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Original Article

Eosinophil Cationic Protein Shows Survival Effect on H9c2 Cardiac Myoblast Cells with Enhanced Phosphorylation of ERK and Akt/GSK-3β under Oxidative Stress

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Eosinophil cationic protein (ECP) is well known as a cationic protein contained in the basic granules of activated eosinophils. Recent studies have reported that ECP exhibits novel activities on various types of cells, including rat neonatal cardiomyocytes. Here we evaluated the effects of ECP on rat cardiac myoblast H9c2 cells. Our results showed that ECP enhanced the survival of the cells, in part by promoting the ERK and Akt/GSK-3β signaling pathways. ECP attenuated the cytotoxic effects of H_2O_2 on H9c2 cells as well as the production of reactive oxygen species, the number of apoptotic cells and caspase 3/7 activity in the cells. In conclusion, ECP activated the ERK and Akt/GSK-3β pathways, resulting in anti-oxidative effects on H9c2 cells that attenuated apoptosis.

Key words: ECP, reactive oxygen species, Akt, ERK

Eosinophil cationic protein (ECP) is one of the cationic proteins present within the basic granules of activated eosinophils, which play important roles in allergic diseases such as bronchial asthma [1]. Since activated eosinophils have been shown to degranulate ECP in bronchial asthma, ECP has been considered a biomarker for this condition [2]. The relationship between the diversity and the role of ECP has been described at the molecular level [3–6]. Based on these earlier studies, ECP was considered to function as an RNase because of the structural similarity between the 2 molecules. Thus, it has been considered that ECP is toxic only to cells. However, more recent reports have demonstrated that ECP has different effects on different cells types. For instance, ECP has been shown to induce the formation of stress fibers in BALB/c 3T3 fibroblasts and to increase the rate of autonomic pulsation in mouse-derived neonatal myocytes. In addition, ECP enhances the differentiation of the embryonal carcinoma cell line, P19CL6 cells, to cardiomyocytes. Recombinant ECP was shown to increase an atrial natriuretic factor (ANF) expression in rat neonatal cardiomyocytes. These findings suggest that ECP might play important multiple roles in myocytes/myoblasts.

Cardiovascular disease (CVD) is a major cause of death worldwide. Many novel therapeutic techniques

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have been developed and used to treat patients with CVD. However, an important issue in treating this disease is the ischemia/reperfusion injury caused by reperfusion therapy. Ischemia/reperfusion injury is caused by the production of oxidative molecules, including reactive oxygen species (ROS) [7–8]. Since ROS can damage myocytes and worsen the prognosis of patients with CVD [9], suppression of ROS production should be important to prevent ischemia/reperfusion injury, including acute myocardial infarction [10–12]. Accordingly, we examined the effect of ECP on H9c2 cells, a line of rat myoblasts, under an oxidative stress condition.

Materials and Methods

Reagents and cell culture. Recombinant human ECP was expressed in Escherichia coli and prepared as previously described [3]. The rat myoblast H9c2 cells were purchased from the American Type Culture Collection (ATCC, CRL-1446) (Manassas, VA, USA). H9c2 cells (between 5 to 15 passages) were cultured in Dulbecco's modified Eagle medium (DMEM; high glucose) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin [13–15]. Cells were cultured at 37℃ under 5% CO_2 and 20% O_2 in a humidified chamber. LY294002, a phosphatidylinositol 3-kinase inhibitor, was purchased from Cell Signaling (Danvers, MA, USA), and API-2, an Akt inhibitor, also known as Triciribine, was purchased from Calbiochem (La Jolla, CA, USA). These were then dissolved in DMSO, and used at the final concentrations previously reported [14, 16]. All other reagents were purchased from Sigma unless otherwise specified.

Cell viability assay. The viability of H9c2 cells was determined using a standard 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium (MTS) viability test (CellTiter AQueous One Solution cell proliferation assay kit; Promega, Madison, WI, USA) according to the manufacturer's instructions [13, 17].

In the initial experiment, H9c2 cells at 2×10^3/well were cultured on 96-well plates and allowed to recover for 24 h. The cells were then stimulated with ECP in the range of 0.2 to 2 μM for 72 h. Then 20 μL of the MTS assay reagent was added to each well and further incubated for 1 h. The number of surviving cells was measured using a plate-reading luminometer (Powerscan; DS Pharma Biomedical, Osaka, Japan) at 490 nm/650 nm as previously described [18].

