Flumazenil preconditions cardiomyocytes via oxygen radicals and $K_{\text{ATP}}$ channels

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Flumazenil preconditions cardiomyocytes via oxygen radicals and $K_{\text{ATP}}$ channels. Am J Physiol Heart Circ Physiol 279: H1858–H1863, 2000.—We determined whether flumazenil mimics ischemic preconditioning in chick cardiomyocytes and examined the role of intracellular reactive oxygen species (ROS) and ATP-dependent potassium ($K_{\text{ATP}}$) channels in mediating the effect. Chick ventricular myocytes were perfused with a balanced salt solution in a flow-through chamber. Cell viability was quantified using propidium iodide, and ROS generation was assessed using the reduced form of 2,7’-dichlorofluorescin (DCFH). Cells were exposed to 1 h of simulated ischemia and 3 h of reoxygenation. Preconditioning was initiated with 10 min of ischemia followed by 10 min of reoxygenation. Alternatively, flumazenil was added to the perfusate for 10 min and removed 10 min before the start of ischemia. Flumazenil (1 and 10 μM) and preconditioning reduced cell death [54 ± 5%, n = 3; 26 ± 4%, n = 6 (P < 0.05); and 20 ± 2%, n = 6 (P < 0.05), respectively, vs. 57 ± 7%, n = 10, in controls] and increased DCFH oxidation (an index of ROS production) [0.35 ± 0.11, n = 3; 2.64 ± 0.69, n = 8 (P < 0.05); and 2.46 ± 0.52, n = 6 (P < 0.05), respectively, vs. 0.26 ± 0.05, n = 9, in controls]. Protection and increased ROS signals with flumazenil (10 μM) were abolished with the thiol reductant N-(2-mercaptopropionyl)-glycine (2-MPG, 800 μM), an antioxidant (83).

MATERIALS AND METHODS

Cardiomyocyte preparation. Ten-day-old embryonic chick ventricular myocytes were prepared using a method first described by Barry et al. (2) and modified by Vanden Hoek et al. (41). Briefly, hearts were harvested and placed in Hank’s balanced salt solution lacking magnesium and calcium (Life Technologies, Grand Island, NY). Ventricles were minced and myocytes were dissociated through deggregation with trypsin (0.025%, Life Technologies) applied four to six times at 37°C with gentle agitation. Isolated cells were then transferred to a solution with trypsin inhibitor for 8 min, filtered through a 100-μm mesh, centrifuged for 5 min at 1,200 rpm at 4°C, and finally resuspended in a nutritive medium described previously by Chandel et al. (5) and Duranteau et al. (9). Resuspended cells were placed in a petri dish in a humidified atmosphere containing 95% air and 5% CO$_2$. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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modified incubator (5% CO₂-95% air at 37°C) for 45 min to promote early adherence of fibroblasts. Nonadherent cells were counted with a hemacytometer, and viability was measured using trypan blue (0.4%). Approximately 1 × 10⁶ cells in the nutritive medium were pipetted onto coverslips (25 mm) and incubated for 3–4 days, after which synchronous contractions of the monolayer were noted. Experiments were performed on spontaneously contracting cells at 3 or 4 days after isolation.

Perfusion system. Glass coverslips containing spontaneously beating chick myocytes were placed in a stainless steel, 1-ml flow-through chamber (Penn Century, Philadelphia, PA). The chamber was sealed with thin water gaskets to minimize oxygen exchange between the chamber wall and the perfusate and then mounted on a temperature-controlled platform (at 37°C) on an inverted microscope. A water-jacketed glass equilibrium column mounted above the microscope stage was used to equilibrate the perfusate to known oxygen tensions (PO₂). The standard perfusion media consisted of a buffered salt solution (BSS) containing (in mM) 177 NaCl, 4.0 KCl, 18 NaHCO₃, 0.8 MgSO₄, 1.0 NaH₂PO₄, 1.21 CaCl₂, and 5.6 glucose, which was equilibrated for 1 h before the experiment by bubbling with a gas mixture of 21% O₂-5% CO₂-74% N₂. A simulated ischemia solution, composed of BSS containing no glucose but with 2-deoxy-D-glucose (20 mM) added to inhibit glycolysis, was bubbled with a gas mixture of 20% CO₂-80% N₂ for 1 h before the experiments. The pH of the perfusion solution was routinely verified (normoxic BSS: pH 7.4, simulated ischemic BSS: pH 6.8). Stainless steel or low-oxygen-solubility polymer tubing connected the equilibrium column to the flow-through chamber to minimize ambient oxygen transfer into the perfusate. In previous studies, the low PO₂ in the chamber was confirmed under conditions identical to those of experiments that used an optical phosphorescence-quenching method (24, 42) (Oxyspot, Medical Systems, Greenvale, NY).

