Caspase-8-mediated BID Cleavage and Release of Mitochondrial Cytochrome c during \(N^\omega\)-Hydroxy-L-arginine-induced Apoptosis in MDA-MB-468 Cells

ANTAGONISTIC EFFECTS OF L-ORNITHINE*

Received for publication, April 15, 2002, and in revised form, July 23, 2002
Published, JBC Papers in Press, July 26, 2002, DOI 10.1074/jbc.M203648200

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We have previously reported that \(N^\omega\)-hydroxy-L-arginine (NOHA), a stable intermediate product formed during the conversion of L-arginine to nitric oxide, induced apoptosis in MDA-MB-468 cells, and this action was antagonized in the presence of L-ornithine. We also reported that apoptosis induced by NOHA in this cell line could not be explained on the basis of a reduction of intracellular polyamines. In the current study, we investigated other potential mechanism(s) by which NOHA may have induced apoptosis in this cell line. We observed that NOHA initially activated caspase-8 and induced cleavage of BH\(_3\) interacting domain. This was followed by release of cytochrome c and subsequently, activation of downstream caspases-9 and -3 to cleave poly(ADP-ribose) polymerase. We also observed that NOHA induced a rapid and persistent hyperpolarization of the mitochondrial membrane potential rather than depolarization indicating that the release of cytochrome c by NOHA was by a mechanism independent of the mitochondrial transition pore. Exogenous l-ornithine did not inhibit NOHA-induced caspase-8 activation and cleavage of BH\(_3\) interacting domain but acted at the mitochondrial level and inhibited the NOHA-induced cytochrome c release and apoptosis.

Metabolic products of arginine modulate the growth of many types of cancer cells. Arginine is converted by arginase to ornithine, the only source of synthesis of the polyamines putrescine, spermidine, and spermine in mammalian cells, which are essential for cell proliferation and regulation of the cell cycle (1, 2). On the other hand, arginine is also catalyzed by the enzyme nitric-oxide synthase (NOS)\(^3\) to form \(N^\omega\)-hydroxy-L-arginine (NOHA) as an intermediate that subsequently forms nitric oxide (NO) (3). NO causes cytostasis (4–6) and apoptosis of cancer cells (7–10) and also affects the cell cycle (4). Thus, depending on the predominant metabolic pathway for arginine present in cells, products of arginine metabolism can cause either cell proliferation or cytostasis followed by apoptosis. We (11) and others (12) have previously demonstrated that polyamines are essential for proliferation of some breast tumor cells and that inhibition of polyamine biosynthesis led to inhibition of cell proliferation followed by apoptosis (11). The polyamine biosynthesis occurs from arginine, which initially is converted by arginase to ornithine. Ornithine is then converted to putrescine by ornithine decarboxylase (13). S-Adenosylmethionine decarboxylase (SAMDC), which converts S-adenosylmethionine (SAM) to decarboxylated SAM, is required for the conversion of putrescine to spermidine (13). Inhibitors of ornithine decarboxylase and SAMDC have been shown to reduce proliferation of various types of malignant cells (14, 15) including breast cancer cells (16), and this was accompanied by a reduction in intracellular polyamine levels.

We have previously demonstrated that breast cancer cell lines that predominately expressed arginase had a higher rate of proliferation when compared with cell lines that predominately expressed NOS (11), thereby demonstrating how products of arginine metabolism may modulate cell proliferation. NOHA inhibited arginase in MDA-MB-468 cells, and this initially led to cytostasis followed by activation of caspase-3. This was accompanied by a decrease in intracellular polyamine levels. As this action of NOHA was abolished in the presence of exogenous l-ornithine, we speculated that the action of NOHA in inducing apoptosis in this cell line was most likely due to a reduction in intracellular polyamine levels. However, in this cell line, when we added difluoromethyl ornithine, an inhibitor of ornithine decarboxylase, or SAM-486A, an inhibitor of SAMDC, either alone or in combination, there was a reduction in intracellular polyamine content, but only cytostasis was observed, not apoptosis (17). On this basis, we concluded that the apoptotic action of NOHA in MDA-MB-468 cells cannot be explained solely on the basis of a reduction in intracellular polyamine levels and that other mechanisms must also be considered. The present work was, therefore, undertaken to elucidate the mechanism(s) involved by which NOHA induced apoptosis in this cell line. MDA-MB-468 cells were selected for our study as this cell line expresses high arginase activity with very little NOS (11). Thus, the effects of NOHA can be studied without any confounding influence of endogenous NOHA and NO produced by these cells.

