Role of reactive oxygen species in acetylcholine-induced preconditioning in cardiomyocytes

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Yao, Zhenhai, J iankun Tong, Xiaohui Tan, Changqing Li, Zuohui Shao, W oo Ch an Kim, Terry L. Vanden Hoek, Lance B. Becker, C. Alvin Head, and Paul T. Schumacker. Role of reactive oxygen species in acetylcholine-induced preconditioning in cardiomyocytes. Am. J. Physiol. Heart Circ. Physiol. 277 (Heart Circ. Physiol. 46): H2504–H2509, 1999.—We examined the ability of ACh to mimic ischemic preconditioning in cardiomyocytes and the role of ATP-sensitive potassium (KATP) channels and mitochondrial reactive oxygen species (ROS) in mediating this effect. Chick embryonic ventricular myocytes were studied in a flow-through chamber while flow rate, pH, PO2, and Pco2 were controlled. Cell viability was quantified with propidium iodide (5 µM), and production of ROS was measured using 2,7′-dichlorofluorescin diacetate. Data were expressed as means ± SE. Preconditioning with 10 min of ischemia followed by 10 min of reoxygenation or 10 min of ACh (1 mM) followed by a drug-free period before 1 h of ischemia and 3 h of reoxygenation reduced cell death to the same extent (preconditioning 19 ± 2% [n = 6, P < 0.05] vs. controls 42 ± 5% [n = 9]). Like preconditioning, ACh increased ROS production threefold before ischemia (0.60 ± 0.16 [n = 7, P < 0.05] vs. controls, 0.16 ± 0.03 [n = 6]; arbitrary units). Protection and increased ROS production during ACh preconditioning were abolished with 5-hydroxydecanoate (5-HD, 100 µM), a selective mitochondrial KATP channel antagonist, and the thiol reductant 2-mercaptoethanol (2-MPG, 1 mM), an antioxidant. These results demonstrate that activation of mitochondrial KATP channels and increased ROS production from mitochondria are important intracellular signals that participate in ACh-induced preconditioning in cardiomyocytes.

ISCHEMIC PRECONDITIONING is an endogenous protective mechanism in which brief periods of myocardial ischemia and reperfusion render the myocardium resistant to a subsequent, more sustained ischemic insult (24). Preconditioning has been shown to occur in a variety of animal species as well as in humans (5, 6). Since the first description of this phenomenon by Murry et al. (24) in 1986, numerous studies have been performed to elucidate the mechanism (6). However, its cellular basis is still not fully understood.

The neurotransmitter ACh antagonizes the cardiac-stimulatory effects of the sympathetic nervous system (17). We previously demonstrated (35, 36) that ACh mimicked ischemic preconditioning in anesthetized dogs. Liu and Downey (19) showed that ACh mimics preconditioning in isolated rabbit hearts. However, the cellular targets of ACh responsible for the initiation of preconditioning in the intact heart are not clear. Therefore, the present study was designed to determine whether ACh mimics ischemic preconditioning in cultured cardiomyocytes.

It has been suggested that mitochondrial reactive oxygen species (ROS) participate in the initiation of hypoxic preconditioning in cardiomyocytes (8, 29, 32). Thus our second aim was to determine whether ROS signals are important components of the coupling mechanism by which ACh mimics preconditioning.

Finally, a large body of evidence reveals that opening of ATP-dependent potassium (KATP) channels is a central mechanism involved in preconditioning initiated by ischemia and ACh (8, 10, 13). Cardiac KATP channel activation was also important in ACh-induced preconditioning in anesthetized dogs (35, 36). Activation of the KATP channel of mitochondria appears to be more important than that of sarcolemma in mediating preconditioning (11, 12, 17, 20, 37). We wanted to determine the role of mitochondrial KATP channel activation in intracellular ROS production and its relation to ACh-induced cardioprotection. For this purpose, we administered the specific mitochondrial KATP-Channel antagonist 5-hydroxydecanoate (5-HD) with ACh in our cardiocyte model of ischemic reperfusion (14). 5-HD was chosen because this compound is thought to selectively compete with ATP at its binding site within the KATP channel and to have a higher affinity for the channel in mitochondria than does glibenclamide (14).

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. (33). Briefly, hearts were harvested and placed in Hanks’ balanced salt solution without magnesium and calcium (Life Technologies, Grand Island, NY). Ventricles were minced, and myocytes were dissociated four to six times with trypsin and EDTA. The myocytes were centrifuged at 300 g for 5 min, and the supernatant was aspirated. The myocytes were then resuspended in modified Hanks solution, and 5-mL aliquots were placed in 60-mm glass dishes. After 30 min, the cells were washed with serum-free medium. The cells were then incubated in serum-free medium for 1–2 h before addition of the drug.

