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Arachidonic Acid Activates K^+-Cl^--cotransport in HepG2 Human Hepatoblastoma Cells

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K^+-Cl^--cotransport (KCC) has been reported to have various cellular functions, including proliferation and apoptosis of human cancer cells. However, the signal transduction pathways that control the activity of KCC are currently not well understood. In this study we investigated the possible role of phospholipase A_2 (PLA_2)-arachidonic acid (AA) signal in the regulatory mechanism of KCC activity. Exogenous application of AA significantly induced K^+ efflux in a dose-dependent manner, which was completely blocked by R-(+)-[2-n-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl]oxy]acetic acid (DIOA), a specific KCC inhibitor. *N*-Ethylmaleimide (NEM), a KCC activator-induced K^+ efflux was significantly suppressed by bromoenol lactone (BEL), an inhibitor of the calcium-independent PLA_2 ($iPLA_2$), whereas it was not significantly altered by arachidonyl trifluoromethylketone ($AACOCF_3$) and *p*-bromophenacyl bromide (BPB), inhibitors of the calcium-dependent cytosolic PLA_2 ($cPLA_2$) and the secretory PLA_2 ($sPLA_2$), respectively. NEM increased AA liberation in a dose- and time-dependent manner, which was markedly prevented only by BEL. In addition, the NEM-induced ROS generation was significantly reduced by DPI and BEL, whereas $AACOCF_3$ and BPB did not have an influence. The NEM-induced KCC activation and ROS production was not significantly affected by treatment with indomethacin (Indo) and nordihydroguaiaretic acid (NDGA), selective inhibitors of cyclooxygenase (COX) and lipoxygenase (LOX), respectively. Treatment with 5,8,11,14-eicosatetraynoic acid (ETYA), a non-metabolizable analogue of AA, markedly produced ROS and activated the KCC. Collectively, these results suggest that $iPLA_2$-AA signal may be essentially involved in the mechanism of ROS-mediated KCC activation in HepG2 cells.

Key Words: K^+-Cl^--cotransport, Reactive oxygen species, Arachidonic acid, Phospholipase A_2, *N*-Ethylmaleimide, HepG2 cells

INTRODUCTION

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). 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).

The alkylating agent *N*-ethylmaleimide (NEM) has been shown to activate KCC in erythrocytes (Lauf et al., 1992;

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ABBREVIATIONS: AA, arachidonic acid; $AACOCF_3$, arachidonyl trifluoromethylketone; BEL, bromoenol lactone; BPB, *p*-bromophenacyl bromide; COX, cyclooxygenase; DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescin diacetate; DIOA, R-(+)-[2-n- butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl]oxy]acetic acid; DPI, diphenylene iodonium; ETYA, 5,8,11,14-eicosatetraynoic acid; Indo, indomethacin; KCC, K^+-Cl^--cotransport; LOX, lipoxygenase; NDGA, nordihydroguaiaretic acid; NEM, *N*-ethylmaleimide; PBFI/AM, potassium-binding benzofuran isophthalate aceoxylmethyl ester; PLA_2, phospholipase A_2; ROS, reactive oxygen species.

Adragna et al., 2004). Since inhibition of protein phosphatases prevents the swelling- and NEM-induced KCC activation (Bize et al., 1998; Kaji and Tsukitani, 1991; Starke and Jennings, 1993), dephosphorylation of the cotransporter has been suggested to be required for its activation. On the other hand, many studies using inhibitors of protein tyrosine kinase have proved evidence that tyrosine phosphorylation may be also involved in controlling the KCC activity (Flatman et al., 1996; Weaver and Cossins, 1996). Although these phosphorylation and dephosphorylation reactions may play an important role in the mechanism of KCC activation, the signal transduction pathways that control the activity of KCC are currently not well understood.

