Original Paper

Protective Role of Cardiac CFTR Activation Upon Early Reperfusion Against Myocardial Infarction

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Abstract
Background: The cardiac isoform of the cystic fibrosis transmembrane conductance regulator (CFTR) was shown to be activated by β-adrenergic or purinergic stimulation and involved in cell volume regulation after osmotic swelling. Also, cardiac CFTR was reported to be essential in the mechanism by which ischemic preconditioning protects against ischemia/reperfusion(I/R)-induced injury of the heart. Here, we explored the possibility that activation of cardiac CFTR can provide protection against I/R-induced myocardial infarction, even after ischemic attack. ***Methods:*** The hearts of wild-type mice were subjected to 30- or 40-min left coronary artery occlusion followed by 2-h or 2-day reperfusion *in vivo*, and myocardial infarction was examined under a variety of conditions. Neonetal rat ventricular myocytes in primary culture were subjected to hypoxia/reoxygenation *in vitro*, and necrotic cell death was examined. ***Results:*** The infarct size was much greater in CFTR knockout mice than in wild-type mice. Intravenous infusion of a number of putative CFTR activators upon reperfusion prominently reduced the size of myocardial infarction in wild-type but not CFTR-deficient mice. This protective effect was abolished by co-administration of a CFTR inhibitor. CFTR activators ameliorated, in a manner sensitive to a CFTR inhibitor, release of myocardial-specific creatine kinase isoenzyme to the serum in mice subjected to I/R *in vivo*. Necrotic death of cultured neonatal rat ventricular myocytes subjected to hypoxia/reoxygenation *in vitro* was ameliorated by CFTR activators or CFTR gene overexpression but aggravated by a CFTR inhibitor or CFTR gene silencing. ***Conclusion:*** It is concluded that activation of endogenous myocardial CFTR upon early reperfusion is involved in protection against necrotic myocardial injury induced by I/R *in vivo* and *in vitro*. Cardiac CFTR may serve as a target accessible even after ischemic attack for pharmacotherapeutic intervention in I/R-induced myocardial infarction.

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Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl⁻ channel activated by protein kinase A (PKA) and protein kinase C (PKC), and is a regulator of a number of other ion channels [1, 2]. CFTR is principally expressed in a variety of epithelial tissues and plays an essential role in transepithelial Cl^- and water movement [1]. A splice variant of CFTR, in which the product of the 5th exon is deleted, is expressed in cardiac myocytes [3]. Electrophysiological studies have provided evidence for functional activation of the cardiac CFTR Cl⁻ channel by stimulation of the β-adrenergic receptors [4, 5] and purinergic receptor [6].

Activation of cardiac CFTR Cl^- currents under β-adrenergic stimulation was shown to be involved in the hypoxia-induced early shortening of action potentials in perfused and paced rabbit hearts [7]. It was also found that under glucose-free hypoxic conditions, molecular and functional expression of sarcolemmal CFTR was transiently enhanced in neonatal rat ventricular myocytes [8]. Cardiac CFTR Cl^- conductance activated by β-stimulation after osmotic swelling in isolated guinea pig ventricular myocytes was shown to play an essential role in the process of cell volume regulation called regulatory volume decrease (RVD) [9]. Persistent cell swelling coupled to impaired RVD has been implicated in the induction of necrotic cell death in a number of pathophysiological contexts including ischemia [10]. Enhanced RVD due to anion channel-mediated Cl^- efflux was actually found to be a key mechanism by which ischemic preconditioning (IPC) protects against ischemic cell injury in rabbit ventricular myocytes [11, 12]. Furthermore, in isolated mouse hearts subjected to ischemia/reperfusion (I/R) insult, cardiac CFTR was shown to be essential for IPC-induced protection against necrosis [13] or apoptosis [14] and also for cardioprotection mediated by postconditioning (POC) [14]. The question therefore arises as to whether activation of cardiac CFTR after ischemic attack can also protect cardiac myocytes against the necrotic cell death induced by I/R. The purpose of this study is to answer this question using *in vivo* I/R experiments in the hearts of wild-type (WT) and CFTR knockout (CFTR⁻/⁻) mice and using *in vitro* hypoxia/reoxygenation experiments in rat ventricular myocytes in primary culture.

Here, we show that activation of endogenous myocardial CFTR upon early reperfusion exerts a protective action, which is unrelated to preconditioning, against necrotic myocardial injury by I/R *in vivo* and *in vitro*. Cardiac CFTR may serve as pharmacotherapeutic target even after an ischemic attack to protect against myocardial infarction.

Materials and Methods

Animals

Animal experiments were performed in accordance with the guidelines of the Physiological Society of Japan. All procedures involving animals were approved in advance by the Ethics Review Committee for Animal Experimentation of the National Institutes of Natural Sciences. WT male C57BL/6J mice (9–10 weeks old) and CFTR⁻/⁻ *B6.129P2-Cftr^tm1Unc* mice were obtained from CLEA Japan, Inc. (Tokyo, Japan) and Jackson Laboratory (ME, USA), respectively, and used in *in vivo* experiments. Wistar rats were obtained from Japan SLC, Inc. (Shizuoka, Japan) and used in *in vitro* experiments. The animals were housed with regulated lighting (light/dark cycle of 12 h/12 h) at 24 ± 1°C. Homozygous CFTR⁻/⁻ mice were generated by mating heterozygous CFTR⁺/⁻ siblings. Genotyping of littermates was performed by PCR on tail DNA using a 3-primer assay. To prevent CFTR⁻/⁻ mice from dying due to ileus, they were fed with a liquid diet (Liquidiet F3107: Bioserve, NJ, USA) using glass liquid feeders until 9 to 13 weeks after breast-feeding for 15 days, as reported previously [15].

Myocardial ischemia/reperfusion in vivo

The *in vivo* experiments were performed using WT and CFTR⁻/⁻ mice under the constant conditions of ~24°C, ~50% moisture and <1005 hPa. The mice were anesthetized with sevoflurane (Maruishi Pharmaceutical, Osaka, Japan) vaporized by 20% oxygen and 80% dinitrogen monoxide using a rodent

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respirator (Model 683: Harvard Apparatus, MA, USA). The temperature around the heart was measured by a thermistor and maintained at 36.4 ± 0.4°C by perfusing the thorax with warm saline via an automatic temperature controller (TC324B: Warner Instruments, CT, USA). The left coronary artery was ligated with an 8-0 silk suture for 30 or 40 min. Ischemia was confirmed by bleaching of the myocardium and the ECG pattern monitored via a DEN-751S Amplifier (Unique Medical, Tokyo, Japan). Reperfusion was initiated by releasing the ligature and visually confirmed. Reperfusion was continued for 2 h or 2 days. In sham-operated animals, the suture was placed beneath the left coronary artery without ligation. To administer chemical agents, the right cervical vein was cannulated. The test chemicals were administered by a one-shot injection of a given dose followed by a 42-min infusion of an additional one-third of the same dose per hour (protocol-i in Fig. 1A), by a 12-min infusion of a given dose (protocol-ii in Fig. 1A; protocols in Figs. 2A and 5A), or by a one-shot injection of a given dose alone (protocol-iii in Fig. 1A), using a syringe for one-shot injections and a syringe-pump (KDS-100: Muromachi Kikai, Tokyo, Japan) for continuous infusions.

