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Hypoxia followed by reoxygenation induces secretion of cyclophilin A from cultured rat cardiac myocytes☆

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Abstract

We previously reported that hypoxia followed by reoxygenation (hypoxia/reoxygenation) rapidly activated intracellular signaling such as mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated protein kinase (ERK) 1/2, p38MAPK, and stress-activated protein kinases (SAPKs). To investigate the humoral factors which mediate cardiac response to hypoxia/reoxygenation, we analyzed the conditioned media from cardiac myocytes subjected to hypoxia/reoxygenation by two-dimensional electrophoresis and mass spectrometry. We identified cyclophilin A (CyPA) as one of the proteins secreted from cardiac myocytes in response to hypoxia/reoxygenation. Hypoxia/reoxygenation induced the expression of CyPA and its cell surface receptor CD147 on cardiac myocytes in vitro. This was also confirmed by ischemia/reperfusion in vivo. Recombinant human (rh) CyPA activated ERK1/2, p38MAPK, SAPKs, and Akt in cultured cardiac myocytes. Furthermore, CyPA significantly increased Bcl-2 in cardiac myocytes. These data strongly suggested that CyPA is released from cardiac myocytes in response to hypoxia/reoxygenation and may protect cardiac myocytes from oxidative stress-induced apoptosis.

Keywords: Apoptosis; Autocrine; Cardiac myocyte; CD147; Cyclophilin; Hypoxia; Ischemia; Reoxygenation; Reperfusion; Signal transduction

We previously reported that both hypoxia and hypoxia followed by reoxygenation (hypoxia/reoxygenation) rapidly and sequentially activated mitogen-activated protein kinase kinase kinase (MAPKKK) activity of Raf-1, MAP kinase kinase (MAPKK), MAPKs ($p44^{mapk}$ and $p42^{mapk}$) (also called extracellular signal-regulated protein kinase [ERK]1 and ERK2, respectively), and S6 kinase ($p90^{rsk}$) as well as Src family tyrosine kinases (c-Src and c-Fyn) and $p21^{ras}$, which are upstream mediators of MAPK pathway [1,2]. We also reported that both hypoxia and hypoxia/reoxygenation rapidly activated stress-activated MAPK signaling cascades involving $p65^{PAK}$, p38MAPK, and stress-activated protein kinases (SAPKs) in cultured rat cardiac myocytes [3]. Activation of these signaling cascades results in the expression of various genes coding for growth factors, cytokines, cell-adhesion molecules, and so on, which may play a role in the adaptation to these stresses or lead to further cell damage known as reperfusion injury. In this study, to investigate the humoral factors which mediate cardiac response to hypoxia/reoxygenation, we analyzed the conditioned media from cardiac myocytes subjected to hypoxia/reoxygenation and found that cyclophilin A (CyPA) was secreted from cardiac myocytes and might

☆ *Abbreviations:* BPB, bromophenol blue; CBB, Coomassie brilliant blue; CyPA, cyclophilin A; 2-D, two dimensional; DMEM, Dulbecco's modified Eagle's medium; DTE, dithioerythritol; ERK, extracellular signal-regulated protein kinases; HIV, human immunodeficiency virus; IEF, isoelectric focusing; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; MS, mass spectrometry; PBS, phosphate-buffered saline; PDA, piperazine diacrylamide; SAPK, stress-activated protein kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Ser, serine; TCA, trichloroacetic acid; TRITC, tetramethyl rhodamine isothiocyanate; TSA, tyramide signal amplification; Tyr, tyrosine; Thr, threonine; VSMC, vascular smooth muscle cell.

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play a role in protecting cardiac myocytes from oxidative stress-induced cell injury.

Materials and methods

Cell culture. Primary cultures of ventricular cardiac myocytes were prepared from neonatal rats as previously described [1]. They were cultured for two days until they were confluent and then serum-starved for 24 h before use.

Hypoxia and reoxygenation. Hypoxic condition (95% N_2, 5% CO_2, and less than 0.1% O_2) was achieved by using an anaerobic jar equipped with a new type AnaeroPack (disposable O_2 absorbing and CO_2 generating agent, Mitsubishi Gas Chemical, Japan) and an indicator to monitor oxygen depletion as described previously [1]. By placing flasks, which contain phosphate-buffered saline (PBS), in an anaerobic jar overnight, the PBS was balanced with the hypoxic atmosphere. Cultured cardiac myocytes were subjected to a hypoxic condition by immediately replacing the medium with the hypoxic PBS in an anaerobic jar. To keep hypoxic conditions, all the procedures were performed in a glove bag filled with 95% N_2 and 5% CO_2. After incubating in a hypoxic condition for 60 min, the cells were reoxygenated by immediately replacing the hypoxic PBS with normoxic PBS for the indicated time periods. We collected the supernatant PBS after 10 min of reoxygenation as reoxygenation-conditioned PBS. We also collected the supernatant PBS after 10 min of incubation with non-stimulated cardiac myocytes under normoxia as control-conditioned PBS.