Previously we have reported that KCC is functionally present in HepG2 human hepatoblastoma cells, and that reactive oxygen species (ROS) are implicated in the mechanism of KCC activation (Kim and Lee, 2001). Arachidonic acid (AA) has been reported to play a role in the generation of ROS in a variety of cells (Shiose and Sumimoto, 2000; Luchtefeld et al., 2003; Kim and Dinauer, 2006). AA is released from membrane phospholipid by three main types of phospholipase A_2 (PLA_2), the low-molecular-weight secreted PLA_2 ($sPLA_2$), the calcium-dependent cytosolic PLA_2 ($cPLA_2$) and calcium-independent PLA_2 ($iPLA_2$) (Leslie, 2004). Thus, in this study we investigated whether AA has a role in ROS-mediated KCC activation in HepG2 cells. In addition, we examined more specifically which subtype(s) of PLA_2 is(are) involved in the mechanism of AA-mediated KCC activation.

METHODS

Materials

The HepG2 human hepatoma cell line was purchased from American Type Culture Collection (Rockville, MA). The powders for Eagle's minimum essential medium, trypsin solution, sodium pyruvate, NEM, BEL, BPB, Indo, NDGA, DPI, and all salt powders were obtained from Sigma-Aldrich (St. Louis, MO). DIOA, $AACOCF_3$ and ETYA were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). [^{3}H]AA was from GE Healthcare (Buckinghamshire, UK). PBFI/AM and DCFH-DA were from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum 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% fetal bovine serum, 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.

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 h 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.

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). Cells were incubated at 37°C with 3 μCi [^{3}H]AA for 18 h. Over this time, cells incorporated an average of 80% of the added [^{3}H]AA. After incubation, cells were washed three times with Tyrode solution containing 3.6% fatty-acid-free bovine serum albumin to remove unincorporated [^{3}H]AA. Then, the cells were incubated at 37°C for 15 min before being subjected to experimental conditions. At the end of the stimulation period, the supernatant was obtained. [^{3}H]AA released was quantified by liquid scintillation spectrometry.

Measurement of intracellular ROS

Relative changes in intracellular ROS in HepG2 cells were monitored using a fluorescent probe, DCFH-DA (LaBel et al., 1992). DCFH-DA diffuses through the cell membrane readily and is hydrolyzed by intracellular esterases to nonfluorescent 2',7'-dichlorofluorescin (DCFH), which is then rapidly oxidized to highly fluorescent DCF in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly (Shen et al., 1996). Cells were washed twice and resuspended at a concentration of 4×10^5 cells/ml in Hank's solution. For loading DCFH-DA into the cells, cells were incubated with the dye for 2 h at a final concentration of 5 μM at 37°C. Fluorescence was monitored at 530 nm with excitation wavelength of 485 nm in a stirred quartz cuvette.

Data analysis

All experiments were performed four times. Data were expressed as mean±standard error of the mean (SEM) and were analyzed using 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

Activation of KCC by AA in HepG2 cells

To identify whether AA activates KCC in HepG2 cells, we examined the effect of AA on K^+ efflux which is regarded as a hallmark of KCC activation (Kim and Lee, 2001; Adragna et al., 2004). AA (1~10 μM) induced a slow and sustained decrease in $[K^+]_i$ in a concentration-dependent manner which was significantly prevented by treatment with DIOA (100 μM), a specific KCC inhibitor (Garay et al., 1988), as depicted in Fig. 1. These results indicate that

AA can activate KCC the HepG2 cells.

Role of PLA_2-AA signal in the mechanism of KCC activation

AA is 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). Thus, to examine the possible role of PLA_2-AA signals in the mechanism of KCC activation, we investigated the effects of known PLA_2 inhibitors on the K^+ efflux induced by NEM which has been shown to activate KCC in numerous cells, including HepG2 cells (Lauf et al., 1992; Kim and Lee, 2001; Adragna et al., 2004). In these experiments we used $AACOCF_3$, BEL and BPB as inhibitors of $cPLA_2$, $iPLA_2$ and $sPLA_2$, respectively (Narendra Sharath Chandra et al., 2007). As illustrated in Fig. 2, BEL (10 μM) significantly inhibited the NEM (100 μM)-induced K^+ efflux. However, $AACOCF_3$ (10 μM) and BPB (10 μM) did not have an influence on the NEM-induced K^+ efflux. The effects of these drugs on the KCC activity may not be due to their non-specific actions changing cell volume, because we added them to the cells as a form of the concentrated stock solution. Thus, these results suggest that $iPLA_2$-AA signal may play an essential role in the mechanism of KCC activation.

