doi:10.4149/neo_2009_02_108

β-arrestin2 inhibits opioid-induced breast cancer cell death through Akt and caspase-8 pathways

M. ZHAO[1, 2], G. ZHOU[2*], Y. ZHANG[2], T. CHEN[1, 2], X. SUN[5], C. STUART[1], G. HANLEY[3], J. LI[2], J. ZHANG[2], D. YIN[1, 4 *]

Departments of [1] Internal Medicine, [3] Laboratory Animal Resources, and [4] Department of Pharmacology, James Quillen College of Medicine, East Tennessee State University, Johnson City, TN37614, USA, [2] Institute of Pathology and Pathophysiology, Shandong University School of Medicine, Jinan250012, Shandong, P.R. China, [5] Department of Obstetrics and Gynecology, Peking University People's Hospital, Beijing100044, P.R. China, e-mail: mmaya777@gmail.com

Received July 18, 2008

β-arrestins, a family of regulatory and scaffold proteins, are well-known negative regulators of G-protein-coupled receptors (GPCRs) including opioid receptors. Recent studies have shown that β-arrestin2 plays a potential role in inhibiting cell death. It has been reported that opioids such as morphine induce cell death at high concentrations (>500 μM for 24 hours), which is similar to morphine plasma concentrations in cancer patients receiving chronic morphine treatment for pain relievers. However, the role of β-arrestin2 in opioid-induced cell death remains to be elucidated. We report here that β-arrestin2 significantly blocks morphine-induced number of cell death in human breast cancer MCF-7 and MDA-MB231 cells. Suppression of endogenous β-arrestin2 by specific RNA interfering (RNAi) and morphine treatment significantly attenuates the levels of phosphorylated Akt compared with inhibition of β-arrestin2 or morphine treatment alone. However, blockade of morphine-induced cell death by β-arrestin2 seems to be dependent on the inhibition of caspase-8, as inhibition of β-arrestin2 and morphine treatment significantly enhanced the levels of cleaved caspase-8. These studies show for the first time that β-arrestin2 blocks morphine-induced cell death through anti-apoptotic Akt and pro-apoptotic caspase-8 pathways. Therefore, targeting β-arrestin2 may be useful for treating side effects of opioids as pain relievers for cancer patients.

Keywords: Breast cancer, β-arrestin, opioid. Akt, caspase-8,cell death

Opioids have been used for centuries as pain relievers for cancer patients, but their abuse has deleterious physiological effects beyond addiction [1] All three opioid receptor types, μ, δ, and κ have been identified on breast cancer cells [2]. Previous studies have reported that opioids inhibit tumor cell proliferation and tumor growth in various models [2, 3]. It is generally believed that at low concentration of morphine treatment, such as concentration less than 10 μM *in vitro*, could promote cell survival [4] while relatively high dose of morphine, such as concentration higher than 500 μM, could induce cell death [2, 4]. We recently reported that opioids induce pro-apoptotic cell death receptor Fas expression and promotes Fas-mediated apoptosis [5, 6].

Arrestins, which consist of four classes, visual arrestin, cone arrestin, β-arrestin 1 and β-arrestin2, play a fundamental role in G protein coupled receptors (GPCRs) regulation [7]. β-arrestin2, an universally expressed member of arrestin family in many tissues [8] is a key negative regulator and scaffold of GPCR signaling [7]. Recent reports reveal that β-arrestins function as adapters to connect the receptors to the cellular trafficking machinery, such as scaffolding GPCRs with Src, mediating GPCR activation of extracellular-signal-regulated kinases (ERK1/2) and JNK3 pathways [9]. Growing evidence indicates that β-arrestins play an anti-apoptotic function [7, 10]. As a subfamily of GPCRs, the opioid receptors are also functionally modulated by β-arrestin2 [11]. β-arrestin2 is able to differentially regulate three members of opioid receptor family [11]. The published results from knockout mice deficient in β-arrestin2 [12] and in μ opioid receptor (MOR) [13] have shown that β-arrestin2 regulate the function of MOR *in vivo*. Recently, Haberstock-Debic et al reported that a cellular mechanism by which β-arrestin2 modulate the physiological effects of morphine *in vivo* [14]. However, the importance of β-arrestin2 in opioid-induced cell death in breast cancers remains to be elucidated. Here we show that anti-apoptotic effects of β-arrestin2 on opioids-induced breast cancer cell