* This work was supported in part by Palomba Weingarten, the Alleghara Charach Cancer Research Fund, and United States Public Health Service Grant CA-78357 (to G. C). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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\(^3\) The abbreviations used are: NOS, nitric-oxide synthase; AMC, 7-amino-4-methylcoumarin; ACF, 7-amino-4-trifluoromethylcoumarin; BID, BH\(_3\) interacting domain; tBID, truncated BID; CMX-Ros, chloromethyl X-Rosamine; fmk, fluoromethyl ketone; MMF, mitochondrial membrane potential; NOHA, \(N^\omega\)-hydroxy-L-arginine; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; SAMDC, S-adenosylmethionine decarboxylase; TNF-\(\alpha\), tumor necrosis factor-\(\alpha\); TNF-R1, tumor necrosis factor-receptor 1.
Cayman Biochemicals (Ann Arbor, MI). L-Ornithine hydrochloride was purchased from Alexis Biochemicals (San Diego, CA). Z-DEVD-fmk, Z-IETD-fmk, Z-LEHD-fmk, and Z-VAD-fmk were purchased from Calbiochem. Antibodies were obtained from the following suppliers: rabbit polyclonal anti-caspase-3 and mouse monoclonal anti-cytochrome c antibodies were from Santa Cruz Biotechnology (San Diego, CA); and anti-cytochrome c oxidase antibody was from CLONTECH (Palo Alto, CA). BID antibody was a kind gift from Dr. S. Korsmeyor (Harvard Medical School). Mitotracker Red chloromethyl X-Rosamine (CMX-Ros) was purchased from Molecular Probes (Eugene, OR). Valinomycin and gramicidin were purchased from Sigma.

Cell Culture—Human breast cancer cell line MDA-MB-468 (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium containing 10% nonessential amino acids, 2 mM l-glutamine, 1 μg/ml insulin, and 10% fetal bovine serum. For experimental purposes, cells were grown in 5% fetal bovine serum, allowed to seed overnight, and treated with drugs for various durations. Cells were lysed in lysis buffer as previously described (7). The lysates were used for caspase-3 (3 μg), caspase-9 (6 μg), and caspase-8 (15 μg) assays using respective substrates. The released specific cleavage of respective substrates becomes fluorescent were quantified using a fluorometer (VersaFluroTM, Bio-Rad) with emission at 440 nm for AMC substrates (7) and at excitation 380 nm and emission at 440 nm for Z-VAD-fmk and Z-DEVD-fmk, respectively.

Western Analysis—Cells were lysed by a Western blot with anti-caspase-3 antibody and anti-PARP antibody (1:1000) overnight followed by subsequent incubation with 1:1000 dilutions of horseradish peroxidase-linked F(ab)2 fragment secondary antibody (Amersham Biosciences) for 1 h. Immunoreactive bands were visualized by the enhanced chemiluminescence detection system (Amersham Biosciences).

Detection of Cytochrome c Release into the Cytosol—Cytochrome c release into the cytosol was detected as described previously with minor modifications (7). Briefly, 6 × 10^6 cells were harvested and washed with phosphate-buffered saline (PBS). The cells were suspended in Buffer A (20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1,5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose, 1 μM protease inhibitor mixture) and homogenized by Dounce homogenizer; unbroken cells and nuclei were removed by centrifugation at 1,000 × g for 10 min at 4 °C. The supernatant was further centrifuged at 10,000 × g for 20 min. The supernatant was saved as a cytosolic fraction while the precipitate was suspended in Buffer A containing 0.5% (v/v) Nonidet P-40 and was saved as the mitochondrial fraction. The mitochondrial and cytosolic fractions were analyzed by Western blot with an anti-cytochrome c monoclonal antibody or with an anti-cytochrome c oxidase antibody.