No involvement of AA metabolites in the mechanism of KCC activation

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 KCC activation, we investigated the effects of Indo, a non-selective COX inhibitor (Bakalova et al., 2002) and NDGA, a general LOX inhibitor (Tang et al., 1996) on the K^+ efflux induced by NEM. As depicted in Fig. 3, pretreatment with either Indo (30 μM) or NDGA (50 μM) failed to affect the NEM (100 μM)-induced K^+ efflux. These results suggest that AA metabolites may not have a role in the mechanism of KCC activation.

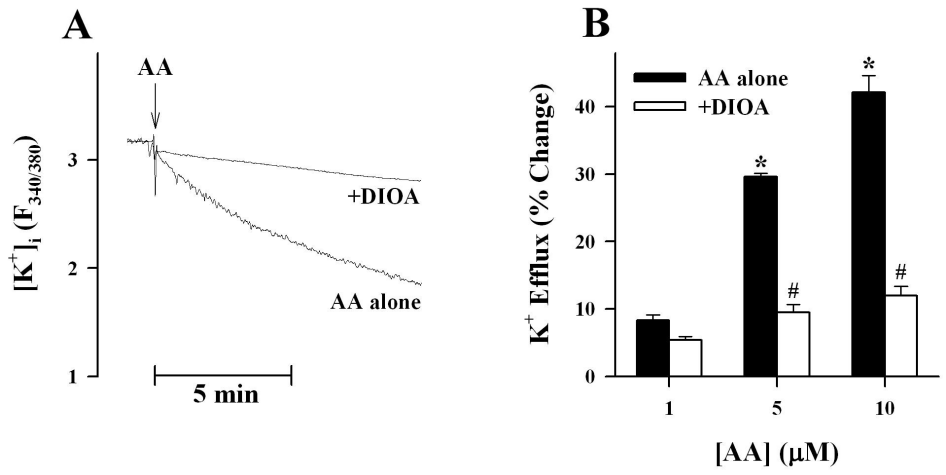

Fig. 1. AA activates KCC in HepG2 human hepatoblastoma cells. The data (A) show changes in $[K^+]_i$ as a function of time, measured by using the K^+-sensitive fluorescent dye PBFI/AM. In the data, PBFI fluorescence ratios are directly proportional to $[K^+]_i$. The arrow shows the time point for addition of AA (10 μM). DIOA (100 μM), a KCC inhibitor, was added 10 min before AA treatment. Quantitative changes (B) were expressed as percent changes of the maximum decrease in PBFI fluorescence ratio 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, #$p < 0.05$ compared to AA alone.