* Corresponding author

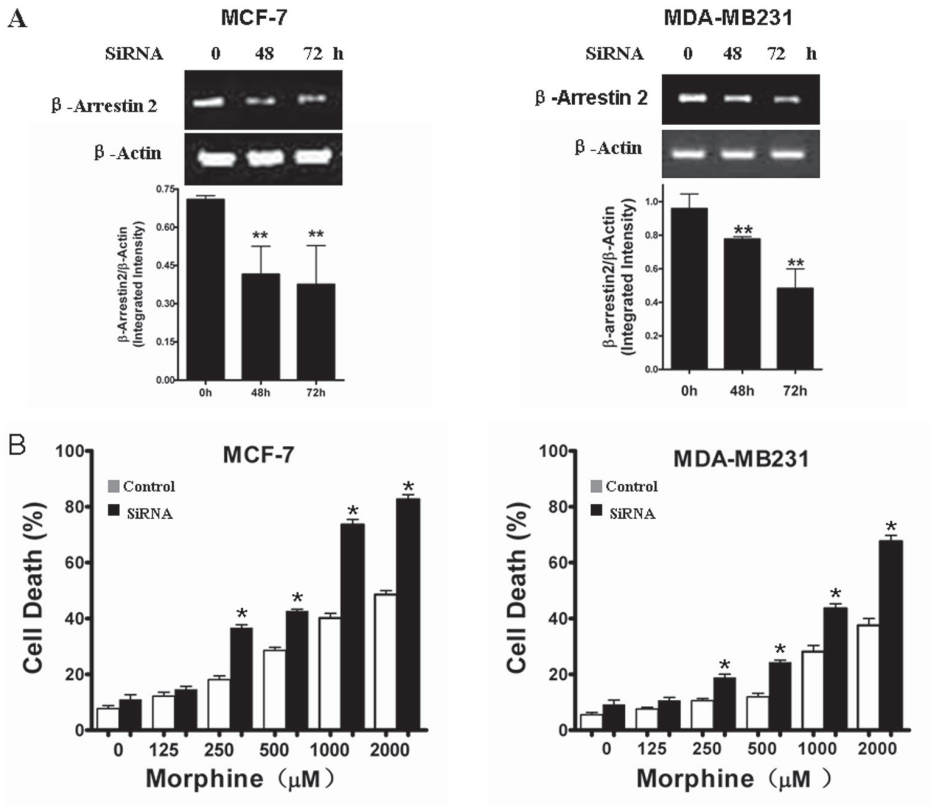

Figure 1. Effects of β-arrestin2 on morphine-mediated cell death in MCF-7 and MDA-MB231 cells. (A) Suppression of the endogenous expressed β-arrestin2 using RNA interference technology in MCF-7 and MDA-MB231 cells. MCF-7 and MDA-MB231 cells were transfected with β-arrestin2 RNAi plasmid or control plasmid separately. After transfection 48 h and 72 h, the cells were harvested and total RNA was extracted and expression of β-arrestin2 was determined by RT-PCR as described under "Materials and Methods". β-actin served as a control for loading and nonspecific RNA interference effects. Photographs of representative bands are shown on the left. Data are means of three independent experiments. ** $p < 0.01$ versus transfected control plasmid. (B) Morphine dose-response of inhibition of β-arrestin2 in MCF-7 and MDA-MB231 cells. After transfection of β-arrestin2 RNAi plasmid or control plasmid 48 h, the cells were treated with various doses of morphine for 12 h. Cell death was evaluated by staining with trypan blue and counted by blinded observers. Data are means ±SD of four to six experiments. *$p < 0.01$ versus transfected control plasmid.

death are mediated by activation of anti-apoptotic Akt and inhibition of pro-apoptotic caspase-8 pathways.

