Involvement of Cyclooxygenase-2 in Carbachol-Induced Positive Inotropic Response in Mouse Isolated Left Atrium

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Received June 1, 2009; accepted September 10, 2009

ABSTRACT

The mouse heart is expected to have characteristic contractile properties. However, basic information on the function of the mouse heart has not been accumulated sufficiently. In this study, the involvement of cyclooxygenase (COX)-2 in carbachol (CCh)-induced inotropic response was investigated in mouse isolated left atrium. Influences of CCh and their mechanisms of action on developed tension elicited by electrical stimulation were examined pharmacologically. The presence of COX-2 in atrium was examined by Western blotting and immunohistochemical analysis. CCh (3 μM for 15 min) produced a biphasic inotropic response: a transient decrease in contractile force followed by a late increase. Atropine suppressed the biphasic inotropic response to CCh. A nonselective COX inhibitor, indomethacin, and a selective COX-2 inhibitor, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) inhibited the positive response. A COX-1 inhibitor, valeryl salicylate, did not affect the positive response. The positive response was almost completely abolished in the endocardial endothelium-deprived atria. Existence of COX-2 in endocardial endothelium was confirmed by Western blotting and immunohistochemical analysis. The present study indicated that the CCh-induced positive inotropic response was mediated by PGs, possibly PGE2 and PGF2α, released in part from endocardial endothelium. Furthermore, for the first time, we demonstrated that the production of PGs depended in part on COX-2 in endocardial endothelium through the muscarinic M3 receptor stimulation.

Acetylcholine (ACh), which generally elicits a negative inotropic response of heart muscles in many animal species, elicits a biphasic inotropic response in mouse atria: a transient decrease in contractile force followed by a late increase (Nishimaru et al., 2000). The transient negative phase is the standard M2 receptor-mediated pertussis toxin (PTX)-sensitive response. Although it was reported that the late positive response is mediated by M3 receptor (Kitazawa et al., 2009) and production of prostaglandins (PGs) (Tanaka et al., 2001), precise mechanisms of this positive inotropic response to muscarinic stimulation in mouse left atrial tissue remained to be clarified. Therefore, the purpose of the present study is to confirm the biphasic response induced by a muscarinic agonist, carbachol (CCh), in mouse left atria and to examine the mechanisms underlying the biphasic response, especially relating to PG production. We confirmed that the negative and the positive inotropic response to CCh in mouse left atria was mediated by the standard M2 receptor-mediated PTX-
sensitive process in cardiac tissue and by PGs released from the endocardial endothelium, respectively. Furthermore, for the first time, we revealed that the production of PGs depended on cyclooxygenase (COX)-2 through the muscarinic M3 receptor stimulation.

Materials and Methods

Animals. This study was conducted in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) as adopted by the U.S. National Institutes of Health and the Kitasato University. Mice, male ddY strain (CLEA, Tokyo, Japan), were housed with a 12-h light/dark cycle and fed laboratory food and water ad libitum. Left atria were isolated under sodium pentobarbital (50 mg/kg i.p. injection) anesthesia. PTX (180 μg/kg, 24 h)-treated left atrium. E, CCh responses in the presence of atropine (10 nM for 5 min) in PTX-treated preparation. F, CCh responses in endothelium-denuded preparation by the treatment with Triton X-100 (0.1%, approximately 10 s).

Measurement of Contractile Force. Left atrium was placed horizontally in a 20-ml tissue bath filled with Krebs-Henseleit solution: 119 mM NaCl, 4.8 mM KCl, 24.9 mM NaHCO3, 1.2 mM KH2PO4, 2.5 mM MgCl2, and 10 mM glucose. The solution was gassed with 95% O2/5% CO2 and maintained at 35 to 36°C. The atrium was driven by rectangular current pulses via a pair of platinum electrodes (field stimulation, 1 Hz, 5 ms, 1.5 times threshold voltage) generated by an electronic stimulator for an equilibration period of at least 90 min as described previously (Hara et al., 2007). Isometric contraction was recorded with a force-displacement transducer (TB-651T; Nihon Kohden, Tokyo, Japan) and monitored with a computer-supported data acquisition system (PowerLab; BioResearch Center, Nagoya, Japan). The usual application of CCh (3 μM) was for 15 min. In a part of the experiments, atrium was denuded of its endocardial endothelium before contractile force measurement according to the method of Tanaka et al. (2001). In brief, Triton X-100 was dissolved in Krebs-Henseleit solution at a concentration of 0.1%, and 0.1 ml of this solution was gently injected into the lumen of the atrium with a syringe. After approximately 10 s, the lumen was gently washed with Krebs-Henseleit solution.

