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β-arrestin2 mediates β-2 adrenergic receptor signaling inducing prostate cancer cell progression

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Abstract. The expression of the β-2 adrenergic receptor (β2AR), one of the stress-inducible receptors, has been reported to be closely correlated with malignant tumors. Prostate cancer is the most common non-cutaneous cancer among males, accompanied with increased castration levels and β2AR activation in patients. However, the role of β2AR activation in prostate cancer cells and its underlying mechanisms are not fully understood. Here, we found that β2AR activation promoted cell proliferation and cell migration through increasing cellular adenylyl cyclase (cAMP) levels and ERK1/2 activation in LNCaP and PC3 prostate cancer cells. Moreover, the scaffold protein β-arrestin2 was found to be involved in the β2AR-mediated activation of ERK1/2 and cell proliferation using stable overexpressing β-arrestin2 LNCaP (LNCaP-βArr2) cells. Furthermore, enhanced β-arrestin2/c-Src complex formation by β2AR activation was observed in LNCaP-βArr2 cells. In addition, the c-Src inhibitor could block this enhanced complex formation and suppressed cell proliferation. This study demonstrates that βArr2 is involved in prostate carcinogenesis induced by stress and provides potential therapeutic targets for cancer.

Introduction

Stress, one of the important factors to affect human health, has been shown to promote tumorigenesis (1). It is well established that chronic behavioral stress could activate β-adrenergic receptors (βARs), especially β2AR (2). There are three βAR subtypes (β1AR, β2AR and β3AR), each of which is coupled to Gs α and further stimulate the intracellular cyclic AMP (cAMP) levels. β1AR and β2AR are broadly expressed throughout tissues of the body, while β3AR is found predominantly in adipocytes (3). Both β1AR and β2AR are expressed in human prostate tissues (4). β2AR is a member of a large family of G-protein-coupled receptors (GPCRs). The traditional G-protein-dependent signal by β2AR is to activate extracellular-signal regulated kinase 1/2 (ERK1/2), which is the fast response signal after isoproterenol (Iso) stimulation (5). In addition, several studies have revealed that chronic behavioral stress resulted in higher levels of tissue catecholamines, greater tumor burden, and a more invasive pattern of ovarian cancer growth in a mouse model, and these effects were mediated primarily through the β2AR activation of cAMP-protein kinase A (PKA) signaling in cancer cells (6). Sastry *et al* reported that epinephrine reduced sensitivity of cancer cells to apoptosis through interaction with β2ARs, which is also related to phosphorylation and inactivation of the proapoptotic protein BAD by the cAMP-dependent protein kinase (7). All the evidence suggests that β2AR activation is a key event in tumorigenesis.

Prostate cancer is one of the most common non-cutaneous malignant tumors among American men (8). It is a slow-growing disease that mostly affects older men. More than 65% of prostate cancers are found in men over 65 years of age, and rarely occurs in men younger than 40 years. Among prostate cancers, the level of plasma testosterone, the stimulant of the androgen receptor, has potent stimulatory effects on androgen-sensitive parameters in the rat prostate (9). Ramos-Jiménez *et al* demonstrated that histamine augments β2AR-induced cAMP accumulation in the human prostate cancer cells DU-145 independently of known histamine receptors (9). Additionally, stimulation of endogenous GPCRs *in vitro*, including those for lysophosphatidic acid (10,11), bombesin (12), and bradykinin (13), induces mitogenic signaling and growth of prostate cancer cells. However, mechanisms underlying these observations are not fully understood.

Taken together, β2AR activation is indicated to have a critical role in tumorigenesis. However, the role of β2AR activation in prostate cancer cells and its underlying mechanisms have not been well addressed. In this study, we showed that β2AR activation promoted prostate cancer cell proliferation and cell migration through increasing cellular cAMP level and

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ERK1/2 activation. β-arrestin2 was shown to be involved in the β2AR-induced ERK activation and cell proliferation, and the formation of β-arrestin2/c-Src complex was a key factor in this process. All these findings may elucidate the mechanism of prostate cancer with stress and provide a potential target for this cancer.

Materials and methods

Reagents. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction and BrdU incorporation kits were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Antibodies against pERK, ERK2, β-arrestin2, HA, c-Src and GAPDH were obtained from Cell Signaling (Danvers, MA) and R&D Systems (Minneapolis, MN). Other reagents were obtained from Sigma-Aldrich and Fisher Scientific (Springfield, NJ) unless indicated.