Materials and methods

Cell culture and reagents. MCF-7 and MDA-MB231 human breast cancer cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and were cultured in Dulbecco's modified Eagle's medium(DMEM), supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/ streptomycin at 37°C in a humidified 5% CO2 atmosphere. Anti-Akt, anti-phosphorylated Akt, caspase-8 antibodies were from Cell Signaling Technology. β-arrestin2 RNA interference (RNAi) plasmid was kindly provided by Dr. Gang Pei (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Shanghai, China).

Transient gene transfection. MCF-7 and MDA-MB-231 cells were transiently transfected using lipofectamine PLUS™ Transfection Reagent (Invitrogen) according to the manufacturer's instructions. For all transfection experiments, pcDNA3 enhanced green fluorescent protein (EGFP) plasmid was used as a control. Transfection efficiencies of the cells were routinely 85~95%, as determined by observing the expression of GFP under fluorescence microscope.

Cell death assay. Cells (2×10^5) were cultured in 12-well plates and transfected with indicated plasmids. Forty eight hours after transfection the cells were treated with different doses of morphine for 12 h. Cells were harvested and stained with trypan blue, and counted by blinded observers.

Reverse transcription-polymerase chain reaction (RT-PCR). Cells were cultured in 6-well plates at 5×10^5 cells per well. Forty eight hours and 72 h after transfection, cells were harvested and total RNA was extracted with the RNeasy Kit (Qiagen) and RT-PCR was performed as described previously [15]. The primer sequences were as follows: β-arrrestin 2, 5'-GTC GAG CCC TAA CTG CAA G-3' (forward) and 5'-ACA AAC ACT TTG CGG TCC TTC-3' (reverse). β-Actin, 5'-CAT GTA CGT TGC TAT CCA GGC-3' (forward) and 5'-CTC CTT