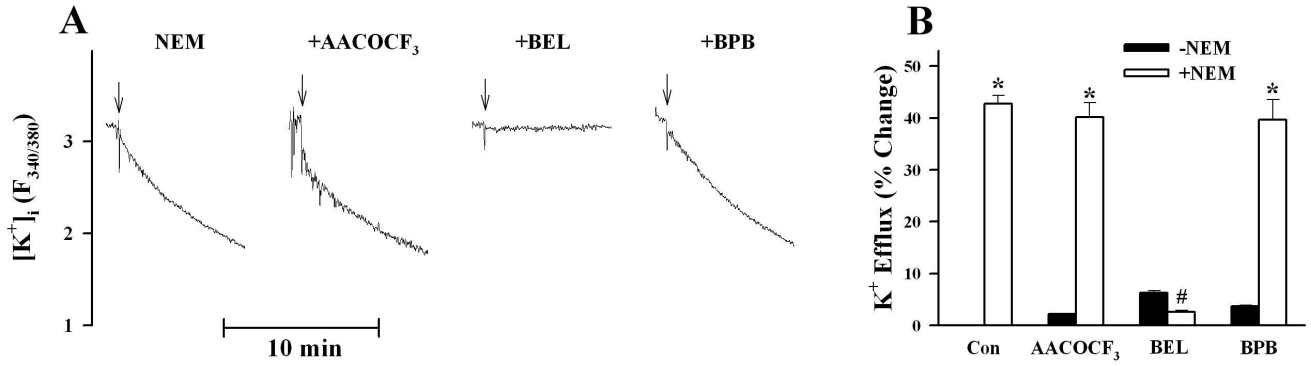

Fig. 2. Effects of PLA_2 inhibitors on the KCC activation induced by NEM in HepG2 human hepatoblastoma cells. The data (A) show changes in $[K^+]_i$ as a function of time, measured by using the K^+-sensitive fluorescent dye PBFI/AM. The arrows show the time points for addition of NEM (100 μM). $AACOCF_3$ (10 μM), BEL (10 μM) and BPB (10 μM) were added 10 min before NEM treatment. 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, #$p < 0.05$ compared to NEM alone.

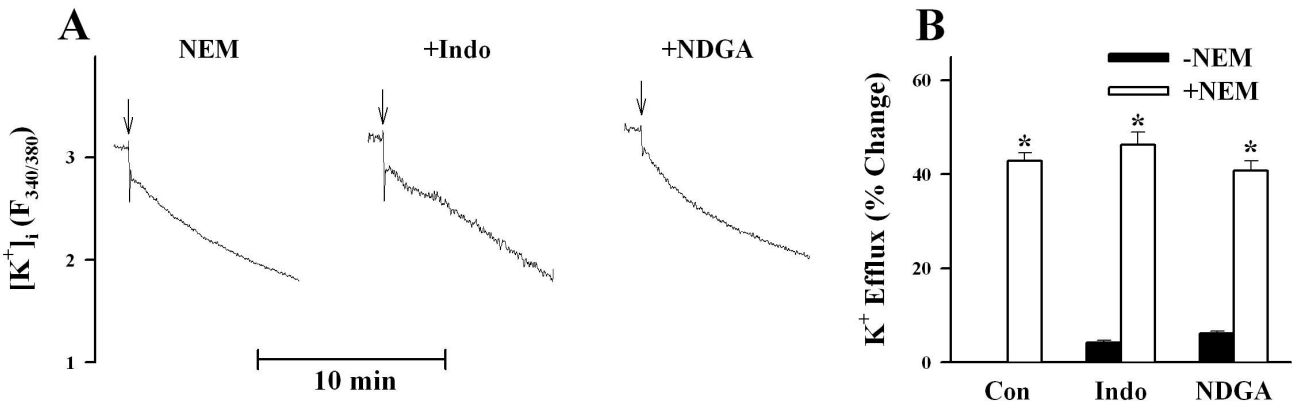

Fig. 3. Effects of inhibitors of COX and LOX on the KCC activation induced by NEM in HepG2 human hepatoblastoma cells. The data (A) show changes in $[K^+]_i$ as a function of time, measured by using the K^+-sensitive fluorescent dye PBFI/AM. The arrows show the time points for addition of NEM (100 μM). Indo (30 μM) and NDGA (50 μM) were added 10 min before NEM treatment. 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.