Cell culture. Prostate cancer LNCaP and PC3 cells were purchased from the American Type Cell Collection (ATCC, Manassas, VA), and maintained in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 1.26 g/liter glucose.

LNCaP cells were seeded in 60-mm-diameter cell dishes to ~60-70% confluence, and then transfected with 3.0 μg pcDNA3.1-HA-βArr2 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Forty-eight hours later, cells were firstly selected in the medium containing 1000 μg/ml G418 (Gibco, Carlsbad, CA), and then maintained in the medium with 500 μg/ml G418. LNCaP cells stably expressing β-arrestin2 were designated as LNCaP-βArr2 cells.

Cell proliferation. Briefly, $1x10^4$ LNCaP, PC3 or LNCaP-βArr2 cells were seeded in 96-well plates and cultured. After overnight starvation, cells were incubated with various concentrations of Iso for an estimated time, and then harvested for counting with trypan blue staining, MTT or BrdU assay according to the manufacturer's instructions. Cells were cultured with 10% FBS as a positive control. For the negative control, cells were pre-treated with 1 μM propranolol (Pro), the antagonist of β2AR for 30 min, then followed by 5 μM Iso for 24 h. For MTT, 20 μl MTT (5 mg/ml) was added to each well, and cells were cultured at 37˚C for 4 h. The supernatant was removed and 150 μl DMSO were added to dissolve the crystal. For the BrdU assay, cells were incubated with 1 μg/ml BrdU at 37˚C for 4 h. Then the absorbance was determined by using a microplate spectrophotometer (Bio-Rad, Hercules, CA) at 570 nm for MTT and at 450 nm for BrdU.

Cell migration. The Transwell migration assay of LNCaP or PC3 cells was performed as previously described with some modification. Twenty-four hours prior to the assay, 500 μl serum-free medium (phenol-free RPMI-1640 with 0.5% BSA) was added to the bottom well. After starvation, $1x10^5$ cells were seeded in the upper chamber of each 8 μm-pore Transwell (Corning, Acton, MA) and stimulated with different concentrations of Iso. Cells were pretreated with 1 μM Pro as the negative control. Four hours later, cells were fixed with 4% paraformaldehyde (PFA), stained with 0.1% crystal violet in 20% ethanol, and the migrated cells were counted from 5 randomly different fields (x100)/chamber. Migrated cells on the bottom side of the filter were quantified by using the NIH image J software.

Cellular cAMP level. Cellular cAMP levels were detected by enzyme immunoassay (EIA) (Sigma-Aldrich). In brief, $2x10^5$ LNCaP cells were seeded in a 24-well plate for 24 h. After overnight starvation, cells were stimulated with various concentrations of Iso for 15 min. The cells were pretreated with 1 μM Pro for 15 min as the negative control. The final concentration of 1 mM 3-isobutyl-1-methylxanthine was added to prevent further hydrolysis by phosphodiesterases. The supernatant was carefully removed, the cells were dissolved with 0.1 M hydrochloric acid (HCl), and the protocol of the kit was followed. The absorbance at 450 nm was determined by using a microplate spectrophotometer (Bio-Rad).

Immunofluorescence. LNCaP cells were pre-incubated with FBS-free medium on glass coverslips in 6-well plates. After overnight incubation, cells were exposed to 5 μM Iso at 37˚C for 5 min, carefully washed, fixed with 4% PFA, permeabilized, washed, blocked, washed, and incubated with human anti-rabbit p-ERK primary antibody (1:200) at 37˚C for 2 h in the dark. Cells were then incubated with Cy3-rabbit secondary antibody (1:1000) for another 1 h at 37˚C, washed, and sealed with 50% glycerin. Observations were performed using a Zeiss laser scanning (LSM) 510 confocal microscope (Zeiss Inc., Thornwood, NY). Images were exported and generated.

