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NO-Evoked Macrophage Apoptosis Is Attenuated by cAMP-Induced Gene Expression

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Abstract

Background: Previous work has suggested that an increase in expression of cyclooxygenase-2, concomitant formation of E-type prostanoids, and in turn intracellular cAMP conveys macrophage resistance against apoptosis.
Materials and Methods: We analyzed the effects of lipophilic cAMP analogs on nitric oxide (NO)–induced apoptosis in RAW 264.7 macrophages and human primary monocyte-derived macrophages. Parameters comprised DNA fragmentation (diphenylamine assay), annexin V staining of phosphatidylserine, caspase activity (quantitated by the cleavage of a fluorogenic caspase-3-like substrate Ac-DEVD-AMC), and mitochondrial membrane depolarization ($\Delta\Psi$), analyzed using $DiOC_6(3)$. Western blots detected accumulation of the tumor suppressor protein p53, relocation of cytochrome c, and expression of the antiapoptotic protein Bcl-X_L. A cAMP response-element decoy approach confirmed cAMP-dependent gene induction.
Results: We verified resistance of murine and human macrophages against NO donors such as *S*-nitrosoglutathione or spermine-NO by pre-exposing cells to lipophilic cAMP analogs or by pretreatment with lipopolysaccaride, interferon-γ, and N^G-nitroarginine-methyl-ester for 15 hr. Cellular prestimulation decreased NO-evoked apoptosis, as apoptotic parameters were basically absent. Macrophage protection was not achieved during a short period of preexposure, i.e., 1 hr. To verify gene induction as the underlying protective principle, we treated RAW cells with oligonucleotides containing a cAMP-responsive element in order to scavenge cAMP response element–binding protein prior to its promoter-activating ability. Decoy oligonucleotides, but not an unrelated control oligonucleotide, weakened cAMP-evoked protection and re-established a p53 response following NO addition.
Conclusion: Gene induction by cAMP protects macrophages against apoptosis that occurs as a result of excessive NO formation. Decreasing programmed cell death of macrophages may perpetuate inflammatory conditions in humans when macrophages become activated in close association with innate immune responses.

Introduction

Cyclooxygenase-2 (Cox-2), also known as prostaglandin (PG) H synthase, catalyzes the rate-limiting steps in the formation of prostaglandin endoperoxides (1). Prostanoids function as extracellular or intracellular messengers (1,2), and classic examples, such as $PGF_{2\alpha}$ or PGE_2, activate adenylyl cyclase through interaction with plasma membrane G protein–coupled receptors (3), thereby causing an increase in cyclic adenosine monophosphate (cAMP).

Activation of macrophages is often associated with Cox-2 expression (4–7). To achieve macrophage activation, a standard protocol of lipopolysaccharide/interferon-γ (LPS/IFN-γ) stimulation is carried out, which in turn leads to Cox-2 expression and prostaglandin formation (PGE_2), followed by a cAMP increase. Recently, we showed that stimulation of macrophages with LPS/IFN-γ while

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blocking nitric oxide (NO)-synthase confers resistance to a cytotoxic response initiated by exogenously added NO donors (8). Cox-2 expression was determined a necessary component in the signaling cascade leading to this protection by reducing resistance by the Cox-2-specific inhibitor NS-398 and by Cox-2 antisense transfection (9).

Overexpression of Cox-2 in several pathological conditions such as colon carcinoma or rheumatoid arthritis points to a causative role of Cox-2 in tumor initiation and/or promotion. Furthermore, in line, the chronic use of nonsteroidal anti-inflammatory drugs such as aspirin reduces the incidence of colon cancer (10) and use of anti-inflammatory drugs such as sulindac abrogate tumorigenesis in experimental murine models of colon cancer (11). Often, tumor prostanoid synthesis is enhanced, and the growth of some established tumor cell lines is inhibited in vitro and in athymic mice by Cox-2 inhibitors. Cox-2 knockout experiments provide further evidence for the requirement of Cox-2 in the development of some cancers, e.g., colon cancer (12–15). One mechanism for tumor development under conditions of Cox-2 overexpression may stem from inhibition of apoptosis as shown in gastrointestinal epithelial cells (16). However, the signal-transducing pathways that convey Cox-2-evoked cell protection and/or decrease apoptosis remain elusive. Since attenuated macrophage apoptosis may contribute to inflammatory conditions such as sepsis or atherosclerosis, it is pertinent to gain insights into underlying signaling mechanisms.

We sought to elucidate how lipophilic cAMP analogs decrease NO-elicited apoptosis. The aim of this study was to determine whether protective principles operate in both murine and human macrophages and to establish pro-apoptotic parameters relative to the interference evoked by a cAMP response. Our experiments suggest that cAMP-mediated gene activation in murine and human macrophages is the predominant underlying protective principle.

Materials and Methods

Materials

Diphenylamine, bovine serum albumin (BSA), and LPS (*E. coli* serotype 0127:B8) were purchased from Sigma (Deisenhofen, Germany). L-N^G-nitroarginine methyl ester (L-NAME) was from Alexis (Grünberg, Germany). Recombinant murine IFN-γ, 8-bromo-cAMP (8-Br-cAMP), and dibutyryl-cAMP (db-cAMP) were provided by Boehringer Mannheim (Mannheim, Germany). RPMI 1640, cell culture supplements, and fetal calf serum (FCS) were ordered from Biochrom (Berlin, Germany). *N*-acetyl-Asp-Glu-Val-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was bought from Biomol (Hamburg, Germany), and spermine-NONOate (Sp-NO) was obtained from Bio-Trend (Köln, Germany). The p53-antibody (PAb248) was kindly provided by H. Stahl, Homburg/Saar, Germany. All other chemicals were of the highest grade of purity and are commercially available.

