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Current Research in Pharmacology and Drug Discovery

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Regulation of bFGF-induced effects on rat aortic smooth muscle cells by β_3 -adrenergic receptors



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ARTICLE INFO

Keywords:

CL316,243

VSMC proliferation

VSMC migration

ERK/AKT phosphorylation

Neointima

ABSTRACT

Background: Basic fibroblast growth factor (bFGF)-mediated vascular smooth muscle cell (VSMC) proliferation and migration play an important role in vascular injury-induced neointima formation and subsequent vascular restenosis, a major event that hinders the long-term success of angioplasty. The function of β_3 -adrenergic receptors (β_3 -ARs) in vascular injury-induced neointima formation has not yet been defined.

Objectives: Our current study explored the possible role of β_3 -ARs in vascular injury-induced neointima formation by testing its effects on bFGF-induced VSMC migration and proliferation.

Methods: β_3 -AR expression in rat carotid arteries was examined at 14 days following a balloon catheter-induced injury. The effects of β_3 -AR activation on bFGF-induced rat aortic smooth muscle cell proliferation, migration, and signaling transduction (including extracellular-signal-regulated kinase/mitogen activated protein kinase, ERK/MAPK and Protein kinase B, AKT) were tested.

Results: We found that vascular injury induced upregulation of β_3 -ARs in neointima. Pretreatment of VSMCs with a selective β_3 -AR agonist, CL316,243 significantly potentiated bFGF-induced cell migration and proliferation, and ERK and AKT phosphorylation. Our results also revealed that suppressing phosphorylation of ERK and AKT blocked bFGF-induced cell migration and that inhibiting AKT phosphorylation reduced bFGF-mediated cell proliferation.

Conclusion: Our results suggest that activation of β_3 -ARs potentiates bFGF-mediated effects on VSMCs by enhancing bFGF-mediated ERK and AKT phosphorylation and that β_3 -ARs may play a role in vascular injury-induced neointima formation.

1. Introduction

Vascular smooth muscle cell (VSMC) migration and proliferation are the key determinants of vascular injury-induced neointima formation and subsequent vascular restenosis (Ross, 1993; Caplice et al., 2003). The phenotypic changes of VSMCs are mainly induced by several growth factors, including bFGF (Adam et al., 1995; Lai et al., 1996). bFGF acts as

a mitogenic and motogenic factor for VSMCs by stimulating the downstream effectors, including ERK/MAPK and AKT (Lindner et al., 1991; Shigematsu et al., 2000; Kato et al., 2019).

β_3 -ARs were originally discovered in adipocytes and function to increase energy expenditure and nutrient consumption (Emorine et al., 1989; Cypess et al., 2015) and later were found in other tissues, including cardiac muscle, endothelial, vascular smooth muscle, and adventitia cells

Abbreviations: bFGF, basic fibroblast growth factor; β_3 -AR, β_3 -adrenergic receptor; ERK/MAPK, extracellular-signal-regulated kinase/mitogen activated protein kinase; PI3K/AKT, phosphatidylinositol 3-kinase and Akt (protein kinase B); VSMC, vascular smooth muscle cell.

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<https://doi.org/10.1016/j.crphar.2022.100094>

Received 8 December 2021; Received in revised form 21 February 2022; Accepted 25 February 2022

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(Gauthier et al., 1996; Moniotte et al., 2001; Moniotte et al., 2001; Leblanc and Tabrizchi, 2018). Stimulation of β_3 -ARs induces proliferation of brown adipocytes and several other types of cells, such as choroidal, airway smooth muscle, neuronal progenitor, and myometrial smooth muscle cells (Anesini and Borda, 2002; Steinle et al., 2003; Steinle et al., 2005; Jhaveri et al., 2010; Romana-Souza et al., 2011; Hadi et al., 2013). Although β_3 -ARs are expressed in cardiovascular system, the function of β_3 -ARs has not been defined. In angioplasty-associated vascular restenosis, some studies suggest that activation of β_3 -ARs may play an inhibitory role in the injury-induced growth of vascular smooth muscle cells and endothelial cells by inducing endothelial cell apoptosis and nitric oxide formation and inhibiting the expression of proinflammatory genes (Brehm et al., 2001; Wolf et al., 2007), whereas others found that blockade of β_3 -ARs completely abolished endothelial cell migration induced by a β_3 -AR agonist (Pauwels et al., 1988; Kou and Michel, 2007; Frazier et al., 2011), indicating an undefined role of β_3 -ARs in vascular injury-induced neointima formation.

