Biphasic Erk1/2 activation sequentially involving Gs and Gi signaling is required in beta3-adrenergic receptor-induced primary smooth muscle cell proliferation

Tarik Hadi, Marina Barrichon, Pascal Mourtialon, Maeva Wendremaire, Carmen Garrido, Paul Sagot, Marc Bardou, Frédéric Lirussi

ARTICLE INFO

Article history:
Received 19 October 2012
Received in revised form 2 January 2013
Accepted 18 January 2013
Available online 4 February 2013

Keywords:
Proliferation
Erk1/2
G protein coupled receptor
Beta-3 adrenergic receptor
Cyclins
Protein kinases

ABSTRACT

The beta3 adrenergic receptor (B3-AR) reportedly induces cell proliferation, but the signaling pathways that were proposed, involving either Gs or Gi coupling, remain controversial. To further investigate the role of G protein coupling in B3-AR induced proliferation, we stimulated primary human myometrial smooth muscle cells with SAR150640 (B3-AR agonist) in the absence or presence of variable G-protein inhibitors. Specific B3-AR stimulation led to an Erk1/2 induced proliferation. We observed that the proliferative effects of B3-AR require two Erk1/2 activation peaks (the first after 3 min, the second at 8 h), Erk1/2 activation at 3 min was mimicked by forskolin (adenylyl-cyclase activator), and was resistant to pertussis toxin (Gi inhibitor), suggesting a Gs protein signaling. This first signaling also required the downstream Gs signaling effectors PKA and Src. However, Erk1/2 activation at 8 h turned out to be pertussis toxin-dependent, and PKA-independent, indicating a Gi signaling pathway in which Src and PI3K were required. The pharmacological inhibition of both the Gs and Gi pathway abolished B3-AR-induced proliferation. Altogether, these data indicate that B3-AR-induced proliferation depends on the biphasic activation of Erk1/2 sequentially induced by the Gs/PKA/Src and Gi/Src/PI3K signaling pathways.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

First identified in the brown adipose tissue [1], the beta3 isofrom of adrenergic receptor (B3-AR) has been extensively studied for its potential interest in the treatment of obesity and type II diabetes. Further work demonstrated its expression in other tissues, such as the brain, retina, urinary bladder, prostate, and blood vessels. These findings opened the way for new potential therapies for different conditions such as bladder instability, hypertension, or depression [2,3]. B3-AR is a G protein coupled receptor (GPCR) that can be coupled to a Gs [4-6] or a Gi protein [7,8]. In smooth muscle cells, B3-AR, like other beta-adrenergic receptors, is most commonly reported to be positively coupled to a Gs protein, leading to the activation of adenylate cyclase, the production of cyclic AMP (cAMP) and subsequent relaxation [4,9].

We previously described the expression of B3-AR in human near-term myometrium, the smooth muscle layer of the uterus, where it is able to induce cAMP-dependent relaxation via Gs protein coupling [4,10-12]. Moreover, the B3-AR is also able to inhibit the production of cytokines (such as TNFα, IL-6, and IL-8) to prevent not only myometrial cell apoptosis [13] but also extracellular matrix remodeling [14], two common features of uterine preparation for labor onset [15–19]. Another common feature of labor onset is the switch in the phenotype of myometrial smooth muscle cells from a proliferative to a hypertrophic state, which allows the synthesis of contractile material [20]. Maintaining uterine smooth muscle cells in a proliferative state could therefore be a promising target for the prevention of preterm labor.

The B3-AR is able to induce the proliferation of several cell types (chondrocytes, brown adipose tissue cells, airway smooth muscle cells, and neuronal progenitors) [8,21-24] via the transient activation of Erk1/2. However, the signaling downstream of B3-AR stimulation, which leads to Erk1/2 activation, still remains controversial since B3-AR G protein coupling appears to be tissue-dependent. For example, Bronnikov et al. [25] and Lindquist et al. [26] both proposed Gs-coupling in brown adipocytes, while Steinele et al. [8], Anesini et al. [21] and Robay et al. [27] proposed Gi-coupling in chondrocytes,
murmur fibroblasts and heterologous A549 B3-AR expressing cells, respectively. Many studies have linked cAMP signaling to Erk1/2 activation, including after B3-AR stimulation, via either PKA or Src protein kinase activation [28,29]. However, other authors have shown that B3-AR-induced Erk1/2 activation is sensitive to pertussis toxin, suggesting coupling with a Gi-protein [7,30,31]. In this case, the proposed signaling involves the activation of an Src/P3K-dependent pathway [8]. The ability of PKA to induce a Gs/Gi coupling switch by phosphorylating GPCRs, leading to receptor internalization and subsequent desensitization, has been reported, but the B3-AR lacks the PKA phosphorylation consensus sequence, which is present on the B2-AR, for instance [9,32–34]. However, in a murine model of B3-AR-transfected T1/2 preadipocytes, it has been suggested that the activation of Src protein kinase family could trigger the coupling of the B3-AR with Gs and Gi simultaneously [35].

