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PDE4 inhibitor, roflumilast protects cardiomyocytes against NO-induced apoptosis via activation of PKA and Epac dual pathways

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Abstract

Myocyte apoptosis plays an important role in myocardial infarction and cAMP is crucial in the regulation of myocyte apoptosis. Phosphodiesterase-4 (PDE4) inhibitor blocks the hydrolysis of cAMP via inhibition of PDE4 and is attractive candidate for novel anti-inflammatory drugs. However, its function in cardiovascular diseases and cardiomyocyte apoptosis is unclear. Therefore, we investigated whether roflumilast, a PDE4 inhibitor, exerts protective effect against NO-induced apoptosis in both of H9c2 cells and neonatal rat cardiomyocytes (NRCMs), focusing on cAMP downstream molecules such as protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac). According to our data, intracellular cAMP was increased by roflumilast treatment in H9c2 cells and NRCMs. Roflumilast inhibited SNP-induced apoptosis and this effect was reversed by PKA specific inhibitor H-89 and KT-5720. In addition, PKA specific activator N^6-benzoyladenosine 3′,5-cyclic monophosphate (N^6Bz-cAMP) mimicked the effects of roflumilast. CREB phosphorylation by roflumilast was also inhibited by H-89, indicating that roflumilast protects SNP-induced apoptosis via PKA-dependent pathway. Roflumilast increased Epac1/GTP-Rap1 and the protective effect was abolished by Epac1 siRNA transfection, demonstrating that Epac signaling was also involved in this protective response. In support, Epac specific activator 8-(4-chlrorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate (8CPT-2Me-cAMP) protected SNP-induced apoptosis. PI3K/Akt inhibitor LY294002 blocked roflumilast-induced Akt phosphorylation and protective effect. Furthermore, inhibition of Epac1 with siRNA had no effect on roflumilast-induced CREB phosphorylation, whereas inhibited Akt phosphorylation, implicating that Akt phosphorylation was regulated by Epac pathway. In addition, it was also observed that rolipram and cilomilast exert similar effects as roflumilast. In summary, our data indicate that roflumilast protects NO-induced apoptosis via both cAMP–PKA/CREB and Epac/Akt-dependent pathway. Our study suggests a possibility of PDE4 inhibitor roflumilast as a potential therapeutic agent against myocardial ischemia/reperfusion (I/R) injury.

Keywords: Phosphodiesterase-4 (PDE4) inhibitor; Roflumilast; I/R; PKA; Epac; Nitric oxide (NO)

1. Introduction

Cardiomyocyte apoptosis has important pathophysiological consequences contributing to functional abnormalities. It has been reported in a variety of cardiovascular diseases, including myocardial infarction, end-stage heart failure and arrhythmogenic right ventricular dysplasia [1–3]. cAMP signaling in cardiomyocytes is crucial in the regulation of myocytes apoptosis and cardiac remodeling. Recent *in vitro* and *in vivo* studies have demonstrated that an increase of cAMP inhibits apoptosis in cardiomyocytes and reduces mortality in acute myocardial infarction [4], suggesting that it has an important role in normal physiological adaptation. In classic signaling cascades, increased production of cAMP leads to activation of protein kinase A (PKA), which in turn causes phosphorylation/activation of cAMP response element (CRE) binding protein (CREB) and subsequent gene expression through CRE-mediated transcription [5]. cAMP-mediated activation of PKA alone, however, cannot account for cAMP's survival effect in all cell types. In neuron and gastric epithelial cells, antiapoptotic effect by cAMP is PKA dependent [6,7], whereas in hepatocytes and β cells the survival effect of cAMP is PKA

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independent [8,9]. Although PKA activation by cAMP analogue protects the myocardium *in vivo* [10], exact roles and underlying mechanisms of cAMP in cardiomyocyte apoptosis are not fully understood.

While most studies of cAMP signaling have focused on protein kinase A (PKA), cAMP has been shown to regulate gene transcription, cellular proliferation, and cytokine signaling through PKA-independent pathway [11,12]. One of such cAMP activated PKA-independent pathway involves guanine nucleotide exchange factors (GEFs also designated as Epac) for small GTPases Rap1 and Rap2. It has been demonstrated that cAMP activated-Epac, in turn, directly activates Rap1 and this does not involve PKA activation [13]. Recent studies reported that Epac is involved in cell adhesion [14,15], neurite extension [16], and regulation of insulin secretion and β-cell apoptosis [17]. In the heart, activation of Epac induces cardiomyocytes hypertrophy through the activation of Rac and calcineurin/NFAT signaling pathway [18]. However, it was not elucidated the role of Epac in cardiomyocytes apoptosis at this moment.

However, the use of cAMP analogs is often difficult to apply in the clinical setting. Alternative methods of upregulating the cAMP and its downstream molecules may lie in the use of phosphodiesterase (PDE) inhibitors. PDEs are family of hydrolases that catalyzes the hydrolysis of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), thus regulating the intracellular cAMP and cGMP gradients [19]. PDEs belong to a complex and diverse superfamily of at least 11 structurally related gene families (PDE1 to PDE11) [20]. At least PDE1, PDE2, PDE3, PDE4 and PDE5 isoforms are expressed in myocardium, of which PDE3 and PDE4 represent about 90% total cAMP–PDE activity and contributes to the regulation of cAMP levels in rat cardiomyocytes [21], thus it maybe also be important in the regulation of specific signaling pathways and cardiac function. In particular, PDE4 localized cytochemically on sarcolemma of the cardiac myocytes in rat [22] and the subcellular localization of PDE4D related to Z-line of sacomere is closely involved in regulation of the myocytes contraction [21]. Furthermore, reduction of PDE4D3 activity resulted in increased PKA-mediated phosphorylation of ryanodine receptor (RyR2, the major intracellular Ca^{2+}-release channel in the heart) in PDE4D knockout mice, rendering the channels "leaky" and contributing to heart failure and arrhythmias [23]. It has been reported that pharmaceutical inhibition of PDE4 exerts beneficial effects on improvement of cardiac contractility during endotoxemia [24]. As it is well known that cAMP inhibits activities of many inflammatory and immunomodulatory cells, PDE4 inhibitors show pronounced anti-inflammatory effects in various animal models (e.g., for asthma and other allergic disease, rheumatoid arthritis, multiple sclerosis, and others) [25]. Therefore, it has been proposed as a new therapeutic approach for variety of inflammatory diseases such as asthma [26]. Rolipram is a specific PDE4 inhibitor whose therapeutic utility has been investigated in the treatment of depression [27] and also has the capacity to suppress inflammatory process. It was recently reported that rolipram antagonizes IL-4 activated signaling in isolated human T cells [28]. However, despite the large effort of the pharmaceutical industries to identify selective PDE4 inhibitors, for only a few of them effectiveness in patients has been reported. Among these, roflumilast, most potent and advanced PDE4 inhibitor so far, has been demonstrated to be an effective anti-inflammatory agent in many inflammatory diseases, including asthma, collagen-induced arthritis and bowel disease [29,30]. It was recently reported that roflumilast inhibits LPS-induced inflammatory mediators via inhibition of NF-*k*B, p38 MAPK and JNK in macrophage [31] and leukocytes–endothelial interaction by inhibiting adhesion molecule expression [32]. Although roflumilast exhibits several beneficial effects in inflammation, the functional role in regulation of cardiomyocyte apoptosis and cardiovascular disease has not been fully explored.

