Choline Produces Cytoprotective Effects Against Ischemic Myocardial Injuries: Evidence for the Role of Cardiac M₃ Subtype Muscarinic Acetylcholine Receptors

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Key Words

Ischemia • Myocytes • Apoptosis • Arrhythmias • Muscarinic acetylcholine receptors • M₃-receptor • Signal transduction

Abstract

Background/Aims: Accumulating evidence indicates the presence of functional M₃ subtype of acetylcholine muscarinic receptors (M₃-mAChR), in addition to the well-recognized M₂-mAChR, in the heart of various species including man. However, the pathophysiological role of the cardiac M₃-mAChR remain undefined. This study was designed to explore the possible role of M₃-mAChR in cytoprotection of myocardial infarction and several related signaling pathways as potential mechanisms. **Methods:** Studies were performed in a rat model of myocardial infarction and in isolated myocytes. **Results:** We found that choline relieved myocardial injuries during ischemia or under oxidative stress, which was achieved by correcting hemodynamic impairment, diminishing ventricular arrhythmias and protecting cardiomyocytes from apoptotic death. The beneficial effects of choline were reversed by the M₃-selective antagonists but not by the M₂-selective antagonist. Choline/M₃-mAChR acti-

vated several survival signaling molecules (antiapoptotic proteins Bcl-2 and ERKs), increased endogenous antioxidant reserve (SOD), and reduced apoptotic mediators (proapoptotic proteins Fas and p38 MAPK) and intracellular Ca²⁺ overload. **Conclusion:** Choline improves cardiac function and reduces ischemic myocardial injuries via stimulating the cardiac M₃-mAChRs which in turn result in alterations of multiple signaling pathways leading to cytoprotection. The findings suggest M₃-mAChR as a new target for drug development for improving cardiac function and preventing cardiac injuries during ischemia/reperfusion.

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Introduction

Myocardial ischemia leads to a cascade of metabolic events which are interrelated and are caused by hypoxia, acidosis, oxidative stress, calcium overload, decreases in survival signaling molecules and increases in death signaling mediators, etc. The ischemic injuries are typically (1) contractile dysfunction, (2) electrical disturbance, and (3) cell loss.

The heart is often regarded as an “adrenergic” organ because β -adrenergic agonists are powerful stimulants of cardiac contractility. Muscarinic receptors mediate parasympathetic control of heart function and the primary effects are opposite to β -adrenergic stimulation. Activation of cardiac mAChRs by vagal stimulation decreases cardiac work, which may have a protective effect against ischemic injury [1-2]. It is generally believed that muscarinic regulation of the heart is accomplished merely via modulation of adrenoceptor activity. And probably because of this view, studies on mAChRs in the pathophysiological process of myocardial ischemia/reperfusion have been sparse and the potential role of mAChRs might have thus been underestimated.

In contrast to most peripheral tissues where multiple subtypes of muscarinic acetylcholine receptor (mAChR) coexist, the myocardium has been traditionally considered to possess a single mAChR subtype: the M_2 receptors [1, 3-4]. Yet this “Single- M_2 -Receptor” concept has been challenged and the “Multiple Subtypes” concept is emerging. We first identified the M_3 subtype of mAChRs (M_3 -mAChRs) in mammalian hearts of various species including humans, dogs, and guinea-pigs, with pharmacologic, functional and molecular biology data [5-13]. The presence of functional M_3 -mAChRs in the heart was subsequently supported by several other laboratories [14-23] and the finding was extended to rats [17, 20] and mice [19]. We have also discovered that stimulation of cardiac M_3 receptors by mAChR agonists such as choline, tetramethylammonium and pilocarpine activates a novel K^+ channel coupled to G_q protein (we named this K^+ current I_{KM3} , meaning M_3 receptor-mediated delayed rectifier K^+ current). Activation of I_{KM3} by stimulation of M_3 receptors causes shortening of cardiac action potential duration (APD) and slowing of heart rate [5, 7]. However, these were observed only in atrial cells and tissues. The pathophysiological roles of M_3 -mAChRs in ventricular myocytes and tissues remained unclear. And in none of the previous studies on myocardial ischemia has the potential role of M_3 -mAChRs been investigated.