In the present study, we used an in-vitro approach to test the hypothesis that the B3-AR is able to induce the proliferation of myometrial cells.

2. Materials & methods

2.1. Materials

LPS (Escherichia coli 055:B5, ref L2880), U0126 (ref U120-1), 8-Br-cAMP (ref B7880), Forskolin (from coleus forskohlii, ref F6886), pertussis toxin (ref P7208), melittin (from honey bee venom, ref M2272 genistein (synthetic, ref G6649), PP2 (ref P0042), H-89 (dihydrochloride hydrate, ref B1427), Triciribine (hydrate, ref T8390), wortmannin (from Penicillium funiculosum, ref W1628) and propranolol (hydrochloride, ref P0884) were purchased from Sigma-Aldrich. PKA inhibitor peptide (ref V5681) was purchased from Promega. SAR150640 was a gift from Sanofi-Midy Research Centre, Exploratory Research Department, Sanofi-Aventis S.p.A. SAR150640 was dissolved in a mixture of 30% absolute ethanol, 2% dimethyl sulfoxide (DMSO) and distilled water for the 10⁻³ M solution and thereafter diluted in distilled water. The final maximal bath concentration was 0.3% for ethanol and 0.02% for DMSO.

2.2. Primary cell lines

Myometrial cell lines were established from human myometrial biopsies obtained from women undergoing elective Caesarean delivery for uncomplicated pregnancies with cephalopelvic disproportion, prior to the onset of labor (Centre Hospitalier et Universaire de Dijon, Gynecology & Obstetrics Department). The biopsies were cut into small strips (1 mm³) and cultured in DMEM supplemented with 20% FBS, 100 IU/ml ESA, 37°C 5% CO₂. After 2 to 3 weeks of culture, the myometrial smooth muscle cells had reached confluence. The ex- plants were then gently removed; the cells harvested and put into new culture plates, containing 10 ml DMEM 10% FBS 100 IU/ml ESA. All of the experiments were performed on myometrial cell lines from the 3rd to the 7th passage. This study was approved by the local ethics committee (CPP, Dijon, France) and regulatory authorities, and written informed consent was obtained from each donor.

2.3. Stimulation protocol

The myometrial cells were seeded in Ø100 mm dishes (2×10⁵ cells/10 ml), 6-well plates (10⁶ cells/3 ml) and 24-well plates (10⁴ cells/1 ml) for flow cytometry, Western blotting and immuno-fluorescence analyses, respectively. The cells were allowed to adhere for 24 h in complete medium, 37°C, 5% CO₂, before 48 h starvation with low glucose (1 g/l) DMEM without phenol red and l-glutamine to synchronize the cells in the G0 phase of the cell cycle, and to reach a basal MAPK phosphorylation level. After starvation, the cells were stimulated for variable durations (3 min to 48 h) and with variable concentrations of SAR150640 (10⁻⁵ to 10⁻³ M), as indicated in the figure legends. Negative and positive controls were performed for each experiment using 100 µl of SAR150640 vehicle (H₂O 20% ethanol, 2% DMSO) and 10% FBS supplemented medium, respectively. Short stimulations (less than 1 h) were performed in the starvation medium, to avoid MAPK phosphorylation induced by the addition of fresh medium. Longer stimulations (1 to 48 h) were performed in Opti-MEM without antibiotics. All inhibitors were added 1 h prior to SAR150640 treatments, as indicated in the individual experiments, except for melittin, which was added at the time of SAR150640 treatment.