Cell Culture

The mouse monocyte/macrophage cell line RAW 264.7 was maintained in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (complete RPMI). All experiments were performed with complete RPMI. NO donors, 8-Br-cAMP, and db-cAMP were dissolved in twice-distilled water. Appropriate vehicle controls were performed.

Monocyte Isolation and Culture

Cells were isolated from 50 ml buffy coats (Blutbank Erlangen, Erlangen, Germany). Blood was diluted 1:2 with phosphate-buffered saline (PBS) and layered on a Ficoll-Isopaque-gradient (p = 1077 g/ml). The interphase containing peripheral blood mononuclear cells (PBMC) was obtained following centrifugation (800 $\times$ g, 20 min). Cells were recovered and washed twice in PBS, and monocytes were isolated using magnetic cell sorting (MACS) technology (Miltenyi Biotec, Bergisch Gladbach, Germany) as described by the supplier to deplete $CD14^-$ cells. In brief, 10^7 total PBMC were resuspended in 60 μl PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin (PBS/E/B). Then 20 μl FcR blocking reagent was added, followed by supplementation with 20 μl hapten-antibody cocktail (composed of CD3, CD7, CD19, CD45RA, CD56, and anti-IgE antibodies) per 10^7 cells, which was mixed and incubated for 5 min on ice. After washing twice with 20$\times$ PBS/E/B, cells were resuspended in 60 μl PBS/E/B and 20 μl FcR blocking reagent (per 10^7 cells), followed by addition of 20 μl MACS Anti-Hapten MicroBeads. Cells were mixed, incubated for 15 min on ice, washed with 20$\times$ PBS/E/B, and resuspended in 500 μl PBS/E/B per 10^8 cells. The

LS^+ column was placed in the magnetic field of a MidiMACS separator and washed with 3 ml PBS/E/B prior to addition of the cells; unlabeled cells passed through. Effluent was collected as the negative fraction that contains the enriched monocyte fraction. Flow cytometry confirmed that this population was 90–95% pure ($CD14^+$ vs. $CD14^-$) (17,18). Cells were grown in Teflon bags (Süd-Laborbedarf GmbH, Gauting, Germany) in RPMI 1640 containing 10% heat-inactivated human antibody serum (Sigma) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). After 6 days of culture, monocytes acquired a macrophage-like phenotype (19) and were used for the experiments. Teflon bags were used to culture the monocyte/macrophage population to avoid adherence (20).

Quantitation of DNA Fragmentation

DNA fragmentation was measured by the diphenylamine assay as previously reported (21). Briefly, following incubations, cells were scraped off the culture plates, resuspended in 250 μl 10 mM Tris, 1 mM EDTA, pH 8.0 (TE buffer), and incubated with an additional volume of lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 4°C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 13000 × *g*. Pellets were resuspended in 500 μl TE buffer and samples were precipitated by adding 500 μl 10% trichloroacetic acid at 4°C. Samples were pelleted at 1400 × g 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 (22). The percentage of DNA fragmentation was calculated as the ratio of DNA content in the supernatant to the amount in the pellet.

Flow Cytometric Analysis of Annexin V Staining

Apoptosis in primary human monocytes/macrophages that is accompanied by phosphatidylserine exposure was analyzed by incubating cells with fluorescein isothiocyanate–labeled annexin V (Beckman Coulter Immunotech, Krefeld, Germany) as described previously (23,24) using an annexin V detection kit (Beckman Coulter Immunotech). At least 10,000 cells were accumulated for analysis.

S-nitrosoglutathione Synthesis (GNSO)

GNSO was synthesized and characterized as previously described (25).

Cytochrome c Release

Mitochondrial cytochrome c release was determined by Western blot analysis of cytosolic cytochrome c, essentially as described previously (26). Following individual incubations, cells were collected and centrifuged at 700 × g for 3 min. Cell pellets were resuspended in 250 μl PBS at room temperature, followed by addition of 250 μl digitonin sucrose solution (150 μM digitonin, 500 mM sucrose) for 30 sec and subsequent centrifugation (10,000 × g for 1 min). Cytosolic supernatants were transferred to tubes and analyzed for protein content. Then 40 μg of protein from each sample was subjected to 12.5% SDS-PAGE and Western blot analysis using the anti-cytochrome c monoclonal antibody (MAb) 7H8.2C12 (PharMingen Europe, Hamburg, Germany) and enhanced chemiluminescence (ECL). To confirm integrity of the outer mitochondrial membrane after digitonin permeabilization, control experiments were performed. Treatment of nonapoptotic cells with the digitonin sucrose solution for up to 10 min did not promote any cytochrome c release into the supernatant.

Measurement of Mitochondrial Membrane Potential ($\Delta\psi$)

Following individual incubations, cells were loaded with 40 nM of the fluorochrome 3,3′-dihexyloxacarbocyanide iodide ($DiOC_6(3)$) (Molecular Probes, Leiden, The Netherlands) for 10 min, after which the dye is accumulated in mitochondria that contain an intact membrane potential (27). After agonist treatment, $\Delta\psi$ was measured on a Coulter Epics XL flow cytometer. At least 10,000 cells were accumulated for analysis. Results are given here in percent of total cells with decreased $\Delta\psi$.

Measurement of Lactate Dehydrogenase (LDH) Release

The integrity of the plasma membrane was determined by measuring LDH activity released into the culture medium. LDH activity was monitored 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 percent 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.

