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Opening of Mitochondrial ATP-Sensitive Potassium Channels Evokes Oxygen Radical Generation in Rabbit Heart Slices[1]

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The purpose of this study was to determine whether ATP-sensitive potassium channel (K_{ATP} channel) activation generates oxygen free radicals in the rabbit heart. We assayed malondialdehyde (MDA) in rabbit heart slices *in vitro* as an indicator of oxygen free radical generation. The K_{ATP} channel openers, pinacidil and cromakalim, significantly increased MDA production in a concentration-dependent manner. MDA formation also increased linearly with incubation time in the presence of K_{ATP} channel openers. The K_{ATP} channel blockers, glibenclamide and 5-hydroxydecanoate (5-HD), decreased K_{ATP} channel opener-induced MDA formation in a concentration-dependent manner. When Fe^{2+} was administered to heart slices that had been pretreated with K_{ATP} channel openers, a marked elevation in MDA was observed, compared to heart slices that were treated with Fe^{2+} alone. A positive linear correlation between Fe^{2+} and MDA level was observed. The MDA levels of heart slices subjected to anoxia for 15 min remained unchanged until reperfusion. When the heart slices were reoxygenated for 30 min, a marked increase in MDA formation was observed. However, in the presence of glibenclamide and 5-HD, reperfusion following anoxia did not result in increased MDA. These results suggest that the opening of mitochondrial K_{ATP} channels in rabbit heart slices evokes oxygen free radical generation *via* a Fenton-type reaction.

Key words: heart slices, malondialdehyde, mitochondrial K_{ATP} channels, oxygen free radicals.

Ischemic preconditioning in the heart was initially described as an adaptive response to brief episodes of ischemia that had the effect of decreasing necrosis during subsequent prolonged ischemia (*1*). Ischemic preconditioning was attributed to a variety of elements (*2*) including (a) protective triggers released in response to the preconditioning stimulus; (b) protein kinases that integrate and relay the signal initiated by the triggers; and (c) effectors that are modulated by the transduced signal and confer myocardial protection.

During ischemia-reperfusion, a variety of factors are released as a consequence of metabolic and neurohormonal changes, and these act as triggers of ischemic preconditioning. In particular, reactive oxygen species generated during brief ischemia/reperfusion have been recognized as potential initiators of preconditioning (*3*). Evidence for this role comes from intact heart studies, where exposure to superoxide or H_2O_2 induce a preconditioning-like protection (*3*), and from studies demonstrating that antioxidants abolish the induction of preconditioning (*4*).

Studies in rabbits have shown that K_{ATP} channel blockers, such as glibenclamide and 5-hydroxydecanoate (5-HD), abolish cardioprotection induced by ischemic preconditioning (*5*), thus implicating K_{ATP} channels as effectors. Additionally, Tokube *et al.* (*6*) reported that K_{ATP} channel opening is induced by oxygen free radicals produced *via* the xanthine oxidase reaction. Moreover, Yao *et al.* (*7*) have shown an association between oxygen free radicals and mitochondrial K_{ATP} channel opening in chick cardiac myocytes. Since free radicals are known triggers of ischemic preconditioning (*3*), it has been suggested that mitochondrial K_{ATP} channel opening before ischemia generates oxygen free radicals which in turn mediate cardioprotection (*8*).

A recent, and as yet poorly understood, development in this field is the discovery of a relationship between cardiac K_{ATP} channel opening and oxygen free radicals during brief hypoxia or ischemia induction. Thus, the purpose of our study was to clarify whether K_{ATP} channel activation in the rabbit heart generates oxygen free radicals. We measured the formation of malondialdehyde (MDA) in rabbit heart slices *in vitro* as an indicator of oxygen free radical generation.

MATERIALS AND METHODS

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). The Local Ethical Committee on Animal Experiments, Inje University, granted permission for the study.

