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Role of reactive oxygen species in apoptosis induced by *N*-ethylmaleimide in HepG2 human hepatoblastoma cells

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Abstract

We have previously reported that *N*-ethylmaleimide induces apoptosis through activation of K^+, Cl^--cotransport in HepG2 human hepatoblastoma cells. In this study, we investigated the role for reactive oxygen species as a mediator of the apoptosis induced by *N*-ethylmaleimide. *N*-ethylmaleimide induced a significant elevation of intracellular level of reactive oxygen species. Treatment with antioxidants (*N*-acetyl cysteine, *N*,*N*′-diphenyl-*p*-phenylenediamine) which markedly suppressed generation of reactive oxygen species, significantly inhibited the *N*-ethylmaleimide-induced activation of K^+, Cl^--cotransport and apoptosis. Inhibitors of NADPH oxidase (diphenylene iodonium, apocynin, D-(+)-neopterine) also significantly blunted the generation of reactive oxygen species, activation of K^+, Cl^--cotransport and apoptosis induced by *N*-ethylmaleimide. These results suggest that reactive oxygen species generated through activation of NADPH oxidase may play a role in the *N*-ethylmaleimide-induced stimulation of K^+, Cl^--cotransport and apoptosis in HepG2 cells.

Keywords: *N*-ethylmaleimide; Reactive oxygen species; Apoptosis; NADPH oxidase; K^+, Cl^--cotransport; HepG2 cell

1. Introduction

The K^+, Cl^--cotransport has been first described in erythrocytes as a swelling-activated K^+ efflux mechanism (Cossins and Gibson, 1997; Lauf et al., 1992). Functional and physiological evidence has shown that K^+, Cl^--cotransport exists in various tissues, such as epithelia (Greger and Schlatter, 1983), 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). K^+, Cl^--cotransport appears to have many important cellular functions; regulation of cell volume (Lauf et al., 1992), transepithelial salt absorption (Amlal et al., 1994), myocardial K^+ loss during ischemia (Yan et al., 1996), regulation of neuronal Cl^- concentration (Rivera et al., 1999) and renal K^+ secretion (Ellison et al., 1985). Recently, we have also reported that K^+, Cl^--cotransport exists and functions as a mediator of apoptotic cell death in HepG2 human hepatoma cells (Kim et al., 2001).

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). On the other hand, induction of apoptosis of cancer cells is regarded as one of the most important methods for cancer treatment (Kornblau, 1998).

Excessively produced reactive oxygen species may result in cellular damage through their interaction with cellular macromolecules and structures (Yu, 1994). Reactive oxygen species appear to act as an important signaling molecule in the processes of apoptotic cell death (Bohler et al., 2000)

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and cell differentiation (Suzukawa et al., 2000). The mechanisms of these actions of reactive oxygen species include activation of proteases and nucleases (Yu, 1994), altered gene expression (Schiaffonati and Tiberio, 1997) and changes in membrane permeability (Yu, 1994). Particularly, recent studies have implicated that reactive oxygen species may be involved in the regulation of activity of K^+, Cl^--cotransport in erythrocytes (Muzyamba et al., 2000).

Thus, the main purpose of the present study was to investigate whether reactive oxygen species are involved in the mechanism of apoptosis associated with activation of K^+, Cl^--cotransport in HepG2 human hepatoblastoma cells. In addition, we examined more specifically whether activation of NADPH oxidase plays a part as an upstream signal for the generation of reactive oxygen species and induction of apoptosis.

2. Materials and methods

2.1. Cell culture

The HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). HepG2 cells were grown at 37 °C in a humidified incubator under 5% CO_2/95% air in an Eagle's minimum essential medium (MEM) 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 confluence, the cells were subcultured following trypsinization.

2.2. 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) to determine the percentage of nuclei with a hypodiploid content indicative of apoptosis (Bombeli et al., 1997).

2.3. Measurement of intracellular reactive oxygen species

Relative changes in intracellular reactive oxygen species in HepG2 cells were monitored using a fluorescent probe, 2′,7′-dichlorofluorescin diacetate (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 2′,7′-dichlorofluorescein (DCF) in the presence of reactive oxygen species. The DCF fluorescence intensity is proportional to the amount of reactive oxygen species 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.

