ORIGINAL PAPER

# β-Arrestin prevents cell apoptosis through pro-apoptotic ERK1/2 and p38 MAPKs and anti-apoptotic Akt pathways

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**Abstract** Our previous studies have shown that β-arrestin 2 plays an anti-apoptotic effect. However, the mechanisms by which β-arrestin contribute to anti-apoptotic role remain unclear. In this study, we show that a deficiency of either β-arrestin 1 or β-arrestin 2 significantly increases serum deprivation (SD)-induced percentage of apoptotic cells. β-arrestin 2 deficient-induced apoptosis was inhibited by transfection with β-arrestin 2 full-length plasmid, revealing that SD-induced apoptosis is dependent on β-arrestin 2. Furthermore, in the absence of either β-arrestin 1 or β-arrestin 2 significantly enhances SD-induced the level of pro-apoptotic proteins, including cleaved caspase-3, extracellular-signal regulated kinase 1/2 (ERK1/2) and p38, members of mitogen-activated protein kinases (MAPKs). In addition, a deficiency of either β-arrestin 1 or β-arrestin 2 inhibits phosphorylation of Akt. The SD-induced changes in cleaved caspase-3, ERK1/2 and p38 MAPKs, Akt, and apoptotic cell numbers could be blocked by double knockout of β-arrestin 1/2. Our study thus demonstrates that β-arrestin inhibits cell apoptosis through pro-apoptotic ERK1/2 and p38 MAPKs and anti-apoptotic Akt signaling pathways.

**Keywords** β-Arrestin · Caspase-3 · ERK1/2 · p38 · Akt · Apoptosis

**Abbreviations**

| | |
|---|---|
| KO | Knockout |
| DKO | Double knockout |
| SD | Serum deprivation |
| TUNEL | Terminal deoxynucleotidyl transferase biotin dUTP nick end labeling |
| ERK1/2 | Extracellular-signal regulated kinase 1/2 |
| MAPKs | Mitogen-activated protein kinases |

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## Introduction

β-Arrestins 1 and 2, member of arrestins family, are multifunctional scaffold/adaptor protein that play a fundamental role in G protein-coupled receptor (GPCR) modulation [1, 2]. Both β-arrestins 1 and 2, two universally expressed members of arrestin family in many tissues [1], are key negative regulators and scaffolds of GPCR signaling [3, 4]. Recent studies have shown that β-arrestins function as adaptors to connect the receptors to the cellular trafficking machinery, such as scaffolding GPCR activation of extracellular-signal regulated kinase 1/2 (ERK1/2) and JNK3 signaling pathways [5, 6]. In addition, β-arrestin 1/2

interact with these signaling molecules by regulating the phosphorylation, ubiquitination and/or subcellular distribution of their binding partners. Moreover, growing evidences reveal that β-arrestin plays a fundamental role in preventing apoptosis [7–9]. For example, stimulation of various GPCRs causes apoptosis in the absence of, but not in the presence of β-arrestins. This indicates an important role for the β-arrestins in anti-apoptotic signaling [8]. However, it seems that the function of arrestins, in some conditions, is not limited to anti-apoptosis, since they are also indicated to be involved in pro-apoptosis [10]. Furthermore, our previous studies have shown that β-arrestin 2 modulates cell proliferation and cell apoptosis through the activation of Akt [11].

Akt (also known as protein kinase B, PKB), a serine/threonine kinase, modulates cell proliferation, cell apoptosis, and inflammation. Activated Akt in turn phosphorylates a variety of proteins involved in survival and apoptotic pathways leading to diminish cell apoptosis. Activated Akt phosphorylates several downstream targets including glycogen synthase kinase-3 beta, which is a crucial regulator in cell survival and apoptosis. We have previously reported that Akt plays an anti-apoptotic role [12, 13]. ERK1/2 and p38, members of mitogen-activated protein kinases (MAPKs) family, are important cellular protein kinases. They can be activated by a series of extracellular signals and then induce cell responses, including cell proliferation, differentiation, survival and apoptosis. It has been reported that p38 plays a pro-apoptotic role [14–16]. p38 seems to sensitize cells to apoptosis by up-regulating Bax [17], a pro-apoptotic member of Bcl-2 family. Activation of ERK1/2 modulates different cell responses, either pro-apoptosis or anti-apoptosis depending on the cell type and stimulus [18, 19]. However, the roles of Akt, ERK1/2, and p38 in β-arrestin 1/2 mediated apoptosis remain to be established.