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Chemicals—Pinacidil (RBI, Natick, MA) and cromakalim (Sigma, St. Louis, MO) were freshly prepared immediately before the start of the experiments and diluted in the test solution to obtain the indicated final concentrations. Glibenclamide and 5-HD were prepared as 5 mM stock solutions in 5% dimethyl sulfoxide (DMSO). An appropriate volume of each stock solution was added to Cross-Taggart medium to achieve the desired concentration immediately before use, as described in the text. At these concentrations, the solvent alone had no effect on the level of lipid peroxidation. All chemicals used in this study, unless specified otherwise, were from Sigma.

Preparation of Heart Slices—New Zealand white rabbits weighing 1.0–1.5 kg each were anesthetized by injection of pentobarbital sodium (50 mg/kg body weight) and heparin (300 IU/ml) into the marginal ear vein. As soon as deep anesthesia was achieved, as evidenced by lack of eye-blink and foot withdrawal reflexes, the heart was rapidly isolated via thoracotomy and the aorta was cannulated. The dissected heart was mounted on a Langendorff apparatus and gently squeezed during retrograde perfusion with oxygenated normal Tyrode solution containing 143 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.5 mM glucose, and 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (adjusted to pH 7.4 with NaOH) for 5-6 min, until all signs of blood were eliminated. Thin (0.4 to 0.5-mm-thick) slices of heart were prepared using a Stadie-Riggs microtome, and stored in ice-cold modified Cross-Taggart medium containing 130 mM NaCl, 10 mM KCl, 1.5 mM $CaCl_2$, 5 mM glucose, and 20 mM Tris-HCl (adjusted to pH 7.4 with NaOH). The average weight of the heart slices used was 53.3 ± 3.5 mg (n = 132).

Lipid Peroxidation Assay—Lipid peroxidation was estimated by measuring the content of malondialdehyde (MDA) according to the thiobarbituric acid assay (*9*). Lipid peroxidation is generally accepted as an indicator of oxidative stress as a result of free radical overproduction and depletion of antioxidant reserves. The test compounds (pinacidil, cromakalim, glibenclamide and 5-HD) were applied for various incubation times after all slices were stabilized for 30 min under aerobic conditions, after which MDA production was measured immediately. Heart slices were homogenized in ice-cold 1.15% KCl (5% w/v). A 0.2-ml aliquot of homogenate was mixed with 50 μl of 8.1% sodium dodecyl sulfate, and the mixture was incubated for 10 min at room temperature. Acetic acid (375 μl of a 20% solution, pH 3.5) and 375 μl of 0.6% thiobarbituric acid were added and the mixture was heated for 60 min in a boiling water bath, and then cooled to room temperature. After adding 1.25 ml of a 15:1 mixture of *n*-butanol and pyridine, the contents were vigorously vortexed and centrifuged at 1,000 rpm for 5 min. The absorbances at 535 and 520 nm of the upper, colored layer were measured in a spectrophotometer (Hitachi, U-2000, Tokyo) and compared with freshly prepared 1,1,3,3-tetraethoxypropane standards. The final values are expressed as percentages of the control group values.

Anoxic Conditions—The partial pressure O_2 (P_{O_2}) was measured using a blood gas analyzer (Nova Stat Profile-3, Nova Bomedical, Waltham, MA, USA). The oxygenation of the incubation medium was maintained by a continuous flow of 100% O_2 to obtain a P_{O_2} between 27 and 31 kPa. For the induction of simulated anoxia, the medium was bubbled with 100% N_2 for morethan 3 h; the P_{O_2} was approximately 0.2 kPa. The anoxia/reoxygenation experiments were carried out at 37°C.

Statistical Analysis—All values are expressed as the arithmetic mean ± SE. The differences between mean values were analyzed by Student's t test, and ANOVA with Fisher's *post hoc* test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of K_{ATP} Channel Openers on MDA Formation—Malondialdehyde (MDA) is a final product of lipid peroxidation and can be used to evaluate oxygen free radical generation. The effects of pinacidil, a K_{ATP} channel opener, on MDA formation were examined over a concentration range of 1–100 μM in heart slices incubated for 60 min at 37°C. As shown in Fig. 1A, pinacidil at a concentration above 10 μM caused a concentration-dependent increase in MDA production. In Fig. 1B, the MDA level at each concentration of pinacidil is expressed relative to the control value (in the absence of pinacidil). Likewise, cromakalim, another K_{ATP} channel opener, significantly increased MDA formation in a concentration-dependent manner with maximal MDA production when 10 μM cromakalim was added (1,325 ± 114 pmol/mg protein, n = 13 slices, $p < 0.05$).