2.4. Cell proliferation analysis

After stimulation, myometrical cell proliferation was assessed by flow cytometry, using propidium iodide labeling. Briefly, the culture medium was removed and the cells were harvested and plated in 15 ml conical tubes. The pellets were re-suspended in 250 µl of trysin buffer (Spermin tetrahydrochloride, citrate, and trypsin) and incubated for 13 min at room temperature (RT), away from light. 200 µl of RNase buffer (Spermin tetrahydrochloride, citrate, trypsin inhibitor, RNase from bovine pancreas) were then added, and the samples were incubated for 10 min at RT, away from light. 25 µl of propidium iodide (PNN-1001, Invitrogen) was finally added before flow cytome- try analysis. 250 µl of cell preparation was then placed in 96-well plates, and analyzed with a guava EasyCyte® plus flow cytometer. A primary gating was performed on a Size-Granularity dot plot to discriminate cells from debris, and a secondary gating was performed on a Red-Area–Red-Width dot plot to discriminate single cells from doublets (see Supplementary methods). Cell proliferation was obtained by measuring the volume of each preparation needed to reach 10000 events in the secondary gate. Cell-cycle phase was evaluated by measuring propidium iodide labeling intensity, which is proportional to the DNA contained in each cell, and the S-phase ratio was quantified using modfit LT 3.3® software.

2.5. Western blotting analysis

After treatments, the plates were immediately put on ice, the medium was aspirated, and cells were lysed directly in the well by the addition of 100 µl of cold RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 1% Sodium deoxycholate, 0.1% SDS, protease and phosphatase inhibitor cocktail). Lysates were scraped and transferred to 1.5 ml conical Eppendorf tubes, and sonicated for 10 s. After the addition of 25 µl 5 × laemmli buffer (0.5 M Tris–HCl, pH 6.8, 10% (w/v) SDS, 20% glycerol, 5% β-Mercaptoethanol, 0.5% Bromophenol blue (w/v)), the samples were heated for 4 min at 95°C. 30 µg of total proteins were then separated on 10% polyacrylamide gel, and transferred to PVDF membranes. After transfer, non-specific binding sites were blocked by incubating the membranes for 1 h at RT in TBS-tween 0.1%, non-fat dry milk 5% and BSA 0.1%. The membranes were then incubated for 1 h 30 min at RT with the primary antibodies, rabbit polyclonal anti-cyclinD1, rabbit polyclonal anti-cyclinE, rabbit polyclonal anti-Erk1/2, rabbit polyclonal anti-Akt, rabbit polyclonal anti-Phospho-Erk1/2, rabbit polyclonal anti-Phospho-Akt (Cellsignaling technologies, #2978, #4129, #9102, #9272, #9101, #9271, respectively), and rabbit polyclonal anti-GAPDH (SC-25778, Santacruz Biotechnologies), (1/1000 dilution in the blocking solution). The primary antibodies were detected by incubating the membranes for 1 h with horseradish peroxidase-coupled secondary donkey anti-rabbit antibody (SC-2357, Santacruz Biotechnologies, CA, USA), (1/10,000 dilution in the blocking solution), and chemoluminescence induced by incubating for 1 min in ECL reagent (SC-2048, Santacruz Biotechnologies). The membranes were then exposed to Kodak films for 3 to 7 min. The films were finally digitized using a GS-800 densitometer, and the bands were quantified with Quantity one® software. For cyclin expression, the quantified bands were normalized with GAPDH, and for phosphorylation assays, the bands were normalized with the total form of the corresponding protein.
2.6. Immuno-fluorescence

Before stimulation, the cells were first cultured in 24-well plates containing sterile 0.13 mm microscope coverslips. After stimulation, the plates were immediately put on ice, the medium was aspirated, and the cells fixed directly in the well by the addition of 1 ml cold PBS 2% paraformaldehyde solution, and incubated for 5 min at 4 °C. After fixation, the cells were blocked and permeabilized by adding 1 ml PBS, 1% BSA and 0.1% saponin solution and incubated for 10 min at RT. The cells were then incubated overnight at 4 °C with primary polyclonal anti-phospho-Erk1/2 rabbit antibody (1/200 dilution in the blocking solution). Primary antibodies were detected using an Alexafluor 488 coupled donkey anti-rabbit antibody (A-21206, Invitrogen), (1/500 dilution in PBS 10% blocking solution), incubated for 30 min at RT, away from light. Nuclear labeling was also performed by incubating the cells in a PBS, DAPI (1 ng/ml) solution for 30 s at RT, away from light. The coverslips were then gently mounted, and mounted onto a superfrrost slide, in a Dako anti-fading mounting medium. The slides were finally observed with a Nikon (E400, Eclipse) epifluorescence microscope. Five pictures were taken in random fields for each labeling, and analyzed using imageJ software.