Two-dimensional gel electrophoresis. We concentrated the reoxygenation-conditioned PBS and control-conditioned PBS by using centripreps (YM-10; Millipore, Bedford, MA, USA) and collected the fractions of molecular weight >10 kDa from them. Immobiline dry strips at pH 3–10 (7 cm), IPG buffer, PlusOne silver staining kit, and Coomassie brilliant blue (CBB-R350) were purchased from Amersham Biosciences (Uppsala, Sweden). For the first-dimensional isoelectric focusing (IEF), IPGphor strips (7 cm) at pH 3–10 were used. The concentrated-conditioned PBS (50 μg/125 μl) in a microtube (Treff AG, Schweiz, Switzerland) was diluted with Milli Q water (375 μl) and deproteinized with the 500 μl of 40% trichloroacetic acid (TCA) (final concentration 20%). The mixtures were allowed to stand on ice for more than 1 h and centrifuged at 13,000 rpm for 10 min. The supernatants were discarded and the precipitates were washed with cold ether three times to remove excess TCA. The final precipitates were dissolved in the IEF solution containing 9 M urea, 4% CHAPS, 65 mM dithioerythritol (DTE), 2% IPG buffer, pH 3–10, and bromophenol blue (BPB). The dried IPG strips were rehydrated overnight in the sample solution. Then, IEF was performed with the following steps; increasing voltage 30 V for 7 h, 60 V for 7 h, from 60 to 200 V for 0.5 h, from 200 to 500 V for 0.5 h, from 500 to 1000 V for 0.5 h, from 1000 to 8000 V for 0.5 h, and held at 8000 V for 1 h, i.e., a total of 11.5 kV h. Before loading on a 2-D SDS–PAGE, the IPG strips were immersed in 5 ml solution containing 50 mM Tris–HCl (pH 8.5), 6 M urea, 30% glycerol, 2% SDS, 150 mM DTE, and 0.005% BPB and were shaken slowly for 10 min at room temperature in order to reduce the inner and intra-disulfide bonds of cysteinyl residues. And then, the reduced proteins were alkylated with 5 ml of 300 mM acrylamide at room temperature for 10 min. For the 2-D sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), we used a gel ($110 \times 110 \times 1$ mm; Nihon Eido, Tokyo, Japan), which was prepared for separating gel (10% acrylamide and 2.6% piperazine diacrylamide [PDA]) and for stacking gel (4% acrylamide and 2.6% PDA). The prerun was performed at 24 mA for 45 min to remove the excess reagents and adjust gel condition. The IPG strips were placed onto the surface of a stacking gel. At first, the 2-D SDS–PAGE commenced at 6 mA for 30 min in order to release proteins from the IPG strips and to stack those into the 2-D gel. Subsequently, the proteins were separated at 12 mA for 3.25 h. The proteins in the gels were stained with the silver staining kit or the CBB R-350 reagent kit and profiled with the image analyzer, Master Scan (Scanalytics, Billerica, MA, USA).

In gel digestion. The proteins on the 2-D SDS–PAGE were subjected to in gel digestion as described previously [4]. The spots were excised manually using a razor blade, placed in microtubes, washed with H_2O (10 min, 37 °C, five times), and destained in 100 μl of 50% CH_3CN and 100 mM ammonium bicarbonate (pH 8.5) for 10 min at 37 °C until colorless. The gels were dehydrated in 100 μl CH_3CN in a microtube for 10 min at 37 °C and was dried in Micro Vac MV-100 (Tomy, Tokyo, Japan) for 5 min. The dried residue was rehydrated by adding 50 μl of 0.001% trypsin in 100 mM ammonium bicarbonate (pH 8.5) and incubated overnight at 37 °C. The incubation mixture in the microtube was centrifuged, and the residue was extracted with 50% CH_3CN and 0.1% trifluoroacetic acid, and centrifuged again. The residue was further extracted with 15% isopropyl alcohol, 20% formic acid, 25% CH_3CN, 40% H_2O, and finally with 80% CH_3CN. The supernatant and all of the extracts were successively dried in a single microtube, and the residue was dissolved in 6 μl of 0.1% formic acid. Aliquots were used for protein identification by mass spectrometry.