Measurement of Mitochondrial Membrane Potential by Flow Cytometry—1 × 10^6 cells were harvested, 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. Valinomycin (10 μM), a potassium ionophore, which serves as a hyperpolarization control, and gramicidin (10 μM), a relatively non-selective ionophore, which serves as a depolarization control were also included in our experiments in order to validate our results for mitochondrial membrane potential (MMP) assay.

RESULTS

Time Course of Caspase-3 and PARP Induction by NOHA and Antagonistic Effect of L-Ornithine—We have shown previously that NOHA when used alone induced significant caspase-3 activity in MDA-MB-468 cells after 48 h, which was blocked in the presence of exogenous l-ornithine. To understand the precise mechanism(s) by which NOHA induced apoptosis in this cell line, we studied the time course of caspase-3 induction after NOHA treatment. We observed a 4-fold induc-
tion of caspase-3 with NOHA (1 mM) at 32 h, which increased subsequently by 10-fold at 48 h (Fig. 1A). Exogenous L-ornithine (1 mM) added along with NOHA was able to block this NOHA-induced caspase-3 induction. We also assessed caspase-3 activation by Western blot analysis by observing its proteolytic cleavage (Fig. 1B). We observed a 17-kDa band corresponding to cleaved caspase-3 as early as 32 h, the intensity of which increased further at 48 h of NOHA treatment. This cleaved band (17 kDa) was undetectable in samples that received exogenous L-ornithine along with NOHA. We observed that PARP, a caspase-3 substrate, was cleaved as early as 32 h, generating a cleaved 85-kDa fragment (Fig. 1C). At 48 h, there was complete fragmentation of 117-kDa PARP into cleaved fragment (85 kDa). L-Ornithine was able to block this NOHA-induced cleavage as well as proteolytic cleavage of caspase-9.

Time Course of Cytochrome c Release into the Cytosol with NOHA and Effect of Exogenous L-Ornithine—To study the upstream sequence of events during NOHA-induced apoptosis, we assessed the time course of cytochrome c release into the cytosol.

Inhibitors of Caspase-3 or Caspase-9 Block the Induction of NOHA-induced Caspase-3 Activation—To further confirm the role of caspase-9 and -3 in NOHA-induced apoptosis, we studied the effect of their inhibitors. We used Z-DEVD-fmk and Z-LEHD-fmk as caspase-3 and -9 inhibitors, respectively. These inhibitors at 25 μM concentration completely inhibited the NOHA-induced activation of caspase-3 and -9 inhibitors, respectively. This suggests that caspase-9 and -3 play a distinct role in NOHA-induced apoptosis.

Time Course of Caspase-9 Induction by NOHA and Antagonistic Effect of Exogenous L-Ornithine—Activation of caspase-3 could be either via death receptor signaling or due to caspase-9 activation through mitochondrial release of cytochrome c. We observed that caspase-9 was activated during NOHA-induced apoptosis. There was a 2-fold induction of caspase-9 at 24 h followed by a 4-fold induction at 32 h, and it increased further to ~9-fold at 48 h (Fig. 2A). Western blot analysis further indicated that at 32 h there was a proteolytic cleavage of caspase-9 that further increased at 48 h (Fig. 2B). L-Ornithine was able to block this NOHA-induced activation as well as proteolytic cleavage of caspase-9.

