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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 A_2 (PLA_2)-arachidonic acid (AA) signals in the mechanism of the NEM-induced apoptosis. In these experiments we used arachidonyl trifluoromethylketone ($AACOCF_3$), bromoenol lactone (BEL) and *p*-bromophenacyl bromide (BPB) as inhibitors of the calcium-dependent cytosolic PLA_2 ($cPLA_2$), the calcium-independent PLA_2 ($iPLA_2$) and the secretory PLA_2 ($sPLA_2$), respectively. BEL significantly inhibited the NEM-induced apoptosis, whereas $AACOCF_3$ 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 $iPLA_2$ may mediate the NEM-induced apoptosis in HepG2 cells.

Keywords: *N*-Ethylmaleimide, Arachidonic acid, Apoptosis, K^+, Cl^--cotransport, Phospholipase A_2, 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.

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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; Clària, 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 A_2 (E.C. 3.1.1.4, PLA_2) 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, bromoenol 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 ($AACOCF_3$) and 5,8,11,14-eicosatetraynoic acid (ETYA) were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). [^{3}H]Arachidonic acid ([^{3}H]AA) was from GE Healthcare (Buckinghamshire, UK). Potassium-binding benzofuran isophthalate aceoxylmethyl 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% CO_2/95% 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 (3×10^8 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 4×10^5 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-Keul's test for individual comparisons. *p* values less than 0.05 are considered statistically significant.

RESULTS

Role of calcium-independent phospholipase A_2 ($iPLA_2$) 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 PLA_2-AA signals in the NEM action, we investigated the effects of specific inhibitors of three different types of PLA_2 on the NEM-induced apoptosis using flow cytometry. In these experiments we used $AACOCF_3$, BEL and BPB as inhibitors of the calcium-dependent cytosolic PLA_2 ($cPLA_2$), the calcium-independent PLA_2 ($iPLA_2$) and the secretory PLA_2 ($sPLA_2$), respectively (Narendra Sharath Chandra *et al*., 2007). As depicted in Fig. 1, BEL (10 μM) significantly inhibited the NEM-induced apoptosis, whereas $AACOCF_3$ (10 μM) and BPB (10 μM) did not. These results suggest that $iPLA_2$ activity may be required for the NEM-induced apoptotic cell death in the HepG2 cells.

AA release by NEM is due to activation of $iPLA_2$

To confirm whether NEM indeed increases AA release being implicated in apoptosis of HepG2 cells, as shown

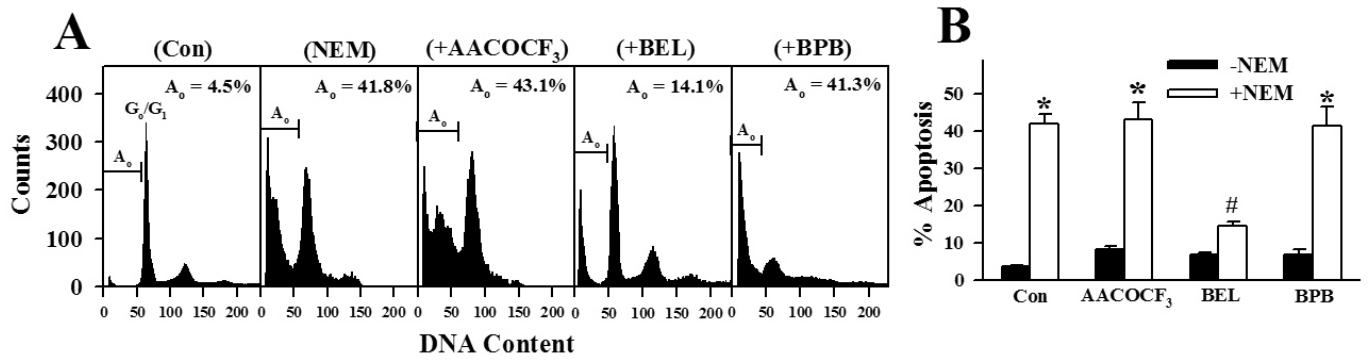

Fig. 1. Effects of PLA_2 inhibitors on the apoptosis induced by NEM in HepG2 human hepatoblastoma cells. In the experiments of (A) the cells were incubated with or without NEM (100 μM) for 4 hr. $AACOCF_3$ (10 μM), BEL (10 μM) and BPB (10 μM) were added 30 min before NEM application. The number of apoptotic cells was measured by flow cytometry as described in text. The region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation. In bar graphs (B) the data represent the mean values of four replicates with bars indicating S.E.M. $^*p<0.05$ compared to control condition in which the cells were incubated with NEM-free medium. $^{\#}p<0.05$ compared to NEM alone.

