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Mechanism of Apoptosis Induced by Diazoxide, a K^+ Channel Opener, in HepG2 Human Hepatoma Cells

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The effect of diazoxide, a K^+ channel opener, on apoptotic cell death was investigated in HepG2 human hepatoblastoma cells. Diazoxide induced apoptosis in a dose-dependent manner and this was evaluated by flow cytometric assays of annexin-V binding and hypodiploid nuclei stained with propidium iodide. Diazoxide did not alter intracellular K^+ concentration, and various inhibitors of K^+ channels had no influence on the diazoxide-induced apoptosis; this implies that K^+ channels activated by diazoxide may be absent in the HepG2 cells. However, diazoxide induced a rapid and sustained increase in intracellular Ca^{2+} concentration, and this was completely inhibited by the extracellular Ca^{2+} chelation with EGTA, but not by blockers of intracellular Ca^{2+} release (dantrolene and TMB-8). This result indicated that the diazoxide-induced increase of intracellular Ca^{2+} might be due to the activation of a Ca^{2+} influx pathway. Diazoxide-induced Ca^{2+} influx was not significantly inhibited by either voltage-operative Ca^{2+} channel blockers (nifedipine or verapamil), or by inhibitors of Na^+, Ca^{2+}-exchanger (bepridil and benzamil), but it was inhibited by flufenamic acid (FA), a Ca^{2+}-permeable nonselective cation channel blocker. A quantitative analysis of apoptosis by flow cytometry revealed that a treatment with either FA or BAPTA, an intracellular Ca^{2+} chelator, significantly inhibited the diazoxide-induced apoptosis. Taken together, these results suggest that the observed diazoxide-induced apoptosis in the HepG2 cells may result from a Ca^{2+} influx through the activation of Ca^{2+}-permeable non-selective cation channels. These results are very significant, and they lead us to further suggest that diazoxide may be valuable for the therapeutic intervention of human hepatomas.

Key words: Diazoxide, Apoptosis, HepG2 cells, K^+ channels, Intracellular Ca^{2+}

INTRODUCTION

Apoptosis is a highly organized cell death process that is characterized by ultrastructural modification (cytoskeletal disruption, cell shrinkage, and membrane blebbing), nuclear alteration (chromatin condensation and internucleosomal DNA cleavage), and biochemical changes (activation of proteases) (Kidd, 1998). Loss of apoptosis resulting from genetic changes is likely to be critical components of carcinogenesis (Schulte-Hermann *et al.*, 1997). Many studies have demonstrated that carcinogenic processes are significantly linked to the derangement of apoptosis-signaling pathways (Adams and Cory, 1998; Kastan *et al.*, 1995). An accumulating body of evidence suggests that apoptosis induction of cancer cells is useful for the treatment of cancer (Kornblau, 1998) and all the known anti-cancer agents work by inducing apoptosis in cancer cells (Kamesaki, 1998). Radiation therapy for cancer is also based on apoptosis induction of cancer cells (Crompton, 1998). Although the apoptotic signaling pathways are not completely known, intracellular Ca^{2+} seems to be regarded as an important regulator of apoptosis (McConkey and Orrenius, 1996).

Potassium channels that are ubiquitous in eukaryotic cells; they have diverse cellular functions, such as the regulation of resting membrane potential, frequency of action potential and membrane potential repolarization rates (Hille, 1992). A variety of studies have also shown that K^+ channel activity is involved in regulation of cellular proliferation (Nilius and Wohlrab, 1992; Rouzaire-Dubois and Dubois, 1998; Wang *et al.*, 1998) and differentiation (Cameron *et al.*, 1998; Shirihai *et al.*, 1998). Among the cloned sub-types of K^+ channels (Aguilar-Bryan *et al.*, 1998; Edwards and Weston, 1995), the voltage-dependent

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K^+ channels, ATP-sensitive K^+ channels (K_{ATP} channels), and Ca^{2+}-activated K^+ channels appear to influence cellular proliferation in a variety of cell types (Kamleiter *et al.*, 1998; Liu *et al.*, 1998; Woodfork *et al.*, 1995). Regulation of cell proliferation by K^+ channels appears to occur at the level of cellular DNA synthesis, because the inhibition of K^+ channel activity synchronizes cells in G_0/G_1 (Deutsch, 1990). In addition, K^+ channels have been found to regulate intracellular signal transduction events that concern mitogenic activity (Stanley, 1999). Since uncontrolled cellular proliferation is a main feature of cancer cells in terms of therapeutic management, many attempts have been made to control the proliferation of cancer cells by modulating K^+ channel activities (Lepple-Wienhues *et al.*, 1996; Liu *et al.*, 1998; Woodfork *et al.*, 1995). Many drugs that inhibit these K^+ channels have been shown to suppress proliferation of cancer cells (Rouzaire-Dubois and Dubois, 1991; Wondergem *et al.*, 1998; Xu *et al.*, 1996).

An accumulating body of evidence implies that K^+ channels play a role in apoptotic cell death (Avdonin *et al.*, 1998; Yu *et al.*, 1998). The apoptosis-inducing effects of *Drosophila* gene products, Reaper and Grim, result from their stable inactivation of voltage-gated K^+ channels (Avdonin *et al.*, 1998). In neuronal cells, K^+ channels appear to be involved in amyloid -induced cell death (Yu *et al.*, 1998). A voltage-dependent K^+ channel blocker, 4-aminopyridine, has been shown to induce apoptosis in human brain tumor cells (Chin *et al.*, 1997). The ability of the anti-Fas antibody to induce apoptosis was completely nullified by diminishing the normal K^+ electrochemical gradient in Jurkat cells (Bortner *et al.*, 1997). Apoptotic cells have a much lower intracellular K^+ concentration, as compared with normal cells (Barbiero *et al.*, 1995; Hughes *et al.*, 1997). Furthermore, apoptosis of thymocytes was suppressed by an increase in extracellular K^+ concentration in a dose-dependent manner (Hughes *et al.*, 1997).