Therefore, the aim of this study was to investigate whether the PDE4 inhibitor roflumilast could modulate NO-induced cardiomyocytes apoptosis, focusing on PKA- and Epac-dependent pathways. Here, for the first time, we report that cAMP elevation by roflumilast induced two different signaling pathways, namely PKA-dependent CREB phosphorylation and Epac-dependent Akt phosphorylation, rendering protection from cardiomyocytes apoptosis.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), α-MEM, fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY). Roflumilast and cilomilast were synthesized by Korea Research Institute of Chemical Technology (KRICT; Taejon, Korea). Rolipram, sodium nitroprusside (SNP), H-89, Direct cAMP enzyme immunoassay kits, and dibutyryl cyclic AMP (db-cAMP) were purchased from Sigma (St Lois, MO, USA). KT-5720, adenosine 3′,5′-cyclic monophosphate, 8-(4-chlorophenylthio)-2′-*O*-methyl-, sodium salt (8CPT-2Me-cAMP), and adenosine 3′,5′-cyclic monophosphate, N^6-Benzoyl-sodium salt (N^6Bz-cAMP) were obtained by Calbiochem (La Jolla, CA, USA). Lipofectamine™ RNAiMAX was obtained by Invitrogen (Carlsbad, CA, USA). Specific antibodies against Akt and p-Akt^{ser473} were the products of Santa Cruz (Santa Cruz, CA, USA) and CREB, p-CREB^{ser133}, Epac1 (cAMP-GEF) were purchased from Upstate Cell Signaling Solution (Charlottesville, VA, USA). Rap1 activation kit was obtained by Stressgen (Victoria, BC, Canada). All other reagents were the products of Sigma chemical unless indicated otherwise (St. Louis, MO, USA).

2.2. Isolation of neonatal rat cardiomyocytes (NRCMs) and cell culture

Hearts from 1–3 days old Sprague Dawley rat (obtained from Orient, Seoul, Korea) were washed in 1× PBS buffer (Mg^{2+}, Ca^{2+} free). Hearts were minced with collagenase type II (0.8 mg/ml, Gibco-BRL) in sterile HBSS and incubated for 5 min. The pellet was collected in HBSS containing 0.8 mg/ml collagen and incubated for 5 min. The supernatant was collected in α-MEM containing 10% FBS and digestion steps were repeated up to 12 times. Cardiomyocytes were purified from fibroblasts using repeated centrifugation step. Finally, NRCMs were resuspended and cultured in α-MEM containing 10% FCS, penicillin G (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. More than 90% of cells were cardiomyocytes (positive for troponin-I and beating feature). The H9c2 embryonal rat heart-derived cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in DMEM with 10% FBS, penicillin G (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells from passages 20 through 30 were used for all studies. To achieve quiescence, cells were starved for 24 h in 0.1% FBS.

2.3. Cell viability assay

Cell viability was determined by a mitochondrial tetrazolium assay (MTT). Subconfluent cells in 12 well plates were incubated with various chemicals for 1 h before exposed to SNP (1.5 mM) for additional 24 h. After SNP stimulation, 100 μl/well of MTT solution (5 mg/ml in PBS) was added and the cells were incubated for 3 h. The medium was aspirated and replaced with 300 μl/well of 0.2% HCl in isopropanol. The plates were shaken for 20 min, then optical density (OD) was measured at 570 nm using the microplate reader (Boehringer Mannheim, IN, USA).

2.4. Assay for cAMP

H9c2 cells (5×10^6 cells/well) were incubated for 15 min in media alone or with roflumilast in the indicated concentrations. db-cAMP (200 μM) was also incubated for 15 min as a positive control. After washing the cells, the medium 0.1 M HCl was added for cell lysis and centrifuged for 10 min at 600 ×g, then use the supernatant directly in the assay. Acetylation of the sample is needed for the sensitivity of cAMP (in case, very low levels of cAMP) as provided by direct cAMP enzyme immunoassay kit. The enzyme immunoassay kit was used according to the manufacturer's instruction.

2.5. FITC-annexin V and propidium iodide double staining by flow cytometry

Double staining with FITC-annexin V and propidium iodide (PI) was performed for flow cytometry analysis. Cells were washed with PBS and resuspended in a binding buffer. FITC-annexin V (1 μg/ml) and PI (20 μg/ml) were added. The mixture was incubated for 10 min in the dark and FACS analysis was done using FACSVantage system (Becton Dickinson, San Jose, CA).

2.6. Western blot analysis

Protein samples of whole cell lysate (30 μg) were mixed with an equal volume of 2× SDS sample buffer, boiled for 5 min, and then separated by 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to nitrocellulose membrane. The membranes were blocked in 5% non-fat dry milk for 1 h, rinsed, and incubated with specific antibodies against Epac1, CREB, p-CREB, Akt and p-Akt in Tris-buffered saline (TBS) containing Tween-20 (0.1%) overnight at 4 °C. Primary antibody was removed by washing the membranes 3 times in TBS-T, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:1000–2000). Following 3 times of washing in TBS-T, immuno-positive bands were visualized by ECL and exposed to X-ray film (Amersham, Piscataway, NJ).

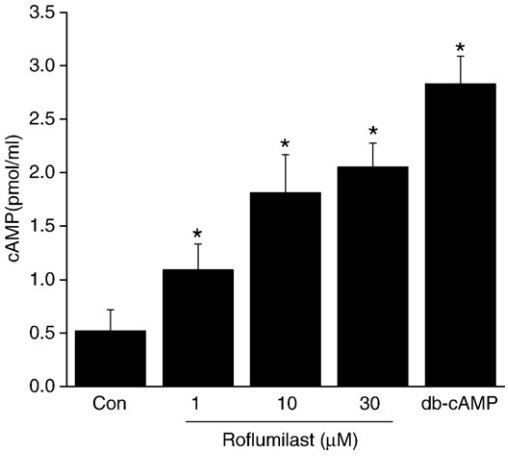

Fig. 1. Effects of roflumilast on intracellular cAMP levels. H9c2 cells were incubated for 15 min with media alone and indicated concentration of roflumilast. Lysates of the cells were assayed using cAMP enzyme immunoassay. Data shown are mean±SD of two experiments (each performed in duplicates). *$p<0.05$ versus control.

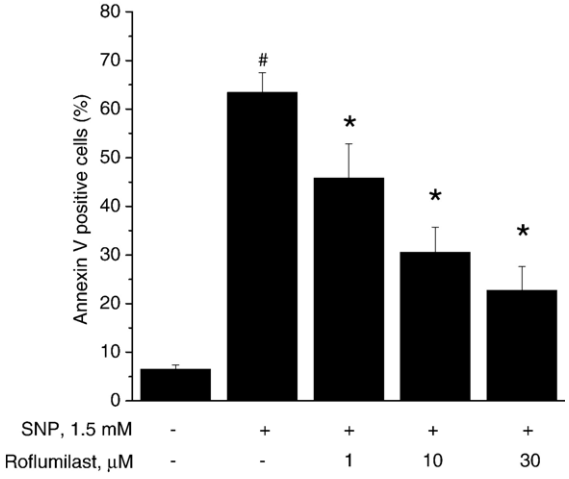

Fig. 2. Roflumilast protects H9c2 cells from subsequent SNP-induced apoptosis. Cells were pretreated with various concentration of roflumilast for 1 h then, incubated with 1.5 mM SNP for 24 h. After the SNP treatment, cells were washed and tested for apoptosis by FITC-annexin V. Data shown are mean±SD of four experiments (each performed in duplicates). #$p<0.05$ versus untreated control, *$p<0.05$ versus 1.5 mM SNP-treated group.

2.7. Analysis of Rap1 activation

Rap1 activation was performed according to the manufacturer's instruction. Briefly, H9c2 cells (5×10^6 cells/well) were incubated with roflumilast (30 μM) and 8CPT-2-Me-cAMP (300 μM) for 30 min and rinsed with TBS. The protein lysates were incubated with GTPγS (0.1 mM) and GDP (1 mM) for 30 min at 30 °C. The reaction was stopped by adding 1 M $MgCl_2$ (60 mM). For precipitation of Rap1, cell lysates transferred to spin cup containing with GST-RalGDS-RBD and incubated at 4 °C for 1 h with gentle shaking. Spin cup was centrifuged at 7200 g for 10–30 s and washed the resin by adding of lysis/binding/wash buffer and re-centrifuged. The immunoprecipitates were added 2× SDS sample buffer and processed for PAGE (12%) and immunoblotting with Rap1 antibodies.

