Arachidonic Acid Mediates Apoptosis Induced by N-Ethylmaleimide in HepG2 Human Hepatoblastoma Cells

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Abstract—We have previously reported that N-ethylmaleimide (NEM) induces apoptosis through activation of K⁺, Cl⁻-cotransport (KCC) in HepG2 human hepatoblastoma cells. In this study we investigated the possible role of phospholipase A₂ (PLA₂)-arachidonic acid (AA) signals in the mechanism of the NEM-induced apoptosis. In these experiments we used arachidonyl trifluoromethylketone (AACOCF₃), bromoenol lactone (BEL) and p-bromophenacyl bromide (BPB) as inhibitors of the calcium-dependent cytosolic PLA₂ (cPLA₂), the calcium-independent PLA₂ (iPLA₂) and the secretory PLA₂ (sPLA₂), respectively. BEL significantly inhibited the NEM-induced apoptosis, whereas AACOCF₃ and BPB did not. NEM increased AA liberation in a dose-dependent manner, which was markedly prevented only by BEL. In addition AA by itself induced K⁺ efflux, a hallmark of KCC activation, which was comparable to that of NEM. The NEM-induced apoptosis was not significantly altered by treatment with indomethacin (Indo) and nordihydroguaiaretic acid (NDGA), selective inhibitors of cyclooxygenase (COX) and lipoxygenase (LOX), respectively. Treatment with AA or 5,8,11,14-eicosatetraynoic acid (ETYA), a non-metabolizable analogue of AA, significantly induced apoptosis. Collectively, these results suggest that AA liberated through activation of iPLA₂ may mediate the NEM-induced apoptosis in HepG2 cells.

Keywords: N-Ethylmaleimide, Arachidonic acid, Apoptosis, K⁺, Cl⁻-cotransport, Phospholipase A₂, HepG2 cell

INTRODUCTION

Apoptosis is characterized by condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of proteases and endonucleases, enzymatic cleavage of the DNA into oligonucleosomal fragments and segmentation of the cells into membrane-bound apoptotic bodies (Kidd, 1998). Apoptosis plays a critical role in maintenance of tissue homeostasis by the selective elimination of excessive cells (Song and Steller, 1999). In particular, genetic mutations culminating in disturbance of apoptosis or derangement of apoptosis-signaling pathways seem to be an essential factor of carcinogenesis (Wang, 1999; Lowe and Lin, 2000; Rodriguez-Nieto and Zhivotovsky, 2006). On the other hand, induction of apoptosis of cancer cells is regarded as one of the most important strategies for cancer treatment (Kornblau, 1998; Fulda and Debatin, 2004; Papenfuss et al., 2008).

Since K⁺, Cl⁻-cotransport (KCC) has been first described in red blood cells as a swelling-activated K⁺ efflux mechanism (Lauf et al., 1992; Cossins and Gibson, 1997), functional and physiological evidence has also shown for the existence of KCC in various types of tissues (Adragna et al., 2004), such as epithelia (Greger and Schlatter, 1983; Amlal et al., 1994), endothelium (Perry and O’Neill, 1993), vascular smooth muscle (Adragna et al., 2000), heart (Yan et al., 1996), skeletal muscle (Weil-Maslansky et al., 1994), and neurons (Rivera et al., 1999). Therefore, KCC has been implicated not only in regulatory volume decrease (Lauf et al., 1992), but also in transepithelial salt absorption (Amlal et al., 1994), myocardial K⁺ loss during ischemia (Yan et al., 1996), blood pressure control (Adragna et al., 2006), regulation of neuronal Cl⁻ concentration (Rivera et al., 1999), and renal K⁺ secretion (Ellison et al., 1985). Interestingly, recent reports have suggested that KCC is expressed in a variety of human cancer cells.
KCC has been reported to down-regulates E-cadherin/β-catenin complex formation by inhibiting transcription of E-cadherin gene and accelerating proteosome-dependent degradation of β-catenin protein, which promotes epithelial-mesenchymal transition, thereby stimulating tumor progression (Hsu et al., 2007a). In addition, upregulation of KCC has been shown to be required for proliferation and invasiveness induced by insulin-like growth factor 1 in breast cancer cells (Hsu et al., 2007b), and cervical and ovarian cancer cells (Shen et al., 2004). On the other hand, KCC activation appeared to induce apoptotic cell death in hepatoma cells (Kim et al., 2001).

