Diazoxide-mediated Preconditioning against Apoptosis Involves Activation of cAMP-response Element-binding Protein (CREB) and NFκB*

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Treatment of various types of cells with the mitochondrial ATP-sensitive K⁺ channel opener, diazoxide, preconditioning cells to subsequent injuries and inhibits apoptosis. The mechanism of such preconditioning is not well understood. We have studied the effect of diazoxide pretreatment on mitochondrial morphology and function in HL60 cells and on susceptibility of these cells to apoptosis. We have found that diazoxide pretreatment inhibited etoposide-induced apoptosis and mitochondrial dysfunction. Diazoxide induced moderate mitochondrial swelling and increase in the cytosolic fraction of mitochondrial intermembrane proteins including cytochrome c without any significant effect on the oxidative phosphorylation function or membrane potential. Possibly as an adaptive response, total protein and mRNA levels of cytochrome c and of the anti-apoptotic Bcl-2 family member, Bcl-xl, increased. These effects coincided with activation of the transcription factors cAMP-response element-binding protein (CREB) and NFκB. The gene encoding cytochrome c carries the cAMP-response element (CRE), and the gene encoding Bcl-xl carries both the CRE and NFκB response elements. The inability of etoposide to trigger apoptosis in preconditioned cells was most likely because of pro-survival signaling by CREB and NFκB, which included up-regulation of cytochrome c and Bcl-xl. All described effects were reversed by a specific mitochondrial ATP-sensitive K⁺ channel inhibitor, 5-hydroxydecanoate, proving the specificity of the action of diazoxide. Preconditioning was also reversed by a specific NFκB inhibitor, SN50, proving the importance of this transcript factor for the phenomenon of preconditioning. CREB and NFκB were activated most likely in response to an observed elevation in cytosolic calcium following diazoxide treatment. We, therefore, conclude that diazoxide-mediated preconditioning against apoptosis involves activation of the pro-survival transcription factors CREB and NFκB.

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1 The abbreviations used are: mitoK-ATP, mitochondrial ATP-sensitive K⁺ channel; 5-HD, 5-hydroxydecanoate; Dz, diazoxide; TPP⁺, tetrakis(4-chlorophenyl)phosphonium; PBS, phosphate-buffered saline; CREB, cAMP-response element-binding protein; CRE, cAMP-response element; pCREB, phosphorylated CREB; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester.

In various types of cells, opening of the mitochondrial ATP-sensitive K⁺ (mitoK-ATP) channels leads to enhanced resist-
**EXPERIMENTAL PROCEDURES**

**Reagents**—Most chemicals were obtained from Sigma unless otherwise indicated.

**Cell Culture and Treatment**—HL60 cells were grown in RPMI 1640 media supplemented with a 10% fetal bovine serum and 1% penicillin/streptomycin (all media components were from Invitrogen) to the density of 1 x 10^6/ml. Cells were treated with either 30 μM diazoxide (Dz) in the absence or presence of 600 μM 5-hydroxydecanoate or with MeSO as a vehicle control. After 1 h, some aliquots of cells were washed with PBS and then treated with 50 μM etoposide. Some cells were treated with diazoxide in the presence of 50 μM SN50 or 10 μM H59 (both from Calbiochem). To chelate cytosolic calcium, some cells were loaded with 6 μM BAPTA-AM (Tod Labs) for 30 min at 37 °C, washed in PBS, and then treated with Dz.