Mass spectrometry. Peptide mapping was carried out using API QSTAR Pulsar (I) hybrid mass spectrometer system with a microliquid chromatograph (Magic 2002, Michrom BioResource, Aurburn, CA, USA). The QSTAR pulsar hybrid mass spectrometer system consists of the apparatus of nanoelectrospray ionization and the quadrupole-time of flight (ESI-TOF) mass spectrometer. Mass accuracy was ±0.1 mass unit. Conditions of mass spectrometry (MS) were as follows; ion spray voltage of 2.0–2.8 kV, voltage for the electron multiplier of 2400 V, nitrogen for curtain gas of 10, nitrogen for collision gas of 10, and collision energy of 20–55 eV for MS/MS analysis. Conditions of micro-LC were as follows; Magic C18 column (0.2 mm, inner diameter × 50 mm) and elution with 0.1% formic acid (solvent A) and 0.1% formic acid in 90% CH_3CN (solvent B) using a program of 3% solvent B for 2 min, gradient at 2.1%/min for 45 min, 100% solvent B for 5 min, and flow rate of 2.5 μl/min. Proteins in-gel digested on 2-D SDS–PAGE were identified with LC-MS/MS using the PROWL (ProFound) and Mascot search engines, and NCBI database.

Ischemia and reperfusion. Rats (male, 250–280 g) were subjected to coronary artery ligation by techniques previously described [5]. Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally), intubated, and ventilated with room air (tidal volume, 20 ml/kg at rate of 60/min) with a respirator (SN-480-7, Shinano Manufacturing, Tokyo, Japan). After lateral thoracotomy and pericardiectomy, a 6–0 silk stitch was placed near the intramyocardial location of the left coronary artery beneath the left atrial appendage. We performed coronary artery occlusion by pressing a short length of tube over the ends of the suture and clamping it firmly against the heart. We achieved reperfusion by removing the clamp. The standard limb lead II electrocardiogram was monitored continuously. We confirmed the ischemia and reperfusion of the regional myocardium by following the changes of the ST segment level on the electrocardiogram and observing the change in the color of the myocardium.

Immunohistochemistry. We used tyramide signal amplification (TSA) technology for fluorescence (TSA-Direct [Green], NEN Life Science Products, according to the manufacturer's instructions). Rats were killed at each time point after myocardial ischemia/reperfusion. Cryostat sections (6-μm thick) of heart ventricles were prepared, air-dried, and fixed in acetone for 5 min. After washing in PBS, the sections were incubated with rabbit polyclonal anti-CyPA antibody (Upstate Biotechnology, NY, USA) or goat polyclonal anti-CD147 antibody (G-19; Santa Cruz Biotechnology, CA, USA) for 1 h at 37 °C. After washing in PBS, the sections were incubated with biotinylated anti-rabbit or goat IgG antibody (Vector Laboratories, CA) for 1 h at 37 °C. After washing in TNT buffer (0.1 mol/L Tris–HCl, pH 7.5,

0.15 mol/L NaCl, and 0.05% Tween 20), the sections were blocked with TNB buffer containing a blocking reagent for 30 min and then incubated with streptavidin–horseradish peroxidase for 30 min. After washing in TNT buffer, the sections were incubated with fluorescein–tyramide for appropriate time (3–10 min), washed in TNT buffer, and then examined, and photographed under a fluorescence microscope.

Immunocytochemistry. For immunocytochemical analysis, to distinguish cardiac myocytes from non-muscle cells (mainly consisted of fibroblasts), we performed double-staining for cardiac myosin and CyPA or CD147 using a mouse anti-cardiac myosin monoclonal antibody (mAb) (CMA19) [6] and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG antibody as described previously [7]. The procedures for staining of CyPA and CD147 were the same as for the tissue samples.

Western blot analyses for phosphorylation of ERK1/2, p38MAPK, SAPKs, and Akt. Cardiac myocytes were treated with 10 nM of recombinant human (rh) CyPA (Sigma Chemical, MO, USA) for the indicated time periods, then the culture media were aspirated immediately and cardiac myocytes were frozen in liquid nitrogen. The cells were lysed on ice with buffer A and the cell lysates were centrifuged, as described previously [3]. The supernatants were suspended in Laemmli's sample buffer. Aliquots of the samples were subjected to Western blot analyses using a rabbit polyclonal phospho-specific anti-ERK1/2 (Thr202/Tyr204), p38MAPK (Tyr182), SAPKs (Thr183/Tyr185), or Akt (Ser473) antibody (New England Biolabs, MA, USA), respectively. Aliquots of the same samples were also subjected to Western blot analyses using a rabbit polyclonal control anti-ERK1/2, p38MAPK, SAPKs, or Akt antibody (New England Biolabs), respectively. The antibody–antigen complexes were developed with chemiluminescence using alkalinephosphatase (New England Biolabs).