A number of G protein-coupled receptors have the ability to regulate apoptosis, either initiating a pro- or an anti-apoptotic signal depending on the receptor subtype and cell type in which the receptor is expressed [24-25]. Among those receptors mediating anti-apoptotic signals is the mAChR family. Muscarinic receptors in cerebellar granule neurons have been shown to protect against apoptotic cell death induced by culturing in non-depolarizing conditions [26] and the effect is attributable to the M_3 subtype action because the M_3 -preferring

antagonist 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) completely eradicated the survival-promoting effects of carbachol. A most recent study confirmed the anti-apoptotic ability of the M_3 -mAChRs and found that the poly-basic region within the short C-terminal tail confers the cytoprotection property of the M_3 -mAChRs [27]. We have shown that choline can activate cardiac M_3 -mAChRs [5, 10-12]. Intriguingly, choline regulates apoptotic cell death in several cell types and choline deprivation has been shown to induce apoptosis in postmitotic primary neuronal cultures of fetal rat cortex and hippocampus [28-32]. In light of the ability of choline to activate M_3 -mAChRs, it is tempting to speculate that the cytoprotection conferred by choline might be mediated by the M_3 -mAChRs. We therefore proposed that the cardiac M_3 -mAChRs inhibit apoptosis occurring in ischemia/reperfusion and contribute to the beneficial effects of mAChRs. To test this hypothesis, we explored the possible role of M_3 -mAChR in cytoprotection against ischemic myocardial injuries and several related signaling pathways (MAP kinases, oxidative stress and $[Ca^{2+}]_i$) as potential mechanisms underlying the function of M_3 -mAChR, using choline as a preferential cardiac M_3 -mAChR agonist and 4-DAMP as a preferential M_3 -antagonist.

Materials and Methods

Rat Model of Myocardial Infarction

Male Wistar rats of 230-270 g were randomly divided into four groups: control, ischemia (MI), ischemia-choline (MI-C), ischemia-choline/4-DAMP groups, respectively. The animals were heparinized (300 U) and anesthetized with sodium pentobarbital (40 mg/kg) via i.p. and were ventilated using a small animal ventilator at a frequency of 70/min and tidal volume of 3 ml. The chest was surgically opened and body temperature maintained at 37°C by placing the animal on a heating pad. The standard limb lead ECG was continuously recorded, together with arterial pressure, on a recorder (Nihon Kohden RM 6200; Tokyo). A left thoracotomy was performed at the 4th rib and a segment of saline-soaked 5-0 sutures was looped around the left anterior descending (LAD) coronary artery, near its origin from the left coronary artery. The ends of the suture were passed through a one-inch segment of double-barreled polyethylene tubing. The ends of the tubing were rounded so that a smooth surface abuts the coronary artery. The tubing was gently placed next to the heart and secured by three knots tied on the suture at the external end of the tubing length, with the last knot up against the end of the tubing. The tubing and suture assembly were then externalized and the thorax closed. Occlusion was accomplished by withdrawing the distal knot 2 mm away from the tubing end. This permits reproducible occlusion of the LAD

without tearing the artery. The occlusion was applied for 6 hrs. Successful occlusion was confirmed by the increase of the amplitude of the R wave of lead I during the first few seconds of each occlusion and elevation of S-T segment of lead II, and a 20-30% decline in the arterial blood pressure compared to the pre-ischemic values [33-34]. Choline Cl (5 mg/kg, i.v.) was administered 15 min before occlusion. For choline/4-DAMP group, 4-DAMP (0.5 µg/kg), or other agents as to be specified in the later sections, was administered 15 min before choline. All the measurements were made after 60 min infarction. Use of animals was in accordance with the regulations of the ethic committees of Harbin Medical University and Montreal Heart Institute.

Measurements of Infarct Size

Having been removed from the animals after 60 min infarction, ventricular tissues were dissected and kept overnight at -4°. Frozen ventricles were sliced into 2 mm thick sections, and then incubated in 1% triphenyltetrazolium chloride at 37° in 0.2 M Tris buffer (pH 7.4) for 30 min. While the normal myocardium was stained brick red, the infarcted areas remained unstained. Size of the infarcted area was estimated by the volume and weight as a percentage of the left ventricle [35].