2.4. Measurement of intracellular K^+ concentration

Intracellular K^+ levels were monitored with the K^+-sensitive fluorescent dye, K^+-binding benzofuran isophthalate acetoxylmethyl ester (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 $150 \times g$ 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 intracellular K^+ concentration are reported as the 340:380 fluorescence ratios.

2.5. Materials

The powders for MEM, trypsin solution, sodium pyruvate and all the chemical compounds otherwise indicated, were obtained from Sigma (St. Louis, MO). DCFH-DA and PBFI/AM were from Molecular Probes (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).

2.6. 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.

3. Results

3.1. Role of reactive oxygen species in the activation of K^+, Cl^--cotransport and apoptosis induced by N-ethylmaleimide

To explore the role of reactive oxygen species in the mechanism of actions of *N*-ethylmaleimide, we first examined whether it increases the level of intracellular reactive

oxygen species in HepG2 cells using DCF fluorescence. *N*-ethylmaleimide (100 μM) markedly enhanced the generation of reactive oxygen species in the cells, as shown in Fig. 1A. This effect of *N*-ethylmaleimide was profoundly suppressed by treatment with antioxidants, *N*-acetyl cysteine (50 mM) and *N*,*N*′-diphenyl-*p*-phenylenediamine (10 μM), as illustrated in Fig. 1A. Secondly, we investigated whether reactive oxygen species generated by *N*-ethylmaleimide mediate its activation of K^+, Cl^--cotransport and apoptosis in HepG2 cells which we have previously reported (Kim et al., 2001). Antioxidants, *N*-acetyl cysteine (50 mM) and *N*,*N*′-diphenyl-*p*-phenylenediamine (10 μM), significantly inhibited the *N*-ethylmaleimide-induced intracellular K^+ efflux indicative of activation of K^+, Cl^--cotransport (Kim et al., 2001), as shown in Fig. 1B. They also significantly prevented the cells from apoptosis induced by *N*-ethylmaleimide assessed using flow cytometric analysis by determining a hypodiploid DNA content stained with propidium iodide, as depicted in Fig. 1C and D. Taken together, these results imply that reactive oxygen species may mediate the *N*-ethylmaleimide-induced activation of K^+, Cl^--cotransport and apoptosis in HepG2 cells.

3.2. Role of NADPH oxidase in the generation of reactive oxygen species, activation of K^+, Cl^--cotransport and apoptosis induced by N-ethylmaleimide

Next, we tried to determine the mechanism by which *N*-ethylmaleimide produced reactive oxygen species and the role of this mechanism in the activation of K^+, Cl^--cotransport and apoptosis. Particularly, we focused on the NADPH oxidase as a major mediator of production of reactive oxygen species, as the enzyme has been previously shown to exist and generate reactive oxygen species in the response to an anticancer agent in HepG2 cells (Lee et al., 2000). Treatment with various inhibitors of the NADPH oxidase, diphenylene iodonium (O'Donnell et al., 1993), apocynin (Stolk et al., 1994) and D-(+)-neopterine (Kojima et al., 1993), completely suppressed the generation of reactive oxygen species induced by *N*-ethylmaleimide, as shown in Fig. 2A. They also remarkably inhibited the *N*-ethylmaleimide-induced activation of K^+, Cl^--cotransport, as depicted in Fig. 2B. In addition, they also significantly prevented the induction of apoptosis by *N*-ethylmaleimide, as illustrated in Fig. 2C and D. These results support that *N*-

