

Mitochondrial K_{ATP} channel openers activate the ERK kinase by an oxidant-dependent mechanism

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Samavati, Lobelia, Martha M. Monick, Salih Sanlioglu, Garry R. Buettner, Larry W. Oberley, and Gary W. Hunninghake. Mitochondrial K_{ATP} channel openers activate the ERK kinase by an oxidant-dependent mechanism. *Am J Physiol Cell Physiol* 283: C273–C281, 2002. First published March 13, 2002; 10.1152/ajpcell.00514.2001.—Extracellular signal-regulated kinases (ERKs) are key regulatory proteins that mediate cell survival, proliferation, and differentiation. Reactive oxygen species (ROS) may play a role in activation of the ERK pathway. Because mitochondria are a major source of ROS, we investigated whether mitochondria-derived ROS play a role in ERK activation. Diazoxide, a potent mitochondrial ATP-sensitive K^+ (K_{ATP}) channel opener, is known to depolarize the mitochondrial membrane potential and cause a reversible oxidation of respiratory chain flavoproteins, thus increasing mitochondrial ROS production. Using THP-1 cells as a model, we postulated that opening mitochondrial K_{ATP} channels would increase production of ROS and, thereby, regulate the activity of the ERK kinase. We found that opening mitochondrial K_{ATP} channels by diazoxide induced production of ROS as determined by an increased rate of dihydroethidium and dichlorofluorescein fluorescence. This increased production of ROS was associated with increased phosphorylation of ERK kinase in a time-dependent fashion. The MEK inhibitors PD-98059 and U-0126 blocked ERK activation mediated by diazoxide. *N*-acetylcysteine, but not diphenylethidium, attenuated ERK activation mediated by diazoxide. Adenovirus-mediated overexpression of manganese superoxide dismutase, which is expressed in mitochondria, decreased the rate of dihydroethidium oxidation as well as ERK activation. We conclude that mitochondrial K_{ATP} channel openers trigger ERK activation via mitochondria-derived ROS.

reactive oxygen species; cell metabolism; electron transport chain

A VARIETY OF STUDIES suggest that low levels of reactive oxygen species (ROS) can exert a physiological role in cell signaling and cell proliferation (32). In particular, ROS are important signaling molecules for activation of mitogen-activated protein kinases (MAPKs) (4, 30, 51). MAPKs are a family of serine/threonine protein kinases that regulate a wide array of cellular pro-

cesses. Three major classes of MAPKs have been identified: extracellular signal-regulated kinases (ERK), Jun NH₂-terminal kinases (JNK), and p38 MAPKs. JNK and p38 MAPKs participate in the cellular response to environmental stress and are cumulatively known as stress-activated protein kinases (24, 28, 47). In contrast, ERK activation is associated with cell survival and cell proliferation in response to growth factors such as platelet-derived growth factor and epidermal growth factor (47). The classic pathway of ERK activation is characterized by the sequential phosphorylation of upstream kinases, namely, Raf-1 and mitogen-activated kinase kinase (MEK) (4, 24, 28, 30, 47). Recently, it has been shown that the ERK pathway can be activated in response to ROS such as H₂O₂ (29, 30).

ROS are generated by several pathways during cell metabolism. Mitochondria generate cellular energy in the form of ATP by the process of oxidative phosphorylation through an elaborate electron transport chain (ETC), in which O₂ accepts electrons and is reduced to water (22, 56). The released energy from the flow of electrons from NADH and FADH₂ through the ETC to O₂ is used to pump protons across the mitochondrial inner membrane and create a transmembrane electrical potential ($\Delta\Psi$). Through this process, a proton motive force is generated as a result of a pH gradient and $\Delta\Psi$. ATP is synthesized when protons flow back to the mitochondrial matrix. It has been reported that, in normal conditions, 1–2% of consumed O₂ undergoes incomplete reduction and generates superoxide anion (O₂^{•-}) (14). The delicate balance between production and removal of O₂ radicals is maintained by an array of enzymatic and nonenzymatic antioxidants. Superoxide dismutases (SOD) convert O₂^{•-} to H₂O₂, which is then reduced by glutathione peroxidase and catalase to water (34). In the presence of transition metals, H₂O₂ can be converted via the Fenton reaction (9, 10, 25) to the highly toxic hydroxyl radical ([•]OH). An imbalance between highly reactive O₂ radicals and the free radical scavenging pool can lead to oxidative stress. However, the generation of low, subtoxic levels of ROS in cells appears to modulate a number of signal transduction

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pathways. Recent studies have shown a key role of intact mitochondrial ETC and mitochondria-derived ROS in tumor necrosis factor- α - and H_2O_2 -mediated signal transduction (15).

The amount of mitochondria-derived ROS can be modified by inhibition of the ETC, change of pH (ΔpH), and reduction of $\Delta\Psi$. Recently, an ionic channel highly selective for K^+ has been identified in the inner membrane of rat liver mitochondria (33). This channel is blocked by ATP and sulfonyleurea derivatives (27, 52). It has been postulated that mitochondrial ATP-sensitive K^+ (K_{ATP}) channels play a role in mitochondrial $\Delta\Psi$ and ΔpH and regulation of mitochondrial volume (52). Several pharmacological agents stimulate ion flux through the K^+ channels in mitochondrial membranes. Among these drugs, diazoxide opens mitochondrial K_{ATP} channels 2,000 times more effectively than the surface K_{ATP} channels in cardiac myocytes and rat liver (23). A number of studies have shown that mitochondrial K_{ATP} channel openers (KCO, diazoxide and pinacidil) can mimic the effect of ischemic preconditioning (IPC), thus protecting cardiac tissue against prolonged ischemic episodes (3). Their protective effect was abolished by coadministration of antioxidants (13).