Western blot analysis and immunoprecipitation. Cells were treated with various treatments and lysed in buffer with 20 mM pH 7.5 Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 20 mM β-glycerophosphate, 10% glycerol, 0.5 mM dithiothreitol (DTT), 20 mM NaF, 1 mM sodium orthovanadate ($NaVO_3$), PMSF and leupeptin. Then the lysate was clarified by centrifugation and the protein was quantified by using the BCA protein assay with BSA standard. Proteins (50 μg) were denatured and separated by SDS-PAGE for each lane. After electrotransfer, the membrane was probed with antibodies against p-ERK1/2 (1:1000), ERK2 (1:2000), β-arrestin (1:1000), HA (1:3000), c-Src(1:1000) or GAPDH (1:3000). Subsequently, blots were incubated with secondary antibody (Sigma-Aldrich), developed with the enhanced chemiluminescence (ECL) system (Amersham, Arlington, CA) and quantified by an NIH Imaging system.

For immunoprecipitation, conjugated agarose beads (Sigma-Aldrich) were added to the LNCaP-βArr2 cell lysates and incubated with anti-HA at 4˚C overnight. Then the beads were washed and the pellets were collected by centrifugation. The sample buffer (6% SDS, 10% glycerol, 5% β-mercaptoethanol, 25 mM, pH 6.5 Tris-HCl) was added to the bead pellets, boiled, followed by the Western blot procedures. The equal amount of total cell lysates was verified with the expression of c-Src and βArr2 (HA-tagged).

Statistical analysis. Each experiment was performed in triplicate and repeated twice in independent conditions. The statistical analysis was performed with SPSS 11.5 software for Windows 2000. Results are expressed as mean ± SEM. Data were analyzed by one-way or two-way analysis of variance

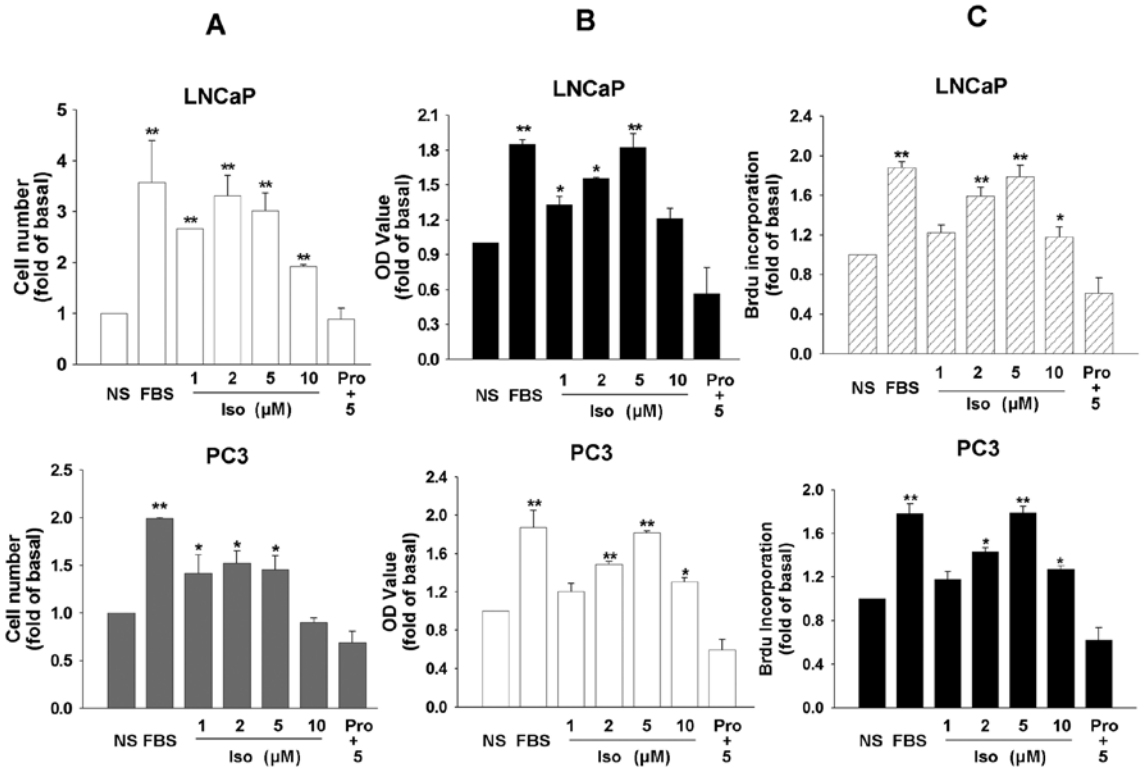

Figure 1. β2AR activation promotes prostate cancer cell proliferation. (A) Cell count, (B) MTT and (C) BrdU assay results for LNCaP or PC3 cells exposed to different concentrations of Iso for 24 h. The positive control was treated by 10% FBS and the negative control was pre-incubated with 1 μM Pro for 30 min before 5 μM Iso exposure. The cell number, the value of OD570 nm or OD450 nm in each non-stimulated group (NS) was recognized as 1.0-fold. The values indicate the fold change compared to the NS group. Data are expressed as the mean ± SEM (n=2). *P<0.05, **P<0.01, vs. the corresponding NS group.

