Original Paper

Acetylcholine Attenuates Hydrogen Peroxide-Induced Intracellular Calcium Dyshomeostasis Through Both Muscarinic and Nicotinic Receptors in Cardiomyocytes

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Key Words

Acetylcholine • Oxidative stress • Hydrogen peroxide • Calcium transient

Abstract

Background/Aims: Oxidative stress induced intracellular Ca^{2+} overload plays an important role in the pathophysiology of several heart diseases. Acetylcholine (ACh) has been shown to suppress reactive oxygen species generation during oxidative stress. However, there is little information regarding the effects of ACh on the intracellular Ca^{2+} regulation in the presence of oxidative stress. Therefore, we investigated the effects of ACh applied before or after hydrogen peroxide (H_2O_2) treatment on the intracellular Ca^{2+} regulation in isolated cardiomyocytes.

Methods: Single ventricular myocytes were isolated from the male Wistar rats for the intracellular Ca^{2+} transient study by a fluorimetric ratio technique. **Results:** H_2O_2 significantly decreased both of intracellular Ca^{2+} transient amplitude and decay rate. ACh applied before, but not after, H_2O_2 treatment attenuated the reduction of intracellular Ca^{2+} transient amplitude and decay rate. Both atropine (a muscarinic acetylcholine receptor blocker) and mecamylamine (a nicotinic acetylcholine receptor blocker) significantly decreased the protective effects of acetylcholine on the intracellular Ca^{2+} regulation. Moreover, the combination of atropine and mecamylamine completely abolished the protective effects of acetylcholine on intracellular Ca^{2+} transient amplitude and decay rate. **Conclusion:** ACh pretreatment attenuates H_2O_2 -induced intracellular Ca^{2+} dyshomeostasis through both muscarinic and nicotinic receptors.

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Introduction

Oxidative stress has been shown to play an important role in the pathophysiology of several pathological conditions including acute myocardial infarction (MI) [1-3]. In MI patients, timely myocardial reperfusion of coronary blood flow is necessary to resuscitate the ischemic or hypoxic myocardium [4, 5]. Reperfusion facilitates cardiac myocytes salvage and decreases cardiac morbidity and mortality. However, reperfusion of an ischemic myocardium may result in paradoxical cardiac dysfunction, a phenomenon known as "ischemia/reperfusion (I/R) injury" [5]. The hydrogen peroxide (H₂O₂) is one of the key mediators underlying cellular oxidative stress during I/R [5]. The formation of H₂O₂ during I/R injury results in intracellular Ca²⁺ overload due to increased lipid peroxidation and sulfhydryl group oxidation in the I/R heart [1]. Therefore, any therapeutic approach which can prevent intracellular Ca²⁺ overload and improves intracellular Ca²⁺ regulation would be beneficial for reperfused heart.

Acetylcholine (ACh) has been shown to suppress reactive oxygen species generation during oxidative stress [6, 7]. Moreover, previous studies reported that parasympathetic activation by vagus nerve stimulation (VNS)-induced ACh release exerted cardioprotective effects against I/R injury [8-11]. Moreover, our recent study demonstrated that VNS reduced the occurrence of ventricular fibrillation and decreases myocardial infarction size in I/R model [12, 13]. Furthermore, VNS could improve cardiac mitochondrial function which was impaired in I/R hearts [12]. Despite these beneficial effects, there is little information regarding the effects of ACh on the intracellular Ca²⁺ regulation in the presence of oxidative stress. In this study, we investigated the effects of ACh applied before or after H₂O₂ treatment on intracellular calcium regulation in isolated cardiomyocytes. We hypothesized that ACh can attenuate intracellular Ca²⁺ dyshomeostasis caused by H₂O₂ in cardiomyocytes.

Materials and Methods

Animal preparation and study protocol

This study was approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University. All the animals were fed with normal rat chow and water ad libitum before the study. Male Wistar rats (250-300 g) were used. The rats were deeply anesthetized with thiopental (0.5 mg/kg; Research institute of antibiotics and biotransformations, Rostok, Czech Republic) after which the hearts were removed for single ventricular myocyte isolation [14]. The isolated cardiomyocytes were used in each study protocol for the measurement of intracellular Ca²⁺ transient. Data were compared between the experimental groups.