In this study, we tested the hypothesis that opening mitochondrial K_{ATP} channels leads to ERK activation via the generation of mitochondria-derived ROS. We demonstrated in THP-1 cells, a monocytic cell line, that opening mitochondrial K_{ATP} channels leads to ROS production and activation of ERK. Furthermore, we observed that ERK activation is blocked by antioxidants, as well as adenovirus-mediated overexpression of Mn-SOD, which is expressed in mitochondria. In contrast, diphenyleneiodonium (DPI), which effectively inhibits cell membrane-associated NADPH oxidase, had no effect on diazoxide-mediated ERK activation. These studies demonstrate that agents that open mitochondrial K_{ATP} channels activate ERK kinase in part via mitochondria-derived ROS.

MATERIALS AND METHODS

Cell culture. The THP-1 cell line was obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 medium supplemented with gentamicin and 10% fetal calf serum (FCS; Life Technologies). The tissue culture medium was changed every 3 days.

Chemicals. Diazoxide, pinacidil, and *N*-acetylcysteine (NAC) were purchased from Sigma Chemical (St. Louis, MO). Diazoxide and pinacidil were dissolved in DMSO before each experiment. NAC was dissolved in water corrected to pH 7.3 with equimolar bicarbonate solution. Dihydroethidium (DHE) and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR). PD-98059, U-0126, and DPI were obtained from Calbiochem (La Jolla, CA). Mn-SOD antibody was obtained from Upstate Biotechnology (Lake Placid, NY).

Infection with recombinant adenovirus constructs. Recombinant adenovirus vectors expressing enhanced green fluorescent protein (AdEGFP) or Mn-SOD (AdMnSOD) (61) were used for these studies. All adenovirus vectors were purchased from the Vector Core Facility at the University of Iowa. Recombinant adenovirus stocks were stored in 10 mM Tris

with 20% glycerol at $-80^\circ C$. The particle titers of adenovirus stocks were typically 10^{13} DNA particles/ml. DNA particle-to-infectious unit ratio was 100. Multiplicity of infection (MOI) is given in infectious units. Adenovirus infection was performed for 2 h at $37^\circ C$ in RPMI 1640 medium without FCS. After infections, an equal volume of RPMI 1640 medium containing 20% fetal bovine serum was added to reach a serum concentration of 10%, and the infections were continued for 36–48 h. We studied various MOI to determine the most effective concentration of virus particles for infection of THP-1 cells. Adenovirus infection at an MOI of 50 in THP-1 cells resulted in $>95\%$ infection (determined by fluorescence of cells infected with AdEGFP vector) without any effect on cell viability at 48 h as measured by trypan blue staining (data not shown).

Measurement of intracellular ROS. Intracellular oxidant production was monitored with two different fluorescence probes: DHE and DCFH-DA. Intracellular O_2^- generation was monitored by measuring the change of fluorescence from intracellular DHE oxidation (Molecular Probes, Eugene, OR) (48, 55). DHE enters the cells and is oxidized by O_2^- or $\cdot OH$ to yield the red fluorescent ethidium, which then binds to DNA, causing amplification of the red fluorescence signal. DHE (10 μM) was added to the cell suspension in PBS for 30 min after each experiment, and the fluorescence was determined with a FACScan (Becton-Dickinson, San Jose, CA) flow cytometer equipped with a 488-nm argon ion laser and supplied with Cell Quest software. DHE fluorescence was measured using 488-nm excitation, and emission was measured using a 585 ± 42 nm band-pass filter. Measurement of propidium iodide (PI) fluorescence to assess membrane integrity was made by using the same excitation wavelength, and emission was determined with a 650-nm long-pass filter.

DCFH-DA fluorescence reflects intracellular ROS. Oxidation by ROS, in particular H_2O_2 and $\cdot OH$, yields the fluorescence product DCF. DCFH-DA was dissolved in DMSO and diluted in PBS to a working concentration of 5 μM . After 30 min of incubation of THP-1 cells in 5 μM DCFH-DA at $37^\circ C$, the cells were washed twice with PBS and exposed to 100 μM pinacidil or 100 μM diazoxide for different times. Fluorescence levels were monitored by flow cytometry analysis. DCF fluorescence was measured by analyzing $\geq 10,000$ events by using a FACS flow cytometer (Becton-Dickinson), as described previously (35).

Western analysis. Western analysis for the presence of specific proteins or for phosphorylated forms of proteins was performed on whole cell sonicates and lysates from THP-1 cells (37–39). Protein (30–100 μg) was mixed 1:1 with $2\times$ sample buffer (20% glycerol, 4% SDS, 10% β -mercaptoethanol, 0.05% bromophenol blue, and 1.25 M Tris-HCl, pH 6.8; all from Sigma Chemical), loaded onto a 10% SDS-polyacrylamide gel, and run at 40 V for 2 h. Cell proteins were transferred to nitrocellulose (ECL, Amersham, Arlington Heights, IL) overnight at 30 V. Equal loading of proteins on the blots was evaluated using nonphosphorylated antibody or Ponceau S, a staining solution designed for detecting proteins on nitrocellulose membranes (Sigma Chemical). Images of the Ponceau S stain are included to demonstrate equal loading of the samples. The nitrocellulose was then blocked with 5% milk in TTBS (Tris-buffered saline with 0.1% Tween 20) for 1 h, washed, and then incubated with the primary antibody [antiphosphorylated ERK (Sigma Chemical) and Mn-SOD antibody (Upstate Biotechnology)] overnight at $4^\circ C$. The blots were washed four times with TTBS and incubated for 1 h with horseradish peroxidase-conjugated anti-IgG antibody (Jackson Laboratories, West Grove, PA). Immunoreactive bands were developed using a chemiluminescent sub-

strate (ECL Plus or ECL, Amersham). An autoradiograph was obtained, with exposure times of 10 s to 2 min.