Determination of Fluorogenic Caspase-3-like Activity

Cells (2×10^6) were incubated as indicated, recovered from cultured plates, and centrifuged (1200 × g, 4°C, 5 min). Cell pellets were resuspended in lysis buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mM EDTA) and left on ice for 30 min. Following sonification (Branson sonifier, 10 sec, duty cycle 100%, output control 1) and centrifugation (10,000 × g, 10 min, 4°C), protein was determined with the direct current (DC) protein assay. Cell supernatants (30 μg protein) were incubated in 100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml leupeptin at 30°C with 12 μM of the caspase-3-like substrate Ac-DEVD-AMC. Substrate cleavage and accumulation of AMC was followed fluorometrically with excitation at 360 nm and emission at 460 nm during a 120-min incubation period. Substrate cleavage during the linear phase of the reaction was quantitated by internal AMC standards. Enzyme activity was expressed as nM AMC/min/mg protein (nM/min × mg).

Transfection

RAW 264.7 cells were transiently transfected by exposition to cAMP responsive element (CRE) oligonucleotides, containing the CRE consensus site derived from the c-*fos* promoter (28), and to an unrelated oligonucleotide as a control. The 5′-terminal fluorescein-labeled oligonucleotides were stabilized by phosphothiorate linkages (Eurogentec, Seraing, Belgium). Following is the CRE sequence used (consensus sequence underlined):

5′-G AT ATT GCC TGA CGT CAG AG-3′
3′-TAA CGG ACT GCA GTC TCT CG-5′

One day before transfection, cells were seeded at a density of 1×10^6 cells/well in 6-well plates. Oligonucleotides (3 μM) were added 24 hr prior to cell stimulation. After changing the medium, cell stimulation was performed as indicated. Transfection efficiency was determined by counting labeled cells by fluorescence microscopy.

Western Blot Analysis

Cell lysis was achieved with lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM phenylsulfonylmethylfluorid, pH 8.0) and sonication (Branson sonifier; 20 sec, duty cycle 100%, output control 60%), followed by centrifugation (10,000 × g, 5 min), and protein determination was made with the Lowry method (29). Protein (150 μg) was resolved on 10% polyacrylamide gels and blotted onto nitrocellulose sheets following standard methodology (30). Equal loading and correct protein transfer to nitrocellulose were routinely quantitated by Ponceau S staining. Filters were incubated with the anti-p53 antibody (1:10) or anti-Bcl-X_L (1:500, Dianova, Hamburg, Germany) overnight at 4°C. Horseradish peroxidase (HRP)–conjugated polyclonal antibodies (1:5000) were used for ECL detection (Amersham).

Statistical Analyses

Each experiment was performed at least three times and statistical analysis was performed using the two-tailed Student's *t*-test. Normal distribution of all data is assured. Results are expressed as mean values ± SD. Otherwise representative data are shown.

Results

NO Donor–Induced Apoptosis in Macrophages

In corroboration with earlier reports, we noticed NO-mediated apoptosis in RAW 264.7 macrophages. The NO donors GSNO (1 mM) or Sp-NO (0.25 mM) promoted 25–30% DNA fragmentation during an 8-hr incubation period. Variations in the amount of individual NO donors needed to promote apoptosis are related to the kinetics of NO release and may further refer to the redox species being formed (31,32). Therefore, the use of different NO donors is advised. GSNO may be considered the most physiological NO donor that releases either NO radical or nitrosonium ion. In contrast, spermine-NO is supposed to release NO radicals, independently, of any cofactors. In our experiments, DNA cleavage under control conditions was below 5% and was quantitated using the diphenylamine assay. Proapoptotic parameters occur in a time-dependent manner and can