(ANOVA) statistical program. P<0.05 was considered to be statistically significant.

Results

Activation of the β-2 adrenergic receptor promotes prostate cancer cell progression. To investigate the role of β2AR activation in prostate cancer cells, we used isoproterenol (Iso), the agonist of β2AR, and observed the subsequent effects both in LNCaP and PC3 cells. As indicated in Fig. 1A, exposure to different concentrations of Iso (1-10 μM, respectively) for 24 h increased survival cell numbers, compared with the corresponding non-stimulated (NS) LNCaP and PC3 cells. However, before Iso exposure, the pre-incubation with propranolol (Pro), the antagonist of β2AR, did not increase the survival cell numbers (P>0.05). Moreover, MTT (Fig. 1B) and BrdU incorporation assays (Fig. 1C) showed similar results with increased survival in Iso-treated cells, compared with their corresponding NS cells. The enhancement effect was maximal at 5 μM. No significant differences in cell viability were observed between the cells pre-treated with Pro or without Iso stimulation both in LNCaP and PC3 cells. These results indicate that β2AR activation enhances prostate cancer cell proliferation, which could be blocked by β2AR inhibitor.

We further determined the effect of β2AR activation on cell migration. As presented in Fig. 2, exposure to Iso (1-10 μM) increased the number of migrated cells in a dose-dependent manner both in LNCaP and PC3 cells. However, cells pre-incubated with Pro before Iso exposure showed no significant

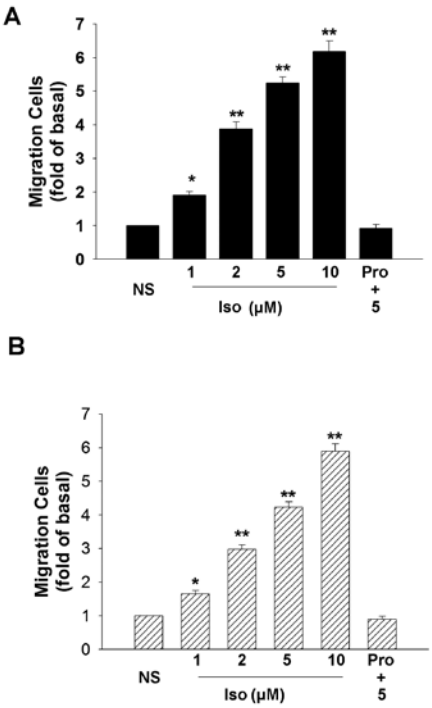

Figure 2. β2AR activation promotes prostate cancer cell migration. Transwell migration assay for LNCaP cells (A) or PC3 cells (B) stimulated with different concentrations of Iso for 4 h. The migrated cells were counted from 5 randomly different fields (x100)/chamber. The cells was pre-incubated with 1 μM propranolol (Pro) for 30 min before exposure to 5 μM Iso as the negative control. The number of each non-stimulated (NS) group was recognized as 1.0-fold. Values indicate the fold-change compared with the NS group. Data are expressed as the mean ± SEM (n=2). *P<0.05, **P<0.01, vs. the corresponding NS group.