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 PLA_2 is involved in the process, we studied the effects of these PLA_2 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, $AACOCF_3$ (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 $iPLA_2$.

AA activates KCC

From results obtained from above experiments it is sug-

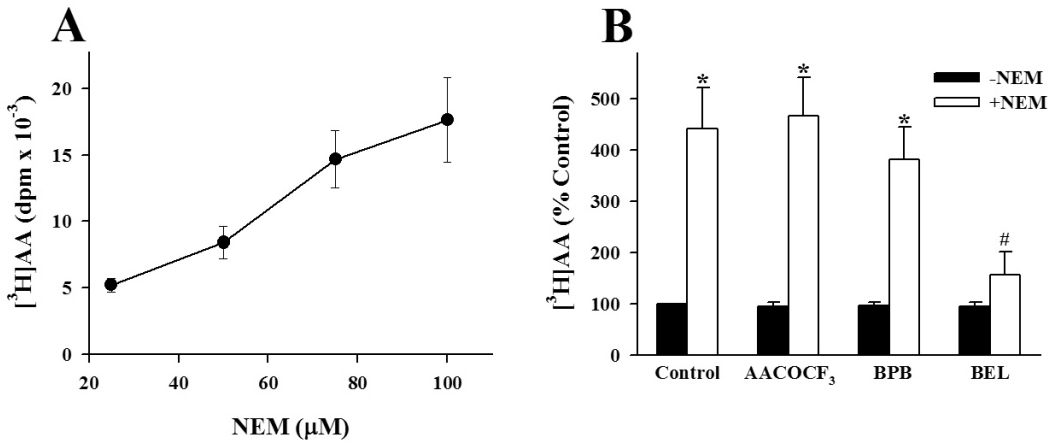

Fig. 2. Dose-dependent [^{3}H]AA release induced by NEM (A) and the effects of PLA_2 inhibitors on the NEM-induced [^{3}H]AA release (B) in HepG2 human hepatoblastoma cells. HepG2 cells were labeled with medium containing [^{3}H]-AA and then treated with either vehicle or NEM for 60 min at a designated concentration. Assay for [^{3}H]AA release was done by scintillation counting method as described in text. In these experiments $AACOCF_3$ (10 μM), BEL (10 μM) and BPB (10 μM) were used as a specific inhibitor of the $cPLA_2$, $iPLA_2$ and $sPLA_2$, 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.

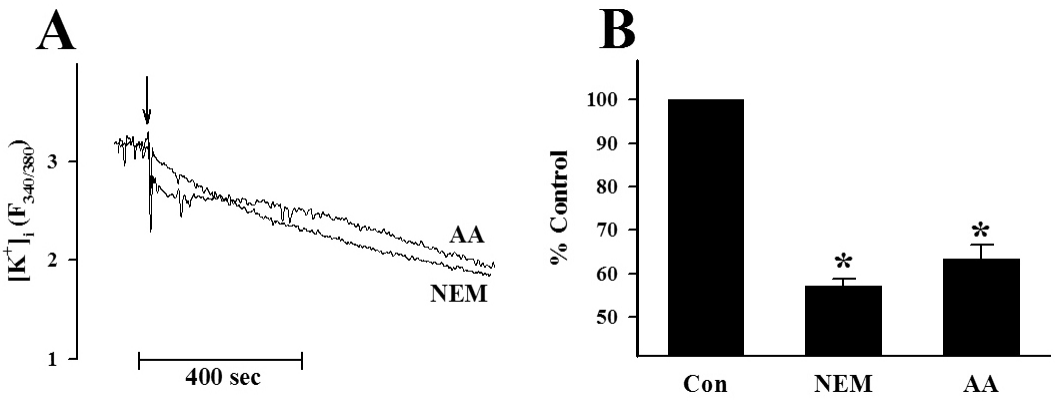

Fig. 3. Effects of NEM and AA on KCC activity in HepG2 human hepatoblastoma cells. The data (A) show changes in intracellular K^+ concentration as a function of time, measured by using the K^+-sensitive fluorescent dye PBFI/AM. PBFI fluorescence ratios are directly proportional to the intracellular K^+ 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).