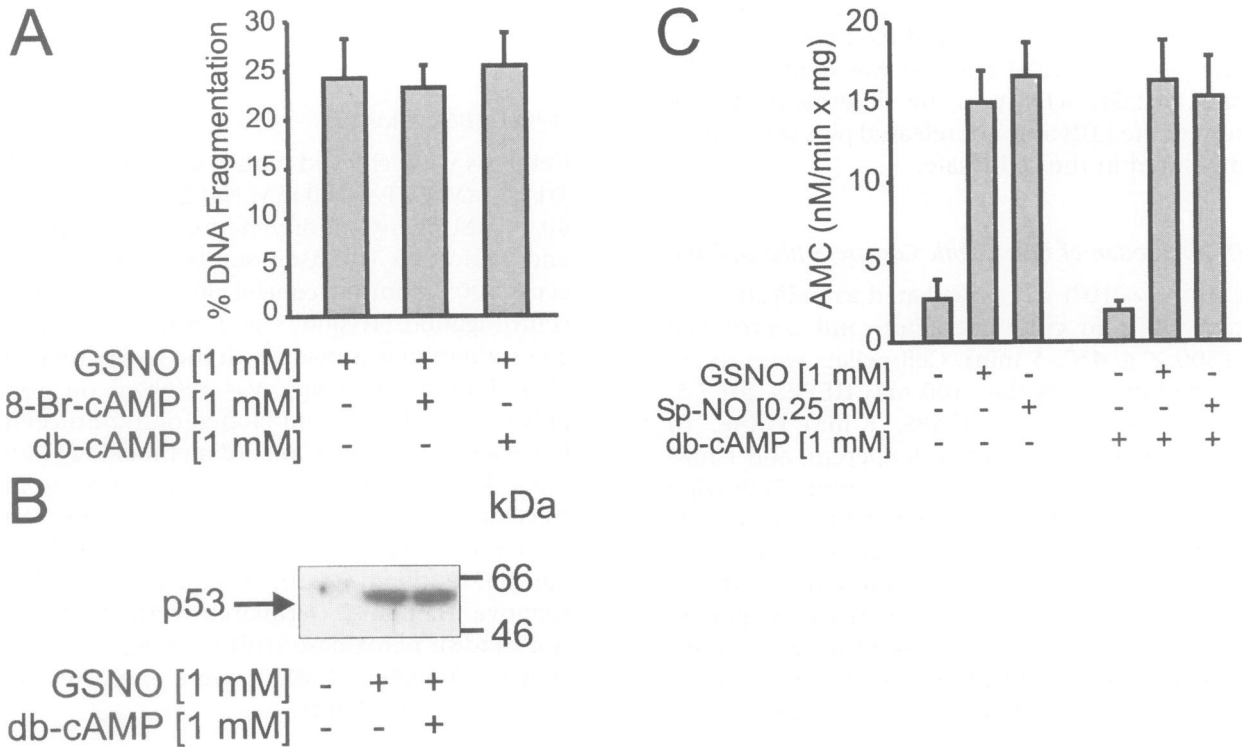

Fig. 1. Apoptotic parameters are unaffected by short-term lipophilic cAMP preincubation. RAW 264.7 macrophages were prestimulated for 1 hr with 1 mM 8-Br-cAMP or db-cAMP prior to the addition of NO donors. (A) DNA fragmentation in response to 1 mM GSNO was measured after 8 hr and quantitated with the diphenylamine assay. (B) p53 accumulation in response to 1 mM GSNO was determined after 4 hr by Western blot analysis. (C) Caspase-3-like cleavage of Ac-DEVD-AMC was analyzed after 4 hr following NO donor addition. Data are mean values ± SD of three individual experiments. For details and statistical analysis see Materials and Methods.

be followed accordingly. DNA fragmentation is considered a late apoptotic parameter and requires incubation periods of roughly 8 hr, while other parameters such as p53 accumulation or caspase-3 activation can be assessed much earlier. The absence of LDH release underscored plasma membrane integrity during these incubations and thus allowed a clear distinction between apoptosis and necrosis.

cAMP Confers Resistance Against NO Donors in RAW264.7 Macrophages

In a first set of experiments we exposed RAW 264.7 macrophages for 1 hr to lipophilic cAMP analogs such as 8-Br-cAMP or db-cAMP prior to initiation of apoptosis by 1 mM GSNO. cAMP analogs (1 mM) are supposed to enter the cell and be effective at those concentrations. GSNO evoked around 20–25% DNA fragmentation during an 8-hr incubation period but neither preincubation with 8-Br-cAMP nor the application of db-cAMP reduced DNA degradation under these conditions (Fig. 1A).

We also noticed unaltered p53 accumulation as a result of GSNO exposure in samples that received db-cAMP beforehand (Fig. 1B). Similar results were obtained with Sp-NO (data not shown). DNA fragmentation and p53 accumulation were indistinguishable in samples irrespective of their pretreatment with lipophilic cAMP derivates. To verify these results, we analyzed caspase-3 activation by cleavage of Ac-DEVD-AMC, a fluorogenic caspase-3-like substrate. Cells were pretreated with db-cAMP for 1 hr followed by exposure to 1 mM GSNO or 0.25 mM Sp-NO for 4 hr. NO donors promoted caspase-3 activation irrespective of liphophilic cAMP analog prestimulation (Fig. 1C). These results provide further evidence that liphophilic cAMP analogs were unable to protect macrophages against NO donors when incubated for only 1 hr.

In further experiments we extended the preexposure period with liphophilic cAMP analogs. Preincubation of macrophages for 15 hr with either 1 mM 8-Br-cAMP or 1 mM db-cAMP conferred resistance against 1 mM GSNO-induced DNA fragmentation. Cells with the addition of cAMP analogs showed around 5% DNA fragmentation compared to 25% of uninhibited controls (Fig. 2A). A similar degree of protection was achieved with the combination of Sp-NO/cAMP analogs. Sp-NO-elicited DNA fragmentation of 25% was drastically reduced to roughly 5% by the liphophilic cAMP pretreatment. Prolonged preincubation of macrophages with cell-permeable cAMP derivatives blocked DNA fragmentation by 60% to 80%. Significant reduction was also obtained by LPS/IFN-γ/NAME preactivation, an established protocol to promote Cox-2 expression and concomitant cAMP-elevating prostanoid formation (9,33). We then sought to correlate suppressed DNA fragmentation with the NO donor–elicited p53 response (Fig. 2B). Western blot analysis revealed no or only minor expression of p53 in controls, but showed massive protein accumulation within a 4-hr treatment with 1 mM GSNO (Fig. 2B, lane 3).

Addition of lipophilic cAMP analogs for 15 hr blocked GSNO-promoted p53 accumulation (Fig. 2B, lanes 5 and 6), with db-cAMP or 8-Br-cAMP being similarly effective. For comparison we found reduced p53 immunoreactivity following LPS/IFN-γ/NAME prestimulation for 15 hr (Fig. 2B, lane 4). We further verified these results by following caspase-3 activation. Thus, cells were pretreated with db-cAMP for 15 hr followed by the addition of 1 mM GSNO or 0.25 mM Sp-NO for 4 hr (Fig. 2C). Under these conditions, NO-evoked caspase-3 activation was largely attenuated (80–90%) by db-cAMP (Fig. 2C). We then followed cAMP-evoked protection by measuring the mitochondrial membrane potential ($\Delta\Psi$) using $DiOC_6(3)$ (Fig. 2D). Control macrophages exhibited a high $\Delta\Psi$, which was significantly lowered when 1 mM GSNO was added for 8 hr. Under the influence of GSNO, 26% of the cells showed a decrease in $\Delta\Psi$ that was not evident when cells were preexposed to db-cAMP.