Statistical analysis. Data were analyzed with GraphPad Prism software (GraphPad) using Student's paired *t*-test. *P* < 0.05 was considered to be significant.

RESULTS

Mitochondrial KCO activate ERK. To evaluate the effect of opening mitochondrial K_{ATP} channels, we performed the following experiments. THP-1 cells were grown in 10% FCS and then cultured in 1% FCS 16 h before each experiment to decrease the baseline ERK activity. To demonstrate that opening mitochondrial K_{ATP} channels activates ERK, cells were stimulated with 100 μ M diazoxide or 100 μ M pinacidil for various periods of time. Garlid et al. (23) showed that, at this concentration, diazoxide preferentially opens the mitochondrial K_{ATP} channels and not the cell surface K^+ channels. After protein extraction, 30 μ g of cell lysates were subjected to SDS-PAGE and standard Western blotting. ERK activity was assessed using the diphosphorylated ERK antibody. We found that ERK activity increased as early as 5 min and was maximally active at 30 min (Fig. 1). Western blot analysis of total ERK demonstrated equal loading of the proteins on the blots and no increase in total ERK. There was no significant difference between diazoxide and pinacidil in the time response or fold increase of ERK activation. Pretreatment of cells for 30 min with 5 μ M PD-98509 or U-0126, MEK inhibitors (2), before treatment with diazoxide, abolished the KCO-mediated ERK activation (Fig. 2). These data demonstrate that agents that open mitochondrial K_{ATP} channels can activate ERK and that the MEK inhibitors abolish this activation.

ERK phosphorylation is oxidant dependent. It has been demonstrated that blocking of the diazoxide effect by antioxidants blocked the ischemic preconditioning effect of KCO in cardiac myocytes (21, 44). To determine whether the phosphorylation of ERK is oxidant dependent, we preincubated the THP-1 cells with 30 mM NAC for 90 min before exposure to 100 μ M diazoxide or 100 μ M pinacidil. NAC is a glutathione precursor and a potent scavenger of ROS (46). We found that NAC attenuated the activation of ERK mediated by pinacidil and diazoxide in a time-dependent fashion (Fig. 3). By contrast, preincubation of THP-1 cells with the NAD(P)H oxidase inhibitor DPI (20 μ M) 90 min before diazoxide exposure failed to block the ERK activation (Fig. 4). These results suggest that the activation of ERK mediated by mitochondrial KCO is oxidant dependent and that the ROS were not derived from membrane-associated NADPH oxidase or mitochondrial complex I.

Exposure to mitochondrial KCO generates intracellular ROS. It has been postulated that opening of mitochondrial K_{ATP} channels causes reversible oxidation of flavoproteins and possible inhibition of respiratory chain complex II (26, 45). This inhibition may cause univalent electron transfer to O_2 and thus generate O_2^- . To further study whether opening the mitochondrial K_{ATP} channels leads to production of ROS,