In subsequent studies, 50 μM pinacidil was used, since

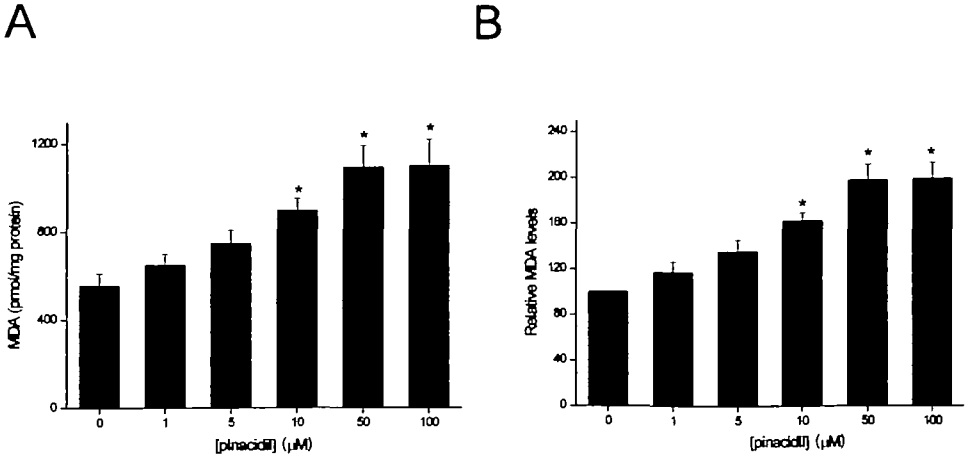

Fig. 1. **A: The relationship between increasing pinacidil concentrations and MDA levels in rabbit heart slices. B: The histogram shows the level of MDA relative to the control value for MDA in the absence of pinacidil.** Rabbit heart slices were treated with various concentrations of pinacidil for 60 min at 37°C. The bars represent the SE. Ten heart slices were examined at each concentration of pinacidil. *$p < 0.05$ compared to control (in the absence of pinacidil).

this concentration yielded substantial MDA formation (Fig. 1). Furthermore, MDA formation increased linearly with incubation time in the presence of 50 μM pinacidil (Fig. 2). A standard incubation time of 45 min was selected for the subsequent experiments. In addition, 100 μM cromakalim significantly increased MDA levels after 45 min (1,243 ± 128 pmol/mg protein, $n = 10$ slices, $p < 0.05$).

Effect of Glibenclamide on MDA Formation—Glibenclamide (10 μM), a K_{ATP} channel blocker, was used to treat heart tissue in the presence of 50 μM pinacidil, and MDA formation was measured (Fig. 3). The basal level of MDA in the control was 556 ± 55 pmol/mg protein ($n = 20$ slices). Pinacidil (50 μM) significantly increased the level of MDA formation after 45 min (1,098 ± 100 pmol/mg protein, $n = 20$ slices). As shown in Fig. 3A, glibenclamide significantly decreased the MDA level induced by pinacidil to 600 ± 80 pmol/mg protein after 45 min of incubation ($n = 20$ slices, $p < 0.05$). Glibenclamide decreased pinacidil-induced MDA formation in a concentration-dependent manner over a concentration range of 1–50 μM (Fig. 3B; $n = 11$ slices for each concentration). The minimal concentration of glibenclamide that blocked MDA formation induced by 50 μM pinacidil was 5 μM ($p < 0.05$). When heart slices were exposed to 50 μM pinacidil in the presence of 50 μM glibenclamide, the MDA level was not different from the control (593 ± 87 pmol/mg protein, $n = 17$ slices, $p > 0.05$).