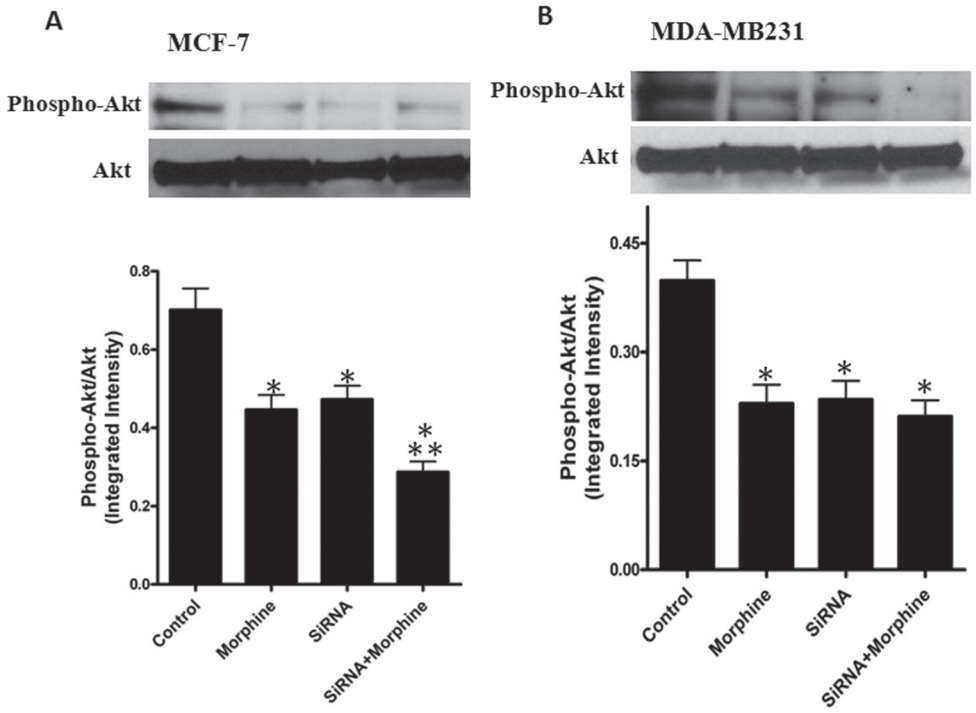

Figure 2. Suppression of β-arrestin2 significantly enhances morphine-induced reduction of levels of phospho-Akt. MCF-7 cells (A) and MDA-MB231 cells (B) were transfected with β-arrestin2 RNAi plasmid and control plasmid. After transfection for 48 h, the cells were treated with 2 mM morphine for 3 h. Cell lysates were probed for levels of total Akt and phospho-Akt by Western blot analysis. Photographs of representative bands in Akt are shown at the top. Data are means of three independent experiments.* $p < 0.01$ compared with transfected control plasmid. ** $p < 0.01$ compared with morphine treatment alone or transfected β-arrestin2 RNAi plasmid alone.

AAT GTC ACG CAC GAT-3' (reverse). PCR products were resolved on 2% agarose gels containing ethidium bromide and photographed under ultraviolet illumination. Photographs of gels were captured to a Macintosh PowerBook G4 laptop computer (Apple Computer Inc.), and densitometry of the PCR product bands was performed with AlphaEaseFC software.

Western blot analysis. Western blotting was performed as described previously[6] using several antibodies. Briefly, equal numbers of cells (1×10^6) were lysed in RIPA Lysis Buffer, which was composed of 1% Nonidet P-40, 50 mM HEPES (pH 7.4), 150 mM NaCl, 500 μM orthovanadate (Fisher Scientific, Fairlawn, NJ), 50 mM $ZnCl_2$, 2 mM EDTA, 2 mM PMSF, 0.1% SDS, and 0.1% deoxycholate. The lysates were separated by 10% SDS-PAGE then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then incubated at room temperature in a blocking solution composed of 5% skim milk powder dissolved in 1 × TBS (10 mM Tris, pH 8.0, and 140 mM NaCl) for 1 h. The membrane was then incubated with the blocking solution containing first antibody overnight at 4 °C. Total Akt, anti-phospho-Akt at Ser^{473}, and caspase-8 were detected with antibody total Akt (1:1000 dilution), phospho-Akt (1:1000 dilution), and caspase-8 (1:800 dilution). After washing three times with TBS for 5 min, the blot was then incubated with a second antibody. The blot was again washed three times with TBS before the membranes were analyzed by the ECL system (Amersham Pharmacia). The same membranes were stripped and re-probed with β-actin as loading controls. The signals were quantified by scanning densitometry and computer-assisted image analysis.

Statistical analysis. All data were presented as the mean ± SD. The data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni tests to determine where differences among groups existed. Differences were considered statistically significant for values of $p < 0.05$.

Results

Suppression of β-arrestin2 expression exerts an additive effect on morphine-induced cell death in human breast cancer cells. We found that morphine induces cell death [5, 6]. Previous studies have suggested that β-arrestin2 has an anti-apoptotic effect [7.10]. To determine whether β-arrestin2 is involved in morphine-induced cell death, we used RNA-mediated interference (RNAi) to determine the effect of endogenous β-arrestin2 on morphine-induced cell death.

We transfected human breast cancer MCF-7 and MDA-MB231 cells with β-arrestin2 RNAi plasmid or control plasmid. The expression of endogenous β-arrestin2 in MCF-7 cells was markedly reduced compared with the control. Similar results were obtained from MDA-MB231 cells (Fig. 1A). Further ex-