2.8. Small interfering RNA (siRNA) and transfection of siRNA

Small interfering RNA (siRNA) against endogenous Epac1 mRNA (GeneBank accession no. NM_021690) was designed and synthesized by Samchully Pharm. Co., Ltd (Seoul, Korea). siRNA was constructed with the following sequences: 5′-GUCAUCUCUUGGCAACGUACCCUdAdC-3′ and 5′-GUAGGGUACGUUGCCAAGAGAUGACGG-3′. The oligonucleotides were annealed according to the Samchully pharm's protocol. The siRNA was transfected into H9c2 cells using Amaxa transfection kit and NRVCMs using Lipofectamine™ RNAiMAX according to the manufacturer's instruction. The 10 μg (H9c2 cells) and 30 nM (NRVCMs) of siRNA were transfected to the cells, respectively, and incubated for 36–48 h prior to the treatment of compounds. Efficiency of siRNA was evaluated by western blot using a specific antibody against Epac1.

2.9. Statistical analysis

Data are represented as means±SD of more than three separate experiments. The significance of difference from the respective control for each experimental test condition was assessed by using Student's *t*-test for each paired experiments. A p value <0.05 was considered as significant.

3. Results

3.1. Effects of roflumilast on cAMP accumulation in H9c2 cells

We first examined the effect of roflumilast on cAMP production in H9c2 cells. As expected, treatment with roflumilast for 15 min increased intracellular cAMP levels. db-cAMP (200 μM) as a positive control was also increased cAMP levels (Fig. 1).

3.2. Roflumilast inhibits NO-induced apoptosis in H9c2 cells

Since it was previously reported that high concentration nitric oxide (NO) induces apoptosis in H9c2 cells [33], we confirmed NO donor SNP-induced apoptosis. In our system, SNP treatment induced apoptosis in a concentration-dependent manner (data not shown). As shown in Fig. 2, roflumilast treatment concentration dependently prevented SNP-induced apoptosis, determined by annexin V staining.

3.3. PKA-dependent protective effect of roflumilast against NO-induced apoptosis in H9c2 cells

Next, we determined whether roflumilast protects SNP-induced apoptosis in a PKA-dependent manner. As shown in Fig. 3A, roflumilast protected SNP-induced apoptosis in a concentration-dependent manner, and this protective effect was optimal at 30 μM roflumilast. db-cAMP (cAMP activator) also inhibited SNP-induced apoptosis (data not shown). To analyze the role of PKA in roflumilast-induced protection, we employed specific inhibitors of PKA, H-89 and KT5720. Incubation with H-89 (10 μM) and KT5720 (5 μM) before roflumilast addition, significantly reversed the protective effects of roflumilast. To further confirm the involvement of PKA, we examined common PKA substrate CREB as an indicator of PKA activation. As shown in Fig. 3B, roflumilast (30 μM) was able to induce CREB phosphorylation and its effect was inhibited by H-89. To directly assess the involvement of PKA in SNP-induced apoptosis, we next examined the effect of N^6Bz-cAMP, a specific activator for

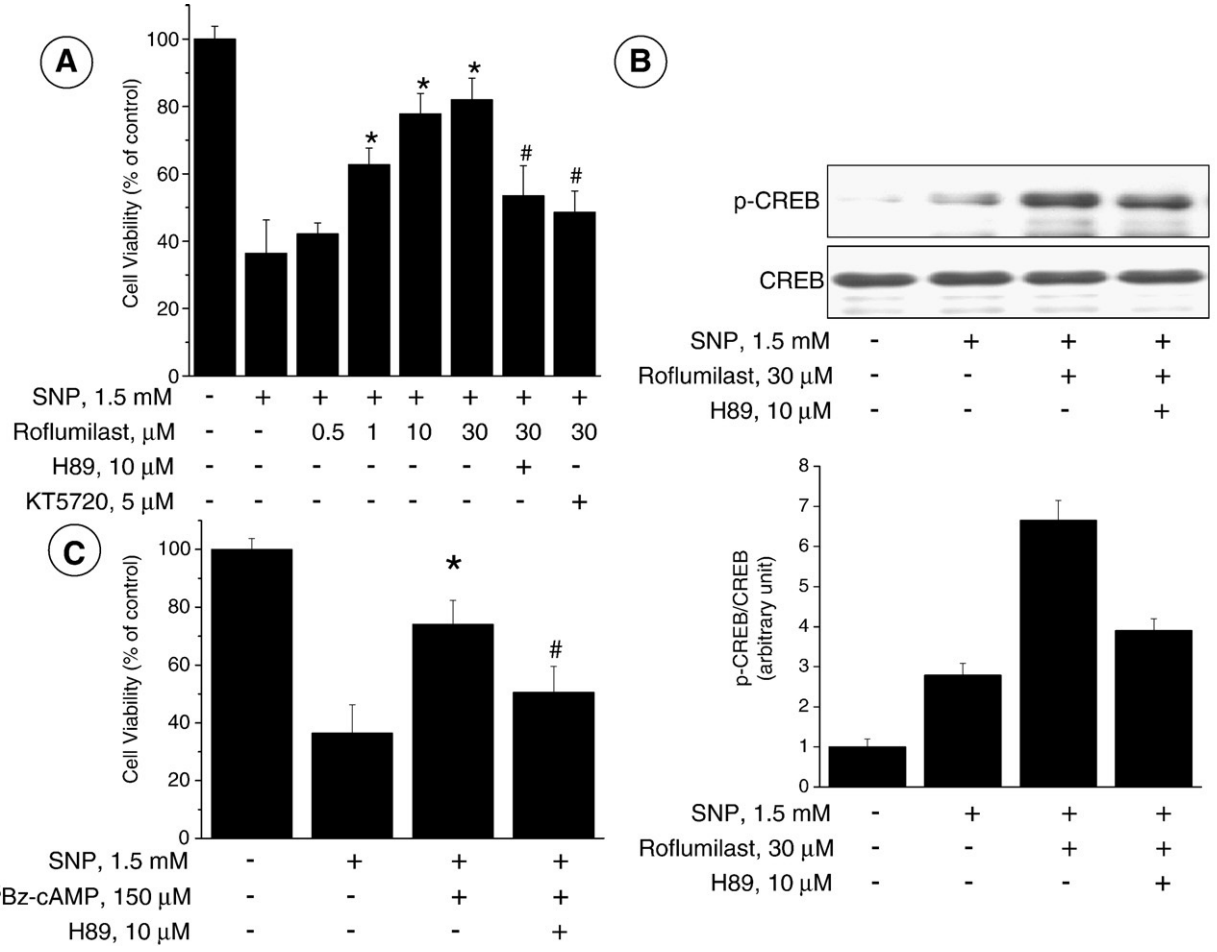

Fig. 3. Roles of PKA signaling induced by roflumilast on NO-induced apoptosis. H9c2 cells were pre-incubated with either by roflumilast (0.5–30 μM) or N^6Bz-cAMP (150 μM) for 1 h in the absence or presence of H-89 (10 μM) and KT-5720 (5 μM), and then SNP (1.5 mM) was treated for indicated times. (A) and (C), Twenty four hours later, cell viability was measured using MTT assay. (B) Thirty minutes later, p-CREB and CREB levels were measured in cellular extracts using western blot. Data shown are mean ± SD of four experiments (each performed in duplicates). *$p<0.05$ versus SNP-treated group, $^{\#}p<0.05$ versus SNP plus roflumilast (30 μM) or N^6Bz-cAMP (150 μM) -treated group. Immunoblots shown are representative of four independent experiments.

PKA. According to our data, N^6Bz-cAMP treatment (150 μM) mimicked the protective effect of roflumilast, while H-89 reversed effects of N^6Bz-cAMP (Fig. 3C). These results imply that the protective effects of roflumilast require PKA signaling.

