Signal transduction of flumazenil-induced preconditioning in myocytes

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Yao, Zhenhai, Bradley C. McPherson, Huiping Liu, Zuohui Shao, Changqing Li, Yimin Qin, Terry L. Vanden Hoek, Lance B. Becker, and Paul T. Schumacker. Signal transduction of flumazenil-induced preconditioning in myocytes. Am J Physiol Heart Circ Physiol 280: H1249–H1255, 2001.—The objective of this study was to examine the role of oxygen radicals, protein kinase C (PKC), and ATP-sensitive K+ (KATP) channels in mediating flumazenil-produced preconditioning. Chick cardiomyocyte death was quantified using propidium iodide, and oxygen radical generation was assessed using 2’,7’-dichlorofluorescin oxidation. Preconditioning was initiated with 10 min of ischemia followed by 10 min of reoxygenation. Alternatively, flumazenil was infused for 10 min and removed 10 min before ischemia. Flumazenil (10 μM) and preconditioning increased oxygen radicals [1,693 ± 101 (n = 3) and 1,567 ± 98 (n = 3), respectively, vs. 345 ± 53 (n = 3) in control] and reduced cell death similarly [22 ± 3% (n = 5) and 18 ± 2% (n = 6), respectively, vs. controls 49 ± 5% (n = 8)]. Protection and increased oxygen radicals by flumazenil were abolished by pretreatment with the antioxidant thiol reductant 2-mercapropionyl glycine (2-MPG) as an antioxidant be-

ous cardioprotection yet identified (7). It would be beneficial if this powerful myocardial protection could be mimicked pharmacologically without the deleterious effects of preconditioning ischemia. Several compounds, including adenosine, ACh, and nitric oxide donors, have been shown to mimic the protective effects of preconditioning (7, 33). Reactive oxygen species (ROS), protein kinase C (PKC), and mitochondrial ATP-sensitive K+ (KATP) channels are major intracellular messengers of ischemic preconditioning (3, 14, 29). However, the signaling pathway of this endogenous cardioprotection is not fully understood.

Flumazenil is a benzodiazepine receptor antagonist that is used clinically to reverse apnea and loss of consciousness associated with oversedation (5, 18). It protects against cerebral ischemia (1, 17). Recent results from our laboratory and others suggest that ROS and KATP channels are potential cellular targets involved when flumazenil produces ischemic preconditioning in cardiomyocytes (36). We wanted to further explore the mechanism of flumazenil-induced preconditioning, specifically the role of ROS, PKC, and KATP channels, in intracellular signal transduction. For this purpose, we administered the thiol reductant 2-mercapropionyl glycine (2-MPG) as an antioxidant before and during the flumazenil infusion to attenuate ROS signals. In addition, we administered two PKC inhibitors and a KATP channel antagonist after the flumazenil infusion to test the hypothesis that ROS activate PKC and the KATP channel.

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 later modified by Vanden Hoek et al. (31). Briefly, hearts were harvested and placed in Hanks’ balanced salt solution lacking magnesium and calcium (Life Technologies, Grand Island, NY). Ventricles were minced, and myocytes were dissociated by four to six rounds of trypsin degradation (0.025%; Life Technologies) at 37°C with gentle agitation. Then isolated cells were transferred to