**Cell Fractions and Whole Cell Lysates**—Nuclear fractions were prepared using the NE-PER nuclear extraction kit (Pierce) according to the manufacturer’s protocol. Cytosolic fractions were prepared without the cell homogenization step to avoid possible homogenization-related release of mitochondrial proteins because of membrane fragility. Instead cells were incubated in permeabilization buffer (195 mM mannitol, 65 mM sucrose, 2 mM HEPES, pH 7.4, 0.05 mM EGTA, 0.5 mg/ml bovine serum albumin, 0.01% digitonin) for 5 min on ice and spun down (Hermle microtube rotor, 13,000 rpm, 5 min, +4 °C). The low ionic strength of the permeabilization buffer prevented any additional release of mitochondrial intermembrane proteins as was tested experimentally. Cell lysates were prepared by washing cells in PBS at room temperature, resuspending them in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% Triton X-100) at 10 x 10^6 cells/ml on ice for 15 min, and sonicating them for 30 s on ice. Cell fractions and lysates were stored at −20 °C until needed.

**Detection of Apoptosis**—Apoptotic cells were detected by staining nuclei with 1 μg Hoechst 33342 (Molecular Probes). Stained nuclei were visualized using a fluorescence microscope. The number of condensed, i.e. apoptotic nuclei versus uniformly stained, i.e. normal nuclei were counted.

**Mitochondrial Membrane Potential**—Mitochondrial membrane potential (∆Ψm) was measured in digitonin-permeabilized cells (18) using the TPP^+ uptake method as described in detail in Ref. 19.

**Oxygen Consumption**—Oxygen consumption was assayed using a Clark-type oxygen sensor (20) in a sealed thermostated chamber as described previously (21). Cells at a concentration of 1 x 10^6/ml were loaded into the chamber at +37 °C. After equilibration, the chamber was sealed, and a reading was taken for 10 min, after which 1% dithionite was added to calibrate the oxygen sensor.

**Electron Microscopy**—Ten million cells per sample were washed in PBS containing 0.1% BSA and incubated with P. buffer (0.1 M Na2HPO4) overnight at +4 °C, washed in P, buffer, and stained with 1% OsO4 in P, buffer for 30 min. After three washes in P, buffer, cells were resuspended in 4% melted agarose and spun down (bucket centrifuge, 3500 rpm, 1 min). Agarose-trapped cell pellets were solidified for 1 h at +4 °C, cut into 1-mm3 pieces, dehydrated sequentially with 25, 50, 75, 90, and 100% ethanol (20 min each), and embedded in Spurr’s resin. Cryostat slices were then prepared and visualized using a Hitachi H-7100 transmission electron microscope.

**Adenylate Kinase Activity**—Adenylate kinase in cytosolic fractions was determined enzymatically as described previously (22). To measure adenylate kinase, 0.1 ml of the cytosolic fraction was added to 0.9 ml of a reaction mixture containing 150 mM KCl, 100 mM triethanolamine, pH 7.4, 16 mM MgSO4, 10 mM EDTA, 0.2 mM NADH, 1.2 mM ATP, 0.29 mM phosphoenolpyruvate, 7 units/ml pyruvate kinase, and 20 units/ml lactate dehydrogenase. After the signal stabilized, 1.4 mM AMP was added. The enzyme-dependent rate of NADH oxidation (V_{NADH}) in a coupled reaction was measured using a Hitachi spectrofluorometer. Excitation was at 340 nm, and emission was measured at 450 nm. The enzyme concentration was then calculated using standard V_{NADH} versus [adenylate kinase] calibration curve.

**Western Blotting**—Protein samples (30 μg of total protein) were electrophoresed against protein ladder standards and electrobotted onto nitrocellulose. After blocking with 5% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 for 2 h, membranes were incubated overnight with primary antibodies diluted 1:2000. Antibodies used were anti-cytochrome c (Pharminex), anti-holocytochrome c (Cruz), anti-pCREB (Upstate Biotechnology), and anti-pCREB (Upstate Biotechnology). Membranes were then washed and incubated for 1 h with corresponding horseradish peroxidase-conjugated secondary antibodies diluted 1:50,000. After washing, membranes were developed using chemiluminescent substrate West- Pico (Pierce), photographed, scanned, and analyzed using Adobe Photoshop software. To verify consistency of loading, membranes were either reversibly stained with Ponceau S before blocking or stripped after the development and reprobed with anti-β-actin antibody (ICN).