Next, H9c2 cells at 5×10^3/well were cultured on 96-well plates and allowed to recover for 24 h. The cells were then stimulated with 1 μM of ECP and H_2O_2 in the range of 0 to 600 μM. The media were changed after 6 h and the MTS assay reagent was added. The number of surviving cells was measured as previously described. All samples were read in triplicate.

Protein extraction, Western blot analysis, and antibodies. To extract proteins, the cells were washed once with PBS and then scraped from the plates. The cells were then lysed in 150 μL of CelLytic™ M with a protease inhibitor (complete mini; Roche, Mannheim, Germany) and phosphatase inhibitor (phosphatase inhibitor cocktail; Sigma). After a gentle shaking incubation at 4℃ for 15 min, the samples were collected using a cell scraper and centrifuged to collect the supernatants. The protein concentration of the cell extracts was determined using a protein assay kit (Bio-Rad Japan, Tokyo, Japan). Sixty-five micrograms of total protein was used for the Western blot analysis, as described previously [19]. Briefly, each sample was mixed with 6× sample buffer and subjected to SDS-PAGE using a 10% separating gel. After SDS-PAGE, the proteins were transferred to PVDF membranes (Bio-Rad Japan), left overnight at 4℃, and then blocked for 1 h in 5% nonfat dried skim milk in TBS containing 0.05% Tween 20 (TBS-T). The membranes were hybridized at 4℃ overnight with an anti-p44/42 MAPK (Erk1/2) antibody (used at 1 : 1,000 dilution; Cell Signaling), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (used at 1 : 1,000 dilution; Cell Signaling), anti-Akt antibody (used at 1 : 1,000 dilution; Cell Signaling), anti-phospho-Akt (Ser473) (D9E) XP® antibody (used at 1 : 2,000 dilution; Cell Signaling), anti-glycogen synthase kinase 3β (GSK-3β) (27C10) antibody (used at 1 : 1,000 dilution; Cell Signaling), and anti-phospho-GSK-3β (Ser9) (5B3) antibody (used at 1 : 1,000 dilution; Cell Signaling), respectively. After 3 stringent washes with TBS-T for 10 min each at room temperature, the membranes were incubated with the peroxidase-conjugated goat anti-rabbit secondary antibody (used at 1 : 2,500 dilution; MP Biomedicals, Aurora, OH, USA). Following 3 successive washes with TBS-T, the

immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL plus; GE Healthcare). Signals were detected with an LAS1000 Imager System (Fuji Film, Tokyo, Japan) and the densitometry was performed with Image J software (W. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA) and normalized to the signal intensity of the control antibody of each sample in each experiment [13].

ROS production. The production of ROS was measured using the cell-permeable fluorogenic probe 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA) based on the ROS-dependent oxidation of DCFH to DCF, according to the manufacturer's protocol (OxiSelect™ ROS Assay Kit; Cell Biolabs Inc., San Diego, CA, USA). In brief, H9c2 cells (1×10^4 cells/well) were cultured in a black 96-well plate. The medium was changed to serum-free medium, and then, after 24 h, the serum-free medium was removed and replaced with 100 μL of 1 × DCFH-DA solution. The cells were incubated at 37℃ for 60 min. The DCFH-DA solution was removed, and the cells were washed with PBS twice and then exposed with H_2O_2 in the range of 100 to 600 μM and 1 μM of ECP for 6 h. Fluorescence was measured using a plate-reading luminometer at 480 nm/530 nm.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. H9c2 cells (5×10^4) were plated on a chamber slide and the serum-free medium was replaced after 48 h. 1 μM of ECP was added and cells were exposed to 200 μM of H_2O_2 at 37℃ for 10 min. The cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) at RT for 1 h. The cells were then washed with PBS twice, supplemented with 0.1% sodium citrate and 0.1% TritonX-100, and incubated at 4℃ for 5 min. After washing with PBS twice, a TUNEL reaction solution was added and the cells were incubated at 37℃ for 1 h. Finally, the cells were washed with PBS three times and fluorescent signals were examined under a fluorescent microscope (BioZero; Keyence, Osaka, Japan) [15]. The number of TUNEL-positive cells was counted in 10 high power fields. All samples were read in triplicate.

Caspase 3/7 assay. Caspase 3/7 activity levels were measured using an assay kit (Caspase-Glo 3/7 Assay; Promega) according to the manufacturer's instructions. Briefly, H9c2 cells (1×10^4) were plated into 96-well plates in 100 μL DMEM with 10% FBS. H9c2 cells were exposed to H_2O_2 in the range of 100 to 200 μM at the indicated concentrations and incubated with 1 μM of ECP or without ECP for 2 h. Caspase 3/7 activities were measured using a plate-reading luminometer [18]. All samples were read in quadruplicate.