Infarct size determination

At the end of reperfusion, the left coronary artery was re-ligated in the same location as before, and Evans blue dye (1.5%) was infused into the left ventricle from the apex cordis in order to distinguish the unstained ischemic zone (i.e., the area at risk) from the blue-stained non-ischemic zone. The heart was excised and sliced into 1-mm-thick cross-sections. *Ex vivo* incubation of the heart sections in 1.5% 2,3,5-triphenyltetrazolium chloride (TTC: Wako Pure Chemical, Osaka, Japan) for 5 min at 37°C allowed differentiation between red-stained viable and pale (unstained) infarcted areas. The left ventricular area (LVA), the area at risk (AAR) and the area of infarction (INF) for each slice were measured with ImageJ software (NIH, MD, USA). The INF/AAR value was determined and used as a measure of infarct size.

Hemodynamics

Effects of chemical agents on the hemodynamic parameters of the heart were examined during the I/R process in a separate group of WT mice. Heart rate and arterial blood pressure were recorded via the left groin artery with a disposable transducer (MLT0670: AD Instruments) connected to an amplifier (ML110: AD Instruments).

Creatine kinase assay

To monitor myocardial necrotic injury, the level of serum creatine kinase MB isoenzyme (CK-MB) of total creatine kinase (CK, EC 2.7.3.2) in blood samples collected via the carotid was measured by the immunoinhibition assay using the CK-MB isoenzyme kit (L-Type CK-MB(2): Wako Pure Chemical) and by an agarose gel electrophoresis method using the CK isozyme kit (Titan Gel CK: Helena Laboratories, TX, USA). The CK-MB values measured by immunoinhibition assay showed a very good correlation (the coefficient: $r = 0.99749$) with those measured by an electrophoresis method (data not shown, n = 6). Therefore, most CK-MB measurements were performed by the immunoinhibition assay. The serum was stored at −80°C until the measurement and used for assays within one month.

Western blot for in vivo specimens

Hearts were excised from WT mice before and after ischemia induced by ligation of the left coronary artery with or without subsequent reperfusion. The left ventricle was then quickly trimmed, frozen in liquid nitrogen and powdered. The sample of ~80 mg was immersed in ice-cold lysis buffer containing (in mM) 10 Tris-HCl (pH 7.3), 1 EDTA, 1 EGTA, 4 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (an irreversible serine protease inhibitor, Pefabloc: Roche Diagnostics, Basel, Switzerland), 0.4 N-(benzyloxycarbonyl) leucinylleucinylleucinal (a specific, potent, reversible, and cell-permeable proteasome inhibitor, MG132: Sigma-Aldrich), 1 sodium orthovanadate (Sigma-Aldrich) and 10 µl/ml protease inhibitor cocktail (Sigma-Aldrich), and incubated on ice for 30 min. The tissue was then homogenized using a pre-cooled homogenizer by repeating 8 strokes 5 times at intervals of 30 s. The same homogenization procedure was repeated 5 times more after adding an equal volume of sucrose buffer containing (in mM) 10 Tris-HCl (pH 7.3), 1 EDTA, 1 EGTA, 500 sucrose, 4 pefabloc, 1 sodium orthovanadate and 10 µl/ml protease inhibitor cocktail. The lysates were centrifuged at 3,000×g for 15 min at 4°C, and the supernatants were centrifuged again at 100,000×g for 30 min at 4°C. The high-speed pellets were dissolved in a 3.5×volume of SDS sample buffer containing 100 mM DTT (Nacalai Tesque, Kyoto, Japan) and 10 µl/ml protease inhibitor cocktail. After heating at 65°C for

3.5 min, the samples were run on a 7.5% SDS-PAGE gel and transferred onto PVDF membranes (Millipore, MA, USA). The blocking and reaction with antibody were performed using an advanced ECL kit (Amersham Biosciences, NJ, USA). The blots were incubated with rabbit polyclonal anti-CFTR antibodies (Alomone Labs, Jerusalem, Israel) overnight at 4°C, and then with horseradish peroxidase-conjugated secondary anti-rabbit antibodies (Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature. Enhanced chemiluminescence was detected by a Typhoon 9400 (GE Healthcare Biosciences, NJ, USA). The protein assay was performed with a 2-D Quant Kit (GE Healthcare Biosciences). As an internal standard for quantitative comparison, Na,K-ATPase $\alpha 1$ was simultaneously detected using an anti-Na,K-ATPase $\alpha 1$ antibody (Santa Cruz Biotechnology). As a positive control for CFTR, rat embryonic heart H9c2 cells transfected with CFTR were employed. H9c2 cells (ATCC number CRL-1446) were grown in DMEM supplemented with 10% fetal bovine serum and 4 mM glutamine. Transfection of the pCINeo-IRES-GFP vector containing a CFTR insert into H9c2 cells was carried out using both Lipofectamine LTX and PLUS (Invitrogen, CA, USA) according to the manufacturer's instructions. After 48 h of transfection, cells were harvested, dissolved in SDS sample buffer, heated at 65°C for 3.5 min, and applied to an SDS-PAGE gel for Western blot.

Ventricular myocytes in primary culture

Primary cultures of neonatal rat ventricular myocytes were prepared as described previously [8]. In brief, the hearts were excised from neonatal rats (day 3), and the ventricles were isolated and minced in 0.05% trypsin-EDTA. After removing more easily attaching non-myocardial cells, the dissociated cells were cultured in Medium 199 (M199: GIBCO-Invitrogen, CA, USA) supplemented with 10% fetal bovine serum. To inhibit fibroblast proliferation, bromodeoxyuridine (0.1 mM) was added to the culture medium continuously except during the experiment period.

Simulated myocardial ischemia/reperfusion in vitro

The *in vitro* experiments were performed using cultured rat ventricular myocytes maintained at 37°C and 95% air *plus* 5% CO_2. After replacing the culture medium with ischemia buffer, the cells were subjected to severe hypoxia by placement in a low-oxygen chamber in which air was substituted with argon. Since the partial pressure of oxygen (pO_2) in the ischemic heart *in vivo* was reported to rapidly reach (within 10 min) a level of less than 2 mmHg [16], we used ischemia buffer composed of one part oxyrase (Oxyrase Inc., OH, USA) and nine parts glucose-free buffer solution containing (in mM) 108 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 5 HEPES and 2.5 Tris (pH adjusted to 7.4 with $NaHCO_3$; osmolality adjusted to 300 mosmol/kg-H_2O with mannitol). This hypoxia treatment using oxyrase caused the pO_2 value, which was monitored by a pO_2 probe (LICOX CMP Monitor: GMS mbH, Mielkendorf, Germany), to fall much more rapidly (to less than 2 mm-Hg within 20 min) compared with a hypoxia treatment without oxyrase (to less than 2 mm-Hg after over 200 min). Reperfusion was performed by replacing ischemia buffer with Dulbecco's Modified Eagle's Medium (DMEM) containing 1% fetal bovine serum in an incubator equilibrated with 95% air *plus* 5% CO_2. Test chemical agents were added directly to ischemia buffer and DMEM.

