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Opening of ATP-sensitive potassium channels causes generation of free radicals in vascular smooth muscle cells

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Abstract Recent evidence suggests that opening of mitochondrial K_{ATP} channels in cardiac muscle triggers the preconditioning phenomenon through free radical production. The present study tested the effects of K_{ATP} channel openers in a vascular smooth muscle cell model using the fluorescent probe MitoTracker (MTR) Red^{TM} for detection of reactive oxygen species (ROS). Rat aortic smooth muscle cells (A7r5) were incubated with 1 μM reduced MTR (non-fluorescent) and the MTR oxidation product (fluorescent) was quantified. Thirty-minute pretreatment with either diazoxide (200 μM) or pinacidil (100 μM), both potent mitochondrial K_{ATP} channel openers, increased fluorescent intensity (FI) to 149 and 162 % of control ($p < 0.05$ for both), respectively, and the K_{ATP} channel inhibitor 5-hydroxydecanoate (5HD) blocked it. Valinomycin, a potassium-selective ionophore, raised FI to 156 % of control ($p < 0.05$). However, 5HD did not affect the valinomycin-induced increase in FI. Inhibition of mitochondrial electron transport (myxothiazol) or uncoupling of oxidative phosphorylation (dinitrophenol) also blocked either valinomycin- or diazoxide-induced increase in FI, and free radical scavengers prevented any diazoxide-mediated increase in fluorescence. Finally the diazoxide-induced increase in fluorescence was not blocked by the PKC inhibitor chelerythrine, but was by HMR 1883, a putative surface K_{ATP} channel blocker. Thus opening of K_{ATP} channels increases generation of ROS via the mitochondrial electron transport chain in vascular smooth muscle cells. Furthermore, a potassium-selective ionophore can mimic the effect of putative mitochondrial K_{ATP} channel openers. We conclude that potassium movement through K_{ATP} directly leads to ROS production by the mitochondria.

Key words Vascular smooth muscle – diazoxide – K_{ATP} channel – reactive oxygen species – A7r5 cells – myxothiazol – valinomycin

Introduction

Historically, reactive oxygen species (ROS) are regarded as toxic byproducts of aerobic metabolism, and tissue damage following reexposure to oxygen after periods of ischemia or hypoxia has been recognized for many years (25). Indeed, in vascular smooth muscle cells ROS are generated in a number of pathologic conditions such as hypoxia/reoxygenation (11, 12, 44), atherosclerosis (42), and hypertension (1). However, increasing evidence now implies that ROS may also have important physiological roles as intracellular messengers (21). For example, ROS appear necessary for cell survival inasmuch as suppression of endogenous intracellular H_2O_2 inhibits vascular smooth muscle cell proliferation and promotes apopto-

sis (35). Moreover, production of ROS is a critical step in the signal transduction triggered by angiotensin II in vascular smooth muscle cells (38).

A variety of intracellular enzymes have been implicated in the production of ROS. For example, NAD(P)H oxidase, xanthine oxidase, microsomal cytochrome P-450, and enzyme complexes of the mitochondrial electron transport chain can all produce ROS (16, 21). Recent data indicate that activation of K_{ATP} channels may also initiate production of ROS in cardiac myocytes. Using a ROS-sensing microprobe Obata et al. (27) demonstrated that mitochondrial K_{ATP} channel (mK_{ATP}) activators increase hydroxyl radical formation in rat myocardium, and this increase could be blocked by the highly selective mK_{ATP} blocker 5-hydroxydecanoate (5HD). ROS production in the heart is also involved in the preconditioning mechanism of cardioprotection (2, 36). Yao et al. (43) showed that a burst of ROS occurred when chick cardiomyocytes were preconditioned with acetylcholine and that 5HD could eliminate the burst. Furthermore, it is known that the K_{ATP} channel activator diazoxide protects isolated hearts against infarction from ischemia/reperfusion injury (3, 15) and that diazoxide's protection can be blocked with a ROS scavenger (10, 29). In the heart, diazoxide is a potent and selective activator of the mK_{ATP} channel (14). 5HD abolished preconditioning's protection in isolated rat hearts (15) and prevented diazoxide-induced cardioprotection in isolated rabbit hearts (29). Most recently in a non-muscle cell line derived from human atrium (6) and in rat cardiomyocytes (10), it was demonstrated that preconditioning with diazoxide was protective and this protection was correlated with increased ROS production. Finally, in intact rabbit hearts we found that the infarct-sparing effect of preconditioning triggered by either bradykinin, morphine, acetylcholine, or phenylephrine could be aborted by infusion of either 5HD or the free radical scavenger N-2-(mercaptopropionyl) glycine (MPG) (7). Collectively these observations suggest a novel signal transduction pathway in which receptor occupancy opens mK_{ATP} causing the production of ROS that then protect the heart.