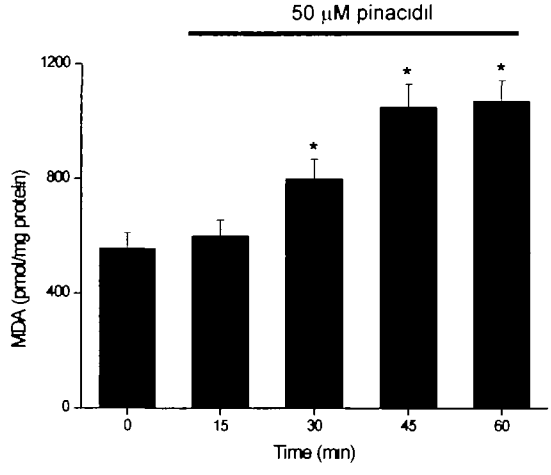

Fig. 2. **Time course of pinacidil-induced MDA formation in rabbit heart slices.** The pinacidil concentration was 50 μM. Results are shown as the mean ± SE of ten slices. *$p < 0.05$ compared to control (in the absence of pinacidil).

Effect of 5-Hydroxydecanoate (5-HD) on MDA Formation—The effects of 5-HD, a selective mitochondrial K_{ATP} channel blocker, on heart slices were also examined. As shown in Fig. 4A, 100 μM 5-HD significantly decreased the MDA production induced by 50 μM pinacidil from 1,098 ± 100 to 800 ± 80 pmol/mg protein after 45 min ($n = 13$ slices, $p < 0.05$). 5-HD also decreased pinacidil-induced MDA formation in a concentration-dependent manner over a concentration range of 1–1,000 μM (Fig. 4B; $n = 15$ slices for each concentration). The minimal concentration of 5-HD that blocked MDA formation induced by 50 μM pinacidil was 100 μM ($p < 0.05$). In addition, the application of glibenclamide and 5-HD decreased cromakalim (100 μM)-induced MDA formation (820 ± 95 pmol/mg protein, $n = 15$ slices, $p < 0.05$).

Relationship between Fe^{2+} Addition and Pinacidil-Induced MDA Formation—Heart slices were treated with Fe^{2+} to investigate whether pinacidil-induced MDA formation proceeds *via* a Fenton-type reaction. When Fe^{2+} was administered to the pinacidil (50 μM)-pretreated heart slices, a marked increase in MDA formation was observed as compared with the MDA levels in heart slices treated with Fe^{2+} alone (Fig. 5). A positive linear correlation ($r^2 = 0.986$) between Fe^{2+} concentration and the MDA level was observed. When analogous experiments were performed with cromakalim (100 μM)-pretreated heart slices, a positive linear correlation ($r^2 = 0.989$) between Fe^{2+} concentration and MDA level was also observed (data not shown).

Effects of Glibenclamide and 5-HD on MDA Formation in Anoxic-Reoxygenated Rabbit Heart Slices—The effects of glibenclamide and 5-HD on MDA formation in anoxic-reoxygenated heart slices were examined (Fig. 6). Heart slices were subjected to anoxia for 15 min followed by reoxygenation for 30 min. The levels of MDA were unchanged during anoxia (520 ± 30 and 510 ± 35 pmol/mg protein in control and anoxic heart slices, respectively). Following reoxygenation, a marked elevation in MDA level to 800 ± 40 pmol/mg protein was observed ($p < 0.05$, compared to the anoxia-induced MDA values). However, in the presence of 10 μM glibenclamide, reoxygenation did not significantly