Propidium iodide staining and caspase assay

Necrotic and apoptotic cell death was monitored by propidium iodide (PI) uptake and caspase 3/7 activation 120 and 300 min after reperfusion, respectively, in cultured cardiomyocytes plated on a 98-well plate coated with Cell Matrix (Type I-C: Nitta Gelatin, Osaka, Japan) at a density of 8×10^5/ml at 37°C and 5% CO_2. PI staining was performed by incubating the cells with 0.5 µg/ml PI (Dojindo, Kumamoto, Japan) and 30 µg/ml Hoechst 33342 for 10 min. The percent of PI-positive cells to total (around 3000) Hoechst 33342-positive cells was determined with an In Cell Analyzer 1000 (GE Healthcare Biosciences, NJ, USA). Caspase 3/7 activity was measured using an Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, WI, USA) according to the manufacturer's instructions. For the positive control, activation of caspase 3/7 was induced by applying 4 µM staurosporine (STS) (Sigma Aldrich, MO, USA) for 300 min.

Overexpression of CFTR gene

For CFTR overexpression in neonatal rat ventricular myocytes in primary culture, the vector construction was made as previously described [17]. In brief, the coding regions of rat CFTR cDNA were subcloned into a bicistronic vector, pCINeo-IRES-GFP. As a negative control, mock transfection was made with pCINeo-IRES-GFP vector without CFTR insert. To facilitate the transfection, MATra magnet-assisted

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transfection system (IBA, Göttingen, Germany) was employed. Cardiomyocytes were seeded on a 96-well plate coated with Cell Matrix (Type I-C) at a density of 8×10^5/ml. Mixture of DNA (0.4 μg/well), MA Lipofection Enhancer reagent (0.2 μl/well) and IBAfect reagent (0.6 μl/well) was prepared according to the manufacturer's protocol. The mixture of 25 μl was add to each well then the culture plates were placed on the MATRa magnet plate for 20 min at room temperature. Cells were then kept in the incubator and studied on day 3 post-seeding (*i.e.* 2 days after transfection).

To assess the effect of transfection with CFTR cDNA in cultured neonatal rat ventricular myocytes, immunoblotting was performed. After 48 h of transfection with cDNA of CFTR or vector alone, the cardiomyocytes were harvested, dissolved in SDS sample buffer, heated at 65°C for 3.5 min, and applied to an SDS-PAGE gel.

Knockdown of CFTR gene

CFTR gene knockdown was performed with small interfering RNA (siRNA) in neonatal rat ventricular myocytes in primary culture. Cardiomyocytes were plated on a 96-well plate coated with Cell Matrix (Type I-C) at a density of 8×10^5/ml. On day 1 post-seeding, cells were transfected with 25 nM siRNA against rat CFTR using HiPerfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Gene-specific siRNA against CFTR was purchased from Sigma-Aldrich: sense (5′ CUGAUAGUCUUGGUUAUUUTT 3′) and antisense (5′ AAAUAACCAAGACUAUCAGTT 3′). Cells were studied on day 3 post-seeding (*i.e.* 2 days after transfection with siRNA). Fluorescence-labeled non-targeting siRNA (ALL STAR: Qiagen) was used as a negative control. In control experiments, almost all cells were found to be fluorescence-positive one day after transfection, showing successful transfection.

To assess siRNA-induced changes in the CFTR gene expression in cultured neonatal rat ventricular myocytes, semi-quantitative RT-PCR was performed. Total RNA was isolated from control or gene-specific siRNA-transfected cells using SEPASOL (Nacalai Tesque, Kyoto, Japan). Contamination of genomic DNA in total RNA was removed by treatment with DNase1 (RT-grade: NIPPON GENE, Tokyo, Japan). Total RNA was used for synthesis of cDNA using oligo-dT primer (Invitrogen, CA, USA) and GoScript™ Reverse Transcription System (Promega). Sequences of primers to amplify rat CFTR and GAPDH as an internal control are as follows: CFTR (forward, 5′-ATAGCACACTGAACATCACCG-3′; reverse, 5′-TGAAGAGGCTCAGAGCAAGC-3′), and GAPDH (forward, 5′-CATGCCGCCTGGAGAAACCTGCCA-3; reverse, 5′-GGGCTCCCCAGGCCCCTCCTGTTG-3′).

Test chemical reagents

The following chemicals were applied to test the effects on I/R injury *in vivo* and/or simulated I/R injury *in vitro*. Genistein was purchased from Wako Pure Chemical (Osaka, Japan), milrinone, gemfibrozil, daidzein, and staurosporine (STS) were from Sigma-Aldrich (MO, USA), AG18 (tyrphostin A23) and ICI 182780 were from Tocris Bioscience (MO, USA) as well as CFTRinh-172 was from Calbiochem (CA, USA), and these chemicals were dissolved in DMSO to obtain their stock solutions. Dibutyryl cyclic AMP (dbcAMP) (Sigma-Aldrich) and phorbol 12-myristate 13-acetate (PMA: Wako Pure) were dissolved in water and ethanol, respectively, to make their stock solutions. The final vehicle concentrations were less than 0.1%. The total doses of genistein, milrinone, PMA, gemfibrozil and CFTRinh-172 given for *in vivo* experiments were 1.2 or 1.5 mg/kg-BW, 50 or 62 μg/kg-BW, 4.8 or 5.9 μg/kg-BW, 43 mg/kg-BW and 0.7 mg/kg-BW, respectively; and the maximal concentrations they reached in the plasma were calculated to be 100 or 123 μM, 2.8 or 3.5 μM, 180 or 222 nM, 1.4 mM, and 20 μM, respectively, if accumulated without degradation. The concentrations of these reagents applied for *in vitro* experiments were as follows: 50 μM genistein, 2.8 μM milrinone, 100 nM PMA and 10 μM CFTRinh-172. These concentrations were selected on the basis of the pharmacological information, as follows: The reported IC_{50} values of genistein, milrinone, PMA, CFTRinh-172, and gemfibrozil are 39.7 μM [18], 2.4 μM [19], 0.01 μM [20], 1.16 μM [21], and 250 μM [22], respectively. The reported concentrations for the maximal effects of genistein, PMA, and CFTRinh-172 are 100 μM [18], 0.1 to 1 μM [23, 24], and 10 to 50 μM [24, 25], respectively. The reported dose for *in vivo* administration via intravenous infusion of milrinone is 50 μg/kg-body weight (BW) for clinical intervention in human ischemic disease [26], and that of CFTRinh-172 is 0.6 to 3 mg/kg-BW for antidiarrheal efficiency in rodents [27]. It must be noted that the blocking effect of CFTRinh-172 was reported to be selective to the CFTR channel compared to other types of anion channel [28], but was not so far examined about its selectivity against cardiac cation channels, as done for another CFTR inhibitor, GlyH-101 [29]. Since the IC_{50} value of AG18 for tyrosine kinase inhibition and the effective concentration to inhibit a chloride channel

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Table 1. The ratio of area at risk per left ventricular area (AAR/LV: %). Data are given as mean ± SEM. All the data administered with chemicals are not significantly different from the vehicle data (Dunnet-*t* test; *p* > 0.05).

	vehicle	genistein	milrinone+PMA		
WT:	Protocol-i	protocol-ii	protocol-i	protocol-ii	protocol-iii
protocols-Figure 1A	66.56 ± 5.09	73.93 ± 3.35	65.57 ± 2.69	72.37 ± 5.43	73.81 ± 4.96
experiments-Figure 1C	(n = 16)	(n = 8)	(n = 12)	(n = 7)	(n = 6)
WT:	vehicle	genistein	gemfibrozil	CFTRinh-172	genistein+ CFTRinh-172
protocol-Figure 2A	73.62 ± 2.51	74.30 ± 2.44	77.70 ± 4.27	72.20 ± 3.53	80.09 ± 4.01
experiments-Figure 2C	(n = 16)	(n = 12)	(n = 7)	(n = 7)	(n = 10)
CFTR⁻ᐟ⁻:	vehicle	genistein			
protocol-Figure 2A	69.08 ± 6.12	80.17 ± 4.86			
experiments-Figure 2D	(n = 7)	(n = 7)			

were reported to be 62 μM [30] and 100 μM [31], respectively, AG18 was applied for the present *in vitro* experiments at 100 μM.

