Cytosolic Ca\(^{2+}\)-Induced Apoptosis in Rat Cardiomyocytes via Mitochondrial NO-cGMP-Protein Kinase G Pathway

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ABSTRACT

Previously, we showed that in adult rat cardiomyocytes, nitric oxide (NO) donors stimulate mitochondrial cGMP production, followed by cytochrome c release, independently of the mitochondrial permeable transition pore. We investigated whether mitochondrial cGMP-induced cytochrome c release from cardiac mitochondria is Ca\(^{2+}\)-sensitive. Mitochondria and cardiomyocytes were prepared from left ventricles of male Wistar rats. The cytosolic Ca\(^{2+}\) concentration was adjusted with Ca\(^{2+}\)-EGTA buffers. Cytochrome c released from mitochondria was measured by Western blotting. Cardiomyocyte apoptosis was assessed by Annexin V staining. From mitochondria was measured by Western blotting. Cardiomyocyte apoptosis was assessed by Annexin V staining.

Introduction

Cardiomyocyte apoptosis can be induced by myocardial infarction and by its reperfusion treatment (Olivetti et al., 1996). Although reperfusion therapy for acute myocardial infarction prevents the extension of infarct size, these beneficial effects are limited by reperfusion injury with myocardial inflammation, which activates inducible nitric oxide (NO) synthase, by cardiomycyte apoptosis (Frangogiannis et al., 2002; Moens et al., 2005). As reperfusion-induced apoptosis may play a central role in cardiac remodeling, prevention of ischemia-reperfusion injury requires a good understanding of the mechanism of NO-induced cardiomyocyte apoptosis (Taimor et al., 2000).

Cytochrome c release from mitochondria is an important step in caspase-3 activation, which is required for apoptosis (Reed and Paternostro, 1999). The mitochondrial membrane permeability transition pore (MPTP), which has an adenine nucleotide translocator in the inner membrane and a voltage-dependent anion channel 1 (VDAC1) in the outer membrane, has been proposed as the means of cytochrome c release (Crompton, 2000; Kinnally et al., 2011). However, the relationship between MPTP formation and cytochrome c release is unclear (Kokoszka et al., 2004; Baines et al., 2007). The pore diameter formed in VDAC1 monomer (20–30 Å) is too small for penetration of cytochrome c (about 12 kDa) (Bayrhuber et al., 2008; Ujwal et al., 2008). One model suggests that VDAC1 responds to apoptotic stimuli with conformational changes and oligomerization to form pores large enough to accommodate cytochrome c, leading to induction of apoptosis (Zalk et al., 2005; Keinan et al., 2010).

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**ABBREVIATIONS:** Carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DIDS, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene; diS-C\(_3\)(5), 3,3'-dipropylthiodicarbocyanine iodide; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; MPTP, mitochondrial permeable transition pore; mtNOS, mitochondrial NO synthase; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (NO-sensitive guanylyl cyclase inhibitor) and voltage-dependent anion channel (VDAC) inhibitor, 4,4'-diisothiocyanato-2,2'-disulfonic acid stilbene, but not by cyclosporin A (mitochondrial permeable transition pore inhibitor). Furthermore, this was significantly and dose dependently inhibited by 0.3–3 \(\mu\)M KT5823 (protein kinase G inhibitor). At the cellular level, intracellular perfusion of cardiomyocytes with buffered Ca\(^{2+}\) (1 \(\mu\)M) also induced apoptosis, which was inhibited in the presence of ODQ.

A membrane-permeable cGMP analog, 8-Br-cGMP, but not cGMP itself, mimicked buffered Ca\(^{2+}\) actions in both cardiac mitochondria and cardiomyocytes. We further confirmed an increase in protein kinase G activity by adding cGMP in mitochondrial protein fraction. Our results suggest that mitochondrial NO-cGMP pathway-induced cytochrome c release from cardiac mitochondria, triggered by increased cytosolic Ca\(^{2+}\), occurs through VDAC via the stimulation of an undiscovered mitochondrial protein kinase G.
We have shown that NO donors, to increase mitochondrial cGMP concentration, induce cytochrome c release from mitochondria, followed by cardiomyocyte apoptosis; we also showed that 1H-[1,2,4]oxadiazolo[4,3-α]quinazolin-1-one (ODQ), an NO-sensitive guanylyl cyclase inhibitor, inhibits cardiomyocyte apoptosis under ischemia-reperfusion conditions (Seya et al., 2007). However, NO and cGMP do not induce mitochondrial swelling or membrane depolarization. These results suggest that a NO-cGMP pathway in cardiac mitochondria induces cytochrome c release through an unidentified MTPP-independent mechanism. Two molecular mechanisms after cGMP production in mitochondria are conceivable: 1) direct stimulation of VDAC by cGMP and 2) cGMP-induced stimulation of other proteins, such as protein kinase G (PKG), a specific receptor of cGMP (Gill et al., 1976).