2.7. B3-AR silencing

The B3-AR was silenced in myometrial cells using the siRNA reagent system (SC-45064, Santa Cruz Biotechnologies) following the manufacturer’s instructions. Briefly, myometrial cells were seeded on 6-well plates and transfected after reaching 60–80% confluence, for 8 h. The B3-AR was knocked down using three specific sequences (B3-AR siRNA, Santa Cruz Biotechnologies, SC-1050110), and a control was performed using non-specific sequences control siRNA A (SC-37007, Santa Cruz Biotechnologies). The cells were reseeded with 1 ml transfection medium for 18 additional hours before the stimulation protocol. For flow cytometry analysis, cells were seeded and starved for 24 h, then transfected for 24 additional hours in absence of FBS before stimulation with SAR150640.

2.8. Statistical analysis

Differences among groups were determined by ANOVA followed by Bonferroni’s multiple comparison test. Statistical analysis was carried out using SigmaStat version 3 (GraphPad Software). All differences were considered significant when p<0.05.

3. Results

3.1. B3-AR stimulation induces HM-SMC proliferation

We first aimed to characterize in detail the proliferative effects of B3-AR stimulation in a primary culture of myometrial smooth muscle cells. We thus used SAR150640, a highly selective B3-AR agonist (Ki = 73nM), that was shown to have no affinity for B1- and B2-AR in more than 60 human models (including the human myometrium) [12]. In line with the proliferative effects observed in other cell types, we found that 48 h of stimulation with SAR150640 induced a dose-dependent increase in cell number (Fig. 1A) and S-phase ratio (Fig. 1B), with a maximal effect at 10−7 M showing a 215 ± 3.611 increase in cell number, when compared with unstimulated cells.

To better characterize the cell-cycle phase involved in this effect, we studied cyclin expression and observed, after 8 h of stimulation, a 4.4 ± 1.0-fold increase in the relative expression of cyclin D1 (G0 to G1 transition phase) and a 4.7 ± 1.2-fold increase in the relative expression of cyclin E (G1 to S transition phase) vs. untreated controls (Fig. 1C, D, E).

Specifity of the B3-AR-mediated effect of SAR150640A was assessed using two complementary approaches. First, we used propranolol, a commonly used beta-blocker that has been described to have a high affinity for the B1- and B2-ARs, and a lower affinity for the B3-AR. Thus, using propranolol at two concentrations, 10−7 and 10−5 M will inhibit B1- and B2-ARs only, or all B-AR subtypes respectively [36]. We observed that, whereas inhibiting B1- and B2-AR with propranolol 10−7 M had virtually no effect on SAR150640-induced proliferation, propranolol 10−5 M antagonized the effects of SAR 150640 on cell number increase (Fig. 1F). We also observed a strong repressive effect of B3-AR specific siRNA on SAR150640-induced cell number increase (Fig. 1G) and cyclin D1 and E overexpression (Fig. 1H, I, J). Collectively, these data indicate that SAR150640 induces B3-AR specific myometrial smooth muscle cell proliferation by inducing the early steps of the cell cycle (G0/G1 then G1/S transition).

3.2. B3-AR stimulation by SAR150640 induces biphasic Erk1/2 activation, and final Akt activation

As it is generally reported that B3-AR induces cell proliferation, via either Erk1/2 or Akt activation, we next measured the abundance of the phosphorylated forms of Erk1/2 and Akt in response to B3-AR stimulation in myometrial smooth muscle cells. We performed a time course stimulation and observed first a strong (8.24±1.55-fold increase vs. CTL) induction of Erk1/2 phosphorylation in response to SAR150640, which appeared at 2 min and peaked after 3 min of stimulation (Fig. 2A, B). This Erk1/2 phosphorylation was associated with its translocation into myometrial smooth muscle cell nuclei (Fig. 2C) indicating the full activation of Erk1/2. We then observed that this first activation phase was followed by a strong and prolonged decrease in Erk1/2 activation (Fig. 2D, E).

Finally, we observed that SAR150640 induced a second and lower wave of Erk1/2 phosphorylation (1.6±0.02-fold increase vs. CTL) after 8 h of stimulation (Fig. 2F, G). No induction of Erk1/2 was observed after 24 or 48 h of stimulation.

Interestingly, while SAR150640 did not induce Akt phosphorylation during the short (Fig. 2A, B) or medium time-course experiments (Fig. 2D, E), a moderate increase in Akt phosphorylation was observed after 48 h of stimulation (1.57±0.18-fold increase vs. CTL, Fig. 2F and G).