Measurements of Hemodynamic Function

Hemodynamic function was assessed before and 60 min after infarction by determining left ventricular systolic pressure (LVSP) and end diastolic pressure (LVEDP), and time derivatives of pressure was measured during contraction (+dP/dt) and relaxation (-dP/dt) recorded on a polygraph. Left ventricular developed pressure (LVDP) was calculated from the difference between the systolic and diastolic pressures. Hemodynamic parameters were monitored throughout the experimental period.

Scoring of Arrhythmia

The Curtis and Walker [36] arrhythmia scoring method was employed. In brief, 0=no arrhythmia; 1=ventricular premature beats (VES) and/or ventricular tachycardia (VT) of <10-s duration; 2=VES and/or VT of 11-30 s; 3=VES and/or VT of 31-90 s; 4=VES and/or VT of 91-180 s, or reversible ventricular fibrillation (VF) of <10 s; 5=VES and/or VT of >180 s, or reversible VF of >10 s; 6=irreversible VF.

Measurements of Myocardial SOD and MDA

Plasma Cu-Zn SOD activity was measured by the nitroblue tetrazolium (NBT) reduction by $O_2^{\cdot -}$ generated by the xanthine/xanthine oxidase system. The activity was calculated by the difference between the absorbance of sample and blank (all reagents are present except the supernatant sample) at 560 nm. One Cu-Zn SOD unit was defined as the enzyme amount causing 50% inhibition in the NBTH₂ reduction rate. SOD activity is expressed as unit/mg protein.

Plasma malondialdehyde (MDA), a presumptive marker of oxidant-mediated lipid peroxidation, was quantified to estimate the extent of overall lipid peroxidation. The activity of MDA was measured using a commercial kit (Lipid Peroxidation Assay Kit, Calbiochem) and is expressed as µM/ml plasma.

Isolation and Culture of Ventricular Myocytes

Neonatal rats were sacrificed in cool 75% ethanol and the hearts were removed. Single ventricular myocytes were isolated by enzyme digestion (0.1% trypsin and 0.1% collagenase). The myocytes were plated onto 96-well plates or 10 cm Petri-dishes (for Western blot) and grew at 37°C in DMEM/F12 medium containing 10% FBS. Cells were cultured for 2 days before experiments. H_2O_2 was added to the culture medium at a final concentration of 200 µM to induce apoptosis. In experiments involving drug treatment, cells were pre-incubated for 30 min with choline or other drugs in the culture medium, followed by 8-hrs incubation with H_2O_2 in the presence of the drugs. The cells were then harvested for further studies.

Detection and Quantification of Apoptosis

Morphological changes of cells were examined under a light microscope: apoptotic cells display significant cell shrinkage, membrane blebbing and nuclear condensation [37-41].

ELISA The Cell Death Detection ELISA kit (Roche Molecular Biochemicals) was employed to quantify DNA fragmentation on the basis of antibody detection of free histone and fragmented DNA, as detailed elsewhere [37-41]. The intensity of absorbance at 405 nm was measured, relative to that of substrate solution (blank), with a microplate reader (Rainbow, Tecan).

TUNEL DNA fragmentation of individual cells was detected in situ by TUNEL (terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling) with the In Situ Cell Death Detection kit, Fluorescein (Roche Molecular Biochemicals) [37-41]. TUNEL staining was analyzed with a confocal microscope (Zeiss).

Immunohistochemistry Analysis

Left ventricles were frozen in isopentane (pre-cooled in liquid nitrogen), embedded in Cryomatrix (Shandon), and cut with a cryostat into 8-15 µm thick tissue sections. The sections were laid out on gelatin coated slides, fixed for 30 min in 4% paraformaldehyde, and incubated in 1% TritonX100 for 30 min. After three washes in PBS, the sections were incubated overnight with primary antibodies (anti-Bcl-2 or anti-Fas antibodies; Research Diagnostics) at 4°C, washed with PBS, and incubated with secondary antibodies conjugated with FITC for 1 h. Slides were examined with a confocal microscope (Zeiss).