In this study, we evaluated the mechanisms by which β-arrestins modulate cell apoptosis. Specifically we determined the involvement of the pro-apoptotic ERK1/2 and p38, and anti-apoptotic Akt signaling pathways. We have found that β-arrestin 1/2 attenuates cell apoptosis via pro-apoptotic ERK1/2 and p38 MAPKs and anti-apoptotic Akt signaling pathways.

## Materials and methods

### Reagents

Antibodies: pro and cleaved caspase-3, total and phospho-Akt (serine 473), total and phospho-ERK1/2, total and phospho-p38, were purchased from cell signaling technology (Beverly, MA). GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Cell culture and transfection

All cells were cultured in DMEM medium (Gibco BRL, Gaithersburg, MD) supplemented with 10 % fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1 % Penicillin/Streptomycin. Cultures were incubated at 37 °C and 5 % $CO_2$ in a fully humidified incubator. Wild type (WT) primary mouse embryonic fibroblasts (MEFs), β-arrestin 1 knockout (KO) MEFs, β-arrestin 2 KO MEFs, and β-arrestin 1/2 double KO (DKO) MEFs were kindly provided by Dr. Robert J. Lefkowitz, Duke University Medical Center, Durham, NC. The β-arrestin 2 full-length plasmid and control plasmid were kindly provided by Dr. Gang Pei. Shanghai Institutes for Biological Science, China. β-arrestin 2 KO MEFs were transfected with β-arrestin 2 full-length plasmid and control vector using Lipofectamine 2000 (Invitrogen Corporation, Carlsland, CA) to create B2 cells as described previously [20]. After transfection 24 h, ∼95 % transfection efficiency was observed using a fluorescent microscopy (Motic Company, Richmond, Canada). 48 h after transfection, the medium was replaced with the basal medium for later serum deprivation (SD) experiments [21, 22].

### Quantification of apoptosis by terminal deoxynucleotidyl transferase biotin dUTP nick end labeling (TUNEL) assay

Apoptotic cells were determined by TUNEL assay using an in situ cell death detection kit (Roche Diagnostic, Indianpolis, IN) as described in our previous publications [12, 23]. The 3′-OH ends of fragmented nucleosomal DNA were specifically labelled in situ in the presence of exogenously added terminal transferase biotin-labelled dUTP, and were detected with alkaline-peroxidase-conjugated anti-fluorescein antibody. Cells were fixed with ice cold 4 % paraformaldehyde for 60 min and then incubate in permeabilisation solution (0.1 % Triton X-100, 0.1 % sodium citrate) for 8 min on the ice. After washing with phosphate buffered saline, 50 μl/well TUNEL reaction mixture were added on the sample and then incubated in a humidified atmosphere for 60 min at 37 °C in the dark. 100 μl substrate solution was placed on the sample following convert-AP incubation. Finally, after washing and mounted with glass coverslip, cells were observed under light microscopy. The percentage of apoptotic cells was calculated by counting approximated 500 cells at least five randomly chosen microscopic fields (magnification 200×).

### Western blot analysis

Western blot was performed as described previously [23, 24]. Briefly, experimental cells were harvested and lysed in

RIPA Lysis Buffer. The lysates were separated by 10 % SDS-PAGE then transferred to Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The membrane was then incubated at room temperature in blocking solution for 1 h, followed with the blocking solution containing first antibody overnight at 4 °C. After washing three times with TBS for 5 min, the blot was incubated with a second antibody. The blot was again washed three times with TBS before being exposed to the SuperSignal West Dura Extended Duration substrate (Pierce Biotechnology, Rockford, IL). Band intensity was quantified by densitometric analyses using a densitometer.

## Statistical analysis

The results were presented as mean ± SD. The data were analyzed using one-way analysis of variance followed by Bonferroni tests to determine where difference among group existed. Differences were considered statistically significant for values of $p < 0.05$.