Statistical analysis. All the data are shown as the mean ± S.D. Between-group variations were assessed by the two-tailed unpaired *t*-test. For multiple comparisons, analysis of variance (ANOVA) was performed and post-hoc analysis with Bonferroni's test was employed. P values < 0.05 were considered significant.

Results

We first examined the effects of ECP on H9c2 cells by MTS assay. ECP in the range of 0.2 to 2 μM slightly enhanced the proliferation of H9c2 cells (Fig. 1). We then examined the phosphorylation of ERK and Akt/GSK-3β by ECP in H9c2 cells (Fig. 2). ECP enhanced the phosphorylation of ERK, Akt and GSK-3β in a dose-dependent manner suggesting activation of both the ERK and PI3K/Akt/GSK-3β signaling pathways.

To elucidate the role of ECP in the survival effect of H9c2 cells, we investigated the response of H9c2 cells under oxidative stress in the presence of ECP.

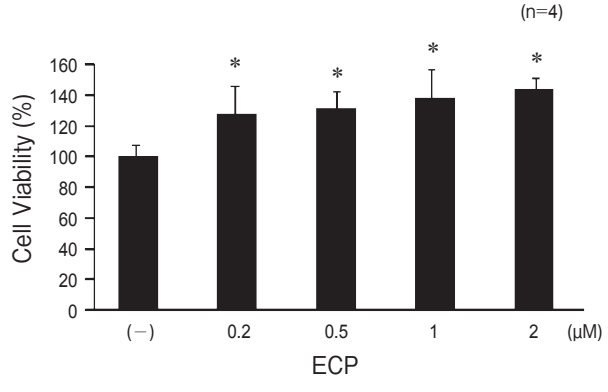

Fig. 1 ECP enhanced the proliferation of H9c2 cells. H9c2 cells were incubated with various concentrations of ECP and cell viability was measured by an MTS assay. The number of cells without ECP was indicated as 100%. An asterisk indicates a significant difference from the cells without ECP at the $p < 0.05$ level. All samples were read in triplicate.