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were placed in a petri dish in a humidified incubator (5% CO2, with trypsin inhibitor for 8 min, filtered through a 100-µm (0.025%, Life Technologies) degradation 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 x 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 day 3 or 4 after isolation.

Perfusion system. Glass coverslips containing spontaneously beating chick myocytes were placed in a stainless steel flow-through chamber (1-ml volume, Penn Century, Philadelphia, PA). The chamber was sealed with thin water gaskets 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 perfusion column mounted above the microscope stage was used to equilibrate the perfusate to known PO2. The standard perfusion medium consisted of a buffered saline solution (BSS; in mM: 117 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 was composed of BSS containing no glucose, with 2-deoxyglucose (20 mM) added to inhibit glycolysis, which was bubbled with a gas mixture of 20% CO2-80% N2 for 1 h before the experiments. 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 (Oxyspot, Medical Systems, Greenvale, NY; Refs. 21, 34).

Cell viability determination. An inverted microscope, equipped for epifluorescent illumination, included a xenon light source (75 W), a 12-bit digital cooled charge-coupled device camera (Princeton Instruments), a shutter and filter wheel (Sutter), and appropriate excitation and emission filter cubes. The microscope also was equipped with Hoffman-modified phase illumination to accentuate surface topology, facilitating the measurement of contractile motion (see Contractile function). Fluorescent cell images were obtained using a x10 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 on loss of membrane integrity (3). 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 cells, allowing PI to enter cells so that the maximum PI value was obtained. 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). The membrane-permeant diacetate form of DCFH (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 (28). ROS in the cells led to oxidation of DCFH, yielding the fluorescent product 2',7'-dichlorofluorescein (DCF) (27). The probe DCFH in cardiomyocytes is readily oxidized by H2O2 or hydroxyl radical but is relatively insensitive to superoxide (7, 32, 33). Fluorescence was measured using an excitation wavelength of 480 nm, dichroic 505-nm long pass, and 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. Background was identified as an area without cells or with minimal cellular fluorescence. Intensity values are reported as percentage of initial values after background value was subtracted.

Chemicals. ACh, 2-mercaptoethanol (2-MPG), and 5-HD were purchased from Sigma Chemical (St. Louis, MO). ACh, 2-MPG, and 5-HD were dissolved in BSS buffer before administration. Myxothiazol, PI, and DCFH-DA were purchased from Molecular Probes.

Experimental design. Five groups of cardiomyocytes (control, preconditioning, ACh, 2-MPG+ACh, and 5-HD+ACh) were studied to determine whether ACh could mimic ischemic preconditioning and whether mitochondrial KATP channel activation with increased ROS production was involved. Cells in the nonpreconditioned groups were subjected to 60 min of ischemia followed by 3 h of reoxygenation. Preconditioned cells underwent a single 10 min of ischemia followed by 10 min of reoxygenation before being subjected to the ischemia-reperfusion protocol used for the nonpreconditioned controls. In nonpreconditioned cells, saline (control series) or 1 mM ACh (for groups that were ACh-, 2-MPG+ACh-, or 5-HD+ACh treated) was added to the perfusate for 10 min instead of the 10-min ischemic period in the preconditioned cells. In addition, the 5-HD+ACh-treated and 2-MPG+ACh-treated cells received 100 µM of 5-HD and 1 mM of 2-MPG, respectively, in perfusate during the 1-h period of baseline before 60 min of ischemia.

Five additional series of studies (saline, ACh, 2-MPG+ACh, myxothiazol+ACh, and 5-HD+ACh) were also used to examine the role of ROS signals in mediating the effect of ACh, to determine whether mitochondria are the source of ACh-induced ROS production, and to determine the role of mitochondrial KATP channel activation in ROS production.

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

RESULTS

Cell death. Percent cell death (%PI uptake) was monitored continuously throughout each experiment, and data are summarized in Fig. 1. After 3 h of reoxygenation, cell death was 42.3 ± 5.0% (n = 9) in the control series and was markedly reduced by preconditioning [18.9 ± 1.9% (n = 6); P < 0.05] or ACh [21.4 ± 4.5% (n = 6); P < 0.05] (Fig. 1A). In contrast, pretreatment with either 2-MPG (1 mM) or 5-HD (100 µM) totally abolished the protective effects of ACh [47.1 ± 5.9% (n = 6) and 36.9 ± 6.6% (n = 5), respectively; Fig. 1, B and C]. Infusion of either 1 mM of 2-MPG or 100
µM of 5-HD alone had no effect on increasing cell death compared with the controls (data not shown).

Contractile function. Cell contraction was assessed continuously throughout the experiments. Ischemia (60 min) resulted in 100% loss of spontaneous contractile motion. Return of spontaneous contractile motion was assessed after 1 h of reoxygenation in all series.