gested that NEM increases AA release through activation of $iPLA_2$, 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

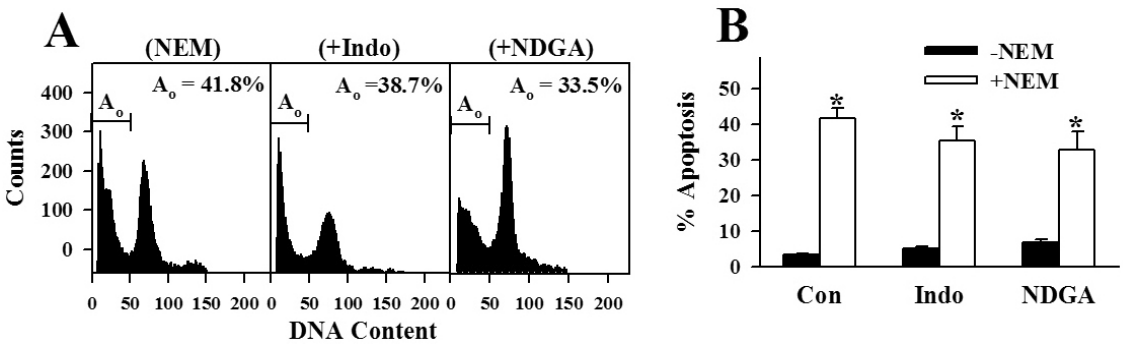

Fig. 4. Effects of inhibitors of COX and LOX on the apoptosis induced by NEM in HepG2 human hepatoblastoma cells. In the experiments of (A) the cells were incubated with or without NEM (100 μM) for 4 hr. Indo (30 μM), a COX inhibitor and NDGA (50 μM), a LOX inhibitor were added 30 min before NEM application. The number of apoptotic cells was measured by flow cytometry as described in text. The region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation. In bar graphs (B) the data represent the mean values of four replicates with bars indicating S.E.M. $*p < 0.05$ compared to control (Con) condition in which the cells were incubated with NEM-free medium.

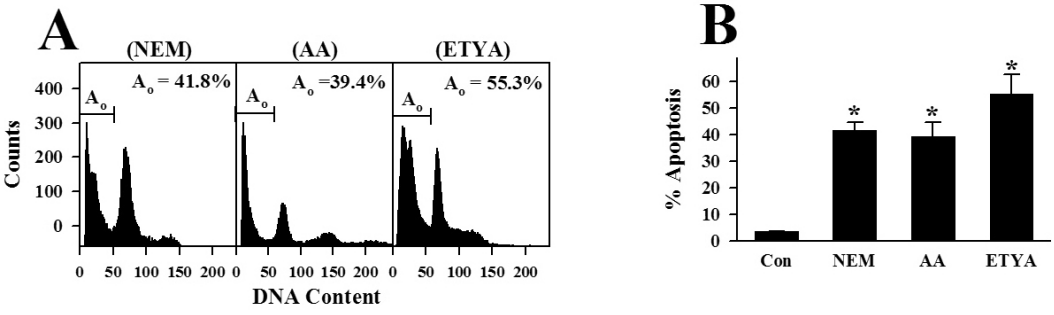

Fig. 5. Effects of AA and ETYA on the apoptosis induced by NEM in HepG2 human hepatoblastoma cells. In the experiments of (A) the cells were incubated with or without NEM (100 μM), AA (10 μM) and ETYA (10 μM), a non-metabolizable analogue of AA. The number of apoptotic cells was measured by flow cytometry as described in text. The region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation. In bar graphs (B) the data represent the mean values of four replicates with bars indicating S.E.M. $*p < 0.05$ compared to control (Con) condition in which the cells were incubated with a drug-free vehicle.

apoptosis. This effect of AA was comparable to the NEM (100 μM)-induced response. In addition, treatment with ETYA (10 μM) 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 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 $iPLA_2$ 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 $AACOCF_3$ (the $cPLA_2$ inhibitor) and BPB (the $sPLA_2$ inhibitor), but by BEL (the $iPLA_2$ inhibitor) (Fig. 2B); (ii) the NEM-induced apoptosis was also significantly inhibited only by the $iPLA_2$ inhibitor, $AACOCF_3$ (Fig. 1); (iii) AA directly activated KCC (Fig. 3).

