[CANCER RESEARCH 63, 5470–5479, September 1, 2003]

Nitric-Oxide-induced Bax Integration into the Mitochondrial Membrane Commits MDA-MB-468 Cells to Apoptosis: Essential Role of Akt[1]

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ABSTRACT

We have previously reported that nitric oxide (NO) induces apoptosis in MDA-MB-468 cells through its action on the mitochondria and the release of cytochrome *c*. In this study, we investigated the critical events that must occur after which these cells are committed to apoptosis. We used the long-acting NO donor DETA-NONOate, which, at a concentration of 1 mM, releases NO in the range produced by activated macrophages. Depolarization of mitochondrial membrane potential (MMP) occurred at 4 h of DETA-NONOate treatment, which returned to control values and which was followed by another wave of depolarization at 24 h. There was a 2-fold increase of cytochrome *c* in the cytosol at 6 h, but it was not until 36 h that the level of cytochrome *c* was increased by 15-fold. Although the initial release of cytochrome *c* from the mitochondria could be inhibited by cyclosporin A or by bongkrekic acid, the later release continued even in its presence. We observed that the later release of cytochrome *c* at 36 h was independent of MMP depolarization but was dependent on Bax integration into the mitochondrial membrane, which committed the cells to apoptosis. We also observed a decline in the levels of cytosolic phospho-Akt at 16–24 h of DETA-NONOate treatment. We also conclude that decrease in phospho-Akt is an essential event upstream from Bax integration in MDA-MB-468 cells.

INTRODUCTION

A central and essential feature in apoptosis or programmed cell death is the dysregulation of the mitochondrial membrane integrity that leads to the redistribution of cytochrome *c* and other apoptotic regulatory proteins into the cytosol (1–3). Subsequently, cytochrome *c* in the cytoplasm interacts directly with apoptosis-activating factor-1 (APAF-1) leading to the ATP-dependent formation of a macromolecular complex knows as the apoptosome (4, 5). This complex recruits and activates caspase-9. Activated caspase-9 can activate additional caspase-9 molecules, as well as caspase-3 and -7, resulting in the morphological features of apoptosis.

It is generally assumed that after the loss of the outer mitochondrial membrane integrity and the release of cytochrome *c* from the mitochondria to the cytosol, the cells are committed to apoptosis (6). The mechanism(s) by which there is loss of outer mitochondrial membrane integrity by various apoptotic agents have, therefore, been the focus of studies by numerous investigators (7, 8). Most studies indicate that the initial event observed after application of the apoptotic stimulus is a decrease in the inner transmembrane potential. This is followed by an increase in the permeability of the outer mitochondrial membrane by forming the PTPC[3] and subsequent release of cytochrome *c* from the intermembranous space into the cytosol. However, we (9) and others (10) have observed that the collapse of the inner MMP is not a universal early event that triggers the release of cytochrome *c* leading to apoptosis, and moreover is not always an essential part of the central apoptotic machinery.

One of the other mechanism(s) by which the release of cytochrome *c* occurs is mediated by the Bcl-2 family, including Bax and Bid, which promote apoptosis, whereas the antiapoptotic members of the Bcl-2 family of proteins, including Bcl-2 and Bcl-x_L, prevent the release of cytochrome *c* from the mitochondria (11). The antiapoptotic members such as Bcl-2 and Bcl-x_L and proapoptotic members such as Bak localize predominantly at the mitochondrial membrane and regulate the mitochondrial membrane integrity and cytochrome *c* release (12). On the other hand, other proapoptotic members such as Bax, Bid, and Bad reside in the cytoplasm in healthy cells (13). In response to apoptotic stimuli, these proapoptotic members redistribute to the mitochondria and promote cytochrome *c* release. Bax translocation to the mitochondria occurs in response to a wide variety of apoptotic stimuli and may, therefore, serve as a key interaction point for various apoptotic signals (14). It has been suggested that after translocation to the mitochondria, Bax induces cytochrome *c* release either by forming a pore through oligomerization or by opening a channel called voltage-dependent anion channel (VDAC) via a direct interaction. Unlike that observed with apoptotic signals, some cell survival signals inhibit Bax translocation to the mitochondria (15).

Translocation of Bax from the cytosol to the mitochondria, can be modulated by the PI3K-Akt pathway, which is one of the important pathways that play a critical role in cell survival (16, 17). Akt kinases are major downstream targets of growth receptor tyrosine kinases that signal via PI3K. Akt kinase activation is a multiple-step process involving both membrane translocation and phosphorylation (18). Akt kinase is a well-established survival factor and prevents release of cytochrome *c* from the mitochondria to the cytosol by phosphorylating and inactivating the proapoptotic factors Bad and procaspase-9.

Nitric Oxide (NO) modulates the signal transduction pathways that can modulate apoptosis, and both pro- and antiapoptotic effects have been reported, depending on both the cell type and the NO concentration. Antiapoptotic effects are associated with low levels (10 nM to 1 μM), whereas high NO concentration (>1 μM) as produced by activated macrophages can lead to apoptosis (19). Excess NO production has been implicated in diseases such as septic shock, autoimmune disease, cerebral infarction, and diabetes mellitus, in which NO-mediated apoptosis is often observed (20). Although, NO-induced apoptosis is initiated by DNA or mitochondrial damage (21), the cascade of events leading to apoptosis has not been fully elucidated.

Received 3/28/03; revised 5/27/03; accepted 6/10/03.

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[1] Supported in part by Palomba Weingarten, the Allegra Charach Cancer Research Fund, and USPHS Grant CA-78357 (to G. C.). Flow cytometry was performed in the core facility of UCLA Jonsson Comprehensive Cancer center and Center for AIDS Research Flow Cytometry Core Facility, which is supported by NIH Awards CA-16042 and AI-28697 and by the Jonsson Cancer Center, UCLA AIDS Institute, and UCLA School of Medicine.

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[3] The abbreviations used are: PTPC, permeability transition pore complex; AMC, 7-amino-4-methyl coumarin; AFC, 7-amino-4-trifluoromethyl coumarin; CMX-Ros, Chloromethyl X Rosamine; DFF-45, DNA fragmentation factor 45; $DiOC_6$, dihexyloxacarbocyanine; FBS, fetal bovine serum; fmk, fluoromethylketone; MMP, mitochondrial membrane potential; PMSF, phenylmethylsulfonyl fluoride; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; RIPA, radioimmunoprecipitation assay (buffer); PARP, poly(ADP-ribose) polymerase; p-Akt, phospho-Akt; PI3K, phosphatidylinositol 3′-kinase.

The primary objective of this study was to elucidate the mechanism by which NO induces apoptosis using a human breast cancer cell line MDA-MB-468. This cell line was selected for our studies because these cells express high arginase activity with very little nitric oxide synthase (22). Thus, the effects of exogenous NO can be studied without any confounding influence of NO produced by these cells. We used DETA-NONOate, an NO donor with a relatively long half-life (20 h at 37°C), which, at a concentration of 1 mM, releases a steady state of NO in the range produced by activated macrophages (23). Because NO has a very short biological half-life, we also wanted to assess the cascade of events that must occur after which the cells are committed to apoptosis. We achieved this by exposing the cells to the NO donor for varied periods of time and then observing the reversible events and the cascade of events that must occur for the cells to be committed to apoptosis.

MATERIALS AND METHODS

Chemicals. DETA-NONOate was purchased from Cayman Chemicals (Ann Arbor, MI). Fluorogenic substrate Ac-DEVD-AMC (caspase-3), and Ac-IETD-AFC (caspase-8) from PharMingen (San Diego, CA). Cyclosporin A and bongkrekic acid from Sigma Aldrich. Caspase-9 substrate, Ac-LEHD-AMC was purchased from Alexis Biochemicals (San Diego, CA). The caspase-3 inhibitor (II) Z-DEVD-fmk, caspase inhibitor V, and caspase-9 inhibitor Z-LEHD-fmk were purchased from CalBiochem (San Diego, CA). Antibodies used were from the following suppliers: goat polyclonal anti-DFF-45 (Sc 6867), goat polyclonal anti-Akt1/2 (Sc 1619), rabbit polyclonal anti-caspase-9 (H-83) from Santa Cruz Biotechnology; rabbit polyclonal anti-caspase-3 (65906E), mouse monoclonal anti-cytochrome *c* (65981A), rabbit polyclonal anti-p-Akt (559029), mouse monoclonal anti-Bax (556467) from PharMingen; cytochrome *c* oxidase from Clontech (Palo Alto, CA); rabbit polyclonal ERK1/2 MAP kinase (9102) from New England Biolabs; rabbit polyclonal p38 MAPK (9212) from Cell Signaling Technology; and mouse monoclonal anti-Bcl-2 (Ab-1) from Calbiochem. MitoTracker Red CMX-Ros was purchased from Molecular Probes (Eugene, OR).