Additional experiments in RAW 264.7 macrophages revealed decreased cytochrome c relocation in response to 1 mM GSNO or 0.25 mM Sp-NO when cells were pretreated with db-cAMP (Fig. 3A). A decreased expression of the anti-apoptotic protein Bcl-X_L that occurred after the addition of NO was absent when macrophages were exposed to db-cAMP beforehand (Fig. 3B).

cAMP Confers Resistance Against GSNO in Human Macrophages

To verify the efficiency of lipophilic cAMP analogs in primary macrophages, we isolated human monocytes, then differentiated them to macrophages. We analyzed the first set of examinations by annexin V staining (Fig. 4A); annexin binds to phosphatidylserine exposed on the outer leaflet of the plasma membrane of apoptotic cells. In controls we saw 1–2% annexin-positive cells. This proportion increased to 37% after 4 hr in GSNO-treated cells and was brought back to control values by pretreatment with cAMP analogs. Annexin V staining was exclusively determined in propidium-negative cells, thus eliminating annexin binding in necrotic populations. We went on to show p53 accumulation in human macrophages in response to GSNO (Fig. 4B). Accumulation of the tumor suppressor was hindered by cAMP pretreatment.

Exposure of human macrophages to GSNO resulted in caspase activation, which is another indicator of apoptosis. Prestimulation of human macrophages for 15 hr with db-cAMP attenuated NO-initiated caspase activation (Fig. 4C). Furthermore, breakdown of $\Delta\Psi$ that occurred in human macrophages as a result of GSNO application was decreased by the addition of db-cAMP (Fig. 4D), and 3–5% of control cells showed a decrease in $\Delta\Psi$. Within 8 hr GSNO brought these values up to 26%, whereas db-cAMP attenuated NO-evoked $\Delta\Psi$ breakdown. We conclude that preactivation of murine or human macrophages for an extended time with cAMP analogs inhibits NO-induced apoptosis.

The Role of Cox-2 in Macrophage Protection

Apparently, in order to inhibit NO-induced apoptosis—i.e., DNA fragmentation, p53 accumulation, annexin V binding, caspase-3 activation, cytochrome c relocation, $\Delta\Psi$ breackdown, and down-regulation of Bcl-X_L—lipophilic cAMP analogs require a long preincubation period. The extended presence of cAMP that is needed to attenuate apoptosis suggests that protection is achieved by cAMP-mediated gene induction. Obviously, Cox-2 expression is not responsible for cAMP-induced protection, which is in contrast to LPS/IFN-γ/NAME prestimulation (9). Although Cox-2 expression occurs after cAMP