Thus, the purpose of this study was to determine whether diazoxide, a K^+ channel opener, induces apoptosis in HepG2 human hepatoblastoma cells. In terms of the mechanism of action, we specifically focused on the role of intracellular Ca^{2+} signals that are commonly involved in the mechanism of apoptosis (McConkey and Orrenius, 1996).

MATERIALS AND METHODS

Materials

The HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). Powdered Eagle's minimum essential medium (MEM) and Earle's basal salt solution (EBSS), diazoxide, glibenclamide, tolbutamide, verapamil, nifedipine, dantrolene, 3,4,5-trimethoxybenzoic acid-8-(diethylamino)-octyl ester (TMB-8), bepridil, benzamil, ethylene glycol-bis-(aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), trypsin solution, sodium pyruvate, probenecid, propidium iodide (PI), ribonuclease A and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). Potassium-binding benzofuran isophthalate aceoxylmethyl ester (PBFI/AM), 1-(2,5-carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2'-amino-methylphenoxy)-ethane-*N,N,N'N'*-tetraacetoxylmethyl ester (Fura-2/AM) and bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid/acetoxymethyl ester (BAPTA/AM) were purchased from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). BAPTA/AM, Fura-2/AM and PBFI/AM were prepared as stock solutions in dimethyl sulfoxide (DMSO), then they were diluted with aqueous medium to the final desired concentrations. The stock solution of drugs was 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 a 5% CO_2 / 95% air mixture in a MEM supplemented with 10% FBS, 200,000 IU/L penicillin, 200 mg/L of streptomycin and 1 mM sodium pyruvate. The culture medium was replaced every other day. After attaining confluence, the cells were subcultured following a trypsinization treatment.

Flow cytometry assays

For the flow cytometry analysis, HepG2 cells were collected and washed twice with PBS buffer, pH 7.4. After fixing in 80% ethanol for 30 min, the cells were washed twice, and resuspended in PBS buffer (pH 7.4) containing 0.1% Triton X-100, 5 mg/L PI and 50 mg/L ribonuclease A for DNA staining. The cells were then analyzed by a FACScan (BIO-RAD, Hercules, CA) and 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).

The normal lipid organization of the plasma membrane is altered soon after apoptosis is initiated. Thus, annexin-V binding was also employed as an indicator of apoptosis (Vermes *et al.*, 1995) to demonstrate the loss of phospholipid asymmetry and the presence of phosphatidylserine on the outer layer of the plasma membrane. Annexin-V binding was analyzed using a commercial kit (Boehringer Mannheim Biochemicals, Mannheim, Germany). The cells were washed in cold PBS, and then resuspended in binding buffer. An aliquot of cell suspension (500 μL) was exposed to Annexin-V-FLUOS. The cells were gently vortexed, incubated at room temperature for 20 min in the dark, and

then analyzed by FACScan within 1 h of staining.

Intracellular K^+ measurement

Intracellular K^+ levels were monitored with the K^+-sensitive fluorescent dye, PBFI/AM (Minta and Tsien, 1989). The cells were washed and then 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 the measurement of PBFI fluorescence was used and fluorescence was monitored at 500 nm with excitation wavelengths of 340 and 380 nm in a stirred quartz cuvette. In the results, the relative changes in intracellular K^+ concentration are reported as the 340:380 fluorescence ratios.

Intracellular Ca^{2+} measurement

Aliquots of the HepG2 cells were washed in EBSS. 5 μM Fura-2/AM was then added and the cells were incubated for 30 min at 37°C. Unloaded Fura-2/AM was removed by centrifugation at 150×*g* for 3 min. The cells were resuspended at a density of 2×10^9 cells/L in Krebs-Ringer buffer (KRB) containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES, 6 mM glucose and 2.5 mM probenecid (pH 7.4). The Fura-2-loaded cells were maintained at 25°C for 90 min before fluorescence measurement. For each experiment, a 0.5 mL aliquot of Fura-2-loaded cells was equilibrated to 37°C in a stirred quartz cuvette. Fluorescence emission (510 nm) was monitored with an excitation wavelength cycling of between 340 and 380 nm using a Hitachi F4500 fluorescence spectrophotometer. At the end of an experiment, the maximum and minimum fluorescence values at each excitation wavelength were obtained by first lysing the cells with 20 mg/L digitonin for the maximum fluorescence value and then adding 10 mM EGTA for the minimum fluorescence value. After obtaining these maximum and minimum values, the 340:380 nm fluorescence ratios were converted into free Ca^{2+} concentrations using a software, F-4500 Intracellular Cation Measurement System, provided by Hitachi.

Data analysis

All the experiments were performed four times. The data were expressed as means standard error of the mean (SEM) and the results were then 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

Induction of apoptotic cell death by diazoxide

The effect of diazoxide on apoptosis in HepG2 cells was examined by using two independent flow cytometric analyses. Diazoxide induced a loss of phospholipid asymmetry, resulting in the appearance of phosphatidylserine on the outer layer of the plasma membrane; this was detected by annexin-V binding, as depicted in Fig. 1A. In addition, diazoxide also induced DNA fragmentation in a concentration-dependent manner, as was assessed by determining the hypodiploid DNA content stained with propidium iodide (Fig. 1B). When taken together, these results indicate that diazoxide induced apoptotic cell death in HepG2 cells.