Cell Culture. Human breast cancer cell line MDA-MB-468 was obtained from American Type Culture Collection. These cells were cultured in DMEM containing 10 mM nonessential amino acids, 2 mM L-glutamine, 1 μg/ml insulin, and 10% FBS. For experimental purpose, cells were grown in 5% FBS, allowed to seed overnight, and treated with drugs for various time periods.

TUNEL Assay. The TUNEL assay was performed using ApoAlert DNA Fragmentation Assay kit from Clontech as described previously (22). Briefly, cells (3×10^6) were collected by centrifugation, washed twice with PBS, and fixed in 1% formaldehyde-PBS at 4°C for 20 min. The cells were collected, washed with PBS, and stored overnight in 70% ethanol. The cells were centrifuged, washed, and gently resuspended in equilibration buffer. Then nucleotide mixture and terminal deoxynucleotidyltransferase (Tdt) enzyme were added, and cells were incubated at 37°C for 1 h. Cells were washed and analyzed in a Becton Dickinson Flow Cytometer.

Caspase Assay. Cells were lysed in insect cell lysis buffer {50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), 10% sucrose, 5 mM DTT, and 1× protease inhibitor} for 30 min at 4°C. The lysates were used for caspase-3 (3 μg); caspase-9 (6 μg), and caspase-8 (15 μg) assays using respective substrates. The released AMC (for caspase-3 and caspase-9) and AFC (for caspase-8), which, after specific cleavage of their respective substrates, become fluorescent, were quantified using a fluorometer (Versa Fluro; Bio-Rad) with excitation at 380 nm and emission at 440 nm for AMC substrates and at excitation 410 nm and emission at 510 nm for AFC substrate respectively (9).

Western Analysis. For analysis of cytosolic proteins, cells were lysed in cell lysis buffer [50 mM HEPES (pH 7.5); 1 mM DTT, 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.1 mM PMSF] and were incubated at 4°C for 30 min. Protein concentration was measured using Bio-Rad protein assay dye concentrate. For analysis of total cellular protein, the cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP40, and 0.5% sodium deoxycholate) containing 0.5% SDS; and protein concentrations was measured using BCA dye concentrate (Pierce). Lysates (30 μg) were resolved electrophoretically on 10% SDS-polyacrylamide gel and electrotransferred to a polyvinylidine difluoride membrane (Bio-Rad) using a tank blot procedure (Bio-Rad Mini Protean II). The membranes were incubated with respective primary antibodies and 1:1000 dilutions of respective horseradish peroxidase-linked F(ab) fragment secondary antibody (Amersham Corp., Piscataway, NJ) for 1 h. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection system (Amersham). as described previously (22, 24).

Detection of Cytochrome *c* Release into the Cytosol. Cytochrome *c* release into the cytosol was detected as described previously with minor modifications (9, 24). Briefly, 6×10^6 cells were harvested and washed with PBS. The cells were suspended in Buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM Sucrose, and 1× protease inhibitor cocktail] and were homogenized by Dounce homogenizer; unbroken cells and nuclei were removed by centrifugation at $1,000 \times g$ for 10 min at 4°C. The supernatant was further centrifuged at $10,000 \times g$ for 20 min. The supernatant was saved as a cytosolic fraction, and the precipitate was suspended in Buffer A containing 0.5% (v/v) NP40 and was saved as the mitochondrial fraction. For further analysis of proteins that were integrated into the mitochondria, the mitochondrial fraction was suspended in 0.1 M Na_2CO_3 (pH 11.5) at 4°C for 30 min and was subjected to ultracentrifugation at 100,000*g* for 1 h. The mitochondrial and cytosolic fractions were analyzed by Western blot with an anti-cytochrome *c* monoclonal antibody (7H8 2Cl2) or with an anti-cytochrome *c* oxidase antibody.

Measurement of MMP by Flow Cytometry. One $\times 10^6$ cells were harvested after various treatments, washed twice with cold 1× PBS and incubated with 100 nM MitoTracker Red CMX-Ros dye at 37°C for 15 min in the dark, washed twice with cold PBS, and analyzed immediately by flow cytometry, as described previously (9).

Antisense Treatment. Antisense oligonucleotides directed against the coding region of human *Bax* gene (GeneBank accession no. AF339054) were custom-synthesized from Life Technologies, Inc. (Gaithersburg, MD). Phosphorothioate-modified 20-mer antisense 5′-TGCTCCCCGGACCCGTCCAT-3′ and scrambled mismatched control oligonucleotide 5′-TGCTCCGGCCACCGCTCACT-3′ were used in this study. Twenty h after seeding, oligonucleotides were delivered at a 1:1 complex with the lipofectin transfection reagent into subconfluent MDA-MB-468 cells. Lipofectin-oligonucleotide complex, prepared to provide final oligonucleotide concentrations of 2 and 6 μM were added for 16 h in serum- and antibiotic–free medium and incubated at 37°C in a chamber with 5% CO_2. Subsequently, cells were washed and incubated in complete serum containing medium for 24 h, and expression of Bax was measured by Western blot analysis.

Bcl-2 Overexpression. Full-length human Bcl-2 gene cloned into the *Xho*I and *Xba*I site of C3-EGFP was a kind gift from Richard J. Youle (NIH, Bethesda, MD). MDA-MB-468 cells were plated at a density of 3×10^5 cells/well in six-well plates. After 24 h, cells were transiently transfected using the LipofectAMINE-plus (Life Technologies, Inc.) as described by the manufacturer, using 1 μg plasmid DNA (C3-EGFP-Bcl-2) and 8 μl of LipofectAMINE/well. After 5 h in serum-free medium, an equal volume of medium containing 2× normal FBS was added. Transfected cells were then cultured for an additional 24 h and were visualized by microscopy. Cells were selected in the presence of 600 μg/ml G418 for 5 weeks for obtaining stably transected cells. During selection period, medium was changed and fresh G418 was added every 72 h. Bcl-2 overexpression in this cell line was confirmed by Western analysis using total lysate.

Statistical Analysis. All of the values are expressed as mean ± SE. Each value is the mean of at least three separate experiments in each group. The differences in the effects of drug treatment when compared with control values were analyzed by one- or two-way ANOVA as appropriate. *P*s ≤ 0.05 were considered significant.

RESULTS

MDA-MB-468 Cells Require More Than 24-h Exposure to DETA-NONOate (1 mM) for Induction of Apoptosis. To understand the sequence of events that occur during NO-induced apoptosis, we determined the minimum exposure time to NO that was required

to induce apoptosis in MDA-MB-468 cells. Cells after exposure to DETA-NONOate for various time periods were either harvested or washed and allowed to grow in the absence of the NO donor for another 24 h. This experiment was designed to assess the minimum time of exposure to NO that was required to commit the cells to apoptosis. For our control experiments, we used oxidized DETA-NONOate, which does not release NO (23). Induction of apoptosis was assessed by the presence of fragmented DNA using TUNEL assay as described in "Materials and Methods" and also by morphological changes such as rounding of the cells observed under microscope. Cells exposed to NO for 24 h or less did not show any significant fragmentation of DNA after they were kept in NO-free medium for another 24 h (Fig. 1*A*). In contrast, cells that were exposed to NO for 36 h or more continued to undergo apoptosis even after they were washed and kept in NO-free medium, as seen with the increased percentage of fragmented DNA (Fig. 1*A*). The induction of apoptosis was also confirmed by Western analysis of cleavage pattern of DFF-45, a known substrate of caspase-3. Viable cells contained intact DFF-45, which appeared as a band of kDa 45,000, whereas cells undergoing apoptosis had fragmented DFF-45 (25). No cleavage of DFF-45 was seen in cells exposed to DETA-NONOate until 24 h, whereas increased cleavage was seen after more prolonged exposure to NO, and this persisted even when the cells were incubated in a NO-free medium for another 24 h (Fig. 1*B*). This indicated that MDA-MB-468 cells had to be exposed to NO for at least 36 h to induce apoptosis. When DETA-NONOate was added with cPTIO (50 μM), a NO scavenger (23), fragmentation of DNA and cleavage of DFF-45 were not observed even at 48 h, indicating that the effects of DETA-NONOate were caused by the NO released (data not shown).