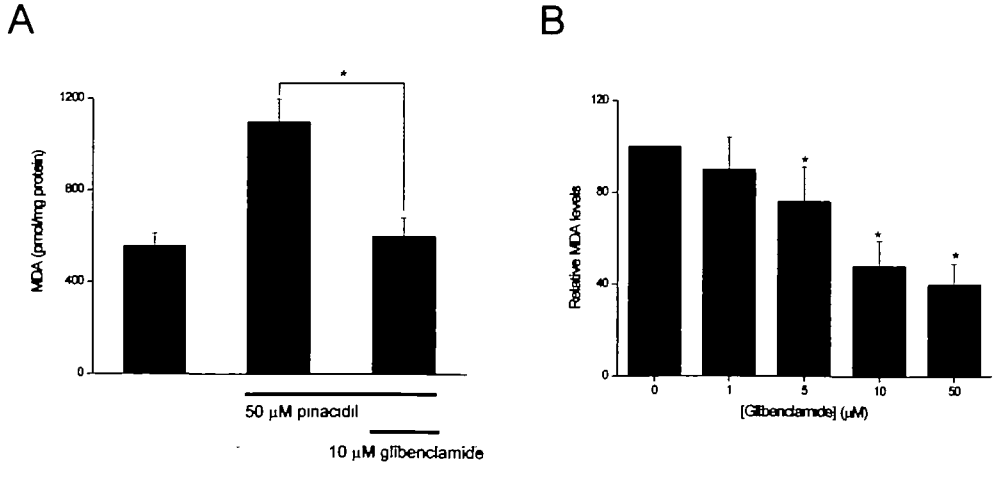

Fig. 3. **A: The effect of glibenclamide (10 μM) on pinacidil (50 μM)-induced MDA formation.** Results are shown as the means ± SE of twenty slices. **B: The effects of different concentrations of glibenclamide on pinacidil (50 μM)-induced MDA formation in rabbit heart slices.** The incubation time was 45 min. The MDA levels were calculated as percentages of the baseline value (100%) measured before the application of glibenclamide. Results are shown as the mean ± SE of eleven slices. *$p < 0.05$ compared with the pinacidil-only treatment group.

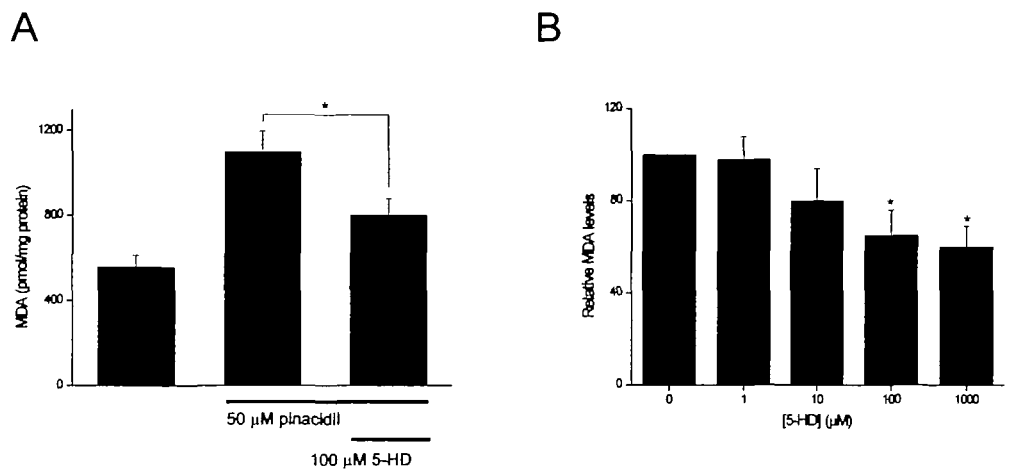

Fig. 4. **A: The effect of 5-HD (10 μM) on pinacidil (50 μM)-induced MDA formation.** Results are shown as the mean ± SE of thirteen slices. **B: The effect of different 5-HD concentrations on pinacidil (50 μM)-induced MDA formation in rabbit heart slices.** The incubation time was 45 min. The MDA levels were calculated as percentages of the baseline value (100%) measured before the application of 5-HD. Results are shown as the mean ± SE of fifteen slices. $*p < 0.05$ compared with the pinacidil-only treatment group.