mitochondria; ischemia; potassium channels; GABA receptors; signal transduction

BRIEF PERIODS OF ISCHEMIA and reperfusion make the myocardium resistant to a subsequent prolonged ischemic insult (22). Known as ischemic preconditioning, this phenomenon provides the most powerful endoge-
Table 1. Composition of balanced salt solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Normal</th>
<th>Simulated Ischemia</th>
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<tbody>
<tr>
<td>NaCl, mM</td>
<td>117.0</td>
<td>113.65</td>
</tr>
<tr>
<td>KCl, mM</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>NaHCO₃, mM</td>
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<td>21.35</td>
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<tr>
<td>MgSO₄ (anhydrous), mM</td>
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<td>0.76</td>
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<tr>
<td>NaH₂PO₄·H₂O, mM</td>
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<td>1.00</td>
</tr>
<tr>
<td>CaCl₂, mM</td>
<td>1.21</td>
<td>1.21</td>
</tr>
<tr>
<td>KCl, N</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.60</td>
<td>5.60</td>
</tr>
<tr>
<td>2-Deoxy-D-glucose, mM</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Osmolarity</td>
<td>291</td>
<td>313</td>
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a solution with trypsin inhibitor for 8 min, filtered through a 100-μm mesh filter, centrifuged for 5 min at 1,200 rpm at 4°C, and finally resuspended in a medium described previously (4, 8, 31). Resuspended cells were placed in a petri dish in a humidified incubator (5% CO₂-95% air at 37°C) for 45 min to promote early adherence of fibroblasts. Nonadherent cells were counted with a hemocytometer, and viability was measured using trypan blue (0.4%). Approximately 1 × 10⁶ cells in 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 a Kynar film (McMaster-Carr, Elmhurst, IL) placed between the coverslip and the metal hypoxic chamber to minimize O₂ exchange between the chamber wall and the perfusate and then mounted on a temperature-controlled platform (37°C) on an inverted microscope. A water-jacketed glass equilibration column mounted above the microscope stage was used to equilibrate the perfusate to known P₀₂. The standard perfusion media (Table 1) were equilibrated for 1 h before the experiment by bubbling with 21% O₂-5% CO₂-74% N₂. A simulated ischemia solution (Table 1) composed of balanced salt solution (BSS) containing no glucose with 2-deoxyglucose (20 mM) added to inhibit glycolysis was bubbled with 20% CO₂-80% N₂ for 1 h before the experiments. The pH of the perfusion solution was routinely verified (pH 7.4 for normoxic BSS and pH 6.8 for simulated ischemic BSS). Stainless steel or low-O₂-solubility polymer tubing connected the equilibration column to the flow-through chamber to minimize ambient O₂ transfer into the perfusate. In previous studies, the low Po₂ in the chamber was confirmed under conditions identical to those of experiments using an optical phosphorescence quenching method (21, 24, 32) (Oxyspot, Medical Systems, Greenvale, NY).

Cell viability. An inverted microscope equipped for epifluorescent illumination included a xenon light source (75 W), a 12-bit cooled charge coupled device camera (Princeton Instruments), a shutter and filter wheel (Sutter), and appropriate excitation and emission filter cubes. Fluorescent cell images were obtained using a ×10 objective lens (Nikon 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, on loss of membrane integrity (29, 31). 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 cell membrane integrity of all cells, allowing PI to enter. Percent 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 probe 2',7'-dichlorofluorescin (DCFH) (DCFH-DA) was continuously present in the perfusate at 5 μM. Within the cell, esterases cleave the acetate groups on DCFH-DA, thus trapping the reduced probe (DCFH) intracellularly (24). ROS generation in the cells leads to the oxidation of DCFH, yielding the fluorescent product DCF (24). The probe DCFH in cardiomyocytes is readily oxidized by H₂O₂ or hydroxyl radical but is relatively insensitive to superoxide (O₂⁻) (8, 29, 31). Fluorescence was measured using an excitation wavelength of 480 nm, dichroic 505-nm-long pass, and emitter band pass of 535 nm (Chroma Technology) with neutral density filters to attenuate the excitation light intensity. Fluorescence intensity was assessed using the probe 2',7'-dichlorofluorescin (DCFH).

Fig. 1. Schematic diagram of the experimental protocol used to determine whether flumazenil (10 μM) mimics ischemic preconditioning (Pre) and whether reactive oxygen species (ROS), protein kinase C (PKC), and the mitochondrial ATP-sensitive K⁺ (K⁺ATP) channel are involved. 2-Mercaptopropionyl glycine (2-MPG) is an antioxidant, Go-6976 is a specific PKC inhibitor, 5-hydroxydecanoate (5-HD) is a selective mitochondrial K⁺ATP channel antagonist, and phorbol 12-myristate 13-acetate (PMA) is a PKC activator. Cell death, expressed as a percentage of propidium iodide (PI) uptake, was monitored throughout the experiment. Flu, flumazenil; Che, chelerythrine.