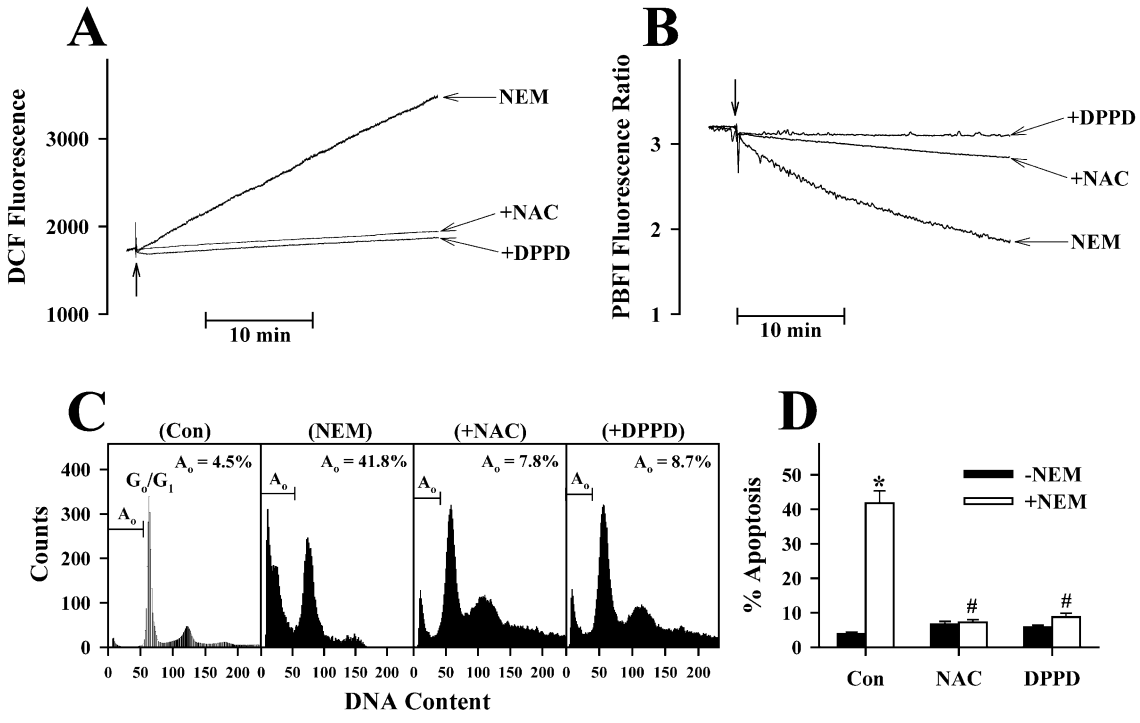

Fig. 1. Effects of antioxidants on the generation of reactive oxygen species (A), K^+, Cl^--cotransport (B) and apoptosis (C and D) induced by *N*-ethylmaleimide in HepG2 human hepatoblastoma cells. The data (A) show changes in levels of reactive oxygen species as a function of time, which was measured by DCF fluorescence method. The arrow shows the time point for addition of *N*-ethylmaleimide (NEM; 100 μM). *N*-acetyl cysteine (NAC; 50 mM) and *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD; 10 μM) were added 10 min before NEM treatment. The data (B) 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 NEM (100 μM). NAC (50 nM) and DPPD (10 μM), antioxidants, were added 10 min before NEM treatment. In the experiments of (C), the cells were incubated with or without NEM (100 μM) for 4 h. NAC (50 mM) and DPPD (10 μM) were given 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_o/G_1 peak, designated A_o, was defined as cells undergoing apoptosis-associated DNA degradation. In bar graphs (D), 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.