## Results

### Effect of $\beta$-arrestin 1 and 2 on SD-induced apoptosis

It has been reported that $\beta$-arrestin 1 suppresses cell apoptosis upon stimulation of insulin-like growth factor 1 [9]. To examine the role of $\beta$-arrestin 1 in SD-induced cell apoptosis, WT and $\beta$-arrestin 1 or 2 KO MEFs were treated with SD for 12, 24 and 48 h. Apoptotic cells were determined by TUNEL assay. We found that both SD and either $\beta$-arrestin 1 deficient (Fig. 1a) or $\beta$-arrestin 2 deficient (Fig. 1b) induced apoptosis in a time dependent manner. Importantly, significantly greater cell apoptosis was observed when $\beta$-arrestin 1 or $\beta$-arrestin 2 deficient MEFs were exposed to SD than WT MEFs were exposed to SD (Fig. 1a, b). To further determine the role of $\beta$-arrestin 2 in SD-induced cell apoptosis, we transfected $\beta$-arrestin 2 full-length plasmid and control vector into $\beta$-arrestin 2 KO MEFs and then detected the cell apoptosis. As shown in Fig. 1c, transfection with $\beta$-arrestin 2 full-length plasmid strongly rescued the number of apoptosis in a deficiency of $\beta$-arrestin 2 MEFs. Taken together, our results suggest that both $\beta$-arrestin 1 and 2 play an anti-apoptotic role in SD-induced cell apoptosis.

### $\beta$-Arrestin 1 and 2 DKO prevents from cell apoptosis

We have shown that a deficiency of either $\beta$-arrestin 1 or 2 promotes cell apoptosis (Fig. 1). We next determined whether there is a synergistic effect of $\beta$-arrestin 1 and 2 DKO MEFs on induction of cell apoptosis. Surprisingly, we found that deficiencies of both $\beta$-arrestin 1 and 2 dramatically inhibit SD-induced apoptosis (Fig. 2). These results suggest that $\beta$-arrestin 1 and 2 DKO plays an anti-apoptotic effect.

### $\beta$-Arrestin 1 and 2 affect the activation of caspase-3 in the presence of SD

The cleaved caspase-3 is an established specific marker and one of the key executioners for apoptosis [25]. Therefore, we detected caspase-3 activation in WT MEFs, $\beta$-arrestin 1 or 2 KO MEFs, $\beta$-arrestin 1/2 DKO MEFs following SD treatment. As shown in Fig. 3, the level of caspase-3 activation was significantly higher in the absence of $\beta$-arrestin 1 or 2 than in WT cells following SD treatment for 12 and 24 h, further suggesting that $\beta$-arrestin 1 or 2 plays an anti-apoptotic effect in SD-induced cell apoptosis. Intriguingly, there is no significant difference between WT and $\beta$-arrestin 1/2 DKO MEFs in the level of cleaved caspase-3 following SD treatment (Fig. 3), further suggesting that in the absence of both $\beta$-arrestin 1 and 2 plays a pro-apoptotic role.

### Effect of $\beta$-arrestin 1 and $\beta$-arrestin 2 on the levels of phospho- ERK1/2 following SD treatment

The Ras/Raf/ERK signaling pathway plays a key role in cell functions. ERK1/2 activity will mediate different cell responses, including cell proliferation, differentiation and apoptosis in vitro and in vivo [18]. Activation of ERK1/2 is associated with the apoptotic pathway, which relies on the activation of initiator caspase-3, 8, 9 [18]. To examine whether $\beta$-arrestin 1 and/or 2 affect phospho-ERK1/2 following SD treatment. ERK1/2 phosphorylation was determined in WT, $\beta$-arrestin 1or 2 KO, $\beta$-arrestin 1/2 DKO MEFs. As shown in Fig. 4, we observed that the levels of phospho-ERK1/2 were significantly higher in $\beta$-arrestin 1 or 2 KO MEFs than in WT MEFs. However, there is no significant difference between WT and $\beta$-arrestin 1/2 DKO MEFs in the levels of phospho-ERK1/2 (Fig. 4). These data showed that SD-mediated apoptosis through an ERK1/2 pathway.