**Real-time Reverse Transcription PCR**—At the indicated times, 2 x 10^6 cells of each experimental group were taken, and total cellular RNA was prepared using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. The concentration of prepared RNA was assayed by UV absorption at 260 nm. Fifty ng of total RNA was reverse transcribed into cDNA using SuperScript first-strand synthesis kit (Invitrogen), according to the manufacturer’s protocol. One μl of the cDNA was subjected to real-time PCR using the following sets of primers: cytochrome c (5′-GGG TTA GTG TTA GAA AGG CAA GAA G-3′ and 5′-CAA AAG TTA TTT TTG TTC GGA TTA G-3′), Bel-1 (5′-GAG ACC CCC AGT GCC ATC AAT G-3′ and 5′-CCG GAA GAG TCC ATT CAC TAC CTG TCC-3′), and actin (5′-GGG CGA CGC CCC TGG AAC CAT GTT GCC CCC T-3′ and 5′-GCT TGA TGT CCT GAT TGT TGC TGG TGC TGC CAT ATC TCC ATG CC-3′). Real-time PCR was performed as described previously (23) using the RotorGene real-time DNA amplification system (Corbett Research) and the double strand-specific fluorescent dye SYBR Green to monitor DNA synthesis. PCR products were subjected to a melting curve analysis, and the data were analyzed with the RotorGene analysis software. The levels of cytochrome c or Bel-1 were normalized to mRNA levels of actin.

**CREB and NFκB Transcriptional Activities**—Transcriptional activities of CREB and NFκB were determined using a heterologous promoter-reporter luciferase assay. To assay CREB, cells were co-transfected with the CREB firefly luciferase reporter vector pCREB-Luc (Invitrogen) and pRL-SV40. To assay NFκB, cells were co-transfected with the NFκB reporter vector pNFκB-Luc (Promega). Luciferase activity was measured using the dual luciferase reporter assay system (Promega) according to the manufacturer’s protocol as described previously (24).

**Cytosolic Calcium Measurements**—Free cytosolic calcium was measured in living cells with the calcium-sensitive fluorescent probe Fura-2 AM as described previously (25). Cells were loaded with 3 μM Fura-2 AM for 30 min at 37 °C, washed, and resuspended in regular media at 1 x 10^6/ml. After 1 h of treatment with MeSO (Ct), Dz, or Dz + 5-HD, the cell suspension was loaded into a round, stirred 37 °C thermostated cuvette in a Hitachi spectrofluorometer, and Fura-2 fluorescence was measured at 505 nm (excitation) at 340 and 380 nm. The Fura-2 340/380 excitation ratio was used as a measure of a free cytosolic calcium.

**RESULTS**

**Pretreatment with Diazoxide Protects Cells against Apoptosis**—As discussed above, earlier studies have shown that pretreatment with diazoxide often protects cells and inhibits apoptosis induced by various stimuli. To determine whether pretreatment with diazoxide preconditioned cells against apoptotic stimuli in our model system, we incubated HL60 cells with 30 μM diazoxide for 1 h, washed, and then added the potent apoptosis inducer, etoposide, to a final concentration of 50 μM. Then, 1.5 and 3 h later, the percentage of apoptotic cells was measured by Hoechst 33342 staining. A subset of cells was co-incubated with a specific mitoK-ATP channel inhibitor, 5-HD. Fig. 1A shows that apoptosis was greatly inhibited by pretreatment with diazoxide, the effect being partially reversed by co-incubation with 5-HD. During apoptosis in the described system, mitochondrial function is progressively impaired (18). To assess mitochondrial function in cells pretreated with diazoxide and then induced with etoposide, we measured mitochondrial membrane potential using the TPP^+ uptake method (19) and the rate of respiration, V_{O2}, using the oxygen electrode (20) at the indicated time points. As shown in Fig. 1B, pretreatment with diazoxide prevented the decrease in mitochondrial membrane potential and respiration and, therefore, protected mitochondrial function against subsequent apoptotic injury. This effect was again partially reversed by 5-HD. We, therefore, confirmed that treatment of
HL60 cells with Dz protects them against apoptosis-related injury.