An experimental design that endogenously stimulates mitochondrial cGMP production with second messengers such as NO or intracellular Ca\(^{2+}\) is needed to confirm these exogenous results. More details of the molecular mechanism of mitochondrial NO-cGMP pathway-induced cytochrome c release also need to be resolved.

Mitochondrial NO synthase (mtNOS) was found in cardiac muscle and shown to be similar to constitutive NOS isoforms, based on Ca\(^{2+}\) sensitivity and constitutive expression (Kanai et al., 2001; Kanai et al., 2004; Dedkova and Blatter, 2009). Cardiac mtNOS reportedly plays various physiological and pathophysiologilcal roles, namely, modulating oxidative phosphorylation and myocardial contractility and an implied role in radiation-induced cell damage via the NO-cGMP pathway (Balligand et al., 1993; Mohan et al., 1996).

In this study, we spotlighted the role of cytosolic Ca\(^{2+}\) as a typical activator of mtNOS, resulting in production of endogenous NO and then cGMP. When extramitochondrial Ca\(^{2+}\), which mimicked cytosolic Ca\(^{2+}\), was kept at 1 \(\mu\)M using Ca\(^{2+}\)-EGTA buffer, cytochrome c was released from cardiac mitochondria without mitochondrial swelling and membrane depolarization. These conditions reconfirmed the existence of the mitochondrial NO-cGMP pathway and revealed an undiscovered mitochondrial PKG that plays an important role in cytochrome c release through VDAC.

Materials and Methods

Materials. Cyclosporine A, 4,4′-diisothiocyanate-2,2′-disulfonic acid stilbene (DIDS), ODQ, N\(^{ω}\)-monomethyl-l-arginine (l-NMMA), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), KIT5823, 8-Br-cGMP, dimethyl sulfoxide (DMSO), annexin V-fluorescein isothiocyanate (FITC), and propidium iodide (PI) were obtained from Wako Pure Chemicals (Osaka, Japan). All chemicals used were of the highest purity commercially available. Solutions were made fresh at sufficiently high concentrations that only very small aliquots had to be added to assay tubes or culture media. The final DMSO concentration was 0.1% in the experiments using mitochondrial suspension and 0.11% in those using primary cultured cardiomyocytes. These DMSO concentrations had no effect on cells or assays. Primary mouse antibody against rat cytochrome c and goat anti-mouse IgG conjugated with Alexa Fluor 680 as secondary antibody were bought from Invitrogen (Carlsbad, CA).

Ethics Statement. The animal experiments were carried out in a humane manner after receiving approval from the Guidelines for Animal Experimentation, Hirosaki University (guideline no. 17, revised 2011) and in accordance with the regulation for animal experiments in our university and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan (law no. 105, 1973; the latest amendment on August 30, 2011). The investigation also conforms to the Guide for Care and Use of Laboratory Animals US (NIH Publication No. 85-23, revised 1996).

Isolation of Cardiomyocytes and Mitochondria. The animals were maintained for 1 week at a temperature of 22 ± 2°C and a relative humidity of 57% on a 12-h light/12-h dark schedule (lights on 08:00–20:00) and had free access to water and food. Ventricular myocytes were isolated from adult Wistar rats (n = 18, 350–400 g) by collagenase perfusion (Ono et al., 2000; Seya et al., 2007). Briefly, each rat was anesthetized with pentobarbital sodium (50 mg/kg i.p.). After endotracheal intubation, artificial respiration was instituted using a Harvard respirator. The adequacy of anesthesia was monitored from the disappearance of righting reflex or pedal withdrawal reflex. Each heart was isolated, the aorta was cannulated under artificial respiration, and both were quickly removed as a unit and hung on a Langendorff-type apparatus to start the coronary perfusion with a Ca\(^{2+}\)-free Tyrode’s solution. Hearts were then perfused with Ca\(^{2+}\)-free Tyrode’s solution containing 0.04% (w/v) collagenase (200 units/mg). After enzyme treatment, left ventricle tissue was dissected, gently agitated, and filled with normal Tyrode’s solution. Isolated cardiomyocytes were used for apoptosis studies.

Rat cardiac mitochondria were prepared as described previously (Seya et al., 2007). Briefly, heparinized adult Wistar rats (n = 23, 350–400 g) were anesthetized with pentobarbital sodium (50 mg/kg i.p.). The adequacy of anesthesia was monitored from the disappearance of righting reflex or pedal withdrawal reflex. Heats were then isolated and homogenized, and mitochondria were isolated in a medium of 250 mM sucrose, 10 mM tris-HCl, and 1 mM EGTA, pH 7.4, by differential centrifugation of heart homogenates. Mitochondria were resuspended in 250 mM sucrose and 10 mM tris-HCl (pH 7.4) and stored in ice. The crude mitochondrial suspension was further purified using centrifugation (82,000 g for 200 min at 4°C) in a continuous sucrose gradient (1.1–1.6 M sucrose in 1 mM EGTA and 5 mM potassium phosphate, pH 7.4). The resulting fractions were collected, resuspended in homogenization buffer, centrifuged (10,000g for 15 min at 4°C), and resuspended.