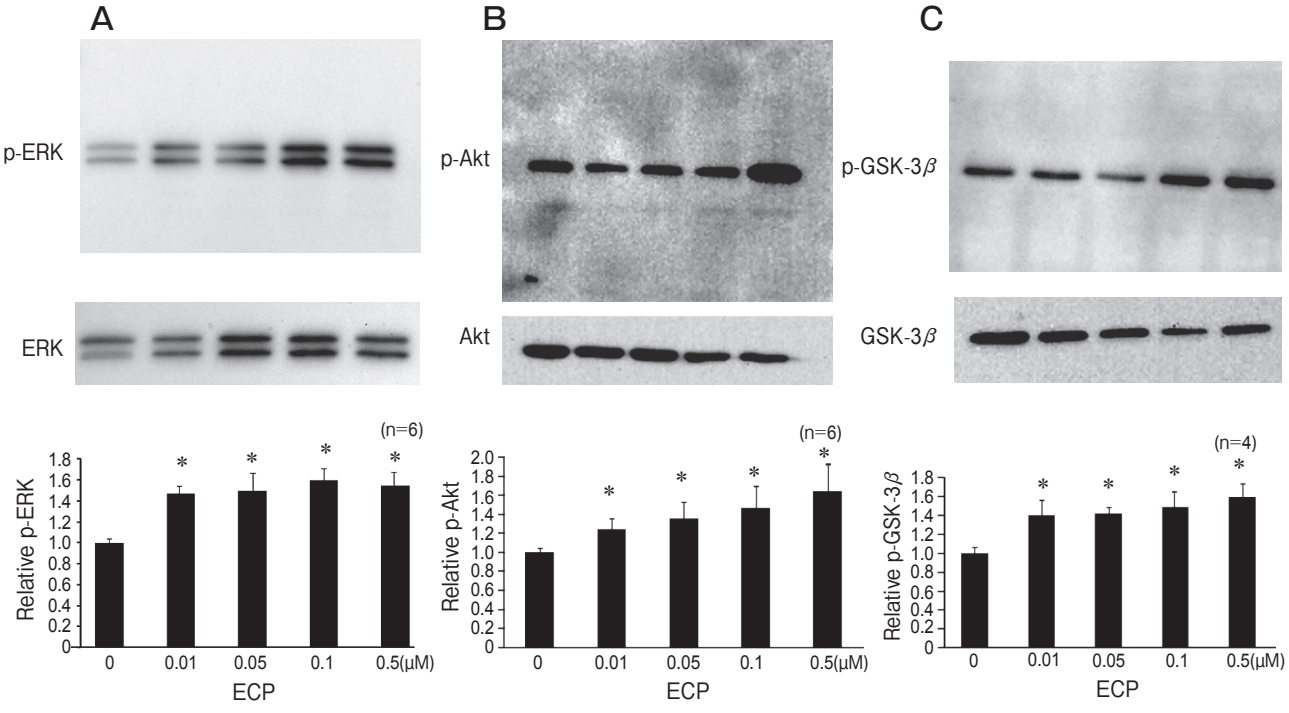

Fig. 2 ECP augmented the phosphorylation of ERK and Akt/GSK-3β. (**A**) The effects of ECP on the phosphorylation of ERK (**A**), Akt (**B**) and GSK-3β (**C**) were evaluated by Western blotting. The results of densitometric analysis for the quantification of the phosphorylation of each blotting are aligned at the bottom of the figure. An asterisk indicates a significant difference from the control at the $p < 0.05$ level.

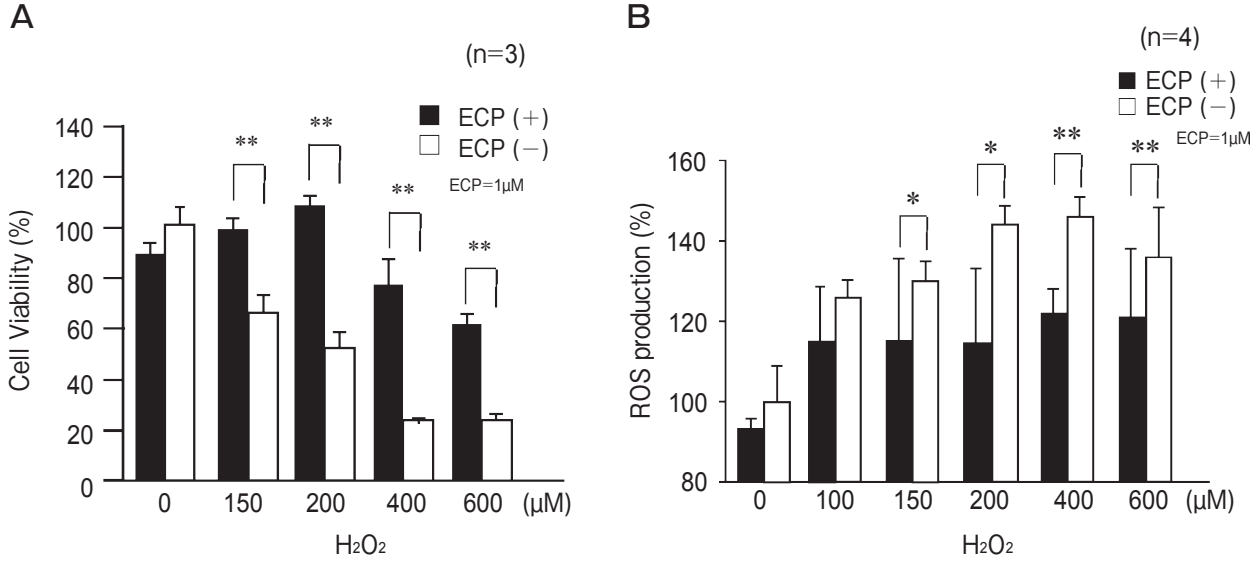

Fig. 3 ECP attenuated the cell death and the production of ROS. H9c2 cells were incubated with 1μM of ECP (closed boxes) and without ECP (open boxes). (**A**) The viability of H9c2 cells exposed to various concentrations of H_2O_2 was measured by an MTS assay. All samples were read in triplicate. (**B**) The production of ROS in H9c2 cells exposed to various concentrations of H_2O_2 was measured by a DCFH-DA assay. All samples were read in quadruplicate. An asterisk indicates a significant difference from the cells without ECP at the $p < 0.05$ level and double asterisk indicates a significant difference from the cells without ECP at the $p < 0.01$ level.