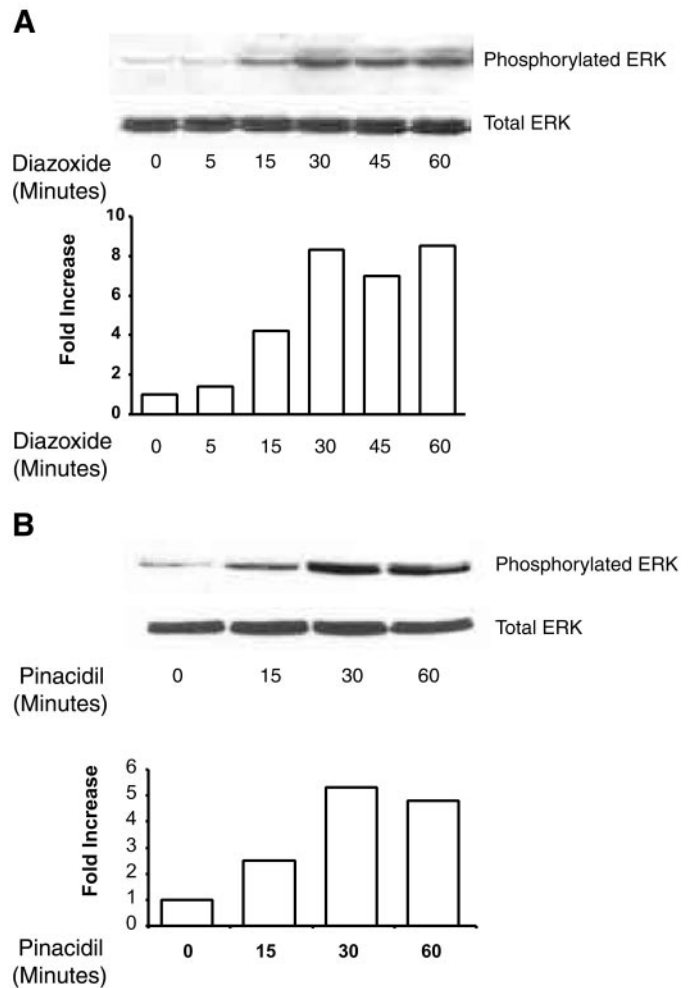


Fig. 1. Mitochondrial ATP-sensitive K^+ (K_{ATP}) channel openers activate extracellular signal-regulated kinase (ERK). THP-1 cells were exposed to 100 μ M diazoxide (A) or 100 μ M pinacidil (B) for different periods of time. Total protein lysates (30 μ g) were subjected to 10% SDS-PAGE and Western blot analysis. Blots were evaluated for phosphorylated (active) and total ERK. Primary antibody concentrations of 1:1,000 and secondary antibody concentrations of 1:20,000 were used. Immunoreactive bands were visualized with chemiluminescence. Densitometry of the phospho-ERK is shown as fold increase [mean optical density (OD) units for control sample \div mean OD units for experimental sample]. Results are representative of 4 experiments.

we used two different oxidant-sensitive fluorescence probes: DHE (10 μ M) in conjunction with the PI (5 μ M) fluorescence probe to assess membrane integrity and DCFH-DA. After culturing THP-1 cells with 100 μ M pinacidil or 100 μ M diazoxide for various times, we measured the change in DHE fluorescence with the FACScan (Fig. 5). On treatment with KCO, the rate of DHE fluorescence increased as early as 1 min. It reached a peak at 30 min and after 180 min slowly decreased to baseline values. These studies suggest that mitochondrial KCO increases ROS production in THP-1 cells. The time course of ROS production after exposure to KCO mirrored the time course of ERK activation in these cells. In Fig. 6, we show the kinetics of ROS production after exposure to diazoxide com-

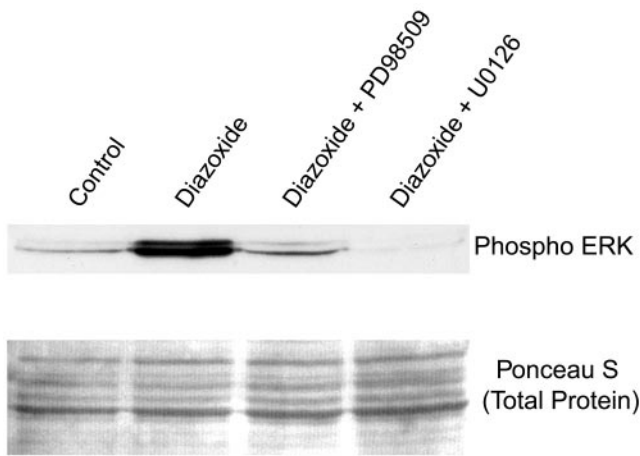


Fig. 2. Mitogen-activated kinase kinase (MEK) inhibitors block the diazoxide-mediated ERK activation. THP-1 cells were cultured in 5 μ M PD-98509 or 5 μ M U-0126 for 30 min before treatment with 100 μ M diazoxide for 30 min. Total protein lysates (30 μ g) were subjected to 10% SDS-PAGE and Western blot analysis. Blots were evaluated for phosphorylated (active) and total ERK. Primary antibody concentrations of 1:1,000 and secondary antibody concentrations of 1:20,000 were used. Immunoreactive bands were visualized with chemiluminescence. Equal loading of gels was demonstrated with Ponceau S stain for total protein. Results are representative of 3 different experiments.

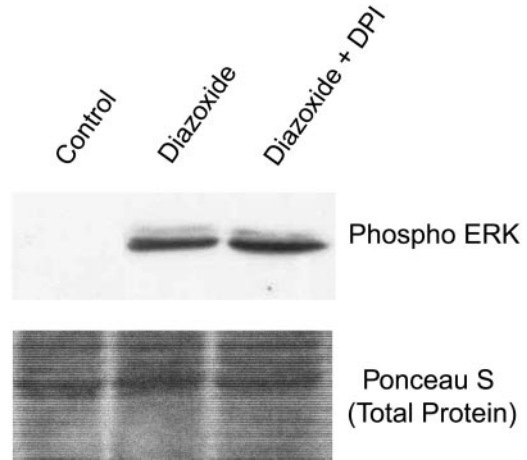


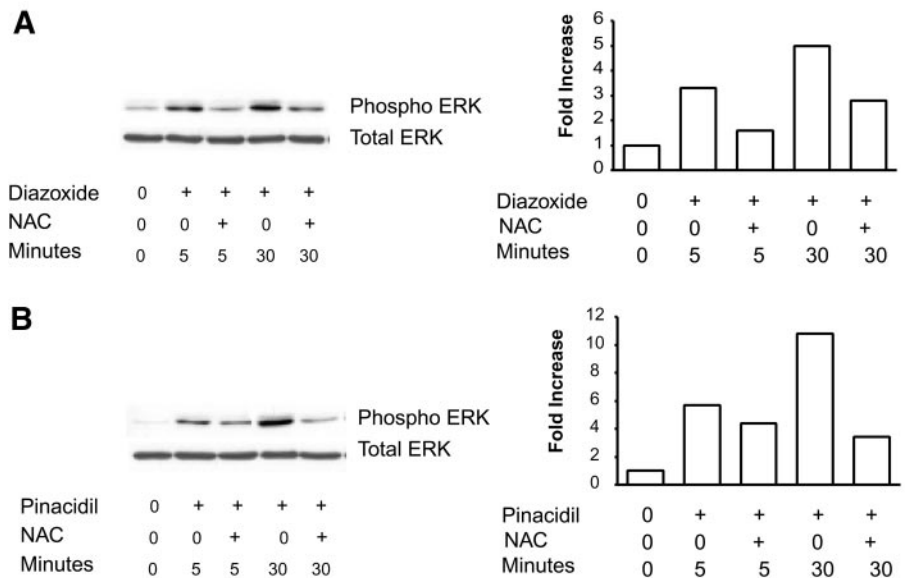
Fig. 4. Effect of diphenyleneiodonium (DPI) on diazoxide-mediated ERK activation. THP-1 cells were preincubated for 90 min with 20 μ M DPI and then cultured with 100 μ M diazoxide for 30 min. Total cell protein was isolated, and protein lysates (30 μ g) were loaded for Western blot analysis. Blots were evaluated for phosphorylated (active) ERK and total ERK protein. Primary antibody concentrations of 1:1,000 and secondary antibody of 1:20,000 were used. Immunoreactive bands were visualized with chemiluminescence. Equal loading of gels was demonstrated with Ponceau S stain for total protein. Result was reproducible in 3 different experiments.