Western blot analyses for Bcl- 2 and Bcl-X. Cardiac myocytes were treated with 50 nM rhCyPA for the indicated time periods. The procedures for preparing the Western blot samples were the same as described above. Aliquots of the samples were subjected to Western blot analyses using mouse anti-Bcl-2 or -Bcl-X mAb (Transduction Laboratories, Lexington, KY, USA). Aliquots of the same samples were subjected to Western blot analysis using a goat polyclonal anti-actin (I-19) antibody (Santa Cruz Biotechnology). The antibody–antigen complexes were developed with chemiluminescence using alkalinephosphatase.

Results

Cultured cardiac myocytes secrete CyPA in response to hypoxia/reoxygenation

Fig. 1 shows the results of 2-D gel electrophoresis of control conditioned PBS (panel A) and reoxygenation-conditioned PBS (panel B) stained with silver. A protein spot (M_r 18.2 kDa and p*I* 8.4) indicated by an arrow (panel B) was not present in control-conditioned PBS (panel A) and seemed to appear in response to hypoxia/reoxygenation. LC-MS/MS analysis of the protein spot identified CyPA.

Hypoxia/reoxygenation induces expression of CyPA and CD147 on cultured cardiac myocytes in vitro

Next, we examined whether cardiac myocytes express CyPA and its cell surface receptor CD147 under normal condition and in response to hypoxia/reoxygenation. Fig. 2 shows double-stained cultured cardiac myocytes under normal condition or subjected to hypoxia/reoxygenation. Panels A, B, and E show the staining pattern specific for CyPA. Panel F shows the staining pattern specific for CD147. Panels C, D, G, and H which correspond to panels A, B, E, and F, respectively, show the staining pattern specific for cardiac myosin heavy chain and indicate that most of the cells were cardiac myocytes. There was only weak expression of CyPA on cardiac myocytes under normal condition (panel A). No significant change in the expression of CyPA was seen on cardiac myocytes subjected to hypoxia for 60 min (panel B). Most of the cardiac myocytes subjected to hypoxia for 60 min followed by reoxygenation for 10 min moderately to strongly expressed CyPA on their

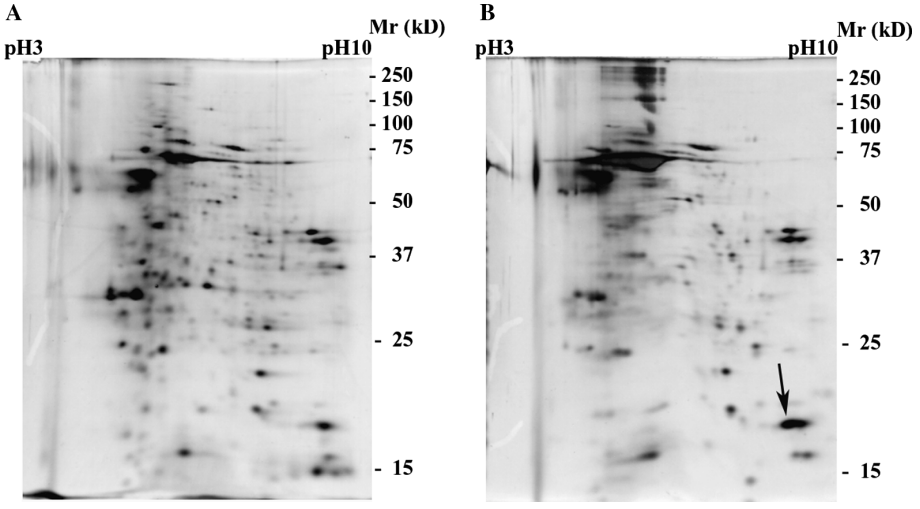

Fig. 1. 2-D gel electrophoresis of control-conditioned PBS (A) and reoxygenation-conditioned PBS (B) stained with silver. An arrow indicates a protein spot (B), which is not present in control-PBS (A).