Statistical analyses

Data are expressed as means ± SEM and were analyzed with the SPSS software package (11.0J: SPSS Japan Inc., Tokyo, Japan). For *in vivo* data, statistical multiple comparisons were performed by Peritz's closed testing procedure with the Kruskal-Wallis H statistic. Significance was judged so that the overall experimentwise error rate became <0.05. For *in vitro* data, one-way ANOVA analysis followed by the post-hoc Dunnett's *t* test was performed among multiple groups, and *t*-test was performed between two groups.

Results

Myocardial ischemia/reperfusion injury in vivo is ameliorated by CFTR activators administered upon reperfusion

To examine a possible role of CFTR during a time period that included 10-min pre-ischemia, 30-min ischemia and 2-min early reperfusion phases, we first observed the effects of intravenous administration of CFTR activators by a one-shot injection followed by a 42-min continuous infusion of CFTR activators on I/R injury, as shown in the *in vivo* experimental protocol given in Fig. 1A (protocol-i). The isoflavonoid genistein (1.5 mg/kg-body weight (BW)), which is known to activate CFTR anion channels in cardiomyocytes [32, 33], markedly decreased the myocardial infarction (pale area unstained with TTC) produced by 30-min ischemia followed by 2-day reperfusion (Fig. 1B). Co-administration of an inhibitor of phosphodiesterase type III (PDEIII), milrinone (62 μg/kg-BW), which should increase intracellular cyclic AMP thereby stimulating PKA, and a PKC activator, phorbol 12-myristate 13-acetate (PMA: 5.9 μg/kg-BW), also provided prominent protection of the ventricle from I/R injury (Fig. 1B). As quantitatively summarized in Fig. 1C, genistein reduced the infarct size assessed by INF/AAR by half, and milrinone *plus* PMA reduced it by one third, without significantly affecting the ratio of the area at risk to the left ventricular area (AAR/LVA) in either case (Table 1).

There are several time periods during which these chemicals exerted a protective action on the ventricle from I/R injury: the 10-min pre-ischemia, 30-min ischemia, early reperfusion, and late reperfusion phases. To discriminate amongst the possibilities, we next compared the effects of milrinone *plus* PMA administered by protocol-i with the effects of the same chemicals at an 80% dose administered by a 12-min infusion starting 10 min before reperfusion (protocol-ii: Fig. 1A), and by a one-shot injection given 10 min after starting reperfusion (protocol-iii: Fig. 1A). As summarized in Fig. 1C, protocol-ii resulted

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Fig. 1. Effects of intravenous administration of CFTR activators on myocardial I/R injury *in vivo* in WT mice. (A) Experimental protocols. At end of 2-day reperfusion, infarct size was measured after staining with TTC. Chemicals were intravenously applied by a one-shot injection followed by continuous infusion (protocol-i), by a continuous infusion (protocol-ii) or by a one-shot injection (protocol-iii). (B) Representative cross-sections of TTC-stained left ventricles from animals administered vehicle alone, genistein or milrinone *plus* PMA by protocol-i. (C) Mean infarct size, calculated as the INF/AAR values (± SEM: bar; n = 6–16), after treatment with vehicle alone, genistein or milrinone *plus* PMA administered by protocol-i, and by protocol-ii and protocol-iii for milrinone *plus* PMA. *$p < 0.05$ and NS: $p > 0.05$ between two data designated.
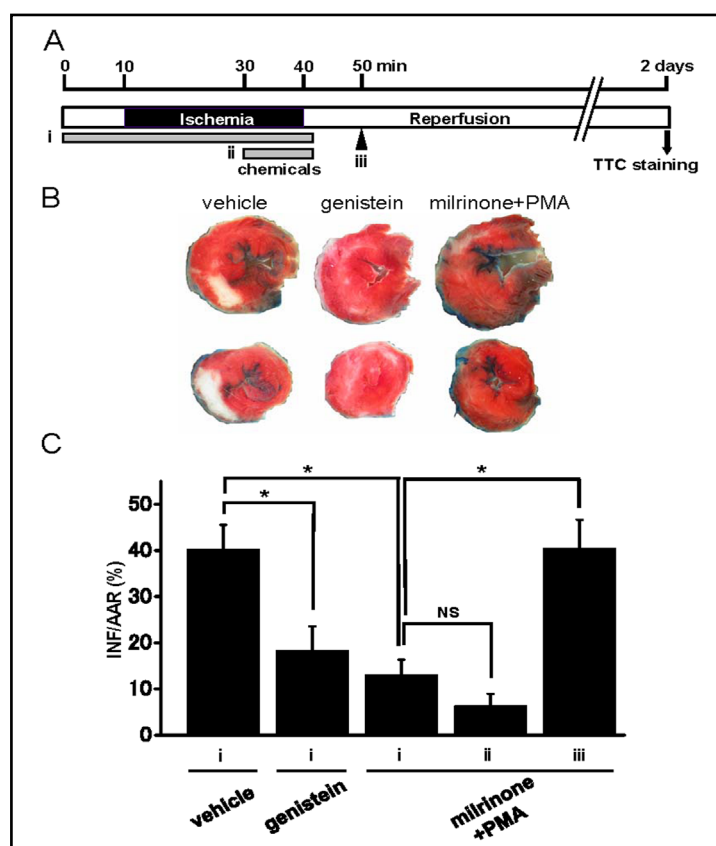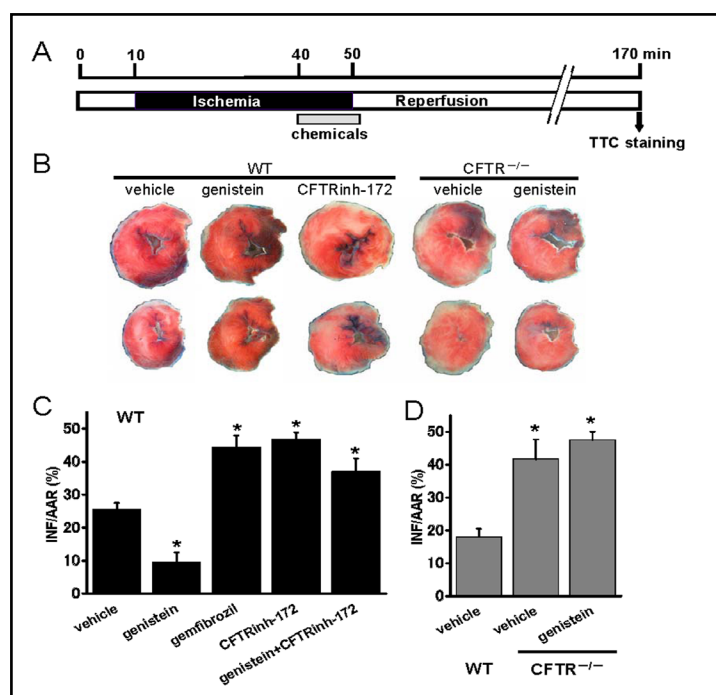