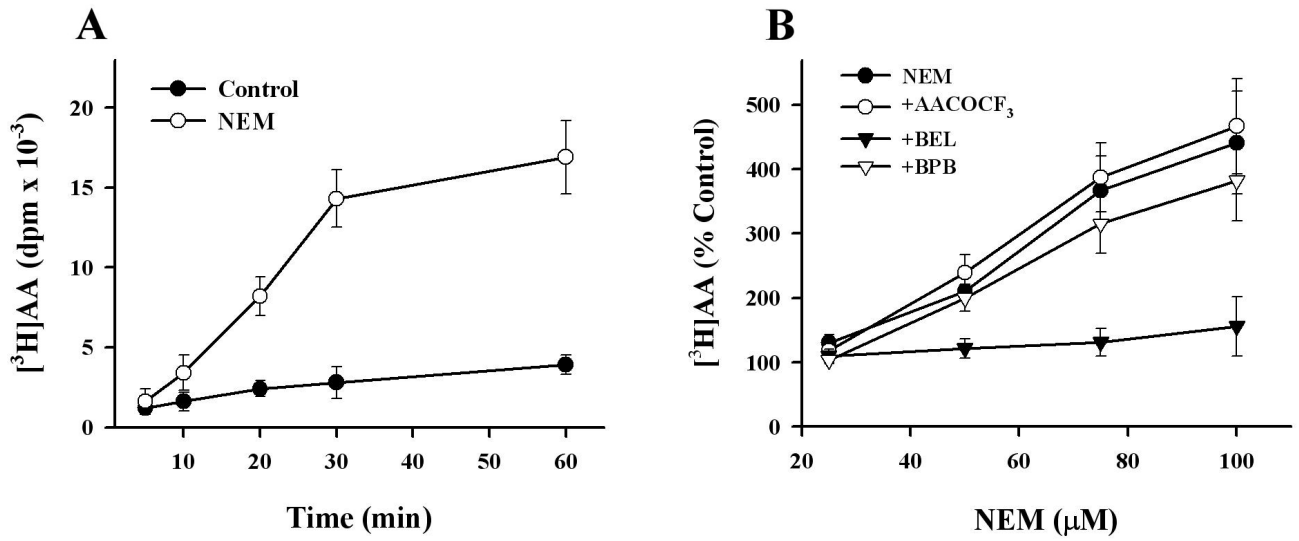

Fig. 4. Time-course of [^{3}H]AA release induced by NEM (A) and the effects of PLA_2 inhibitors on the NEM-induced [^{3}H]AA release (B) in HepG2 human hepatoma cells. (A) HepG2 cells were labeled with medium containing [^{3}H]AA and then treated with either vehicle or NEM (100 μM) for a designated time. Assay for [^{3}H]AA release was done by scintillation counting method as described in Method section. (B) NEM was treated with or without various drugs for 60 min. 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.

NEM, a KCC activator induces AA release through activation of iPLA$_2$

The results from above experiments implicate that $iPLA_2$-AA signal may mediate KCC activation induced by NEM. To confirm this role of $iPLA_2$-AA signal, we examined whether NEM, a KCC activator indeed liberates AA measuring [^{3}H]AA released into the surrounding medium from HepG2 cells labeled with [^{3}H]AA using liquid scintillation spectrometry. As shown in Fig. 4A, NEM (100 μM) profoundly increased AA release within 30 min in the cells. To further identify which subtype of PLA_2 is involved in the process, we studied the effects of specific inhibitors of three different types of PLA_2 on the NEM-induced AA release. As depicted in Fig. 4B, BEL (10 μM) significantly inhibited the NEM-induced [^{3}H]AA liberation, whereas $AACOCF_3$ (10 μM) and BPB (10 μM) did not. These results imply that NEM-induced KCC activation may be due to $iPLA_2$-mediated AA liberation in HepG2 cells.

Role of iPLA$_2$-AA signal in ROS-mediated KCC activation

It has been previously shown that ROS are involved in the mechanism of NEM-induced KCC activation (Kim and Lee, 2001). To clarify the role of AA in ROS-mediated KCC activation, we investigated the effects of the PLA$_2$ inhibitors on the NEM-induced ROS production. As illustrated in Fig. 5, BEL (10 μM) significantly inhibited the NEM (100 μM)-induced ROS generation measured by DCF fluorescence. However, AACOCF$_3$ (10 μM) and BPB (10 μM) did not have an influence. In addition, DPI, an inhibitor of the NADPH oxidase (Kim et al., 2000) almost completely suppressed the NEM-induced ROS increase, which is consistent with the results of a previous study (Kim and Lee, 2001), demonstrating that the NADPH oxidase mediates the NEM-induced ROS production. Taken together, these results indicate that iPLA$_2$-AA signal may be essentially involved in the NADPH oxidase-mediated ROS production and in turn, KCC activation.

AA metabolites did not have a role in the mechanism of KCC activation as shown in Fig. 3. To clarify whether AA metabolites are not involved in the NEM-induced ROS production, we investigated the effects on the NEM-induced ROS production of Indo and NDGA, COX and LOX inhibitors, respectively. As depicted in Fig. 6, pretreatment with either Indo (30 μM) or NDGA (50 μM) failed to affect the NEM (100 μM)-induced ROS generation. These results further suggest that AA metabolites may not play a role in ROS production associated with KCC activation.