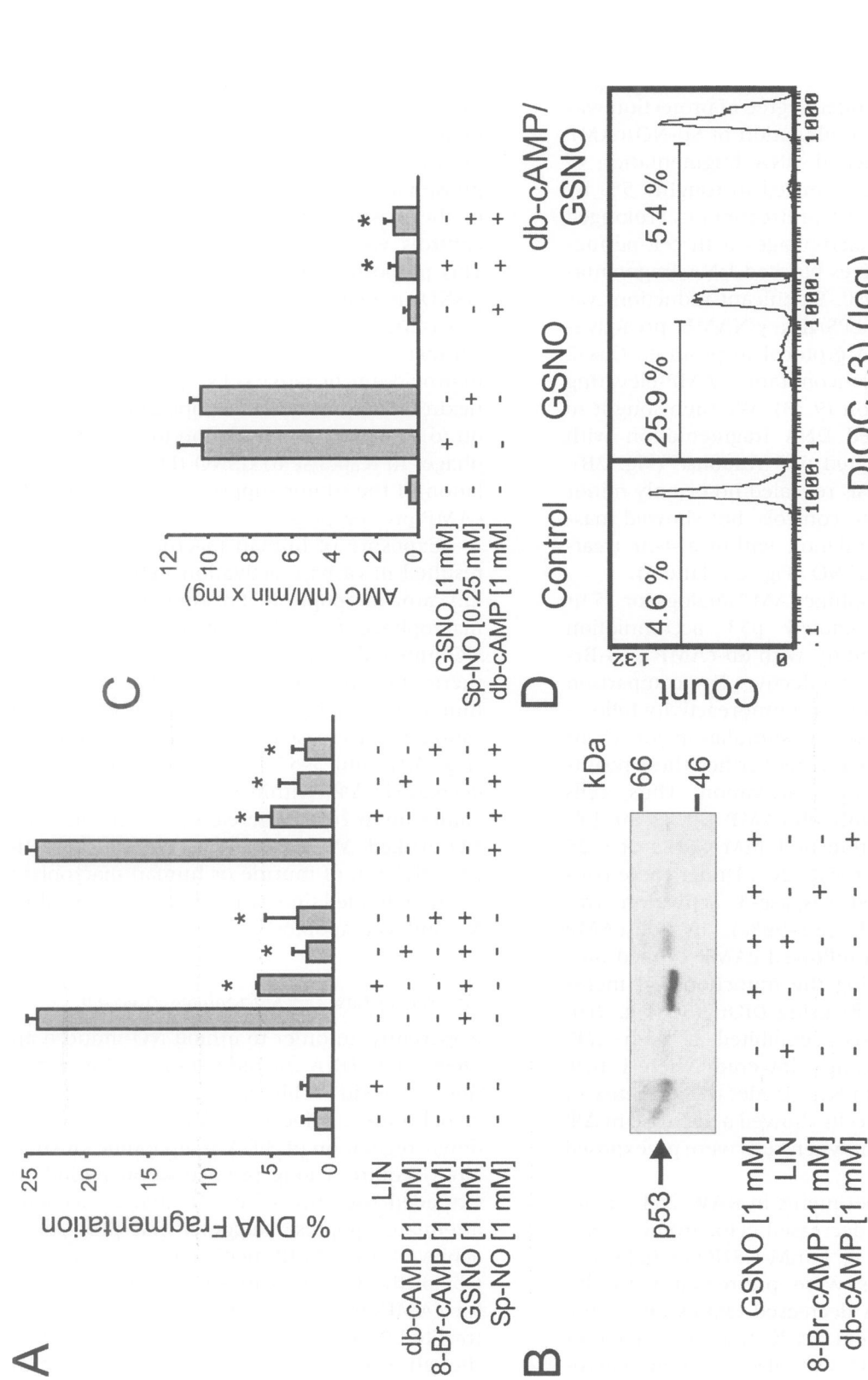

Fig. 2. Long-term lipophilic cAMP preincubation decreased apoptotic parameters. RAW 264.7 macrophages were prestimulated for 15 hr with 1 mM 8-Br-cAMP, or 1 mM db-cAMP, with a combination of LPS (10 μg/ml), IFN-γ (100 U/ml), and NAME (1 mM) (LIN) prior to the addition of NO donors, or remained as controls. (A) DNA fragmentation was initiated with 1 mM GSNO or 0.25 mM Sp-NO and quantitated after 8 hr with the diphenylamine assay (*$p \leq 0.05$ versus NO-treated controls). (B) p53 expression in response to 1 mM GSNO was determined after 4 hr by Western blot analysis. (C) Caspase-3-like activity was measured after 4 hr by the cleavage of Ac-DEVD-AMC. (D) Mitochondrial membrane potential ($\Delta\psi$) was assessed after 8 h by the $DiOC_6(3)$ method in response to 1 mM GSNO. Histograms show single-color labeling. Representative results are shown. For details and statistical evaluations see Materials and Methods.

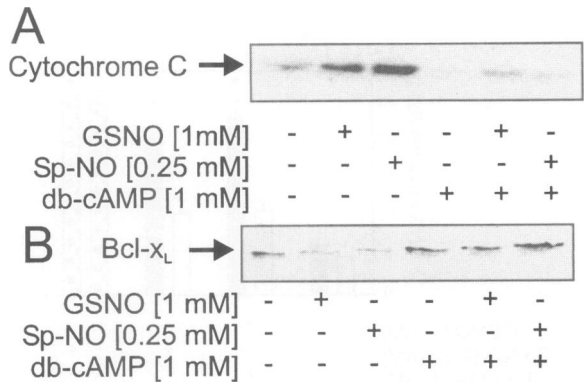

Fig. 3. Long-term lipophilic cAMP treatment decreased cytochrome c release and Bcl-X_L down-regulation. RAW 264.7 macrophages, preincubated with 1 mM db-cAMP for 15 hr, were exposed to 1 mM GSNO or 0.25 mM Sp-NO for 6 hr. Translocation of cytochrome c (A) and expression of Bcl-x_L (B) were analyzed by Western blot analysis as described in Materials and Methods. Western blots are representative of three similar experiments.

stimulation (data not shown), the Cox-2-specific inhibitor NS-398 (10 μM) left cAMP-evoked cell protection unaltered and only blocked LPS/IFN-γ/NAME–mediated protection (Fig. 5). NS-398 reversed protection in LPS/IFN-γ/NAME–prestimulated cells, thus allowing 25% DNA breakdown, which is comparable to GSNO-treated macrophages.

In contrast, the addition of NS-398 left cAMP-induced protection unaltered. Therefore, Cox-2 expression is a prerequisite for LPS/IFN-γ/NAME– but not cAMP-evoked resistance against NO-mediated apoptosis. To prove the hypothesis that gene induction as a result of cAMP action is responsible for cellular protection, we used a decoy oligonucleotide approach to reverse protection.