### Effect of $\beta$-arrestin 1 and $\beta$-arrestin 2 on the levels of phospho-p38 in SD-induced apoptosis

It was reported that p38 involves in cell survival and apoptosis [15]. However, it is not known whether p38 participate in $\beta$-arrestin-mediated apoptosis following SD treatment. We examined the levels of phospho-p38 in WT, $\beta$-arrestin 1 or 2 KO, $\beta$-arrestin 1/2 DKO MEFs following SD treatment. As shown in Fig. 5, $\beta$-arrestin 1 or 2 deficient cells have a significantly higher level of phospho-p38 than WT cells. In addition, both $\beta$-arrestin 1 and 2 deficient did not change the level of phospho-p38 (Fig. 5).
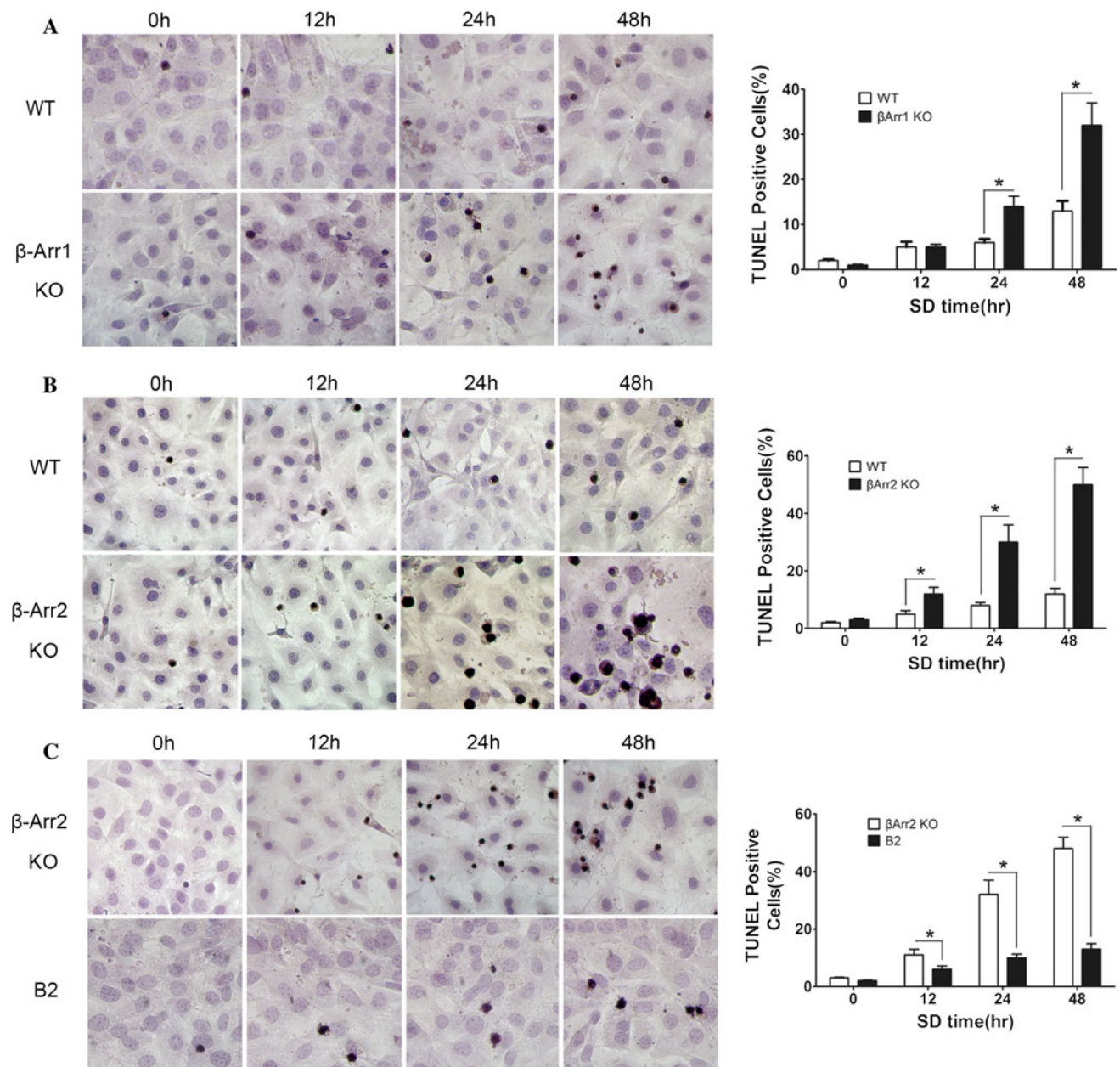
**Fig. 1** $\beta$-Arrestin 1 and $\beta$-arrestin 2 plays an anti-apoptosis role in SD-induced apoptosis. WT, $\beta$-arrestin 1 KO (**a**) and $\beta$-arrestin 2 KO (**b**) MEFs were treated with SD for 12, 24 and 48 h. Apoptotic cells were detected by TUNEL assay. **c** $\beta$-Arrestin 2 full-length plasmid and control plasmid were transfected into $\beta$-arrestin 2 KO MEFs. After 48 h transfection, the cells were treated with SD for the indicated times and then determined apoptosis using TUNEL assay. TUNEL-stained cells (*dark cells*) are shown at above. Magnification ×200. The *bar* graph shows the percentage of apoptotic cells. All data are representative of three independent experiments. *$p < 0.01$