3.4. Roflumilast activates Epac–Rap1 signaling in H9c2 cells

Recent studies have shown that Epac was identified as one of cAMP targets and Rap1 specific GEF in a PKA-independent manner [13]. We therefore hypothesized that Epac–Rap1 signaling pathway may be involved in roflumilast-induced protective effects in H9c2 cells. To test this hypothesis, we examined whether roflumilast activated Rap1 by assaying GTP-Rap1. As shown in Fig. 4A, roflumilast treatment (30 μM) upregulated Epac1, which was somewhat dependent upon time and this increase was declined at 3 h. The cAMP agonist, 8CPT-2Me-cAMP (300 μM), designed to specifically activate the Epac but not PKA, also induced Epac1 expression. Moreover, roflumilast treatment for 30 min activated GTP-Rap1 by ~2.5-fold compared to unstimulated cells without affecting total Rap1 level. 8CPT-2Me-cAMP also activated GTP-Rap1 (Fig. 4B).

3.5. The protective effect of roflumilast against NO-induced apoptosis is also Epac dependent

Because we observed Epac–Rap1 activation in response to roflumilast, it is possible that roflumilast inhibits NO-induced apoptosis by activating Epac–Rap1. To address this possibility, we examined the effect of silencing Epac1 gene expression by siRNA on protective effect of roflumilast. Under our experimental conditions, the maximal silencing of Epac1 was observed with 10 μg of siRNA (up to 71% reduction, Fig. 5A), and therefore we have used this concentration of Epac1 siRNA in all our experiments. In Fig. 5B, we have shown that Epac1 siRNA partially reduced roflumilast-induced protective effect compared to normal H9c2 cells. These results suggest that roflumilast protects NO-induced apoptosis through an Epac signaling pathway.

3.6. The protective effects of roflumilast involves Akt phosphorylation in H9c2 cells

The Akt cascade is known to mediate cellular survival. Thus, we tested the involvement of Akt. As shown in Fig. 6A, Akt phosphorylation was induced by roflumilast (30 μM) treatment

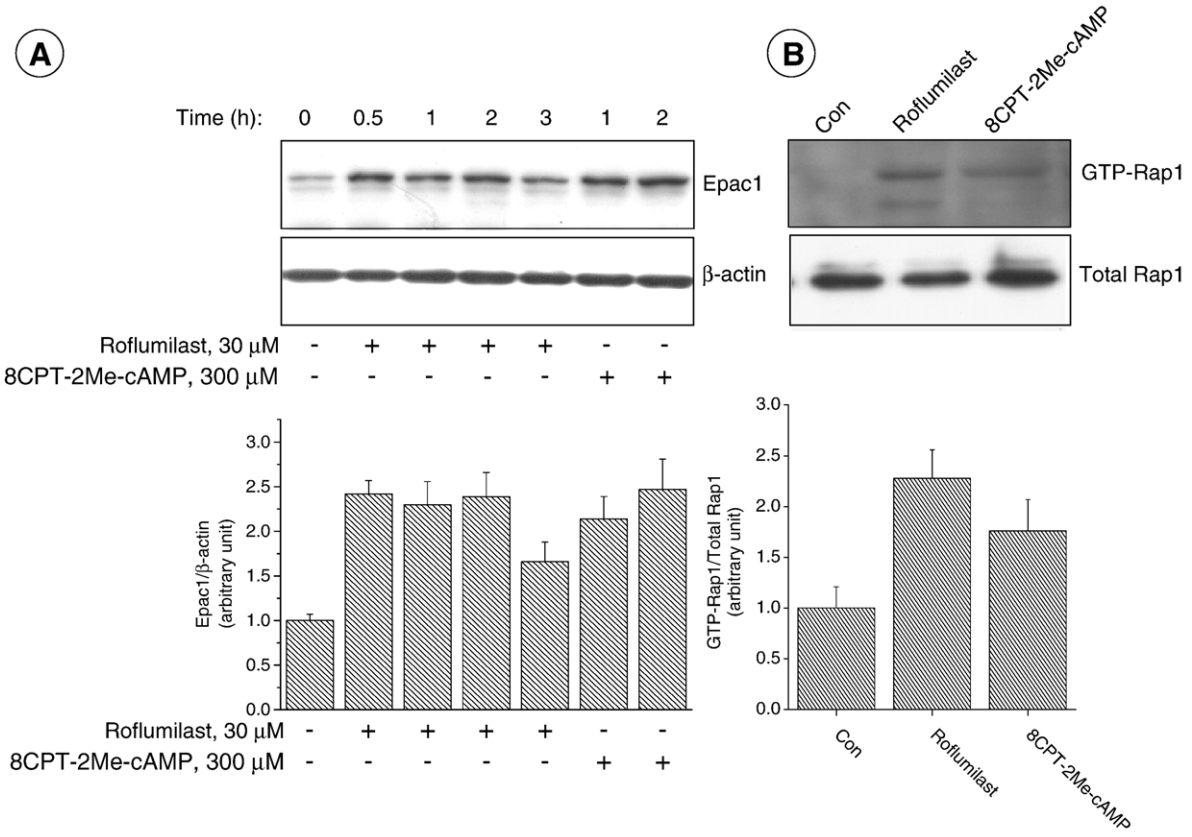

Fig. 4. Upregulation of Epac1 and Rap1-GTP in roflumilast-treated H9c2 cells. (A) Cells were pretreated with roflumilast (30 μM) and 8CPT-2Me-cAMP (300 μM) for indicated time points. Total protein was isolated and subjected to western blot analysis for Epac1. Epac1 expression was quantified by β-actin expressions. (B) Cells were pretreated with roflumilast (30 μM) and 8CPT-2Me-cAMP (300 μM) for 30 min. The activated GTP-bound Rap 1 was determined by using a pull-down assay with a glutathione-agarose conjugate of Ral GDS binding domain and detected by immunoblotting with Rap1 antibodies. See "Materials and methods" for detail. Immunoblots shown are representative of two (B) and three (A) independent experiments.

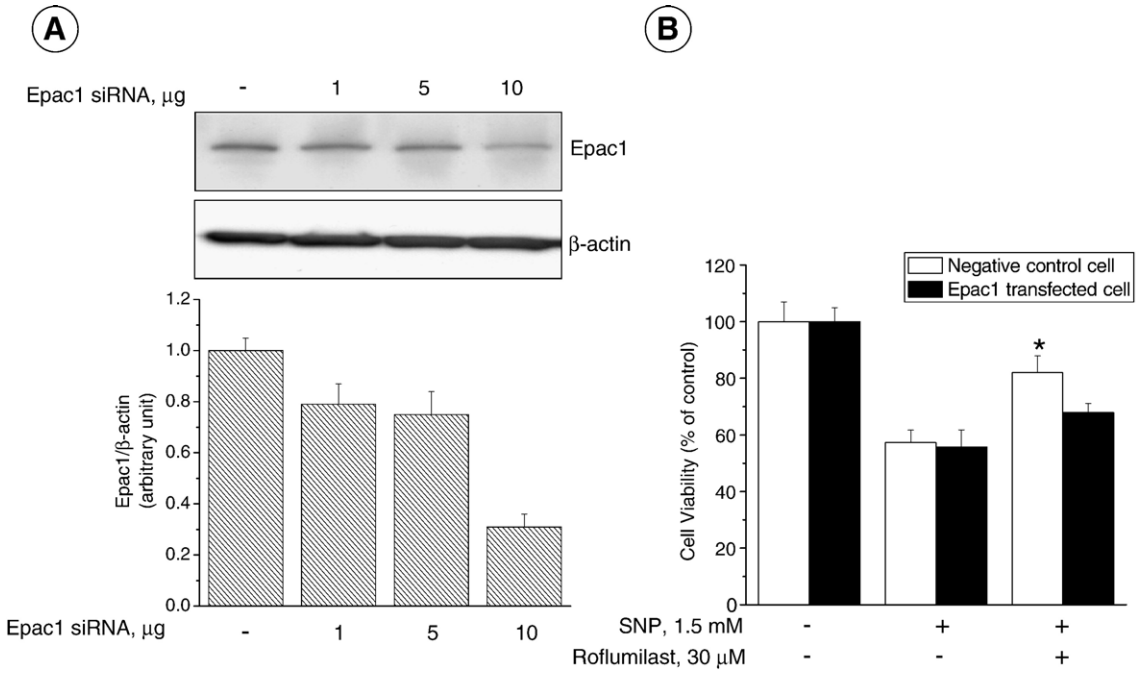

Fig. 5. Roles of Epac signaling induced by roflumilast on NO-induced apoptosis. (A) Cells were transfected with a dose-dependent Epac1-specific siRNA (1–10 μg) for 36 h. Then, total protein was isolated and subjected to western blot analysis for Epac1. Epac1 expression was quantified by β-actin expressions. (B) Cells were transfected with non-targeting control and Epac-specific siRNA (10 μg) for 36 h and pre-incubated with roflumilast (30 μM) for 1 h and then, treated with SNP (1.5 mM) for 24 h. Cellular viability was determined by MTT assay. Data shown are mean ± SD of four experiments (each performed in duplicates). *$p < 0.05$ versus SNP-treated control. Immunoblots shown are representative of two independent experiments.