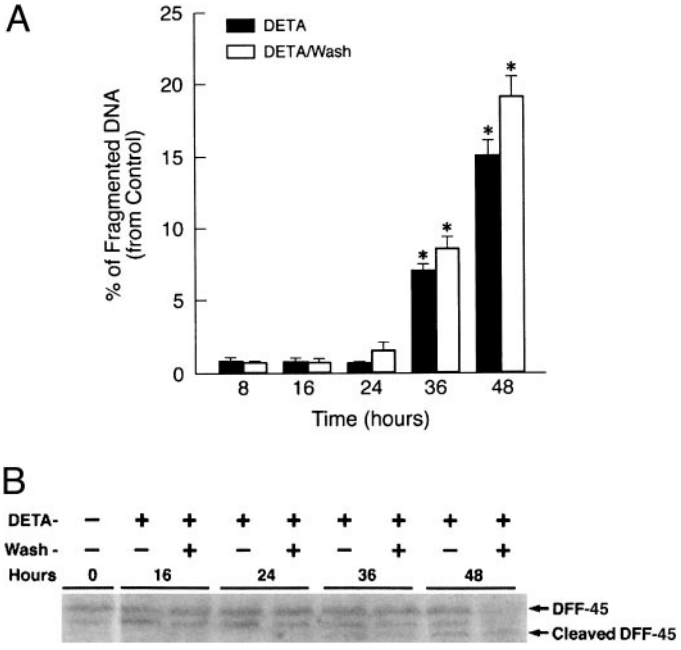

Fig. 1. The time course of DNA fragmentation and cleavage of DFF-45 by DETA-NONOate in MDA-MB-468 cells. In *A,* cells, treated with DETA-NONOate (1 mM) for various time periods, were either harvested (*DETA, black columns*) or washed and allowed to grow in DETA-NONOate-free medium for another 24 h (*DETA/Wash, white columns*). TUNEL assay was performed with the samples obtained from both sets of experiments as described in "Materials and Methods." Results are expressed as the mean of three different experiments ± SE. ∗, the values were significantly different from the values obtained at 8 h; $P < 0.01$. In *B,* cells were treated with DETA-NONOate (1 mM) as described in *A,* and Western analysis was performed with 30 μg of total cell lysates using anti-DFF-45 antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

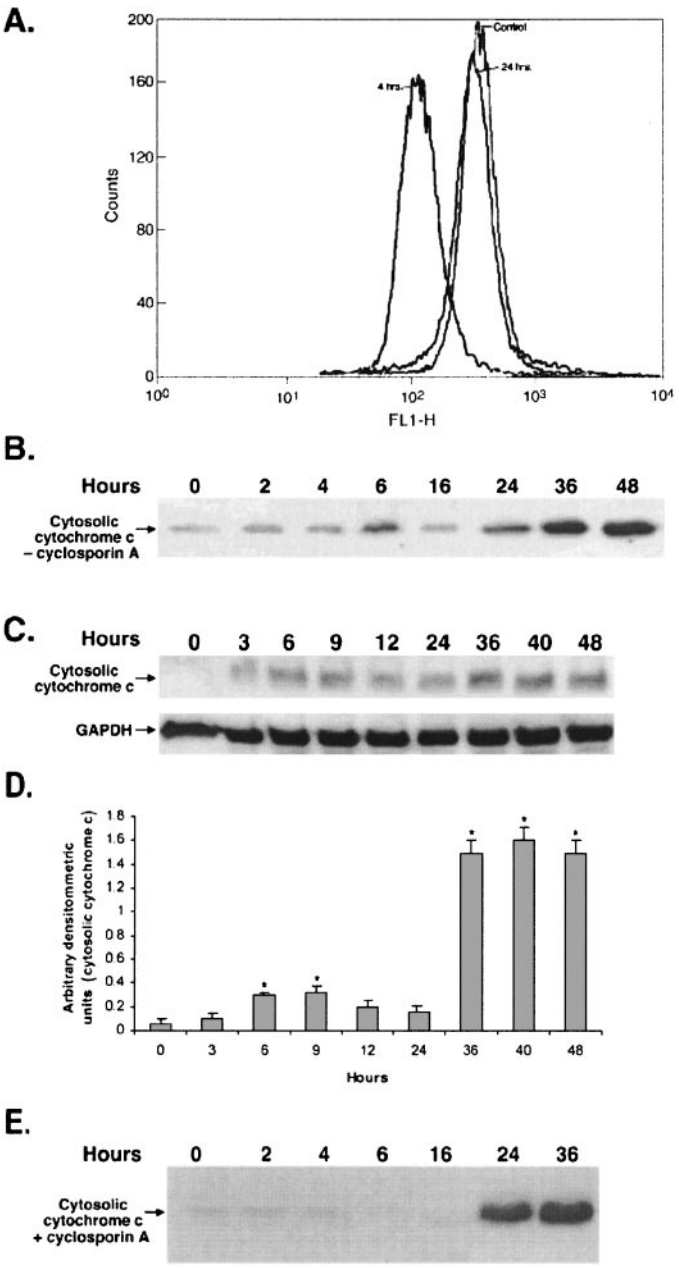

Fig. 2. Effect of DETA-NONOate on the MMP and the time course of cytochrome *c* release in the absence or presence of cyclosporin A. In *A,* cells (1×10^6) treated with DETA-NONOate (1 mM) for various time periods were analyzed for changes in MMP as described in "Materials and Methods" by flow cytometry using CMX-Ros dye. Trace from one of three such experiments is shown. In *B,* cells (3×10^6), treated with DETA-NONOate (1 mM) for various time periods, were subjected to cell fractionation as described in "Materials and Methods." Cytosolic fractions were analyzed by Western blot with 10 μg of protein using anti-cytochrome *c* antibody. In *C,* cells (3×10^6) treated with DETA-NONOate (1 mM) for various time periods were subjected to cell fractionation. Cytosolic fractions were analyzed by Western blot with 15 μg of protein using anti-cytochrome *c* and GAPDH antibody. *D,* densitometric units of cytosolic cytochrome *c* from *C.* Results are expressed as the mean of three different experiments ± SE. ∗, the values are significantly different from the control values; $P < 0.01$. In *E,* cells (3×10^6), treated with either DETA-NONOate (1 mM) alone or in the presense of cyclosporin A (1 μM) for various time periods, were subjected to cell fractionation, and cytosolic fractions were analyzed by Western blot using anti-cytochrome *c* antibody as in *B.*

Depolarization of MMP Was Observed at 4 h Whereas Maximum Release of Cytochrome *c* Occurred between 36 and 48 h. We assessed the changes in MMP and the time course of cytochrome *c* release from the mitochondria after exposure of MDA-MB-468 cells to NO. Depolarization of MMP has been associated with the release of cytochrome *c* into the cytosol followed by the activation of

caspase-9 and -3 to finally execute apoptosis. To understand the relationship between depolarization of MMP and cytochrome *c* release into the cytosol, we performed time course experiments after exposure of these cells to NO. The cells were treated with a lypophilic fluorochrome dye, Mito Tracker Red CMX-Ros, and changes in MMP were analyzed by flow cytometry. Cells exposed to NO initially underwent a maximum magnitude of depolarization of MMP as early as 4 h after exposure, after which the membrane potential was restored to control values, followed by another smaller wave of depolarization at 24 h (Fig. 2*A*). When the cells were treated with another Mito Tracker dye $DiOC_6$, similar results as observed with CMX-Ros were obtained (data not shown). To assess cytochrome *c* released from the mitochondria into the cytosol, cell fractionation was performed, and the cytosolic fractions were analyzed by Western analysis. We observed a 2-fold increase of cytochrome *c* in the cytosol at 6 h, a reduction at 16 h, whereas at 36 and 48 h, there was a 15-fold increase in their levels in the cytosol (Fig. 2*B*). To further confirm that the cytochrome *c* release was biphasic, experiments were done at more time points between 6 and 36 h. We consistently observed a moderate increase in cytosolic cytochrome *c* at ~6–9 h from control value, followed by a decrease at ~12 to 24 h and then a significant increase in cytochrome *c* at 36–48 h (Fig. 2*C*). Cyclosporin A (26) and bongkrekic acid (27), which are known to block PTPC, were effective in blocking the initial phase of NO-induced cytochrome *c* release from the mitochondria but were ineffective in blocking the later phase of cytochrome *c* release that occurred at 24 and 36 h (Fig. 2*D*).