While our primary interest is in cardiomyocytes, an immortalized adult cardiac myocyte cell culture is currently unavailable. We therefore decided to pursue this line of investigation using an immortalized A7r5 rat aortic smooth muscle cell line. Vascular smooth muscle cells, like cardiac cells, contain mitochondria and express muscarinic M_2 receptors (32). The present study was designed to address the following questions: 1) Do K_{ATP} channel openers induce ROS production in this model? 2) Does imitating mK_{ATP} opening with a potassium-selective ionophore also induce the generation of ROS in these cells? 3) Does ROS induced by K_{ATP} channel opening come from the mitochondrial respiratory chain? and 4) Does diazoxide-dependent ROS production require activation of protein kinase C (PKC)?

Methods

Cell culture

A7r5 cells were obtained from ATCC (Manassas, VA) and seeded onto chambered coverglass slips (Nunc, Inc., Naperville, IL). DMEM with 10% fetal calf serum (FCS), glucose (4.5 g/dl), pyruvate (0.11 g/l), penicillin (5 units/ml), streptomycin (5 mg/ml), gentamycin (10 mg/ml) and fungizone (1 ml/l) was used as growth medium. Experiments were performed on days 2 – 3 following splitting. Cells from at least two independent passages were studied per group. For the actual experiments, the fortified DMEM used as growth medium was replaced with DMEM without FCS or phenol red.

ROS detection

Treated cells were exposed to the experimental agent for 30 or 40 min as listed below before being exposed to reduced MitoTracker (MTR) Red™ to detect free radical production. Reduced MTR (1 μM) was added to the medium and the cells were kept at 37 °C in 5% CO_2 in the dark for 15 min. Cells were then washed three times with DMEM to remove excess MTR, and the chambered coverslips transferred to a Nikon TMS-F inverted microscope with Nikon CF-Fluor 20X/0.75 objective, 350 W Xenon lamp, filter wheel (Sutter), integrating CCD camera (Cohu Electronics Model 4410) for quantification of cell fluorescence. Cells were illuminated with 560 nm light and fluorescent emission measured at 610 nm. Images were integrated for 15 video frames (0.5 s) and analyzed using Intracellular Imaging® Incyt software (Intracellular Imaging, Cincinnati, OH). Individual cell fluorescence was measured in 4 – 5 random fields per chamber and data averaged per field. For each group, 4 – 6 chambers were generally analyzed. Experiments with the different agents as described below were always performed in parallel with their respective controls. Cells in all groups were kept in the incubator (37 °C with 5% CO_2) during the loading period. Measurements were made at 37 °C in room air.

Protocols

To test the effects of K_{ATP} channel openers on ROS production, cells were incubated with either diazoxide (200 μM) (n = 4) or pinacidil (100 μM) (n = 4) for 30 min prior to the addition of reduced MTR. In a small number of pilot studies, we tested diazoxide doses of 20, 200 and 2000 μM. While all doses seemed to cause an increase in fluorescence, the 200 μM dose seemed to give the most robust response. Since 200 μM has been a concentration

commonly used by others, we continued our experiments with this one.

The effects of free radical scavengers and of a mK_{ATP} inhibitor were tested. In the MPG, Tiron and 5HD groups, cells were exposed to either MPG (400 μM) (n = 5), 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron, 10 mM) (n = 4) or 5HD (1 mM) (n = 4) starting 40 min prior to staining with MTR. In the diazoxide + MPG group (n = 5), cells were incubated with MPG (400 μM) alone for 10 min, and then the medium was changed to include both diazoxide (200 μM) and MPG (400 μM). After 30 min, reduced MTR was added. Similar protocols were used in the diazoxide + Tiron (n = 4) and diazoxide + 5 HD (n = 4) groups.