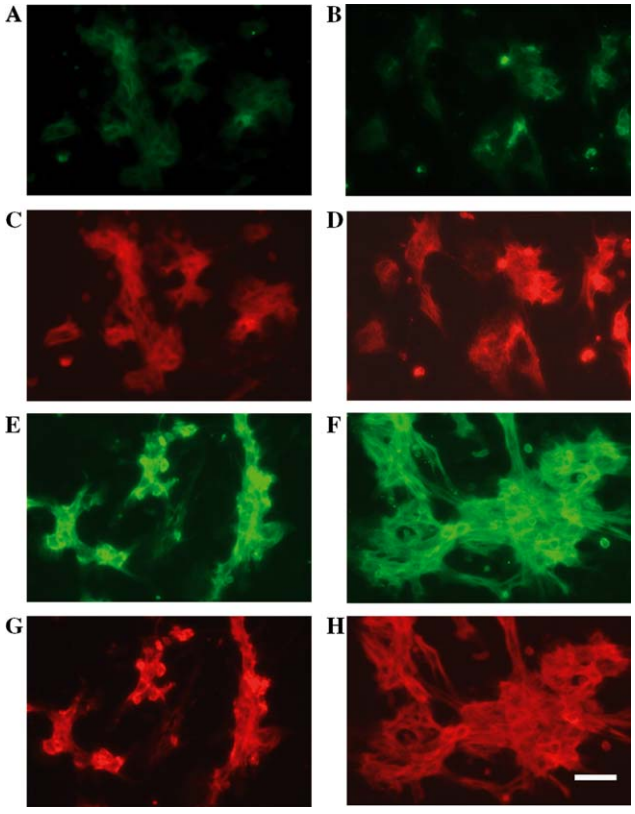

Fig. 2. Immunocytochemical study of cultured cardiac myocytes for CyPA and CD147. (A, B, E, and F) Myocytes under normal condition (A), myocytes subjected to hypoxia for 60 min (B), and myocytes subjected to hypoxia for 60 min followed by reoxygenation for 10 min (E), stained with anti-CyPA antibody, and labeled with FITC. Myocytes under normal condition (F) stained with anti-CD147 antibody and labeled with FITC. Panels C, D, G, and H which correspond to panels A, B, E, and F, respectively, show the staining pattern specific for cardiac myosin heavy chain and labeled with TRITC. Bar = 50 μm.

surfaces (panel E). Strong expression of CD147 was seen on cardiac myocytes under normal condition (panel F). The expression levels of CD147 on cardiac myocytes were not significantly changed by hypoxia or by hypoxia/reoxygenation in vitro (data not shown).

Ischemia/reperfusion induces expression of CyPA and CD147 on cardiac myocytes in vivo

To confirm the expression of CyPA and CD147 on cardiac myocytes in vivo, we examined their expression in ventricular tissues from sham-operated rats and rats subjected to myocardial ischemia/reperfusion. In sham-operated rats and rats subjected to myocardial ischemia for 30 min, there was only weak or almost no expression of CyPA on cardiac myocytes (Figs. 3A and B, respectively). In rats subjected to myocardial ischemia for 30 min followed by reperfusion for 15 min, there was a clear expression of CyPA on most of the cardiac myocytes (Fig. 3C). In sham-operated rats and rats subjected to myocardial ischemia for 30 min, there was a moderate expression of CD147 on most of the cardiac myocytes (Figs. 3D and E, respectively). Myocardial ischemia for 30 min followed by reperfusion for 30 min significantly increased the expression of CD147 on most of the cardiac myocytes (Fig. 3F).

CyPA activates ERK1/2, p38MAPK, SAPKs, and Akt in cultured cardiac myocytes

To investigate whether CyPA transduces signals through CD147 and stimulates cardiac myocytes, we examined whether rhCyPA phosphorylates MAPK family members ERK1/2, p38MAPK, and SAPKs, as well as Akt in cultured cardiac myocytes. As shown in Figs. 4A, B, and C, rhCyPA significantly phosphorylated ERK1/2, p38MAPK, and SAPKs, indicating the activation of these kinases. The phosphorylation was led to a maximum level biphasically at 2–5 min and 30 min for ERK1/2 and SAPKs, and at 5–10 min and 30 min for p38MAPK. rhCyPA also significantly phosphorylated

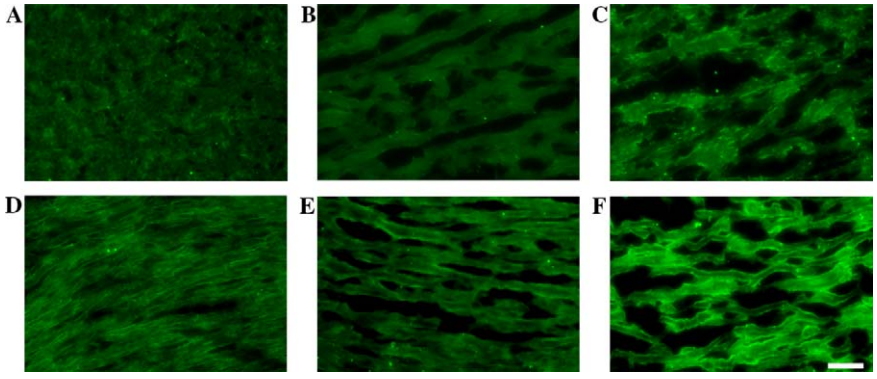

Fig. 3. Immunohistochemical study of ventricular myocardium for CyPA and CD147. Sham-operated myocardium (A), myocardium subjected to ischemia for 30 min (B), and myocardium subjected to ischemia for 30 min followed by reperfusion for 15 min (C) were stained with anti-CyPA antibody. Sham-operated myocardium (D), myocardium subjected to ischemia for 30 min (E), and myocardium subjected to ischemia for 30 min followed by reperfusion for 30 min (F) were stained with anti-CD147 antibody. Bar = 50 μm.