pared with the kinetics of ERK activation. The similar time course of these two events suggests a direct link between intracellular ROS production and ERK activation.

DCFH-DA diffuses into cells easily, where it is hydrolyzed by intracellular esterases to liberate DCFH. On reaction with oxidizing species, DCFH forms its two-electron oxidation product, the highly fluorescent compound DCF (35). The fluorescence intensity can be easily measured by flow cytometry. The oxidation of DCFH is predominantly thought to occur as a result of a reaction with H_2O_2 or $\cdot OH$ (6). THP-1 cells were

treated with DCFH-DA as described in MATERIALS AND METHODS, and cells were incubated with 100 μ M pinacidil or 100 μ M diazoxide for various times. Cells were harvested, and the fluorescence intensity was assessed with FACSscan. Figure 7 demonstrates that incubation of THP-1 cells with pinacidil or diazoxide led to an increase of ROS production as measured by fluorescence intensity of DCF by flow cytometry (an increase of positive cells from 6–7% to 60% after 30 min of exposure to pinacidil). As a composite, these studies suggest that diazoxide and pinacidil increase intracellular ROS, which coincides with ERK activation.

Fig. 3. *N*-acetylcysteine (NAC) attenuates ERK activation mediated by diazoxide and pinacidil. Cells were preincubated for 90 min with 30 mM NAC. After 90 min, 100 μ M diazoxide (A) or 100 μ M pinacidil (B) was added for 5, 30, or 60 min. Total cell protein was then isolated, and protein lysates (30 μ g) were loaded for Western blot analysis. Blots were evaluated for phosphorylated (active) ERK and total ERK protein. Primary antibody concentrations of 1:1,000 and secondary antibody of 1:20,000 were used. Immunoreactive bands were visualized with chemiluminescence. Densitometry of the phospho-ERK is shown as fold increase (mean OD units for control sample \div mean OD units for experimental sample). Results are representative of 4 experiments.



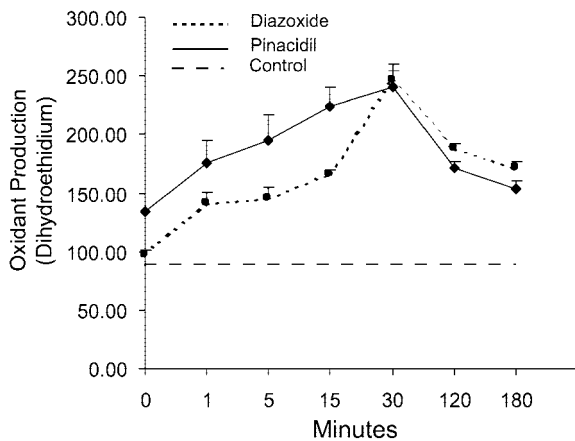


Fig. 5. Pinacidil and diazoxide increase generation of reactive oxygen species (ROS) in THP-1 cells. THP-1 cells were cultured with 100 μ M pinacidil or 100 μ M diazoxide for 1–180 min. At the end of the culture period, cells were incubated with dihydroethidium (DHE, 10 μ g/ml) for 30 min. ROS levels were determined by ethidium fluorescence intensity on the basis of mean fluorescence of 10,000 cells measured with FACScan. Exposure of THP-1 cells to 100 μ M pinacidil ($n = 4$) or 100 μ M diazoxide ($n = 3$) was associated with a transient increase of ROS. Values are means \pm SE of 4 different experiments for pinacidil and 3 different experiments for diazoxide. $P < 0.001$ by paired t -test at 30 min in both groups.