The effect of valinomycin, a potassium-selective ionophore, on ROS production was determined. In this group (n=6), valinomycin (25 nM) was present for 30 min prior to adding reduced MTR. In the valinomycin + 5HD group (n = 6), cells were incubated with 5HD (1 mM) alone for 10 min, and then with 5HD (1 mM) + valinomycin (25 nM) for 30 min.

In order to evaluate the involvement of mitochondria the effects of site III electron transport inhibition and of uncoupling were tested. In the myxothiazol group (n = 4), cells were treated with myxothiazol (0.2 μM) for 40 min prior to staining. In the diazoxide + myxothiazol group (n = 4), cells were first incubated with myxothiazol (0.2 μM) alone for 10 min, and then myxothiazol (0.2 μM) and diazoxide (200 μM) for 30 min before addition of MTR. In the DNP group (n = 4), cells were treated with 2,4-dinitrophenol (DNP, 200 μM) for 40 min. In the diazoxide + DNP group (n = 4), cells were incubated first with DNP (200 μM) alone for 10 min, and then with DNP (200 μM) and diazoxide (200 μM) for 30 min. This same timing was used in valinomycin + DNP (n = 6) and valinomycin + myxothiazol (n = 4) groups to examine the effects of uncoupling and electron transport blockade on the mitochondrial action of a non-receptor potassium ionophore.

To determine whether DNP was influencing MTR uptake through changes in membrane potential, an additional group was studied in which oxidized rather than reduced MTR was used in control cells and cells exposed to DNP (n = 10).

We tested whether the cardiomyocyte sarcolemmal K_{ATP} channel inhibitor HMR 1883 affected diazoxide-induced ROS production. In the HMR group (n = 4), cells were incubated with HMR 1883 (10 μM) for 40 min. In the diazoxide + HMR group (n = 4), cells were treated first with HMR 1883 (10 μM) alone for 10 min, and then with HMR 1883 (10 μM) and diazoxide (200 μM) for 30 min.

Finally, the dependence of mitochondrial ROS production on PKC activation was determined. Cells were incubated with either diazoxide (200 μM) (n = 6) or chelerythrine (chel) (2 μM) (n = 6), a selective PKC antagonist, alone for 40 min. In the diazoxide + chel group (n = 6), cells were treated first with chelerythrine (2 μM) for 10 min, and then exposed to the combination of chelerythrine (2 μM) and diazoxide (200 μM) for 30 min.

Examination of surface K_{ATP} channels

We attempted to see if diazoxide had any affect on the surface K_{ATP} channels of A7r5 cells. Whole-cell membrane currents were examined using conventional patch-clamp techniques. Coverslips were placed in 35 mm culture dishes containing modified Tyrode's saline (in mM: 130 NaCl, 4.74 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, 10 glucose; pH adjusted to 7.4 with NaOH) on the stage of an inverted microscope (Zeiss, Axiovert 100). Glass borosilicate pipettes (Corning Glass Co., #7052) were fire-polished and filled with internal recording solution (in mM: 135 KCl, 10 HEPES, 10 EGTA, 1.2 $MgCl_2$, 4 ATP; pH adjusted to 7.4 with KOH) to obtain 3 – 4 MΩ whole-cell recording electrodes. High resistance (~10 gigaohm) seals were formed under visual control using gentle suction and the underlying membrane was ruptured to record whole cell currents. After brief stabilization (2 – 3 min), the membrane potential was held at –80 mV and currents were elicited by 400 ms voltage steps incremented by +20 mV from –140 to –20 mV. This protocol activates K_{ATP} channels and other inward rectifying K^+ channels with little effect on other K^+ currents (8). The voltage stepping protocol was repeated 3 times at 1 min intervals to insure stability and then 100 μM pinacidil, a K_{ATP} channel opener, was introduced into the bath. Current traces were recorded with and without P/4 leak subtraction at 1 min intervals for an additional 30 min. Data were recorded (Axopatch 1D, Axon Instruments) and digitized (Digidata 1200, Axon Instruments) and the output was stored in a Pentium III microcomputer for off-line analysis using pCLAMP 6.0 software.