Inhibitors of Caspase-3 or Caspase-9 Block the Induction of NOHA-induced Caspase-3 Activation—To further confirm the role of caspase-9 and -3 in NOHA-induced apoptosis, we studied the effect of their inhibitors. We used Z-DEVD-fmk and Z-LEHD-fmk as caspase-3 and -9 inhibitors, respectively. These inhibitors at 25 μM concentration completely inhibited the NOHA-induced activation of caspase-3 and -9 inhibitors, respectively. This suggests that caspase-9 and -3 play a distinct role in NOHA-induced apoptosis.
tosol and correlated this with the time course of caspase-9 and -3 activation. We isolated mitochondrial and cytosolic fractions from cells after different treatments as described under "Materials and Methods." We observed the release of cytochrome c into the cytosol as early as 32 h, which further increased by 4-fold after 48 h of NOHA treatment. However, simultaneous treatment of cells with L-ornithine blocked this release of cytochrome c into the cytosol (Fig. 3A). We also confirmed that our cytosolic fractions had no mitochondrial contamination by probing the membrane with anti-cytochrome-c oxidase antibody (data not shown).

Measurement of MMP by Flow Cytometry—Disruptions in MMP are measured with a number of cationic lipophilic fluorochromes including MitoTracker Red CMX-Ros and flow cytometry (18). Fig. 3B (top panel) shows the validation of the method using a negative control. Addition of dye, CMX-Ros (100 nM), caused a shift in the fluorescence compared with the negative control. Valinomycin, a control for hyperpolarization, further shifted the fluorescence to the right, an indication of hyperpolarization of the MMP. Gramicidin, a control for depolarization, shifted the intensity of fluorescence to the left indicative of depolarized MMP. NOHA induced hyperpolarization of the MMP as early as 3 h, and this hyperpolarization persisted until 48 h, and L-ornithine did not change this NOHA-induced hyperpolarization (Fig. 3B). Similar results were found when we used other dyes like dihexyloxacarbocyanine or rhodamine 123 (data not shown). Thus our results show that release of cytochrome c into the cytosol was not due to the decrease in the MMP.

Time Course of Caspase-8 Induction by NOHA and Effect of L-Ornithine—We studied the time course of activation of caspase-8, an upstream caspase, usually known to act upstream of mitochondrial events. Recent reports have suggested that activation of caspase-8 followed by truncation of BID leads to release of cytochrome c that is independent of decrease in MMP. Fig. 4A shows that caspase-8 activity was induced by 2-fold at 8 h of NOHA treatment and reached a maximum of 7-fold after 48 h. Exogenous L-ornithine did not inhibit the NOHA-induced activation of caspase-8 (data not shown). We observed a similar pattern of caspase-8 proteolytic cleavage by NOHA in Western blot analysis as shown in Fig. 4B.

Time Course of BID Cleavage after NOHA Treatment and Effect of Exogenous L-Ornithine—We studied the time course of BID cleavage in order to correlate the sequence of events during NOHA-induced apoptosis. Fig. 4C shows a slight cleavage of BID at 4 h, and maximum cleavage was observed at 32 h. A cleaved band could be observed at 15 kDa (p15 BID) corresponding to the truncated BID (tBID). Again, exogenous L-ornithine did not inhibit the NOHA-induced BID cleavage, and the time course of caspase-8 activation was correlated with the time course of BID cleavage.

Effect of Different Caspase Inhibitors on the NOHA-induced Cytochrome c Release into the Cytosol—To confirm that caspase-8 activation works upstream of the mitochondrial
events leading to cytochrome c release, we performed experiments where inhibitors of different caspases were incubated along with NOHA at concentrations reported in different cell lines to block their activation after 48 h. Fig. 5A shows that NOHA-induced cytochrome c release into the cytosol could be prevented by the caspase-8 inhibitor or a pan-caspase inhibitor but not by caspase-3 or caspase-9 inhibitors.

**TNF-α Potentiates the Action of NOHA in Inducing Caspase-8 Activity—**Caspase-8 is activated in many cell lines after activation of the TNF-R1/Fas pathway. We therefore assessed the role of TNF-α during NOHA-mediated caspase-8 activation. TNF-α was not able to activate the caspase-8 or -3 in MDA-MB-468 cells. However, when combined with NOHA (1 mM), we observed that TNF-α potentiated the action of NOHA in inducing caspase-8 (and caspase-3) activity at a much earlier time point. Fig. 5B shows that after 4 h there was a 2-fold induction in the caspase-8 activation with a combination of TNF-α and NOHA compared with the caspase-8 activation with NOHA alone, which further increased to ~3.5-fold after 8 h of NOHA treatment.