Collectively, our data indicates that p38 involves in $\beta$-arrestin 1 and 2 mediated cell apoptosis.

### Effect of $\beta$-arrestin 1 and 2 on the levels of phospho-Akt following SD

It has been reported that $\beta$-arrestin 1 and 2 modulate Akt activation [9, 26, 27]. It has been established that Akt plays a critical role in cell proliferation and apoptosis [28]. In the present study, we determined the levels of phospho-Akt (serine 473) in WT, $\beta$-arrestin 1 or 2 KO, $\beta$-arrestin 1/2 DKO MEFs following SD treatment. We found that the levels of phospho-Akt in $\beta$-arrestin 1 or 2 KO MEFs were significantly lower compared to in WT cells. However, there is no obviously difference in levels of phospho-Akt between WT and $\beta$-arrestin 1/2 DKO MEFs. Together, these data support the idea that $\beta$-arrestin 1 and 2 exert anti-apoptosis though an Akt pathway.
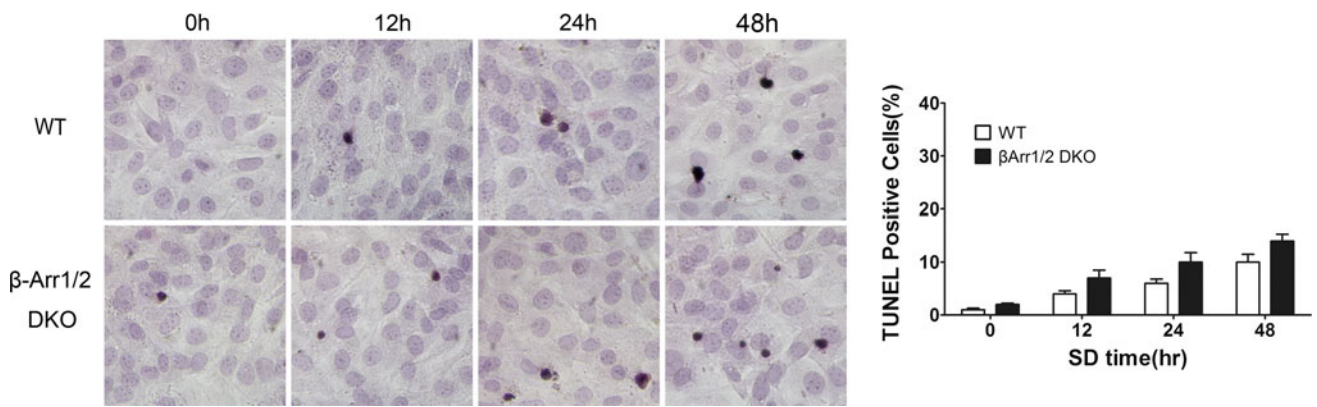
**Fig. 2** DKO of $\beta$-arrestin 1 and 2 prevent from cell apoptosis in MEFs. WT and $\beta$-arrestin 1/2 DKO MEFs were treated with SD for indicated times. Apoptotic cells were determined using TUNEL assay. All data are representative of three independent experiments. *$p < 0.01$