Chemicals

HMR 1883 was a gift from Aventis Pharmaceuticals, Parsippany, NJ. FCS and fungizone were obtained from Gibco, Grand Island, NY. MTR was purchased from Molecular Probes, Eugene, OR, valinomycin from ICN, Costa Mesa, CA and myxothiazol from Fluka, Milwaukee, WI. All other chemicals were purchased from Sigma Co., St. Louis, MO. MTR, diazoxide, pinacidil, valinomycin, myxothiazol, and DNP were first dissolved in small amounts of DMSO, and then diluted in warm medium. The final DMSO content of the medium was < 1 %. This concentration of DMSO and even a doubling of it had no effect on fluorescence. All other agents were dissolved in water or warm medium.

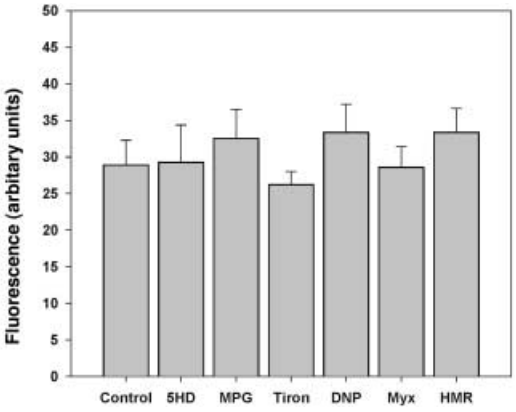

Fig. 1 The absolute fluorescence of A7r5 cells exposed to the six drugs used in this study. None caused any increase in fluorescence over that seen in untreated cells (*DNP* 2,4-dinitrophenol; *5HD* 5-hydroxydecanoate; *HMR* HMR 1883; *MPG* N-(2-mercaptopropionyl) glycine; *Myx* myxothiazol).

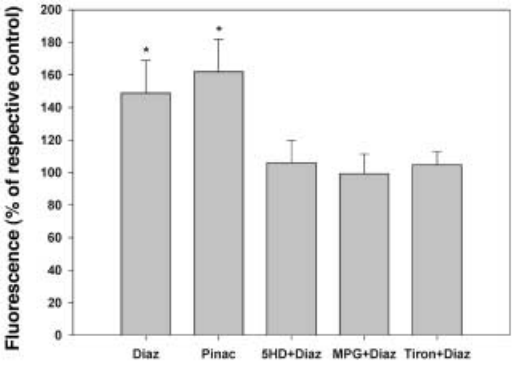

Fig. 2 Both of the mK_{ATP} openers, diazoxide (Diaz) and pinacidil (Pinac), increased FI. The mKATP blocker 5-hydroxydecanoate (5HD) as well as the scavengers N-(2-mercaptopropionyl) glycine (MPG) and Tiron blocked that increase. In the first two bars fluorescence was expressed as a percentage of that seen in cells incubated in fresh medium only. In the remaining three bars fluorescence was compared to that in cells exposed to the blocker/scavenger drug alone. *$p < 0.05$ vs respective control.

Statistics

Mean field averages (arbitrary units) ± S.E.M. are shown for Fig. 1. Experimental cell fluorescence normalized for fluorescence in respective control cells is presented in all other figures. Baseline fluorescence intensities (FI) of all inhibitor control groups (Fig. 1) were analyzed with one-way ANOVA to test for differences. Unpaired t-tests were used to test for the effects of diazoxide, pinacidil, and valinomycin on FI when these agents were combined with the various inhibitors and antagonists. Bonferroni's correction was used when multiple comparisons were made. P values of less than 0.05 were considered significant.

Results

Under the light microscope, cell size and morphology were not significantly different among the groups before and after treatment and staining with MTR. There were no differences in FI among the inhibitor control groups. After staining with MTR, cells treated with either MPG, Tiron, 5HD, myxothiazol, DNP, or HMR 1883 showed no differences in FI from cells treated only with medium (Fig. 1).

Both diazoxide and pinacidil significantly increased FI to 148.8 ± 20.1 and 161.9 ± 20.1% of control (Fig. 2). The potent mK_{ATP} channel blocker 5HD blocked diazoxide's increase in FI. When diazoxide was combined with either of the free radical scavengers, MPG (hydroxyl radical scavenger) or Tiron (superoxide scavenger), FI was not different from that seen in cells treated with MPG or Tiron alone, indicating that ROS were responsible for the increase in FI following diazoxide exposure. The potassium-selective ionophore valinomycin also significantly increased FI (Fig. 3). The increase in FI could not be abolished by 5HD, indicating that mK_{ATP} channels were not involved in valinomycin's effect.