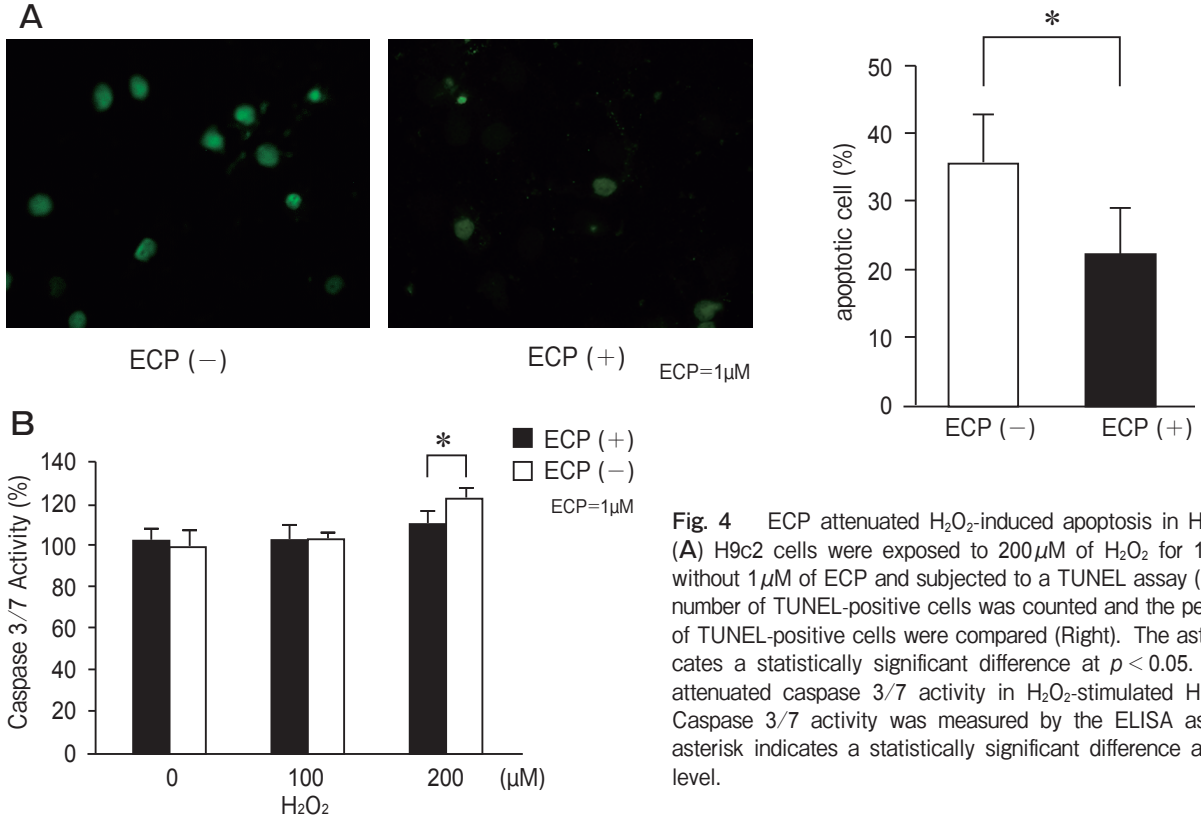

Fig. 4 ECP attenuated H_2O_2-induced apoptosis in H9c2 cells. (**A**) H9c2 cells were exposed to 200 μM of H_2O_2 for 1h with or without 1 μM of ECP and subjected to a TUNEL assay (Left). The number of TUNEL-positive cells was counted and the percentages of TUNEL-positive cells were compared (Right). The asterisk indicates a statistically significant difference at $p < 0.05$. (**B**) ECP attenuated caspase 3/7 activity in H_2O_2-stimulated H9c2 cells. Caspase 3/7 activity was measured by the ELISA assay. The asterisk indicates a statistically significant difference at $p < 0.05$ level.