Our current study explored the role of β_3 -ARs in bFGF-induced vascular smooth muscle cell proliferation and migration (markers of phenotypic and behavioral changes of VSMCs during vascular injury) by determining whether activation of β_3 -ARs affects bFGF-mediated mitogenic and motogenic activity of VSMCs in relation to neointima formation following vascular injury, a surrogate of restenosis.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), ABC kit, and U0126 were purchased from Thermofisher (Waltham, MA). Porcine pancreatic collagenase type I and elastase were purchased from Worthington (Lakewood, NJ). Wortmannin and antibodies directed against ERK (Cat# 05-1152), phospho-ERK (Cat# 05-481), AKT (Cat # 9272), and phospho-AKT (Ser473, Cat # 9271) were purchased from Cell Signaling technology (Beverly, MA). β_3 -adrenergic receptor antibody was purchased from Novus (Cat.# NLS4198). Male Sprague-Dawley rats were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA). CL316,243 and SA59230A were purchased from Cayman Chemicals (Ann Arbor, MI). 2F Fogarty arterial embolectomy catheters were purchased from Edward Lifesciences (Irvine, CA). All other reagents were obtained from Sigma Aldrich (St. Louis, MO).

2.2. Rat carotid artery injury

Male Sprague-Dawley rats (Hilltop), weighing 400–450 g, were anesthetized via an intraperitoneal injection of a mixture of xylazine (5 mg/kg) and ketamine (60 mg/kg) (Chang et al., 2006). Rat carotid artery injury was generated by following the published procedure (Chang et al., 2004). A moderate right common carotid artery injury was created by inserting and withdrawing a 2F Fogarty arterial embolectomy balloon catheter 4 times. The uninjured left common carotid arteries served as a control. The animal protocol was approved by the A.T. Still University Animal Care and Use Committee following the NIH Guide for the Care and Use of Laboratory Animals.

2.3. Immunostaining of β_3 -ARs

Rats were anesthetized with overdosing sodium pentobarbital (120 mg/ml) 14 days after the injury. The rats were fixed with 4% formaldehyde and carotid arteries were dissected, processed using a Leica TP1020 semi-enclosed benchtop tissue processor (Leica Microsystems, Nussloch, Germany), and embedded in paraffin using a Leico embedding station. The carotid arteries were sectioned using a Leica RM2255 microtome. Cross-sections of 8 μ m thickness were stained for β_3 -ARs using ABC kit and a β_3 -AR antibody (primary antibody dilution was 1:100). Images were captured with a Leica DFC400 digital microscope

camera mounted on a Leica DM4000B microscope using Surveyor Software (Objective Imaging Ltd., Cambridge, United Kingdom).

2.4. Cell culture

VSMCs were isolated from the thoracic aortae of 100–125 g male Sprague-Dawley rats by enzymatic dissociation following the published procedure (Brown et al., 1999). Cells were grown in DMEM/F12 containing 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B and maintained at 37 °C, 95% air, and 5% CO₂ in a humidified atmosphere. The experiments were conducted with subcultured cells (passages 5–10) because these cells show morphological and functional changes similar to the cells isolated from neointima following vascular injury (Schwartz, deBlois et al., 1995; Bochaton-Piallat et al., 1996). The cells not only express genes that represent the characteristics of developmental stages of smooth muscle cells but are also able to replicate and expand, which are the major events occurring in the process of neointima formation following vascular injury (Chamley-Campbell et al., 1981; Schwartz et al., 1986; Hedin et al., 1991; Majesky et al., 1992). Therefore, subcultured vascular smooth muscle cells are the appropriate model to study the cellular characteristics of neointima cells in injured vessel walls.

2.5. Cell migration

Cell migration was measured by a wound healing assay (Chang et al., 2006). Briefly, confluent cells were subjected to a scratch with a 10 μ l sterile pipette tip to create a cell-free area (gap). Pictures were captured before and after 24-h treatment with specific agents (e.g., bFGF) using a microscope connected to a digital camera from Scion Corporation (Frederick, MD) and cell-free areas were measured using Image J software. Cell migration is expressed as percentage changes of the cell-free area after 24-h treatment with the agents. 5 μ M of hydroxyurea was used to inhibit cell proliferation (Sarkar et al., 1996).