No involvement of K^+ channels in the diazoxide-induced apoptosis

Since diazoxide is known to act as an activator of ATP-sensitive K^+ (K_{ATP}) channels (Challinor-Rogers and McPherson, 1994), we examined the role of K^+ channels in the observed apoptosis-inducing action of diazoxide. To do this, we measured the change of intracellular K^+ concentration using PBFI fluorescence (Minta and Tsien, 1989). As is shown in Fig. 2A, diazoxide (150 μM) did not

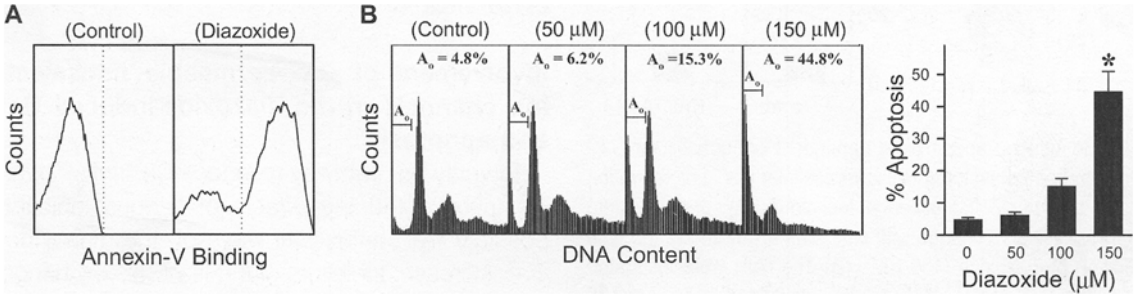

Fig. 1. Diazoxide induces apoptotic cell death of HepG2 human hepatoblastoma cells. (A) After cells were treated with or without diazoxide (150 μM) for 24 h, the cells were stained with Annexin-V-FLUOS and analyzed by flow cytometry. (B) The cells were incubated with diazoxide for 24 h at each designated concentration and the number of apoptotic cells was measured by flow cytometry. 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 the bar graphs, the data represent the mean values of four replications with bars indicating SEM. *p<0.05, as compared to the control.

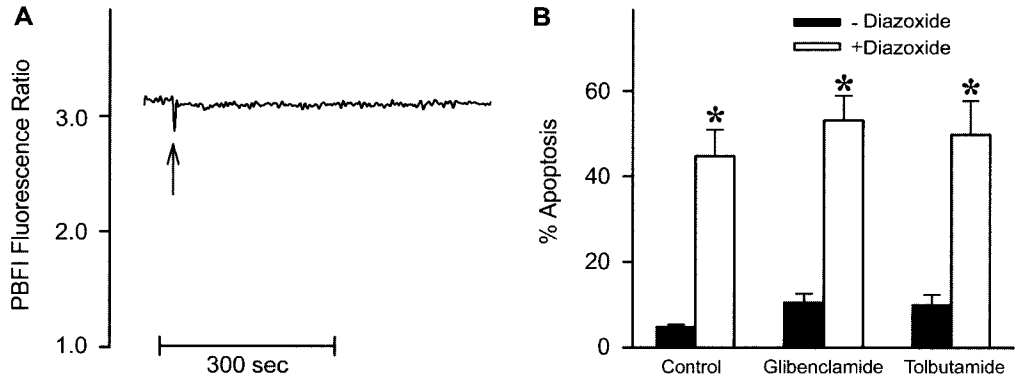

Fig. 2. No involvement of K^+ channels was found for the diazoxide-induced apoptosis in HepG2 human hepatoblastoma cells. (A) The data show changes in intracellular K^+ concentration as a function of time, which was measured by using the K^+-sensitive fluorescent dye PBFI/AM. The arrows show the time point for addition of diazoxide (150 μM). (B) The cells were incubated with diazoxide (150 μM) for 24 h. Inhibitors of K_{ATP} channels (100 μM glibenclamide, 10 μM tolbutamide) were added 30 min before diazoxide treatment. The number of apoptotic cells was measured by flow cytometry. The data represent the mean values of four replications with bars indicating SEM. *p<0.05, as compared to the control.

significantly alter the intracellular K^+ concentration. In addition, treatment with the inhibitors of K_{ATP} channels (100 μM glibenclamide, 100 μM tolbutamide) did not significantly suppress the diazoxide-induced apoptosis, as is shown in Fig. 2B. These results strongly suggest that activation of K_{ATP} channels and the subsequent changes in intracellular K^+ concentration may not be involved in the apoptosis induced by diazoxide.

Role of Ca^{2+} influx in the diazoxide-induced apoptosis

To examine the role of intracellular Ca^{2+} signaling in the apoptosis induced by diazoxide, we assessed the intracellular Ca^{2+} concentration as a function of time using the Fura-2 fluorescence technique (Grynkiewicz *et al.*, 1985). As is shown in Fig. 3A, diazoxide (150 μM) induced a rapid and sustained increase in intracellular Ca^{2+} concentration. To determine the source of the diazoxide-induced intracellular Ca^{2+} increase, we measured intracellular Ca^{2+} concentration using a nominal Ca^{2+}-free medium containing 100 μM EGTA. This experimental protocol can effectively reduce the extracellular free Ca^{2+} concentration, and therefore, blunt the available Ca^{2+} influx. Under these conditions, the cellular Ca^{2+} response to diazoxide (150 μM) was markedly reduced, as is illustrated in Fig. 4A. However, inhibitors of intracellular Ca^{2+} release from internal stores (50 μM dantrolene and 20 μM TMB-8) did not significantly influence intracellular Ca^{2+} increase induced by diazoxide, as is shown in Fig. 4A. Furthermore, treatment with BAPTA/AM, an intracellular Ca^{2+} chelator, and EGTA significantly suppressed the diazoxide-induced apoptosis, whereas dantrolene and TMB-8 did not affect the diazoxide-induced apoptosis, as is shown in Figs. 3B and 4B, respectively. Collectively, these results suggest that Ca^{2+} influx from the extracellular sites may mediate the apoptosis induced by diazoxide.