Myxothiazol was used to impair mitochondrial electron transport at cytochrome b-c_1. When combined with myxothiazol, diazoxide no longer increased FI of the cells (107.9 ± 10.5%, Fig. 4). These data indicate that the mitochondrial respiratory chain is the major source for diazoxide-induced ROS production. Figure 1 revealed that uncoupling the mitochondria with DNP caused no increase in FI. Once in the uncoupled state, the addition of diazoxide no longer caused an increase in FI (99.4 ± 8.3%) (Fig. 4). Additionally both DNP and myxothiazol prevented valinomycin from increasing FI (Fig. 3).

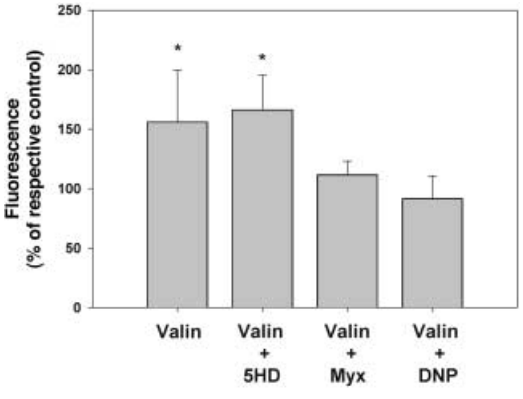

Fig. 3 Valinomycin (Valin), which acts as a potassium ionophore at the mitochondrial membrane, increased FI. 5-Hydroxydecanoate (5HD) could not block the increase in FI but myxothiazol (myx) or the uncoupler 2,4-dinitrophenol (DNP) could. FI was normalized to that from cells incubated in medium alone, while the Valin + 5HD, Valin + Myx, and Valin + DNP data were normalized to those from cells exposed to only 5HD, Myx, or DNP, resp. *$p < 0.05$ vs respective control.

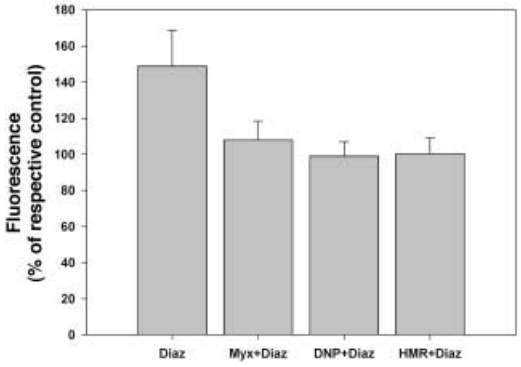

Fig. 4 The increase in FI from diazoxide (Diaz) was blocked by either the electron transport blocker myxothiazol (Myx), the uncoupling agent 2,4-dinitrophenol (DNP), or the surface K_{ATP} channel blocker HMR 1883 (HMR). In the first bar, fluorescence was normalized to cells in fresh medium only. In the remaining panels fluorescence was normalized to cells treated with the tool drug alone. *p < 0.05 vs respective control.

Because DNP affects mitochondrial membrane potential and MTR uptake might be affected by such a change in potential, we were concerned that blockade of the increase in fluorescence induced by diazoxide might have been influenced artifactually by a change in potential rather than interruption of diazoxide's ability to generate free radicals. Thus we tested the effect of DNP in cells treated with oxidized rather than reduced MTR. The former is fully fluorescent and not sensitive to free radicals. DNP had no effect on cell fluorescence when oxidized MTR was used (FI = 53.1 ± 2.9 arbitrary units in untreated vs. 55.3 ± 3.2 with DNP, p = ns). These results indicate that DNP was not suppressing FI in the above studies through changes in mitochondrial membrane potential.

The mK_{ATP} inhibitor 5HD effectively prevented the diazoxide-induced increase in FI (Fig. 2), which is compatible with the hypothesis that it was the opening of potassium channels on the mitochondria that had stimulated ROS production. To further test for possible involvement of surface K_{ATP} channels, we used HMR 1883, a putative selective blocker of those channels. HMR 1883 abolished the diazoxide-induced increase in FI (100.4 ± 8.8%).