Caspase-3 and -9 Activation Was Detected As Early As 8 h but Reached Its Maximum at 36 h. Caspase-3 has been established as an effector caspase whose activation commits cells to apoptosis by cleavage of important intracellular substrates like DFF-45 and PARP. The activation profile of caspase-3 on exposure to DETA-NONOate was measured using Ac-DEVD-AMC as substrate. After exposure of the cells to NO, activation of caspase-3 was detected as early as 8 h (2-fold), but it was only at 36 h that caspase-3 activity increased 8-fold. The 2–3-fold increased caspase-3 activity found in MDA-MB-468 cells that were in contact with DETA-NONOate for 24 h or less, decreased to control levels within 24 h after the cells were kept in NO-free medium (Fig. 3*A*). This was confirmed by Western analysis when cleaved fragments of caspase-3 were no longer observed after the cells were kept in NO-free medium (Fig. 3*B*). In contrast, after exposure to NO for 36 and 48 h, cleaved fragments persisted even after the cells were kept in NO-free medium for another 24 h (Fig. 3*B*). Fluorometric analysis using Ac-LEHD-AMC as substrate revealed a 2-fold activation of caspase-9 at 8 h, but at 36 h, its activity sharply increased by 10-fold (Fig. 3*C*). This initial low activity was also reflected in Western analysis in which pro-caspase-9, detected as a band of M_r 55,000, was found partially cleaved to a M_r 45,000 band at 8 h (data not shown). Inhibition of caspase-9 by its specific inhibitor, LEHD-CHO, completely inhibited caspase-3 activity (Fig. 3*D*). No significant activation of caspase-8 was found after treatment with the DETA-NONOate (data not shown). It, therefore, appears that after exposure of MDA-MB-468 cells to NO, activation of caspase-9 and -3 was detected as early as 8 h after exposure but peaked only at 36–48 h.

Translocation of Cytosolic Bax to Mitochondrial Membrane at 24–36 h. Because depolarization of MMP was not associated with the peak release of cytochrome *c* into the cytosol after exposure of MDA-MB-468 cells to NO, we assessed other possible mechanisms for the cytochrome *c* release into the cytosol at later time points. It has recently been reported that apoptosis is stimulated by the insertion of

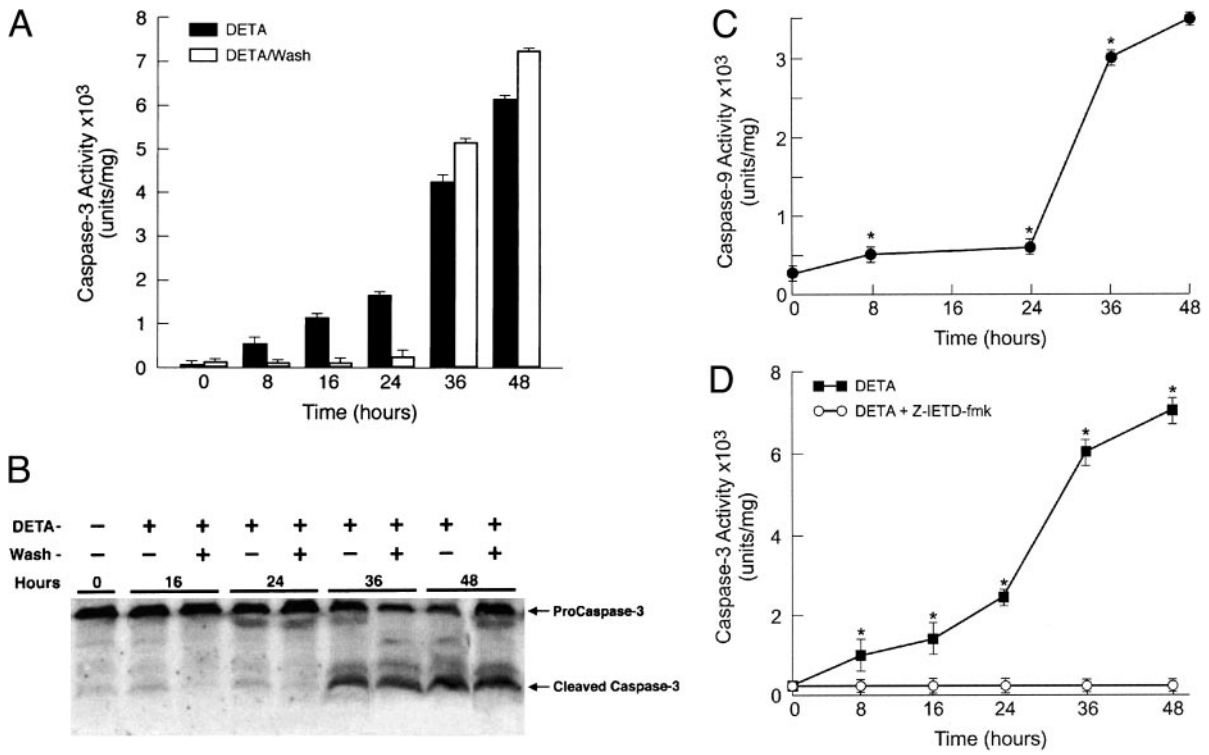

Fig. 3. Cells were treated with DETA-NONOate (1 mM), as described in Fig. 1*A*. In *A,* caspase-3 activation was measured using 3 μg of total cell lysate. Results are expressed as the mean of three different experiments ± SE. Two-way ANOVA analysis was performed, followed by Bonferroni post-tests to look at significant differences between the different groups. *, the washout values after DETA-NONOate treatments (*DETA/Wash, white columns*) are significantly different from those that were treated with DETA-NONOate without any washout (*DETA, black columns*). In *B,* Western analysis was performed using 30 μg of total cell lysate and anti-caspase-3 antibody. In *C*, caspase-9 activation was measured using 6 μg of total cell lysate. Results are expressed as the mean of three different experiments ± SE. *, the values are significantly different from the control; $P < 0.01$. In *D,* cells were treated either with DETA-NONOate (1 mM) alone or in the presence of a caspase-9 inhibitor Z-IETD–fmk for various time periods, and caspase-3 activity was measured using 3 μg of total cell lysate. Results are expressed as the mean of three different experiments ± SE. *, the values are significantly different from the control; $P < 0.01$.

Bax, a cytosolic protein, from cytosol into the mitochondrial membrane (14). Cell fractionation studies were done, and levels of Bax in the cytosol and mitochondria were monitored after treatment with DETA-NONOate at various time points. In control cells, Bax was mainly localized in the cytosol or loosely associated with the mitochondria, with none found integrated into the mitochondrial membrane. Integrated proteins in the mitochondrial membrane were assessed by treating the mitochondrial fractions of the cells with 100 mM Na_2CO_3 (pH 11.5), followed by ultracentrifugation. After 24 h of exposure to NO, there was a significant change in the intracellular localization of Bax, which was integrated into the mitochondrial membranes (Fig. 4*A, top panel*). Native gel revealed only higher oligomers of Bax integrated in the mitochondrial membrane at later time points of DETA-NONOate treatment (Fig. 4*C*). Cytosolic Bax was found to decrease after 24 h of DETA-NONOate treatment (Fig. 4*A, middle panel*).

Antisense Bax Oligonucleotides Suppressed the DETA-NONOate-induced Apoptosis in MDA-MB-468 Cells. We observed that, after exposure of these cells to NO for 24 h, Bax translocated to the mitochondrial membrane, and the cells were committed to apoptosis. This suggested that Bax could be playing a central role during NO-induced apoptosis. To further confirm this, we performed antisense experiments to significantly decrease the intracellular Bax expression in MDA-MB-468 cells and assess the effect of NO on these cells. We observed that antisense Bax oligonucleotides (6 μM) decreased the intracellular Bax levels by 80% (Fig. 5*A*), whereas antisense Bax-scrambled oligonucleotides were unable to decrease the Bax levels to a significant degree (Fig. 5*B*), indicating the specificity of antisense Bax oligonucleotides. Control-scrambled oligonucleotide and Bax-antisense treated cells were exposed to DETA-NONOate for