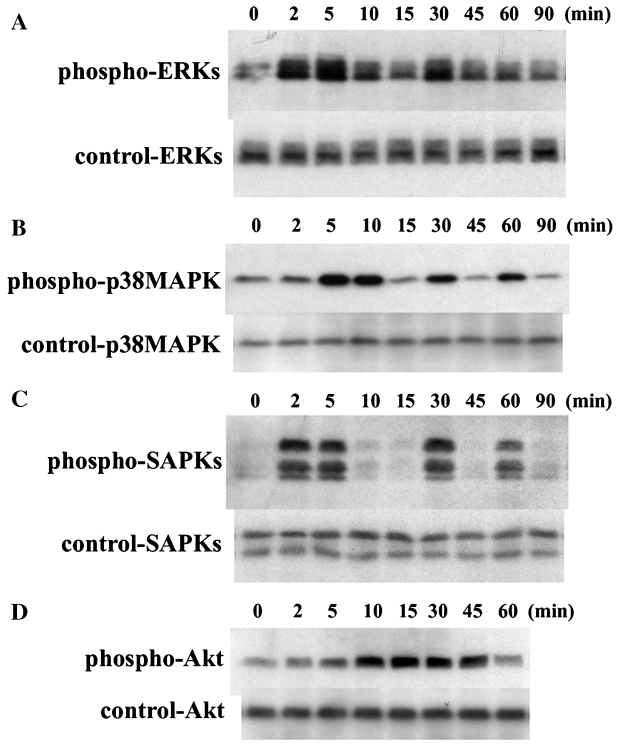

Fig. 4. Recombinant human (rh) CyPA phosphorylates ERK1/2, p38MAPK, SAPKs, and Akt. Serum-starved cardiac myocytes were treated with recombinant human (rh) CyPA (10 nM) for the indicated time periods and lysed in buffer A. The cell lysates were centrifuged and the supernatants were subjected to Western blot analyses using a phospho-specific ERK1/2 (Thr202/Tyr204) (A), p38MAPK (Tyr182) (B), SAPKs (Thr183/Tyr185) (C), or Akt (Ser473) (D) antibody, respectively. Aliquots of the same samples were also subjected to Western blot analyses using a rabbit polyclonal control anti-ERK1/2 (A), p38MAPK (B), SAPK (C), or Akt (D) antibody. The antibody–antigen complexes were developed with chemiluminescence using alkalinephosphatase. The experiments were performed at least in triplicate. The results shown are from one typical experiment.

Akt with a maximum level at 15–30 min. We confirmed that almost equal amounts of ERK1/2, p38MAPK, SAPKs, and Akt proteins were electrophoresed in each reaction by Western blot analyses using control anti-ERK1/2, -p38MAPK, -SAPKs, and -Akt antibodies (phosphorylation-state independent) (Fig. 4).

CyPA increases the expression of Bcl-2 in cultured cardiac myocytes

Because CyPA activates Akt in cardiac myocytes, next, we examined whether CyPA increases anti-apoptotic proteins such as Bcl-2 and Bcl-X_L in cardiac myocytes. As shown in Fig. 5, CyPA significantly increased Bcl-2 with a maximum level at 16 h, whereas CyPA did not significantly change the levels of Bcl-X_L and Bcl-X_S. Western blot analysis using an anti-actin antibody as an internal standard showed that almost equal amounts of samples were electrophoresed in each reaction (Fig. 5).

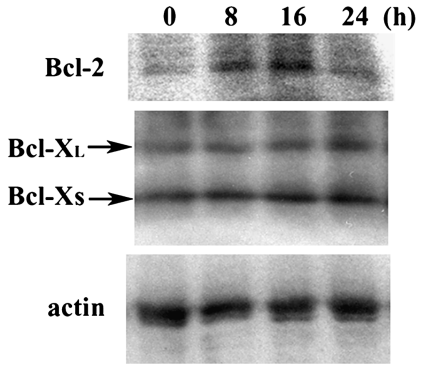

Fig. 5. Effects of recombinant human (rh) CyPA on the expression of Bcl-2 and Bcl-$X_{S/L}$. Serum-starved cardiac myocytes were treated with rhCyPA (50 nM) for the indicated time periods and lysed in buffer A. The cell lysates were centrifuged and the supernatants were subjected to Western blot analyses using an anti-Bcl-2 or -Bcl-X mAb. Aliquots of the same samples were also subjected to Western blot analysis using an anti-actin antibody. The antibody–antigen complexes were developed with chemiluminescence using alkalinephosphatase. The experiments were performed at least in triplicate. The results shown are from one typical experiment.