2.6. Cell proliferation

Cell proliferation was measured by using resazurin, an indirect method to measure cell proliferation by monitoring viable cell number (Riss et al., 2004; Wolf et al., 2012; Ivanov et al., 2014; Ren et al., 2015; Tapias et al., 2015). Live cells reduce resazurin to resorufin, a fluorescent end product of which intensity reflects the number of living cells. Briefly, following the treatment, cells were incubated with serum-free medium containing 15 μ g/ml resazurin for 2 h and the absorbance was read at Ex = 530/30 nm and Em = 590/20 nm.

2.7. ERK and AKT phosphorylation

At the end of experiments, cells were lysed in lysis buffer containing 188 mM Tris-HCl (pH = 6.8), 1 mM EDTA, 15% glycerol, 1% SDS, 2 mM sodium vanadate, protease inhibitors cocktail (Sigma Aldrich, 1:200 dilution), and serine/threonine phosphatase inhibitor cocktail (Sigma Aldrich, 1:100 dilution). Phosphorylated ERK and AKT were measured by Western blot analysis. Band densities were measured using Image J software and the levels of ERK and AKT phosphorylation are expressed as the ratio of phosphorylated ERK to total ERK and phosphorylated AKT to total AKT. It should be noted that originally, we probed the membrane with anti-phospho-ERK antibody first, stripped the membrane, and then reprobed it with anti-ERK antibody. We found that the phospho-ERK signals was much stronger than the total ERK signal and it was impossible to strip and reprobe for the total ERK after phospho-ERK detection. We then reversed the order by probing the membrane for total ERK first, then stripping and reprobing the membrane with anti-phospho-ERK antibody. In the case of AKT phosphorylation detection, we measured AKT phosphorylation first by using phospho-AKT (Ser473) monoclonal antibody, then stripped and reprobed the membrane for total AKT.

Antibody dilutions used for detecting phospho-ERK, total ERK, and total AKT were 1:1000. The antibody dilution for phospho-AKT was 1:500.

2.8. Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by a post hoc test and expressed as mean \pm SD. A p value of less than 0.5 was considered as a significant difference.

3. Results

3.1. β_3 -ARs were highly expressed in the neointima following vascular injury

Studies revealed that β_3 -ARs are upregulated in cardiac muscle under several pathological conditions (Cheng et al., 2001; Moniotte et al., 2001; Moniotte et al., 2007) and this upregulation is implicated in protecting cardiomyocytes from damages caused by ischemia/reperfusion and oxidative stress (Gauthier et al., 1998; Angelone et al., 2008; Aragon et al., 2011; Calvert et al., 2011; Sorrentino et al., 2011; Niu et al., 2012; Watts et al., 2013; Belge et al., 2014; Garcia-Prieto et al., 2014). However, no studies have been conducted to investigate the expression levels of β_3 -ARs in injured blood vessels. Here, we used a rat carotid artery injury model to examine the expression of β_3 -ARs. We created a moderate carotid artery injury by controlling the times of insertion and withdrawal of an inflated balloon. The expression of β_3 -ARs was checked by immunostaining at the 14th day following the injury. As shown in Fig. 1, β_3 -ARs were highly expressed in newly formed intima, especially in the area that is close to the lumen. The results were confirmed in three different rats.

3.2. Pretreatment of VSMCs with a selective β_3 -AR agonist, CL316,243, enhanced bFGF-induced cell proliferation

Our next experiments were designed to test the possible role of β_3 -ARs in vascular injury-induced neointima formation by using VSMCs isolated from rat aorta. Because VSMC proliferation and migration are the

determinants of neointima formation (Ross, 1993; Caplice et al., 2003) and bFGF is one of the major contributors in this process (Adam et al., 1995; Lai et al., 1996), we examined the effect of β_3 -AR activation on bFGF-induced VSMC proliferation by using CL316,243, a selective β_3 -AR agonist. Several β_3 -AR agonists are currently available, including BRL 37, 344, BRL 35,135, and CL316,243. Among the agonists, CL316,243 is most potent and has a higher selectivity for β_3 -ARs (Dolan et al., 1994). Importantly, CL316,243 has no or minimal effects on β_1 -ARs and β_2 -ARs at concentrations less than 100 μ M (Dolan et al., 1994).