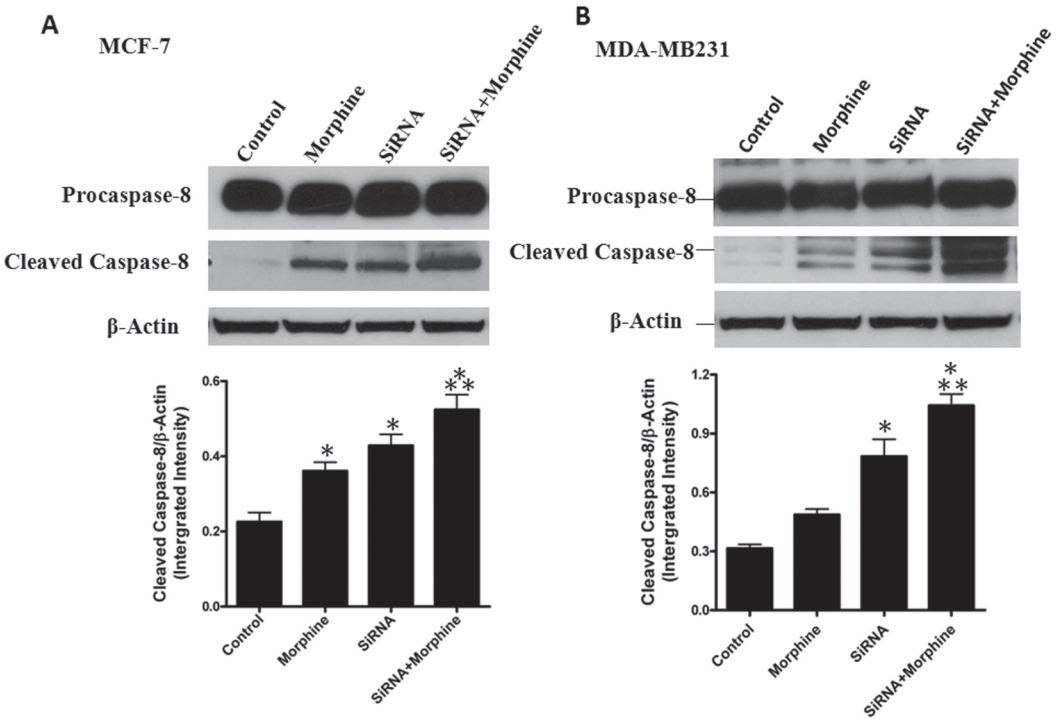

Figure 3. β-arrestin2 prevents morphine-induced cell death through caspase-8 pathway. MCF-7 cells (A) and MDA-MB231 cells (B) were transfected with β-arrestin2 RNAi plasmid or control plasmid. After transfection 48 h, the cells were treated with 2 mM morphine for 3 h. The levels of procaspase-8 and cleaved caspase-8 were analyzed by Western blotting with anti-caspase-8 antibody. Data are means of three independent experiments.*$p < 0.05$ versus transfected control plasmid. **$p < 0.01$ versus morphine treatment alone.

periments showed that when the expression of β-arrestin2 was reduced by β-arrestin2 RNAi, morphine-induced cell death was significantly increased in MCF-7 and MDA-MB231 cells compared with cells transfected with control plasmid (Fig. 1B). These results indicate that β-arrestin2 is a potential inhibitor of morphine-induced cell death.

β-arrestin2 inhibits morphine-induced breast cancer cell death through anti-apoptotic Akt pathway. Akt is a well-established anti-apoptotic protein.[16] It has been reported that β-arrestin2 plays a critical role in the regulation of Akt by dopamine in the nervous system.[17] We therefore questioned whether Akt participates in β-arrestin2 inhibition of morphine-induced cell death. Morphine treatment at 2 mM was selected from a previous publication[2] and was used in the subsequent experiments. The levels of total Akt and phospho-Akt at Ser^{473} were determined by Western blot analysis in MCF-7 cells (Fig. 2A) and in MDA-MB231 cells (Fig. 2B). We showed that the level of phospho-Akt was significantly reduced by either morphine treatment alone or suppression of β-arrestin2 expression by RNAi. Interestingly, inhibition of β-arrestin2 by RNAi and morphine treatment significantly exerts an addictive effect on reduction of phospho-Akt level. Collectively, these data clearly show that β-arrestin2 affects morphine-induced cell death in an Akt-dependent manner.