Determination of Various Mitochondrial Functions. To confirm mitochondrial viability, we measured the respiratory control ratio using a Clark-type oxygen electrode (Gilson, Middleton, WI); we used mitochondria maintaining respiratory control ratio value ≥ 6. Protein concentration in the mitochondrial suspension was determined by the Bradford method (Bradford, 1976).

To assess mitochondrial membrane function, mitochondrial swelling was determined by adding mitochondrial suspension (0.05 mg of protein per milliliter) to Ca\(^{2+}\)-EGTA buffered medium and measuring decreased absorbance at 540 nm (Seya et al., 2007; Seya et al., 2009). Mitochondrial membrane potential was assessed by adding mitochondrial suspension (0.1–0.2 mg of protein per milliliter) to Ca\(^{2+}\)-EGTA buffered medium containing 0.5 \(\mu\)g/ml diS-C3(5) and recording fluorescence intensity at 670 nm in a fluorescence spectrophotometer (FP-750, JASCO Co., Japan) following excitation at 620 nm.

Cytochrome c release from mitochondria was measured as described in a previous article (Seya et al., 2007). In brief, mitochondria (0.05–0.1 mg protein/ml) were incubated in a medium of 0.15 M KCl, 5 mM KH\(_2\)PO\(_4\), 5 mM succinic acid, and 10 mM HEPES (pH 7.4 adjusted with 1 M tris) at 30°C. Ten to 30 min after drug administration, the reaction medium was centrifuged at 7,000g for 10 min at 4°C. Supernatant was treated by the trichloroacetic acid method, and medium was then centrifuged at 20,000g; cytochrome c levels in precipitate were measured by Western blot (Tash and Means, 1987).

The PKG activity in mitochondria was spectrophotometrically measured by cGMP administration using a cyclic GMP-dependent protein kinase assay kit obtained from Cyclex Co., Ltd. (Nagano, Japan). The mitochondrial protein fraction was prepared from mitochondria (60–100 mg/ml) by sonication for 90 s at 150 W and
50% duty cycles (Sonifier; Branson Sonic Power, Danbury, CT) and then centrifugation (10,000g for 20 min at 4°C).

Endogenous cGMP Production by Constitutive Activation of mtNOS. To activate mtNOS, mitochondria were continuously exposed to extramitochondrial Ca$^{2+}$, with the concentration kept at 1 μM, using Ca$^{2+}$-EGTA buffer (0.15 M KCl, 5 mM KH$_2$PO$_4$, 1 mM EGTA and 10 mM HEPES (pH 7.4 adjusted with 1 M tria). The calculated amount of CaCl$_2$ (0.972 mM) was added to the medium up to the desired Ca$^{2+}$ concentration (1 μM), which was calculated using CALCON, which is based on Goldstein's algorithm (Goldstein, 1979). After up to 30 min of calcium loading with 1 μM Ca$^{2+}$, cytochrome c released from mitochondria was measured by Western blotting, as mentioned previously (Seya et al., 2007).

Detection of Apoptotic Cells by Patch-Clamp Method. A small piece of ventricular tissue obtained from collagenase digestion of rat heart was dissected and gently agitated in the recording chamber (0.5 ml in volume) filled with the normal Tyrode’s solution. Experiments were carried out at room temperature. Current and voltage signals of cardiomyocytes were recorded using a patch-clamp amplifier (EPC-7; List, Darmstadt, Germany). Patch pipettes were pulled with a micropipette puller (model P-97; Sutter Instrument Co., Novato, CA) from glass capillaries (Corning #7052). The pipettes had resistances of 3–5 MΩ when filled with the internal solution (for diastolic calcium solution (0.07 μM): 110 mM K-aspartate, 20 mM KCl, 5 mM Na$_2$ATP, 5 mM MgCl$_2$, 5 mM EGTA, 5 mM HEPES, 0.1 mM Na$_2$GTP, 1.5 mM CaCl$_2$ (pH 7.2 adjusted with KOH); and for high-capacitance solution (1 μM): 110 mM K-aspartate, 20 mM KCl, 5 mM Na$_2$ATP, 5 mM MgCl$_2$, 5 mM EGTA, 5 mM HEPES, 0.1 mM Na$_2$GTP, 4.3 mM CaCl$_2$ (pH 7.2 adjusted with KOH). After seal formation (1–8 GΩ), patch membranes were ruptured by applying suction. Under current-clamp mode, resting membrane potentials and action potentials were measured; myocytes showing resting potentials below −70 mV and typical action potentials for rats were used to measure fluorescence. Cells were then voltage-clamped at −40 mV, and reagents were added to the recording chamber to give concentrations of annexin V-FITC 25 μg/ml and PI 1 μg per milliliter. Thereafter, bright-field optical images and fluorescence images excited by 560 nm wavelengths for FITC and PI, respectively, were captured every 10 min under a fluorescence microscope (IX71; Olympus, Tokyo, Japan). Cells treated with 1 mM H$_2$O$_2$ served as a positive control. The number of apoptotic cells (FITC-linked annexin V positive cells) was calculated by comparing the rate of apoptotic cells relative to total cells. In each experiment, average values of 50–100 cells were taken as the experiment’s results.