Fig. 2. Effects of intravenous administration of a CFTR activator (genistein) and a CFTR inhibitor (CFTRinh-172 or gemfibrozil) on myocardial I/R injury *in vivo* in WT and CFTR$^{-/-}$ mice. (A) Experimental protocol employed in this experiment. At end of 120-min reperfusion, the hearts were stained with TTC. (B) Representative cross-sections of TTC-stained left ventricles of WT mice and CFTR$^{-/-}$ mice administered genistein or CFTRinh-172 under I/R. (C and D) Mean infarct size, calculated as the INF/AAR values (± SEM: bar; n = 7–16 for C and 7–8 for D), after treatment with vehicle alone and chemicals in WT mice (C) and CFTR$^{-/-}$ mice (D). *$p < 0.05$ *vs* the vehicle data in WT mice.

in a protective effect similar to that of protocol-i, without significantly affecting the AAR/LVA value (Table 1), whereas protocol-iii exhibited no protective effect. Since the chemicals administered by protocol-ii may not reach the tissue site during the ischemic period but may reach it only after reperfusion, these data indicate that the early reperfusion phase is the time period during which the tissue site is sensitive to these chemicals.
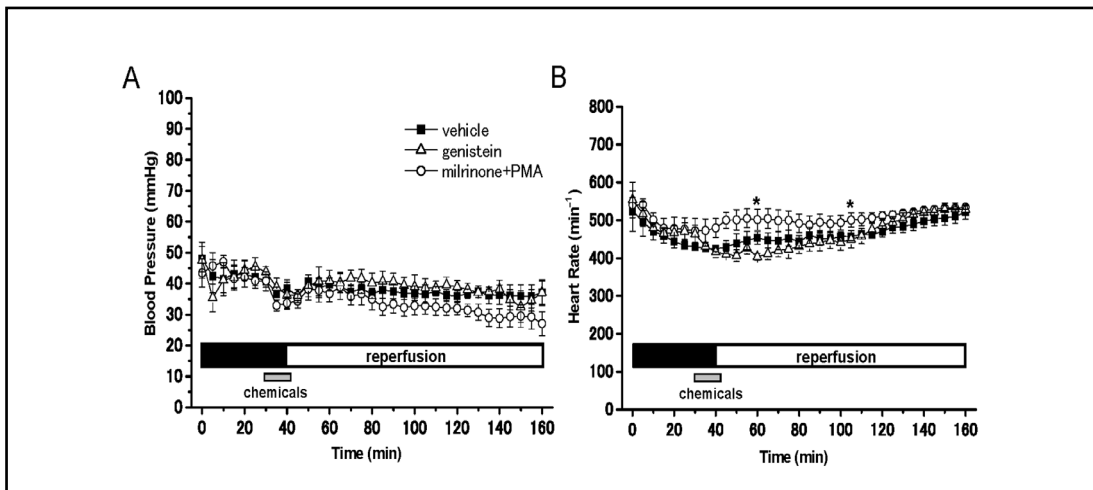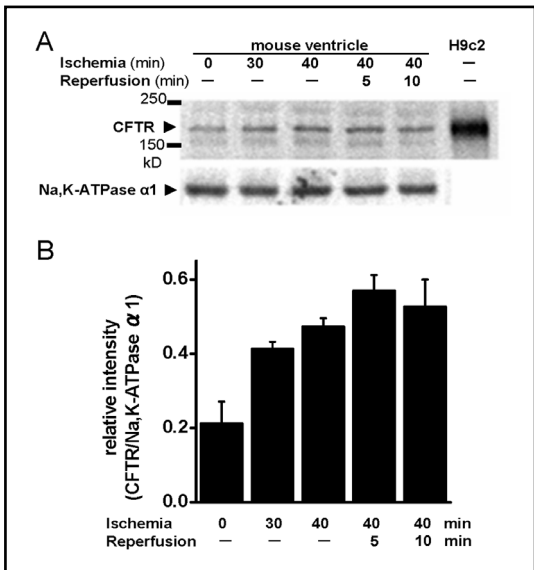
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Fig. 3. Effects of intravenous administration of genistein and milrinone *plus* PMA on time-dependent changes in blood pressure and heart rate in mice subjected to I/R *in vivo*. (A) Blood pressure. (B) Heart rate. *Insets*: Experimental protocol employed in this experiment. Error bars represent SEM (n = 4–5). *p < 0.05 *vs* the vehicle data at a given time point. It is noted that time-dependent changes in blood pressure were not significantly affected by genistein or milrinone *plus* PMA (A), and that those in heart rate were not significantly affected by genistein (B, *triangles*). Milrinone *plus* PMA slightly induced an enhancing effect on the heart rate, though the effect was statistically significant only at 60 and 105 min (B, *circles*).
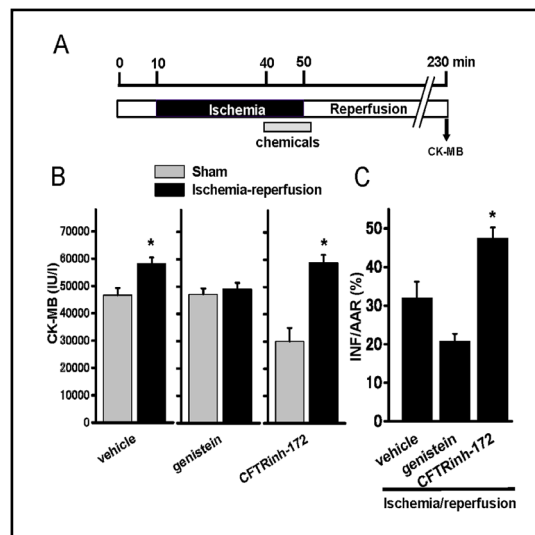
Fig. 4. Effects of ischemia and ischemia/reperfusion *in vivo* on molecular expression of CFTR in the ventricles of WT mice. (A) Representative Western blot data for CFTR protein and Na,K-ATPase α1 in mouse ventricles before (0 min) and 30 or 40 min after ischemia, followed by no reperfusion or 5- or 10-min reperfusion. The last lane shows CFTR protein signal derived from H9c2 cells transfected with CFTR, which serves as a positive control. (B) The mean values (± SEM, n = 4) for relative intensity of the CFTR bands, normalized with Na,K-ATPase α1 band intensity. There are no significant difference between the data at given time points and the data at 0 min.