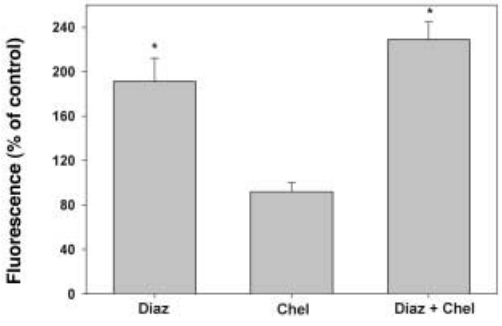

Fig. 5 The increase in fluorescence from diazoxide (Diaz) was blocked by the PKC antagonist chelerythrine (Chel). In all bars fluorescence was normalized to that in cells incubated in fresh medium. *p < 0.001 vs control.

As depicted in Fig. 5 the PKC blocker chelerythrine had no independent effect on fluorescence. Furthermore it did not attenuate the increased ROS production observed following exposure of cells to diazoxide.

The average current recorded at the end of voltage steps to –120 mV after leak subtraction was 48.9 ± 7.6 pA (mean ± SEM). Bath application of 100 μM pinacidil failed to significantly increase inward current at hyperpolarizing potentials (peak current 58.2 ± 9.2 pA, n = 4). Thus we failed to detect any measurable increase in inwardly rectifying current after exposure of cells to the non-selective K_{ATP} channel opener pinacidil.

Discussion

The present data indicate that opening of K_{ATP} channels or permeabilization of membranes to potassium with valinomycin results in increased generation of ROS. The mitochondrial electron transport chain appears to be the major source of diazoxide-induced ROS implicating the mK_{ATP} as the site controlling ROS production. However, in light of the HMR 1883 data, this study cannot completely exclude a possible role of the sarcolemmal K_{ATP} channel in generation of ROS.

A major aim of this study was to establish a convenient model in which K_{ATP}-dependent ROS production could be studied. Such a model would allow us to perform highly mechanistic studies. While our major interest is ROS-dependent protection in the heart, it is unknown how many of the present observations can actually be extrapolated to heart muscle. As is discussed below many of our results do agree with similar experiments done in heart tissue and cells. It is possible to actually test novel observations from the A7r5 model in intact hearts. For example, we would predict that valinomycin should precondition the heart. We cannot discount the possibility, however, that K_{ATP}-dependent ROS production could be fundamentally different between A7r5 cells and heart muscle. If that proves to be the case then these data would pertain to only vascular smooth muscle.

Activation of K_{ATP} channels induces generation of ROS in vascular smooth muscle cells

ATP-dependent K^+ channels have been identified in a variety of tissues including myocardium, pancreatic β-cells, certain types of neurons, various types of nonarterial smooth muscle such as urinary bladder and gallbladder, and vascular smooth muscle (26). Since these

early observations it has become apparent that there are indeed two populations of K_{ATP} channels in cells, sarcolemmal (27) and mitochondrial (20, 30), with differing pharmacological/electrophysiological profiles (14, 24). Garlid and colleagues (14, 15) have measured K_{ATP} channel activity in isolated mitochondria from rat liver and bovine myocardium, and other laboratories have studied channel activity in rat (19) and rabbit (24) cardiomyocytes. These studies have validated the pharmacological tools that can be used to differentiate between the mitochondrial and sarcolemmal channels. In those cell types, diazoxide has been identified as a specific opener (14) and 5HD as a selective closer (15, 22) of mK_{ATP}. Although appropriate studies have not been done to confirm the existence of K_{ATP} channels on mitochondria from A7r5 cells, there is little reason to believe that they would differ from those in heart or liver. The more difficult question is whether diazoxide and 5HD affect the surface channels in the A7r5 smooth muscle cells. Our electrophysiological experiments have demonstrated that the non-selective K_{ATP} channel opener pinacidil had no effect on membrane current. Either A7r5 cells completely lack surface K_{ATP} channels or there are so few of them that their summed current cannot be detected. Our results are consistent with those of Van Renterghem and Lazdunski (39) who reported that a small conductance, Ca^{2+}-activated K^+ channel was the only detectable source of K^+ conductance in A7r5 cells. Because we lack information on the pharmacology of the surface channels of vascular smooth muscle cells, we obviously cannot completely rule out the possibility that surface channels may be affecting our results.