Cell viability. The inverted microscope, equipped for epifluorescent illumination, included a xenon light source (75 W), a 12-bit digital cooled CCD camera (Princeton Instruments), a shutter and filter wheel (Sutter), and appropriate excitation and emission filter cubes. The microscope was also equipped with Hoffman-modified phase illumination to accentuate surface topology, facilitating the measurement of contractile motion (see below). Fluorescent cell images were obtained using a magnification of 10× the objective (Nikon Plan Fluor). Data were acquired and analyzed with Metamorph software (Universal Imaging). Cell viability was quantified with the nuclear stain propidium iodide (PI, 5 μM) (Molecular Probes, Eugene, OR), an exclusion fluorescent dye that binds to chromatin upon loss of membrane integrity (3, 40–41). PI is not toxic to cells over a course of 8 h, permitting its addition to the perfusate throughout the experiments. At the completion of each experiment, digitonin (300 μM) was added to the perfusate for 1 h. Digitonin disrupted the cell membrane integrity of all of the cells, allowing the maximum amount of PI to enter. The percentage of loss of viability (cell death) was then expressed relative to the maximum value after 1 h of digitonin exposure (100%).

Measurement of ROS. ROS generation in cells was assessed using the reduced form of 2',7'-dichlorofluorescin (DCFH) as a probe. The membrane-permeable diacetate form of the DCFH dye (DCFH-DA) was added to the perfusate at a final concentration of 5 μM. Within the cell, esterases
cleave the acetate groups on DCFH-DA, thus trapping the reduced probe (DCFH) intracellularly (35). ROS in the cells leads to the oxidation of DCFH, yielding the fluorescent product DCF− (35, 37). The probe DCFH in cardiomyocytes is readily oxidized by H2O2 or the hydroxyl radical but is relatively insensitive to superoxide (9, 40, 41). Fluorescence was measured using an excitation wavelength of 480 nm, a dichroic 505-nm long pass, and an emitter bandpass of 535 nm (Chroma Technology) with neutral density filters to attenuate the excitation light intensity. Fluorescence intensity was assessed in clusters of several cells identified as regions of interest. The background was identified as an area without cells or with minimal cellular fluorescence. Intensity values are reported as the percentages of initial values after the background value was subtracted.

Chemicals. Flumazenil was purchased from Hoffmann-La Roche (Nutley, NJ). N-(2-mercaptopropionyl)-glycine (2-MPG) and 5-hydroxydecanoate (5-HD) were purchased from Sigma Chemical (St. Louis, MO). Flumazenil, 2-MPG, or 5-HD was dissolved in BSS buffer before administration. PI and DCFH-DA were purchased from Molecular Probes.

Experimental design. Four groups of cardiomyocytes (control, preconditioned, and 1 and 10 μM of flumazenil-treated cardiomyocytes) were studied. Control cells were subjected to 60 min of ischemia followed by 3 h of reoxygenation. Preconditioned cells underwent 10 min of ischemia followed by 10 min of reoxygenation before being subjected to the ischemia-reoxygenation protocol used for the controls. In nonpreconditioned cells, an equal volume of BSS buffer (control) or flumazenil (1 or 10 μM) was added to the perfusate for 10 min instead of the 10-min ischemic period in the preconditioned cells.

An additional study series with four groups of cardiocytes (2-MPG (800 μM)−, 2-MPG + flumazenil (10 μM)−, 5-HD (1 mM)−, and 5-HD + flumazenil (10 μM)−treated cardiomyocytes) was used to determine the importance of ROS signals and mitochondrial KATP channels in mediating flumazenil-induced preconditioning. 2-MPG or 5-HD was added to the perfusate during the 1-h period of baseline before 60 min of ischemia.