Previously we have reported that KCC is functionally present in a human hepatoma cell line, and that NEM induced apoptosis through KCC activation (Kim et al., 2001). However, the exact mechanism by which NEM ultimately induces apoptotic cell death is still unclear. Interestingly NEM has been reported to release arachidonic acid (AA) in platelets (Leoncini and Signorello, 1999a). In addition, AA appears to be involved in the induction of apoptosis in a variety of cancer cells (Monjazeb et al., 2005; Claria, 2006; Nakanishi and Rosenberg, 2006). Emerging evidence has shown that elevated intracellular AA can induce cell death via the mitochondrial-mediated apoptosis pathway (Pompeia et al., 2003). Specifically, AA has been shown to induce an extended opening of mitochondrial permeability transition pore in a rat hepatoma cell line, followed by release of cytochrome c and apoptosis (Scorrano et al., 2001). The cytotoxicity of AA may also be mediated through its ability to increase ceramide levels. Jayadev et al. (1994) identified a correlation between TNF-α-induced accumulation of AA and increased levels of ceramide in human promyelocytic leukemia cells. A recent report by Martin et al. (2005) demonstrated that an accumulation of AA caused by the COX-2 inhibitor, DuP-697, activated acidic sphingomyelinase (SMase) to generate ceramide-enriched caveolae within the plasma membrane outer leaflet in HT-29 colon cancer cells. Subsequently, these caveolae enhanced the clustering of a TRAIL-mediated death-inducing signaling complex. Additionally, AA induced apoptosis through a deleterious effect on mitochondria and promotes ROS production in HepG2 cells (Shin and Kim, 2009). Peroxisome proliferator-activated receptor-α (PPAR-α) has also been reported to act as a possible contributor to the growth inhibitory effect of AA in human breast cancer cells (Bocca et al., 2008).

Thus, the main purpose of the present study was to investigate whether AA is involved in the mechanism of apoptosis associated with activation of KCC in HepG2 human hepatoblastoma cells. In addition, we examined more specifically which subtype of phospholipase A2 (E.C. 3.1.1.4, PLA2) is involved in the NEM-induced liberation of AA, and thus induction of apoptosis.

MATERIALS AND METHODS

Materials

The HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). The powders for Eagle's minimum essential medium, trypsin solution, sodium pyruvate, NEM, bromoeno lactone (BEL), p-bromophenacyl bromide (BPB), indomethacin (Indo), nordihydroguaiaretic acid (NDGA) and all salt powders were obtained from Sigma-Aldrich (St. Louis, MO). Arachidonyl trifluoromethylketone (AACOCF3) and 5,8,11,14-eicosatetraynoic acid (ETYA) were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). [3H]Arachidonic acid ([3H]AA) was from GE Healthcare (Buckinghamshire, UK). Potassium-binding benzofuran isophthalate aceoxymethyl ester (PBFI/AM) was from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). The stock solutions of drugs were sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

HepG2 cells were grown at 37°C in a humidified incubator under 5% CO2/95% air in an Eagle's minimum essential medium supplemented with 10% FBS, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization.