Carroll et al. (6) used the fluoroprobe MTR Orange to demonstrate that diazoxide can increase ROS production in a non-muscle cell line derived from human atrium. Proof is also lacking in their study that the K_{ATP} channels that triggered radical production were on mitochondrial membranes. In the present study we used myxothiazol to extend the observations of Carroll et al. (6), and have now demonstrated that the ROS were derived from the electron transport chain in mitochondria. Forbes et al. (10) performed a similar experiment using 2',7'-dichlorofluorescein as the ROS indicator in freshly isolated rat cardiomyocytes. Both diazoxide and pinacidil caused a small but significant increase in ROS production which could be blocked by 5HD. Because Forbes et al. (10) studied cardiac cells, they were able to conclude that their tool drugs were indeed acting on mitochondrial channels, and that the ROS were the result of opening of mK_{ATP}.

These prior studies have all demonstrated increased fluorescence of dyes reacting with ROS following administration of K_{ATP} channel openers. It has been assumed that this observation is the result of increased ROS production. However, there are other possibilities. If the activity of intracellular scavengers such as catalase abruptly diminished or if mitochondrial anion channel activity was altered to permit greater release of superoxide, fluorescence could increase in the absence of increased mitochondrial ROS production. However, there is no precedent for the former. Although some have proposed that opening of anion channels in the cell is important in cardioprotection (9), we were unable to confirm this result (18).

Although much is known about the preconditioning phenomenon, our understanding is far from complete. It has been demonstrated that activation of certain cell surface receptors such as adenosine, opioid, and bradykinin can trigger activation of PKC that then initiates a signaling cascade leading to cardioprotection. But free radicals (2, 36) and even opening of mK_{ATP} (29) can also trigger this process. The present data help to establish the relationship among these entities and emphasize the importance of ROS as intracellular messengers. Our results indicate that opening of K_{ATP} channels do increase the intracellular concentration of ROS which could then initiate downstream cellular events.

Like Carroll et al. (6) we chose a MTR probe over other fluoroprobes. Reduced MTR orange is non-fluorescent until it is oxidixed by ROS. The oxididzed form is concentrated in the mitochondria causing them to be fluorescent (17). In preliminary studies we tested 2',7'-dichlorofluorescein as our fluorochrome as suggested by Forbes et al. (10). While we often saw an increase in fluorescence with diazoxide treatment, the increase was small and in our case inconsistent. The MTR produced a much more robust signal presumably because of its mitochondrial localization. However, several shortcomings have been described for MTR: the most important is the influence of mitochondrial membrane potential on probe uptake (33). While a large fraction of the oxidized probe is covalently bound to SH-groups, incorporation of the remaining fraction into the mitochondria is dependent on the membrane potential. At the concentration used in the present study, diazoxide has been reported to partly uncouple rabbit heart mitochondria (19, 23, 24). Thus, diazoxide also might cause a voltage-dependent release of the oxidized probe. This hypothesis was confirmed by studying cells using 100 nM oxidized MTR which is already fluorescent and insensitive to ROS. In that situation diazoxide often caused a slight decrease in FI (data not shown). However, since this error is in the opposite direction of our present findings of increased FI with diazoxide, it indicates that the MTR data, if anything, underestimate the actual amount of ROS produced by the diazoxide-treated cells. We cannot rule out the possibility that some of the other interventions, however, might have caused a voltage dependent change in FI.

There were other internal confirmations of the method. Uncoupling mitochondria with DNP did not significantly affect FI of oxidized MTR, again showing that the method is not very sensitive to changing mitochondrial voltage. Finally the scavengers MPG and Tiron

also eliminated the diazoxide-induced signal demonstrating that the increased FI represented ROS production.

The source and mechanism of diazoxide-induced free radical production

The finding that myxothiazol and DNP blocked the diazoxide-induced increase in ROS production again confirmed that the increased ROS was mitochondrial in origin. Myxothiazol blocks ROS production at complex III of the electron transport chain. DNP is a protonophoretic uncoupler of oxidative phosphorylation, and uncouplers specifically reduce ROS production of mitochondrial origin (34, 37).