In addition, another series of studies with six groups of cardiomyocytes [control, preconditioned, 1 or 10 μM of flumazenil, 2-MPG (800 μM)−, and 2-MPG (800 μM) + flumazenil (10 μM)−treated cardiomyocytes] was used to examine the increase in ROS signals and its role in mediating the beneficial effect of flumazenil.

The dosages of 2-MPG and 5-HD were chosen on the basis of our preliminary results in which these agents were shown to almost completely abolish the beneficial effects of flumazenil. The maximum dose of 5-HD without demonstrated toxicity was chosen for this study.

Statistical analysis. Data are expressed as means ± SE. Differences between the groups for cell death and ROS production were compared using a two-factor analysis of variance (ANOVA) with repeated measures and Fisher’s least significant difference test. Differences between groups were considered significant if the P value was <0.05.

RESULTS

Cell death. The percentage of cell death (measured by the percentage of PI uptake) was monitored intermittently throughout each experiment, and the data are summarized in Fig. 2. After 3 h of reoxygenation, cell death averaged 56.9 ± 7.0% (n = 10) in the control series and was markedly reduced by preconditioning (20.0 ± 1.9%, n = 6, P < 0.05). Flumazenil (10 μM) markedly reduced cell death, whereas the 1 μM dose of flumazenil had no effect (53.6 ± 5.2%, n = 3, and 25.9 ± 4.1%, n = 6, respectively). The reduction in cell death was similar with flumazenil (10 μM) and preconditioning (25.9 ± 4.1%, n = 6, and 20.0 ± 1.9%, n = 6, respectively) compared with controls (56.9 ± 7.0%, n = 10; Fig. 2A).

Fig. 3. Plots documenting the effects of different protocols on 2',7'-dichlorofluorescein (DCF) oxidation in arbitrary units (A.U.), an index of reactive oxygen species generation. A: in control cells, intensity of DCF fluorescence increased slightly over 1 h (Cont). Infusion of Flu (1 or 10 μM) for 10 min followed by 10 min of a drug-free period increased generation of reactive oxygen species (ROS). B: amount and pattern of increase of ROS signals with the high dose of Flu (10 μM) were similar to those after PRE. C: treatment with the antioxidant 2-MPG (800 μM) alone had no effects on ROS signals but abolished the increase of ROS signals by Flu (2-MPG + 10 μM Flu).
2-MPG and 5-HD had no effects on cell death compared with controls (n = 4 for each series); however, they abolished the beneficial effects of flumazenil (10 μM) (Fig. 2, B and C; n = 6 for each series).

**Intracellular ROS generation.** Figure 3A documents three experiments with control and 1 and 10 μM flumazenil-treated cardiomyocytes. In control cells, the intensity of the DCF fluorescence slightly increased over 1 h. The infusion of 10 μM flumazenil for 10 min followed by 10 min of a drug-free period increased DCF fluorescence, and 1 μM flumazenil had no effect. Flumazenil (10 μM) and preconditioning produced a similar amount and pattern of DCF fluorescence (Fig. 3B). The increase of ROS signals by flumazenil was abolished by treatment with 2-MPG; 2-MPG had no effect on ROS signals by itself (Fig. 3C). Fluorescence increased only 0.26-fold in controls (0.26 ± 0.05, n = 9), 0.35-fold with the low dose of flumazenil (1 μM), but 2.64-fold with the high dose of flumazenil (10 μM) (0.35 ± 0.11, n = 3, and 2.64 ± 0.69, n = 8 (P < 0.05), respectively, vs. 0.26 ± 0.05, n = 9, in controls; Fig. 3B). DCF fluorescence increased similarly with flumazenil (10 μM) and preconditioning (2.64 ± 0.69, n = 8, and 2.46 ± 0.63, n = 6, respectively; Fig. 3B). The increase was abolished by 2-MPG (Fig. 3B). 2-MPG had no effect on DCF fluorescence intensity by itself.