Flow cytometric analysis of apoptosis

For flow cytometric analysis, HepG2 cells were collected and washed twice with phosphate-buffered saline (pH 7.4). After fixation in 80% ethanol for 30 min, cells were washed twice and resuspended in phosphate-buffered saline (pH 7.4) containing 0.1% Triton X-100, 5 μg/ml propidium iodide and 50 μg/ml ribonuclease A for DNA staining. Cells were then analyzed with a FACScan (BIO-RAD, Hercules, CA). At least 20,000 events were evaluated. All histograms were analyzed using WinBryte software (BIO-RAD, Hercules, CA) to determine the percentage of nuclei with a hypodiploid content indicative of apoptosis (Bombeli et al., 1997).
Measurement of AA release

AA release was determined by measuring $[^{3}H]AA$ released into the surrounding medium from HepG2 cell suspensions labeled with $[^{3}H]AA$ (Van Der Zee et al., 1995). Cell suspensions (3×10⁸ cells/ml) were incubated at 37°C with 3 μCi $[^{3}H]AA$ for 18 hr. Over this time, cells incorporated an average of 80% of the added $[^{3}H]AA$. After incubation, cell suspensions were washed three times with Tyrode solution containing 3.6% fatty-acid-free bovine serum albumin (BSA) to remove unincorporated $[^{3}H]AA$. HepG2 cells were incubated at 37°C for 15 min before being subjected to experimental conditions. At the end of the stimulation period, cell suspensions were centrifuged (150 g, 3 min), and the supernatant was obtained. $[^{3}H]AA$ released was quantified by liquid scintillation spectrometry.

Measurement of intracellular K⁺ concentration ([K⁺]i)

Intracellular K⁺ levels were monitored with the K⁺-sensitive fluorescent dye, PBFI/AM (Minta and Tsien, 1989). Cells were washed, and resuspended at a density of 4×10⁶ cells/ml in Krebs-Ringer buffer. The cells were loaded with 5 μM PBFI/AM in Krebs-Ringer buffer containing 0.02% pluronic F-127, a nonionic surfactant, for 2 hr at 37°C. Unloaded dye was removed by centrifugation at 150 g for 3 min. The dual-wavelength excitation method for measurement of PBFI fluorescence was used. Fluorescence was monitored at 500 nm with excitation wavelengths of 340 and 380 nm in a stirred quartz cuvette. In the results relative changes in [K⁺]i were reported as the 340:380 fluorescence ratios.

Data analysis

All experiments were performed four times. Data are expressed as means ± standard error of the mean (S.E.M.) and were analyzed using a one-way analysis of variance (ANOVA) and Student-Newman-Keuls’s test for individual comparisons. p values less than 0.05 are considered statistically significant.

RESULTS

Role of calcium-independent phospholipase A₂ (iPLA₂) in the NEM-induced apoptosis in HepG cells

Previously we have shown that NEM induced apoptosis through KCC activation (Kim et al., 2001). To examine the possible role of PLA2-AA signals in the NEM action, we investigated the effects of specific inhibitors of three different types of PLA2 on the NEM-induced apoptosis using flow cytometry. In these experiments we used AACOCF₃, BEL and BPB as inhibitors of the calcium-dependent cytosolic PLA2 (cPLA₂), the calcium-independent PLA2 (iPLA₂) and the secretory PLA2 (sPLA₂), respectively (Narendra Sharath Chandra et al., 2007). As depicted in Fig. 1, BEL (10 μM) significantly inhibited the NEM-induced apoptosis, whereas AACOCF₃ (10 μM) and BPB (10 μM) did not. These results suggest that iPLA₂ activity may be required for the NEM-induced apoptotic cell death in the HepG2 cells.

AA release by NEM is due to activation of iPLA₂

To confirm whether NEM indeed increases AA release being implicated in apoptosis of HepG2 cells, as shown
above, we examined the effect of NEM on AA release measuring \([^{3}H]\)AA released into the surrounding medium from HepG2 cell suspensions labeled with \([^{3}H]\)AA using liquid scintillation spectrometry. As shown in Fig. 2A, NEM increased AA release in a dose-dependent manner. To further identify which subtype of PLA2 is involved in the process, we studied the effects of these PLA2 inhibitors on the NEM-induced AA release. As illustrated in Fig. 2B, BEL (10 μM) significantly inhibited the NEM (100 μM)-induced AA release. However, AACOCF3 (10 μM) and BPB (10 μM) did not have an influence. These results indicate that NEM can induce AA release from the HepG2 cells through activation of iPLA2.