Statistical Analysis. Group comparisons were performed by analysis of variance with the Student-Newman-Keuls post hoc correction procedure or with Student’s t test. Values are presented as means ± SEM; *P < 0.05 was considered statistically significant.

Results

Buffered Ca$^{2+}$-Induced Cytochrome c Release from Cardiac Mitochondria. To activate mtNOS, the extramitochondrial free Ca$^{2+}$ concentration was kept at 1 μM using Ca$^{2+}$-EGTA buffer. Calcium loading with 1 μM Ca$^{2+}$ for 30 min time dependently and significantly accelerated cytochrome c release from cardiac mitochondria (2.8 ± 0.5-fold of control, Fig. 1A). Diastolic concentration of buffered Ca$^{2+}$ (0.1 μM) did not induce cytochrome c release. Furthermore, continuous exposure to 1 μM free Ca$^{2+}$ did not evoke mitochondrial swelling in cardiac mitochondria (Supplemental Fig. S1). Up to 10 μM free Ca$^{2+}$ did not induce depolarization of the mitochondrial membrane potential (data not shown).

Mitochondrial cytochrome c release induced by buffered 1 μM Ca$^{2+}$ was significantly inhibited by L-NMMA (0.1 mM; Fig. 1A). Cytochrome c release from mitochondria treated with Ca$^{2+}$-EGTA buffer supplemented with 150 mM KCl and 5 mM succinate, which were incubated up to 30 min at 30°C. Cytochrome c release from mitochondria was determined by Western blotting. (A) Change over time in cytochrome c release induced by Ca$^{2+}$ (1 μM; closed circles, 0.1 μM; open circles). Values are means ± S.E.M. of four experiments. Significant differences: *P < 0.05 compared with control; **P < 0.01 compared with 0.1 μM Ca$^{2+}$; (B) Densitometry of band intensities for Control (0.1 μM Ca$^{2+}$), Ca$^{2+}$ (1 μM), Ca$^{2+}$ (1 μM) + L-NMMA (NMMA, 0.1 mM), Ca$^{2+}$ (1 μM) + carboxy-PTIO (PTIO, 0.1 mM), and Ca$^{2+}$ (1 μM) + ODQ (1 μM) after 30-min incubation. Values were normalized to the average value of control and expressed as means ± S.E.M. of four experiments. Significant differences: *P < 0.05 compared with control; **P < 0.01 compared with 0.1 μM Ca$^{2+}$

**Fig. 1.** Buffered Ca$^{2+}$ (1 μM) increased cytochrome c release from mitochondria. Extramitochondrial Ca$^{2+}$ was adjusted with Ca$^{2+}$-EGTA buffer supplemented with 150 mM KCl and 5 mM succinate, which were incubated up to 30 min at 30°C. Cytochrome c release from mitochondria was determined by Western blotting. (A) Change over time in cytochrome c release induced by Ca$^{2+}$ (1 μM; closed circles, 0.1 μM; open circles). Values are means ± S.E.M. of four experiments. Significant differences: *P < 0.05 compared with control; **P < 0.01 compared with 0.1 μM Ca$^{2+}$; (B) Densitometry of band intensities for Control (0.1 μM Ca$^{2+}$), Ca$^{2+}$ (1 μM), Ca$^{2+}$ (1 μM) + L-NMMA (NMMA, 0.1 mM), Ca$^{2+}$ (1 μM) + carboxy-PTIO (PTIO, 0.1 mM), and Ca$^{2+}$ (1 μM) + ODQ (1 μM) after 30-min incubation. Values were normalized to the average value of control and expressed as means ± S.E.M. of four experiments. Significant differences: *P < 0.05 compared with control; **P < 0.01 compared with 0.1 μM Ca$^{2+}$.
a NOS inhibitor), carboxy-PTIO (0.1 mM), an NO scavenger, and ODQ (1 μM), an NO-sensitive soluble guanylyl cyclase inhibitor (Fig. 1B). In the basal condition in the presence of 0.1 μM Ca2+, each inhibitor alone did not affect mitochondrial cytochrome c release (data not shown). It was reported that high concentrations of ODQ (≥10 μM) induce mitochondrial swelling followed by cytochrome c release (Fraser et al., 2006). However, ODQ (1 μM) alone did not affect mitochondrial swelling and cytochrome c release despite its significant (34.2 ± 7.1%) inhibitory effect on the 1 μM Ca2+-induced cytochrome c release.