3.3. Erk1/2, but not Akt-activation mediates B3-AR-induced proliferation

To test the hypothesis that Erk1/2 or Akt are required to trigger B3-AR-induced myometrial smooth muscle cell proliferation, we performed co-stimulations with the MEK1/2 inhibitor, U0126, or with the Akt inhibitor, triciribin. Flow cytometry analysis revealed that the blockade of MEK1/2 fully inhibited the effect of SAR150640 on cell number and S-Phase increase (Fig. 3A, B, respectively). Western blot analysis confirmed the inhibitory effect of MEK on Erk1/2 activation (Fig. 3C) and demonstrated that MEK1/2 inhibition abolished SAR-150640-induced cyclin D1 protein expression (Fig. 3C, D) and partially inhibited cyclin E protein expression (Fig. 3C, E).

Western blot analysis confirmed the inhibitory effects of triciribin on Akt phosphorylation after 48 h of stimulation with SAR150640 (Fig. 3F). However, Akt inhibition had no significant effect on cell proliferation (Fig. 3G).

These data indicate that Erk1/2 (but not Akt) is required for B3-AR stimulation to induce human myometrial smooth muscle cell proliferation, and also that the induction of cyclin D1 and E expression previously observed is under the control of Erk1/2.

3.4. The first phase of Erk1/2 activation is Gs-dependent

Erk1/2 is an important signaling intermediate in cell proliferation that can be activated by a wide variety of effectors. As mentioned, B3-AR-induced activation of Erk1/2 has been linked to the activation of either a Gs/cAMP/PKA or a Gq/Pi3K/Src pathway. We thus aimed to
identify the pathway activated after B3-AR stimulation in human myometrial smooth muscle cells.

We first studied the 2 to 5-minute peak of Erk1/2 activation. In line with our previous work, which demonstrated Gs coupling of the B3-AR in the human myometrium, we observed that both forskolin, a direct activator of adenylyl cyclase, and 8-Br-cAMP, the stable analogue of cAMP, activated Erk1/2 after 3 min of stimulation (Fig. 4A, B). Moreover, co-stimulation with the Gi inhibitor pertussis toxin (PTX, 200 ng/ml) showed that neither forskolin-, nor SAR150640-induced Erk1/2 activation were sensitive to Gi inhibition (Fig. 4C, D).

We also studied the activation of PKA in response to B3-AR stimulation. Western blot analyses revealed that PKA phosphorylation level increased within 2 to 5 min in response to SAR150640 (see Supplemental data 1A) and was released from a peri-nuclear location (corresponding to inactive PKA) into the cytoplasm (see supplemental data 1B). Moreover, PKA activity, monitored using the Promega Peptag...
Assay®, was also increased in response to SAR150640 stimulation within 2 to 5 min (see Supplemental data 1C). We also performed co-stimulations with the toxin melittin, which reportedly inhibits Gs protein, but is a potent activator of Gi[37]. For this reason, it was added concomitantly with SAR150640. Interestingly, we observed that 3 min of co-stimulation with melittin abolished both basal and B3-3AR-induced Erk1/2 phosphorylation (see supplemental data 2A, B). Collectively, these data indicate that the first peak of Erk1/2 activation is Gs-dependent but completely Gi-independent.

Finally, we performed co-stimulations with the inhibitors of PKA and Src, namely H89 (5 μM) and PP2 (10 μM), respectively (Fig. 4E), and observed that both inhibitors abolished SAR150640-induced Erk1/2 phosphorylation at 3 min, indicating a classical Gs/cAMP/PKA/Src activating pathway. Further experiments were carried out using a peptidic inhibitor of PKA (20 μM), also showing an abolition of Erk1/2 phosphorylation (see supplemental data 1D).

3.5. The second wave of Erk1/2 activation is Gi-dependent

The same approach was used to assess the pathway involved in the second wave of Erk1/2 activation, occurring within 8 h of B3-AR stimulation, and we observed that it was abolished by PTX co-stimulation, indicating Gi dependent Erk1/2 activation (Fig. 5A, B). Contrary to what was found during the first peak of Erk1/2 activation, we observed that the second wave of activation remained unaffected by PKA inhibition with H89 (Fig. 5C, D). This suggests that PKA, which is required for
the short-term Erk1/2 activation, is no longer needed to activate Erk1/2 after 8 h of stimulation, and that the first peak of Erk1/2 activation is not needed for the second to occur. In contrast, we observed that Src inhibition with PP2 abolished SAR150640-induced Erk1/2 activation at 8 h (Fig. 5C, D), as was the case for Erk1/2 activation at 3 min, indicating that Src plays a role in both phases of Erk1/2 activation.