Adenovirus-mediated overexpression of Mn-SOD decreases mitochondria-derived ROS. To further localize the subcellular location of KCO-mediated ROS production, we evaluated the effect of overexpression of Mn-SOD. We infected THP-1 cells with adenovirus expressing Mn-SOD. Mn-SOD is located in the mitochondrial matrix and removes O_2^- . Western blot analysis of protein extracts 36 h after adenovirus-mediated gene transfer demonstrated a significant increase of cellular Mn-SOD (Fig. 8A). At 36 h after infection with adenovirus constructs containing EGFP or Mn-SOD, the infected cells were stimulated with pinacidil for 10 min and the DHE assay was performed as described above. THP-1 cells overexpressing Mn-SOD failed to

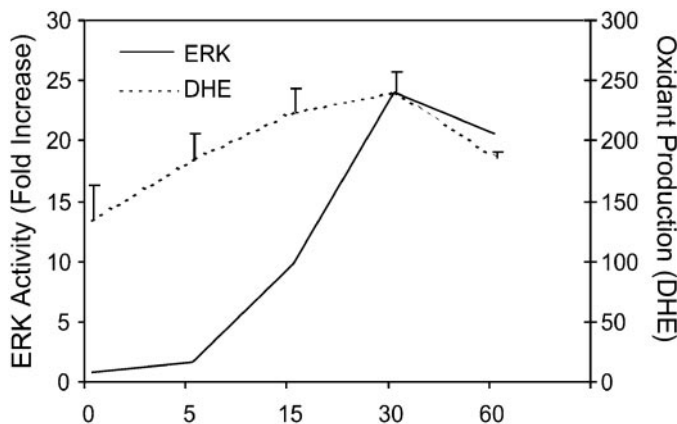


Fig. 6. K_{ATP} channel opener (KCO)-mediated ROS production correlates with KCO-mediated ERK activation. Exposure of THP-1 cells to 100 μ M diazoxide induces ROS production as measured by DHE fluorescence (dashed line) and an increase in ERK activation in a time-dependent fashion. Kinetics of ROS production after exposure to diazoxide suggest that ROS production regulates ERK activation.

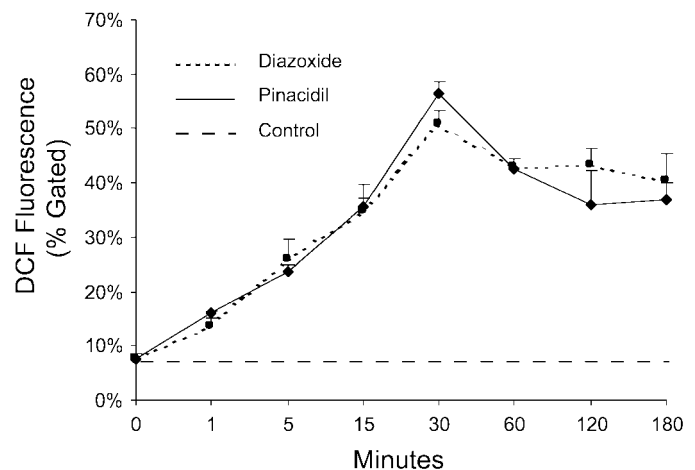


Fig. 7. Pinacidil and diazoxide increase dichlorofluorescein (DCF) fluorescence in THP-1 cells. THP-1 cells were incubated with 5 μ M 2',7'-dichlorofluorescein diacetate for 30 min. After cells were washed twice with PBS, they were exposed to 100 μ M diazoxide or 100 μ M pinacidil for different times. DCF fluorescence based on percent gated cells and mean fluorescence of 10,000 events was measured with FACScan. Values are means \pm SD of 3 separate experiments.

increase the rate of DHE fluorescence after pinacidil treatment compared with control cells infected with AdEGFP (Fig. 8B). These results suggest that Mn-SOD is an effective scavenger of the superoxide generated on exposure to KCO, and they also suggest a mitochondrial origin of the superoxide, since the Mn-SOD is only active in the mitochondrial matrix (60).

Overexpression of Mn-SOD blocks KCO-mediated ERK activation. Because Mn-SOD is able to scavenge superoxide generated after exposure of THP-1 cells to KCO, we determined whether the activation of ERK was triggered by mitochondria-derived ROS. We infected THP-1 cells with AdMnSOD or AdEGFP at an MOI of 50 (61). At 36 h after infection, the cells were treated with 100 μ M pinacidil for 30 min. Total cell protein was isolated, and Western blotting was performed to detect activated ERK (Fig. 9). We observed that Mn-SOD-overexpressing cells significantly decreased ERK activity in response to pinacidil. These results suggest that mitochondria-derived ROS activate the ERK pathway in response to KCO.

DISCUSSION

Our studies demonstrate that KCO can activate the ERK MAPK in THP-1 cells. In this system, activation of ERK is rapid (by 5 min) and reaches a peak at 30 min. This activation is inhibited in cells overexpressing Mn-SOD or cells pretreated with the oxygen radical scavenger NAC. DPI, which inhibits membrane-associated NADPH oxidase, had no effect on KCO-mediated ERK activation. Parallel to these findings, we were able to measure an increase of ROS production in THP-1 cells on treatment with KCO with two different fluorescence probes. An increase in superoxide production, as measured by DHE oxidation, was not detected in cells that overexpressed Mn-SOD. Overexpression of Mn-SOD suppressed KCO-mediated ERK activation.