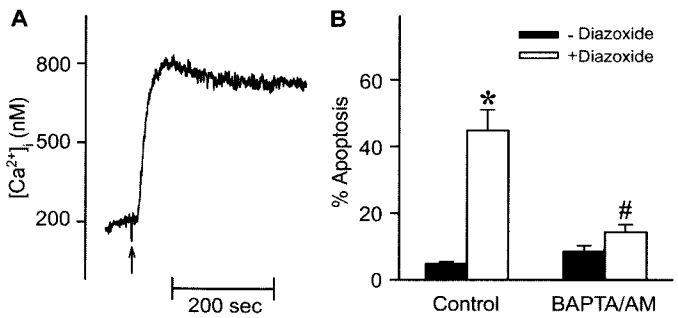

Fig. 3. Diazoxide-induced apoptosis is dependent on intracellular Ca^{2+} in HepG2 human hepatoblastoma cells. Intracellular Ca^{2+} concentration was assessed by Fura-2 fluorescence technique (A), and the data represent intracellular Ca^{2+} change with time. The arrow shows the time point for addition of diazoxide (150 μM). (B) The cells were incubated with diazoxide for 24 h. BAPTA/AM (1 μM) was added to the cells 30 min before treatment with diazoxide (150 μM) and the number of apoptotic cells was measured by flow cytometry. The data represent the mean values of four replications with bars indicating SEM. *p<0.05, as compared to the control. #p<0.05, as compared to the diazoxide alone.

Involvement of Ca^{2+}-permeable nonselective cation channels in the diazoxide-induced Ca^{2+} influx and apoptosis

To verify the pathway to induce Ca^{2+} influx by diazoxide, we investigated the effects of various inhibitors of the possible Ca^{2+} influx pathways on the diazoxide-induced Ca^{2+} increase. Inhibitors of the Na^+/Ca^{2+} exchange (bepridil and benzamil) and voltage-dependent Ca^{2+} channel blockers (verapamil and nifedipine) did not significantly alter the diazoxide-induced intracellular Ca^{2+} increase (Fig. 5A). However, an inhibitor of Ca^{2+}-permeable nonselective cation channels, flufenamic acid, completely inhibited the

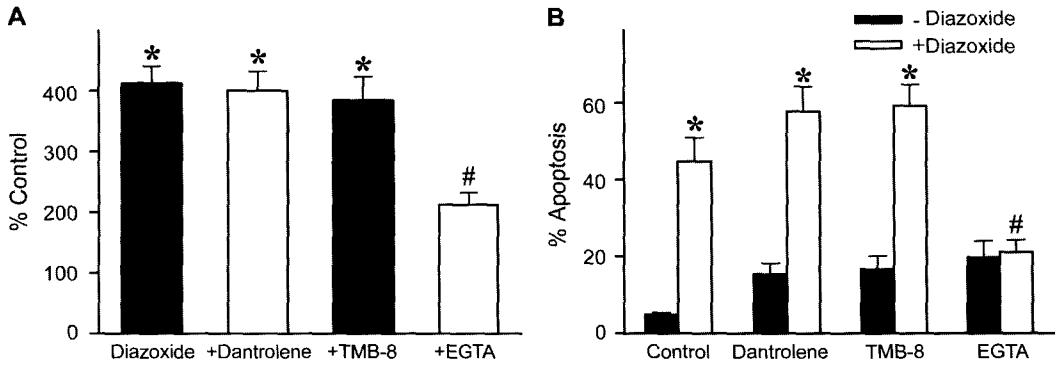

Fig. 4. Activation of Ca^{2+} influx mediates the diazoxide-induced intracellular Ca^{2+} increase and apoptosis in HepG2 human hepatoblastoma cells. Intracellular Ca^{2+} concentration (A) and apoptosis (B) were assessed by the Fura-2 fluorescence technique and flow cytometry, respectively. (A) A nominal Ca^{2+}-free medium containing 100 μM EGTA was used. Dantrolene (50 μM) and TMB-8 (20 μM) were added 5 min before diazoxide application. The data for the diazoxide (150 μM)-induced changes in intracellular Ca^{2+} levels are expressed as a percent of control condition in which the cells were incubated with diazoxide-free medium. (B) Diazoxide (150 μM) was treated for 24 h. Dantrolene (50 μM), TMB-8 (20 μM) and EGTA (1 mM) were added 30 min before diazoxide treatment. The data (A and B) represent the mean values of four replications with bars indicating SEM. *p<0.05, as compared to the control. #p<0.05, as compared to the diazoxide alone.