In the protocol 1, cardiomyocytes were divided into 5 groups (n = 8 cells/rat and 8 rats/group) as shown in Fig. 1A. Cardiomyocytes were divided into 5 groups. In groups I, cardiomyocytes were treated with normal saline solution (NSS) for 5 minutes as a control group. Group II was treated with ACh (10 μM) (Acetylcholine chloride, Sigma-Aldrich, St. Louis, MO, USA) for 5 minutes, and Group III was treated with NSS for 2 minutes followed by H₂O₂ (2 mM) for 3 minutes. Group IV was treated with a muscarinic receptor blocker, atropine (1 μM) for 5 minutes, and Group V was treated with a nicotinic receptor blocker mecamylamine (1 μM) (Sigma-Aldrich, St. Louis, MO, USA) for 5 minutes. The real-time Ca²⁺ transient measurement was performed at the beginning of the study (baseline) and at the end of treatment in each group.

In protocol 2, cardiomyocytes were divided into 5 groups (n = 8 cells/rat and 8 rats/group) as shown in Fig. 1B. Cardiomyocytes were divided into 5 groups. In groups I, they were treated with NSS for 10 minutes followed by H₂O₂ (2 mM) for 3 minutes and served as a control group. Group II was treated with NSS for 5 minutes followed by ACh (10 μM) for 5 minutes followed by H₂O₂ (2 mM) for 3 minutes. Group III was treated with atropine (1 μM) for 5 minutes followed by ACh (10 μM) for 5 minutes and then H₂O₂ (2 mM) for 3 minutes. Group IV was treated with a mecamylamine (1 μM) for 5 minutes followed by ACh (10 μM) for 5 minutes and then H₂O₂ (2 mM) for 3 minutes. Group V was treated with a mecamylamine (1 μM) and atropine (1 μM) for 5 minutes followed by ACh (10 μM) for 5 minutes and then H₂O₂ (2 mM) for 3

Fig. 1. Experimental groups and the treatment protocol of protocol 1 (A), protocol 2: pre-treatment groups (B), protocol 3: post-treatment groups (C), Cardiac myocytes loaded with fluorescent dye Fura-2/AM (D). NSS=normal saline solution, ACh=acetylcholine, MEC=mecamylamine, H_2O_2 =hydrogen peroxide, Ca^{2+} =intracellular calcium measurement.

minutes. The real-time Ca^{2+} transient measurement was performed at the beginning of the study (baseline) and at the end of treatment in each group.

In protocol 3, cardiomyocytes were divided into 5 groups ($n = 8$ cells/rat and 8 rats/group) as shown in Fig. 1C. In this protocol, cardiomyocytes were divided into 5 groups. Cardiomyocytes in groups I were treated with NSS for 5 minutes followed by H_2O_2 (2 mM) for 3 minutes and then NSS for 5 minutes, and served as a control group. Group II was treated with NSS for 5 minutes followed by H_2O_2 (2 mM) for 3 minutes and then ACh (10 μ M) for 5 minutes, and Group III was treated with atropine (1 μ M) for 5 minutes followed by H_2O_2 (2 mM) for 3 minutes and then ACh (10 μ M) for 5 minutes. Group IV was treated with a mecamylamine (1 μ M) for 5 minutes followed by H_2O_2 (2 mM) for 3 minutes and then ACh (10 μ M) for 5 minutes. Group V was treated with the combination of mecamylamine (1 μ M) and atropine (1 μ M) for 5 minutes followed by H_2O_2 (2 mM) for 3 minutes and then ACh (10 μ M) for 5 minutes. The real-time Ca^{2+} measurement was performed at the beginning of the study and after the treatment in all groups.

Calcium transient measurement

Cardiomyocytes were isolated from the hearts of male Wistar rats using a method described previously [15]. In brief, under deep anesthesia, the heart was immediately removed and placed into a modified Langendorff apparatus. The hearts were perfused with modified Krebs solution (130 mM NaCl, 4.5 mM KCl, 1.4 mM $MgCl_2$, 0.4 mM NaH_2PO_4 , 0.75 mM $CaCl_2$, 4.2 mM HEPES, 20 mM taurine, 10 mM creatine and 10 mM glucose, pH 7.3 at 37°C) for 5 minutes, followed by calcium-free solution (100 μ M EGTA) for 4 minutes, Tyrode's solution with collagenase (0.1 mg/ml) for 10 minutes, and modified Krebs solution containing 100 μ M $CaCl_2$ and 1 mg/ml type II collagenase for another 8 minutes. The ventricles were removed from the cannula, cut into small pieces and incubated in 10 ml of collagenase solution gassed with 100% O_2 for 7 minutes at 37°C, with regular triturating. The cardiomyocytes were separated from undigested ventricular tissues by filtering through cell strainer, and were settling into a loose pellet. Then, the supernatant was removed and replaced with modified Krebs solution containing 1% BSA and 500 μ M $CaCl_2$. This process was repeated with modified Krebs solution containing 1 mM $CaCl_2$. After this procedure, the cardiomyocytes were ready for recording [15]. The isolated cardiomyocytes were placed in a modified