Western Blotting. Western blotting was performed as described previously (Yamawaki and Hara, 2008; Yamawaki et al., 2008). Protein lysates were obtained by homogenizing atrial tissues with Triton-based lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 0.1% protease inhibitor mixture; Nacalai Tesque, Kyoto, Japan). Protein concentration was determined by using the bicinchoninic acid method (Pierce, Rockford, IL). Equal amounts of proteins (20 μg) were separated by SDS-polyacrylamide gel electrophoresis (7.5%) and transferred to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI). After blocking with 0.5% skim milk, membranes were incubated with a primary antibody (anti-COX-2, 1:500 dilution) at 4°C overnight, and membrane-bound antibodies were visualized by using peroxidase-conjugated secondary antibody (1:10,000 dilution, 1 h) and the ECL-plus system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Equal loading of protein was confirmed by measuring total actin expression. The resulting autoradiograms were analyzed by using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

Immunofluorescence Staining. Immunofluorescence staining was performed as described previously (Baden et al., 2008). Left atrial tissues were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Thin tissue sections (2 μm) were made and deparaffinized by the standard procedure. After being treated with 10 mM sodium citrate buffer (pH 6.0) for 10 min, the sections were blocked with 5% normal goat serum for 1 h at room temperature, incubated with murine COX-2 antibody (1:100 dilution, overnight) at 4°C, followed by fluorescent-conjugated secondary antibody (Alexa

Fig. 1. Actual traces of the inotropic response to CCh (3 μM) in mouse isolated left atrium. A, control (carbachol, 3 μM). B, CCh responses in the presence of atropine (100 nM for 5 min). C, CCh responses in the presence of 4-DAMP (100 nM for 5 min). D, CCh responses in PTX (180 μg/kg, 24 h)-treated left atrium. E, CCh responses in the presence of atropine (10 nM for 5 min) in PTX-treated preparation. F, CCh responses in endothelium-denuded preparation by the treatment with Triton X-100 (0.1%, approximately 10 s).
Flour 568; 1:2000 dilution, 1 h) (Invitrogen, Carlsbad, CA) at room temperature. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1 μg/ml, 5 min). Images were obtained by using fluorescence microscope (BX-51; Olympus, Tokyo, Japan) equipped with cooled CCD camera (MicroPublisher 5.0 RTV; Roper Japan, Tokyo, Japan).

**Materials.** The following drugs and materials were used: CCh (Wako, Osaka, Japan), atropine sulfate, indomethacin, valeroyl salicylate, NS-398, AH6809, 4-diphenyl-acetoxy-N-methylpiperidine (4-DAMP), and total actin antibody (Sigma-Aldrich, St. Louis, MO), pertussis toxin (Calbiochem, La Jolla, CA), DAPI (Dojindo, Kumamoto, Japan), AL8810, SQ29,548, murine COX-2 antibody (for immunohistochemistry) (Cayman Chemical, Ann Arbor, MI), and COX-2 (C-20) antibody (for Western blotting) (Santa Cruz Biotechnology, Santa Cruz, CA).

**Statistical Analysis.** Data are shown as mean ± S.E.M. Statistical evaluations were performed by using analysis of variance followed by Bonferroni’s test for comparisons in more than three groups and by the Student’s *t* test for comparisons between two groups. A value of *p* < 0.05 was considered statistically significant.