AA activates KCC

From results obtained from above experiments it is sug-

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**Fig. 2.** Dose-dependent \([^{3}H]\)AA release induced by NEM (A) and the effects of PLA2 inhibitors on the NEM-induced \([^{3}H]\)AA release (B) in HepG2 human hepatoblastoma cells. HepG2 cells were labeled with medium containing \([^{3}H]\)-AA and then treated with either vehicle or NEM for 60 min at a designated concentration. Assay for \([^{3}H]\)AA release was done by scintillation counting method as described in text. In these experiments AACOCF3 (10 μM), BEL (10 μM) and BPB (10 μM) were used as a specific inhibitor of the cPLA2, iPLA2 and sPLA2, respectively. These inhibitors were added 10 min before NEM treatment. Results are expressed as percent change of control condition in which cells were treated with a drug-free vehicle. All the data points represent the mean values of four replications with bars indicating SEM. *p < 0.05 compared to control condition in which the cells were incubated with NEM-free medium. **p < 0.05 compared to NEM alone.

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**Fig. 3.** Effects of NEM and AA on KCC activity in HepG2 human hepatoblastoma cells. The data (A) show changes in intracellular K⁺ concentration as a function of time, measured by using the K⁺-sensitive fluorescent dye PBFI/AM. PBFI fluorescence ratios are directly proportional to the intracellular K⁺ level. In all figures the arrows show the time points for addition of either NEM (100 μM) or AA (10 μM). Quantitative changes (B) were expressed as percent changes of the maximum decrease in PBFI fluorescence induced by the drug compared to control condition in which the cells were treated with a drug-free vehicle. Each column represents the mean value of four replications with bars indicating SEM. *p < 0.05 compared to control (Con).
suggested that NEM increases AA release through activation of iPLA₂, which activates KCC, and in turn induces apoptosis. Thus next we tried to verify that AA released by NEM can induce KCC activation. As shown in Fig. 3, AA (10 μM) profoundly induced K⁺ efflux, a hallmark of KCC activation (Lauf et al., 1992; Kim et al., 2001; Adragna et al., 2004). This effect of AA was comparable to the NEM (100 μM)-induced response. These results support that AA may mediate the NEM-induced KCC activation, and thus apoptosis in HepG2 cells.

**AA by itself, rather than its metabolites mediates the NEM-induced apoptosis**

AA serves as the precursor for prostanoid and leukotriene production via the actions of cyclooxygenase (COX) and lipoxygenase (LOX), respectively (Harizi et al., 2008). To clarify the role of these enzyme products in the NEM-induced apoptosis, we investigated the effects of Indo, a non-selective COX inhibitor (Bakalova et al., 2002) and NDGA, a general LOX inhibitor on the NEM-induced apoptosis. As depicted in Fig. 4, pretreatment with either Indo (30 μM) or NDGA (50 μM) failed to affect the NEM (100 μM)-induced apoptosis. These results suggest that AA metabolites may not have a role in the NEM-induced apoptosis in HepG2 cells.

To test whether AA alone rather than its metabolites serves as a mediator for the NEM-induced action, we observed the effects of AA and ETYA, a non-metabolizable analogue of AA on the apoptotic cell death. As depicted in Fig. 5, treatment with AA alone (10 μM) profoundly induced apoptosis.
apoptosis. This effect of AA was comparable to the NEM (100 \( \mu \)M)-induced response. In addition, treatment with ETYA (10 \( \mu \)M) mimicked the response of AA. These results strongly suggest that AA by itself may mediate the NEM-induced apoptosis in HepG2 cells.