Western Blot Analysis

The procedures were essentially the same as described previously [11, 13, 37-41]. Extracted cytosolic and membrane proteins (60 µg) were fractionated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide gels) and transferred onto PVDF membrane. After transfer, the membrane was incubated at 4°C overnight with the primary antibodies against phospho-p38 MAPK, phospho-SAPKs, phospho-ERKs, Bcl-2 and Fas (Research Diagnostics), and against M₃-mAChR (Santa Cruz), respectively, followed by incubation with the secondary antibody (donkey anti-goat, FITC-conjugated antibody) in the

Table 1. Effects of Choline on Hemodynamic Parameters during Coronary Artery Occlusion. HR: heart rate; LVSP: left ventricular systolic pressure; LVEDP: left ventricular end diastolic pressure; dP/dt: time derivatives of pressure. MI: myocardial infarction; Choline: 5 mg/kg (i.v.); 4-DAMP: (0.5 µg/kg, i.v.); Meth: methoctramine (M₂-selective antagonist, 10 µg/kg, i.v.). *p< 0.05 vs. Control; †p<0.05 vs. Sham (MI); ‡p<0.05 vs. choline alone (MI).

Group	HR (beats/min)	LVSP (mmHg)	LVEDP (mmHg)	+dP/dt (mmHg/s)	-dP/dt (mmHg/s)
Control	340 ± 38	125 ± 24	9 ± 1	3988 ± 350	3555 ± 324
Sham (MI)	359 ± 40	143 ± 29*	23 ± 2*	2690 ± 268	2405 ± 201*
Choline (MI)	322 ± 45	126 ± 16	12 ± 2 [†]	3290 ± 335	3006 ± 251 [†]
Choline + 4-DAMP (MI)	353 ± 49	137 ± 15	22 ± 2 [‡]	2815 ± 252	2620 ± 285 [‡]
Choline + Meth (MI)	328 ± 38	128 ± 11	14 ± 2	3305 ± 322	3025 ± 317

blocking buffer. Bound antibodies were detected using chemiluminescent substrate (Western Blot Chemiluminescence Reagent Plus; PerkinElmer Life Science Products). Densities of immunoblotting bands were quantified with the use of a Molecular Imager System (GS-505; Bio-Rad).

Measurement of Intracellular Ca²⁺ Concentration

Cardiomyocytes were incubated in 20 µM Fluo-3AM (Molecular Probes) for 1 hr at 37°C to load cells with Fluo-3AM (acetoxymethyl ester form, Molecular Probes). The cells were washed with PBS three times and centrifuged at 5×g for 15 s, and then re-suspended in serum-free DMEM culture medium and subjected to laser confocal scanning analysis at 500 nm.

Data Analysis

Group data are expressed as mean ± SEM. Statistical comparisons among multiple groups were performed by analysis of variance (ANOVA). If significant effects were indicated by ANOVA, a t-test with Bonferroni correction or a Dunnett's test was used to evaluate the significance of differences between individual means. Otherwise, baseline and drug data were compared by Student's t-test. A two-tailed P<0.05 was taken to indicate a statistically significant difference. The number of experiments, animals, or cells is specified in figure legends.

Results

Choline Improves the Hemodynamic Function of Ischemic Hearts

Table 1 shows the hemodynamic parameters before and after administration of choline after occlusion. Myocardial infarction caused significant increases in left ventricular pressure including both LVEDP and LVSP, as well as reduction of ±dP/dt, indicating impaired cardiac ejection function. Administration with choline (5 mg/kg, i.v.) ameliorated these deleterious changes; LVEDP was

markedly reduced, and LVSP slightly decreased, by choline, and the decreases in ± dP/dt seen with the infarcted rats were diminished by choline. Noticeably, co-administration of the M₃-selective antagonist 4-DAMP (0.5 µg/kg, i.v.) prevented the effects of choline (Table 1). Similar influence by another M₃-selective antagonist p-F-HHSiD (hexahydro-sila-hydrochloride, p-fluoro analog (2 µg/kg, i.v.), on the hemodynamic effects of choline, was also consistently observed in our experiments (data not shown). On the other hand, the M₂-selective antagonist methoctramine (10 µg/kg, i.v.) failed to alter the effects of choline (Table 1).