The return of spontaneous contraction was noted in 3 of 15 experiments in the controls (20%). Return of contractile function was observed in 100% of the preconditioned (6 of 6, P < 0.05) and 77% of the ACh-treated (10 of 13, P < 0.05) groups, significantly higher compared with controls. In contrast, there was no difference observed among series treated with 2-MPG+ACh (0 of 4), 5-HD+ACh (1 of 10), and controls (3 of 15).

Intracellular ROS generation. Figure 2 documents one typical experiment from control and ACh-, 2-MPG+ACh-, myxothiazol+ACh-, and 5-HD+ACh-treated series. In the control cells, intensity of the DCF fluorescence increased slightly over 1 h (Fig. 2A). Infusion of ACh (1 mM) for 10 min followed by 10 min of a drug-free period markedly increased ROS production (Fig. 2A). The increase in ROS production by ACh was abolished by pretreatment with 2-MPG (1 mM; Fig. 2B), myxothiazol (0.2 µM; Fig. 2C), or 5-HD (100 µM; Fig. 2D).

Figure 3 summarizes the results of ROS production at the end of a 10-min drug-free period after ACh. Treatment with ACh increased ROS production 60% above baseline (0.60 ± 0.16, n = 7; P < 0.05; arbitrary units); this increase was only 16% in controls (0.16 ± 0.03, n = 6). More importantly, the ACh-induced ROS burst was completely abolished by pretreatment with 1 mM 2-MPG (0.01 ± 0.04, n = 4), 0.2 µM myxothiazol (0.02 ± 0.07, n = 5), or 100 µM 5-HD (0.09 ± 0.03, n = 5) compared with that in controls (0.16 ± 0.03, n = 6). Finally, treatment with 5-HD (100 µM) alone for 1 h did not affect ROS signals. However, treatment with either 2-MPG (1 mM) or myxothiazol (0.2 µM) resulted in a slightly lower value than that in controls, which may reflect a decrease in baseline production of ROS by mitochondria.

DISCUSSION

The results of the present study clearly demonstrate that 1) 10-min ACh administration reduces cell death as much as 10-min ischemic preconditioning in embryonic chick cardiomyocytes; 2) the cardioprotective effect of ACh correlates with its ability to increase intracellular ROS signals before the start of ischemia; 3) the effect of ACh on cell death reduction and on increase of ROS signals is abolished by either the antioxidant 2-MPG or the specific K<sub>ATP</sub> channel blocker 5-HD, which has a much higher affinity for the K<sub>ATP</sub> channel in mitochondria than in cell membrane (14); and 4) the increased ROS signal with ACh is totally abolished by...
myxothiazol, the mitochondria site III electron transport inhibitor. Our data therefore indicate that ACh mimics ischemic preconditioning via activation of mitochondrial \( K_{ATP} \) channels and release of ROS by mitochondria.

Initially, we observed that 10 min of ischemia preceding 10 min of reoxygenation before 60 min of ischemia and then 3 h of reoxygenation reduced cell death markedly compared with control cells subjected to 60 min of ischemia and 3 h of reoxygenation (18.9 ± 1.9\% vs. 42.3 ± 5.0\%). These findings are in close agreement with those of others (7, 18) who reported reduced cell death in a similar myocyte model. Subsequently, infusion of ACh (1 mM) for 10 min, instead of 10 min of ischemic preconditioning, similarly reduced cell death (21.4 ± 4.5\%). These data agree with our previous in vivo results with ACh and preconditioning (35, 36) and those of others (19, 31) in which ACh reduced myocardial infarct size in anesthetized dogs and isolated rabbit hearts.

Fig. 2. Graphs document 1 representative experiment from all series. In control cells (Cont), intensity of 2',7'-dichlorofluorescin (DCF) fluorescence increased slightly over 1 h (A). Infusion of 1 mM of ACh for 10 min followed by 10 min of a drug-free period increased reactive oxygen species (ROS) generation markedly (A). Increase of ROS signals by ACh was abolished with pretreatment of 1 mM 2-MPG (2-MPG + ACh), 0.2 µM myxothiazol (Myxo + ACh, C), or 100 µM 5-HD (5-HD + ACh, D). A.U., arbitrary units.

Fig. 3. ROS production at the end of a 10-min drug-free period with ACh. ACh markedly increased ROS burst (60% above baseline). In controls, ROS was only 16% higher than the baseline over 1 h. More importantly, ACh-induced ROS burst was completely abolished by pretreatment with 2-MPG (2-MPG + ACh), Myxo (Myxo + ACh), or 5-HD (5-HD + ACh). *P < 0.05 vs. baseline.
al. (32) showed a higher percentage of contractile function recovery with preconditioning in the same myocyte model. These observations reveal that ACh and ischemic preconditioning produce the same extent of cardioprotection in our ischemia-reperfusion model of cardiomyocytes.