**DISCUSSION**

The primary objective of our study was to elucidate the mechanism(s) by which NOHA led to apoptosis of MDA-MB-468 cells and to assess the specific site of action and mechanism(s) by which l-ornithine prevented NOHA-induced apoptosis (Fig. 6). Mitochondria play a central role in the commitment of cells to apoptosis (19, 20). Most studies indicate that the initial event observed following application of the apoptotic stimulus is a decrease in the inner transmembrane potential followed by an increase in the permeability of the outer mitochondrial membrane by forming the permeability transition pores and subsequent release of cytochrome c from the intermembranous space into the cytosol (21). This released cytochrome c then forms a complex with the apoptosis activating factor-1 (Apafl) and procaspase-9 called “apoptosome” to activate caspase-9, which in turn activates caspase-3, a downstream caspase (22, 23). Once activated, caspase-3 cleaves its substrate PARP and, subsequently, leads to fragmentation of DNA (24). In our flow cytometry analysis using the mitochondria-sensitive dye CMX-Ros during NOHA-induced apoptosis, we did not observe any decrease in MMP. Rather, we observed release of cytochrome c from the mitochondria during NOHA-induced hyperpolarization of the MMP. Our method for assessing changes in transmembrane potential was validated using valinomycin, which causes hyperpolarization of MMP and which increased the fluorescence, whereas gramicidin, which causes depolarization of MMP, decreased the fluorescence. The hyperpolarization of MMP caused by NOHA was further confirmed using other dyes like dihexyloxacarbocyanine and rhodamine 123 that showed similar results (data not shown). Our results indicate that the collapse of the inner MMP is not a
universal early event that triggers cytochrome c release leading to apoptosis and moreover is not always an essential part of the central apoptotic machinery. In this regard, our results are similar to those reported by other investigators who observed that HL-60 cells underwent apoptosis in response to the cytotoxic insults of actinomycin D, etoposide, and staurosporine without showing significant loss of mitochondrial inner transmembrane potential (25). We therefore considered other potential mechanisms by which NOHA may affect the permeability of the outer mitochondrial membrane and thereby lead to cytochrome c release into the cytosol independent of a depolarization of the MMP. In this regard two major mechanisms have been proposed (26, 27). One mechanism proposed is that BID, a cytosolic BH3 interacting domain only member of the Bcl-2 family of proteins, is a substrate for caspase-8, which is activated by the FAS/TNF-R1 pathway and cleaves BID at the C-terminal to generate tBID. This tBID then translocates to the mitochondria and induces cytochrome c release either in a Bax-dependent or independent manner (26, 28). More recently, caspase-8-mediated cleavage of BID was also shown to induce N-myristoylation of tBID, and this modification enhanced its targeting to lipid membranes and mitochondria (29). One possible reason for the targeting of tBID to mitochondria has been attributed to its propensity for binding to cardiolipin (30), a membrane lipid unique to mitochondria (31). Another mechanism suggested is that after apoptotic insult, Bax, another multidomain pro-apoptotic cytosolic protein, integrates to the outer mitochondrial membrane and causes cytochrome c release (27) in a BID-independent manner.