Considerable amounts of AA are found esterified in the membranes of mammalian cells and later released via PLA_2 hydrolysis of the acyl bond at the sn-2 position (Waite, 1996). Since AA serves as a precursor for eicosanoids generated by pathways dependent on cyclooxygenases (COX), lipoxygenases (LOX), and cytochromes P450 (CYP) (Jenkins *et al*., 2009), we examined the possible involvement of eicosanoids produced by AA metabolism in the NEM-induced apoptosis. However, the results of this study suggest that AA metabolites may not have a role in the NEM-induced apoptosis, because the NEM-induced effect was not significantly altered by treatment with Indo and NDGA, inhibitors of COX and LOX, respectively (Fig. 4). Therefore we postulated that the NEM-induced apoptosis may be mediated by AA itself. To test this hypothesis, we determined the direct effects of AA and ETYA, a non-metabolizable analogue of AA, on apoptosis in HepG2 cells. The results showing that exogenous treatment with AA significantly induced apoptosis, which was comparable to the NEM-induced effect, and that treatment with ETYA mimicked the response of AA (Fig. 5), strongly suggest that AA by itself may mediate the NEM-induced apoptotic cell death.

NEM appears to produce reactive oxygen species (ROS) in various cells (Leoncini and Signorello, 1999b; Kim and Lee, 2001; Meves *et al*., 2001; Yellaturu *et al*., 2002). Particularly, NEM has been reported to generate ROS by activation of membrane-bound NADPH oxidase (Kim and Lee, 2001) which is known to exist in HepG2 cells (Ehleben *et al*., 1997; Cool *et al*., 1998). Moreover, AA is well known to have an essential role in the activation

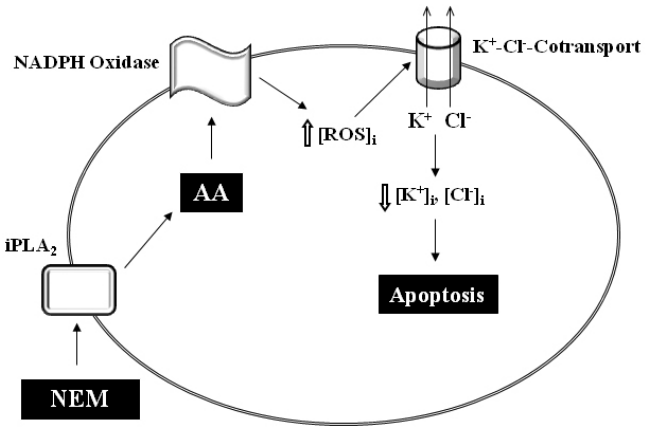

Fig. 6. Proposed role of AA in the mechanism of apoptosis induced by NEM in HepG2 human hepatoblastoma cells.

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 $iPLA_2$ in HepG2 cells.

In this study, however, we did not investigate how NEM induces activation of $iPLA_2$ 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 PLA_2 through elevation of intracellular Ca^{2+} level (Leoncini and Signorello, 1999a). Since activation of $iPLA_2$ does not require elevation of intracellular Ca^{2+} level (Leslie, 2004), involvement of intracellular Ca^{2+} signal may be excluded. Activation of $iPLA_2$ 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 and Rao, 2003), and depletion of intracellular Ca^{2+} store in smooth muscle cells (Wolf *et al*., 1997). Since direct modification of $iPLA_2$ 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 $iPLA_2$ is not known, and remains to be determined in the future studies.

In conclusion, AA produced by activation of $iPLA_2$ 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 $iPLA_2$-AA signal may be a good target for the therapeutic intervention of human hepatoma.

ACKNOWLEDGMENTS

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