and sustained until 3 h. SNP treatment slightly increased Akt phosphorylation and pretreatment with roflumilast for 1 h resulted in a further increase of Akt phosphorylation. Also, Akt phosphorylation by roflumilast was abolished by LY294002 (PI3K inhibitor, 10 μM) treatment (Fig. 6B). Next, we examined whether the protective effect of roflumilast was directly involved in Akt dependent pathway. Pretreatment with roflumilast for 1 h protected cell from NO-induced apoptosis, and this protective effect was readily reversed by LY294002 (Fig. 6C).

3.7. Roflumilast modulates Akt phosphorylation via Epac activation in H9c2 cells

It was previously reported that Epac activation by 8CPT-2Me-cAMP subsequently activates Akt pathway in bile acid and Fas-induced apoptosis in hepatocytes [9]. Our results indicate that roflumilast-induced PI3 kinase/Akt signaling is crucial for the protective effect against NO-induced apoptosis. We next examined whether Epac activation by roflumilast indeed contributes to Akt phosphorylation. As shown in Fig. 7A, the reduction of Epac1 by siRNA abolished roflumilast-induced Akt phosphorylation. By contrast, Epac1 reduction by siRNA did not affect roflumilast-induced CREB phosphorylation, indicating that roflumilast-induced Akt phosphorylation is most likely to be mediated via Epac signaling pathway. Furthermore, 8CPT-2Me-cAMP induced Akt phosphorylation, whereas N^6Bz-cAMP did not (Fig. 7B). This was also confirmed by observing that 8CPT-2Me-cAMP and N^6Bz-cAMP treatment inhibited NO-induced apoptosis, and this protective effect was abolished by PI3 kinase/Akt inhibitor only when 8CPT-2Me-cAMP was used (Fig. 7C). These results suggest that Akt phosphorylation is upregulated by Epac pathway.

3.8. Roles of rolipram and cilomilast on NO-induced apoptosis in H9c2 cells

Our results have indicated that activation of PKA and Epac was essential for roflumilast-induced protective effect on NO-induced apoptosis, it would be important to confirm the physiological relevance of the pathway by another PDE4 selective inhibitor. Therefore, we set out a key series of experiments with rolipram and cilomilast, well known PDE4 inhibitors in H9c2 cells. As shown in Fig. 8, rolipram (30 μM) and cilomilast (30 μM) protected SNP-induced apoptosis in a concentration-dependent manner. Furthermore, similar to roflumilast, rolipram and cilomilast inhibited NO-induced apoptosis via both cAMP–PKA/CREB and Epac/Akt-dependent pathways (Fig. 8).

3.9. Roles of roflumilast and rolipram on NO-induced apoptosis in NRCMs

Because the above findings demonstrated in cardiac myogenic cell line, H9c2 cells, the next series of experiments was carried out in NRCMs. In Fig. 9A, the selective PDE4 inhibitors, roflumilast and rolipram reproduced the protective effect as seen in H9c2 cells. Interestingly, roflumilast affected viability at relatively lower concentration compared to H9c2 cells. Maximum protection occurred at a dose of roflumilast

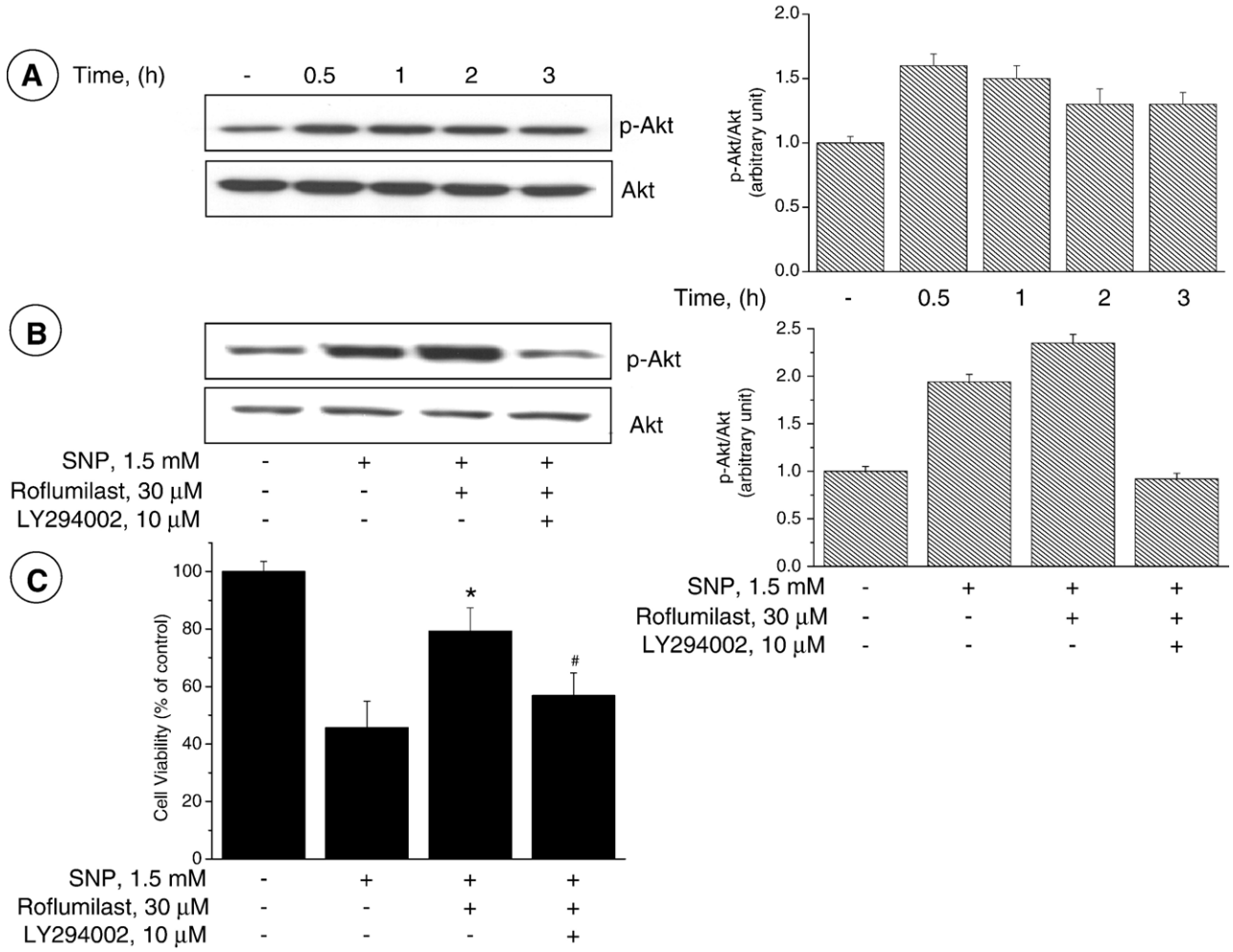

Fig. 6. Effect of roflumilast on Akt signaling against NO-induced apoptosis. (A) Cells were pretreated with roflumilast (30 μM) for indicated time points and (B) pre-incubated with roflumilast (30 μM) for 1 h in the absence or presence of LY294002 (10 μM) and then SNP (1.5 mM) was treated for 30 min. Then total protein was isolated and subjected to western blot analysis for p-Akt/Akt. (C) Cells were pre-incubated with roflumilast (30 μM) for 1 h in the absence or presence of LY294002 (10 μM), then SNP (1.5 mM) was treated for 24 h. Cell viability was analyzed by MTT assay. Data shown are mean ± SD of four experiments (each performed in duplicates). $*p<0.05$ versus SNP-treated control, $^{\#}p<0.05$ versus SNP plus roflumilast-treated group. Immunoblots shown are representative of three independent experiments.