We, therefore, assessed the possibility as to whether NOHA-induced activation of caspase-3 and apoptosis occurred by a BID-dependent or BID-independent mechanism. As caspase-8 activation is required for truncation of BID, we initially assessed the time course of activation of caspase-8, its proteolytic cleavage, and the appearance of tBID and how this correlated with the release of cytochrome c from the mitochondria. In this regard, we observed that activation of caspase-8 was significantly increased after 8 h following NOHA treatment, and peak activation occurred at 32 h (Fig. 4A). Slight truncation of BID was seen as early as 4 h that peaked between 24 and 32 h. Following exposure of the cells to NOHA, the peak release of cytochrome c release coincided with the peak caspase-8 activation and significant truncation of BID. We then considered the possibility as to whether the early and late release of cytochrome c may have been due to BID-mediated integration of Bax at the outer mitochondrial membrane. However, this does not appear to be the case as we were not able to demonstrate Bax integration into the mitochondrial membrane at any time point, and therefore the release of cytochrome c by tBID must have occurred by a Bax-independent mechanism. Activation of caspase-3 started around 32 h and peaked at 48 h following treatment of cells with NOHA. This activation of caspase-3, therefore, was initiated only following caspase-8 activation and truncation of BID, which then induced a massive release of cytochrome c and ultimately was responsible for the activation of caspase-3, thereby committing the cells to the apoptotic pathway. We next assessed the mechanism by which NOHA induced activation of caspase-8. We observed that NOHA along with TNF-α led to a significant activation of caspase-8 activity when compared with cells treated with NOHA or TNF-α alone (Fig. 5B). This action of NOHA therefore may have contributed to the apoptosis as TNF-α is released by this cell line.2

We have demonstrated previously that l-ornithine inhibited NOHA-induced apoptosis. We, therefore, elected to use l-ornithine as a tool to further identify the sequence of events during NOHA-induced apoptosis (Fig. 6). l-Ornithine did not inhibit the NOHA-induced activation of caspase-8 and cleavage of BID, suggesting that the action of l-ornithine must have occurred further downstream. NOHA-induced hyperpolarization of MMP was not changed by l-ornithine indicating that hyperpolarization was not the triggering event that led to cytochrome c release. On the other hand, l-ornithine antagonized the effect of NOHA in releasing cytochrome c from the mitochondria and all subsequent steps downstream leading to apoptosis. It, therefore, appears that the antagonistic effect of l-ornithine during NOHA-induced apoptosis was most likely at the level of the mitochondria where it prevented the release of cytochrome c. In this regard, it is interesting that other investigators have demonstrated (32) that extracellularly applied arginine inhibited neuronal apoptosis induced by multiple stimuli. Furthermore, arginase was identified by mass spectrometry as one of the proteins released from the mitochondria during apoptosis (33). On this basis, it was speculated that the protective effect of arginine in inhibiting neuronal apoptosis was dependent on depletion of arginine metabolized by arginase. As arginase converts arginine to ornithine, it would be interesting to speculate whether l-ornithine was the anti-apoptotic factor in these studies similar to our observation in studies related to NOHA-induced apoptosis. Macrophages as well as myoepithelial cells at the site of breast cancer express NOS (35) and therefore have the capacity to generate both NOHA and NO. Thus NOHA and NO by independent mechanisms may induce apoptosis and therefore complement each other.

In conclusion, our studies indicate that NOHA-induced apoptosis occurs upstream of the mitochondria, most likely by activation of caspase-8 followed by cleavage of BID to tBID. Our studies also indicate that l-ornithine has anti-apoptotic action and acts at the level of the mitochondria to inhibit NOHA-induced release of cytochrome c and thereby apoptosis. Further studies are in progress to assess the precise mechanism(s) by which l-ornithine acts at the level of the mitochondria as an anti-apoptotic factor and whether this is specific to NOHA or whether it may be applicable to some other apoptotic agents that act by activating caspase-8.

Acknowledgments—We thank Janis Cuevas and Svetlana Arutyunova for excellent technical assistance. We thank Dr. Fuyuhiko Tamanoi, Dr. Catherine F. Clarke, and Dr. Jon M. Fukuto for

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2 R. Singh, S. Pervin, and G. Chaudhuri, unpublished observation.
constructive criticisms during the preparation of the manuscript. Flow cytometry was performed in the core facility of UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility that is supported by National Institute of Health Awards CA-16042 and AI-28697, by the Jonsson Cancer Center, the UCLA AIDS Institute, and the UCLA School of Medicine.

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