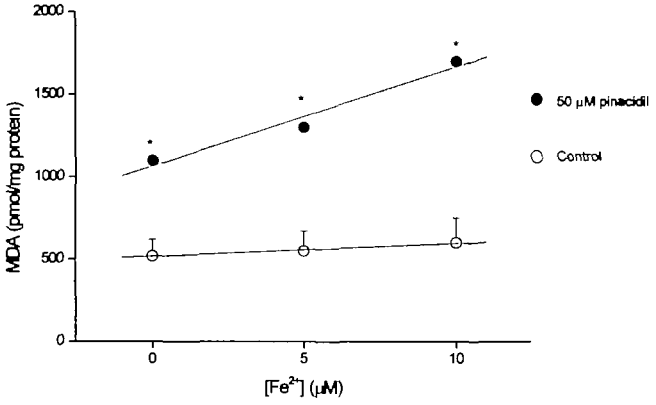

Fig. 5. **Dose-response relationship between [Fe^{2+}] and MDA formation in pinacidil-treated heart slices.** Three different concentrations of Fe^{2+} (0, 5, and 10 μM) were administered to pinacidil (50 μM)-treated heart slices, and the MDA levels were compared for the pinacidil (●), and control (○) groups. Results are shown as the mean ± SE of ten slices. $*p < 0.05$ compared with control.

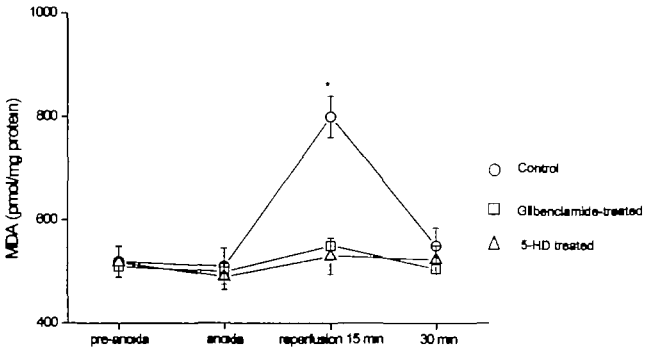

Fig. 6. **MDA production in anoxic-reperfused rabbit heart slices.** Three groups were compared: control (○), glibenclamide-treated (□), and 5-HD-treated heart (△) slices. Results are shown as the mean ± SE of 8 heart slices under each experimental condition. $*p < 0.05$ compared with the glibenclamide- or 5-HD–treated groups.

increase MDA formation (500 ± 30 and 550 ± 35 pmol/mg protein in anoxic and glibenclamide-treated heart slices, respectively). Similar findings were obtained in analogous experiments performed with 100 μM 5-HD (Fig. 6).

DISCUSSION

Ischemic preconditioning is a phenomenon in which single or multiple short periods of ischemia or hypoxia (*10*) result in a marked reduction in the infarct size, extent of stunning and incidence of cardiac arrhythmia that accompany more prolonged periods of ischemia. Although a number of potential factors and signaling molecules mediating this phenomenon have been proposed, the activation of K_{ATP} channels is an important component of ischemic preconditioning (*11*). Two subtypes of KATP channel exist: mitochondrial K_{ATP} channels, located in the inner mitochondrial membrane (*12*), and the surface K_{ATP} channels located in the sarcolemmal membrane (*13*). It was initially assumed that sarcolemmal K_{ATP} channels were responsible for protection. Opening of these channels results in membrane hyperpolarization and shortens phase 3 of the action potential, thereby attenuating energy consumption and reducing intracellular calcium levels to produce a cardioprotective effect (*14*). However, several studies suggest that the sarcolemmal K_{ATP} channel is unlikely to be the site of the cardioprotective effects of ischemic preconditioning and K_{ATP} channel openers. Instead, mitochondrial K_{ATP} channels have been proposed as the effectors of cardioprotection (*15*).