Interestingly, we further observed that 10 min of ACh administration before 10 min of a drug-free period, like preconditioning, significantly increased (threefold) ROS production, which correlates with its effects on cell death reduction and contractile function recovery. ACh-produced attenuated cell death and increased ROS signals were totally abolished with the antioxidant 2-MPG. It has been suggested that biological oxidants regulate intracellular signal transduction (29). ROS are important intracellular signaling components in producing hypoxic preconditioning in cardiomyocytes (7). Our results together with those of others (7, 29) indicate that increased ROS signals play an important role in mediating the protective effects of ACh.

Increased ROS production during hypoxic preconditioning appears to originate from the mitochondrial electron transport system (7). We found that the increase in DCFH oxidation with ACh was markedly attenuated by myxothiazol, a mitochondrial site III electron transport inhibitor. ACh-induced increase in ROS signaling was abolished by the selective mitochondrial K\textsubscript{ATP} channel blocker 5-HD (1, 14, 22, 25). Garlid and coworkers (9) showed that 5-HD specifically interacts with the K\textsubscript{ATP} channel of the mitochondria in cardiomyocytes. Although more experiments are needed to confirm the mechanism by which ACh affects intracellular ROS production, the present data, together with the results of others, suggest that mitochondria are the likely source of ACh-produced ROS signals.

The mechanism by which ACh increases ROS production is unknown. ACh activates potassium channels in cardiomyocytes (26). The specific K\textsubscript{ATP} channel blocker glibenclamide antagonized the negative chronotropic and inotropic effects of ACh in isolated dog hearts (23). We previously found that activation of K\textsubscript{ATP} channels plays a crucial role in ACh-produced preconditioning in anesthetized dogs (35, 36), likely via stimulation of mitochondrial K\textsubscript{ATP} channels (37). Recent evidence from other laboratories reveals that activation of mitochondrial K\textsubscript{ATP} channels is responsible for mediating the preconditioning effect (9, 12, 20). Selective activation of mitochondrial K\textsubscript{ATP} channels with diazoxide protected, whereas blockade of the mitochondrial K\textsubscript{ATP} with 5-HD worsened, cardiac ischemia-reperfusion injury (9, 12). Liu et al. (20) provided direct subcellular evidence that mitochondrial K\textsubscript{ATP} opening is cardioprotective. In our experiments, the administration of the selective mitochondrial K\textsubscript{ATP} channel blocker 5-HD (9, 12, 14, 16, 20) totally abolished ACh-induced cardioprotection and ROS production. These data strongly suggest that ACh activates mitochondrial K\textsubscript{ATP} channels, increasing ROS production and thus mimicking preconditioning.

Although the mechanisms by which ACh opens mitochondrial K\textsubscript{ATP} channels are not understood, it has been suggested that M\textsubscript{2} muscarinic-receptor activation increases potassium channel activity (26). Stimulation of muscarinic M\textsubscript{2} receptors increased potassium channel activity via G\textsubscript{i} proteins in guinea pig cardiomyocytes (15). ACh activated K\textsubscript{ATP} channels via stimulation of M\textsubscript{2} receptors to attenuate myocardial ischemia-reperfusion injury in anesthetized dogs (36); however, the specific cells expressing those receptors were not established. If ACh regulates K\textsubscript{ATP} channels through the muscarinic M\textsubscript{2} receptors and the G\textsubscript{i} proteins on these chick ventricular cells, it could conceivably mimic ischemic preconditioning effects. Data obtained by others with ACh and pertussis toxin in isolated, perfused rabbit hearts and anesthetized rats support this hypothesis (19, 30, 31). Other mechanisms by which ACh activates mitochondrial K\textsubscript{ATP} channels and increases its ROS generation may also exist. The dose of ACh used for the present study is higher than that we previously used in anesthetized dogs (35, 36), and it was chosen on the basis of the preliminary results showing that 1 mM was the minimum dose to mimic preconditioning on cell death reduction. It is possible that a subtype of muscarinic receptors exists in chick embryonic cardiomyocytes that is less sensitive to ACh. Alternatively, ACh might activate mitochondrial K\textsubscript{ATP} channels and increase ROS generation via an effect independent of muscarinic receptors. More experiments are needed to examine these possibilities and to determine the detailed intracellular signaling pathway by which ACh activates mitochondrial K\textsubscript{ATP} channels and increases its ROS production, thus mimicking preconditioning.

In conclusion, this study demonstrates that activation of cardiac mitochondrial ATP-dependent potassium channels increases mitochondrial ROS signaling, thereby activating an important intracellular signaling pathway by which ACh and preconditioning protect against ischemia-reperfusion injury.

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