Effects of DIDS on 1 μM Ca2+-Induced Cytochrome c Release from Cardiac Mitochondria. To confirm the involvement of VDAC in the 1 μM Ca2+-induced cytochrome c release, the effect of DIDS, an inhibitor of VDAC, was investigated. Acceleration of cytochrome c release by 1 μM Ca2+ was significantly inhibited in the presence of DIDS (0.1 mM) (Fig. 2A). However, mitochondrial membrane permeable transition pore (MPTP) inhibitor cyclosporin A did not affect cytochrome c release induced by buffered 1 μM Ca2+.

Effects of Protein Kinase G Inhibitor on 1 μM Ca2+-Induced Cytochrome c Release from Cardiac Mitochondria. We further investigated the role of protein kinase G (PKG) as a major receptor of cGMP in cytochrome c release from cardiac mitochondria exposed to 1 μM Ca2+ for 30 min. KT5823 (0.3–3 μM; PKG-specific inhibitor) dose dependently and significantly inhibited Ca2+-induced cytochrome c release from cardiac mitochondria (Fig. 2B).

Apoptosis of Isolated Ventricular Cardiomyocytes Induced by Continuous Exposure to Buffered 1 μM Ca2+. Effects of ODQ on Ca2+-induced cardiomyocyte apoptosis were investigated using the patch-clamp method, which was performed on morphologically healthy ventricular myocytes. Intracellular perfusion was carried out through patch-clamp electrode. Figure 3A shows representative images of cardiomyocytes intracellularly perfused with 1 μM Ca2+.

**Fig. 2.** Effects of DIDS and KT5823 on 1 μM Ca2+-induced cytochrome c release. Extramitochondrial concentrations of Ca2+ were kept at 1 μM using Ca2+-EGTA buffer, which was supplemented with 150 mM KCl and 5 mM succinate, and then incubated for 30 min at 30°C. Cytochrome c release from mitochondria was determined by Western blotting. (A) Densitometry of band intensities for DIDS (0.1 mM, hatched bar), 1 μM Ca2+ (closed bar), Ca2+ + DIDS (gray bar), or Ca2+ + cyclosporin A (1 μM, horizontal bar). Values are normalized to the average value obtained from control (0.1 μM Ca2+, open bar) and expressed as means ± S.E.M. of three experiments. Significant differences: **P < 0.01 compared with control; #**P < 0.01 compared with value of 1 μM Ca2+ alone. (B) Densitometry of band intensities for 1 μM Ca2+ (closed bar), Ca2+ + KT5823 (0.3–3 μM, gray bars). Values are normalized to the average value obtained from the control (0.1 μM Ca2+, open bar) and expressed as means ± S.E.M. of six experiments. Significant differences: *P < 0.05 compared with control; **P < 0.05 compared with value of the presence of 1 μM Ca2+ alone.

**Fig. 3.** Apoptosis of primary cultured adult rat cardiomyocytes induced by intracellular perfusion of buffered Ca2+ (1 μM). Patch-clamp procedures were performed on morphologically healthy ventricular myocytes. Pipette solution contained either diastolic (0.07 μM) or high calcium (1 μM) solution (see Materials and Methods). Apoptotic cells were analyzed by FITC-linked annexin V-propidium iodide staining. (A) Upper panels, typical phase-contrast view of each cardiomyocyte at 0 min under the following conditions: a, diastolic concentration of Ca2+ (0.07 μM); b, high concentration of Ca2+ (1 μM); c, Ca2+ + 1 μM ODQ. Middle panels, phase-contrast view of each cardiomyocyte at 100 min. Lower panels, representative images of annexin V–FITC/propidium iodide staining. (B) The graph shows relative amplitudes of 1 μM Ca2+-induced cardiomyocyte apoptosis in the diastolic concentration of 0.07 μM Ca2+ (open bar), excess concentration of 1 μM Ca2+ (closed bar), and 1 μM Ca2+ + 1 μM ODQ (gray bar). Fluorescence signal of FITC-linked annexin-V, used as a measure of cardiomyocyte apoptosis, was quantified by normalizing the fluorescence intensity of FITC-linked annexin-V to that of electrode-free cardiomyocytes; relative intensity = (a – b)/c – b, in which a = clamped-cell fluorescence, b = background fluorescence (cell-free area), and c = fluorescence of electrode-free cells (see Materials and Methods). Values are mean ± S.E.M. of 5 (0.07 μM Ca2+), 1 (1 μM Ca2+), or 9 (1 μM Ca2+ + 1 μM ODQ) experiments. **P < 0.01 compared with 0.07 μM Ca2+; #P < 0.05 compared with 1 μM Ca2+ alone.
μM Ca²⁺ in the presence of ODQ. One micromolar (middle panel), but not 0.07 μM (diastolic concentration, left panel) of cytosolic free Ca²⁺, triggered contractions of cardiomyocytes and increased annexin V-FITC fluorescent intensity. However, ODQ (1 μM; right panel) strongly inhibited 1 μM free Ca²⁺-induced annexin V-FITC staining. Fig. 3B summarizes changes in fluorescent intensity of free Ca²⁺-induced annexin V-FITC staining. Excess cytosolic free Ca²⁺ (1 μM), but not diastolic Ca²⁺ concentration (0.07 μM), markedly induced cardiomyocyte apoptosis, which was significantly inhibited in the presence of ODQ (1 μM).