Choline Reduces the Risk of Ischemic Arrhythmias

Coronary artery occlusion resulted in generation of ventricular arrhythmias. Ventricular premature beat (VPB) and ventricular tachycardia (VT) occurred in all six rats studied and VT predisposed into ventricular fibrillation (VF) in 2/5 animals. Administration of choline produced dose-dependent reduction of VPB incidence and VT duration. This antiarrhythmic effect was abrogated or even abolished if the animals were pretreated with the M₃-mAChR selective antagonist 4-DAMP (Figs. 1 and 2) but not by methoctramine.

Ischemic Myocardial Injuries: Infarct Size and Apoptotic Cell Death

Administration of choline reduced the size of infarcted area by ~25% and the effect was diminished by 4-DAMP to inhibit the M₃-mAChRs (Fig. 3).

Programmed cell death (apoptosis) is believed to be one of the deleterious factors leading to cardiac injuries in myocardial infarction or ischemia (MI). We determined

Fig. 1. Effects of choline on ischemic arrhythmias and the potential role of M_3 -mAChRs. (A) Shown are examples of various types ventricular arrhythmias, as labelled. (B-E) Shown are data averaged from six animals for each group. VPB: ventricular premature beat; VT: ventricular tachycardia; Cho: choline, 5 mg/kg (i.v.); Meth: methoctramine (10 μ g/kg, i.v.), 4-DAMP: (0.5 μ g/kg, i.v.). * $p < 0.05$ vs. Control (Ctl); + $p < 0.05$ vs. Choline.

the apoptosis in our ischemic model using TUNEL method with tissue slices from the ischemic zone and the border zone (Fig. 4A). Application of choline (5 mg/kg, i.v.) dramatically reduced apoptotic cell death by 50% and co-application with 4-DAMP abrogated the effect.

Oxidative stress is another important damaging factor in the pathological process of MI. Our experiments with cultured neonatal rat ventricular myocytes demonstrated that H_2O_2 (200 μ M) caused remarkable DNA fragmentation, a hallmark of apoptosis, as indicated by the increased OD value determined by ELISA. Choline produced cytoprotection by decreasing H_2O_2 -induced

DNA fragmentation and the beneficial effect was prevented by 4-DAMP (Fig. 4B-4D).

Apoptotic/Survival Signals and Oxidants/Antioxidants

To shed some lights on the potential mechanisms underlying choline's cytoprotective effects on ischemic myocardium, we measured the cytoplasmic level of the anti-apoptotic signaling molecule Bcl-2 and the membrane density of death receptor Fas by immunohistochemistry. As shown in Fig. 5, MI diminished intracellular Bcl-2 level and upregulated Fas expression in the membrane. Choline

Fig. 2. Effect of choline on infarct size and the potential role of M_3 -mAChRs. (A) Shown are examples of infarcted area of the left ventricular walls (coronal sections). The infarcted area is stained white. (B) Shown are mean data of infarct size from 6 rats. * $p < 0.05$ vs. Control (Ctl); + $p < 0.05$ vs. Choline (Cho). Cho: choline, 5 mg/kg (i.v.); MI: myocardial infarction; Meth: methotramine (10 μ g/kg, i.v.); 4-DAMP: (0.5 μ g/kg, i.v.).

Fig. 3. Effect of choline on cardiomyocytes apoptosis and the potential role of M_3 -mAChRs. (A) Effect of choline on cardiomyocyte apoptosis determined by TUNEL staining in tissue preparations dissected from the ischemic boarder zone. Left panels: examples of the confocal images of TUNEL staining. Right panels: mean data of positive TUNEL staining from 6 rats for each group. Cho: choline, 5

mg/kg (i.v.); 4-DAMP: 0.5 μ g/kg (i.v.); MI: myocardial infarction. * $p < 0.05$ vs. Control (Ctl); + $p < 0.05$ vs. Cho. (B) Anti-apoptotic effects of mAChR agonists (choline, pilocarpine, or tetramethylammonium) in cultured neonatal rat ventricular cells. Apoptosis was induced by H_2O_2 (200 μ M) and quantified by DNA fragmentation with ELISA; the OD (optical density) values represent the degree of DNA fragmentation. Cho: choline, 400 μ M; PC: pilocarpine, 10 μ M; TMA: tetramethylammonium, 1 mM. (C) Concentration-dependent anti-apoptotic effect of choline. (D) Influence of M_3 -mAChR selective antagonist 4-DAMP (2 nM) on the anti-apoptotic effect of choline (400 μ M). * $p < 0.05$ vs. Control (Ctl); + $p < 0.05$ vs. H_2O_2 alone. Data for neonatal cells were averaged from 4 independent experiments.

