetase (38). Consequently, increased p53 levels either may allow DNA repair or may take part in the transducing mechanism leading to apoptosis, either by acting directly on the DNA or by causing a cell cycle G_1 arrest. Endogenously derived NO or exogenously supplied GSNO or SNP is sufficient to cause p53 accumulation. This correlates with the apoptosis-inducing potency of the NO donors. The weaker response with SNAP and the negative signal caused by SIN-1 needs clarification but may point to the involvement of different signaling pathways. Detailed studies are underway to establish p53 accumulation before the appearance of apoptotic markers and to examine the effect of suppressive mechanisms on the level of p53.

Inhibitory pathways in NO-stimulated apoptosis. As with several other systems (39), NO-induced apoptosis in RAW 264.7 cells is largely Ca^{2+} independent but was found to be suppressed by Zn^{2+} (Fig. 5D). Protection was observed at 500 μM, a concentration not toxic to RAW 264.7 macrophages during an 8-hr incubation period. In agreement are reports suggesting that prevention of apoptosis is a function of labile Zn^{2+} and that reduction of this Zn^{2+} pool below a threshold concentration induces apoptosis. The unexpected finding that Ca^{2+} chelators induce DNA fragmentation may also result from simultanous chelation of Zn^{2+} (40). In murine macrophages, cycloheximide and actinomycin D alone induced some DNA fragmentation (17). Our results corroborated those reports, leaving the requirement for protein biosynthesis in this case unanswered.

NO-induced apoptosis is inhibited by PKC and PKA (Figs. 5, A and C, 6, and 8). The role of PKC is controversial. For example, PKC activation inhibits DNA fragmentation in thymocytes (41) and radiation-treated mouse fibroblasts (42) and counteracts DNA fragmentation in U-937 monoblastic leukemia cells induced by ceramide (43). In contrast, it also promotes thymocyte apoptotic death (44). NO-induced DNA fragmentation was clearly suppressed by PKC activation, rather than PKC down-regulation. This is supported by experiments using PKC inhibitors like staurosporine and calphostin C to sensitize macrophages to SNP- and GSNO-induced apoptosis. Considering tumor promoters like TPA as inhibitors of apoptosis (45), interference with the macrophage NO system seems possible. Assuming macrophage NO production as one mechanism of the nonspecific immune response directed against tumor cells, inducing tumor regression, inhibition of NO-induced apoptosis in target cells by PKC activation can be envisioned. Comparably to PKC activation, lipophilic cAMP analogues down-regulate NO-induced cell death. The action of cAMP-elevating agents in suppressing apoptosis is known for other systems as well (46). Under inflammatory conditions, elevated levels of prostaglandin E_2 acting via adenylyl cyclase in autocrine and paracrine fashions are rationalized. As a result, elevated cAMP levels in macrophages may suppress NO-induced apoptosis. LPS and cytokines are known as cyclooxygenase-2- and NOS-inducing agents. Therefore, the balance between apoptosis-promoting and -opposing signals may determine NO toxicity and may regulate the susceptibility of different cells to NO-mediated apoptosis. Macrophage NO, with its cytotoxic activity against pathogens, affects macrophages in a self-destructing loop as well. This process may eliminate activated macrophages by an apoptotic process after massive stimulation and may down-regulate macrophage activity at sites of prolonged elevated NO production.

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