1 μM and rolipram 10 μM, respectively. In all further experiments, roflumilast and rolipram were used at the dose of 1 μM and 10 μM. Similarly to H9c2 cells, phosphorylation of CREB and Akt was abrogated by H-89 and LY294002 treatment, indicating that activation of these two pathways in NRCMs plays an important role in PDE4 inhibitor-induced protection (Fig. 9B). Epac1 gene expression by Epac1 siRNA (30 nM) transfection significantly reduced by up to 79% compared to control cells. In Fig. 9D, knockdown of Epac1 gene expression significantly attenuated PDE4 inhibitor induced protective effects compared to control cells. Furthermore, the reduction of Epac1 abolished roflumilast- and rolipram-induced Akt phosphorylation, however, did not affect CREB phosphorylation (Fig. 9E). These are consistent with results shown in H9c2 cells.

4. Discussion

PDE4 selective inhibitor increases the intracellular cAMP level and suppressed I/R injury in various models. However, its potential in myocardial I/R injury and cardiomyocyte survival remains to be elucidated. In the present study, we explored the potential use of roflumilast as an antiapoptotic drug in cardiomyocyte survival both in the H9c2 cell and neonatal rat cardiomyocytes (NRCMs). We also demonstrated that protective effect of PDE4 inhibitor roflumilast against NO-induced cardiomyocytes apoptosis is mediated via PKA–CREB and Epac–Akt dual pathway.

PDE4 is present in myocardium of various species, although its relative ratio might be different among species [21], and selective pharmacological PDE4 inhibition increased cardiomyocytes cAMP levels. To elucidate its role in cardiomyocytes, we first examined whether the roflumilast elevates cAMP level in H9c2 cells. To date, several reports have been suggested regarding the role of cAMP in apoptosis of cardiac myocytes. An increase of cAMP was shown to promote myocyte survival in case of cardiac I/R injuries via activation of PKA [4,34–36]. In contrast, other studies demonstrated that high dose of Bromo-cAMP (Br-cAMP) induced apoptosis in cardiac myocytes via cAMP–PKA pathway [37]. Although effects of cAMP are conflicted in cardiomyocyte, our data showed that roflumilast

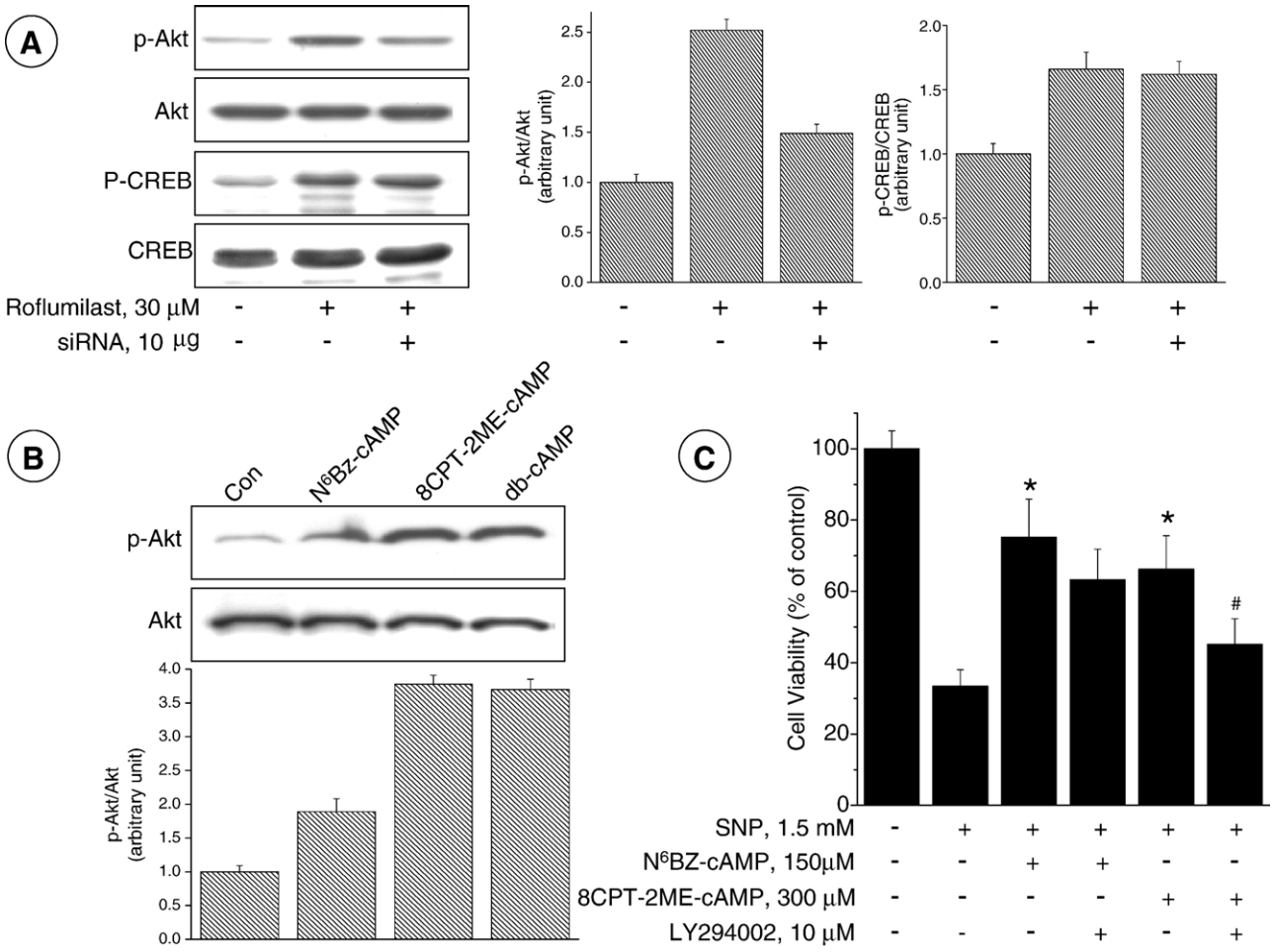

Fig. 7. Roles of Akt phosphorylation by Epac on NO-induced apoptosis in H9c2 cells. (A) Cells were transfected with Epac-specific siRNA (10 μg) for 36 h and then, treated with roflumilast for 30 min. Total protein was isolated and subjected to western blot analysis for p-CRBB/CREB, p-Akt/Akt. (B) Cells were treated with N^6Bz-cAMP (150 μM), 8CPT-2Me-cAMP (300 μM) and db-cAMP for 30 min for western blot analysis. Total protein was isolated and subjected to western blot analysis for p-Akt/Akt. (C) Cells were pre-incubated with N^6Bz-cAMP (150 μM) and 8CPT-2Me-cAMP (300 μM) for 1 h in the absence or presence of LY294002 (10 μM) and then SNP (1.5 mM) was treated for 24 h. Cell viability was also measured using MTT assay. Data shown are mean ± SD of four experiments (each performed in duplicates). *$p < 0.05$ versus SNP-treated control, $^{\#}p < 0.05$ versus SNP plus 8CPT-2Me-cAMP-treated group. Immunoblots shown are representative of three independent experiments.

protects NO-induced apoptosis via cAMP–PKA–CREB pathway. CREB is phosphorylated by PKA and generally mediates antiapoptotic mechanisms through bcl-2 expression in cardiomyocytes [38,39]. Consistent with this notion, our results show that PKA-dependent protective mechanism by roflumilast also involves CREB phosphorylation and this effect was abolished by H-89 and KT5720. Similarly to roflumilast, rolipram and cilomilast inhibited NO-induced apoptosis through activation of PKA–CREB pathway. However, the effects of CREB activation on cardiomyocyte survival and heart failure are controversial. For example, CREB becomes proapoptotic via induction of proapoptotic transcriptional repressor ICER (inducible cAMP early repressor), which antagonizes antiapoptotic molecule expression [40]. Thus, CREB-dependent induction of ICER may be critical for maintaining the balance of cell survival and death.