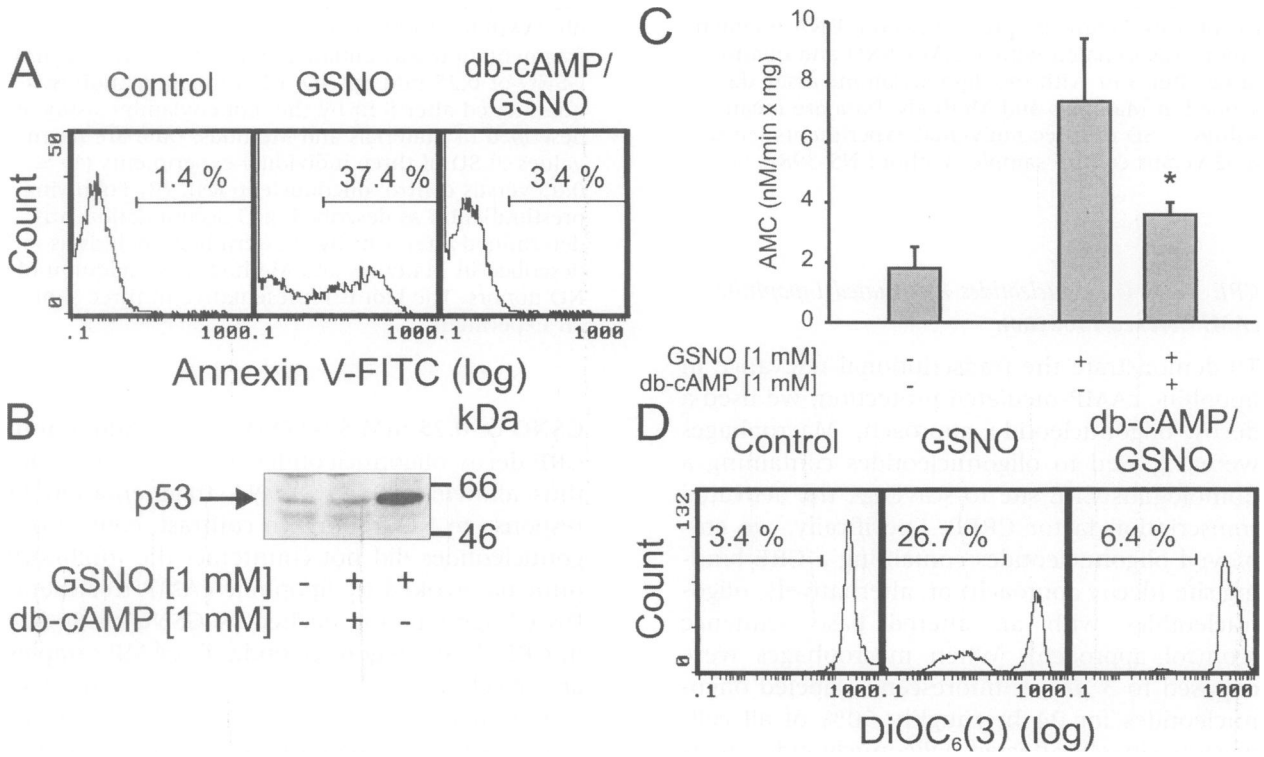

Fig. 4. Long-term lipophilic cAMP preincubation decreased apoptosis in human macrophages. Primary human monocyte-derived macrophages were prestimulated for 15 hr with 1 mM db-cAMP prior to stimulation with 1 mM GSNO, or remained as controls. (A) Annexin V staining was quantitated after 4 hr. Histograms show single-color labeling. (B) Expression of p53 in response to 1 mM GSNO was determined after 4 hr by Western blot analysis. (C) Caspase-3-like activity was measured by the cleavage of Ac-DEVD-AMC after 4 hr. (D) Mitochondrial membrane potential ($\Delta\psi$) was assessed by $DiOC_6(3)$ measurements after 8 hr. Histograms show single-color labeling. Representative results are shown. For details and statistical evaluations see Materials and Methods.

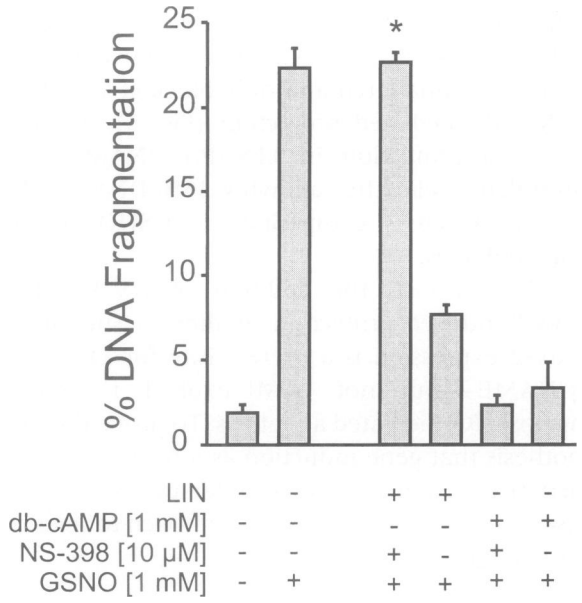

Fig. 5. Cox-2 inhibition by NS-398 did not affect cAMP-mediated protection. RAW 264.7 macrophages in the presence or absence of 10 μM NS-398 were prestimulated for 15 hr with 1 mM db-cAMP, a combination of LPS (10 μg/ml), IFN-γ (100 U/ml), and NAME (1 mM) (LIN), or remained as controls. Following prestimulation, DNA fragmentation was initiated with 1 mM GSNO and quantitated after 8 hr with the diphenylamine assay described in Materials and Methods. Data are mean values ± SD of three individual experiments (*$p \leq$ 0.02 versus control samples without NS-398).

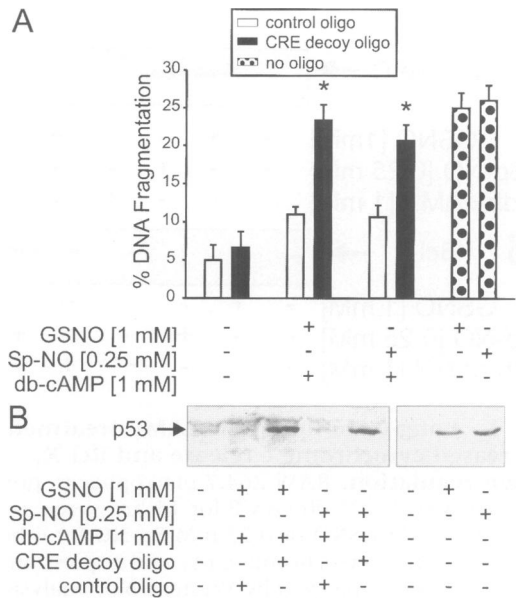

Fig. 6. CRE decoy oligonucleotides decreased cAMP-mediated protection. (A) RAW 264.7 macrophages (1×10^6 cells) were incubated with decoy or control olignucleotides for 24 hr as described under Materials and Methods. After changing the medium cells were prestimulated with 1 mM db-cAMP for 15 hr as indicated. Afterwards, DNA fragmentation was initiated by the addition of 1 mM GSNO or 0.25 mM Sp-NO. DNA fragmentation was quantitated after 8 hr by the diphenylamine assay as described in Materials and Methods. Data are mean values ± SD of three individual experiments (*$p \leq$ 0.05 versus control oligonucleotides). (B) Following prestimulation as described, p53 accumulation was determined after 4 hr by Western blot analysis as described in Materials and Methods after addition of NO donors. The blot is representative of three similar experiments.

CRE Decoy Oligonucleotides Attenuated Lipophilic cAMP-Evoked Protection

To demonstrate the transcriptional relevance in lipophilic cAMP-mediated protection, we used a decoy oligonucleotide approach. Macrophages were exposed to oligonucleotides containing a homologous CRE site to scavenge the activated transcription factor CREB. Specifically, we employed oligonucleotides containing a CRE binding site (decoy approach) or, alternatively, oligonucleotides with an altered base sequence (control approach). When macrophages were exposed to 3 μM of fluorescence-labeled oligonucleotides for 24 hr roughly 50% of all cells were positively affected. Oligonucleotide incorporation was estimated by using a fluorescence microscope and was in line with previous studies (33). After oligonucleotide incorporation, cells were exposed to 1 mM db-cAMP to promote protection against NO donors.