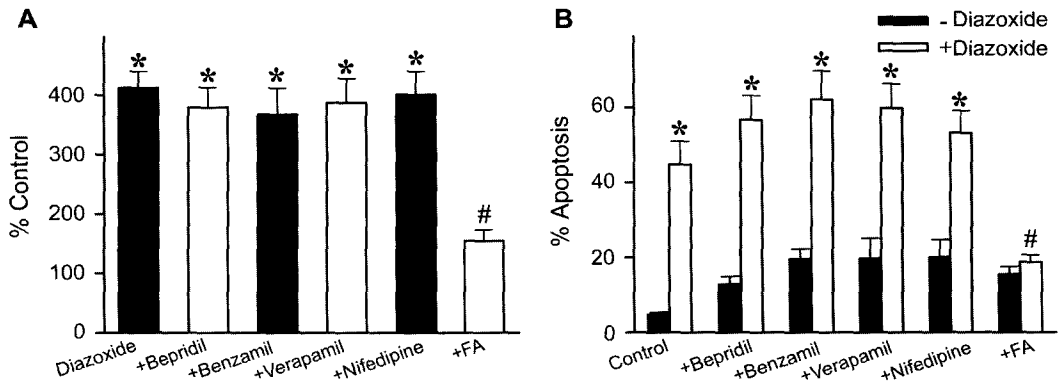

Fig. 5. Involvement of Ca^{2+}-permeable nonselective cation channels in the diazoxide-induced Ca^{2+} influx (A) and apoptosis (B) in HepG2 human hepatoblastoma cells. Experimental procedures and data presentations are the same as Fig. 4. In the experiments inhibitors of Na^+, Ca^{2+}-exchanger (50 μM bepridil, 50 μM benzamil), voltage-sensitive Ca^{2+} channel blockers (100 μM verapamil, 100 μM nifedipine) and an inhibitor of non-selective cation channels (100 μM flufenamic acid) were used. The data (A and B) represent the mean values of four replications with bars indicating SEM. *p<0.05, as compared to the control. #p<0.05, as compared to the diazoxide alone.

diazoxide-induced Ca^{2+} influx (Fig. 5A). These results imply that this type of channels, but not the Na^+/Ca^{2+} exchanger or voltage-dependent Ca^{2+} channels, may mediate Ca^{2+} influx by diazoxide. Furthermore, flufenamic acid, but not bepridil, benzamil, verapamil and nifedipine, profoundly inhibited the diazoxide-induced apoptosis (Fig. 5B). Taken together, Ca^{2+}-permeable nonselective cation channels may be actively involved in the Ca^{2+} influx pathway activated by diazoxide.

DISCUSSION

K^+ channel activity has been implicated in cellular proliferation in a variety of cell types (Nilius and Wohlrab, 1992; Rouzaire-Dubois and Dubois, 1998; Wang *et al.*, 1998). Many agents that inhibit K^+ channels have been shown to suppress cellular proliferation (Rouzaire-Dubois and Dubois, 1991; Wondergem *et al.*, 1998; Xu *et al.*, 1996). The K^+ channel blockers have particularly been used to suppress tumor cell proliferation (Lepple-Wienhues *et al.*, 1996; Nilius and Wohlrab, 1992; Wondergem *et al.*, 1998) and to induce apoptosis (Chin *et al.*, 1997). However, the exact role of the K^+ channels in apoptosis of human tumor cells originated from hepatic tissue has not yet been completely settled. So, the main purpose of this study was to determine whether the modulation of K^+ channel activity by diazoxide has an effect on the apoptotic cell death, and we used HepG2 human hepatoblastoma cells to determine this. The results of the present study have clearly shown that diazoxide induced apoptosis in the HepG2 cells (Fig. 1). Since the only known pharmacological action of diazoxide is to activate K_{ATP} channels

(Challinor-Rogers and McPherson, 1994), the roles of this channel in the diazoxide-induced regulation of intracellular K^+ concentration, as well as apoptosis, were investigated. We found as an unexpected result that diazoxide did not alter intracellular K^+ concentration (Fig. 2A), and that glibenclamide and tolbutamide, inhibitors of K_{ATP} channels did not affect the apoptotic action of diazoxide (Fig. 2B). These results clearly support the idea that K_{ATP} channels may not be involved in the diazoxide-induced apoptosis. Recently, Malhi *et al.* have shown that K_{ATP} channels exist in the HepG2 cells used in this study (Malhi *et al.*, 2000), and these channels were detected by using the method of polymerase chain reaction (PCR). Nevertheless, the total K^+ conductance due to the activation of K_{ATP} channels by diazoxide may be too small to change the intracellular K^+ concentration.

Since intracellular Ca^{2+} has been shown to act as a common mediator of chemical-induced cell death (Harman and Maxwell, 1995) and also to act as a signal transducer in the mechanism of apoptosis (Distelhorst and Dubyak, 1998; McConkey and Orrenius, 1996), we investigated whether an intracellular Ca^{2+} signal is involved in the observed diazoxide-induced apoptosis of the HepG2 cells. Indeed, diazoxide increased the intracellular Ca^{2+} concentration (Fig. 3A), and a treatment with BAPTA/AM, an intracellular Ca^{2+} chelator, significantly suppressed the diazoxide-induced apoptosis (Fig. 3B). This implied that diazoxide may induce apoptosis through a disturbance of the intracellular Ca^{2+} homeostasis mechanism.

Diazoxide seemed to increase Ca^{2+} concentration by way of a Ca^{2+} influx, since the diazoxide-induced increase in the intracellular Ca^{2+} concentration was significantly prevented by using nominal Ca^{2+}-free medium containing 100 μM EGTA. However, the same result was not achieved by the internal Ca^{2+} release blockers, TMB-8 and dantrolene (Fig. 4A). Consistently, the diazoxide-induced apoptosis was significantly prevented by EGTA, but the inhibitors of internal Ca^{2+} release did not affect it (Fig. 4B). This suggests that Ca^{2+} influx may mediate the apoptosis induction by diazoxide. Previously, we have reported that a sustained Ca^{2+} influx mediates apoptotic cell death in HepG2 cells that is induced by various substances, including tamoxifen (Kim *et al.*, 1999) and *tert*-butyl hydroperoxide (Kim *et al.*, 2000).