Fig. 4. Effects of choline on the expression of Bcl-2 (A) and Fas (B) and the potential role of M₃-mAChRs. Bcl-2 and Fas protein expressions were determined by immunohistochemistry for ischemic myocardial slices and by immunocytochemistry for cultured neonatal rat ventricular cells treated with H₂O₂ (200 μ M). Bcl-2 was stained brown in the images. Cho: choline (5 mg/kg, i.v.); MI: myocardial infarction; 4-DAMP (0.5 μ g/kg). * p <0.05 vs. Control (Ctl) untreated group; † p <0.05 vs. MI group; ‡ p <0.05 vs. choline during MI or in presence of H₂O₂. Data were averaged from 8 rats for each group.

Fig. 5. Effects of choline on serum levels of superoxide dismutase (SOD) and malondialdehyde (MDA). Cho: choline (5 mg/kg, i.v.); 4-DAMP (0.5 μ g/kg); MI: myocardial infarction. * p <0.05 vs. Control (Ctl) untreated group; † p <0.05 vs. MI group; ‡ p <0.05 vs. MI group treated with choline. Data shown were averaged from 8 rats for each group.

drastically increased Bcl-2 to the level overshooting the control value in healthy myocardium, and moderately decreased the density of Fas receptors. The effects were reversed by co-administration of 4-DAMP. Similar observations were obtained in the cultured neonatal rat ventricular myocytes in presence of H₂O₂ to induce apoptotic cell death (Fig. 5); H₂O₂ diminished the anti-apoptotic protein Bcl-2 and enhanced death receptor Fas. Choline prevented the deleterious effects of oxidative stress and the effect was antagonized by 4-DAMP.

Fig. 6. Effects of choline on the levels of active forms of MAP kinases. Shown are Western blot results with protein samples extracted from cultured rat neonatal ventricular myocytes.

Averaged data were obtained from 5 independent experiments and the values are normalized to those without choline (Cho). * $p < 0.05$ vs. Control (Ctl) group in the presence of H₂O₂.

Serum level of superoxide dismutase (SOD) is an index of endogenous antioxidant capacity. MI substantially reduced the serum level of SOD as compared with healthy animals. Administration of choline prevented the decrease and the effect of choline was diminished by 4-DAMP applied 10 min before co-administration of choline and 4-DAMP. Malondialdehyde (MDA) level in serum represents the degree of lipid peroxidation. As illustrated in Fig. 6, MI considerably increased MDA. Choline significantly abrogated the increase and the effect was reversed by 4-DAMP.

P38 MAPK and SAPKs are known to transduce apoptotic or death signal, whereas ERK1/ERK2 mediate survival or anti-apoptotic signal in the cell. Our Western blotting analyses demonstrated that the levels of active p38, SAPKs, and ERK2 were minimal or undetectable under normal control conditions and were not affected

by application of choline. By comparison, appreciable active ERK1 was detected in control, which again was not altered by choline. However, in the presence of oxidative stress (H₂O₂ + FeSO₄), the levels of the active p38 and ERK1 were markedly increased. Noticeably, choline decreased the active p38 but strikingly enhanced the active ERK1/ERK2 levels (Fig. 7). The experiments with the protein samples extracted from MI rat demonstrated qualitatively the same results; choline boosted up the levels of active ERKs, but reduced active p38 MAPK (Fig. 7B and 7C).