**Fig. 3** Role of $\beta$-arrestin 1 and 2 in SD-induced caspase-3 activation. WT, $\beta$-arrestin 1 KO, $\beta$-arrestin 2 KO and $\beta$-arrestin 1/2 DKO MEFs were treated with SD for 12 and 24 h. The levels of pro-caspase-3 and cleaved caspase-3 were determined by Western blot. Data are representative of three independent experiments. *$p < 0.01$
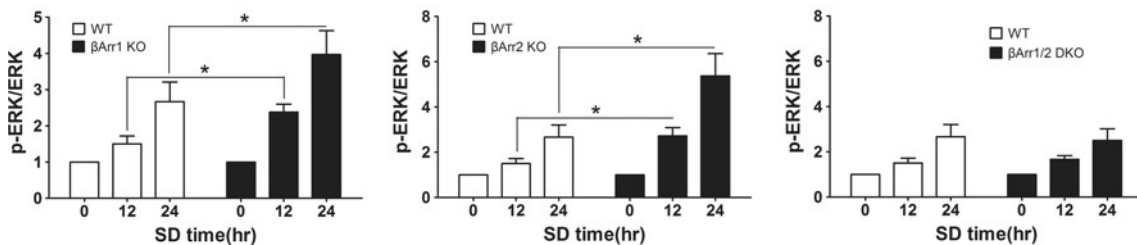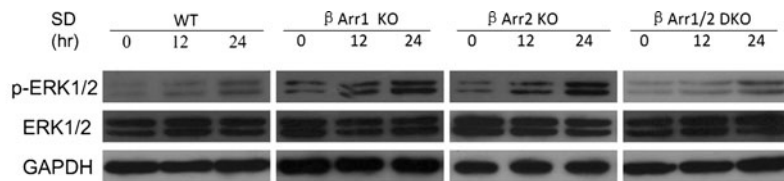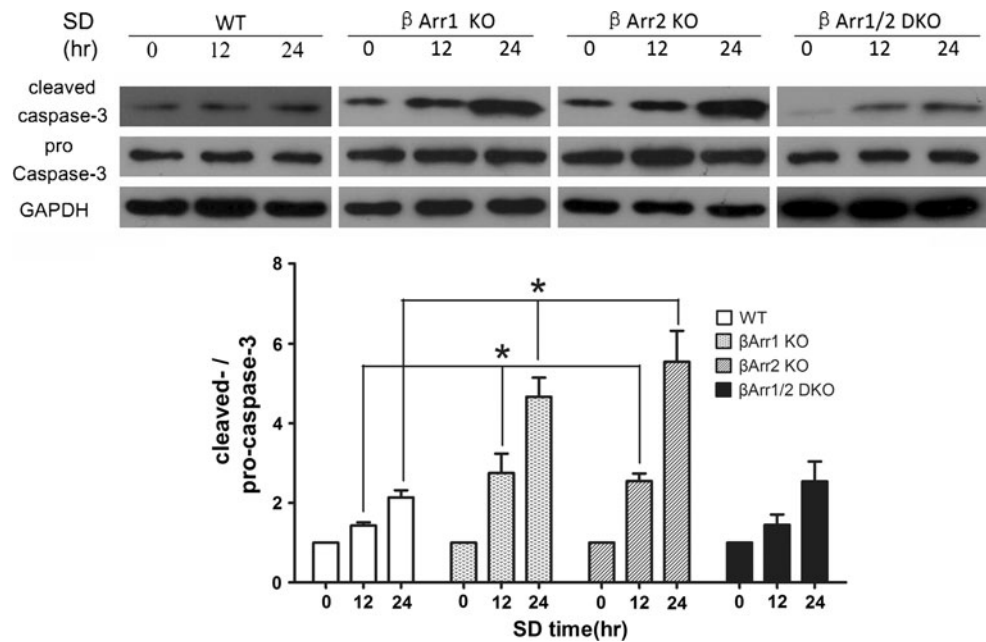