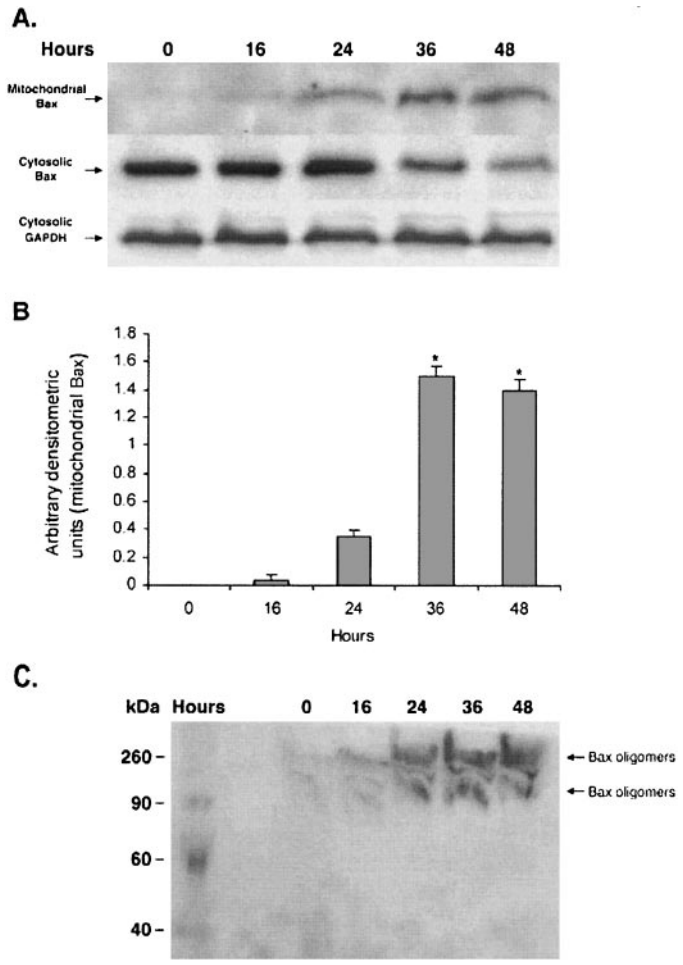

Fig. 4. Time course of translocation of cytosolic Bax to the mitochondrial membrane. *A,* cells were treated with DETA-NONOate (1 mM) for various time periods, and cell fractionation was performed to separate the mitochondrial fraction, which was treated with 100 mM sodium carbonate (pH 11.5) to remove the loosely bound proteins on the surface of the mitochondrial membrane; Western analysis was done using anti-Bax antibody (*top panel*). Cytosolic fractions (10 μg) were analyzed by Western blot using anti-Bax antibody (*middle panel*). *B,* densitometric units of mitochondrial bax from *A*. Results are expressed as the mean of three different experiments ± SE. ∗, the values are significantly different from the control; $P < 0.01$. *C,* cells (3×10^6) were treated with DETA-NONOate (1 mM) for various time periods, and mitochondrial fractions, obtained after alkaline treatment, as in *A,* were electrophoresed on a 8% native polyacrylamide gel. Western analysis was performed using anti-Bax antibody. kDa, M_r in thousands.

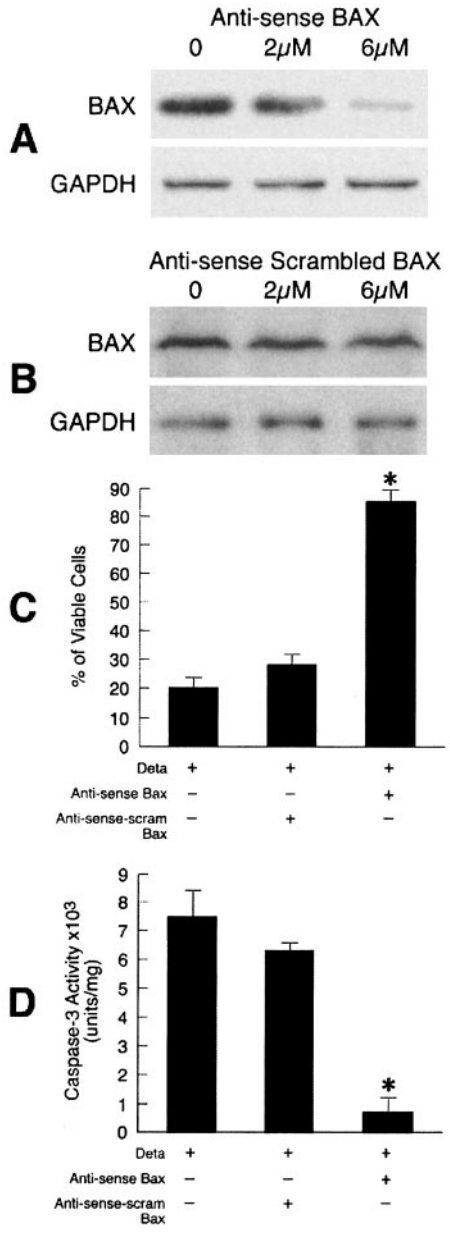

Fig. 5. Antisense Bax expression protects MDA-MB-468 cells from DETA-NONOate-induced cell death and caspase-3 activation. Cells (5×10^5) were seeded overnight on a 6-well plate and were treated with (*A*) phosphorothioate-modified antisense Bax oligonucleotides or (*B*) antisense scrambled Bax oligonucleotides, as described in "Materials and Methods." Western analysis was performed using 30 μg of total cell lysate with either anti-Bax or anti-GAPDH antibody. *C,* cells were treated as in *A* and *B,* and the percentage of viable cells were determined using trypan blue. Results were expressed as the mean of three different experiments ± SE. ∗, the values are significantly different from the control; $P < 0.01$. *D,* 3 μg of total cell lysate obtained after treatment of cells as in *A* and *B* were analyzed for caspase-3 activation. Results were expressed as the mean of three different experiments ± SE. ∗, the values are significantly different from the controls; $P < 0.01$.

48 h; and cell viability was measured, and caspase-3 activity was assayed. Eighty percent of cells treated with antisense Bax oligonucleotides were found viable after 48 h of DETA-NONOate treatment, compared with control cells, which were not so treated (Fig. 5*C*). Cells treated with antisense Bax oligonucleotides were found to have 90% less caspase-3 activity compared with control MDA-MB-468 cells (Fig. 5*D*). Therefore, lowering the Bax levels in MDA-MB-468 cells suppressed the induction of apoptosis after 48 h of exposure to NO.

Overexpression of Bcl-2 Suppressed DETA-NONOate-induced Apoptosis. Bcl-2 overexpression has been reported to interfere with the Bax oligomerization and subsequent integration into the mitochondrial membrane (12). Because Bax integration into the mitochondrial membrane was found to be associated with commitment of these cells to apoptosis in this cell line, we wanted to examine the effect of Bcl-2 overexpression on DETA-NONOate induced apoptosis. Human Bcl-2 was overexpressed in these cells as described in "Materials and Methods," and overexpression was confirmed by Western blot analysis (Fig. 6*A*). MDA-MB-468 cells and MDA-MB-468/Bcl-2 cells were exposed to DETA-NONOate for 48 h, after which cell viability and caspase-3 activity was assayed. After 48 h of DETA-NONOate treatment, 90% of the MDA-MB-468/Bcl-2 cells were found viable as compared with only 15% of control MDA-MB-468 (Fig. 6*B*). Similarly, caspase-3 activity was found suppressed in MDA-MB-468/Bcl-2 cells by 80% as compared with control MDA-MB-468 cells (Fig. 6*C*). Exposure to DETA-NONOate did not increase cytochrome *c* levels in the cytosol of MDA-MB-468/Bcl-2 cells as compared with control MDA-MB-468 cells, in which a significant increase of cytochrome *c* was detected at ~24–36 h of DETA-NONOate treatment (Fig. 6*D*). Furthermore, after exposure to NO, Bax was not found integrated in the mitochondria of MDA-MB-468/Bcl-2 cells, indicating that Bcl-2 interfered with mitochondrial Bax integration (data not shown). To examine the effect of DETA-NONOate on the native forms of Bax in the cytosol, cell fractionation was performed, and cytosolic fractions from MDA-MB-468 and MDA-MB-468/Bcl-2 were run on native gradient gel; Western analysis was then performed. No oligomer of Bax was found in 468/Bcl-2 compared with MDA-MB-468 cells, in which DETA-NONOate-induced oligomers were seen at 16–24 h in the cytosolic fractions, but disappeared at later time points from the cytosol (data not shown).