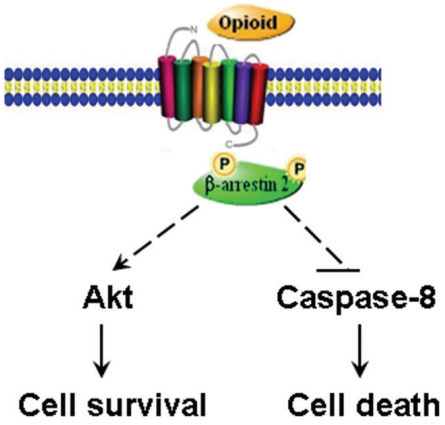

Figure 4. Possible mechanisms for β-arrestin2-inhibiting in morphine-promoted cell death. β-arrestin2 exerts its anti-apoptotic effect through activation of anti-apoptotic gene Akt and suppression of apoptotic gene caspase-8 signaling pathways.

β-arrestin2 blocks morphine-induced cell death through caspase-8 pathway in human breast cancer cells. Caspase activity is an established specific marker for cell death and caspase-8 is a key factor in apoptosis execution [18]. There-

fore, we examined caspase-8 activity in β-arrestin2 and morphine-mediated cell death. As shown in Figure 3A (MCF-7 cells) and Figure 3B (MDA-MB231 cells), a 43/41 kDa cleavage product of caspase-8 was not observed in untreated (control) group. However, the cleaved caspase-8 product appeared with either morphine treatment alone or during inhibition of endogenous β-arrestin2 by RNAi. Interestingly, inhibition of β-arrestin2 significantly enhances the morphine-induced level of the cleaved product of caspase-8 compared with morphine treatment alone. These results suggest that β-arrestin2 blocks morphine-induced cell death through inhibition of caspase-8 pathway.

Discussion

Growing evidence indicates that opioids regulate cell proliferation and cell death in various cells such as neurons and tumor cells. Opioids induce breast cancer cell death at a chronic higher dose (in *vitro* concentrations >500 μM for 24 h) [2]. Although it is well established that several opioid receptor isoforms are expressed on tumor cells [19], the molecular mechanisms by which opioids influence breast cancer cells remain to be elucidated. Because treatment of cancer pain usually requires long-term morphine use with increasing doses, opioid tolerance generally develops, possibly along with pro-apoptotic effects of morphine. In our studies, we investigated the mechanisms of opioid-induced cell death in human breast cancer cells.

β-arrestin2 has been demonstrated as an important modulator of GPCR signaling and plays a role in anti-apoptotic function [10]. After agonist stimulation by opioids such as morphine, β-arrestin2 confers distinct enzymatic activities upon the opioid receptor and thereby mediates growth inhibitory or apoptotic signals. Little is known regarding role of β-arrestin2 in opioid-induced cell death. In the present study, we silenced endogenous β-arrestin2 by RNAi to determine the effect of endogenous β-arrestin2 on morphine-induced cell death in breast cancer cells. We found that inhibition of β-arrestin2 enhanced morphine-induced cell death through pro-apoptotic caspase-8 signaling pathway.

Cell survival factor inhibits cell apoptosis through activating specific signaling pathway(s), including the Akt pathway. In many cell types in culture, transfection of constitutively active Akt (caAkt) prevents cell death while a dominant negative Akt induces pancreatic cancer cell death.[20] Our results showed that the suppression of β-arrestin2 expression by RNAi and morphine treatment dramatically exerts an additive effect on reduction of phospho-Akt level. To the best of our knowledge, this is the first report of β-arrestin2 inhibiting opioid-induced cell death through activation of the cell survival protein Akt and inhibition of pro-apoptotic protein caspase-8. Based on the results presented Figures 1-3, a hypothetical model is presented in Figure 4, describing a proposed scheme for β-arrestin2 as a potential inhibitor of opioid-induced cell death.

We thank Dr. Gang Pei (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) for providing β-arrestin2 RNAi plasmid. This research was supported by National Institutes of Health (NIH) grant DA020120 and East Tennessee State University Research Development Committee (ETSU RDC) 0048 to D. Yin. This work was also supported in part by ETSU RDC grant 07-026M to G. Hanley.

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