Endogenous myocardial CFTR activity is involved in protection against ischemia/ reperfusion injury in vivo

Genistein (1.2 mg/kg-BW) administered by a 12-min infusion again markedly protected against myocardial infarction produced by 40-min ischemia followed by 120-min reperfusion *in vivo* (protocol shown in Fig. 2A), as shown in Fig. 2 (B and C). In contrast to genistein, a 12-min infusion with gemfibrozil (43 mg/kg-BW), which is a blocker of the CFTR channel [22], significantly increased the infarct size (Fig. 2C) without significantly affecting the AAR/LVA value (Table 1). Another CFTR inhibitor, 3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone (CFTRinh-172, 0.7 mg/kg-BW), similarly aggravated I/R injury (Fig. 2, B and C) without significantly affecting the AAR/LVA value (Table 1). Administration of CFTRinh-172 did not induce myocardial infarction

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Fig. 5. Effects of intravenous administration of a CFTR activator (genistein) and a CFTR inhibitor (CFTRinh-172) on the release of CK-MB to the blood plasma in WT mice subjected to I/R or a sham operation. (A) Experimental protocol employed in this experiment. At the end of 180-min reperfusion, the serum level of CK-MB was measured. (B) The mean values for increased serum CK-MB activity (± SEM; n = 3–7) after sham-operation or I/R with treatment with vehicle alone, genistein or CFTRinh-172. The serum CK-MB activity of normal mouse under anesthesia was 224.8 ± 117.1 IU/l (n = 5). Serum CK-MB activities were measured by the immunoinhibition assay. *$p < 0.05$ *vs* the Sham data. (C) The INF/AAR values (± SEM; n = 6) of mice from which serum samples were obtained for CK-MB measurements. * $p < 0.05$ *vs* the vehicle data.

in sham-operated mice (data not shown, n = 3). Furthermore, CFTRinh-172 completely eliminated the protective effect of genistein (Fig. 2C) with little effect on the AAR/LVA value (Table 1). These pharmacological data support the idea that activation of endogenous myocardial CFTR anion channels upon early reperfusion protects against I/R injury *in vivo*.

To avoid ambiguousness associated with pharmacological approaches, an involvement of CFTR endogenously expressed in the mouse heart *in vivo* was next studied by employing CFTR$^{-/-}$ mice. As shown in Fig. 2 (B and D), the infarct size in CFTR$^{-/-}$ mice administered vehicle alone was greater than that in WT mice administered vehicle alone, and reached a size similar to that in WT mice administered a CFTR blocker (Fig. 2C). Moreover, genistein failed to protect against I/R injury in CFTR$^{-/-}$ mice (Fig. 2, B and D), indicating that the actual target of genistein was CFTR under the present experimental conditions.

To examine the possibility that the protective effects of genistein and milrinone *plus* PMA were mediated by changes in the hemodynamics of the heart, blood pressure and heart rate were continuously monitored during a period of 40-min ischemia followed by 120-min reperfusion, with administration of CFTR activators by a 12-min infusion starting 10 min before reperfusion (protocol: see Insets in Fig. 3). Time-dependent changes in blood pressure were never significantly affected by genistein or by milrinone *plus* PMA (Fig. 3A). Although the heart rate was slightly enhanced after a 12-min infusion of milrinone *plus* PMA, that was not significantly affected by genistein (Fig. 3B). These data suggest that the target of these chemicals is myocardial CFTR. Actual expression of the myocardial CFTR protein before and after ischemia/reperfusion was confirmed by Western blot (Fig. 4). The CFTR protein expression never decreased but rather tended to increase by 30 to 40 min of ischemia, and the expression level did not significantly decrease even after 5 to 10 min of reperfusion (Fig. 4).

Taken together, these results are in agreement with the inference that the activity of myocardial CFTR endogenously expressed in the mouse heart is involved in protection against I/R injury *in vivo*.

Myocardial CFTR activity ameliorates necrotic injury induced by ischemia/reperfusion in vivo

Since ischemic myocardial necrotic injury is known to be reflected by increased serum activity of the myocardial-specific isoenzyme of creatine kinase (CK-MB) [34, 35], the level of serum CK-MB was measured after I/R in WT mice *in vivo* in the absence and presence of CFTR activator or inhibitor using the experimental protocol shown in Fig. 5A. Sham-operation *per se* was found to increase the serum CK-MB activity up to around 46000 IU/l
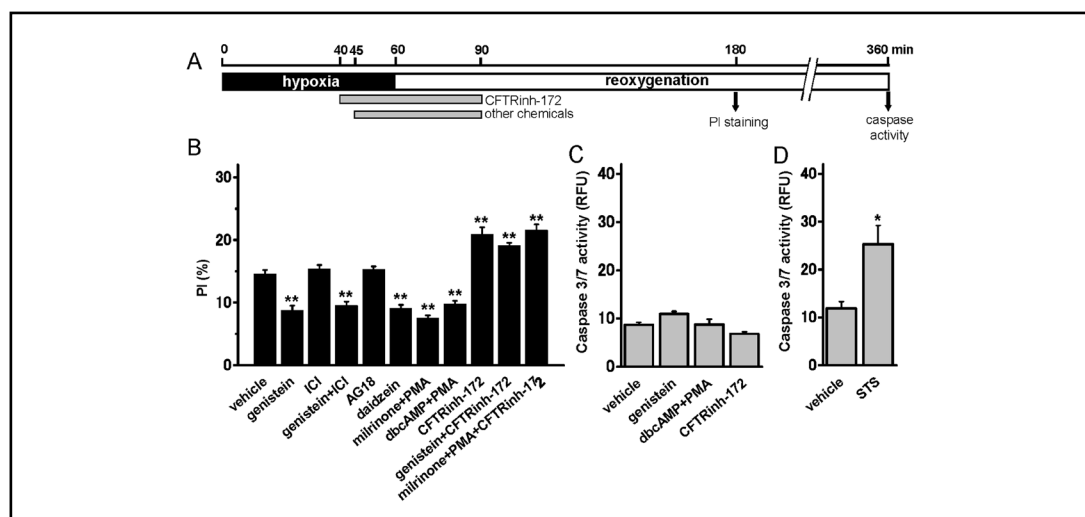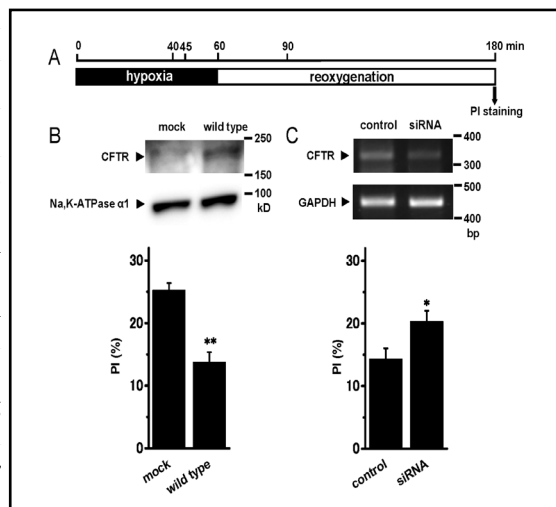
Fig. 6. Effects of extracellular application of chemicals on hypoxia/reoxygenation-induced necrosis and apoptosis in cultured neonatal rat ventricular myocytes. Following chemicals were applied: a CFTR activator (genistein, daidzein, milrinone *plus* PMA or dbcAMP *plus* PMA), a CFTR inhibitor (CFTRinh-172), an estrogen receptor antagonist ICI 182780 (ICI), a protein tyrosine kinase inhibitor (AG18) and an apoptosis inducer (STS). (A) Experimental protocol employed in this hypoxia/reoxygenation experiment. (B) The % of PI-positive necrotic cells measured at the end of 120-min reoxygenation. (C) The caspase 3/7 activity (expressed by relative fluorescence units: RFU) measured at the end of 300-min reoxygenation. (D) The positive control caspase 3/7 activity in cultured cardiomyocytes treated with 4 μM STS for 300 min. Values are means ± SEM (n = 9–18 for B, 8 for C and 3 for D). * $p < 0.05$ and ** $p < 0.001$ *vs* the vehicle data.