Krebs solution containing 1 mM $CaCl_2$. Intracellular Ca^{2+} transient were measured using the CELL^R imaging software (Olympus Soft Imaging Solutions GmbH, Germany). The isolated cardiomyocytes were loaded with Fura-2/AM at the final concentration of 5 μ M [16] and fluorescent intensity was recorded during electrical pacing (1 Hz, 10 ms duration, 15 V) [17]. The ratio of the emissions wavelengths is directly related to the amount of intracellular Ca^{2+} [18]. When Ca^{2+} binds to a ratiometric indicator, it changes the optimum excitation or emission wavelength of the indicator. An elevation of Ca^{2+} concentration induces an increase in Fura-2 emission fluorescence when the indicator is excited at 340 nm, with a corresponding decrease in fluorescence at 380 nm excitation [19, 20]. The experiments were performed in the temperature controlled chamber system at 37°C. The H_2O_2 concentration at 2 mM is a stock solution. The final concentration in the recording chamber is 50 μ M which is widely used to trigger oxidative stress-induced intracellular Ca^{2+} dyshomeostasis in cardiomyocytes [21, 22]. Intracellular Ca^{2+} transient decay rate which approximates the rate of Ca^{2+} uptake into the SR by sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), intracellular calcium transient amplitude which approximates the intracellular Ca^{2+} level during systolic period, and diastolic Ca^{2+} level which approximates the intracellular Ca^{2+} level during diastolic period were recorded before and after treatment.

Statistical analysis

Data were expressed as mean $\pm$ SD. Comparisons of variables were performed using the one-way ANOVA followed by LSD post-hoc test. $P < 0.05$ was considered statistically significant.

Results

ACh at the concentrations of 1, 10 and 100 μ M did not alter the diastolic Ca^{2+} level compared to the baseline. However, all concentrations of ACh significantly decreased the intracellular Ca^{2+} transient amplitude (0.03 ± 0.015 in 1 μ M, 0.02 ± 0.005 in 10 μ M, and 0.03 ± 0.005 in 100 μ M, compared to 0.06 ± 0.001 at baseline) and Ca^{2+} transient decay rate (0.09 ± 0.003 in 1 μ M, 0.09 ± 0.004 in 10 μ M, and 0.06 ± 0.002 in 100 μ M, compared to 0.23 ± 0.02 at baseline). In the first protocol, the effects of H_2O_2 , ACh, atropine and mecamylamine on the intracellular Ca^{2+} transient were investigated. H_2O_2 significantly decreased both of intracellular Ca^{2+} transient amplitude (Fig. 2A) and intracellular Ca^{2+} transient decay rate (Fig. 2B). The intracellular Ca^{2+} diastolic level was not altered (Fig. 2C) compared to the control group in response to all treatments. In addition, ACh at 10 μ M significantly decreased the intracellular Ca^{2+} transient amplitude (Fig. 2A) and Ca^{2+} transient decay rate (Fig. 2B), but did not alter the level of intracellular diastolic Ca^{2+} level (Fig. 2C) compared to the control group. However, atropine and mecamylamine did not alter the intracellular Ca^{2+} transient amplitude, Ca^{2+} transient decay rate and the intracellular diastolic Ca^{2+} level. The effect of H_2O_2 , ACh, atropine and mecamylamine on the intracellular Ca^{2+} transient tracing shape is shown in Fig. 2D.

In the second protocol, the effects of ACh administered prior to H_2O_2 application were investigated. The intracellular Ca^{2+} transient amplitude was significantly decreased in H_2O_2 treated group. However, pretreated with ACh significantly prevent the reduction of the intracellular Ca^{2+} transient amplitude compare to the H_2O_2 treated group, $p < 0.05$ (Fig. 3A). Furthermore, pretreated with ACh significantly increased the intracellular Ca^{2+} transient decay rate compared to the H_2O_2 treated group (Fig. 2B). However, in all experimental groups, ACh did not alter the level of intracellular diastolic calcium (Fig. 3C) compared to the H_2O_2 treated group. The protective effect of ACh was reduced when cardiomyocytes were exposed to the muscarinic receptor blocker (atropine) and nicotinic receptor blocker (mecamylamine) prior to ACh application (Fig. 3B, 3C). Moreover, co-incubation of atropine and mecamylamine completely abolished the cardioprotective effects of ACh on intracellular Ca^{2+} transient amplitude and decay rate. The effects of ACh on Ca^{2+} transient tracing shape is shown in Fig. 3D.