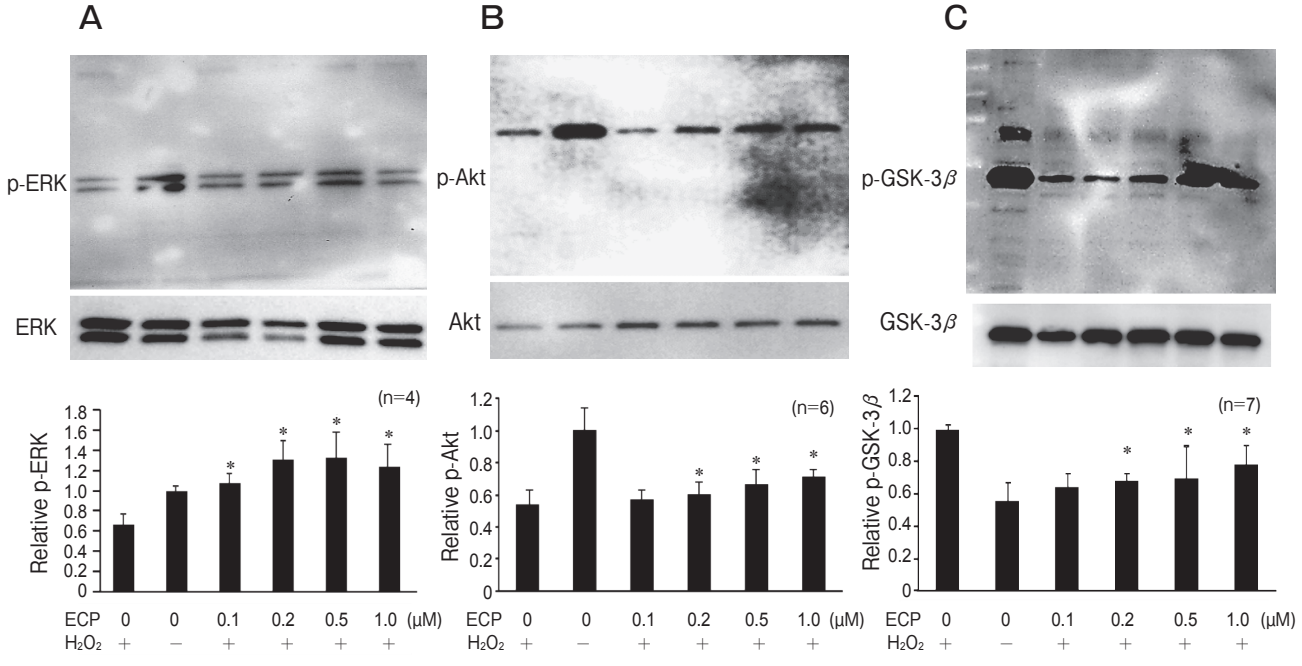

Fig. 5 ECP augmented the cell signaling pathway in H9c2 cells exposed to H_2O_2. H9c2 cells exposed to H_2O_2 were treated with various concentrations of ECP and the phosphorylation of ERK (**A**), Akt (**B**) and GSK-3β (**C**) was examined by Western blotting. The results of densitometric analysis for the quantification of the phosphorylation of each blotting are aligned at the bottom of the figure. The phosphorylation level in H9c2 cells incubated without H_2O_2 and ECP was taken as 1.0. An asterisk indicates a significant difference from the H9c2 cells incubated without H_2O_2 and ECP at the $p < 0.05$ level.