from the normal level (around 225 IU/l), as summarized in Fig. 5B (*Sham*), in agreement with previous reports that CK-MB release was induced by heart surgery, such as coronary bypass grafting and thoracotomy, even in patients in the absence of myocardial infarction [36-38]. After 40-min ischemia followed by 180-min reperfusion *in vivo* (protocol shown in Fig. 5A), the serum level of CK-MB further increased, as shown in Fig. 5B (*Left*), in WT mice administered vehicle alone. Necrotic CK-MB release was reduced to the level of sham-operation by administration of genistein (1.2 mg/kg-BW) by a 12-min infusion starting 10 min before reperfusion (Fig. 5B, *Middle*). Infusion of CFTRinh-172 (0.7 mg/kg-BW for 12 min) caused a more marked increase in the serum CK-MB activity in mice subjected to I/R compared to that observed in sham-operated mice administered CFTRinh-172 for 12 min (Fig. 5B, *Right*). The serum CK-MB levels after I/R with infusion of vehicle, genistein and CFTRinh-172 well correlated with the INF/AAR values (Fig. 5C).

Myocardial CFTR activity ameliorates necrotic injury induced by hypoxia/reoxygenation in vitro

Necrotic cell injury was also monitored by PI uptake in cultured neonatal rat cardiomyocytes in primary culture under simulated I/R *in vitro*, using the experimental protocol depicted in Fig. 6A. After 60-min hypoxia followed by 120-min reoxygenation, the % of PI-positive necrotic cells increased from the baseline value (7.2 ± 0.4%, n = 3) to approximately 15% (Fig. 6B, *vehicle*). However, the necrotic cell injury induced by hypoxia/reoxygenation was reduced to around 8% by a 45-min treatment (from 15 min before to 30 min after reoxygenation) with genistein (50 μM). Genistein is known to exhibit not only a CFTR-activating effect but also a weak estrogenic effect due to estrogen receptor binding [39] and an inhibitory effect on protein tyrosine kinases (PTKs) [40]. However, a selective estrogen receptor antagonist, ICI 182780 (10 μM), failed to affect hypoxia/reoxygenation-induced necrosis of cultured cardiomyocytes and to eliminate the ameliorating effect of

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Fig. 7. Effects of overexpression and knockdown of CFTR gene on hypoxia/reoxygenation-induced necrosis in cultured ventricular myocytes. (A) Experimental protocol employed in this hypoxia/reoxygenation experiment. (B and C) At the end of 120-min reoxygenation, the % of PI-positive necrotic cells were measured in cultured neonatal rat ventricular myocytes transfected with WT CFTR cDNA (B, *bottom panel*) and CFTR siRNA (C, *bottom panel*). Transfection efficiency was tested for CFTR cDNA by Western blotting (B, *upper panel*) and for CFTR siRNA by RT-PCR (C, *upper panel*). Na,K-ATPase α1 and GAPDH were analyzed as loading controls. Values are means ± SEM (n = 8 for B and 9 for C). ** $p < 0.001$ *vs* mock (B) and * $p < 0.05$ *vs* control (C).

genistein when applied together (Fig. 6B, *ICI*). Also, a PTK inhibitor, AG18 (100 µM), failed to mimic the genistein effect (Fig. 6B). Furthermore, the genistein effect was mimicked by daidzein (75 µM) (Fig. 6B), a structural analog of genistein that has little inhibitory effect on PTKs but is capable of activating CFTR anion channels [33]. Thus, it appears that genistein protects against hypoxia/reoxygenation *in vitro* through its action on CFTR channels but not through effects on estrogen receptors or PTKs. Actually, a similar protective effect was induced by treatment with other CFTR-activating chemicals, milrinone (0.6 µg/ml) *plus* PMA (100 nM) and dibutyryl cyclic AMP (dbcAMP) (1 mM) *plus* PMA (100 nM), as summarized in Fig. 6B. Also, treatment with a CFTR inhibitor, CFTRinh-172 (10 µM), increased the number of PI-positive cells to approximately 20% and totally eliminated the ameliorating effects of genistein and milrinone *plus* PMA (Fig. 6B). The activity of caspase 3/7 measured 300 min after reperfusion was not affected by genistein, dbcAMP *plus* PMA or CFTRinh-172 (Fig. 6C), although treatment with an apoptosis inducer STS (4 µM for 300 min) activated caspase 3/7 in cultured neonatal rat cardiomyocytes (Fig. 6D).

To directly demonstrate an involvement of CFTR in cultured rat cardiomyocytes, the effects of transfection with cDNA of or siRNA against CFTR were observed under simulated I/R using the experimental protocol given in Fig. 7A. Necrotic cell injury induced by hypoxia/reoxygenation was ameliorated by overexpression of CFTR gene (Fig. 7B). In contrast, simulated I/R-induced necrotic cell injury was aggravated by CFTR gene silencing (Fig. 7C)

Taken together, it is concluded that the activation of myocardial CFTR anion channels may protect against necrotic, but not apoptotic, injury induced by I/R *in vivo* and by simulated I/R *in vitro*.

Discussion

Molecular remodeling of a number of ion channels including CFTR is known to be induced in cardiac myocytes subjected to ischemic cardiomyopathy [41]. In fact, our previous study demonstrated that molecular and functional expression of cardiac CFTR did not decrease but becomes transiently upregulated by simulated ischemia *in vitro* in neonatal rat ventricular myocytes in primary culture [8]. Also, in the present study, molecular expression of the cardiac CFTR protein was found to never decrease but tend to be enhanced *in vivo* in the mouse ventricle after ischemia (Fig. 4).

Genistein, which is the best-studied potentiator of CFTR activity [33], effectively reduced I/R injury *in vivo* in the mouse heart (Figs. 1 and 2) and simulated I/R injury *in vitro* in rat cardiomyocytes (Fig. 6). The *in vivo* genistein effect was found to be abolished

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by a CFTR channel inhibitor, CFTRinh-172 (Fig. 2C). Also, as shown in Fig. 6B, the protective effect of genistein on hypoxia/reoxygenation-induced injury *in vitro* in rat cardiomyocytes was eliminated by CFTRinh-172 and was not mimicked by an estrogen receptor antagonist, ICI 182780, or a PTK antagonist, AG18. It could, however, be mimicked by a genistein analog, daidzein, which is known to activate CFTR and not inhibit PTKs. Moreover, genistein failed to affect I/R injury *in vivo* in CFTR-deficient mice (Fig. 2D). These results indicate that the target of genistein is cardiac CFTR.

Putative PKA and PKC phosphorylation sites are highly conserved in both the epithelial and cardiac isoforms of CFTR, and synergistic interactions between PKA- and PKC-mediated phosphorylation play an important role in the activation of epithelial [42] and cardiac CFTR [43]. In the present study, the combined application of a PDEIII inhibitor, milrinone, and a PKC activator, PMA, was found to reduce I/R-induced myocardial infarction *in vivo* (Fig. 1) and hypoxia/reoxygenation-induced cardiomyocyte death *in vitro* (Fig. 6). The protective effect of milrinone *plus* PMA *in vitro* was abolished by CFTRinh-172 and was mimicked by dbcAMP *plus* PMA (Fig. 6). This effect of milrinone on cardiac CFTR may well explain previous observations that pre-ischemic administration of milrinone protects against I/R-induced myocardial injury [44, 45].