8-Br-cGMP-Induced Cytochrome c Release from Cardiac Mitochondria. The effects of 8-Br-cGMP on isolated cardiac mitochondria were examined to determine whether the mechanism of Ca²⁺-induced cytochrome c release from mitochondria involves cGMP production. Exposure to 8-Br-cGMP (300 μM) for 30 min accelerated the release of cytochrome c from cardiac mitochondria in a time-dependent manner (Fig. 4A). However, cGMP itself failed to release cytochrome c from mitochondria. This release was inhibited in the presence of DIDS, an inhibitor of VDAC, but not cyclosporin A, a mitochondrial MPTP inhibitor (Fig. 4B). In addition, continuous exposure of 8-Br-cGMP evoked neither membrane potential change nor swelling in cardiac mitochondria, suggesting that it is independent of MPTP activation (data not shown; Seya et al., 2007).

Figure 4C illustrates that KT5823 (0.3–3 μM), a PKG-specific inhibitor, dose-dependently and significantly inhibited 8-Br-cGMP-induced cytochrome c release from cardiac mitochondria, similar to the blockade of Ca²⁺ (1 μM)-induced cytochrome c release (Fig. 2B).

8-Br-cGMP-Induced Apoptosis of Isolated Ventricular Cardiomyocytes. To confirm that mitochondrial cGMP can induce cardiomyocyte apoptosis, isolated ventricular cardiomyocytes were exposed to a membrane-permeable cGMP analog, 8-Br-cGMP, extracellularly. Figure 5A shows representative images of annexin V-FITC staining of apoptotic cardiac myocytes after treatment for 6 h with 8-Br-cGMP (300 μM). Continuous exposure of cardiomyocytes to 8-Br-cGMP (300 μM), but not cGMP (300 μM), induced significant cardiomyocyte apoptosis. Figure 5B shows changes over time in the ratio of apoptotic cells after 8-Br-cGMP administration,

Fig. 4. Cytochrome c release from cardiac mitochondria induced by 8-Br-cGMP. Extramitochondrial concentration of 8-Br-cGMP or cGMP (each 0.3 mM) was incubated for 30 min at 30°C using isolated cardiac mitochondria. Cytochrome c release from mitochondria was determined by Western blotting. (A) Changes over time in cytochrome c release induced by 8-Br-cGMP (closed circles) and cGMP (open squares). Control was represented as open circles. Values are means ± S.E.M. of three experiments. *P < 0.05 compared with control at the corresponding time. (B) Densitometry of band intensities for the control (open bar), 0.3 mM 8-Br-cGMP; (c) in the presence of 0.3 mM 8-Br-cGMP + KT5823 (0.3 mM). Significant differences: **P < 0.01 compared with control; ***P < 0.001 compared with value in the presence of 8-Br-cGMP alone. (C) Inhibitory effect of KT5823 on 8-Br-cGMP- (closed circles) and cGMP-(open circles) induced cardiomyocyte apoptosis. Values are expressed as means ± S.E.M. of five (8-Br-cGMP) or four (cGMP) experiments. *P < 0.05 compared with cGMP alone at corresponding time. (C) Inhibitory effect of KT5823 (gray bar) on the 8-Br-cGMP (0.3 mM)-induced cardiomyocyte apoptosis (closed bar) after 6 h of incubation. Values for apoptotic cells were calculated by comparing rates of apoptotic cells relative to total cell number. Values are means ± S.E.M. of five (8-Br-cGMP) or four (cGMP) experiments. *P < 0.05 compared with control (open bar) or KT5823 alone (horizontal bar). **P < 0.01 compared with value in the presence of 8-Br-cGMP alone.