**Results**

**Influences of Anticholinergic Drugs and PTX on CCh-Induced Contractile Response in Mouse Isolated Left Atrial Preparation.** CCh (3 μM for 15 min) produced a biphasic inotropic response: an early decrease in contractile force followed by a late increase in mouse isolated left atrial preparation (Fig. 1A). The contractile force decreased to 26.2 ± 2.8% of the initial value at 1 min and increased to 116.0 ± 8.6% at 7 min (*n* = 5) (Fig. 2A). Atropine (100 nM) almost completely inhibited the biphasic response to CCh (Figs. 1B and 2A). A selective muscarinic M3 receptor antagonist, 4-DAMP (10 nM), inhibited only the positive inotropic responses without any influence on the negative response to CCh. Atropine (100 nM) almost completely inhibited the positive inotropic action (Figs. 1C and 2B). The same extent of the negative inotropic response lasted for almost 15 min until the end of observation. In the PTX-treated preparations, CCh
produced only the positive inotropic response even in the early phase (Fig. 1D). The peak of the positive inotropic response was significantly augmented. Atropine (10 nM) inhibited the positive inotropic response to CCh in the PTX-pretreated atria (Figs. 1E and 2C).}

**Influences of Prostanoids Receptor Antagonists on CCh-Induced Contractile Response in Mouse Isolated Left Atrial Preparation.** A selective inhibitor for PGE$_2$ (EP) receptor, AH6809 (1 μM), and a selective inhibitor for PGF$_2$α (FP) receptor, AL8810 (1 and 3 μM), preferentially and significantly suppressed the positive inotropic response to CCh. The blockade by either antagonist did not affect the negative response to CCh (Fig. 3, A and B). A selective inhibitor for thromboxane A$_2$ (TP) receptor, SQ29,548 (1 μM), did not influence either negative or positive inotropic response to CCh (Fig. 3C). In another set of experiments, we confirmed the effects of these selective prostanoid antagonists. PGE$_2$ (1 μM) produced the positive inotropic responses in mouse left atria (149.0 ± 4.7% at 11 min after PGE$_2$ application, n = 7). Pretreatment with 1 μM AH6809 for 15 min significantly but slightly inhibited the PGE$_2$-induced positive inotropic response (136.9 ± 4.7%, n = 9, p < 0.05). PGF$_{2α}$ (10 nM) produced the positive inotropic response (174.2 ± 14.7% at 9 min after PGF$_{2α}$ application, n = 6). Pretreatment with AL8810 (1 μM) for 15 min significantly suppressed the PGF$_{2α}$-induced positive inotropic response (121.4 ± 3.2%, n = 6, p < 0.05).

**Influences of COX Inhibitors on CCh-Induced Contractile Response in Mouse Isolated Left Atrial Preparation.** A nonselective COX inhibitor, indomethacin (3 μM), almost completely suppressed the positive inotropic response to CCh without any influence on the negative inotropic response (Fig. 4A). A selective COX-1 inhibitor, valeryl salicylate (500 μM), did not affect the biphasic inotropic response (Fig. 4B). A selective COX-2 inhibitor, NS-398 (1–10 μM), suppressed only the positive inotropic response in a concentration-dependent manner (Fig. 4C).

**Influences of Endocardial Endothelium on CCh-Induced Contractile Responses in Mouse Isolated Left Atrial Preparation.** In the atrium that was denuded of its endocardial endothelium by the treatment with 0.1% Triton X-100, the positive inotropic response to CCh was signifi-
cantly reduced, but the negative inotropic response was maintained (Figs. 1F and 5A). After the treatment with Triton X-100, the positive inotropic response to 10 nM PGF2α/H9251 was confirmed in the same preparations. The same degrees of contractions to PGF2α/H9251 were observed between control and Triton X-treated preparations (Fig. 5B).

**Localization of COX-2 in Mouse Isolated Left Atrial Preparation.** The presence of COX-2 in the lysates of left atrial preparation were examined by Western blotting (Fig. 6). Control in this figure means the time-matched (7 and 30 min) atrial preparation in which the same procedures were conducted as those in Fig. 6A without CCh. At the peak (7 min) of the contractile response to CCh, COX-2 expression decreased slightly but not significantly compared with control (Fig. 6, Ca and Da). After 30 min, COX-2 expression was not different in the presence or absence (control) of CCh (Fig. 6, Cb and Db). In the freshly dissected left atrial preparations, COX-2 was expressed, but the expression level seemed lower compared with the preparations exposed to an electrical stimulation (Fig. 6B). It also appeared that expression of COX-2 increased in a time-dependent manner (Fig. 6, B and Ca and b), suggesting that the electronic stimulation to the atrium might augment the COX-2 expression. Immunohistochemical analysis revealed the presence of COX-2 staining at the endocardial endothelium located around the surface of the cardiac muscles (Fig. 7, A and B, arrowheads). The staining was not present in a section of the same sample without COX-2 antibody (Fig. 7, C and D). The presence of COX-2 staining was confirmed in two other samples (data not shown).