In the third protocol, we investigated the effects of ACh administered after H_2O_2 application on intracellular Ca^{2+} transient. Our results showed that in cardiomyocytes treated

Fig. 2. The effect of acetylcholine, atropine and mecamlamine on the intracellular Ca^{2+} transient amplitude (A), and intracellular Ca^{2+} transient decay rate (B) and intracellular diastolic Ca^{2+} level (C). The effects of ACh on Ca^{2+} transient tracing shape (D). * $p < 0.05$ vs. NSS, † $p < 0.05$ vs. ACh. NSS=normal saline solution, ACh=acetylcholine, MEC= mecamlamine, H_2O_2 = hydrogen peroxide, Ca^{2+} = intracellular calcium measurement.

Fig. 3. The effect of pre-treated cardiac myocytes with acetylcholine on the intracellular Ca^{2+} transient in isolated cardiac myocytes under H_2O_2 -induced oxidative stress. ACh significantly attenuated the reduction of intracellular Ca^{2+} transient amplitude (A) and increased intracellular Ca^{2+} transient decay rate (B) but did not alter intracellular diastolic calcium level (C) compared with H_2O_2 group. The protective effects of ACh were abolished by ACh receptor blocking agents. The effects of ACh on Ca^{2+} transient tracing shape (D). * $p < 0.05$ vs. NSS, † $p < 0.05$ vs. H_2O_2 , ‡ $p < 0.05$ vs. ACh. NSS=normal saline solution, ACh= acetylcholine, MEC= mecamlamine, H_2O_2 = hydrogen peroxide, Ca^{2+} =intracellular calcium measurement.

Fig. 4. The effect of post-treated cardiac myocytes with acetylcholine on the intracellular Ca^{2+} transient in isolated cardiac myocytes H_2O_2 -induced oxidative stress. ACh did not improve intracellular Ca^{2+} transient amplitude (A), intracellular Ca^{2+} transient decay rate (B) and intracellular diastolic Ca^{2+} level (C) compared with H_2O_2 group. The effects of ACh on Ca^{2+} transient tracing shape (D). NSS= normal saline solution, ACh= acetylcholine, MEC= mecamlamine, H_2O_2 = hydrogen peroxide, Ca^{2+} = intracellular calcium measurement.

with H_2O_2 followed by ACh, the intracellular Ca^{2+} transient amplitude (Fig. 4A), intracellular Ca^{2+} transient decay rate (Fig. 4B) and the intracellular Ca^{2+} diastolic level (Fig. 4C) did not alter, compared to cardiomyocyte treated with H_2O_2 followed by NSS. Furthermore, atropine and mecamlamine or co-incubation of atropine and mecamlamine did not alter the intracellular Ca^{2+} transient amplitude (Fig. 4A), intracellular Ca^{2+} transient decay rate (Fig. 4B) and intracellular Ca^{2+} diastolic level (Fig. 4C). The effects of ACh given after H_2O_2 application in cardiomyocytes on Ca^{2+} transient tracing shape is shown in Fig. 4D.

Discussion

The major findings of the present study are as follows. In cardiomyocytes under H_2O_2 -induced oxidative stress, pre-treated cardiomyocytes with ACh (1) did not alter the intracellular diastolic Ca^{2+} level, (2) attenuated the reduction of intracellular Ca^{2+} transient amplitude and intracellular Ca^{2+} transient decay rate are caused by H_2O_2 , (3) both atropine (a muscarinic acetylcholine receptor blocker) and mecamlamine (a nicotinic acetylcholine receptor blocker) significantly decreased the protective effects of ACh on the intracellular Ca^{2+} regulation, (4) the combination of muscarinic and nicotinic blockers completely abolished the protective effects of ACh on intracellular Ca^{2+} transient amplitude and decay rate. Finally, ACh given after H_2O_2 application did not improve intracellular Ca^{2+} regulation impaired by oxidative stress.