The cultured smooth muscle cells were pretreated with 0.1, 1, 2, or 5 μ M CL316,243 for 24 h followed by the treatment with or without 15 ng/ml bFGF in presence or absence of the same concentrations of CL316,243 for additional 24 h. The rationale of choosing 15 ng/ml of bFGF in our current study was based on the results from previous studies that this concentration produced significant induction of human iliac and rat aortic smooth muscle cell proliferation (Weiss and Maduri, 1993; Axel et al., 1997). Cell proliferation was measured after 24 h of bFGF treatment. As shown in Fig. 2, CL316,243 alone did not alter the cell proliferation at any of the concentrations tested. Although all the concentrations of CL316,243 tested seem to increase bFGF-induced cell proliferation, only 5 μ M of CL316,243 produced significant enhancement on bFGF-induced cell proliferation (about 16%).

We also discovered that cell death occurs when the concentration of CL316,243 reaches 10 μ M (data not shown here). Therefore, all of our experiments used concentrations less than 10 μ M.

Since 5 μ M CL316,243 produced the maximal effect, we used that concentration to test if the selective β_3 -AR antagonist, SR59230A, could prevent the effect of CL316,243 on bFGF-induced cell proliferation. As shown in Fig. 3, 5 μ M CL316,243 alone did not affect smooth muscle cell proliferation, but significantly potentiated the bFGF-induced effect on cell growth. SR59230A at the concentrations tested had no effect on its own, but significantly decreased the effect of CL316,243 on bFGF-induced proliferation in a dose-dependent manner. A similar effect was observed with 10 μ M of SR59230A, at which a higher concentration (10 μ M) of caused cell death (data not shown here).

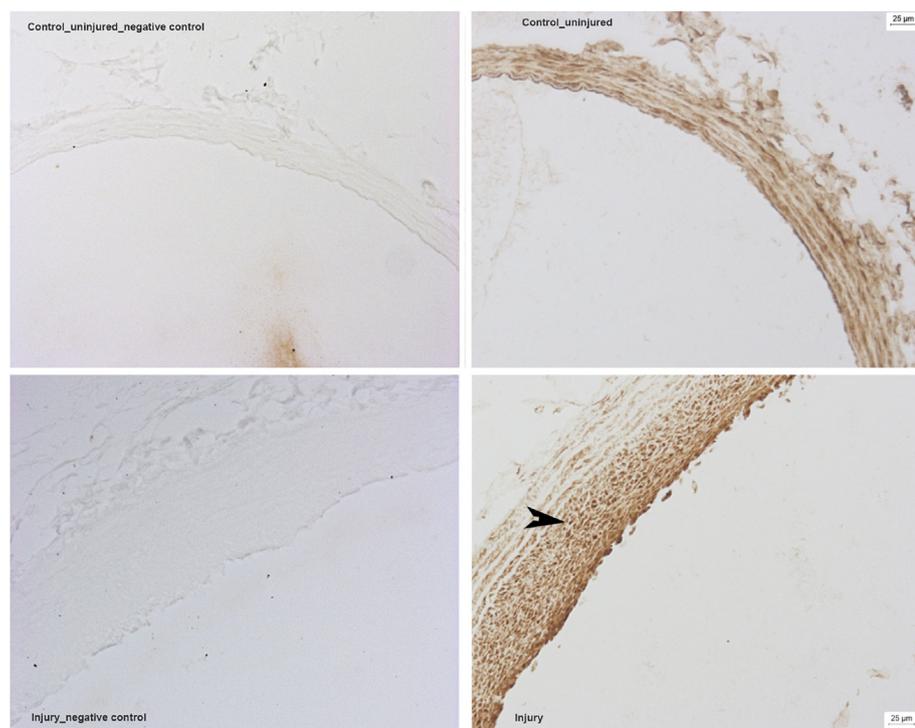


Fig. 1. β_3 -ARs were highly expressed in neointima. Vascular injury was induced by 2F balloon catheter via inserting and withdrawal 4 times. Rats were sacrificed 14 days after the injury. The carotid arteries were fixed, collected, embedded in paraffin, sectioned to 8 μ m, and stained with β_3 -AR antibody using an ABC kit. Pictures show the β_3 -AR expression in the carotid artery sections from one of the three independent rats. The arrow directs to the brown particles showing the positive staining. Left panel: Negative controls are the sections that were stained without β_3 -AR antibody but all reagents included in the ABC kit. Right panel: sections were stained with all ABC kit reagents as well as β_3 -AR antibody. Control uninjured: uninjured left common carotid artery; Injury: injured right common carotid artery. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