**Discussion**

In the present study, we showed that CCh produced a biphasic inotropic response: a transient decrease in contractile force followed by a late increase in mouse isolated left atrial preparation. The same biphasic response by application of ACh in mouse isolated left atria was reported previously (Nishimaru et al., 2000), and the authors showed that the muscarinic M2 receptor-Gi system mediated the negative inotropic response. By using the selective pharmacologic inhibitors, we determined that the late positive inotropic re-
A response to CCh was caused by PGs, possibly PGE$_2$ and PGF$_{2\alpha}$. The production of PGs depends on COX-2 in endocardial endothelium through muscarinic M$_3$ receptor stimulation.

Because atropine completely inhibited the biphasic response to CCh, the muscarinic receptor mediates both inotropic responses. It is interesting to note that in the PTX-pretreated atria, CCh produced the markedly increased positive inotropic response compared with atria without PTX treatment, and no negative response was observed. Atropine antagonized the augmented positive response. Because it is well known that PTX works as the uncoupler of the G$_i$ protein-related response, the CCh-induced negative response is

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**Fig. 5.** Influences of endocardial endothelium on CCh-induced contractile responses in mouse isolated left atrial preparation. A, in the atrium, which was denuded of its endocardial endothelium by Triton X-100 (0.1%), the positive inotropic response to CCh was significantly reduced. B, the positive inotropic response to PGF$_{2\alpha}$ (10 nM) was confirmed in the same preparations. *, $p < 0.05$ compared with control.

**Fig. 6.** Changes in COX-2 expression in mouse isolated left atria. Actual traces of the inotropic effect of CCh (3 $\mu$M) in the mouse isolated left atrium (A): a, the peak of the positive inotropic responses induced by CCh (7 min); b, 30 min after CCh treatment. Western blotting examples of freshly dissected left atrial preparations (B) and at the time shown in A, a and b, and C. Equal loading of protein was confirmed by measuring total actin expression. Control: time-matched (7 and 30 min) control without CCh. COX-2 expression is shown as -fold increase relative to control (D).
the muscarinic M_R receptor-G_ protein-coupled reaction. After activation of cardiac M_R receptor in atrium, the βγ subunit of the G_ protein directly activates the inwardly rectifying muscarinic K channel, i.e., the ACh receptor-operated K channel (Yamada et al., 1998). Although the M_R receptor is a predominant muscarinic receptor subtype presented in heart, the presence of low levels of non-M_R muscarinic receptors including M_ receptor subtype was reported (Pönicke et al., 2003; Wang et al., 2004; Myslivecek et al., 2008). Muscarinic M_R-receptor blockade by 4-DAMP almost completely abolished the positive response to CCh with slight inhibition of the negative response in the present study. It was recently demonstrated in the M_ receptor knockout mice that M_R receptors mediate positive inotropic responses in mouse atria (Kitazawa et al., 2009). The negative response persisted for a long duration in the 4-DAMP-treated and the endothelium-deprived mouse atria similar to the response observed in rat and guinea pig. These results suggest that the biphasic inotropic response to CCh in the mouse atria consisted of the positive inotropic response over the long-lasting negative response. Thus, the augmented positive response in the PTX-treated preparation was explained by blockade of the negative response through the inhibition of M_ receptor-G_ protein mechanism.