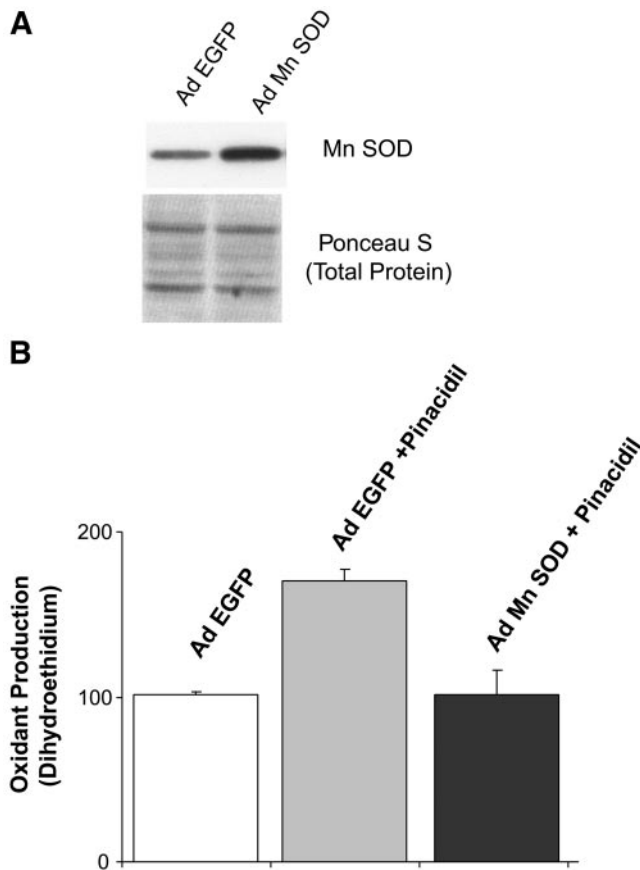


Fig. 8. Overexpression of manganese superoxide dismutase (Mn-SOD) decreases ROS generation in response to pinacidil. THP-1 cells were infected with adenovirus constructs expressing Mn-SOD (AdMnSOD) at a multiplicity of infection of 50. At 36 h after infection, 10 μ g of total cell lysates were subjected to standard SDS-PAGE and Western blot analysis. Blot was assessed for Mn-SOD protein. Primary antibody concentrations of 1:1,000 and secondary antibody of 1:20,000 were used. Immunoreactive bands were visualized with enhanced chemiluminescence. Equal loading of gels was demonstrated with Ponceau S stain for total protein (see Fig. 6A). THP-1 cells were infected with Mn-SOD or enhanced green fluorescence protein (EGFP) adenovirus constructs (AdEGFP) with multiplicity of infection of 50. After 36 h, cells were incubated with 100 μ M pinacidil for 30 min. ROS amounts were determined by ethidium fluorescence intensity on the basis of mean fluorescence of 10,000 cells. Each column represents the result of 3 different independent measurements (B). Rate of DHE oxidation in Mn-SOD-overexpressing cells after pinacidil exposure compared with EGFP-overexpressed cells after pinacidil decreased significantly ($P < 0.001$).

Taken together, our results suggest that KCO activate ERK via mitochondria-derived ROS.

The activation of ERK is a critical signal for cell survival, proliferation, and differentiation. Receptors for peptide growth factors, such as platelet-derived growth factor, epidermal growth factor, and insulin, are protein tyrosine kinases that undergo phosphorylation in response to ligand binding. This triggers sequential phosphorylation and activation of the Ras-Raf-MEK-ERK pathway (8, 11, 24, 47). Recent studies support the notion that the ERK pathway can be redox regulated (29, 30). The activation of a number of cell surface receptors elicits rapid production of ROS (4, 5).

Furthermore, extracellular administration of H_2O_2 can activate the MAPK pathway (30). Recently, Bogoyevitch and co-workers (7) showed that intact mitochondrial transport function is essential for signaling by H_2O_2 in cardiac myocytes. Our studies show that ERK activation in response to KCO also depends on mitochondria-derived ROS.

In designing this study, we took advantage of observations using the IPC model and the knowledge that compounds that open mitochondrial K_{ATP} channels are capable of mimicking the effect of IPC. It is known that ROS can function as second messengers during IPC (18, 40, 54) and that KCO, which are specific for mitochondrial K_{ATP} channels, are able to simulate the preconditioning effect by generating ROS (3, 44). Most previous studies have been performed in intact hearts and have shown that the protective effect of ischemic preconditioning, as well as KCO, is abolished by administration of free radical scavengers, implying a role for ROS as signaling molecules. The mechanisms by which the mitochondrial KCO might alter ROS generation are not well understood. It has been suggested that K^+ influx into the mitochondrial matrix would

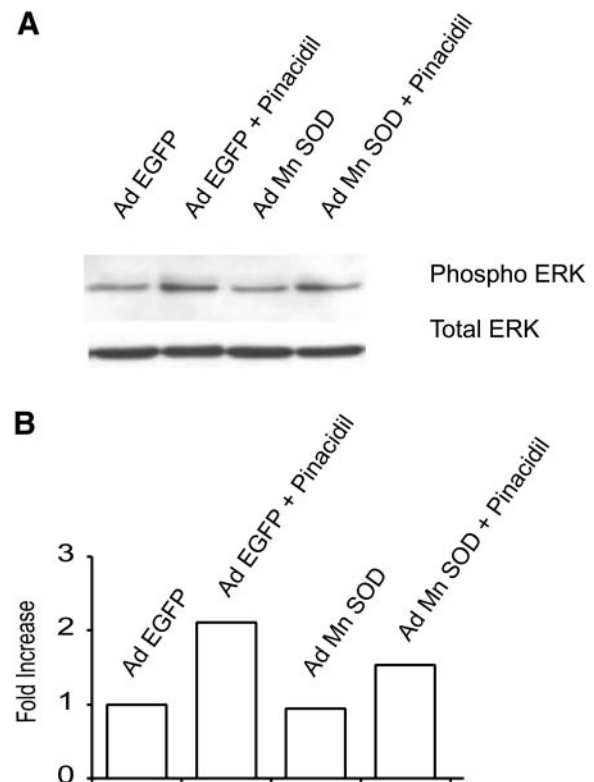


Fig. 9. Overexpression of Mn-SOD decreases ERK activation mediated by pinacidil. A: after 36 h, cells were incubated with 100 μ M pinacidil for 30 min. Total protein lysates (30 μ g) were then resolved on standard SDS-PAGE. Blots were evaluated for phosphorylated (active) ERK and total ERK protein. Primary antibody concentrations of 1:1,000 and secondary antibody of 1:20,000 were used. Immunoreactive bands were visualized with chemiluminescence. B: densitometry of phospho-ERK as fold increase (mean OD units for control sample \div mean OD units for experimental sample). Results are representative of 4 experiments.