As the B3-AR-mediated Gi/PI3K/Src-dependent activation of Erk1/2 had already been described, we also performed co-stimulations using the PI3K inhibitor wortmannin (WTM). We observed that WTM abolished Erk1/2 phosphorylation observed after 8 hours of SAR150640 stimulation (Fig. 5C, D), whereas no effect of PI3K inhibition was seen on the 3-minute Erk1/2 phosphorylation peak (data not shown).

Finally, melittin per se induced a strong activation of Erk1/2 at 8 h, with no further enhancement in cases of co-stimulation with SAR150640 (see supplemental data 2C, D). The Gi protein inducing effects of melittin probably explains this strong induction of Erk1/2 phosphorylation observed after 8 h of stimulation. Collectively, the results indicate that the second peak of Erk1/2 activation depends on a Gi/PI3K/Src-dependent mechanism, and is Gi and PKA independent.

3.6. Gi and Gs activation are both required to induce myometrial cell proliferation

As we identified two B3-AR dependent pathways leading to Erk1/2 activation, at two different time points, we finally aimed to identify which pathway was required to induce myometrial smooth muscle cell proliferation. We performed 48 h of co-stimulation with either a

---

**Fig. 3.** Erk1/2 but not Akt activation is required for SAR150640-induced proliferation. A, B, C, D & E: Erk1/2 inhibition: Cells were treated with SAR150640 (10^−7 M) for 8 or 48 h in the absence or presence of U0126 (2 × 10^−5 M). A: Cell number: Numbers are expressed as percentages versus control, and represented as mean values±SEM. N=4. *=p<0.05 vs. all. B: S-phase ratio: Total percentages obtained with Modfit® are represented as mean values±SEM. n=4. ¥=p<0.05 vs. all. C: Cyclin expression: Presented blots are representative of 4 independent experiments D & E: Quantification: 34 kDa cyclin D1 band and 48 kDa cyclin E band were digitized and quantified with Quantity one. Relative quantities vs. control are represented as mean values±SEM, n=4. *=p<0.05 vs. all. F & G: Akt inhibition: Cells were treated with SAR150640 (10^−7 M) for 48 h in the absence or presence of triciribin (2×10^−5 M). F: p-Akt blot: Presented blot is representative of 4 independent experiments. G: Cell number: Numbers are expressed as percentages versus control, and represented as mean values±SEM. N=4. *=p<0.05 vs. respective control.
Gs or a Gi inhibitor. We observed that the blockade of either Gs or Gi protein, by melittin and PTX, respectively, abolished SAR150640-induced proliferation (Fig. 6A, B), indicating that both pathways are required for B3-AR stimulation to induce proliferation. Therefore, both waves of Erk1/2 activation are needed to obtain myometrial smooth muscle cell proliferation.

When H89 was used, we observed that PKA, whose activation is not involved in the second wave of Erk1/2 phosphorylation (Fig. 5C), is needed to trigger the final proliferative effect (Fig. 6C), once again, similar results were obtained using the PKA peptide inhibitor (see Supplemental data 1E). In contrast, PI3K inhibition by WTM, which had no impact on the first phase, but blocked the second wave of Erk1/2 activation, also blocked SAR150640-induced proliferation (Fig 6C). As expected, the inhibition of Src, which is involved in Erk1/2 activation at both 3 min and 8 h, also abolished the proliferative effects of B3-AR stimulation (Fig. 6C). Thus, we demonstrated that the blockade of Src, PI3K, and/or PKA blocked B3-AR-induced proliferation of human myometrial cells, confirming that both the Gs/cAMP/PKA and Gi/PI3K/Src pathway are required.

Finally, to further support these results, we performed a sequential inhibition of the two Erk1/2 activation peaks using U0126 (A schematic representation of the experiment is presented on Fig. 6D). We observed that inhibition of either the first or the second Erk1/2 activation lead to a full blockade of SAR150640-induced cell proliferation (Fig. 6E), confirming the requirement of the two Erk1/2 activation peaks in the B3-AR-induced proliferative signaling.