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<tbody>
<tr>
<td>Go-6976</td>
<td>FLU + Go-6976</td>
<td>Che</td>
<td>FLU + Che</td>
<td>PMA</td>
</tr>
<tr>
<td>5-HD</td>
<td>FLU + 5-HD</td>
<td>PMA</td>
<td>PMA</td>
<td>PMA</td>
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**Baseline** | **Ischemia** | **Reperfusion**

[Diagram showing FLU or PRE]
controls (n = 5) compared using a two-factor ANOVA with repeated mea-

between groups for cell death and ROS production were

298 ± 6 arbitrary units (AU), respectively, vs. 345 ± 6 arbitrary units (AU) in controls (n = 3 in each). Flumazenil generated the same amount of ROS as did preconditioning. Treatment with 2-MPG (800 μM) alone had no marked effects on ROS signals but abolished the increase in ROS signals by flumazenil. B: flumazenil-produced ROS were abolished by ebselen, a glutathione peroxidase that converts H2O2 to H2O. The precursor of H2O2 is superoxide (O2−). Superoxide dis-
mutase (SOD) is an enzyme in cytosol that catalyzes conversion of O2− to H2O2. Interestingly, diethyldithiocarbamic acid (DDC), a cy-

tosol Cu,Zn-SOD inhibitor, attenuated ROS production of flumazenil. Furthermore, myxothiazol, a mitochondria electron transport inhibitor, did not affect ROS generation by flumazenil. These data indicate that ROS produced by flumazenil are mainly H2O2 radicals that originate in cytosol and that cytosol SOD and glutathione peroxidase have important roles in their generation and degrad-

addition, DCFH is more readily oxidized by H2O2 than by O2−. Furthermore, myxothiazol (0.5 μM), a mito-

chondria electron transport inhibitor, did not affect ROS generation by flumazenil. These data indicate that ROS produced by flumazenil are mainly H2O2 radicals that originate in cytosol and that cytosol SOD and glutathione peroxidase have important roles in their generation and degrad-

Hflumazenil mimics ischemic preconditioning via ROS. In the control series, 60 min of ischemia and 3 h of reperfusion resulted in cell death of 49 ± 5% (n = 8).
Preconditioning with 10 min of ischemia and 10 min of reoxygenation before ischemic insult markedly reduced cell death (18 ± 2%, n = 6). Infusion of flumazenil (10 μM) for 10 min followed by 10 min of a drug-free period reduced cell death to the same extent (22 ± 3%, n = 5) as did preconditioning. The preconditioning effect of flumazenil was abolished by the antioxidant 2-MPG administered during the equilibrium before ischemic insult (52 ± 10%, n = 6; Fig. 3).

2-MPG reduces cell death when administered during ischemia-reperfusion. The antioxidant 2-MPG reduced cell death significantly when administered during ischemia-reperfusion [25 ± 6% (n = 5) vs. 52 ± 10% (n = 3); Fig. 4]. These results further confirm that 2-MPG functions as a free radical scavenger in our model.

ROS signals stimulate PKC. When administered during ischemia and reperfusion (after flumazenil infusion), specific PKC inhibition with Go-6976 (0.1 μM) or chelerythrine (2 μM) virtually abolished the protection of flumazenil [43 ± 7% (n = 6) and 54 ± 7% (n = 4); Fig. 5]. PKC activation with 0.2 μM PMA mimicked

![Graph](http://ajpheart.physiology.org/)

**Fig. 3.** Effects of different protocols on cell death expressed as a percentage of PI uptake. Preconditioning and flumazenil (10 μM) significantly reduced cell death compared with nonpreconditioned control cells. Treatment with the antioxidant 2-MPG (800 μM) alone had no effect on cell death but abolished the protective effects of flumazenil (2-MPG + Flu). *P < 0.05 vs. controls.

![Graph](http://ajpheart.physiology.org/)

**Fig. 4.** Cell death, expressed as a percentage of PI uptake, was assessed at the end of 1 h of ischemia and 3 h of reperfusion. The antioxidant 2-MPG (800 μM) reduced cell death when administered during ischemia-reperfusion (IR). *P < 0.05 vs. controls.