ETYA mimics the effects of AA on ROS production and KCC activation

To confirm that AA itself rather than its metabolites

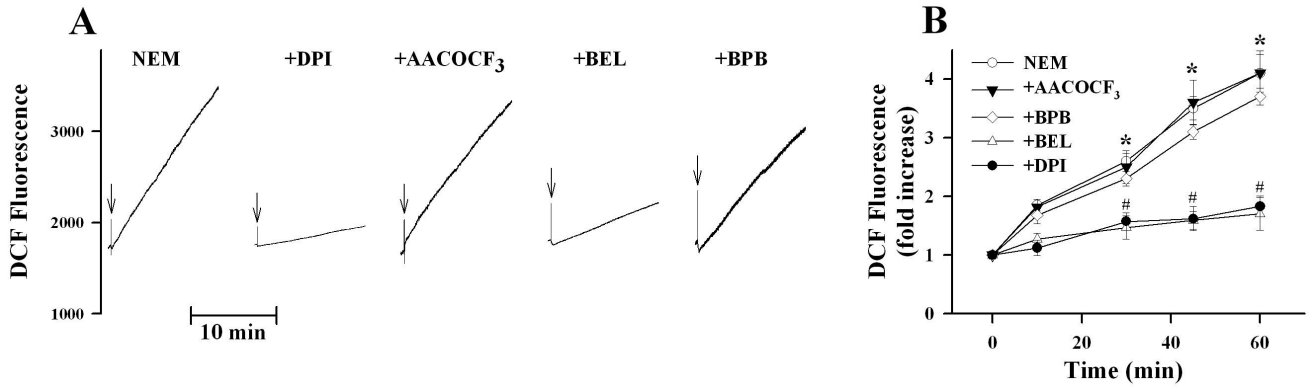

Fig. 5. Effects of PLA$_2$ inhibitors and DPI on the ROS generation induced by NEM in HepG2 human hepatoblastoma cells. The data (A) show changes in ROS levels as a function of time, which was measured by DCF fluorescence method. The arrows show the time points for addition of NEM (100 μM). DPI (50 μM), an inhibitor of NADPH oxidase, and PLA$_2$ inhibitors, AACOCF$_3$ (10 μM), BEL (10 μM) and BPB (10 μM) were added 10 min before NEM treatment. In the data (B) results are expressed as fold increase compared to the initial DCF fluorescence intensity. 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.