CFTR is known to be expressed in vascular smooth muscle cells and to regulate vascular tone in a manner dependent on cyclic AMP [46]. Thus, there is a possibility that activation of vascular CFTR is involved in protection against I/R injury. However, in the present study, blood pressure was not affected by administration of CFTR activators (Fig. 3). Also, the protective effects of CFTR activators were reproduced *in vitro* in the simulated I/R system that used cultured cardiomyocytes and was devoid of vascular smooth muscle cells (Fig. 6).

It seems likely that endogenous activity of CFTR protects against I/R injury even without the administration of any CFTR activators, because a CFTR blocker (gemfibrozil or CFTRinh-172) aggravated I/R-induced myocardial infarction *in vivo* (Fig. 2) and hypoxia/reoxygenation-induced cardiomyocyte death *in vitro* (Fig. 6), though one cannot completely exclude possible non-specific actions of these CFTR activators and blockers. However, the present study showed that myocardial infarction observed in the ventricle of CFTR-deficient mice *in vivo* was more marked than that in the WT mice (Fig. 2), directly supporting an involvement of endogenous CFTR in the protection effect. A similar involvement of endogenous CFTR in cardioprotection against I/R injury was also shown for IPC in the *in vivo* models [13, 14] and for POC in the *ex vivo* models [14]. Such endogenous CFTR activation would be brought about by some endogenous activators which are massively released during ischemia, such as ATP from the cardiomyocytes themselves [47, 48] and catecholamines from sympathetic nerve endings [49].

Myocardial cell death associated with I/R injury occurs by two different mechanisms, necrosis and apoptosis [13, 14, 50]. In the present study, the I/R injury *in vivo* was found to occur in parallel with increases in the level of serum CK-MB, an index of the necrotic injury of cardiomyocytes (Fig. 5), suggesting the loss of membrane integrity largely due to necrosis. *In vitro* experiments in cultured cardiomyocytes using a simulated I/R protocol also showed necrotic cell death characterized by PI stainability and the lack of an increase in caspase 3/7 activity (Fig. 6). An activator of CFTR anion channels (genistein) reduced such necrotic cell damage, while a CFTR antagonist (CFTRinh-172) aggravated necrotic injury after I/R *in vivo* (Fig. 5) and after simulated I/R *in vitro* (Fig. 6). Since there remains a possibility that the CFTR activator and inhibitor exerted non-specific actions as well, the effects of overexpression and knockdown of CFTR gene were tested in the *in vitro* experiments. Transfection with cDNA or siRNA of CFTR into cultured cardiomyocytes was then found to ameliorate or aggravate, respectively, necrotic cell injury induced by hypoxia/reoxygenation (Fig. 7). Thus, it appears that the activity of cardiac CFTR exerts an anti-necrotic, but not anti-apoptotic, effect on I/R injury.

It is noted that myocardial necrosis is a complication, though rare, of cystic fibrosis that leads to sudden, unexpected and fatal cardiac arrest in infants; this was first reported in 1945 [51], and various other cases have been reported thereafter [52]. Recently, it was also

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reported that a 13-month old patient who died of heart failure with myocardial necrosis was retrospectively diagnosed after histological examination as having had cystic fibrosis [53].

Cell swelling is a prominent feature of ischemic myocardial injury, and is due to intracellular hypertonic stress in the myocytes resulting from accumulation of the by-products of anaerobic metabolism. Even after reperfusion, irreversibly injured cells are known to exhibit enormous swelling [54]. This necrotic volume increase (NVI) is known to be associated with an impairment of the RVD mechanism in a number of pathophysiological situations [10]. Volume-sensitive outwardly rectifying anion channels (VSOR) are known to be activated by cell swelling and to be involved in the RVD process in a wide variety of cell types including cardiac myocytes [55]. VSOR activity was implicated in apoptotic injury caused by ischemia/reperfusion in myocardial cells [56, 57]. In the present study, however, an involvement of VSOR in necrotic injury induced by ischemia/reperfusion could be ruled out: necrotic injury was not augmented but reduced by genistein, which is an activator of CFTR but an inhibitor of VSOR [58, 59]. Wang et al. [9] reported that CFTR anion channels are activated by β-adrenergic stimulation and involved in the RVD that occurs after osmotic swelling in guinea pig cardiomyocytes. It is therefore conceivable that activation of CFTR anion channels plays an anti-necrotic role by facilitating the RVD process in cardiomyocytes subjected to ischemia/reperfusion.

Chen et al. [13] reported that cardiac CFTR serves as a crucial mediator in mouse heart IPC on the basis of observations that the IPC-induced protection of mouse heart from I/R-induced myocardial infarction was prevented by a CFTR blocker (gemfibrozil) and by targeted inactivation of the CFTR gene (CFTR$^{-/-}$ mice). Xiang et al. [14] recently provided evidence for a critical role of CFTR in IPC- and POC-mediated cardioprotection against I/R myocardial injury. The post-ischemic early reperfusion phase is a more relevant stage for therapeutic intervention than the stage prior to the ischemic attack, which is the target stage for IPC. Also, it must be noted that IPC and POC would not be practical as a therapeutic intervention for patients. In the present study, it was found that myocardial infarction was prominently reduced by CFTR activators administered by a post-ischemic intravenous infusion given for 12 min from 10 min before to 2 min after reperfusion, but not by injection of the drugs 10 min after starting reperfusion (Fig. 1). Thus, it is likely that the early reperfusion phase is the time window for therapeutic intervention, during which cardiac CFTR may be pharmacotherapeutically targeted to protect against myocardial infarction.

Conclusion

To test a possibility that activation of CFTR after ischemic attack can protect cardiac myocytes against I/R-induced myocardial injury, I/R experiments were performed in the hearts of WT and CFTR$^{-/-}$ mice *in vivo* and in rat ventricular myocytes without and with transfection of CFTR cDNA or siRNA *in vitro*. These experiments indicate that endogenous CFTR activity is involved in cardioprotection against necrotic I/R injury and that the administration of a CFTR activator after ischemic attack and in the early reperfusion phase may protect against necrotic myocardial injury induced by I/R. This study is the first to provide evidence that cardiac CFTR could be targeted by pharmacotherapeutic approaches even after an ischemic attack to protect against myocardial infarction.

Abbreviations

AAR (Area at risk); INF (area of infarction); CK (creatine kinase); CK-MB (creatine kinase MB isoenzyme); CFTR (cystic fibrosis transmembrane conductance regulator); dbcAMP (dibutyryl cyclic AMP); DMEM (Dulbecco's Modified Eagle's Medium); IPC (ischemic preconditioning); I/R (ischemia/reperfusion); LVA (left ventricular area); M199 (Medium 199); pO_2 (partial pressure of oxygen); PDEIII (phosphodiesterase type

III); POC (postconditioning); PI (propidium iodide); PKA (protein kinase A); PKC (protein kinase C); PTK (protein tyrosine kinase); RVD (regulatory volume decrease); siRNA (small interfering RNA); STS (staurosporine); CFTRinh-172 (3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone); TTC (2,3,5-triphenyltetrazolium chloride).

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