![Graph](http://ajpheart.physiology.org/)

**Fig. 5.** Effects of different protocols on cell death expressed as a percentage of PI uptake. Flumazenil significantly reduced cell death compared with nonpreconditioned control cells. Treatment with the PKC inhibitor Go-6976 (0.1 μM; A) or chelerythrine (2 μM; B) alone had no effect on cell death but abolished the protective effect of flumazenil. *P < 0.05 vs. controls.
the protective effect (17 ± 4%, n = 6; Fig. 6). In addition, PKC inhibitors (Go-6976 and chelerythrine), when given before ischemia, had no effects on flumazenil-generated ROS.

**PKC activation opens the mitochondrial K<sub>ATP</sub> channel.** The selective mitochondrial K<sub>ATP</sub> channel antagonist 5-HD, which was given during the ischemia-and-reperfusion period, had no effect on cell death by itself (36), but in the present study, it abolished the protective effect of flumazenil and PMA [46 ± 5% (n = 8) and 43 ± 7% (n = 6), respectively; Fig. 6].

**DISCUSSION**

We made several novel observations in our in vitro cardiomyocyte model of simulated ischemia-reperfusion. First, a transient increase in ROS generation after 10 min of flumazenil infusion correlates with preconditioning effects that reduce cardiocyte death during ischemia and reperfusion. Second, the ROS signals with flumazenil appear to activate PKC, in that PKC inhibition abolished protection by flumazenil. Third, PKC activation opens mitochondrial K<sub>ATP</sub> channels, as indicated by the ability of 5-HD to abolish protection. Our results suggest that an increase in ROS signals leads to the opening of mitochondrial K<sub>ATP</sub> channels via PKC activation. This intracellular signaling is one important pathway by which flumazenil mimics ischemic preconditioning.

Initial data demonstrated that a single 10-min ischemic period before a more sustained ischemia markedly reduced cardiocyte death compared with a single 60-min period of ischemic insult (18 ± 2 vs. 49 ± 5%). Subsequently, an infusion of flumazenil for 10 min, instead of 10 min of ischemia (preconditioning), reduced cardiocyte death to an extent similar to that resulting from preconditioning (22 ± 3 and 18 ± 2%, respectively). In addition to the beneficial effects of ischemic preconditioning or flumazenil in reducing cell death, return of spontaneous contraction was markedly higher in preconditioned and flumazenil-treated cells. These results agree with our previous findings (35, 36) and those of others (29). Flumazenil mimics preconditioning to attenuate ischemia-reperfusion injury.

ROS are key intracellular messengers (27) but can potentially contribute to cell death when generated at high levels. The protection of flumazenil was abolished by pretreatment with 2-MPG, a thio reductant with antioxidant properties. This effect suggests that ROS signals are important second messengers in mediating cardioprotection. However, when 2-MPG was administered during prolonged ischemia-reperfusion, it markedly reduced cell death, likely via degrading free radicals generated during ischemia-reperfusion (15). Interestingly, we also found that flumazenil, similar to ischemic preconditioning, resulted in a marked increase in DCFH oxidation, an index of ROS production. Others have reported that ROS are involved in triggering cardioprotection of ischemic and hypoxic preconditioning (8, 29), ACh (35), and opioids (34). More importantly, we previously demonstrated that 10 min of flumazenil infusion resulted in the same pattern and magnitude of ROS signals observed during ischemic preconditioning (36). Thus these findings suggest that ROS signals are crucial in producing the protection of flumazenil.

Flumazenil-generated ROS are mainly H<sub>2</sub>O<sub>2</sub>, because DCFH is more readily oxidized by H<sub>2</sub>O<sub>2</sub> than by O<sub>2</sub>•. In addition, ROS were abolished by ebselen, a
glutathione peroxidase that converts $H_2O_2$ to $H_2O$. The precursor of $H_2O_2$ is $O_2$. SOD is an enzyme in cytosol that catalyzes this reaction. Interestingly, DDC, a cytosol Cu,Zn-SOD inhibitor, attenuated ROS production. Thus flumazenil-generated ROS are $H_2O_2$ present in cytosol. However, we cannot rule out the possibility that $H_2O_2$ were generated in mitochondria or other subcellular sites and then transported to cytosol. ROS generated by ischemic and hypoxic preconditioning originate from mitochondria (29). It is reasonable to suspect that mitochondria are the source of flumazenil-induced ROS. However, we found that myxothiazol, a mitochondria electron transport inhibitor, did not affect ROS generation by flumazenil. Taken together, our data indicate that ROS produced by flumazenil are mainly $H_2O_2$ radicals that originate in cytosol (Fig. 7). Obviously, the exact mechanism by which flumazenil generates ROS is still not clear and needs further study.