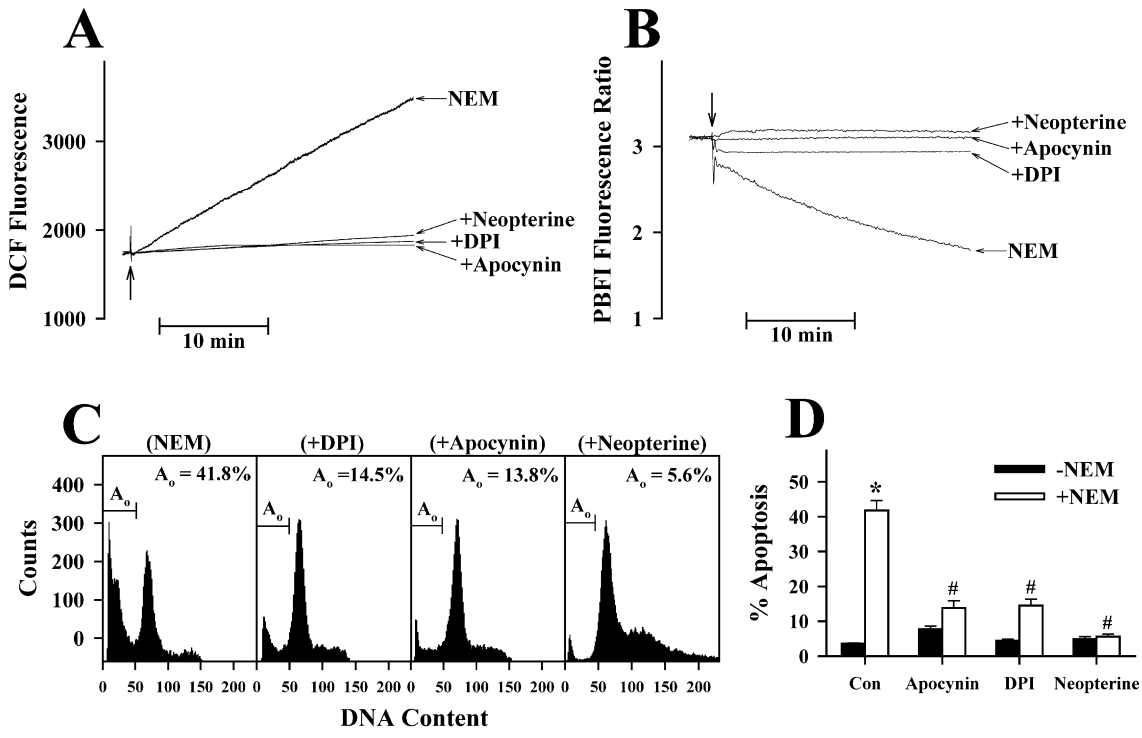

Fig. 2. NADPH oxidase mediates the *N*-ethylmaleimide-induced generation of reactive oxygen species (A), activation of K^+, Cl^--cotransport (B) and apoptosis (C and D) in HepG2 human hepatoblastoma cells. Experimental procedures and data presentations are the same as in Fig. 1. In these experiments, diphenylene iodonium (DPI; 5 μM), apocynin (500 μM) and D-(+)-neopterine (100 μM) were used as an inhibitor of NADPH oxidase. These drugs were given 10 and 30 min before *N*-ethylmaleimide (100 μM) application in the experiments of (A and B) and (C and D), respectively. $^*P<0.05$ compared to control. $^{\#}P<0.05$ compared to *N*-ethylmaleimide alone.

ethylmaleimide may generate reactive oxygen species through the activation of NADPH oxidase, which induces activation of K^+, Cl^--cotransport, leading to induction of apoptosis in HepG2 cells.

4. Discussion

K^+, Cl^--cotransport appears to have many physiological functions, including cell volume regulation (Lauf et al., 1992; Cossins and Gibson, 1997). Additionally, it has pathophysiological roles. Inappropriate activation of K^+, Cl^--cotransport in erythrocytes leads to excessive KCl loss, cell shrinkage and elevation of hemoglobin concentration, leading to deleterious rheological effects, including increased vascular resistance (Stuart and Ellory, 1988). Recently, Shen et al. (2000) have reported that human cervical carcinogenesis is accompanied by up-regulation of K^+, Cl^--cotransport transcripts. We have also reported that the prolonged activation of K^+, Cl^--cotransport could induce apoptosis in HepG2 human hepatoma cells (Kim et al., 2001).