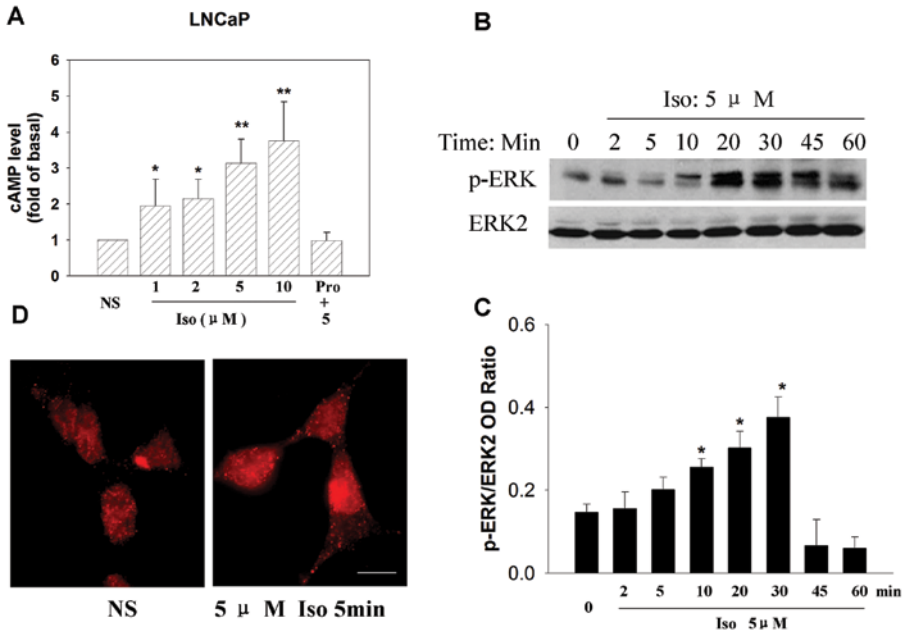

Figure 3. β2AR activation increases cellular cAMP levels and ERK1/2 activation. (A) cAMP-EIA assay for cellular cAMP level in LNCaP cells stimulated with various concentrations of Iso for 15 min. The cells were pre-treated with 1 μM Pro for 15 min as the negative control. The cAMP levels of the non-stimulated (NS) group were set as 1.0-fold. The values indicate the fold-change compared with the NS group. Data were expressed as the mean ± SEM (n=2). *P<0.05, **P<0.01, vs. the NS group. (B) Immunofluorescence assay of p-ERK in LNCaP cells with or without exposure to 5 μM Iso for 5 min. Scale bar, 100 μm. (C) Representative Western blot analysis of p-ERK1/2 level in LNCaP cells with exposure to 5 μM Iso for different time, and ERK2 as the loading control. (D) Quantification of expression data shown in (C) by using Image J software. Data are expressed as the mean ± SEM (n=3). *P<0.05, **P<0.01, vs. LNCaP cells treated by Iso at 0 min.

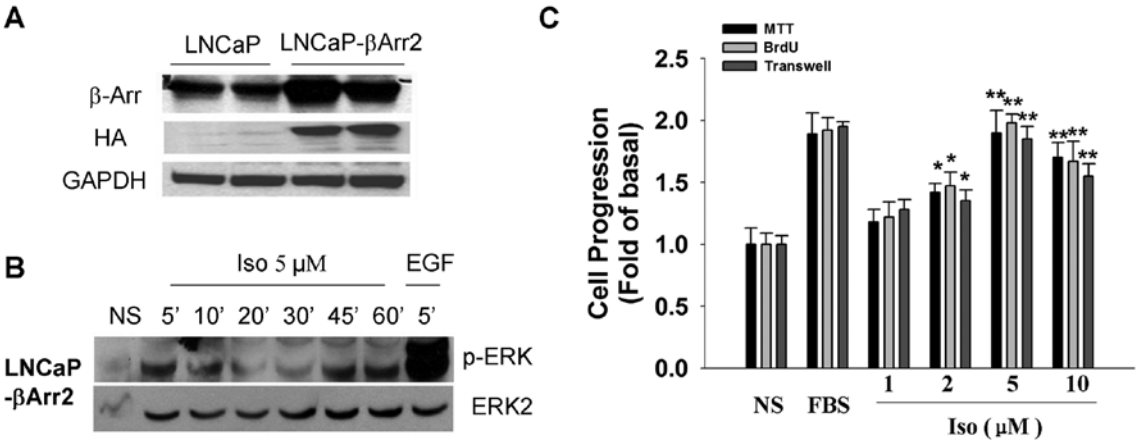

Figure 4. β-arrestin is involved in β2AR activation-enhanced p-ERK and cell proliferation. (A) Representative Western blot analysis of β-arrestin and HA levels in LNCaP-βArr2 cells and GAPDH as the loading control. (B) Representative Western blot analysis of p-ERK levels in LNCaP cells with exposure to 5 μM Iso for different times, EGF as the positive control, and ERK2 as the loading control. (C) The MTT and BrdU assays for LNCaP-βArr2 cells exposed to different concentrations of Iso for 30 min. The positive control was treated with 10% FBS. The value of OD570 nm (for MTT) or OD450 nm (for BrdU) in non-stimulated (NS) LNCaP cells was set as 1.0-fold. The values indicate the fold-change compared with the non-stimulated (NS) LNCaP-βArr2 cells. Data are expressed as the mean ± SEM (n=2). *P<0.05, **P<0.01, vs. the NS group.

increase in the number of migrated cells compared with that of the corresponding NS group (P>0.05). These findings indicate that β2AR activation enhances the prostate cancer cell migration which can be blocked by the β2AR antagonist. Taken together, these data demonstrate that β2AR activation promotes prostate cancer cell progression.