**DISCUSSION**

KCC appears to have many physiological functions, including cell volume regulation (Lauf et al., 1992; Cossins and Gibson, 1997; Adragna et al., 2004). Additionally, it has pathophysiological roles. Inappropriate activation of KCC in erythrocytes leads to excessive KCl loss, cell shrinkage and elevation of hemoglobin concentration (Olivieri et al., 1992; Joiner, 1993), leading to deleterious rheological effects, including increased vascular resistance (Stuart and Ellory, 1988). Interestingly, Shen et al. (2000) have reported that human cervical carcinogenesis is accompanied by up-regulation of KCC transcripts. Recently KCC has been shown to be involved in cancer cell proliferation and invasion (Shen et al., 2004; Hsu et al., 2007a; Hsu et al., 2007b). We have also reported that the prolonged activation of KCC could induce apoptosis in HepG2 human hepatoma cells (Kim et al., 2001).

NEM that reacts with and oxidizes sulfhydryl groups, has been reported to have many cellular actions, such as inhibition of platelet aggregation (Leoncini and Signorello, 1999a), release of AA from platelets (Leoncini and Signorello, 1999b) and endothelial cells (Neve et al., 1995), and modulation of norepinephrine release from hippocampus synaptosomes (Wurster et al., 1990). These actions of NEM may result from the alkylation of specific cysteine residues present in certain signal-coupling proteins, including G-proteins (Hoshino et al., 1990). Particularly, it has long been known to cause strong activation of KCC in erythrocytes (Lauf et al., 1992). Previously, NEM has been shown to induce apoptosis through activation of KCC in HepG2 cells (Kim et al., 2001). However, the exact target molecule(s) involved in the NEM-induced apoptosis has(have) not been clarified. The results of the present study strongly suggest that AA produced by activation of iPLA2 seems to be the upstream signaling molecule whose activation is essentially required for the NEM-induced activation of KCC, and in turn, induction of apoptosis in HepG2 cells. These conclusions are based on (i) NEM profoundly increased AA release in the HepG2 cells (Fig. 2A) and the NEM-induced AA release was significantly inhibited not by AACOCF3 (the cPLA2 inhibitor) and BPB (the sPLA2 inhibitor), but by BEL (the iPLA2 inhibitor) (Fig. 2B); (ii) the NEM-induced apoptosis was also significantly inhibited on-
of the NADPH oxidase (Curnutte, 1985; Block et al., 2006; Hii and Ferrante, 2007; Kim et al., 2008). Thus one can easily speculate that NEM may generate ROS through activation of NADPH oxidase induced by AA released by activation of iPLA2 in HepG2 cells.

In this study, however, we did not investigate how NEM induces activation of iPLA2 in HepG2 cells. Although speculated, this may be achieved by either direct structural modification or indirect stimulation of the enzyme. NEM has been reported to activate PL2 through elevation of intracellular Ca2+ level (Leslie, 2004), involvement of intracellular Ca2+ signal may be excluded. Activation of iPLA2 can be specifically regulated by ATP in pancreatic β-cells (Ramanadham et al., 2004), p38 mitogen-activated protein kinase (MAPK) in vascular smooth muscle cells (Yellaturu et al., 2003), and depletion of intracellular Ca2+ store in smooth muscle cells (Wolf et al., 1997). Since direct modification of iPLA2 by NEM and interaction between NEM and these molecules or processes have not been clearly demonstrated yet, these possibilities may not be excluded. At present the exact mechanism of the NEM-induced activation of iPLA2 is not known, and remains to be determined in the future studies.

In conclusion, AA produced by activation of iPLA2 may be the upstream mechanism of increased activity of KCC associated with the NEM-induced apoptosis in HepG2 cells, as shown in Fig. 6. These results further suggest that iPLA2-AA signal may be a good target for the therapeutic intervention of human hepatoma.

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Hii, C. S. and Ferrante, A. (2007). Regulation of the NADPH oxidase (Curnutte, 1985; Block et al., 2006; Hii and Ferrante, 2007; Kim et al., 2008). Thus one can easily speculate that NEM may generate ROS through activation of NADPH oxidase induced by AA released by activation of iPLA2 in HepG2 cells.

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