The present study demonstrated that H_2O_2 application impaired intracellular Ca^{2+} regulation in isolated cardiomyocytes. Both intracellular Ca^{2+} transient amplitude and

decay rate significantly reduced in H₂O₂ treated group. Our findings are consistent with the previous study in the rat ventricular myocyte which demonstrated that H₂O₂ significantly decreased both intracellular Ca²⁺ transient amplitude and the rate of systolic Ca²⁺ removal [23]. The reduction of intracellular Ca²⁺ removal due to the impaired SERCA activity was shown to lead to intracellular Ca²⁺ overload and cardiac dysfunction [23]. Previous study also demonstrated that ACh is required for the maintenance of the cardiac Ca²⁺ cycling proteins [24]. Specifically, lack of ACh significantly reduces intracellular Ca²⁺ transient amplitude and intracellular Ca²⁺ transient decay rate, suggesting that ACh is necessary for the maintenance of intracellular Ca²⁺ transient regulation [24].

The timing of ACh application was also crucial for its protective effect in our study in which early application of ACh prior to H₂O₂ application showed better benefit. This may explain the findings reported previously in a swine model of I/R injury that vagus nerve stimulation (VNS) applied early at the onset of the ischemic period exerts cardioprotection against I/R injury [12, 13]. However, VNS applied at the onset of reperfusion did not produce cardioprotection against I/R injury, suggesting that the timing of VNS application with respect to the onset of myocardial ischemia is an important determinant factor [13].

It has been shown previously that ACh significantly decreased both Ca²⁺ transient amplitude and decay rate by reducing cAMP-PKA-dependent phosphorylation of ryanodine receptors and phospholamban, leading to the reduced open probability of the ryanodine receptor, and slower SERCA-mediated intracellular Ca²⁺ re-uptake into the SR, respectively [25]. However, H₂O₂ has been shown to decrease SERCA and NCX activities [26, 27] by inhibiting PKC activities, leading to the alteration of the intracellular Ca²⁺ homeostasis. Thus, ACh and H₂O₂ mediate the intracellular Ca²⁺ homeostasis through different signaling pathways. Although the potential mechanism by which ACh prestimulation protects cardiomyocytes against H₂O₂-induced intracellular Ca²⁺ dyshomeostasis are unclear, it might be due to the ability of ACh to protect cardiomyocytes from oxidative stress-related damage and prevent cardiac mitochondrial dysfunction as shown by previous studies. In rat's cardiomyoblasts, ACh exerted cardioprotection against oxidative injury through FoxO3a/PGC-1α signaling pathway [28]. Moreover, in endothelial cells, activation of α7 nicotinic acetylcholine receptor protected against oxidant stress damage through reducing vascular peroxidase-1 in a JNK signaling-dependent manner [29]. These protective mechanisms of ACh could be responsible for improved intracellular calcium homeostasis observed in the present study.

The important roles of both muscarinic and nicotinic cholinergic receptors on the intracellular calcium handling have been demonstrated in this study. We found that while either atropine (a muscarinic acetylcholine receptor blocker) or mecamylamine (a nicotinic acetylcholine receptor blocker) significantly decreased the protective effects of ACh on intracellular Ca²⁺ regulation, the combination of atropine and mecamylamine completely abolished the protective effects of ACh on intracellular Ca²⁺ transient amplitude and decay rate. These findings emphasize that ACh exerts the beneficial effect on intracellular Ca²⁺ regulation through both muscarinic and nicotinic receptors.

Study Limitations: In the present study, we only measured the Ca²⁺ transient at the beginning and at the end of the study. Future study is needed to investigate the changes of calcium transient over times. Although we did not assess the sarcoplasmic reticulum calcium content, previous studies demonstrated that the sarcoplasmic reticulum Ca²⁺ content was directly proportionate to intracellular Ca²⁺ transient amplitude and intracellular Ca²⁺ transient decay rate [30, 31].

Conclusion

Acetylcholine protects cardiomyocytes against H₂O₂-induced intracellular Ca²⁺ dyshomeostasis through the activation of both muscarinic and nicotinic receptors. These findings provide the important information regarding the mechanism underlying the cardioprotective effect of ACh against intracellular Ca²⁺ dyshomeostasis under oxidative

stress. Moreover, these roles of ACh may explain the cardioprotective benefits of VNS during I/R injury observed in the preclinical studies.

Disclosure Statement

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the Thailand Research Fund grants TRG5780007 (SP), RSA5880015 (KS) and BRG5780016 (SCC), the NSTDA Research Chair grant from the National Science and Technology Development Agency Thailand (NC), and the Chiang Mai University Center of Excellence Award (NC).

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