N-ethylmaleimide that reacts with and oxidizes sulfhydryl groups, appears to have many cellular actions, for example, release of arachidonic acid (Neve et al., 1995), inhibition of platelet aggregation (Leoncini and Signorello, 1999), and modulation of noradrenaline release from hippocampus neurons (Wurster et al., 1990). These actions of *N*-ethylmaleimide seem to be due to its 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 K^+, Cl^--cotransport in erythrocytes (Lauf et al., 1992). Recently, *N*-ethylmaleimide has been shown to induce apoptosis through activation of K^+, Cl^--cotransport in HepG2 cells (Kim et al., 2001). However, it has not been clarified that this apoptosis-inducing activity of *N*-ethylmaleimide is due to either a direct modification of thiol groups of the K^+, Cl^--cotransport proteins, or modulation of upstream signaling proteins involving in activation of K^+, Cl^--cotransport. The results of the present study strongly suggest that NADPH oxidase seems to be the upstream signaling molecule whose activation is essentially required for the *N*-ethylmaleimide-induced generation of reactive oxygen species, and in turn, activation of K^+, Cl^--cotransport and apoptosis in HepG2 cells. These conclusions are based on (i) antioxidants which completely blunted the generation of reactive oxygen species by *N*-ethylmaleimide (Fig. 1A), significantly prevented activation of K^+, Cl^--cotransport (Fig. 1B) and induction of apoptosis (Fig. 1C and D), and (ii) specific inhibitors of NADPH oxidase significantly suppressed the *N*-ethylmaleimide-induced generation of reactive oxygen species (Fig. 2A), activation of K^+, Cl^--cotransport (Fig. 2B) and apoptosis (Fig. 2C and D).

Oxidative stress is proposed as a common mechanism of apoptosis (Kamata and Hirata, 1999). Reactive oxygen species have been demonstrated to play an essential role in apoptosis induced by the anticancer agent tamoxifen in HepG2 cells (Lee et al., 2000). The results of this study further support this notion that reactive oxygen species act as a common mediator of apoptosis. The major biological process leading to oxygen-derived generation of reactive oxygen species is electron transport associated with mitochondrial membranes (Halliwell, 1989). The membrane-bound NADPH oxidase also appeared to play a role in production of reactive oxygen species in conjunction with apoptosis induced by an anticancer agent (Lee et al., 2000). The results of the present study showing that the *N*-ethylmaleimide-induced generation of reactive oxygen species is due to the activation of NADPH oxidase, further suggest that it may take an important part in the mechanism of production of reactive oxygen species in HepG2 cells. The NADPH oxidase is originally known to produce reactive oxygen species during the respiratory burst in neutrophils (Babior, 1995). It has also been functionally active in non-phagocytic cells, including endothelial cells (Jones et al., 1996), vascular smooth muscle cells (Marshall et al., 1996), neuroepithelial bodies of the lung (Youngson et al., 1997) and type I cells of the carotid body (Kummer and Acker, 1995). Activation of this enzyme proceeds through a multistep assembly at the plasma membrane of several components including the two subunits of cytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$), the small GTP-binding proteins (Rac and Rap1A), and the cytosolic factors ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) (Babior, 1999). These components has also been detected and functionally active in HepG2 cells (Cool et al., 1998; Ehleben et al., 1997).

In this study, we did not determine how *N*-ethylmaleimide activates the NADPH oxidase, and it remains to be studied in the future. Although speculated, Ca^{2+}/calmodulin-dependent protein kinase-II may be possibly involved in the mechanism of actions of *N*-ethylmaleimide. Because it has been shown to activate Tiam1 (Fleming et al., 1999), a Rac1-specific exchange factor, and thus activate Rac1, which is essentially required for the generation of reactive oxygen species by the NADPH oxidase in HepG2 cells (Cool et al., 1998). However, this mechanism may not be the case, since *N*-ethylmaleimide did not increase intracellular Ca^{2+} concentration (data not shown), which is required for the activation of Ca^{2+}/calmodulin-dependent protein kinase-II (Fujisawa, 2001). Numerous reports have also suggested that arachidonic acid seemed to be implicated in the mechanism of activation of the NADPH oxidase in phagocytic cells (Daniels et al., 1998; Shiose and Sumimoto, 2000). Thus, increased release of arachidonic acid may be a possible mechanism by which *N*-ethylmaleimide-induced activation of NADPH oxidase. This possibility awaits to be tested in the future study.

In conclusion, generation of reactive oxygen species through activation of NADPH oxidase may be the upstream mechanism of increased activity of K^+, Cl^--cotransport associated with the *N*-ethylmaleimide-induced apoptosis in HepG2 cells. These results further suggest that NADPH oxidase may be a good target for the therapeutic intervention of human hepatoma.

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