Our results agree with those of others (20, 40) who found that preconditioning reduced cell death in a similar cardiomyocyte model. Kochs et al. (19) reported that flumazenil protects against cerebral ischemia-reperfusion damage. Flumazenil and ischemic preconditioning produced a similar degree of cardioprotection in our ischemia-reperfusion model of cardiomyocytes.
FLUMAZENIL PRECONDITIONS CARDIOMYOCYTES

Subsequently, we observed that preconditioning with 10 min of simulated ischemia resulted in a DCFH-oxidation (ROS) of 246% above the baseline. Others (9, 10) have also reported increased ROS generation with hypoxic preconditioning in a similar myocyte model. ROS may be important in mediating flumazenil-induced preconditioning. Interestingly, 10 min of flumazenil administration followed by 10 min of a drug-free period resulted in a marked ROS production. More importantly, 10 min of ischemia (preconditioning) and 10 min of flumazenil (10 μM) produced the same amount and pattern of ROS signals. The effect on cell death and ROS signals with flumazenil was abolished by 2-MPG, which indicates that ROS signals are important in triggering the protection of flumazenil. Vanden Hoek and co-workers (40) showed that ROS signals are crucial components of the intracellular signaling pathway by which hypoxic preconditioning occurs. The recent results of Yao et al. (43) with acetylcholine in cardiomyocytes suggest that ROS signals are crucial in mediating protection against ischemia-reperfusion injury (43). Biological oxidants may regulate intracellular signal transduction (39).

The sources and intracellular regulation of ROS signals by flumazenil are unknown. Mitochondria appear to be the source of ROS signals with hypoxia- and acetylcholine-induced preconditioning (9, 43). Duranteau et al. (9) and Vanden Hoek et al. (40) demonstrated that an ROS burst during hypoxic preconditioning came from mitochondria. We found that acetylcholine mimics ischemic preconditioning via an increase in mitochondrial ROS production (43). Mitochondrial K\textsubscript{ATP} activation contributes to acetylcholine-induced ROS generation (43). It has previously been shown that an acetylcholine-induced increase in ROS signals was abolished by the selective mitochondrial K\textsubscript{ATP} channel blocker 5-HD (14, 25–30). Benzodiazepine receptors, although not established in chick cardiac myocytes, have been found in mitochondria (1, 31, 34). Inhibition of these receptors affects various ion channels (18), has antistress activity (32, 33), and regulates neurosteroidogenesis (6, 34). Although further experiments are needed to identify the sources and mechanism of ROS generation by flumazenil, the results from the present investigation and those of others with acetylcholine and preconditioning indicate that an intracellular ROS signal is an important pathway by which preconditioning, acetylcholine, and flumazenil protect against ischemia-reperfusion injury.

Downstream signal transduction of intracellular ROS signals includes activation of mitochondrial K\textsubscript{ATP} channels (22, 28, 43). In the current experiments, we observed that the selective mitochondrial K\textsubscript{ATP} channel blocker 5-HD completely blocked the cell death reduction produced by flumazenil. Our previous results and those of others (8, 16, 20, 22, 23, 43) have shown that mitochondrial K\textsubscript{ATP} channel opening is crucial in mediating the protection of preconditioning, acetylcholine, and adenosine in vivo and in vitro. K\textsubscript{ATP} channels are also important in the trigger phase of ischemic preconditioning (10, 29). However, we found that flumazenil-produced ROS were not altered by 5-HD, a specific K\textsubscript{ATP} channel blocker. Taken together, mitochondrial K\textsubscript{ATP} channel activation seems to be only involved in the downstream signal transduction of ROS when mediating the cardioprotection of flumazenil.

The current dose of 5-HD was higher than that used by other studies (20), although a similar cardiomyocyte preparation was used. This is likely because a greater extent of K\textsubscript{ATP} channel activation is produced by flumazenil than by adenosine, opioids, and other stimulants. Therefore, a higher dose of 5-HD is required in the present study. It could also be due to the different ages of chick embryos that were harvested to prepare cardiomyocytes. We used 10-day-old embryos, whereas others used 14-day-old embryos. Finally, we used 10 min of ischemia to elicit preconditioning, whereas 5 min of ischemia was used by others. These variations in experimental protocols may also contribute, at least partially, to the high dose of 5-HD used in this study.

In conclusion, this study demonstrates that flumazenil mimics ischemic preconditioning to reduce cell death. The cardioprotection of flumazenil is mediated through increased intracellular ROS signals with subsequent activation of mitochondrial K\textsubscript{ATP} channels.

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