Extracellular free Ca^{2+} ions may enter into the cells by the following two mechanisms: (*i*) activation of plasma membrane Ca^{2+} channels, and (*ii*) activation of the reverse mode of the Na^+/Ca^{2+} exchange mechanism. There were no significant effects of bepridil and benzamil, known inhibitors of the Na^+/Ca^{2+} exchanger, on the Ca^{2+} influx and apoptosis induced by diazoxide (Figs. 5A and 5B), and this indicates that the Na^+/Ca^{2+} exchanger may not be involved in these actions of diazoxide. In addition, the voltage-dependent Ca^{2+} channel blockers, verapamil and nifedipine did not significantly alter the diazoxide-induced intracellular Ca^{2+} increase and apoptosis (Figs. 5A and 5B). This suggests that there is no involvement of these ion channels in the activity of diazoxide in the HepG2 cells. Recently, Ca^{2+}-permeable nonselective cation channels have been reported to exist in the HepG2 cells (Chen *et al.*, 1997). Since these channels have mediated Ca^{2+} influx in many different types of cells (Fasolato *et al.*, 1993; Zhang *et al.*, 1994), we determined the possible role for this channel in the diazoxide-induced Ca^{2+} influx by using flufenamic acid (FA), a known inhibitor of the channels (Gogelein *et al.*, 1990). FA completely inhibited the diazoxide-induced Ca^{2+} influx (Fig. 5A) and apoptosis (Fig. 5B), indicating that these types of plasma membrane Ca^{2+} channels may act as a major Ca^{2+} influx pathway that is activated by diazoxide.

In this study, we did not determine the downstream mechanism of Ca^{2+} influx linked to the final apoptosis induction, and this mechanism remains to be studied. There is however, an accumulating body of evidence that implies that an intracellular Ca^{2+} signal plays an important role in the mechanism of apoptosis (McConkey and Orrenius, 1996). One of targets for the elevation of intracellular Ca^{2+} concentration is the activation of Ca^{2+}-dependent protein kinases and phosphatases. Activation of calcineurin, a Ca^{2+}/calmodulin-dependent protein serine/threonine phosphatase, by an intracellular Ca^{2+} increase (Bonnefoy-Berard *et al.*, 1994) has been shown to induce apoptosis through regulating the activity of the transcription factor, NF-AT (nuclear factor of activated T cells) (Shibasaki *et al.*, 1997). Direct activation of the Ca^{2+}-dependent neutral proteinase, calpain, may represent another target for the intracellular Ca^{2+} action in apoptosis. Calpain is rapidly activated in apoptotic cells (Squier *et al.*, 1994), and the specific inhibitors of calpain block apoptosis in many different types of cells (Jordan *et al.*, 1997; Squier *et al.*, 1997). Increased intracellular Ca^{2+} activates Ca^{2+}/Mg^{2+}-dependent endonuclease (Cohen and Duke, 1984), and this results in DNA fragmentation, which is the most characteristic biochemical feature of apoptosis (Wyllie *et al.*, 1984). Ca^{2+}-dependent transglutaminase which catalyzes the post-translational coupling of amines to proteins and the crosslinking of proteins, also appears to be a target for Ca^{2+} action. The enzyme is highly activated in apoptotic cells (Fesus *et al.*, 1987), and the overexpression of this enzyme triggers apoptotic cell death (Melino *et al.*, 1994).

Recently, diazoxide appears to activate the mitochondrial ATP sensitive potassium (mitoK_{ATP}) channels (Duchen, 2004), which protects neurons against apoptotic cell death induced by oxidative stress (Teshima *et al.*, 2003). We do not know the exact reason for the differential effects; apoptosis induction in the hepatoma cells observed in this

study, and apoptosis inhibition in the neuronal cells. Although speculated, in the hepatoma cells diazoxide may act primarily on the plasma membrane, rather than the mitoK_{ATP} channels. However, we cant exclude the possibility that the mitoK_{ATP} channels may be absent, or if present, they have no profound influence on the apoptotic process in the hepatoma cells.

In conclusion, diazoxide was shown to induce apoptosis in HepG2 human hepatoblastoma cells. It is very interesting that the modulation of K^+ channel activity may not be involved, whereas Ca^{2+} influx through the activation of Ca^{2+}-permeable nonselective cation channels may play a central role in the diazoxide-induced apoptosis. These results further suggest that diazoxide may be quite valuable in the future for the therapeutic management of human hepatomas.

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REFERENCES

Adams, J. M., and Cory, S.. The Bcl-2 protein family: arbiters of cell survival. *Science*, 281, 1322-1326 (1998).

Aguilar-Bryan, L., Clement, J. P. 4th, Gonzalez, G., Kunjilwar, K., Babenko, A., and Bryan, J., Toward understanding the assembly and structure of K_{ATP} channels. *Physiol. Rev.*, 78, 227-245 (1998).

Avdonin, V., Kasuya, J., Ciorba, M. A., Kaplan, B., Hoshi, T., and Iverson, L., Apoptotic proteins Reaper and Grim induce stable inactivation in voltage-gated K^+ channels. *Proc. Natl. Acad. Sci. U. S. A.*, 95, 11703-11708 (1998).

Barbiero, G., Duranti, F., Bonelli, G., Amenta, J. S., and Baccino, F. M., Intracellular ionic variations in the apoptotic death of L cells by inhibitors of cell cycle progression. *Exp. Cell Res.*, 217, 410-418 (1995).

Bombeli, T., Karsan, A., Tait, J. F., and Harlan, J. M., Apoptotic vascular endothelial cells become procoagulant. *Blood*, 89, 2429-2442 (1997).

Bonnefoy-Berard, N., Genestier, L., Flacher, M., and Revillard, J. P., The phosphoprotein phosphatase calcineurin controls calcium-dependent apoptosis in B cell lines. *Eur. J. Immunol.*, 24, 325-329 (1994).