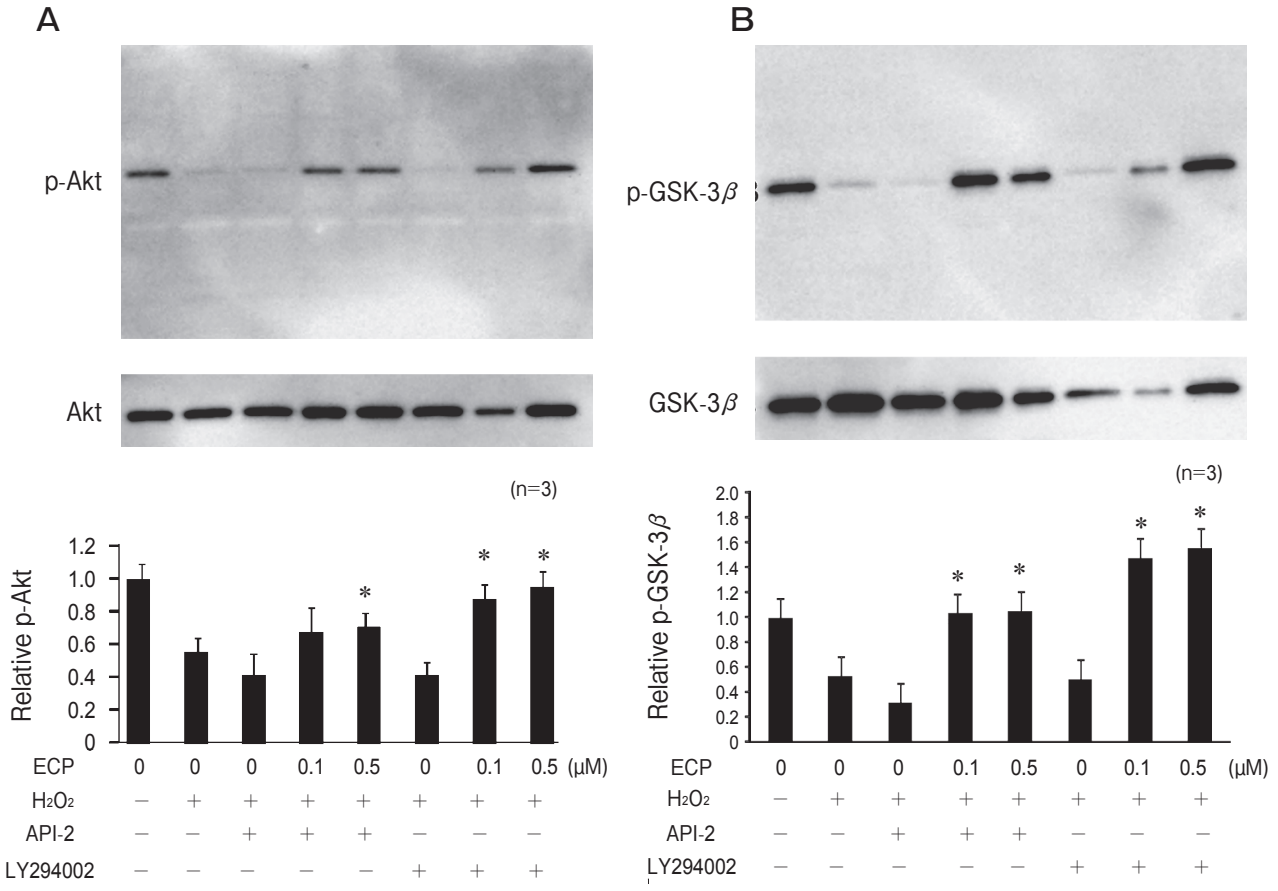

Fig. 6 ECP augmented the phosphorylation of Akt/GSK-3β in H_2O_2-stimulated H9c2 cells. H_2O_2-stimulated H9c2 cells were incubated with or without API-2 and LY294002, and the phosphorylation of Akt (**A**) and GSK-3β (**B**) was evaluated. The results of densitometric analysis for the quantification of the phosphorylation of each blotting are aligned at the bottom of the figure. An asterisk indicates a significant difference from the H9c2 cells without ECP in the presence of H_2O_2 and each inhibitor at the $p < 0.05$ level.

H9c2 cells were incubated in the presence or absence of ECP and then stimulated with H_2O_2. As previously described, when H9c2 cells were incubated for 72 h with 1 μM ECP without H_2O_2, their viability was significantly increased. When H9c2 cells were incubated for 6 h with 1 μM ECP in the absence of H_2O_2, their viability was slightly decreased, although this change was not statistically significant (Fig. 3A). ECP apparently protected H9c2 cells from death by oxidation with H_2O_2. While the number of surviving cells exposed to H_2O_2 decreased in a dose-dependent manner, ECP significantly enhanced the survival of the cells (Fig. 3A). To clarify the protective role of ECP under oxidative stress, we next examined the production of reactive oxygen species by an DCFH-DA assay. The results showed that ECP attenuated the production of ROS in H_2O_2-stimulated H9c2 cells (Fig. 3B).

In the TUNEL assay, a significantly smaller number of TUNEL-positive cells was observed in the ECP-treated H9c2 cells than the H9c2 cells not treated with ECP (Fig. 4A). ECP also attenuated caspase 3/7 activity in H9c2 cells exposed to 200 μM of H_2O_2 (Fig. 4B).

To assess the effects of ECP on cellular signaling in H_2O_2-stimulated H9c2 cells, H9c2 cells were stimulated with 500 μM of H_2O_2 for 1.5 h, then incubated with ECP for 2 h. The results showed that ECP augmented the phosphorylation of ERK, Akt and GSK-3β in H9c2 cells when stimulated with H_2O_2 (Fig. 5).

To confirm the effect of ECP on these signaling

pathways, we examined the effects of ECP on H_2O_2-stimulated H9c2 cells in the presence of 10 nM of API-2 and 20 μM of LY294002. ECP recovered the phosphorylation of both Akt and GSK-3β in H_2O_2-stimulated H9c2 cells while API-2 and LY294002 attenuated the phosphorylation of Akt and GSK-3β without ECP (Fig. 6).