Discussion

In the present study, we have showed that CyPA was one of the proteins secreted from cultured rat cardiac myocytes in response to hypoxia/reoxygenation and that hypoxia/reoxygenation induced the expression of CyPA and its cell surface receptor CD147 [8] on cardiac myocytes. This strongly suggests that secreted CyPA interacts with CD147 on cardiac myocytes in an autocrine fashion and plays a role in activating intracellular signaling which mediates cardiac response to hypoxia/reoxygenation. We also showed that rhCyPA activated MAPK family kinases and Akt, and significantly increased Bcl-2 in cardiac myocytes, suggesting a protective role for CyPA against oxidative stress-induced apoptosis.

CyPA is an immunophilin family protein known to exist intracellularly and is distributed ubiquitously. CyPA is known to be an enzyme with peptidyl–prolyl *cis–trans* isomerase activity and acts as a molecular chaperone in protein folding [9,10]. In fact, CyPA has been shown to bind with cyclosporine A or is incorporated into human immunodeficiency virus type 1 (HIV-1) particles, and plays an essential role in immunosuppressive effect of cyclosporine A as well as HIV-1 infection [11–13]. In addition to the intracellular function, CyPA has also been shown to be secreted by cells in response to various stimuli and play important roles in chemotaxis of neutrophils, monocytes, and eosinophils as well as in protecting host cells from external stresses [14–16]. Jin et al. [16] reported that CyPA was secreted by vascular smooth muscle cells (VSMCs) in response to oxidative stress induced by LY83583, an O_2^- generator, and that secreted CyPA-mediated ERK activation in VSMCs, increased DNA synthesis, and

inhibited nitric oxide-induced apoptosis in VSMCs. The authors also demonstrated that the expression of CyPA was markedly increased in the balloon-injured vascular lesion, suggesting that CyPA acts as an oxidative stress-responsive growth and survival factor for VSMCs. It has been shown that mammalian cells quickly respond and adapt to external stresses such as mechanical load, metabolic changes, and hypoxia/reoxygenation, by expressing a number of various genes, which may have protective or injurious effects on the cells. In particular, cardiac myocytes express various genes coding for growth factors, cytokines, cell-adhesion molecules, and so on, in response to ischemia/reperfusion to adapt to these stresses or lead to further cell damage known as reperfusion injury. Moreover, evidence has accumulated that cardiac myocytes secrete various growth factors such as angiotensin II, transforming growth factor-β1, endothelin-1, atrial natriuretic peptide, and adrenomedullin, which in turn mediate cellular response to external stresses in an autocrine fashion [17–21]. Kitta et al. [22] reported that hepatocyte growth factor protected cardiac myocytes against apoptosis induced by oxidative stresses such as daunorubicin, serum deprivation, and hydrogen peroxide. In the present study, we have demonstrated for the first time that cardiac myocytes secreted CyPA in response to hypoxia/reoxygenation and that secreted CyPA played a role in activating intracellular signaling through CD147, which was upregulated on cardiac myocytes by hypoxia/reoxygenation. Upregulation of CyPA and CD147 on cardiac myocytes was also confirmed by ischemia/reperfusion in vivo, suggesting that the similar mechanism was involved in cardiac response to ischemia/reperfusion in vivo. Although at least several growth factors may be involved in the cardiac response to oxidative stresses, our data strongly suggest that CyPA plays a role in the protection of cardiac myocytes against oxidative stress-induced apoptosis through this autocrine mechanism.

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References

[1] Y. Seko, K. Tobe, K. Ueki, T. Kadowaki, Y. Yazaki, Hypoxia and hypoxia/reoxygenation activate Raf-1, mitogen-activated protein (MAP) kinase kinase, MAP kinases, and S6 kinase in cultured rat cardiac myocytes, Circ. Res. 78 (1996) 82–90.

[2] Y. Seko, K. Tobe, N. Takahashi, Y. Kaburagi, T. Kadowaki, Y. Yazaki, Hypoxia and hypoxia/reoxygenation activate src family tyrosine kinases and $p21^{ras}$ in cultured rat cardiac myocytes, Biochem. Biophys. Res. Commun. 226 (1996) 530–535.

[3] Y. Seko, N. Takahashi, K. Tobe, T. Kadowaki, Y. Yazaki, Hypoxia and hypoxia/reoxygenation activate $p65^{PAK}$, p38 mitogen-activated protein kinase (MAPK), and stress-activated protein kinase (SAPK) in cultured rat cardiac myocytes, Biochem. Biophys. Res. Commun. 239 (1997) 840–844.