Fig. 5. 8-Br-cGMP-induced apoptosis in primary cultured adult rat cardiomyocytes. Extramitochondrial concentration of 8-Br-cGMP or cGMP (each 0.3 mM) applied extracellularly to isolated cardiomyocytes and incubated for 6 h at 30°C. (A) Upper panel: representative images of annexin V–FITC/propidium iodide staining of apoptotic cardiac myocytes after treatment for 6 h with 8-Br-cGMP (300 μM). Continuous exposure of cardiomyocytes to 8-Br-cGMP (300 μM), but not cGMP (300 μM), induced significant cardiomyocyte apoptosis. Figure 5B shows changes over time in the ratio of apoptotic cells after 8-Br-cGMP administration.
which was significantly accelerated after 3 h in the 8-Br-cGMP-treated group. Also, PKG inhibitor KT5823 (3 μM) significantly inhibited 8-Br-cGMP-induced cardiomyocyte apoptosis after treatment for 6 h (Fig. 5C). The positive control, H2O2 (1 mM), time dependently increased the ratio of apoptotic cells to 84.3 ± 7.9% (n = 3) after treatment for 6 h. The ratio of both FITC-linked annexin V and PI-positive cells [necrotic cells, indicated in Fig. 5A(e)] after 6 h of incubation remained at lower levels: 4.0 ± 2.8% (n = 4) for 8-Br-cGMP (300 μM) and 5.3 ± 1.8% (n = 4) for cGMP (300 μM).

**PKG Activity in the Mitochondrial Protein Fraction.**

To confirm the existence of mitochondrial PKG, we measured PKG activity in isolated mitochondrial protein fraction. PKG activity in the mitochondrial protein fraction was significantly increased by the administration of external cGMP (50 μM), which was strongly inhibited in the presence of KT5823 (3 μM) (Fig. 6).

**Discussion**

In the present study, we confirmed that buffered Ca2+ (1 μM) stimulates mtNOS, resulting in endogenous mitochondrial cGMP production followed by cytochrome c release from mitochondria. Intracellular perfusion with buffered Ca2+ (1 μM) significantly induced cardiomyocyte apoptosis. Both cytochrome c release induced by buffered 1 μM Ca2+ and the membrane-permeable cGMP analog 8-Br-cGMP were significantly and dose dependently inhibited by PKG inhibitor. Furthermore, cGMP induced an increase in PKG activity in isolated mitochondrial protein fraction.

In inflammatory diseases, NO is excessively produced by iNOS. We have previously shown that the addition of excess NO donor, which mimics exposure to excess NO, induced mitochondrial cGMP production, followed by cytochrome c release, from rat ventricular cardiomy mitochondria (Seya et al., 2007). Release of cytochrome c was not dependent on swelling or membrane depolarization of mitochondria. However, these results insufficiently explain the role of endogenous mitochondrial NO and then cGMP produced by mtNOS. In this study, we showed that continuous exposure of buffered cytosolic Ca2+ (1 μM) evokes cGMP production in mitochondria followed by cytochrome c release, but not the activation of mitochondrial swelling or membrane depolarization. We confirmed that 30-min exposure of buffered cytosolic Ca2+ (1 μM) increased mitochondrial cGMP to approximately 10–15 pmol per milligram of protein (data not shown). Furthermore, cytochrome c release from mitochondria induced by buffered 1 μM Ca2+ was significantly inhibited by two typical NO inhibitors, namely, the NOS inhibitor L-NMMA and the NOS scavenger carboxy-2-phenyl-4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide. These results suggest that increased mitochondrial calcium concentration activates mtNOS, resulting in endogenous NO followed by cGMP production (Lacza et al., 2003; Seya et al., 2007; Dedkova and Blatter, 2009).

Cytochrome c release from cardiac mitochondria exposed to buffered 1 μM Ca2+ and apoptosis in cardiomyocytes induced by intracellular perfusion of buffered 1 μM Ca2+ were strongly inhibited in the presence of ODQ, demonstrating that cardiomyocyte apoptosis is induced by intrinsic cGMP after exposure to buffered 1 μM Ca2+. These results suggest that NO-induced apoptosis occurs mainly via the mitochondrial NO-cGMP pathway in rat cardiomyocytes. The influence of contaminated cytosolic soluble guanylyl cyclase can be ignored because extramitochondrial addition of a membrane-permeable cGMP analog (8-Br-cGMP), but not cGMP, stimulated cytochrome c release from cardiac mitochondria. We could not demonstrate that 8-Br-cGMP induces cardiac apoptosis using the patch-clamp method because the gigaseal could not be maintained for more than 2 h of reperfusion time.