4. Discussion

The B3-AR has been studied over the past decades for its potential therapeutic interest in several conditions such as cachexia, metabolic syndrome, obesity, diabetes, cardiac diseases, and more recently in...
depression, overactive bladder and preterm labor [5,38]. The proliferative effects of the B3-AR could also be a therapeutic approach in some of these diseases. For example, by inducing hippocampal neuronal progenitor proliferation, the B3-AR may be of interest in the treatment of depression [22]. It is also able to induce choroidal cell proliferation, and could thus prevent damage in cases of diabetic retinopathy [8]. Additionally, the proliferative effect on myometrial smooth muscle cells could be beneficial by maintaining uterine contractile quiescence. The first finding of interest in the present study is that B3-AR stimulation induces the proliferation of human myometrial smooth muscle cells. The second finding is that we propose a new signaling mechanism by which B3-AR stimulation induces a proliferative effect involving biphasic Erk1/2 activation triggered by the sequential activation of the Gs/cAMP/PKA and Gi/PI3K/Src pathways.

4.1. Proliferative effects of the B3-AR

4.1.1. Erk1/2 dependent induction of cyclin D1 and E expression

Among the mitogenic associated protein kinase (MAPK) pathways, which are responsible for both cell cycle induction and regulation, the Erk1/2 pathway has been extensively studied [39–41]. Recent studies have highlighted a strong link between Erk1/2 activation and cyclin D1 and then cyclin E expression, which is involved in G0 to G1 and G1 to S cell cycle phase transition, respectively. Indeed, Erk1/2 activates the transcription factor AP-1 and inactivates the repressor Tob [42,43] [41–43], respectively promoting and inhibiting cyclin D1 expression. In turn, cyclin D1, by activating its targets, the two cyclin-dependent kinases 4 & 6, will lead to the induction of cyclin E expression [44]. In this report, we have shown that SAR150640-induced myometrial smooth muscle cell proliferation depends on the induction of cyclin D1 and E expression, but not of cyclin B1, which is involved in G2 to M transition. Whereas previous papers described, as we have here, an Erk1/2-dependent proliferative effect of the B3-AR [39,42,43], they did not assess its impact on the induced expression of different cyclins. Our results indicate for the first time that the proliferative effect of the B3-AR could be mediated by the specific induction of the early steps, G0/G1 and G1/S transitions, of the cell cycle. Moreover, in line with data from the literature, we observed that Erk1/2 blockade fully inhibits B3-AR-induced cyclin D1 and E expression.

4.1.2. The B3-AR induces a biphasic Erk1/2 activation

Whereas Erk1/2 activation has also been reported to trigger other processes such as apoptosis, inflammation or differentiation [39]. Our team has previously shown that B3-AR stimulation was able to prevent myometrial inflammation and apoptosis. This apparent discrepancy has already been observed, and seems to be due to the kinetics and the degree of Erk1/2 phosphorylation. For example, Kahan et al. [45] reported that the biphasic activation of Erk1/2, with an early (1–10 min) strong peak of activity, followed by a second sustained phase of lower activity (over several hours) is needed to induce proliferation, whereas Tombes et al. reported that the acute and prolonged phosphorylation of Erk1/2 is associated with the induction of cell cycle arrest and inflammation [46]. No formal explanation has been made for this phenomenon; however, it has been suggested that the pathways leading to Erk1/2 phosphorylation, mediated either by B-raf or Raf-1 could trigger these opposite effects [46]. In this study we have reported that B3-AR-dependent Erk1/2 phosphorylation was biphasic with peaks at 3 min and 8 h. Thus, given this biphasic activation with both the anti-inflammatory and anti-apoptotic effects previously observed, these results suggest that B3-AR-induced Erk1/2 activation would maintain myometrial smooth muscle cells proliferation, and thus uterine quiescence. Late Akt activation, at a time where Erk1/2 is no longer activated by B3-AR stimulation, in combination with the fact that its inhibition with Triciribin had no or little effect on B3-AR-induced proliferation, suggests that Akt has a role in the early phase of B3-AR-induced proliferation, as has been suggested for other adrenergic receptors [23,47]. However, it is noteworthy that after 48 h of stimulation, a time at when Akt is activated, but no longer Erk1/2, the proportion of cells in the S-phase is still higher in the SAR150640-stimulated cells than in untreated cells, indicating that the proliferative effect of SAR150640 is sustained for at least 48 h, and suggesting that Akt may play a role in this latter step.
4.2. B3-AR pathway activation