Free radicals have been proposed as crucial regulators of cellular responses in a number of pathological conditions and diseases, especially those involving the cardiovascular system (*16*). In the heart, free radicals participate in the tissue damage observed during ischemia-reperfusion (*17*). The radicals involved in those processes are mainly oxygen-based, such as superoxide, hydroxyl, or peroxyl radicals. The pathophysiological relevance of free radicals is supported by the consistent shortening of action potential duration by oxidative damage following prolonged exposure to ischemia-reperfusion conditions (*18*). In contrast, oxygen free radicals generated from brief ischemia/reperfusion have also been recognized as possible triggers for preconditioning (*3, 19*).

In addition to the interactions between K_{ATP} channels and oxygen free radicals discussed above, our results indicate that the opening of K_{ATP} channels induces oxygen radical generation, as reflected in increased MDA formation in

heart slices (Figs. 1 and 2). Obata *et al.* (*20*) reported previously that glibenclamide, a K_{ATP} channel blocker, attenuates hydroxyl radical formation *via* K^+-induced depolarization. The present study examined MDA formation in response to pharmacological activation and following the inhibition of K_{ATP} channel opening. Although the mechanism by which K_{ATP} channel openers participate in subsequent oxygen free radical generation is obscure, we assessed the activity of K_{ATP} channel openers by measuring MDA formation in heart slices. We tested the effects of K_{ATP} channel openers (pinacidil and cromakalim) on MDA formation in order to investigate K_{ATP} channel–dependent oxygen radical generation. We demonstrated that opening K_{ATP} channels significantly increase MDA formation (Figs. 1 and 2) and that K_{ATP} channel opener–induced MDA formation is abolished by glibenclamide (Fig. 3). We conclude that the opening of K_{ATP} channels evokes the production of oxygen free radicals.

There are many potential sites of oxygen free radical production in the intact heart (*21, 22*). In addition, neutrophils and other humoral inflammatory mediators localized in the microcirculation may release oxygen free radicals in the intact heart (*23*). However, the rabbit heart slices used in the present study contained neither inflammatory nor endothelial cells, nor significant amounts of xanthine oxidase (*24*), so the contribution of non-cardiomyocytes to the measured oxygen free radical generation was negligible. Within cardiac myocytes, oxygen free radical sources include superoxide generation from NAD(P)H or other oxidases such as cytochrome P450 (*22*), nitric oxide synthase under conditions of arginine depletion (*25*), and the mitochondrial electron transport chain (*21*). It has previously been reported that the opening of mitochondrial K_{ATP} channels causes a moderate increase in the mitochondrial production of oxygen free radicals, suggesting that oxygen free radical production is regulated by volume effects derived from the activation of the electron transport system (*19*). Recently, it has been suggested that oxygen free radicals are associated with mitochondrial K_{ATP} channel opening (*7*). In Fig. 3, we demonstrate that glibenclamide, a mitochondrial and sarcolemmal K_{ATP} channel blocker, abolishes K_{ATP} channel opener–induced MDA formation. This result is in agreement with those of Grover and Garlid (*26*). However, because both channels are activated by pinacidil and cromakalim, and inhibited by glibenclamide (*12, 27–31*), it is not possible at this time to ascertain whether the oxygen free radical generation is mediated through mitochondrial or sarcolemmal K_{ATP} channels or both. Thus, specific agents modulating the opening and closing of K_{ATP} channels are in demand. To investigate the role of sarcolemmal or mitochondrial K_{ATP} channels, we have used 5-HD. There is good evidence that 5-HD is selective for mitochondrial over sarcolemmal K_{ATP} channels (*27, 31, 32*), and our present study shows that 5-HD reduces K_{ATP} channel opener-induced MDA formation in a concentration-dependent manner (Fig. 4). These data suggest that oxygen free radical generation requires the activation of mitochondrial K_{ATP} channels. To investigate this possibility, the activity of another K_{ATP} channel opener, cromakalim, was examined. Cromakalim enhances MDA formation in heart tissue slices. In addition, both glibenclamide and 5-HD decrease cromakalim-induced MDA formation. The findings in the present study are in agreement with those in recent reports on rat hearts (*33, 34*) or cultured human atrial derived cardiocytes (*35*). It was reported that in *in vivo* rat hearts, K_{ATP} channel openers increase ROS, as measured with a microdialysis probe perfused with sodium salicylate, which reacts with hydroxyl radicals (*33*). Forbes *et al.* (*34*) also demonstrated that in isolated rat ventricular myocytes, the addition of diazoxide or pinacidil resulted in an increase in reactive oxygen species (ROS), as measured with a ROS-sensitive fluorescent probe 2′,7′-dichlorofluorescin (DCF). They found that 5-HD abolishes the increase in DCF fluorescence by mitochondrial K_{ATP} channel opening. Furthermore, Carroll *et al.* (*35*) demonstrated the ability of diazoxide to cause the generation of ROS directly in human cardiac derived cardiocytes. Taken together, these results suggest that the opening of mitochnodrial K_{ATP} channel leads to ROS production and that this is an important component of ischemic preconditioning.