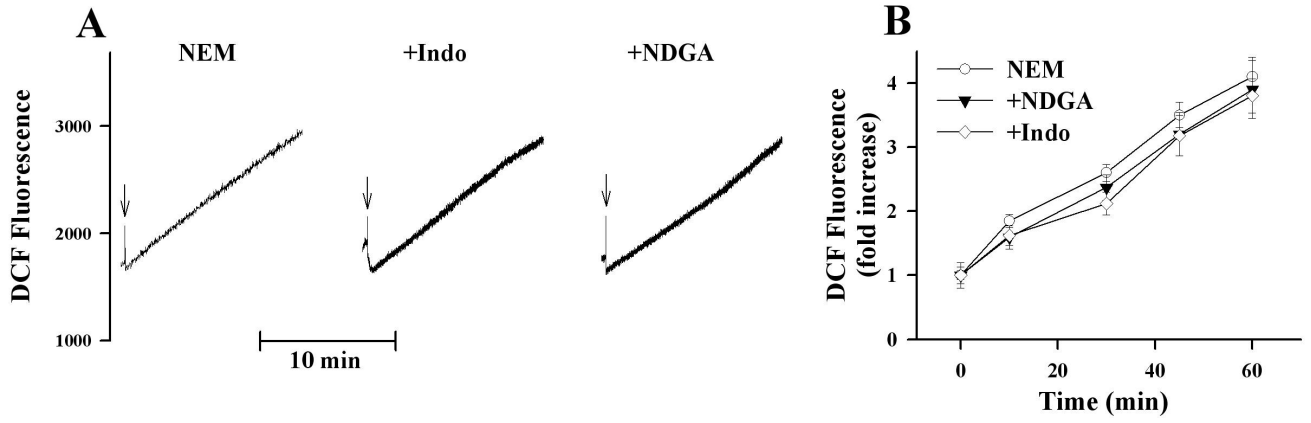

Fig. 6. Effects of inhibitors of COX and LOX on the ROS generation induced by NEM in HepG2 human hepatoblastoma cells. The data (A) show changes in ROS levels as a function of time, which was measured by DCF fluorescence method. The arrows show the time points for addition of NEM (100 μM). Indo (30 μM), a COX inhibitor and NDGA (50 μM), a LOX inhibitor were added 10 min before NEM treatment. In the data (B) results are expressed as fold increase compared to the initial DCF fluorescence intensity. Data points represent the mean values of four replications with bars indicating SEM.

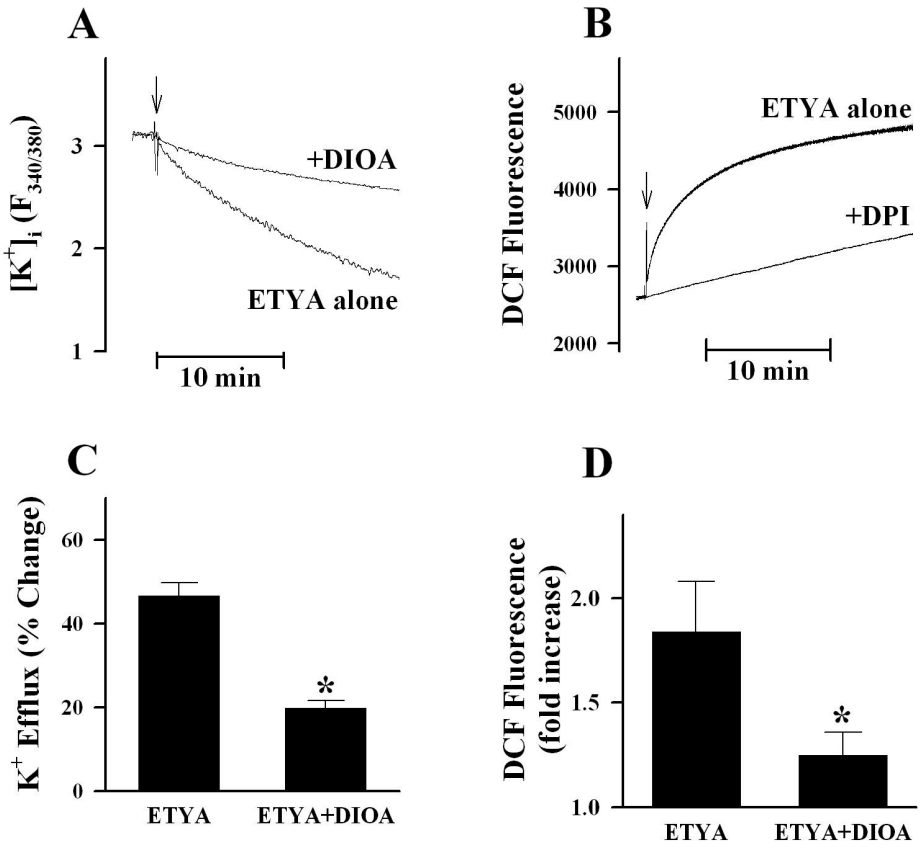

Fig. 7. Effects of ETYA, a non-metabolizable analogue of AA, on the KCC activation (A, C) and ROS generation (B, D) in HepG2 human hepatoblastoma cells. The data (A, B) show changes in $[K^+]_i$ and ROS levels as a function of time, respectively. The arrows show the time points for addition of ETYA (10 μM). In these experiments DIOA (100 μM) and DPI (50 μM), were added 10 min before ETYA treatment. Quantitative changes were expressed as percent changes of the maximum decrease in PBFI fluorescence (C) and fold increase compared to the initial DCF fluorescence intensity (D) 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 ETYA alone.

serves as a key player for ROS-mediated KCC activation, we tested whether ETYA, a non-metabolizable analogue of AA (Kehl, 2001) can mimic the effects of AA on the level of ROS and intracellular K^+ concentration. As depicted in Fig. 7A and C, treatment with ETYA (10 μM) significantly induced K^+ efflux, which was significantly blocked by pretreatment with DIOA, a specific KCC inhibitor. In addition, ETYA (10 μM) profoundly increased ROS level, which was suppressed by pretreatment with DPI, a NADPH oxidase inhibitor. These effects of ETYA were comparable to those of AA shown in Fig. 1. These results strongly suggest that AA itself may be involved in the mechanism of ROS-mediated KCC activation.