Activation of β2AR increases cellular cAMP levels and ERK1/2 activation in prostate cancer cells. The increase in cAMP levels plays an important role in the β2AR-activated cell proliferation, and therefore the effect of β2AR activation on cellular cAMP level in LNCaP cells was measured. As shown in Fig. 3A, compared with the NS LNCaP cells, the cells exposed to various concentrations of Iso for 15 min displayed a significant increase in cellular cAMP levels. The enhancement of this effect appeared to be dose-dependent. No significant change in cAMP levels was observed between the Pro pre-treated cells and the NS cells (P>0.05). These results indicate that β2AR activation promotes cAMP levels in LNCaP cells.

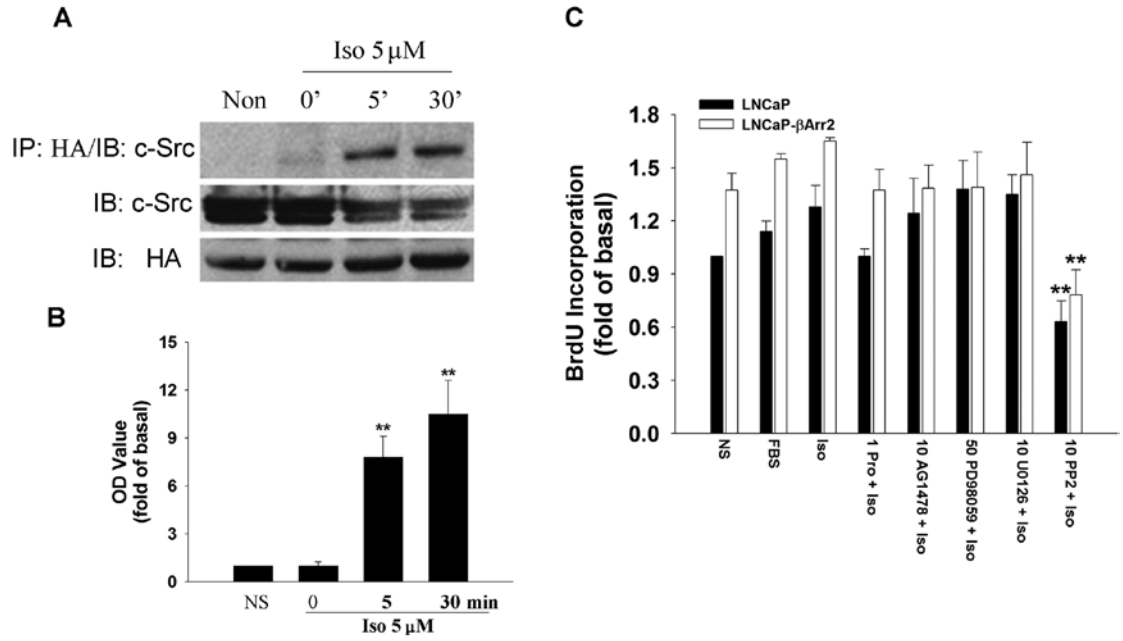

Figure 5. β2AR activation promotes β-arrestin2/c-Src complex formation. (A) Representative immunoprecipitation and Western blot assay for HA and c-Src in LNCaP-βArr2 cells exposed to 5 μM Iso for 0, 5 and 30 min. (B) Quantification of immunoprecipitation data shown in (A) using Image J software. Data are expressed as mean ± SEM (n=3). *P<0.05, **P<0.01, vs. the untreated LNCaP-βArr2 cells. (C) BrdU assay for LNCaP cells and LNCaP-βArr2 cells pre-treated with different inhibitors (1 μM Pro, 10 μM AG1478, 50 μM PD98059, 10 μM UO126 or 10 μM PP2) for 30 min before exposure to 5 μM Iso for 24 h. The OD450 nm value in non-stimulated (NS) LNCaP cells was set as 1.0-fold. The values indicate the fold-change compared with the LNCaP NS group. Data are expressed as the mean ± SEM (n=2). *P<0.05, **P<0.01, vs. the .LNCaP NS group.