Bortner, C. D., Hughes, F. M., Jr, and Cidlowski, J. A., A primary role for K^+ and Na^+ efflux in the activation of apoptosis. *J. Biol. Chem.*, 272, 32436-32442 (1997).

Cameron, J. S., Lhuillier, L., Subramony, P., and Dryer, S. E., Developmental regulation of neuronal K^+ channels by target-derived TGF *in vivo* and *in vitro*. *Neuron*, 21, 1045-1053 (1998).

Challinor-Rogers, J. L., and McPherson, G. A., Potassium channel openers and other regulators of K_{ATP} channels. *Clin. Exp. Pharmacol. Physiol.*, 21, 583-597 (1994).

Chen, W. H., Yeh, T. H., Tsai, M. C., Chen, D. S., and Wang, T. H., Characterization of Ca^{2+}- and voltage-dependent nonselective cation channels in human HepG2 cells. *J. Formos. Med. Assoc.*, 96, 503-510 (1997).

Chin, L. S., Park, C. C., Zitnay, K. M., Sinha, M., DiPatri, A. J. Jr., Perillan, P., and Simard, J. M., 4-Aminopyridine causes apoptosis and blocks an outward rectifier K^+ channel in malignant astrocytoma cell lines. *J. Neurosci. Res.*, 48, 122-127 (1997).

Cohen, J. J., and Duke, R. C., Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.*, 132, 38-42 (1984).

Crompton, N. E., Programmed cellular response in radiation oncology. *Acta Oncol.*, 37 Suppl 11, 1-4 (1998).

Deutsch, C., Potassium channels: basic function and therapeutic aspects. *Prog. Clin. Biol. Res.*, 334, 251-271 (1990).

Distelhorst, C. W., and Dubyak, G., Role of calcium in glucocorticosteroid-induced apoptosis of thymocytes and lymphoma cells: resurrection of old theories by new findings. *Blood*, 91, 731-734 (1998).

Duchen, M. R., Roles of mitochondria in health and disease. *Diabetes*, 53, S96-102 (2004).

Edwards, G., and Weston, A. H., The role of potassium channels in excitable cells. *Diabetes Res. Clin. Pract.*, 8 Suppl, S57-66 (1995).

Fasolato, C., Hoth, M., Matthews, G., and Penner, R., Ca^{2+} and Mn^{2+} influx through receptor-mediated activation of nonspecific cation channels in mast cells. *Proc. Natl. Acad. Sci. USA*, 90, 3068-3072 (1993).

Fesus, L., Thomazy, V., and Falus, A., Induction and activation of tissue transglutaminase during programmed cell death. *FEBS Lett.*, 224, 104-108 (1987).

Gogelein, H., Dahlem, D., Englert, H. C., and Lang, H. J., Flufenamic acid, mefenamic acid and niflumic acid inhibit single nonselective cation channels in the rat exocrine pancreas. *FEBS Lett.*, 268, 79-82 (1990).

Grynkiewicz, G., Poene, M., and Tsien, R. Y., A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, 260, 3440-3450(1985).

Harman, A. W., and Maxwell, M. J., An evaluation of the role of calcium in cell injury. *Annu. Rev. Pharmacol. Toxicol.*, 35, 129-144 (1995).

Hille, B., Ionic Channels of Excitable Membranes, 2nd Ed., Sinauer Associates, Inc., Sunderland, MA, pp. 115-139 and 472-503 (1992).

Hughes, F. M., Jr, Bortner, C. D., Purdy, G. D., and Cidlowski, J. A., Intracellular K^+ suppresses the activation of apoptosis in lymphocytes. *J. Biol. Chem.*, 272,30567-30576 (1997).

Jordan, J., Galindo, M. F., and Miller, R. J., Role of calpain and interleukin-1 beta converting enzyme-like proteases in the

beta-amyloid-induced death of rat hippocampal neurons in culture. *J. Neurochem.*, 68, 1612-1621 (1997).

Kamesaki, H., Mechanisms involved in chemotherapy-induced apoptosis and theirimplications in cancer chemotherapy. *Int. J. Hematol.*, 68, 29-43 (1998).

Kamleiter, M., Hanemann, C. O., Kluwe, L., Rosenbaum, C., Wosch, S., Mautner, V. F., Muller, H. W., and Grafe, P., Voltage-dependent membrane currents of cultured human neurofibromatosis type 2 Schwann cells. *Glia*, 24, 313-322 (1998).

Kastan, M. B., Canman, C. E., and Leonard, C. J., P53, cell cycle control and apoptosis: implications for cancer. *Cancer Metastasis Rev.*, 14, 3-15 (1995).

Kidd, V. J., Proteolytic activities that mediate apoptosis. *Annu. Rev. Physiol.*, 60,533-573 (1998).

Kim, J. A., Kang, Y. S., Lee, S. H., Lee, E. H., and Lee, Y. S., Involvement of Ca^{2+} influx in the mechanism of tamoxifen-induced apoptosis in HepG2 human hepatoblastoma cells. *Cancer Lett.*, 147, 115-123 (1999).

Kim, J. A., Kang, Y. S., Lee, S. H., and Lee, Y. S., Inhibitors of Na^{+}/Ca^{2+} exchanger prevent oxidant-induced intracellular Ca^{2+} increase andapoptosis in a human hepatoma cell line. *Free Radic. Res.*, 33, 267-277 (2000).

Kornblau, S. M., The role of apoptosis in the pathogenesis, prognosis, and therapy of hematologic malignancies. *Leukemia*, 12 Suppl 1, S41-46 (1998).