DETA-NONOate leads to early decrease in Akt protein levels. Recently it has been found that Akt regulates cell survival and apoptosis at a premitochondrial stage by preventing Bax conformation change and its translocation to the mitochondria (16). We, therefore, assessed the levels of cytosolic and total cellular Akt in MDA-MB-468 cells after DETA-NONOate treatment to understand the potential role of Akt in Bax translocation to the mitochondria. Cells treated with DETA-NONOate for various time periods were lysed in cell lysis buffer as described in "Materials and Methods," and the cytosolic fraction was analyzed by Western blot for Akt. We observed a 50% decline of Akt levels in the cytosolic fraction of cells exposed to NO for 16–24 h (Fig. 7*A*). ERK1/2 are components of the MAPK pathway and plays an important role in cell proliferation, whereas p38 MAPK, a stress-regulated kinase, is activated in NO-induced apoptosis. To determine the specificity of DETA-NONOate-induced Akt decline, the lysates were Western blotted for ERK1/2, and p38 kinase. DETA-NONOate specifically inhibited Akt, whereas the levels of p38 kinase and ERK1/2 kinase did not change (Fig. 7*A*). We further analyzed the levels of p-Akt after various treatments with DETA-NONOate and after probing the membrane with p-Akt antibody. We observed that the levels of p-Akt declined similar to the levels of Akt after exposure to DETA-NONOate (Fig. 7*B*). Furthermore, we analyzed the levels of total cellular Akt by Western blot in cell lysates obtained by lysing the

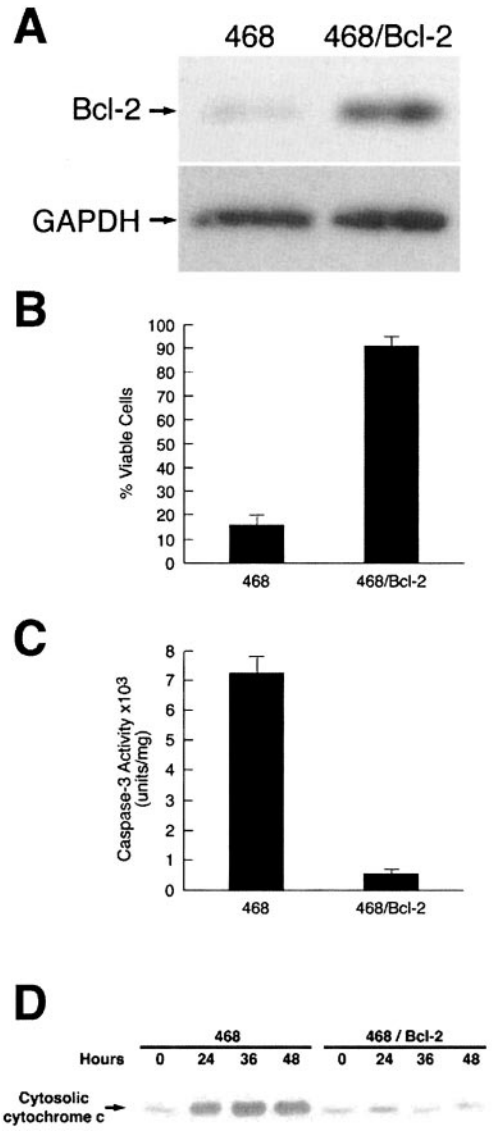

Fig. 6. Bcl-2 overexpression protects MDA-MB-468 cells from DETA-NONOate-induced cell death and caspase-3 activation. In *A,* cells (3×10^5) were seeded overnight on a six-well plate and were transiently transfected with 1 μg of C3-EGFP-Bcl-2 plasmid. Stably transfected cells overexpressing Bcl-2 were selected in the presence of G418 (600 μg/ml) as described in "Materials and Methods," and Western analysis was performed with 10 μg of total cell lysate using anti-Bcl-2 or anti-GAPDH antibody. *B,* control (*468*) and Bcl-2-overexpressing 468 (*468/Bcl-2*) were treated with DETA-NONOate for 48 h, and the percentages of viable cells were determined using the trypan blue method. Results are expressed as the mean of three different experiments ± SE. ∗, the values are significantly different from the control; $P < 0.01$. *C,* 468 and 468/Bcl-2 cells, treated with DETA-NONOate for 48 h, were lysed, and 3 μg of total cell lysates were analyzed for caspase-3 activation. Results are expressed as the mean of three different experiments ± SE. ∗, the values are significantly different from the control; $P < 0.01$. *D,* 468 and 468/Bcl-2 cells were treated with DETA-NONOate for various time periods. Cytosolic fractions, obtained as described in "Materials and Methods," were analyzed by Western blot using anti-cytochrome *c* antibody.

cells with RIPA buffer containing 0.5% SDS. We found that, in the total cell lysate, there was a lag in the decline of Akt levels that started at ~24 h (Fig. 7*C*) comparable with the lag at 16 h in the cytosolic fractions. Fig. 7*D* shows the densitometric analysis of the blot from Fig. 7*C*. DETA-NONOate led to a decline in p-Akt levels at 16–24 h, whereas the total Akt levels started to decrease only at 24 h.

Akt Levels Affected the Sensitivity of DETA-NONOate-induced Apoptosis. To further investigate the role of Akt in NO-induced apoptosis, we varied the Akt levels in the cells by growing them in media containing either 0% or 20% FBS. The cells were then trans-

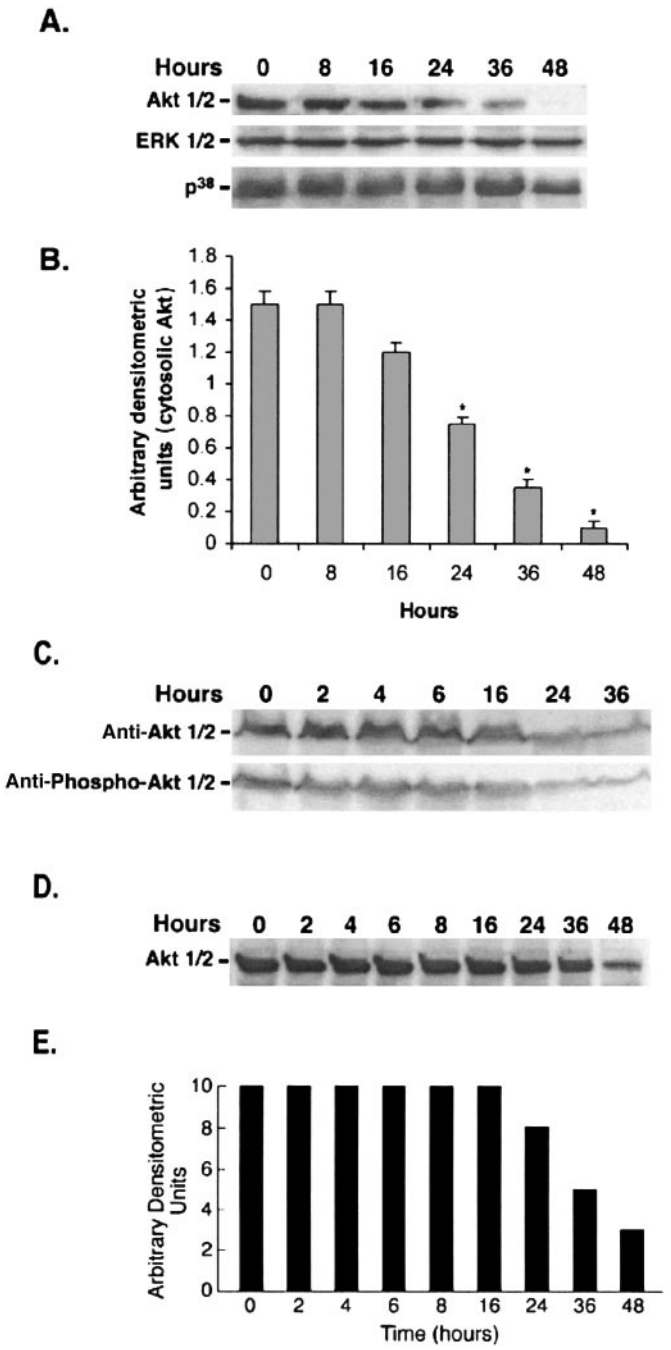

Fig. 7. DETA-NONOate down-regulates Akt protein. Cells (3×10^6), treated with DETA-NONOate for various time periods, were lysed in cell lysis buffer, and 10 μg of the cytosolic fractions were analyzed by Western blot using (*A*) anti-Akt1/2, anti-ERK1/2, and p38 antibodies; (*B*) analysis using densitometric units of cytosolic Akt from *A*. Results are expressed as the mean of three different experiments ± SE. ∗, the values are significantly different from the control; $P < 0.01$. *C,* Western blot analysis using anti-Akt1/2 and anti-p-Akt1/2 antibodies. In *D,* cells, treated with DETA-NONOate for various time periods, were lysed in RIPA buffer containing 0.5% SDS; and 15 μg of total cellular protein were analyzed by Western blot using Akt1/2 antibody. *E,* densitometric analysis of Western blot analysis of *C*.

ferred in medium containing 5% FBS after which these cells were treated with DETA-NONOate for various time periods and prepared for a Western analysis to detect the levels of p-Akt and a caspase-3 assay for the induction of apoptosis. Cells grown in medium without FBS had less p-Akt in control cells compared with cells grown in 20% FBS (Fig. 8*A*), and were more sensitive to DETA-NONOate-induced apoptosis, as detected by a 6-fold increase in caspase-3 activity as early as 24 h (Fig. 8*B*). On the other hand, cells grown in 20% FBS had increased p-Akt levels with no caspase-3 activity at 24 h, whereas only at 48 h was a 5-fold increase in caspase-3 activation seen (Fig. 8*B*). This experiment clearly indicated that the levels of Akt affected the sensitivity of DETA-NONOate-induced apoptosis.