It was reported that application of PGs elicited positive inotropic responses in rat (Canga et al., 1981), guinea pig (Sakuma et al., 1989), and mouse (Tanaka et al., 2001) atrial preparations. Jaiswal et al. (1988) reported that a nonselective COX inhibitor, indomethacin, blocked the output of PGs caused by ACh in the rabbit isolated heart. Consistent with previous reports, indomethacin completely abolished the positive inotropic response to CCh in the present study. The abolishment of the positive inotropic response to ACh by indomethacin in mouse atrial tissue was also seen in the previous report (Tanaka et al., 2001). In the same report, although there was no direct evidence, the importance of PGF_2α, PGE_2, and PGD_2 for the positive inotropic effect to ACh was suggested. More selective inhibitors of PG receptors were used for modulation of CCh-induced responses in the present report. The selective EP receptor antagonist, AH8809 (Woodward et al., 1995), and the selective FP receptor antagonist, AL8810 (Griffin BW et al., 1999), but not the selective TP receptor antagonist, SQ29,548 (Ogletree et al., 1985), markedly inhibited the positive inotropic responses to CCh. Thus, it is suggested that PGs, especially PGE_2 and PGF_2α, formed by CCh stimulation produced the positive inotropic response in mouse isolated left atrial preparation. In another set of experiments, we confirmed the inhibitory effect of the prostanoid receptor antagonists on the inotropic response to PGs. A selective inhibitor for FP receptor, AL8810, significantly inhibited the inotropic response to PGF_2α. On the other hand, the inhibitory effect of AH6809 on the PGE_2-induced inotropic response was slight, although it was statistically significant. Although AH6809 is used as the selective EP receptor antagonist, it was reported that AH6809 had the affinity to various types of prostanoid receptors (Abramovitz et al., 2000). Therefore, the inhibitory effects of AH6809 on the positive inotropic response to CCh (Fig. 3A) might involve not only blockade of PGE_2-induced inotropism but also other kinds of PGs-induced inotropism.

Indomethacin inhibited the positive response to CCh in the present study. Valeroyl salicylate (Bhattacharyya et al., 1995), a selective COX-1 antagonist, did not affect the biphasic response. NS-398, a selective COX-2 antagonist, significantly inhibited the positive response in a concentration-dependent manner. COX-2 is generally acknowledged as an inducible COX. It is impossible to elucidate the induction of COX-2 by CCh application, because appearance of the positive inotropic response was very rapid after CCh application, approximately 2 min in normal condition and within 1 min in PTX-treated preparation. Instead, our results suggest that COX-2 expression was induced in a time-dependent manner. COX-2 expression was induced in a time-dependent manner. COX-2 expression was induced in a time-dependent manner. COX-2 expression was induced in a time-dependent manner.
X-100, CCh-induced positive response was significantly reduced. Tanaka et al. (2001) showed that exogenously applied PGF$_{2\alpha}$ produced the positive inotropic action in atria treated with Triton X-100. The positive inotropic response to PGF$_{2\alpha}$ was confirmed in the same endothelium-denuded preparations in our experiment. Malik and colleagues (Tyagi et al., 1996) reported that ACh induced the release of PGs in isolated ventricular myocytes from rabbit heart. In their preparations, there was no endocardial endothelium. In the present experiment, the slight positive inotropic responses to CCh in endothelium-denuded atria remained up to approximately 25% from the nadir (Figs. 1F and 5A). These slight positive responses would be caused by PGs from cardiomyocyte itself.

Western blot analysis of atrial lysates revealed that COX-2 presented in the preparation of time-matched control, i.e., atria under continuous electrical stimulation without CCh application. Moreover, at the time of peak positive response after CCh application, COX-2 expression seemed to decrease slightly but not significantly. By immunohistochemical staining of the preparation of time-matched control, COX-2 was confirmed in the endocardial endothelium. From these results, it was considered that COX-2 was expressed in the endocardial endothelium of mouse left atria after continuous electrical stimulation and that COX-2 was activated by CCh treatment to synthesize PGs. Thus, COX-2 expressed in endocardial endothelium had an important role in induction of CCh-induced positive response. Furthermore, muscarinic stimulation through M$_3$ receptor on the endothelium achieves its role as a trigger for activation of COX-2. Jaiswal et al. (1989) previously reported that 4-DAMP blocked the PG production by ACh in the isolated perfused rabbit heart. Thus, direct relationships between muscarinic M$_3$ receptor and PG production were considered in heart.

In conclusion, the present study demonstrated that CCh induced both negative and positive inotropic responses in mouse left atria. Consistent with previous reports, the negative response is mediated by muscarinic M$_3$ receptor-G$_i$ system. The late positive inotropic response is mediated by PGs, possibly PGE$_2$ and PGF$_{2\alpha}$, released partly from endocardial endothelium. The production of PGs depends in part on COX-2 in endocardial endothelium through the muscarinic M$_3$ receptor stimulation.

References

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