The cellular response to cAMP may be associated with the cAMP binding proteins such as PKA and Epac. However, the biological basis for divergent cellular responses to cAMP is not fully elucidated. Furthermore, to our knowledge, no study has ever shown the direct effects of Epac on cardiomyocyte apoptosis and clarified underlying mechanisms. An important finding of the present study is that roflumilast induces Epac–Rap1 activation in H9c2 cells. At first, we examined whether Epac activation is also involved in protection against H9c2 cells apoptosis. Our results have demonstrated that 8CPT-2Me-cAMP treatment inhibited NO-induced apoptosis and this was not reversed by H-89. It was previously reported that cAMP activates Epac–Rap1 in a PKA-independent manner and this was possible by using a newly developed cAMP analogue, 8CPT-2Me-cAMP, that selectively activates Epac–Rap1 pathway [13,41]. Since no pharmacological inhibitor of Epac is available, we used Epac1 siRNA system for silencing Epac1. According to our data, protective effect of roflumilast against NO-induced apoptosis was significantly abolished by Epac1 silencing with siRNA. Results of our present study raise the possibility that antiapoptotic effect of cAMP may be involved in activation of cAMP-Epac in cardiomyocytes, and furthermore

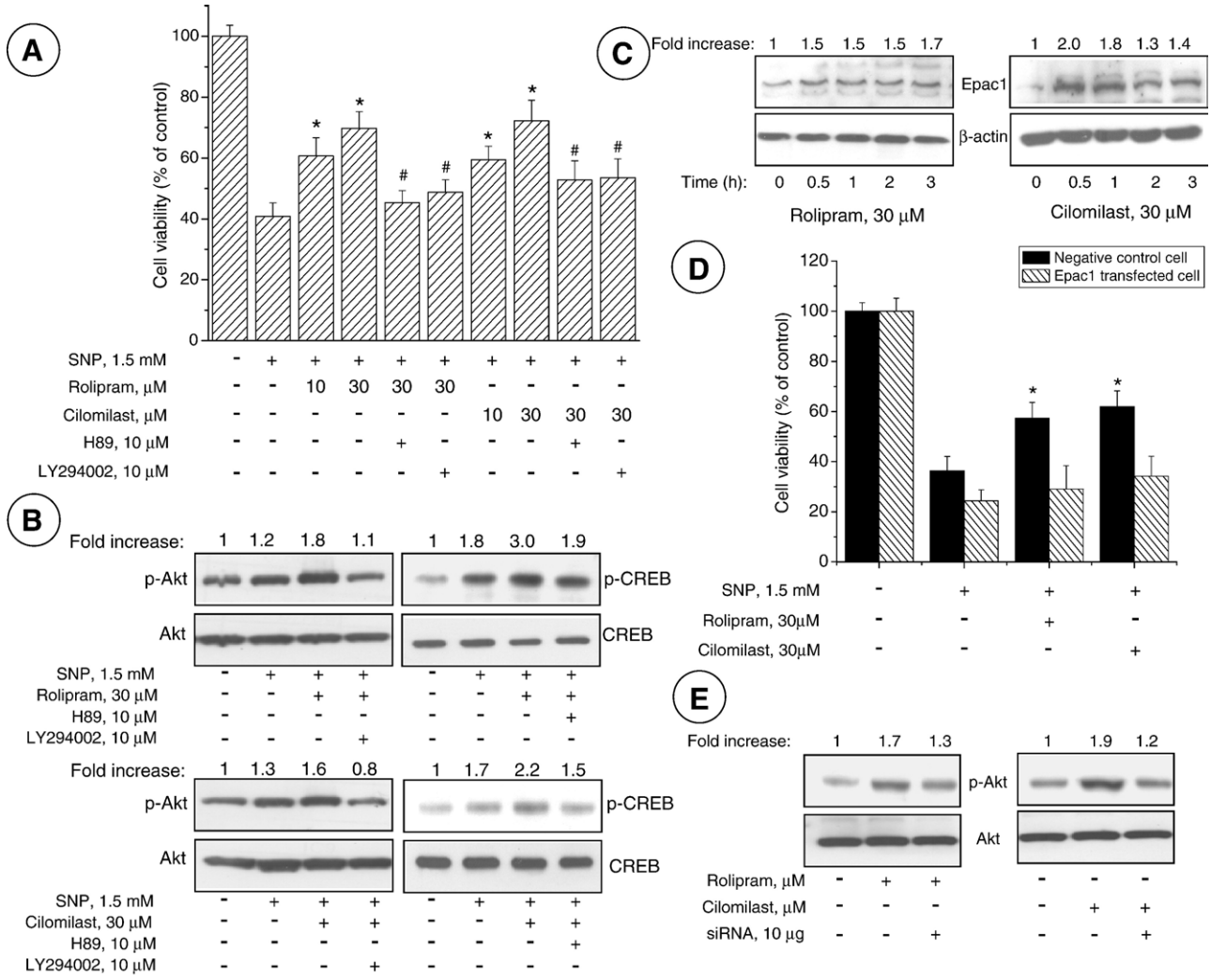

Fig. 8. Roles of rolipram and cilomilast on NO-induced apoptosis in H9c2 cells. H9c2 cells were pre-incubated with either by rolipram (10 or 30 μM) and cilomilast (10 or 30 μM) for 1 h in the absence or presence of H-89 (10 μM) and LY294002 (10 μM), then SNP (1.5 mM) was treated. (A) After 24 h later, cell viability was measured by using MTT assay. (B) After 30 min later, p-Akt/Akt and p-CREB/CREB levels were measured in cellular extracts using western blot. (C) Cells were pretreated with rolipram (30 μM, left panel) and cilomilast (30 μM, right panel) for indicated time points. Total protein was isolated and subjected to western blot analysis for Epac1. Epac1 expression was quantified by β-actin expressions. (D–E) Cells were transfected with non-targeting control and Epac1-specific siRNA (10 μg) for 36 h. Rolipram (30 μM) and cilomilast (30 μM) were pretreated for 1 h and then, treated with SNP (1.5 mM) for 24 h. Cellular viability was determined by MTT assay (D). Rolipram (30 μM, left panel) and cilomilast (30 μM, right panel) were treated for 30 min and western blot analysis was performed (E). Data shown are mean±SD of three experiments (each performed in duplicates). *$p<0.05$ versus SNP-treated group and $^{\#}p<0.05$ versus SNP plus rolipram (30 μM) or cilomilast (30 μM) treated group. Immunoblots shown are representative of two independent experiments.

indicate that protective effect of roflumilast in cardiomyocytes shares both PKA- and Epac-dependent signal pathways. Based on our finding that roflumilast increases the amount of active GTP-bound Rap1, the downstream mediator of Epac, this result raises the possibility that Rap1 activation may mediate the survival effect of cAMP-Epac activation by roflumilast. Rap GTPases, Rap1 and Rap2, are the only known downstream effectors of cAMP-Epac activation described so far. Studies in various cells have suggested that Rap1 activation may be cytoprotective [42–44]. Thus, further studies are needed to examine whether Rap1 is involved in roflumilast-mediated survival in cardiomyocytes.

Recent studies reported that cAMP-induced Akt activation inhibits apoptosis and its activation is due to Epac but not PKA [9]. Another report showed that Epac deletion mutant was unable to phosphorylate Akt [45]. Results of our present study indicated that Akt activation by PDE4 inhibitor is cAMP-Epac-dependent but PKA-independent event in H9c2 cells. Inhibition of Epac pathway fails to induce Akt phosphorylation, and 8CPT-2Me-cAMP mediates Akt activation without PKA involvement. However, the mechanism by which cAMP-Epac/Rap regulates PI3 kinase/Akt activity is not fully understood. Thus, one could speculate that Ras, structurally related to Rap1, binds to and activates the p110α- and 110γ-catalytic subunits of PI3 kinase [46]. Since Ras and Rap1 have identical effecter binding regions [47,48], it has been hypothesized that Rap may bind to Ras effecter such as PI3-kinase.