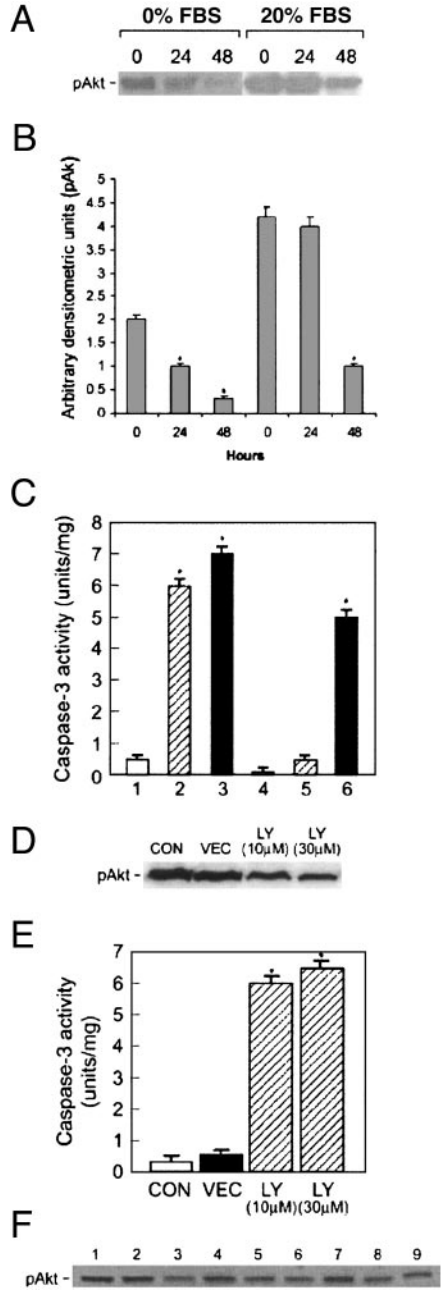

Fig. 8. Decrease of p-Akt is essential in DETA-NONOate-induced apoptosis. Cells initially grown in 0 and 20% FBS were then treated with DETA-NONOate for 24 and 48 h in the presence of 5% FBS and were prepared for (*A*) Western analysis and probed with p-Akt antibody; (*B*) densitometric units of cytosolic p-Akt from *A*. Results are expressed as the mean of three different experiments ± SE. ∗, the values are significantly different from the control; $P < 0.01$. *C,* caspase-3 fluorometric assay as described in "Materials and Methods"; with 0% FBS (control, *Lane 1*), 24-h DETA-NONOate treated (*Lane 2*), and 48-h DETA-NONOate treated (*Lane 3*); with 20% FBS (control, *Lane 4*), 24-h DETA-NONOate treated (*Lane 5*), and 48-h DETA-NONOate treated (*Lane 6*). *D,* cells, treated for 48 h with ethanol (●●●) or LY294002 in different concentrations [*LY* (10 μM) and *LY* (30 μM)], were prepared for Western analysis and probed with p-Akt antibody. *E,* cells, treated as described in *C,* were prepared for caspase-3 fluorometric assay. *F,* cells were treated with control (*Lane 1*); DMSO (*Lane 2*); DETA-NONOate (*Lane 3*); caspase-3 I (II) (25 μM; *Lane 4*); caspase-3 I (II) (10 μM)+DETA-NONOate (*Lane 5*); caspase-3 I (II) (25 μM)+DETA-NONOate (*Lane 6*); caspase-3 I (V) (25 μM; *Lane 7*); caspase-3 I (V) (10 μM)+DETA-NONOate (*Lane 8*); caspase-3 I (V) (25 μM)+DETA-NONOate (*Lane 9*).

We next wanted to examine whether the inhibition of the Akt pathway was sufficient to induce apoptosis in MDA-MB-468 cells. Cell-permeable LY294002, which is a PI3K inhibitor (17), was used to inhibit the Akt pathway. The cells treated with LY294002 (10–30 μM) for 48 h, were prepared for Western analysis and for caspase-3 assay. Cells treated with LY294002, had decreased levels of p-Akt (Fig. 8*C*) and caspase-3 activity was increased by 6-fold (Fig. 8*D*), indicating apoptosis in these cells.

We further wanted to examine whether the activation of caspase-3 led to a decrease in Akt levels as has been found by some investigators (28) in other cell lines. The cells treated with caspase-3 inhibitor (II) (Z-DEVD-fmk) or (V) [Z-VK-X (Biotin)-D-fmk] and DETA-NONOate, were prepared for Western analysis. We found that even in the presence of caspase-3 inhibitor, DETA-NONOate treatment decreased p-Akt levels (Fig. 8*E*).

DISCUSSION

The primary objective of this study was to assess the mechanism(s) by which NO induced apoptosis in a human breast cancer cell line MDA-MB-468. We observed that the maximal percentage of fragmented DNA was observed after exposure of these cells to 1 mM DETA-NONOate for 48 h. DNA fragmentation continued even after the cells were exposed to NO for 48 h and then kept in a NO-free medium for another 24 h. This indicated that these cells were committed to apoptosis after exposure to NO for 48 h, and all of the additional experiments were, therefore, carried out within this time period.

NO-induced apoptosis has been studied in a variety of cell types, and a direct action of NO on the mitochondria leading to membrane depolarization followed by cytochrome *c* release has been suggested as the major mechanism (29, 30). We, therefore, elected to study the possible effect of NO on the depolarization of MMP. In our studies, we did observe an initial depolarization of the MMP as early as 4 h, but the MMP returned to normal values. There was a second phase of depolarization at 24 h, which also was short lived, and the MMP rapidly returned to normal values. The release of cytochrome *c* after the initial depolarization of MMP occurred via the PTPC, because this release was blocked in the presence of both cyclosporin A and bongkrekic acid, which are known blockers of the PTPC. On the other hand, the second phase of cytochrome *c* release was not blocked by these pore blockers, suggesting that formation of PTPC was not involved in this phase of cytochrome *c* release and that other mechanism(s) need to be considered.

Because cytochrome *c* release was followed by the activation of caspase-9 and -3, we next wanted to elucidate whether the pattern of activation of these caspases could be correlated to the pattern of cytochrome *c* release. There was a slight but significant increase in both caspase-9 and caspase-3 activities by 8 h after exposure to NO, and a marked increase in the activities of both of these caspases was observed at 36 and 48 h. This indicated to us that the magnitude of activation of these caspases was dependent on the magnitude of cytochrome *c* release into the cytosol. The activation of caspase-3 was directly caused by activation of caspase-9, because a specific caspase-9 inhibitor completely prevented caspase-3 activation, thus ruling out the possibility of activation of caspase-3 through other pathways.

In view of the different pattern of cytochrome *c* release and activation of the caspase-9 and -3 seen in MDA-MB 468 for up to 24 h of exposure to NO, when compared with that observed after exposure to NO for 36 h or more, we wanted to assess whether the commitment of the cells to apoptosis also differed between these two phases. This was studied by our washout experiments wherein we exposed the cells to NO for variable time periods and then maintained the cells in NO-free medium for 24 h. MDA-MB-468 cells were, therefore, exposed to NO for various periods of time for up to 24 h and then kept in NO-free medium for 24 h. We observed that the caspase-3 activity, proteolytic cleavage of caspase-3, DNA fragmentation, and cleavage of DFF-45 were all significantly less after the removal of NO, when compared with the cells that had been exposed to NO for the same duration of time. This indicated that the progression of cells to apoptosis could be reduced after the removal of the apoptotic stimulus. On the other hand, the cells were committed to apoptosis after exposure of these cells to NO for more than 36 h. It, therefore, appears that certain critical events must occur for the cells to be committed to apoptosis and that it is necessary for MDA-MB-468 cells to be exposed to NO for at least 36 h. Our results, therefore, suggest that the amount of cytochrome *c* release determines whether the cells are committed to apoptosis. This may explain why, after initial release of cytochrome *c* into the cytosol by NO, the removal of the apoptotic stimulus prevents the progression of the cells to apoptosis.