**Fig. 4** Effect of $\beta$-arrestin 1 and 2 on SD-induced ERK1/2 phosphorylation. Total and phospho-ERK1/2 were determined by Western blot in WT, $\beta$-arrestin 1 KO, $\beta$-arrestin 2 KO and $\beta$-arrestin 1/2 DKO MEFs following serum-starved for the indicated times. All data are representative of three independent experiments. *$p < 0.01$
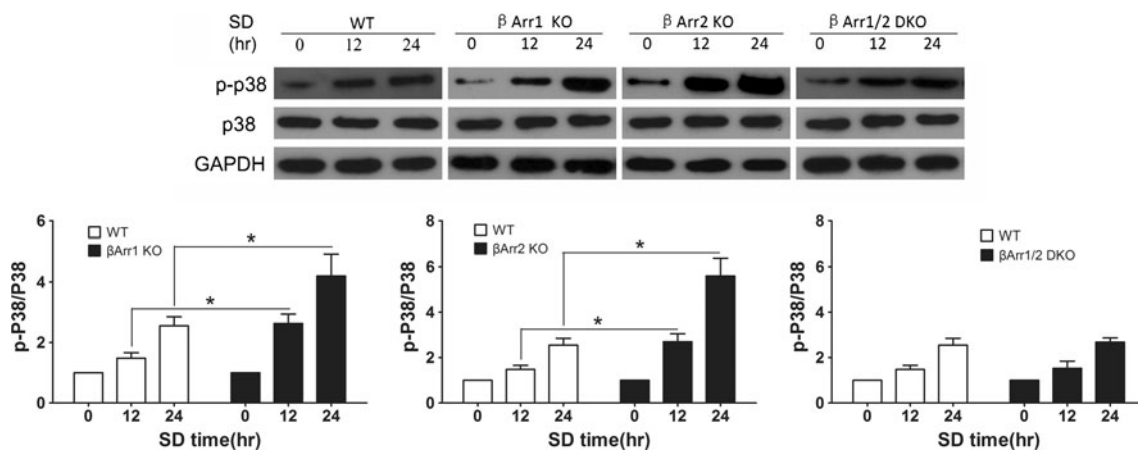
**Fig. 5** Role of β-arrestin 1 and 2 in SD-mediated p38 phosphorylation. WT, β-arrestin 1 KO, β-arrestin 2 KO and β-arrestin 1/2 DKO MEFs were treated with SD for indicated times. Total and phospho- p38 were determined by Western blot. All data are representative of three independent experiments. *$p < 0.01$
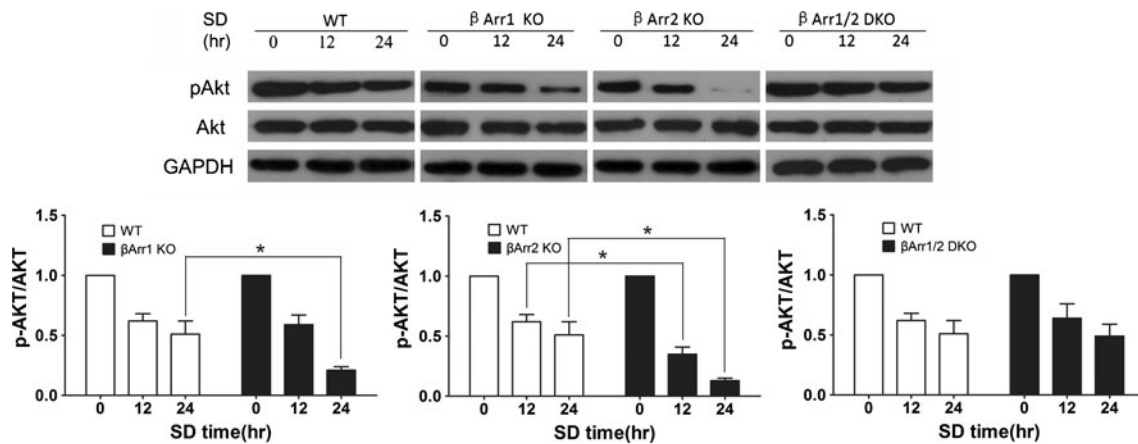