Effect of Diazoxide on Mitochondrial Morphology and Function—To understand how diazoxide preconditions cells, we investigated its effect on mitochondrial morphology and function. We incubated HL60 cells with diazoxide for 1 h and prepared samples for electron microscopy as described previously (18). As seen in Fig. 2A, diazoxide treatment led to the appearance of moderately swollen mitochondria with ballooning cristae. Statistical analysis of electron micrographs (Fig. 2B) revealed that in the control almost 100% of all mitochondria have a normal conformation, whereas after 1 h of treatment with diazoxide, 40% of the mitochondria change conformation from normal to swollen. This was reversible by co-incubation of cells with 5-HD.

To find out whether mitochondrial function was affected after treatment with diazoxide, we measured oxygen consumption and mitochondrial membrane potential. Diazoxide treatment led to slight stimulation of respiration but had no effect on mitochondrial membrane potential (Fig. 2C). This was expected because earlier studies had revealed similar results in isolated mitochondria (8). Therefore, although the diazoxide treatment led to 5-HD-sensitive moderate swelling and remodeling of mitochondria, their oxidative function and membrane potential were not impaired.

Treatment with Diazoxide Results in Increased Cytosolic Fractions of Mitochondrial Intermembrane Proteins—Even moderate swelling and remodeling of mitochondria may lead to rupture of the outer mitochondrial membrane and release of intermembrane proteins. We, therefore, analyzed cytosolic fractions of cells treated with diazoxide for 1 h for the presence of mitochondrial intermembrane proteins cytochrome c and adenylate kinase. Cytosolic and total cytochrome c were assayed by Western blotting of either cytosolic fractions (Fig. 3A) or total lysates using antibody specific to holocytochrome c.
Quantitative analysis of Western blots (Fig. 3B) revealed that on average 5% of total cytochrome c was present in the cytosol after treatment with diazoxide. This represents a very limited release, compared with the one observed during apoptosis, when at least 30% of total cytochrome c was released from the mitochondria (21). Using antibody specific to holocytochrome c prevented detection of apocytochrome c on its way to being imported into mitochondria. Cytosolic adenylate kinase, measured enzymatically as described previously (22), also increased (Fig. 3C), showing nonspecific permeabilization of the outer mitochondrial membrane because of moderate swelling after treatment with diazoxide. It should be noted that the observed moderate increase in cytosolic cytochrome c did not result in the induction of apoptosis for up to 6 h (data not shown).

**Overexpression of Mitochondrial Proteins**—While analyzing cytosolic and total cytochrome c following treatment with diazoxide, we noticed a consistent increase in total cellular cytochrome c (Fig. 4, A and B). To confirm whether such an increase reflected enhanced expression, we performed a real-time reverse transcription PCR analysis (24) (Fig. 4C), and we found that both Bcl-xl protein and Bcl-xl mRNA levels were elevated after treatment with diazoxide. These data indicate that the expression of anti-apoptotic Bcl-2 family protein Bcl-xl is up-regulated in concert with cytochrome c.

Observed overexpression of cytochrome c and Bcl-xl after treatment with diazoxide was reversed by the addition of 5-HD (Fig. 4). All of these measured mitochondrial proteins are nuclear coded. Thus, diazoxide treatment induces mitochondrial-nuclear and nuclear-mitochondrial communication resulting in up-regulation of expression of mitochondrial proteins such as cytochrome c and Bcl-xl.