Following these manipulations, apoptotic DNA fragmentation was initiated with 1 mM GSNO or 0.25 mM Sp-NO (Fig. 6A). Addition of CRE decoy oligonucleotides reversed protection, thus allowing 20–25% DNA fragmentation in response to NO donors. In contrast, control oligonucleotides did not counteract the inhibitory principle evoked by lipophilic cAMP treatment. DNA fragmentation elicited by GSNO or Sp-NO in CRE decoy oligonucleotide/db-cAMP samples and in cells that received no oligonucleotide at all was comparable. Moreover, CRE decoy oligonucleotides restored p53 accumulation in response to 1 mM GSNO or 0.25 mM Sp-NO (Fig. 6B), whereas control oligonucleotides left lipophilic cAMP-initiated suppression of p53 unaltered. Additional control experiments showed oligonucleotides by themselves to be nonapoptotic (data not shown), whereas accumulation of p53 was achieved by the application of GSNO or Sp-NO

(Fig. 6B). These results show conclusively that inducible protection as a result of cAMP action along with an attenuated p53 response is antagonized by oligonucleotides that contain a CRE binding site.

Discussion

Preactivation of murine or human macrophages confers resistance against apoptotic death, which is elicited by NO donors such as GSNO or Sp-NO. Protection is achieved by stimulating cells with lipophilic cAMP analogs for 15 hr and experimentally is shown by blocking caspase-3 activation, cytochrome c relocation, $\Delta\Psi$ breakdown, p53 accumulation, DNA fragmentation, annexin V staining, as well as preserved Bcl-X_L expression. Decoy oligonucleotides containing a CRE binding site reversed inducible resistance, thus supporting the notion that cAMP-mediated gene activation is required to convey protection.

In corroboration with other reports, we noticed typical apoptotic alterations in response to high concentrations of NO (34). Apoptosis was characterized by membrane and mitochondrial alterations as well as variations in the expression of pro- and anti-apoptotic proteins (23,24,26,27,35–37). Observing these apoptotic features led to the classification of RAW 264.7 macrophages as type II cells, which are characterized by mitochondrial alterations and subsequent caspase-3-like protease activation following stress- or chemical-evoked cellular damage (38). For macrophages we established p53 to be at least partly involved in conveying the apoptotic initiating activity of nitric oxide (39).

Previously, we demonstrated that macrophage prestimulation with LPS/IFN-γ/NMMA protected macrophages against NO-mediated apoptosis by promoting Cox-2 expression; a 15 hr preincubation period was required to inhibit apoptosis (9). Expression of Cox-2 in the sequence of events leading to colorectal cancer has been determined and genetic evidence links Cox-2 to tumorigenesis (16,40). The use of Cox-2-selective inhibitors as therapeutic agents for colorectal polyposis and cancer joins Cox-derived metabolites and deregulation of apoptosis together (15). We have shown here by using the specific Cox-2 inhibitor NS-398 that Cox-2, which is also induced by prestimulation of macrophages with lipophilic cAMP analogs, is not responsible for cAMP-mediated protection against apoptosis. Expression of Cox-2 in macrophages is associated with prostaglandin E_2 formation, which in turn may increase intracellular cAMP (41,42). Deactivation of several macrophage responses by cAMP-elevating manipulations, such as adenyly cyclase stimulation, phosphodiesterase inhibition, and/or Cox-2 activation, is known and considered a standard regime for suppressing an early phase of the innate immune response known to be linked to macrophage stimulation (43,44). The action of cAMP-elevating agents in suppressing apoptosis is known for several other systems, i.e., for hepatocytes, neutrophils, or natural killer cell–derived cytotoxicity (45–47). However, the mechanism of cAMP-mediated signal transduction in conferring resistance is largely unknown. Under inflammatory conditions, increased levels of cAMP-elevating prostaglandins acting via adenylyl cyclase may efficiently balance apoptotic initiating and counteracting pathways in cells of the monocyte/macrophage lineage. Our assumption that cAMP is involved in establishing cellular resistance was confirmed by applying lipophilic cAMP analogs that blocked all apoptotic features. Protection from apoptosis was not restricted to cultured murine RAW 264.7 macrophages but also occured in primary human monoctye-derived macrophages. Cellular responses against cAMP are mediated by activation of cAMP-dependent protein kinases (48). The inactive kinase holoenzyme dissociates after the binding of cAMP and liberates the catalytic subunit, which in turn phosphorylates multiple substrates. Phosphorylation may affect structural components or enzyme activities, or alternatively, may promote alterations in gene transcription by phosphorylation and activation of the transcription factor CREB (49,50). Phosphorylated CREB binds to the promoter region of various genes and initiates transcription (51,52). Protection by cAMP-elicited signal transduction has been shown for human cancer cell growth (53,54), radiation resistance of melanoma cells (55), and FAS-mediated apoptosis of primary hepatocytes (46), although in some other systems, cAMP has been reported to act in a pro-apoptotic manner (56,57). Our study verifies the protective mechanism of conveying resistance against NO donors by lipophilic cAMP analog pretreatment. The successful use of decoy oligonucleotides with their ability to scavenge activated CREB, thereby abrogating protection and reestablishing p53 accumulation, confirmed the assumption of cAMP-derived gene induction. Although cAMP-responsive gene products remain elusive, they seem to participate in the regulation of p53, thereby affecting a rather upstream signaling component during NO-initiated apoptosis. An understanding of transducing mechanisms that confer inducible resistance against high doses of NO in

immune-competent cells such as macrophages by cAMP-mediated gene activation may open avenues for pharmacologic intervention.

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