Although the involvement of K_{ATP} channel openers in subsequent oxygen free radical generation remains obscure, some evidence suggests that the opening of K_{ATP} channels evokes oxygen free radical generation *via* a Fenton-type reaction (*33*). We examined the potential link between K_{ATP} channel opener–induced oxygen free radical generation and a Fenton-type reaction. We found a marked elevation in the levels of MDA formation in pinacidil- and Fe^{2+}-treated, compared to only Fe^{2+}-treated, heart slices. This result is consistent with that of others who have found that Fe^{2+} markedly enhances the formation of hydroxyl radicals in cromakalim-treated rat hearts, compared with Fe^{2+} treatment alone (*33*). It is well known that H_2O_2 plus Fe^{2+} generate oxidants that initiate free radical reactions (*36*). The primary oxidant generated by H_2O_2 plus Fe^{2+} is the hydroxyl radical (*37*), which is believed to initiate lipid peroxidation (*38*). Although possible sources of H_2O_2 include leukocytes, coronary endothelial cells, or myocardial mitochondria within the intact heart, the source of H_2O_2 could be myocardial mitochondria under our experimental conditions. Superoxide generated from mitochondria is known to enter the cytosol, where it is converted to H_2O_2 by superoxide dismutase (*39*). Therefore, it seems likely that the critical Fenton reactions occur intracellularly under our experimental conditions. Furthermore, the data of Byler *et al.* (*40*) are consistent with this explanation.

In the present study, we found that MDA formation is elevated in anoxia-reoxygenated heart slices and that anoxia-reoxygenation–induced MDA formation is suppressed in the presence of glibenclamide or 5-HD (Fig. 6). These findings indicate that anoxia-reoxygenation of the myocardium generates oxygen free radicals by activating mitochondrial K_{ATP} channels. It was previously demonstrated that the K_{ATP} channel opener, bimakalim, induces cardioprotection in canine hearts when administered before the onset of or after 60 min of ischemia and throughout the period of reperfusion (*41*), and that mitochondrial K_{ATP} channel opening induces similar cardioprotection (*42, 43*). Early studies suggested that reperfusion of cardiac tissue after prolonged ischemia is associated with persistent electrophysiological and mechanical dysfunction, including reduced action potential amplitude, reduced tension development, and elevated resting tension (*44, 45*). There is considerable evidence that such injury is associated with a marked increase in the generation of oxygen free radicals (*40*). Interestingly, recent experimental evidence suggests

that oxygen free radicals generated from brief ischemia/reperfusion are possible "triggers" in the initiation of preconditioning (*3, 46*). Furthermore, the generation of endogenous reactive oxygen intermediates from cardiomyocytes appears important in preconditioning, and it appears these species are generated in the mitochondria of myocytes (*39*). Although specific signaling targets for oxygen free radicals in preconditioning may include protein kinase C (*47*), p38 mitogen–activated protein kinase (*48*), and K_{ATP} channels (*49*), it remains unclear how oxygen free radicals confer protection. In this regard, further experiments are necessary to determine the relationships among mitochondrial K_{ATP} channel opening, oxygen free radicals and cardioprotection against ischemia-reperfusion injury.

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