**Activation of CREB and NFκB**—The question remained what transcription factor(s) could be responsible for up-regulation of such diverse targets as a component of a mitochondrial respiratory chain, cytochrome c, and a Bcl-2 family member, Bcl-xl. It had been shown in previous studies that CREB protein could be such a factor; CREB can transcriptionally regulate proteins of the mitochondrial respiratory chain as well as Bcl-2 family proteins (9) and other targets. CREB often works in concert with another transcription factor, NFκB (11). NFκB itself has been shown previously to regulate the expression of Bcl-xl (12). We, therefore, decided to test whether these systems were activated after treatment with diazoxide. Fig. 5A shows that in diazoxide-treated cells, there was an increased fraction of phosphorylated CREB (pCREB) as well as an increased nuclear fraction of the p65/RelA regulatory subunit of NFκB.
NFκB. Quantitative analysis of Western blots is shown in Fig. 5B. Increased phosphorylation of CREB and an increased nuclear presence of the p65/RelA subunit of NFκB mark the activation of these factors. It should be noted that in HL60 cells, as in many other cancer cell lines and in contrast to normal non-malignant cell lines, pCREB and nuclear p65/RelA are present even in control cells (26, 27). To further prove that the described transcription factors were indeed activated after diazoxide treatment, we performed heterologous luciferase promoter-reporter assays using either pCREB-luc or pNFκB-luc constructs as described previously (24). The assay confirmed activation of both CREB and NFκB (Fig. 5C). The activation of CREB and NFκB factors after diazoxide treatment was sensitive to 5-HD.

Thus, we observed activation of CREB and NFκB transcription factors after treatment with diazoxide. Their activation was marked by increased phosphorylation and by an increased nuclear fraction of the p65/RelA subunit, respectively. The heterologous luciferase promoter-reporter assay also showed an increase in CREB and NFκB activities.

Activation of NFκB Could Be Caused by Increased Cytosolic Calcium and Is Required for Preconditioning—The unanswered question was: what triggered activation of both CREB and NFκB after treatment with diazoxide? Both of these factors can be activated by elevated cytosolic calcium (16, 17). It has been suggested (28) that diazoxide treatment may result in inhibition of mitochondrial calcium uptake and reduced mitochondrial calcium buffering capacity. As a result, cytosolic calcium may become elevated (29). To test this, we loaded cells with the calcium-sensitive fluorescent probe Fura-2 AM and measured Fura-2 fluorescence in response to diazoxide treatment as described previously (25). The assay showed that cells treated with diazoxide for 1 h exhibit increased cytosolic calcium compared with the control (Fig. 6A). The elevation in cytosolic calcium was reversed by co-treatment with 5-HD.

Chelation of cytosolic calcium with the intracellular calcium chelator BAPTA inhibited both CREB and NFκB (Fig. 6B, bar 4 in each panel) and prevented CREB and NFκB activation after diazoxide treatment (Fig. 6B, bar 5 in each panel).
Diazoxide Treatment Leads to Activation of CREB and NFκB

FIG. 7. Sequence of events triggered by diazoxide and leading to preconditioning (See “Discussion”).

could not check whether BAPTA-mediated inhibition of CREB and NFκB affected sensitivity of Dz-pretreated cells to etoposide because BAPTA itself has a protective effect during apoptosis in the described system (30).

To investigate the importance of each transcription factor for the phenomenon of preconditioning, we incubated cells with diazoxide in the presence of either the CREB inhibitor H89 (31) or the NFκB inhibitor SN50 (15) and then induced apoptosis. Although H89 effectively inhibited CREB (Fig. 6B, top panel, bar 2) and prevented CREB activation after diazoxide treatment (Fig. 6B, top panel, bar 3), it did not reverse preconditioning (data not shown). On the other hand, SN50 not only inhibited NFκB (Fig. 6B, bottom panel, bar 2) and prevented NFκB activation after diazoxide treatment (Fig. 6B, bottom panel, bar 3) but also abolished the effect of preconditioning and restored sensitivity to etoposide as shown in Fig. 6C.