In above results, we mainly showed that PDE4 inhibitors inhibited NO-induced apoptosis via PKA-dependent CREB and Epac-dependent Akt activation in H9c2 cells. To further support

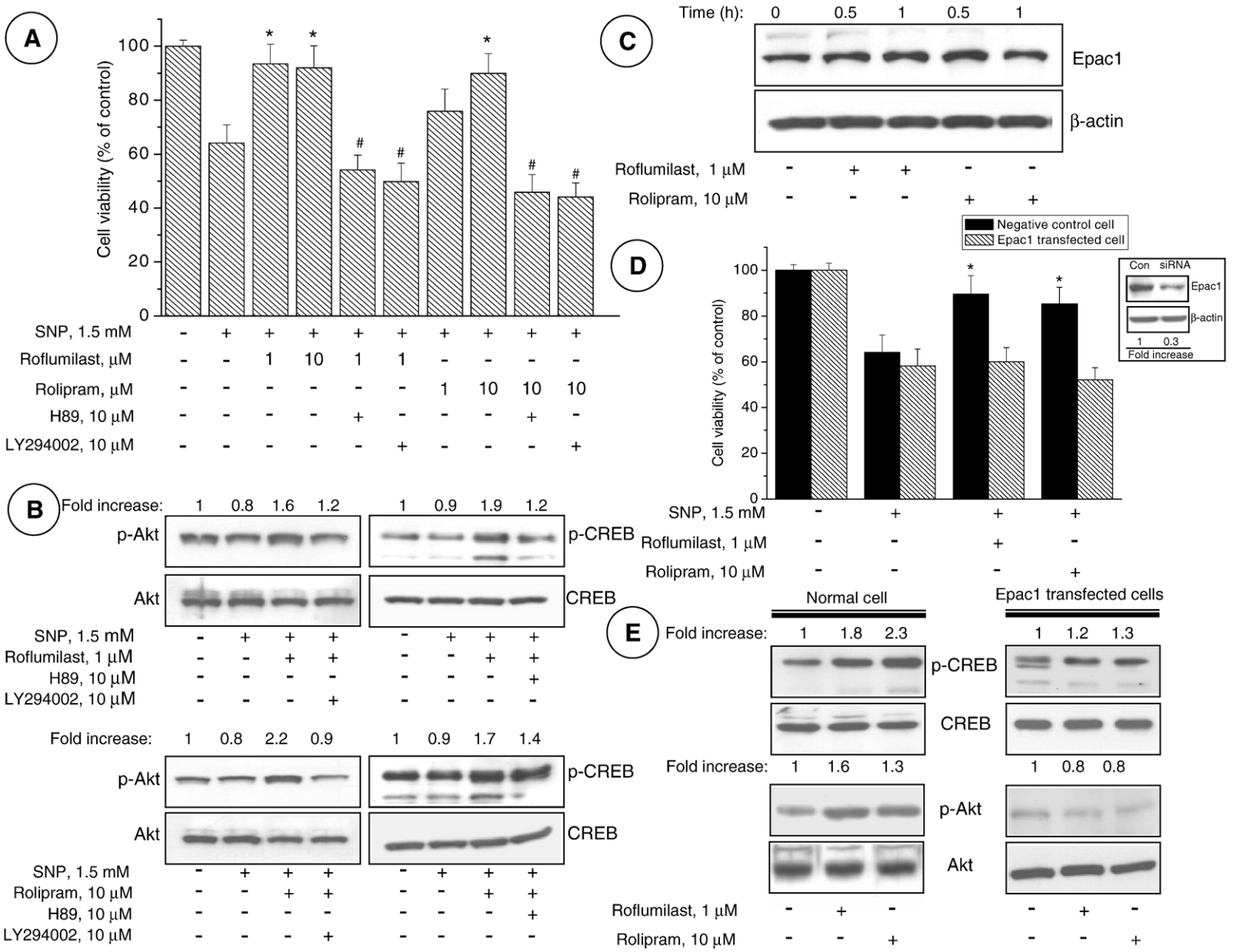

Fig. 9. Roles of roflumilast and rolipram on NO-induced apoptosis in NRVCMs. NRVCMs were pre-incubated with either by roflumilast (1 or 10 μM) and rolipram (1 or 10 μM) for 1 h in the absence or presence of H-89 (10 μM) and LY294002 (10 μM), and then SNP (1.5 mM) was treated. (A) After 24 h later, cell viability was measured by using MTT assay. (B) After 30 min later, p-CREB/CREB and p-Akt/Akt levels were measured in cellular extracts using western blot. (C) Cells were pretreated with roflumilast (1 μM) and rolipram (10 μM) for indicated time points. Total protein was isolated and subjected to western blot analysis for Epac1. Epac1 expression was quantified by β-actin expressions. (D–E) Cells were transfected with non-targeting control and Epac1-specific siRNA (30 nM) for 42 h. Roflumilast (1 μM) and rolipram (10 μM) were pretreated for 1 h and then, treated with SNP (1.5 mM) for 24 h. Cellular viability was determined by MTT assay (D). Roflumilast (1 μM, left panel) and rolipram (10 μM, right panel) were treated for 30 min and western blot analysis was performed (E). Data shown are mean±SD of three experiments (each performed in duplicates). *$p<0.05$ versus SNP-treated group. $^{\#}p<0.05$ versus SNP plus roflumilast (1 μM) or rolipram (10 μM) treated group. Immunoblots shown are representative of two independent experiments.

our finding, studies were performed in NRCMs. As expected, SNP induced apoptosis in NRCMs, however their effect was less potent than H9c2 cells in general, suggesting that NRCMs is more resistant to NO. The protection against NO-induced apoptosis by PDE4 inhibition was shown and similar mechanisms were observed in isolated NRCMs. Maximal inhibition of roflumilast on NO-induced apoptosis occurred at a dose of 1 μM in NRCMs, however, its concentration appeared to be insufficient in H9c2 cells. We do not as yet understand the reason for the discrepancy between H9c2 cells and NRCMs, but differences in NO sensitivity and experimental conditions may account for the differences. Regarding NO sensitivity, SNP induced cell death was lesser at high cell density than that at low cell density in our studies (Cell viability showed up to 58.4% at high cell density and 34.4% at low cell density, respectively). Also, the concentration of roflumilast for protective effect was different according to the cell density. The relatively low concentration of roflumilast was required at high cell density (data not shown). Therefore, several factors including cell type and cell density may be affect the effective concentration of roflumilast.

Myocardial I/R has been implicated in the induction of inducible nitric oxide synthase (iNOS) that leads to increase production of NO, however role of NO in heart has yielded conflicting reports regarding on the severity of I/R injury. It is now well appreciated that high, non-physiological levels of NO actually promote cellular necrosis and apoptosis [49], while the demonstrated cytoprotective effects involve low concentrations of NO [50]. According to these knowledge NO is necessary for the normal cardiac physiology, but it is potentially toxic in excess concentration. Since, as shown in our *in vitro* study, roflumilast inhibited NO-induced apoptosis in cardiomyocyte, further studies are needed to examine whether roflumilast also protects myocardial infarction *in vivo*. Our preliminary study shows that roflumilast reduced infarct size after I/R injury in mice animal model. We are currently working on this issue and it will be addressed in the future study.

Based on these results, we are reporting for the first time that PDE4 inhibitor roflumilast protects cardiomyocytes from NO-induced apoptosis via activation of PKA and Epac dual pathway. Our study provides a new insight into the mechanisms responsible for the pharmacological activity of roflumilast and suggests its possible application as a potent therapeutic agent in preventing I/R injury and cardiovascular failure.

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