Notably, phosphorylation of ERK1/2 (p-ERK1/2), the activated form of ERK1/2, has been reported to be regulated by the β2AR-mediated increase in cAMP levels (14). We thus examined the protein levels of p-ERK1/2 and ERK2 in LNCaP cells with Iso stimulation. As indicated in Fig. 3B and C, p-ERK expression was increased in a time-dependent manner (0-30 min) with 5 μM Iso. The maximal enhancement effect of Iso was observed at 30 min and the p-ERK1/2 levels significantly decreased in cells exposed to Iso at 45 and 60 min compared with the untreated LNCaP cells (P<0.05). Similarly, the immunofluorescence assay revealed that p-ERK1/2 was markedly enhanced in LNCaP cells exposed to 5 μM Iso for 5 min (Fig. 3D). Together, these findings strongly indicate that β2AR activation induces an enhancement of cellular cAMP level and ERK1/2 activation, and thereby may contribute to prostate cancer cell progression.

β-arrestin2 is involved in β2AR-activated ERK1/2 and cell proliferation in prostate cancer cells. β-arrestin mediates desensitization and internalization of β2AR, and also acts as a scaffold protein in the ERK cascade (15). We therefore, evaluated whether β-arrestin is involved in the β2AR-mediated ERK1/2 activation in LNCaP cells. LNCaP-βArr2 cells stably overexpressing β-arrestin2 with HA tag were firstly established, which showed a significantly enhanced β-arrestin2 expression (Fig. 4A). The levels of the active phosphorylated ERK1/2 and ERK2 forms in LNCaP-βArr2 cells were examined after Iso stimulation. The active p-ERK1/2 level showed two high peak times in LNCaP-βArr2 cells at 5, and 45 min with high levels persisting at 60 min after exposure to 5 μM Iso (Fig. 4B). On the other hand, ERK1/2 was highly activated at 30 min and decreased at 45 and 60 min in LNCaP cells exposed to 5 μM Iso (Fig. 3B). These data indicate that β-arrestin2 is involved in the β2AR-activated ERK1/2 in prostate cancer cells.

We next evaluated the relevance of β-arrestin2 in the β2AR-induced cell progression through examining the cell viability of LNCaP-βArr2 cells by using the MTT and BrdU assays, and cell migration by the Transwell assay. The results showed that both the cell proliferation and cell migration of LNCaP-βArr2 were significantly increased after exposure to different concentrations of Iso for 30 min (Fig. 4C), confirming that β-arrestin2 is indeed involved in β2AR-activated ERK1/2 cell progression in prostate cancer cells.

Activation of β2AR enhances the β-arrestin2/c-Src complex formation in prostate cancer cells. c-Src has been shown to be enhanced in the β2AR activation response (16) and it has also been reported to promote cell proliferation in other cells (17). We thus wondered whether c-Src participated in the β2AR activation in prostate cancer cells. Immunopreciptation assay identified a marked increase of the β-arrestin2/c-Src complex formation in LNCaP-βArr2 cells after Iso stimulation for the indicated times, compared with the untreated LNCaP-βArr2 cells (P<0.05, Fig. 5A and B). By contrast, the protein level of c-Src were significantly reduced in LNCaP-βArr2 cells after exposure to Iso for 5 and 30 min, compared with that of untreated cells. We next measured the cell proliferation of LNCaP-βArr2 cells pretreated with different signaling inhibitors (1 μM Pro, 10 μM AG1478, 50 μM PD98059, 10 μM UO126 or 10 μM PP2) for 30 min before exposure to Iso. As presented in Fig. 5C, the proliferation of LNCaP-βArr2 cells was significantly inhibited by PP2, the inhibitor for c-Src. However, the LNCaP-βArr2 cell proliferation was not obviously affected by the MEK, MAPK and PI3K inhibitors. All

the results provide a strong support that the β-arrestin2/c-Src complex formation is a key factor for β2AR-activated ERK1/2 and cell proliferation, and c-Src is involved in these processes.