DISCUSSION

KCC appears to respond to a variety of physiological stimuli in erythrocytes, including cell swelling, H^+ and urea (Lauf et al., 1992; Adragna et al., 2004). In normal high K^+-containing erythrocytes, KCC activation will result in net KCl efflux. KCC may contribute to cell shrinkage following swelling, and has therefore been implicated in regulatory volume decrease (Adragna et al., 2004). In addition to these physiological roles, inappropriate activation of KCC in red blood cells 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). Recently KCC appears to be involved not only in cancer cell proliferation and invasion (Shen et al., 2004; Hsu et al., 2007a; Hsu et al., 2007b), but also in apoptotic cell death (Kim et al., 2001). Therefore, KCC seems to be important in both physiological and pathophysiological processes, but regulatory mechanism of KCC is not much understood. From the results of this study we suggest that $iPLA_2$-AA signal may be importantly involved in the mechanism of KCC activation. As far as we know, we report, for the first time, the role of $iPLA_2$-AA signal in the regulatory mechanism of KCC activity.

ROS have been shown to be required for the NEM-induced KCC activation in HepG2 cells (Kim and Lee, 2001). Other studies have also implicated that ROS are involved in the regulation of KCC activity in red blood cells (Muzyamba et al., 2000; Gibson et al., 2003). NEM appeared to generate ROS by activation of the NADPH oxidase (Kim and Lee, 2001) which is known to exist in HepG2 cells (Ehleben et al., 1997; Cool et al., 1998). In this study we demonstrated that AA itself generated ROS through activation of the NADPH oxidase in HepG2 cells. In other cellular systems AA has also been reported to activate the NADPH oxidase (Curnutte, 1985; Wong et al., 2003; Block et al., 2006; Hii and Ferrante, 2007; Kim et al., 2008). Since eicosanoids can be produced by AA metabolism via the actions of COX and LOX (Harizi et al., 2008), they are supposed to activate the NADPH oxidase. Although COX-1 genetic deletion and a LOX inhibitor have been reported to attenuate activation of the NADPH oxidase (Zhang et al., 2006; Choi et al., 2008), the results of this study suggest that eicosanoids may not play a role in regulation of activity of the NADPH oxidase

in HepG2 cells. Collectively, KCC activity in HepG2 cells seems to be regulated by the NADPH oxidase-mediated ROS generation linked to iPLA$_2$-AA signal.

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), modulation of norepinephrine release from hippocampus synaptosomes (Wurster et al., 1990), and activation of KCC (Lauf et al., 1992; Adragna et al., 2004). 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). Although the dephosphorylation process seems to be required for the NEM-induced KCC activation (Kaji and Tsukitani, 1991; Starke and Jennings, 1993; Bize et al., 1998), the results of this study suggest that it may be due to AA released by activation of iPLA$_2$. In the present study we did not investigate how NEM induces activation of iPLA$_2$, resulting in release of AA in HepG2 cells, and it remains to be determined in the future study. However, it can be speculated that this action of NEM may be achieved by either direct structural modification or indirect stimulation of the enzyme. Although NEM has been shown to activate PLA$_2$ through elevation of intracellular Ca^{2+} level (Leoncini and Signorello, 1999b) in platelets, this mechanism may be excluded, because activation of iPLA$_2$ does not require elevation of intracellular Ca^{2+} level (Leslie, 2004). Specifically, activation of iPLA$_2$ has been reported to be 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). For the mechanism of the NEM-induced iPLA$_2$ activation in HepG2 cells, these possibilities may not be excluded.

In conclusion, in this study we suggest that iPLA$_2$-AA signal may be importantly involved in the mechanism of KCC activation. Considering that KCC has a role in growth and death of human cancer cells, these findings may contribute to understanding of the strategy for management of human cancers.

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