Therefore, NFκB appears to be a major factor responsible for the preconditioning because inhibition of NFκB restored sensitivity to etoposide. Both CREB and NFκB became activated possibly in response to elevation in cytosolic calcium caused directly or indirectly by diazoxide-mediated opening of mitoK-ATP channels.

DISCUSSION

We have studied the effect of diazoxide on preconditioning. The results of our study are summarized in a diagram in Fig. 7. We have found that treatment with diazoxide caused moderate swelling and remodeling in about 40% of all analyzed mitochondrial inner membrane proteins, including cytochrome c. Observed remodeling of mitochondrial cristae could make cytochrome c available for release possibly through a small number of Bax/Bak oligomers constitutively present in the outer mitochondrial membrane. All of this led directly or indirectly to increases in cytosolic calcium, possibly because of disruption of mitochondrial calcium uptake. Such an effect of diazoxide-mediated opening of the mitoK-ATP channels on mitochondrial calcium uptake has been observed in other studies (28). Increased cytosolic calcium could activate the transcription factors CREB and NFκB. Activated CREB and NFκB induced the expression of the anti-apoptotic protein Bcl-xl and the respiratory chain protein cytochrome c. Increased expression of Bcl-xl shifted the ratio of anti- to pro-apoptotic Bcl-2 family proteins in favor of anti-apoptotic. Overexpression of cytochrome c resulted in replenishment of a pool of this vitally important mitochondrial protein, which is otherwise lost during apoptosis. All of this led to protection of mitochondrial function and overall resistance to subsequent apoptotic stimuli.

Recent literature suggests that there are effects of diazoxide and 5-HD not related to mitoK-ATP channels but related rather to metabolic stress. Such data are very important; however they are often applicable to higher concentrations of diazoxide (more than 100 μM) and to models using isolated mitochondria or submitochondrial particles. For instance, Holmuhamedov et al. (32) found that diazoxide at a concentration of 100 μM has protonophoretic properties in planar phospholipid membranes and in isolated mitochondria. However, as shown by Kowaltowski et al. (8), protonophoretic properties of diazoxide at concentrations less than 50 μM are insignificant.

Hanley et al. (33) found that diazoxide dose-dependently inhibits oxidation of succinate in submitochondrial particles. These authors argued that diazoxide may exert its preconditioning effect via inhibition of succinate-dependent respiration and a subsequent moderate increase in reactive oxygen species production and may result in PKC activation. In contrast to Hanley’s hypothesis, Ferranti et al. (34) reported that diazoxide actually reduced reactive oxygen species production by isolated mitochondria. Moreover, in our model system we saw not inhibition but stimulation of respiration by diazoxide (Fig. 2C). Therefore, it is unlikely that the effect was associated with inhibition of succinate dehydrogenase under our conditions.

Kopustinskiene et al. (35) reported that the diazoxide-stimulated respiration was not K+-dependent but was dependent on the mitochondrial ADP/ATP translocase; however, they used twice as much diazoxide (58 μM) and isolated mitochondria. Also, the molecular composition of the mitoK-ATP channel is still unknown, and it has been suggested that the ADP/ATP translocase may be a part of it (36, 37).

Therefore, although there may be K+-independent effects of high concentrations of diazoxide on mitochondria, in our model system we did not see any inhibition of mitochondrial function. On the contrary, our data indicate stimulation of respiration in the absence of depolarization.

The significance of our finding is that it identifies transcription factors CREB and NFκB as important mechanistic components during preconditioning. Activation of these transcription factors links the event specific to mitochondria, i.e. opening of the mitoK-ATP channels, to cytosolic events, i.e. elevation in cytosolic calcium and activation of the transcription factors CREB and NFκB, followed by nuclear response, i.e. increased expression of nuclear coded proteins, and as a result, to overall cellular response, i.e. enhanced resistance to an apoptotic signal.

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