Subsequently, we noticed that the cardioprotection of flumazenil was abolished when Go-6976 or chelerythrine, specific PKC inhibitors, was given after flumazenil infusion. This suggests that ROS with flumazenil activate PKC. ROS signals activate PKC (10, 11), which may mediate cardioprotection of preconditioning (13, 19, 26). Numerous studies (3, 11, 23) have suggested that PKC is a central mediator of preconditioning. In intact rats, inhibition of PKC partially or completely abolished the effects of preconditioning, depending on the strength of the preconditioning stimulus (13). In a similar cardiomyocyte model of simulated ischemia and reperfusion, Liang (19) demonstrated that PKC activation was an important component of signal transduction of ischemic preconditioning and adenosine. Specific isoforms of PKC are more important in producing cardioprotection, and translocation of PKC ($\delta$ and $\epsilon$) may mediate the cardioprotective effect of preconditioning in rats and rabbits (16, 23). In addition, we noticed that PKC inhibition did not affect flumazenil-generated ROS. This observation is consistent with recent results of Vanden Hoek et al. (30), who showed that PKC inhibition with Go-6976 did not affect ROS produced by preconditioning. Therefore, our results and those of others strongly suggest that PKC is an important downstream signal of flumazenil-produced ROS.

ROS signals also lead to activation of mitochondrial $K_{ATP}$ channels (8, 29), which are important in triggering and mediating the protection of preconditioning (14, 20). We found that blockade of mitochondrial $K_{ATP}$ channels with 5-HD after flumazenil infusion abolished its protection, but our previous data showed that when 5-HD was given before and during the flumazenil infusion, free radical signals were not affected (36). Therefore, $K_{ATP}$ channel activation is a downstream signal of flumazenil-induced ROS.

Whether PKC activation opens the $K_{ATP}$ channel or vice versa is unclear (9, 26, 28). We noticed that the specific PKC activator PMA, administered during ischemia and reperfusion, reduced cell death to the same extent as did flumazenil. The protective effects of flumazenil and PMA were blocked by 5-HD given during ischemia and reperfusion, which suggests that flumazenil activates the mitochondrial $K_{ATP}$ channel via PKC. PKC enhances the ability of diazoxide to open the mitochondrial $K_{ATP}$ channel in rabbit cardiomyocytes (25). Our results therefore suggest that PKC activates the mitochondrial $K_{ATP}$ channel in the protection of flumazenil. The mechanism by which mitochondrial $K_{ATP}$ channel activation produces cardioprotection has not been established (3). In a recent review article, Gross and Fryer (14) suggested that activation of the mitochondrial $K_{ATP}$ channel leads to entry of potassium ion into the mitochondria and intramitochondrial depolarization. This action could decrease mitochondrial calcium overload and cause matrix swelling, which enhances mitochondrial energy synthesis and respiration (3).

The mechanism of preconditioning is complicated and not fully established. Multiple signaling pathways, including adenosine, ACh, $K_{ATP}$ channels, ROS, PKC, and other kinases, are involved in the cardioprotection of preconditioning (7, 28). Results of this study addressed only the importance of ROS-PKC-mitochondrial $K_{ATP}$ channel signaling pathway in flumazenil-induced preconditioning protection. The recent study by Vanden Hoek et al. (30) showed that adenosine had no effects on ROS production before ischemia, which further suggests that, in addition to ROS-PKC-$K_{ATP}$ channel signal transduction, other parallel signaling pathways exist by which preconditioning and flumazenil protect cardiomyocytes in ischemia-reperfusion.

In conclusion, we showed that flumazenil mimics ischemic preconditioning to reduce cell death in cultured cardiomyocytes. Transiently increased ROS signals (likely $H_2O_2$) with flumazenil that activate mitochondrial $K_{ATP}$ channels via PKC appear to explain how flumazenil mimics ischemic preconditioning. Our data also suggest that clinical use of flumazenil could be beneficial for patients with ischemic heart disease.

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REFERENCES