Evaluation of the downstream pathway from mitochondrial cGMP production to cytochrome c release is very important. Apoptosis evoked by cGMP was first reported by Wu et al. (1997), who showed that atrial natriuretic peptide activates the receptor guanylyl cyclase followed by an increase in cardiac cGMP level and then apoptosis in neonatal rat cardiomyocytes (Wu et al., 1997). Some studies also report cardiomyocyte apoptosis induced by cardiac cGMP (Giulivi et al., 1998; Arstall et al., 1999; Ghafoorifar et al., 1999). However, the mechanism of cytosolic cGMP-induced apoptosis has been unclear. In our study, cytochrome c release induced by mitochondrial cGMP was independent of mitochondrial swelling and membrane depolarization, which were thought to be important indices of cytochrome c release. Furthermore, cytochrome c release stimulated by mitochondrial cGMP analog 8-Br-cGMP was independently inhibited by ODQ, suggesting that the release did not involve the adenine nucleotide translocase, an essential component of MPTP. Although it is necessary to measure the inhibitory effect of DIDS on 8-Br-cGMP-induced cardiomyocyte apoptosis, DIDS prohibited detection of apoptotic cells because of its high self-absorbance.
swelling, was significantly reduced by the external addition of PKG in isolated mitochondria (Borutaite et al., 2009). These phenomena reflect the sudden influx of excess Ca\textsuperscript{2+} into the mitochondria mimicked ischemia-reperfusion injury. In this study, we stimulated NOS by buffered 1 \textmu M Ca\textsuperscript{2+} to produce NO and then cGMP without mitochondrial depolarization (Supplemental Fig. S1). The bolus administration of 1 \textmu M Ca\textsuperscript{2+} caused neither cytochrome c release nor mitochondrial swelling (data not shown).

The main receptor for cGMP is PKG. In cardiomyocytes, cytosolic PKG activators such as diazoxide and sildenafil, a typical inhibitor of phosphodiesterase type 5, reportedly inhibit MPTP opening and apoptosis by stimulating the mitochondrial K\textsubscript{ATP} channel (Takimoto et al., 2005; Das et al., 2005; Costa et al., 2006; Xi et al., 2009; Borutaite et al., 2009). On the other hand, Taimor et al. (2000) reported that PKG inhibitor inhibited NO-induced cardiomyocyte apoptosis. However, these phenomena occurred by cytosolic but not mitochondrial PKG. In this study, we demonstrated that, in mitochondria, KT5823 (a specific inhibitor of PKG) significantly and dose dependently inhibits cytochrome c release induced both by buffered 1 \textmu M Ca\textsuperscript{2+} and by 8-Br-cGMP (Figs. 2B and 4C). KT5823 also significantly inhibited 8-Br-cGMP-induced cardiomyocyte apoptosis (Fig. 5C). Furthermore, KT5823 also inhibited an increase in PKG activity by adding cGMP in mitochondrial protein fraction (Fig. 6). These results indicate that mitochondrial, but not cytosolic, PKG regulates the opening of VDAC and (by extension) cytochrome c release from mitochondrial intermembrane space, and the possibility of direct stimulation of VDAC by cGMP alone can be excluded. Although 8-Br-cGMP may stimulate both cytosolic and mitochondrial PKG in these experimental procedures, an apoptotic effect induced by the mitochondrial NO-cGMP-PKG pathway may exceed a protective (antiapoptotic) effect induced by the cytosolic NO-cGMP-PKG pathway in cardiomyocytes (Fig. 7). Recently, an atomic-force microscopic study revealed the existence of various VDAC oligomers in outer mitochondrial membrane and also showed the contribution of cytochrome c to VDAC oligomer formation (Zalk et al., 2005; Hoogenboom et al., 2007). Several cytosolic protein kinases having tyrosine residues, such as p38 mitogen-activated protein kinase, and Jun N-terminal protein kinase, are known to regulate the oligomerization of VDAC and cytochrome c release (Keinan et al., 2010). We hypothesized that an undiscovered mitochondrial PKG partially regulates the mitochondrial NO-cGMP-PKG pathway. In this study, we demonstrated that, in isolated mitochondria, cGMP-induced cytochrome c release by excess cytosolic Ca\textsuperscript{2+} was significantly and dose dependently inhibited by inhibitors of PKG. Exogenous administration of 8-Br-cGMP gave results similar to those seen in the Ca\textsuperscript{2+}-stimulated endogenous mitochondrial NO-cGMP pathway. These results suggest that mitochondrial NO-cGMP–induced cytochrome c release from mitochondria occurs through an undiscovered mitochondrial PKG pathway in cardiac myocytes, as depicted in Fig. 7. In future studies, elucidation of the molecular mechanism of mitochondrial NO-cGMP-PKG pathway may lead to therapeutic strategies for pathologic conditions such as cardiac remodeling after myocardial reperfusion in ischemic heart diseases.

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Authorship Contributions

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Other: Furukawa, Okumura, Ono, Seya, Motomura acquired funding for the research.
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