Intracellular Ca²⁺ Content

Intracellular Ca²⁺ overload is one of the deleterious factors causing ischemic myocardial injuries and post-ischemic myocardial stunning. The data in Fig. 9 reveal that oxidative stress caused remarkable increase in [Ca²⁺]_i,

Fig. 7. Effect of choline on intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) overload induced by oxidative stress (H_2O_2 , 200 μM) in cultured neonatal rat ventricular myocytes. $[\text{Ca}^{2+}]_i$ was measured by Fluo-3AM with a confocal microscope. Cho: choline (10 mM) and 4-DAMP (2 nM). * $p<0.05$ vs. Control (Ctl); + $p<0.05$ vs. H_2O_2 alone; and † $p<0.05$ vs. choline in presence of H_2O_2 . Data were averaged from 11 independent experiments.

and this increase was abolished in the cells pretreated with choline (10 mM), measured by Fluo-3AM (Molecular Probes) under a confocal microscope. And the effect of choline was prevented by 4-DAMP, suggesting that activation of the cardiac M_3 -mAChRs can protect the cells from damage caused by oxidative stress-induced intracellular Ca^{2+} overload.

Discussion

This study explored the possible role of M_3 -mAChR in cytoprotection of infarcted myocardium and several signaling pathways (MAP kinases, oxidative stress and $[\text{Ca}^{2+}]_i$) as the potential mechanisms. The results show that administration of choline-Cl relieves myocardial injuries during ischemia or under oxidative stress; this is achieved by correcting hemodynamic impairment, diminishing ventricular arrhythmias and protecting cardiomyocytes from apoptotic death. The beneficial effects of choline are likely mediated by stimulation of the cardiac M_3 -mAChRs which lead to activation of several survival signaling molecules (Bcl-2 and ERKs), increase in endogenous antioxidant reserve, and reduction of apoptotic mediators (Fas and p38 MAPK) and

intracellular Ca^{2+} overload. This represents the first documentation of pathophysiological function and the underlying signal transduction mechanisms of the cardiac M_3 -mAChRs. The findings suggest M_3 -mAChR as a potential approach for the treatment of ischemic heart disease and a new target for drug development for improving cardiac function and preventing cardiac injuries during ischemia/reperfusion.

Role of the M_3 -mAChRs in Mediating Choline's Beneficial Effects Against Ischemic Injuries

Several lines of evidence are in favour of the potential role of the cardiac M_3 -mAChRs in mediating the beneficial effects of choline against ischemic injuries of heart in a rat model of myocardial infarction. First, the present study demonstrates that the M_3 -selective antagonist 4-DAMP, at concentrations known to selectively inhibit M_3 -mAChRs, both reversed the effects of choline on hemodynamic parameters, arrhythmias, apoptosis, Ca^{2+} overload, and the related signaling pathways. Another M_3 -selective antagonist *p*-F-HHSD also restored the changes of hemodynamic parameters of ischemic rats induced by choline. 4-DAMP and *p*-F-HHSD have been widely used to dissect the function of M_3 -mAChRs and also been previously validated with radio-ligand binding studies and functional studies by our laboratory. Second, the M_2 -selective antagonist methoctramine failed to alter the effects of choline. Finally, in accordance with the previous finding [27, 42] we found that activation of M_3 -mAChRs stimulates activities of ERKs, the antiapoptotic signaling molecules.

Multiple Signal Transduction Mechanisms Underlying the Effects of M_3 -mAChRs in the Heart

It is commonly accepted that the odd-numbered mAChR subtypes M_1 - and M_3 -mAChRs are characterized biochemically by stimulation of a large inositol phosphate response while having a small stimulatory effect on adenylate cyclase activity, whereas the even-numbered M_2 - and M_4 -mAChRs isoforms are linked to an inhibition of adenylate cyclase activity and only a modest stimulation of inositol phosphate release. The present study reveals for the first time some yet-unreported signal transduction mechanisms of M_3 -mAChRs, such as Bcl-2, Fas, p38 MAPK, SOD and $[\text{Ca}^{2+}]_i$. More importantly, our results suggest that M_3 -mAChRs accomplish their cellular functions in the heart through multiple signaling pathways. Activation of the cardiac M_3 -mAChRs enhances survival signals and

decreases death signals to reduce apoptosis. By increasing antioxidant capacity (SOD) and decreasing lipid peroxidation (MDA), M₃-mAChRs can exert antiarrhythmic effects. Moreover, limiting intracellular Ca²⁺ overload may help to restore the impaired hemodynamic function of ischemic heart and to diminish the risk of cell death.