4.2.1. Sequential Gs then Gi signaling

The major finding of this study is that the biphasic activation of Erk1/2 in myometrial smooth muscle cells depends on the sequential activation of the Gs and then Gi pathway. This ability of the B3-AR to couple first to a Gs then to a Gi protein could explain the absence of consensus observed regarding B3-AR coupling and subsequent signaling. Interestingly, Bronnikov et al. [25] and Lindquist et al. [26], who both proposed Gs coupling, focused their demonstration on evaluating the increase in cAMP production or Erk1/2 activation after a short time (5 min) of B3-AR stimulation. Moreover, Lindquist et al. [26] demonstrated that this early activation of Erk1/2 was PTX insensitive but did not assess the impact of PTX on final B3-AR-mediated proliferation. In this context, the 3 min signaling for Erk1/2 activation associated with Gs signaling observed in our study is in keeping with their findings. In contrast, authors that have focused on the final effect of the B3-AR rather than on the early activation pathway concluded that Gi coupling was involved. First, Anesini et al. [21] and Robay et al. [27] both investigated the effect of the B3-AR on cells stimulated for more than 15 min to assess the activation of ion channels. In this context, they observed that B3-AR-induced ion channel activation was PTX sensitive and associated with a decrease in cAMP concentrations. The results in our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations. The results of our study are in line with these data, since we observed that the first 3 min signaling was followed by a decrease in cAMP concentrations.

![Figure 6](https://example.com/figure6.png)

**Fig. 6.** Both Gs and Gi activation are required to induce proliferation. Cells were treated with or without SAR150640 (10^{-7} M) for 48 h in the absence or presence of A: Melittin (10^{-7} M), B: Pertussis toxin (200 ng/ml), or C: Protein kinase inhibitors PP2 (5 \times 10^{-5} M), wortmannin (5 \times 10^{-5} M), or H89 (5 \times 10^{-6} M). Cells were labeled with propidium iodide and analyzed by flow cytometry. Cell numbers are expressed as percentages versus control, and represented as mean values±SEM, *=p<0.05 vs. all. D & E: Sequential Erk1/2 inhibition: For early inhibition, cells were stimulated with SAR150640 10^{-7} M in presence of U0126 (2 \times 10^{-5} M), after two hours stimulation, cells were washed out with PBS, then stimulated with SAR150640 10^{-7} M alone for the remaining 46 h. For late inhibition, cells were stimulated for 2 h with SAR150640 10^{-7} M alone, then U0126 (2 \times 10^{-5} M) was added for the remaining 46 h. D: Schematic representation of the sequential inhibition. E: Cell number: Numbers are expressed as percentages versus control, and represented as mean values±SEM. N=4. *=p<0.05 vs. control.
that this Gi/PI3K/Src-induced activation occurred during the second peak of Erk1/2 phosphorylation.

4.2.2. Role of Src

Our study also highlights the involvement of Src in B3-AR-mediated proliferation. The activation pathway of the Src protein kinase family still remains unclear. It has been shown that Src present two phosphorylation sites, Tyr416, and Ser17, both of which enhance Src activity [48]. In the present study, we observed phosphorylation of the Tyr416 site in response to B3-AR stimulation. In contrast, no phosphorylation of Ser17 (PKA-dependent site) was observed (see Supplemental data 1). These data suggest that Src activation occurs independently and upstream of PKA activation. According to our results, Src plays a crucial role in both phases of Erk1/2 activation, which led us to consider its potential role in triggering the switch of coupling, a role that remains to be further elucidated. One argument favoring the hypothesis of a Src-dependent switch has been proposed by Cao et al. [35]. In a murine model of immortalized T1/2 preadipocytes, they observed that over-expressed B3-AR can directly recruit the protein kinase Src, leading to the activation of a Gi protein, and subsequent PI3K-dependent Erk1/2 activation. According to their findings, B3-AR could thus be directly (Gs) and indirectly (Gi) coupled to both G proteins [35].

4.3. Conclusion

In conclusion, these data bring new insights into the proliferative effects driven by B3-AR, and the new sequential activation of Gs and then Gi (summarized in Fig 7) proposed here could explain the discrepancies in the signaling pathways described in previous studies. In light of our previous work, the present study emphasizes the potential value of the B3-AR agonist, SAR150640, in the pharmacological management of preterm labor, as it is able to maintain proliferation and to oppose the triggering mechanisms of labor, namely remodeling [13], inflammation, apoptosis [14] and their consequences (i.e. myometrial contractions).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jbamer.2013.01.019.

Fig. 7. New putative signaling model for B3-AR-induced proliferation.

Acknowledgements

This work was supported in part by the Regional Council of Burgundy (Conseil regional de Bourgogne) grant A01 Proliferation.

References