Lepple-Wienhues, A., Berweck, S., Bohmig, M., Leo, C. P., Meyling, B., Garbe, C., and Wiederholt, M., K^{+} channels and the intracellular calcium signal in human melanoma cell proliferation. *J. Membr. Biol.*, 151, 149-157 (1996).

Liu, S. I., Chi, C. W., Lui, W. Y., Mok, K. T., Wu, C. W., and Wu, S. N., Correlation of hepatocyte growth factor-induced proliferation and calcium-activated potassium current in human gastric cancer cells. *Biochim. Biophys. Acta*, 1368, 256-266(1998).

Malhi, H., Irani, A. N., Rajvanshi, P., Suadicani, S. O., Spray, D. C., McDonald, T. V., and Gupta, S., KATP channels regulate mitogenically-induced proliferationin primary hepatocytes and human liver cell lines: implications for liver growth control and potential therapeutic targeting. *J. Biol. Chem.*, 275, 26050-26057 (2000).

McConkey, D. J. and Orrenius, S., The role of calcium in the regulation of apoptosis. *J. Leukoc. Biol.*, 59, 775-783 (1996).

Melino, G., Annicchiarico-Petruzzeli, M., Piredda, L., Candi, E., Gentile, V., Davies, P. J., and Piacentini, M., Tissue transglutaminase and apoptosis: Sense and antisense transfection studies with human neuroblastoma cells. *Mol. Cell Biol.*, 14,6584-6596 (1994).

Minta, A. and Tsien, R. Y., Fluorescent indicators for cytosolic sodium. *J. Biol. Chem.*, 264, 19449-19457 (1989).

Nilius, B. and Wohlrab, W., Potassium channels and regulation of proliferation ofhuman melanoma cells. *J. Physiol (Lond).*, 445, 537-548 (1992).

Rouzaire-Dubois, B. and Dubois, J. M., K^{+} channel block-induced mammalian neuroblastoma cell swelling: a possible mechanism to influence proliferation. *J. Physiol (Lond).*, 510, 93-102 (1998).

Rouzaire-Dubois, B. and Dubois, J. M., A quantitative analysis of the role of K^{+} channels in mitogenesis of neuroblastoma cells. *Cell Signal.*, 3, 333-339 (1991).

Schulte-Hermann, R., Bursch, W., Low-Baselli, A., Wagner, A., and Grasl-Kraupp, B., Apoptosis in the liver and its role in hepatocarcinogenesis. *Cell Biol. Toxicol.*, 13, 339-348 (1997).

Shibasaki, F., Kondo, E., Akagi, E., and McKeon, F., Suppresion of signaling through NF-AT by interactions between calcineurin and BCL-2. *Nature*, 386, 728-731(1997).

Shirihai, O., Attali, B., Dagan, D., and Merchav, S., Expression of two inward rectifier potassium channels is essential for differentiation of primitive human hematopoietic progenitor cells. *J. Cell Physiol.*, 177, 197-205 (1998).

Squier, M. K. T. and Cohen, J. J., Calpain, an upstream regulator of thymocyte apoptosis. *J. Immunol.*, 158, 3690-3697 (1997).

Squier, M. K. T., Miller, A. C. K., Malkinson, A. M., and Cohen, J. J., Calpain activationin apoptosis. *J. Cell Physiol.*, 159, 229-237 (1994).

Stanley, R. G., Advances in second messenger and phosphoprotein research. Academic Press, Orlando, FL 33, 107-127 (1999).

Teshima, Y., Akao, M., Li, R. A., Chong, T. H., Baumgartner, W. A., Johnston, M. V., and Marban, E., Mitochondrial ATP-sensitive potassium channel activation protectscerebellar granule neurons from apoptosis induced by oxidative stress. *Stroke*, 34, 1796-1802 (2003).

Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C., A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods*, 184, 39-51 (1995).

Wang, S., Melkoumian, Z., Woodfork, K. A., Cather, C., Davidson, A. G., Wonderlin, W.F., and Strobl, J. S., Evidence for an early G_1 ionic event necessary for cell cycle progression and survival in the MCF-7 human breast carcinoma cell line. *J. Cell Physiol.*, 176, 456-464 (1998).

Wondergem, R., Cregan, M., Strickler, L., Miller, R., and Suttles, J., Membrane potassium channels and human bladder tumor cells: II. Growth properties. *J. Membr. Biol.*, 161, 257-262 (1998).

Woodfork, K. A., Wonderlin, W. F., Peterson, V. A., and Strobl, J., Inhibition of ATP-sensitive potassium channels causes reversible cell-cycle arrest of human breast cancer cells in tissue culture. *J. Cell Physiol.*, 162, 163-171 (1995).

Wyllie, A. H., Morris, R. G., Smith, A. L., and Dunlop, D., Chromatin cleavage inapoptosis: Association with condensed chromatin morphology and dependence ormacromolecular synthesis. *J. Pathol.*, 142, 67-67 (1984).

Xu, B., Wilson, B. A., and Lu, L., Induction of human myeloblastic ML-1 cell G_1 arrest by suppression of K^+ channel activity. *Am. J. Physiol.*, 271, C2037-2044 (1996).

Yu, S. P., Farhangrazi, Z. S., Ying, H. S., Yeh, C. H., and Choi D. W., Enhancement of outward potassium current may participate in β-amyloid peptide-induced cortical neuronal death. *Neurobiol. Dis.*, 5, 81-88 (1998).

Zhang, H., Inazu, M., Weir, B., Buchanan, M., and Daniel, E., Cyclopiazonic acid stimulates Ca^{2+} influx through non-specific cation channels in endothelial cells. *Eur. J. Pharmacol.*, 251, 119-125 (1994).