**Fig. 6** Effect of β-arrestin 1 and 2 on SD-mediated Akt phosphorylation. WT, β-arrestin 1 KO, β-arrestin 2 KO and β-arrestin 1/2 DKO MEFs were treated with SD for 12 and 24 h. Total and phospho-Akt (p-Akt) were determined by Western blot. Data are representative of three independent experiments. *$p < 0.01$

## Discussion

β-arrestin 1 and 2, functions as multifunctional scaffold/ adaptor protein for most GPCRs and are widely participated in desensitization and endocytosis of different kinds of cell surface receptors [7, 29, 30]. Recent evidence has shown that β-arrestin 1 and 2 contributes to an anti-apoptotic effect [7–9]. We have reported that overexpression of β-arrestin 2 significantly inhibits opioid-induced apoptosis [20], suggesting that β-arrestin 2 acts as a negative regulator in opioid-induced apoptosis. We have also reported that overexpression of β-arrestin 2 inhibits resveratrol-induced apoptosis, revealing that β-arrestin act as a negative regulator in resveratrol-induced apoptosis [11]. In our current study, we found β-arrestin 1 or 2 deficient cells showed a prominently increased the number of cell apoptosis and activation of the ERK1/2 and p38 MAPKs,

as well as decreased activation of the Akt. Activation of ERK1/2 and p38 kinase leads to cell apoptosis [31–35].

MAPKs are a family of serine/threonine-specific protein kinases in response to extracellular stimuli and regulate various cellular activities, such as cell proliferation, differentiation, cell survival and apoptosis [36]. MAPKs consist of three major subfamilies in mammalian cells [14], including JNK, p38 and ERK. The activation of p38 kinases generally promotes cell apoptosis [15]. We have already reported that p38 is required for opioid- induced microglia apoptosis [12]. We observed that SD stimulus increases p38 activation both in WT and β-arrestin 1 or 2 KO cells (Fig. 5). However, a deficiency of either β-arrestin 1 or 2 significantly enhanced the levels of phospho-p38 than in WT cells (Fig. 5), revealing that inhibition of β-arrestin 1 or 2 increased cell apoptosis through a p38 signaling pathway. Importantly, we found that β-arrestin 1

and 2 DKO could not alter the levels of phospho-p38 compared to WT cells (Fig. 5). The mechanisms by which β-arrestin 1 and 2 DKO contribute to the levels of phospho-p38 remain to be elucidated. While determining the exact mechanism beyond the scope of the current study and will be investigated in future.

ERK1/2 (also known as p42/44 MAPK), is a subfamily kinase in MAPKs. The ERK signaling pathway consists of three kinases (MEKK, MEK, ERK) which play a crucial role in modulation of cell survival and apoptosis [18, 37]. Based on the cell type and stimulus, ERK can modulate either pro-apoptosis or anti-apoptosis [38]. In our present study, we found that knockout of β-arrestin 1 or 2 exerts an additive effect on SD-increased levels of ERK1/2 phosphorylation (Fig. 4). These results suggest that β-arrestin 1 and 2 modulates SD-mediated apoptosis via an ERK1/2 signaling pathway. It therefore appears that β-arrestin 1 or 2 attenuates the SD-mediated MAPK response.

Akt plays a fundamental role in modulation of cell survival and apoptosis [39]. It was reported that β-arrestin 1 mediated activation of phosphatidylinositol 3-kinase (PI3K) leads to the subsequent activation of Akt and anti-apoptosis [9]. In our current study, we showed that the levels of phospho-Akt were significantly lower in the absence β-arrestin 1 or 2 than in WT cells during the SD treatment (Fig. 6). Our results indicate that upon SD stimulation, β-arrestin-dependent Akt activation plays an important role in anti-apoptotic effect.

In summary, our present results suggest that SD induces cell apoptosis via activation of ERK1/2 and p38 and inhibition of Akt. More importantly, our current studies reveal that a deficiency of β-arrestin 1 or 2 increases cell apoptosis through pro-apoptotic ERK1/2 and p38 MAPKs and anti-apoptotic Akt pathways.

**Conflict of interest** The authors declare that they have no conflict of interest.

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