The massive release of cytochrome *c* into the cytosol after exposure of the MDA-MB-468 to NO for 36 h was not associated with depolarization of the mitochondria and was not attenuated by cyclosporin A or bongkrekic acid. On this basis, we concluded that the general concept forwarded by others that NO induces apoptosis by a direct action on the mitochondria leading to depolarization and release of cytochrome *c* through the PTPC (29, 30) was unlikely to be a universal phenomenon. We, therefore, proceeded to elucidate other mechanism(s) by which the second and more important phase of massive cytochrome *c* release occurred and to assess whether NO acted on the premitochondrial initiation phase to commit the cells to apoptosis.

We then assessed whether the second and critical phase of cytochrome *c* release was caused by the integration of Bax into the mitochondrial membrane and whether this integration was responsible for the commitment of the cells to NO-induced apoptosis. We observed that the latter phase of cytochrome *c* release was mediated by Bax integration into the mitochondria because this latter phase was not blocked by the PTPC blockers. A high level of Bax is constitutively expressed in MDA-MB-468 cells, and we did not observe an additional increase in Bax levels by NO. After 24-h exposure of these cells to NO, Bax started migrating from the cytosol to the mitochondria and integrated into the mitochondrial membrane. The migration and integration of Bax into the mitochondrial membrane increased progressively with time, and maximal effects were seen at 48 h. The magnitude of Bax integration into the mitochondrial membrane correlated very well with the second phase of cytochrome *c* release. Because this second phase of cytochrome *c* release was not blocked by the PTPC blocker cyclosporin A or bongkrekic acid, it seems that the integration of Bax into the mitochondrial membrane must have been responsible for the second phase of cytochrome *c* release from the mitochondria, which then led to apoptosis. This was further confirmed by assessing the effects of NO on MDA-MB-468 cells transfected with antisense to Bax and comparing the effects with cells transfected with scrambled antisense to Bax. There was a concentration-related decrease in Bax expression in cells transfected with antisense to Bax, and the amount of Bax expressed in these cells correlated positively with the amount of caspase-3 activity after exposure of these cells to NO and inversely with the number of viable cells.

The mechanism by which overexpression of Bax leads to cytochrome *c* release, and thereby to apoptosis, can vary in different cell types. Induction of the overexpression of Bax in stably transfected Jurkat cells induced the PTPC, which was accompanied by typical features of apoptosis including cytochrome *c* release, caspase activa-

tion, cleavage of PARP, DNA fragmentation, and cell death (31). These events were triggered just by overexpression of Bax. The apoptotic effects were abolished in the presence of a combination of cyclosporin A and aristolochic acid, a phospholipase A2 inhibitor, which suggests that the effects of Bax in Jurkat cells were mediated by PTPC. However, other investigators demonstrated that, although overexpression of Bax in HeLa cells also led to the release of cytochrome *c*, this was not blocked by inhibitors of the PTPC like cyclosporin A or bongkrekic acid (32). These results indicated that the release of cytochrome *c* induced by Bax overexpression in HeLa cells was independent of the PTPC. On these bases, some investigators have proposed that Bax may interact indirectly with one or more proteins that reside in either the inner or the outer mitochondrial membrane and that regulate or constitute the mitochondrial PTPC (32). Alternatively, other investigators have proposed that Bax may itself form a channel that modifies ion fluxes across the mitochondrial membranes (31).

Some investigators have observed that NO increases Bax expression in certain cell lines thereby altering the Bax:Bcl_2 ratio (33–36). However, these investigators did not assess the mechanism by which NO-induced Bax expression led to apoptosis. Murine embryonic fibroblast from $Bax^{-/-}$ mice that were exposed to NO failed to undergo apoptosis (37), indicating that Bax is essential for NO-induced apoptosis in some cell types. In MDA-MB-468 cells, NO did not increase Bax levels further, probably because Bax is already overexpressed in this cell line. However, despite the presence of high levels of Bax in this cell line, apoptosis did not occur until the cells were exposed to an apoptotic agent like NO. This may be a unique feature of some cancer cell lines in which some mechanism may exist to retain Bax in the cytosolic compartment and in which redistribution of Bax to the mitochondria may occur only after exposure of the cells to an apoptotic agent. The precise mechanism by which NO leads to Bax migration is not known, and studies are being carried out to elucidate the mechanism(s) involved.

Our results, therefore, demonstrate that release of cytochrome *c* in the course of apoptosis can occur through two non-mutually exclusive mechanisms as has been suggested by others (38). We have previously demonstrated that N^w hydroxy-L-arginine, an intermediate formed during NO biosynthesis, induced cytochrome *c* release followed by apoptosis in this cell line by cleavage of Bid to tBid (truncated Bid) by a Bax-independent mechanism (9). Integration of Bax into the mitochondrial membrane is, therefore, not an essential prerequisite for the induction of apoptosis in this cell line and may vary with the apoptotic agent used.

The MDA-MB-468 cells constitutively express very little of the antiapoptotic protein Bcl-2. It has recently been reported that expression of Bcl-2 is capable of inhibiting Bax translocation and integration into the mitochondria (13), and overexpression of Bcl-2 has been reported to interfere with Bax oligomerization and subsequent integration into the mitochondrial membrane (14). Our results indicated that overexpression of Bcl-2, which is capable of inhibiting Bax translocation and integration into the mitochondrial membrane, protected MDA-MB-468 cells from the apoptotic actions of NO. On this basis, we concluded that the integration of Bax into the mitochondrial membrane was responsible for the second phase of cytochrome *c* release, and it was critical for these cells to undergo apoptosis after exposures to NO.

It has been demonstrated that Akt regulates cell survival and apoptosis by the suppression of Bax translocation (16, 17). Our results indicate that Akt may be involved in NO-induced apoptosis because the levels of both total and p-Akt kinase were significantly reduced (50%) by 16 h after exposure to NO, whereas the total protein levels of ERK1/2 and p38 kinase did not change. This reduction in Akt kinase by NO was not caused by a decrease in gene expression[4] but was most likely caused by initial redistribution followed by the effects of NO on translational or posttranslational events similar to those that were previously reported by us for cyclin D1 (23). We further confirmed that the decrease in Akt levels was essential for NO-induced apoptosis. This was assessed by varying the levels of Akt in the cells by growing them in media containing 0% and 20% FBS; we found that the sensitivity of DETA-NONOate-induced apoptosis directly correlated with the levels of Akt in the cells. DETA-NONOate led to early induction of apoptosis in cells containing less Akt compared with cells in which Akt levels were high. The importance of Akt levels was emphasized in experiments in which the Akt pathway was inhibited by inhibitor LY294002. Inhibition of the Akt pathway was sufficient to induce apoptosis in these cells. We also observed that the decrease in the p-Akt levels was not caused by activation of the caspase-3, because the decrease was also seen in the presence of caspase-3 inhibitors. These experiments also indicate that the decline in Akt level is an event upstream from Bax integration in NO-induced apoptosis.

In conclusion, our results demonstrate that NO-induced cytochrome *c* release is biphasic. The initial release was preceded by membrane depolarization, but the amount of cytochrome *c* release did not commit the cells to the apoptotic pathway. On the other hand, the second phase of cytochrome *c* release, which was of a much greater magnitude, occurred after Bax migration from the cytosol and oligomerization and integration into the mitochondrial membrane. This second phase of cytochrome *c* release committed the cells to apoptosis. The migration of Bax to the mitochondria was most likely caused by reduction in both the total as well as the phosphorylated form of Akt kinase. This reduction of Akt was attributable to an action of NO on translational or posttranslational events and was not attributable to a decrease in *Akt* gene expression. In this study, we did not assess the precise mechanism(s) by which NO attenuates the expression of Akt kinase. Further work is in progress to elucidate whether NO directly affects the levels of Akt or whether NO affects targets farther upstream from Akt.

ACKNOWLEDGMENTS

We thank Janis Cuevas and Svetlana Arutyunova for their technical assistance.

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