[4] R. Mineki, H. Taka, T. Fujimura, M. Kikkawa, N. Shindo, K. Murayama, In situ alkylation with acrylamide for identification of cysteinyl residues in proteins during one- and two-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis, Proteomics 2 (2002) 1672–1681.

[5] H. Selye, E. Bajusz, S. Grasso, P. Mendell, Simple techniques for the surgical occlusion of coronary vessels in the rat, Angiology 11 (1960) 398–407.

[6] Y. Yazaki, H. Tsuchimochi, M. Kuro-o, M. Kurabayashi, M. Isobe, S. Ueda, R. Nagai, F. Takaku, Distribution of myosin isozymes in human atrial and ventricular myocardium: comparison in normal and overloaded heart, Eur. Heart J. 5 (Suppl. F) (1984) 103–110.

[7] Y. Seko, N. Takahashi, M. Azuma, H. Yagita, K. Okumura, Y. Yazaki, Effects of in vivo administration of anti-B7-1/B7-2 monoclonal antibodies on murine acute myocarditis caused by coxsackievirus B3, Circ. Res. 82 (1998) 613–618.

[8] V. Yurchenko, G. Zybarth, M. O'Connor, W.W. Dai, G. Franchin, T. Hao, H. Guo, H.-C. Hung, B. Toole, P. Gallay, B. Sherry, M. Bukrinsky, Active site residues of cyclophilin A are crucial for its signaling activity via CD147, J. Biol. Chem. 277 (2002) 22959–22965.

[9] A. Galat, Peptidylproline *cis–trans*-isomerases: immunophilins, Eur. J. Biochem. 216 (1993) 689–707.

[10] S.F. Gothel, M.A. Marahiel, Peptidyl–prolyl *cis–trans* isomerases, a superfamily of ubiquitous folding catalysts, Cell Mol. Life Sci. 55 (1999) 423–436.

[11] J. Liu, FK506 and cyclosporine, molecular probes for studying intracellular signal transduction, Immunol. Today 14 (1993) 290–295.

[12] D.A. Fruman, S.J. Burakoff, B.E. Bierer, Immunophilins in protein folding and immunosuppression, FASEB J. 8 (1994) 391–400.

[13] J. Luban, K.L. Bossolt, E.K. Franke, G.V. Kalpana, S.P. Goff, Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B, Cell 73 (1993) 1067–1078.

[14] B. Sherry, N. Yarlett, A. Strupp, A. Cerami, Identification of cyclophilin as a proinflammatory secretory product of lipopolysaccharide-activated macrophages, Proc. Natl. Acad. Sci. USA 89 (1992) 3511–3515.

[15] Q. Xu, M.C. Leiva, S.A. Fischkoff, R.E. Handschumacher, C.R. Lyttle, Leukocyte chemotactic activity of cyclophilin, J. Biol. Chem. 267 (1992) 11968–11971.

[16] Z.-G. Jin, M.G. Melaragno, D.-F. Liao, C. Yan, J. Haendeler, Y.-A. Suh, J.D. Lambeth, B.C. Berk, Cyclophilin A is a secreted growth factor induced by oxidative stress, Circ. Res. 87 (2000) 789–796.

[17] J. Sadoshima, Y. Xu, H.S. Slayter, S. Izumo, Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro, Cell 75 (1993) 977–984.

[18] N. Takahashi, A. Calderone, N.J. Izzo Jr., T.M. Maki, J.D. Marsh, W.S. Colucci, Hypertrophic stimuli induce transforming growth factor-beta 1 expression in rat ventricular myocytes, J. Clin. Invest. 94 (1994) 1470–1476.

[19] Y. Bezie, L. Mesnard, D. Longrois, F. Samson, C. Perret, J.J. Mercadier, S. Laurent, Interactions between endothelin-1 and atrial natriuretic peptide influence cultured chick cardiac myocyte contractility, Eur. J. Pharmacol. 311 (1996) 241–248.

[20] T. Horio, T. Nishikimi, F. Yoshihara, H. Matsuo, S. Takishita, K. Kangawa, Inhibitory regulation of hypertrophy by endogenous atrial natriuretic peptide in cultured cardiac myocytes, Hypertension 35 (2000) 19–24.

[21] F. Yoshihara, T. Horio, T. Nishikimi, H. Matsuo, S. Kangawa, Possible involvement of oxidative stress in hypoxia-induced adrenomedullin secretion in cultured rat cardiomyocytes, Eur. J. Pharmacol. 436 (2002) 1–6.

[22] K. Kitta, R.M. Day, T. Ikeda, Y.J. Suzuki, Hepatocyte growth factor protects cardiac myocytes against oxidative stress-induced apoptosis, Free Radic. Biol. Med. 31 (2001) 902–910.