Increased K^+ influx into mitochondria has the potential to uncouple the respiratory chain due to futile cycling of K^+ via the K^+/H^+ antiporter (13). This has led several groups to propose that opening of mK_{ATP} protects the heart by uncoupling mitochondria (19, 24). Indeed, excessive doses of pinacidil or diazoxide can uncouple mitochondria by virtue of an intrinsic protonophoretic action that is independent of their pharmacological actions on mK_{ATP} (23). However, it has been demonstrated in rat heart mitochondria that K^+ flux through mK_{ATP} is far too low to cause significant uncoupling (23). An interesting feature of our model is that mitochondrial ROS production is potently inhibited by uncoupling (34, 37). The findings that the uncoupling agent DNP blocked the diazoxide-induced increase in ROS production further support the hypothesis that ROS production is under mitochondrial K_{ATP} control.

Interestingly, the potassium ionophore, valinomycin, mimicked the effects of both diazoxide and pinacidil on FI in the present study. As expected, 5HD could not abolish the valinomycin-induced increase in FI, indicating that 5HD was indeed inhibiting diazoxide-induced generation of ROS by acting specifically at the level of a K_{ATP} channel. Importantly, valinomycin was unable to increase fluorescence when mitochondrial electron transport was inhibited with myxothiazol or uncoupled with DNP, indicating that the ROS were coming from the mitochondria. Our data lead us to conclude that it is the movement of potassium per se that is responsible for triggering ROS production. That movement would be both into the mitochondria and out of the cell. We do not know which was the critical event.

If potassium movement into the mitochondria were indeed the triggering event, then the following mechanism, based on studies in isolated mitochondria, seems most likely (4). ROS production increases with increasing matrix pH. Matrix alkalinization will normally accompany K^+ uptake, because there is an imbalance between uptake of K^+ and anionic equivalents. K^+ uptake is driven by proton ejection by electron transport, causing profound alkalinization. This is compensated in part by electroneutral uptake of phosphate and other substrate anions, driven by the pH gradient. However, the cytosolic concentrations of these anions are far lower than that of K^+, and this imbalance will result in a higher matrix pH at the new steady state. The mechanism of increased ROS production is the subject of active investigation in our laboratories.

HMR 1883, a purported selective blocker of sarcolemmal K_{ATP} channels in cardiac myocytes (31), completely abolished ROS production by diazoxide, suggesting that a surface channel may have been involved. However, data from the present as well as a previous study (5) have not been consistent with this exclusive action. Protection from ischemic preconditioning can be abolished by either a free radical scavenger (2, 36) or the mK_{ATP} blocker, 5HD (3, 15). Furthermore, diazoxide, at a dose that exclusively opens mitochondrial channels, duplicates the protection (3). HMR 1883 did not affect the protection from ischemic preconditioning but completely abolished the protection from diazoxide (5). Several explanations could clarify the discrepant findings. One is that HMR 1883 serves as a potent inhibitor or scavenger of ROS production. However, if that were the case, then HMR 1883 should have blocked protection from ischemic preconditioning which it did not (5). A second suggestion is that HMR 1883 somehow directly interferes with diazoxide's ability to cause K_{ATP}-dependent ROS generation, but not that related to surface receptors.

K_{ATP} channels and PKC

In a recent publication the protective effect of diazoxide in rats was abolished by downregulation of PKC following 24 h of exposure to phorbol ester (41). The authors interpreted this observation to indicate that diazoxide activates PKC to open mK_{ATP} even though they did not actually measure any index of channel opening. We tested this hypothesis by measuring the generation of ROS by diazoxide in the presence of the PKC blocker chelerythrine. PKC blockade did not block diazoxide-dependent ROS production suggesting that, at least in A7r5 cells, PKC is not needed for diazoxide to open K_{ATP} (Fig. 5). Indeed Tritto et al. (36) and we (3) have both found that PKC inhibitors block protection triggered by ROS. We interpret these data to indicate that PKC activation is downstream from ROS generation which in turn is downstream from K_{ATP} opening.

Conclusion

These studies further support the hypothesis that a signal transduction pathway exists in the cell in which open-

ing of K_{ATP} channels leads to subsequent production of ROS by mitochondria. These ROS are then thought to act as messengers to initiate signaling cascades leading to cardioprotection or other cellular events. Our data are most compatible with the K_{ATP} channel residing on the mitochondria, but at this time we cannot exclude a sarcolemmal location. While much indirect evidence of this pathway has been generated in the preconditioned heart, these studies show that a K_{ATP}-dependent ROS signal can be directly demonstrated in vascular smooth muscle cells.

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