Anti-Apoptotic Effect of M₃-mAChRs as the Key Feature Against Ischemic Injuries

The anti-apoptotic effect of choline mediated by M₃-mAChRs might be the key property for its cytoprotection against ischemic injuries. Choline alters the activities and levels of several signaling molecules known to be critical to apoptosis, increasing activities of the antiapoptotic mediators Bcl-2, ERKs, and SOD, and decreasing those of the proapoptotic mediators Fas, p38, and intracellular Ca²⁺. The ability of choline and M₃-mAChRs to produce cytoprotection against apoptosis has been previously reported in several non-cardiac cells. Choline deficiency is known to cause apoptosis in neurons, preneuronal PC12 cells, hepatocytes, etc, which can be prevented by choline supplementation [26-32]. The M₃-mAChR-selective inhibitor 4-DAMP has been shown to abolish the cytoprotective action of carbachol against apoptosis in cerebellar granule neurons [24-25]. Indeed, the anti-apoptotic ability of the M₃-mAChRs has recently been further confirmed at the molecular level; the study by Budd *et al* [27] reveals that the poly-basic region within the short C-terminal tail confers the cytoprotection property of the M₃-mAChRs.

Potential Implications of the Study

Choline, a member of the vitamin B complex family, is well known to be a necessary substrate of membrane lipid, and a precursor and metabolite of acetylcholine. Studies have demonstrated that choline is not limited to be a structural component of cellular membrane but it also has many cellular functions. Indeed, choline, used as a nutrition supplement, has received more and more attention because a growing body of evidence indicates that it plays an important role in the prevention of many pathological conditions, such as cirrhosis of the liver, arteriosclerosis, certain deficiencies of brain function and memory, and the pathogenesis of Alzheimer's disease [43-45]. The present study demonstrates that choline improves cardiac function and relieves ischemic injuries, unravelling a novel aspect of choline pharmacology; that is, it also regulates cardiac function. This suggests that apart from

the mechanisms for its actions, choline itself could be considered as a supplement for patients with coronary artery disease. In agreement with our data that choline abrogates the propensity and severity of arrhythmias occurring during myocardial ischemia, a study performed by Podzuweit [46] showed that choline abolished the ventricular tachycardia induced by subepicardial infusion of norepinephrine and Ca²⁺ in open-chest pigs. The fact that choline weakens intracellular Ca²⁺ overload revealed in the present study provides an explanation for Podzuweit's finding.

Another novel finding of the study is that choline produces its beneficial effects likely via activating the cardiac M₃-mAChRs. Although more conclusive data are required to definitely establish the link, M₃-mAChRs as a potential target for the treatment of heart disease is worthy of further investigation, particularly when considering the fact that the M₃-mAChR density increases while the M₂-mAChR density decreases in atria with atrial fibrillation induced by experimental congestive heart failure of dogs [11, 14, 46].

Possible Weakness of the Study

It should be noted that although the concept that functional M₃-mAChRs exist in the heart has been appreciated, use of choline as an M₃-mAChR agonist has not been well justified since the specificity of choline toward M₃-mAChRs has not been definitely established. Yet, most of the previous studies concerning choline and mAChR subtypes demonstrated that choline acts more selectively to M₃-mAChR over other subtypes. Unfortunately, specific agonists towards any of the mAChR subtypes are currently unavailable. Similarly, while 4-DAMP is considered to be selective to M₃-mAChR, it is not specific. Nonetheless, 4-DAMP has been widely used for dissecting M₃-mAChR and studying the function of M₃-mAChRs.

Our present study does not provide any insights into the mechanistic link between the M₃-mAChRs and the various signaling pathways studied. Nevertheless, our data at least indicate for the first time that the cardiac M₃-mAChRs may play an important pathophysiological role in the heart.

Abbreviations

4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide); Cho (choline-Cl); mAChR (muscarinic

acetylcholine receptor); M₃-mAChR (M₃ subtype mAChR); I_{KM3} (M₃-mAChR-mediated K⁺ current); Meth (methoctramine); MI (myocardial infarction); PC (pilocarpine); *p*-F-HHSiD (hexahydro-sila-hydrochloride, *p*-fluoro analog); TMA (tetramethylammonium); VPB (premature beat); VT (ventricular tachycardia); VF (ventricular fibrillation).

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