dissipate the potential across the inner membrane and uncouple the ETC (27, 45, 52). In addition, diazoxide exhibits a direct effect on mitochondrial energy metabolism by inhibition of respiratory chain complex II in liver mitochondria (26). It also enhances respiration and augments electron flux through the Q cycle (31). One interesting aspect of the effect of diazoxide is the reversible depolarization of mitochondrial membrane potential. This might enhance superoxide production without increasing cell death compared with other mitochondrial ETC inhibitors that are very toxic to cells.

In these studies, we showed that the opening of K_{ATP} channels is associated with an early increase in the rate of superoxide production. These findings parallel recent studies showing an increase in mitotracker fluorescence in cardiac myocytes on treatment with diazoxide (13). To test whether the KCO-mediated superoxide generation is mitochondrial in origin, we infected the THP-1 cells with AdMnSOD. Mn-SOD-overexpressing cells showed decreased superoxide production on treatment with KCO. The effect of overexpression of Mn-SOD confirms the mitochondrial origin of KCO-mediated superoxide, since mitochondria-localized Mn-SOD is only known to be active in the mitochondrial matrix and dismutates the fraction of superoxide that arises in this compartment (1, 17, 58).

We also assessed the effect of DPI on ERK activation. DPI is an NAD(P)H oxidase inhibitor (36) that also inhibits superoxide formation from the flavin moiety of nitric oxide synthase (59). It had no effect on KCO-mediated ERK activation. The lack of an effect of DPI on ERK activation on treatment with KCO suggests that NAD(P)H oxidase, mitochondrial complex I, and nitric oxide synthase are unlikely sources of ROS that mediate ERK activation. We are presently determining which mitochondrial ETC complex is important for the increased production of superoxide mediated by KCO.

Although excessive amounts of ROS can lead to tissue injury and inflammation, low levels of ROS may be useful for cell signaling. Quantitative differences in amounts of ROS may have different impacts on the redox environment of the cell and may initiate different signaling pathways (46). This can be demonstrated by comparing the effects of ischemia-reperfusion injury and ischemic preconditioning. In the first instance, ROS triggers signaling pathways that result in cell injury and cell death. In the latter instance, signaling pathways are initiated that protect the cells from subsequent ROS-mediated injury. The extent of ischemia-reperfusion injury can also be diminished by free radical scavengers and overexpression of Mn-SOD (16). The beneficial effect of IPC also is abolished with administration of free radical scavengers (42, 44, 53). Recently, Ozcan and co-workers (43) showed that KCO protect the isolated cardiac mitochondria by attenuation of oxidant stress at reoxygenation (similar to the effect of SOD in the ischemia-reperfusion model). These findings suggest that effects of KCO on isolated mitochondria may be different from those in the whole cell. Our findings are supported by findings of other

investigators that KCO triggers generation of oxygen radicals (13, 21, 44). We hypothesize that low levels of ROS generated during KCO exposure trigger protective cell mechanisms, whereas higher levels of ROS result in tissue injury (ischemia-reperfusion).

An important finding of this study is that KCO, which mimic IPC, trigger ERK activation. ERK activation has been shown to enhance cell survival. Furthermore, free radical scavengers inhibited this effect of KCO. These observations might help elucidate signaling pathways that are activated during IPC. Protein kinase C (PKC) is held as the central mediator of IPC (49, 50). Recent studies suggest that mitochondria-derived superoxide may play a critical role as an upstream activator of PKC (41). In addition, it has been postulated that PKC exerts its protective effect by activating mitochondrial K_{ATP} channels (49). PKC is known to be an activator of the ERK pathway. Thus it is possible that the effect of mitochondria-derived ROS is mediated via activation of one or more PKC isoforms. We are presently investigating these possibilities.

Mitochondria-derived ROS may also affect activation of the ERK pathway at sites other than PKC. The extent of phosphorylation (activation) of the kinases in the ERK pathway reflects the balance between kinases and relevant phosphatases. Activation of kinases and/or inhibition of phosphatases may result in MAPK activation. Recent studies indicate that phosphatases are regulated by a redox mechanism. Most phosphatases contain in their active site cysteine residues and/or metal centers (Fe^{+2} - Zn^{+2}), which are targets of oxidation by various ROS, including superoxide and H_2O_2 (12, 19, 57). The inhibition of phosphatases is potentially important, since phosphatases exhibit a much higher specific activity than the kinases (20).

In summary, our studies show that KCO trigger ERK activation via the production of mitochondria-derived ROS. Although our study was not designed to differentiate between the signaling cascade by superoxide and that by H_2O_2 , our data indicate that mitochondria-derived superoxide is necessary to activate ERK, since Mn-SOD overexpression abolished the effect of KCO. Our primary interest in these studies was to determine whether mitochondria-derived oxidants activate the ERK pathway in monocytic cells. However, these studies have broader implications. They also provide one mechanism (increased expression of the ERK MAPK) to explain how KCO protect cells from subsequent ROS-mediated cell injury.

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