Discussion

The deposition of eosinophil granule proteins in cardiac tissues was previously reported in eosinophilic myocarditis [20]. However, none of the previous reports have shown a molecular role of ECP in diseases, but only described the accumulation of eosinophils to the local organs with the marked increase of ECP level. Only toxicity has been suggested without describing molecular mechanisms. In recent reports on ECP using recombinant ECP, the effect of ECP on the cells was not always cytotoxic; rather, ECP sometimes induced differentiation and strengthened the cytoskeleton in various cell types [4–6]. Taking these facts into consideration, we consider that the functional role of ECP should be revisited to confirm the biological significance of ECP. For this purpose, we prepared a recombinant ECP protein [21] and used it to confirm the RNase activity and bactericidal activity of ECP [3]. In myocytes, ECP has been shown to exert diverse effects. For instance, we have reported that ECP increased the beating rate of rat neonatal cardiomyocytes [5], and accelerated the differentiation of P19CL6 cells into myocytes by activating ERK pathway [6].

As previously described, the intracellular signaling pathway plays a central role in the cell survival [22], and signaling through GSK-3β is also critical for the survival of apoptotic H9c2 cells exposed to H_2O_2 [23]. These results suggest that modulation of the signaling pathway affects cell survival under oxidative stress conditions [24]. In this report we found that ECP enhanced the survival of rat cardiac myoblasts of the cell line H9c2 under oxidative stress by activating the Akt/GSK-3β signaling pathway. H9c2 cells are widely used for *in vitro* analyses, including investigations of oxidative stress. Interestingly, ECP did not show toxic effects on H9c2 cells, but rather exhibited proliferative and protective effects on H9c2 cells. Our results showed that ECP enhanced the phosphorylation of ERK in H9c2 cells incubated with or without H_2O_2. This augmentation of ERK phosphorylation may be one of the molecular mechanisms of ECP on H9c2 cells, as described in P19CL6 cells [5]. ECP was previously shown to enhance the phosphorylation of ERK via FGFR signaling in P19CL6 cells; therefore, the FGFR pathway may be suggested to be involved in the effects of ECP on H9c2.

Since the PI3K-Akt pathway has been reported to protect cells from injury [25], we examined the effect of ECP on Akt-GSK-3β phosphorylation. Interestingly, ECP enhanced the phosphorylation of Akt and the phosphorylation of GSK-3β without any stimulation. When H9c2 cells were exposed to H_2O_2, the phosphorylation of Akt and the phosphorylation of GSK-3β were attenuated, and ECP recovered these phosphorylations. These results indicate that ECP enhances the Akt-GSK-3β signaling pathway in H9c2 cells.

The results of the DCFH-DA assay demonstrated that ECP attenuated the production of ROS. As ROS are known to be toxic to cells, ultimately inducing apoptosis, our data imply that ECP had a protective effect on H9c2 cells under an oxidative stress condition. ECP was previously reported to induce the production of ROS in HL-60 cells and HeLa cells [26]. In contrast, in our study ECP attenuated the production of ROS, and this effect was significant when H9c2 cells were stimulated with H_2O_2 at concentrations between 150 μM and 600 μM. The difference may have been due to the differences in the cell types and ECP concentrations between the 2 studies.

H_2O_2 was previously shown to induce apoptosis in H9c2 cells by promoting the cleavage of caspase [12, 27], and this is the first study to demonstrate the anti-apoptotic effects of ECP. ECP was previously considered to be toxic for cells. For example, Kato *et al.* reported that eosinophil granular proteins damaged bronchial epithelial cells [28]. However, this damage was mainly induced by the major basic protein and eosinophil peroxidase, but not by ECP. Chang *et al.* reported that the recombinant ECP protein induced apoptosis in BEAS-2B cells [29]. In contrast, we here demonstrated the anti-apoptotic effects of ECP in H_2O_2-stimulated H9c2 cells. The molecular mechanism underlying cellular injury by oxidative stress has been investigated, and it is now widely accepted that intracellular signaling pathways including the PI3K-Akt pathway are involved in the pathobiology of oxida-

tive stress [12, 22]. In this report, ECP enhanced the phosphorylation of ERK, Akt, and GSK-3β in H_2O_2-stimulated H9c2 cells. Therefore, we consider that ECP altered the signaling pathway to protect against ROS-induced cellular damage.

Here we demonstrated that ECP is a potent inducer of the phosphorylation of Akt and GSK-3β. Because the Akt signaling pathway plays an important role in cell survival, ECP might be involved in the mechanism of cell survival protecting H9c2 cells by altering the Akt/GSK-3β signaling pathway.

In conclusion, ECP enhances the survival of H9c2 cells through augmented phosphorylation of ERK and the Akt-GSK-3β axis under oxidative stress, which might result in attenuation of the ROS and ROS-induced apoptosis.

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