Discussion

β2ARs, members of the superfamily of GPCRs, are associated with chronic behavioral stress. Accumulating evidence supports that β2AR activation is closely correlated with malignant tumors. The activation of β2AR is followed in a variety of signaling pathways. One of the traditional G-protein-dependent signaling pathways by β2AR is to activate ERK, which is the fast response signal after Iso stimulation (18). In the present investigation, we provide evidence that β2AR activation promotes prostate cancer cell proliferation and cell migration, through increasing cancer cellular cAMP level and ERK activation, further supporting that increased cAMP levels and ERK activation are involved in the β2AR-activated cell progression (14,19).

The classical signaling of ERK activation by β2AR is the Gs-dependent adenylyl cyclase activation. However, Shenoy *et al* illustrated that Iso-stimulated β2AR leads to G protein-independent ERK activation which was dependent on β-arrestin (20). Thus, activation of ERK by β2AR was resolved into two components depending respectively, on Gs-Gi/protein kinase A (PKA) or β-arrestins. G protein-dependent activity was quite transient and rapid, peaked within 2-5 min, and was blocked by a Gi inhibitor and a PKA inhibitor. On the other hand, β-arrestin-dependent activation was slower at onset (peak 5-10 min) and less robust, but it was sustained for longer periods of time and showed little decrement over 30 min. β-arrestin binding to the phosphorylated receptor hinders GPCR (21) and induces conformational changes in β-arrestin (22). GPCRs are classified into two families depending on their association with arrestin post-endocytosis (23-25). Class A receptors dissociate from arrestin after internalization and are rapidly recycled back to the cell surface. By contrast, class B receptors remain associated with arrestin2 after internalization and are eventually subjected to lysosomal degradation. Here, our studies demonstrated that β2AR activated ERK was β-arrestin-dependent, and the presence of β-arrestin delayed the ERK activation rate by β2AR, further supporting that β2AR was a class B receptor and showed a late response to stimulation in prostate cancer cells. Therefore, we highlight the importance of β-arrestin in the ERK activation and cell progression by β2AR activation in prostate cancer cells.

Arrestins are a multifunctional family of proteins (23,26,27), that have four members: β-arrestin1, β-arrestin2, x-arrestin and s-arrestin. β-arrestin1 and β-arrestin2 are ubiquitously expressed in the tissues, whereas x-arrestin and s-arrestin are found exclusively in the visual system. The classical functions of β-arrestins are to mediate desensitization, sequestration, and recycling of GPCR. In addition to regulation of GPCR signals, especially β2AR, β-arrestins have been reported to participate in some tumor-related signaling pathways such as p53/MDM2 and transforming growth factor β1 (TGF-β1) pathways, which function vitally in anti-apoptosis, cell growth, proliferation, and migration (28-30). β-arrestins were previously shown to be involved in the NF-κB pathway, to affect cytokine secretion (31), and to increase MMP9 activity (32) thus, suggesting a potential role in providing a suitable microenvironment for tumor progression. Taken together, β-arrestin participation in β2AR-activated ERK and cell progression in prostate cancer may not only mediate the signals of cell growth, but also affect the tumor microenvironment.

The Src family of non-receptor tyrosine kinases, is a major group of cellular signal transducers (33). It was the first identified and the essential protein tyrosine kinase (34,35), which could modulate various cellular functions including proliferation, survival, adhesion, and migration (33). Many GPCRs are able to stimulate tyrosine phosphorylation, increase Src family tyrosine kinases activity (36-39). c-Src could switch β2AR from adenylyl cyclase coupling to Ras activation and its downstream effectors, MAPK pathway (40,41). In the current study, our data strongly indicate that c-Src plays an essential role in the β2AR-mediated ERK activation and cell proliferation in prostate cancer cells, which enhanced the formation of β-arrestin2/c-Src complex, and the inhibitor of c-Src could block the complex formation and cell proliferation. c-Src recruitment to β-arrestin is obligatory for β2AR activation and cell progression in prostate cancer, and consistent with a previous report that the interaction of β-arrestin and c-Src was critical for colorectal carcinoma cell migration (42), illustrating the potential therapeutic target of c-Src for the tumor.

In summary, our research presented the evidence that β-arrestin2 played the vital role in β2AR-mediated prostate cancer cell progression and ERK activation, and the formation of β-arrestin2/c-Src complex was a key factor for this process. This study could elucidate the mechanism of prostate carcinogenesis induced by stress and provide a perspective therapeutic target for human prostate cancer.

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