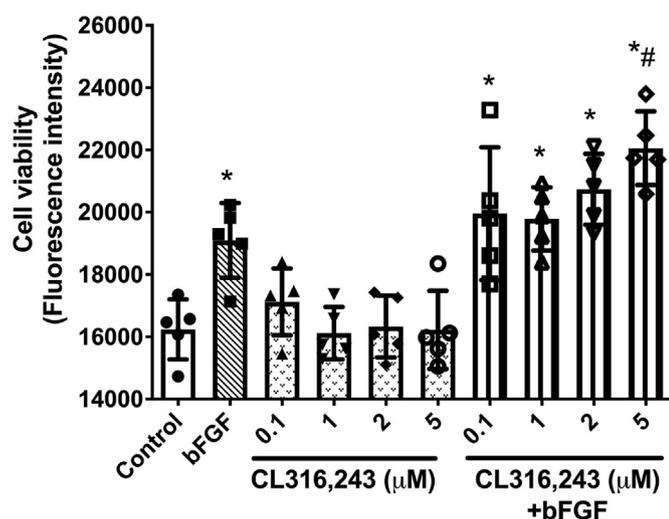


Fig. 2. CL316,243 potentiated bFGF-mediated increase in cell viability. VSMCs were pretreated with different concentrations of CL316,243 in serum-free medium for 24 h followed by a stimulation with 15 ng/ml of bFGF for 24 h in the presence and absence of the same concentrations of CL316,243. Cell viability was measured by using resazurin and expressed as intensity of fluorescence (mean ± SD). Results are from five independent experiments. Data were analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. *p < 0.05, compared to control, #p < 0.05 compared to bFGF alone.

3.3. Pretreatment of VSMCs with CL316,243 potentiated bFGF-induced cell migration

VSMCs were pretreated with 2 μM or 5 μM of CL316,243 for 24 h followed by stimulation with or without 15 ng/ml bFGF for an additional 24 h in the presence or absence of the same concentrations of CL316,243. As shown in Fig. 4, 15 ng/ml bFGF induced a 98.5% increase in cell migration. CL316,243 at the concentration of 2 μM failed to affect bFGF-induced cell migration. However, 5 μM CL316,243 itself significantly promoted cell migration (55% increase compared to control), although the increase was significantly smaller than that induced by bFGF alone (98.5%). Pretreatment of VSMCs with 5 μM CL316,243 synergistically enhanced the bFGF-induced cell migration (coefficient of drug interaction is 0.89).

3.4. Pretreatment of VSMCs with CL316,243 enhanced bFGF-induced phosphorylation of ERK and AKT

The effects of bFGF are mediated by activating the downstream effectors, including the ERK/MAPK and PI3K/AKT cascades (Lindner et al., 1991; Martin, 1998; Powers et al., 2000; Shigematsu et al., 2000; Li et al., 2004; Tang et al., 2008; Kato et al., 2019). In order to understand the mechanism of CL316,243 in enhancing bFGF-induced cell behavioral changes, we tested the effects of CL316,243 on bFGF-induced ERK and AKT phosphorylation. Cells were pretreated with either 2 μM or 5 μM CL316,243 for 24 h followed by stimulation with 15 ng/ml of bFGF for 5 min. The time of bFGF stimulation was determined by our preliminary data showing that a substantial phosphorylation of ERK and AKT was induced beginning at 5 min. Although CL316,243 alone at either concentration did not induce phosphorylation of ERK or AKT, pretreatment of VSMCs with both concentrations of CL316,243 significantly enhanced bFGF-mediated ERK and AKT phosphorylation (Figs. 5 and 6).

3.5. Pretreatment of VSMCs with U0126 or wortmannin inhibited bFGF-induced ERK or AKT phosphorylation in a dose-dependent manner

To understand if ERK and/or AKT pathways are essential for bFGF-induced VSMC proliferation and migration, our next experiments were designed to test if pretreatment of VSMCs with a selective ERK inhibitor, U0126, or a selective AKT inhibitor, wortmannin, blocks bFGF-induced phosphorylation of ERK or AKT. It is well known that U0126 has significantly high selectivity for activated ERK. Its binding affinity for the activated ERK is about 100-fold higher than PD098059, another ERK inhibitor (Favata et al., 1998). Wortmannin is a very potent and specific inhibitor for PI3 kinase/AKT (Huang et al., 2011) with IC50 of 4.2 nM, which is much lower than other inhibitors of PI3kinase, such as LY294002 (Walker et al., 2000). Wortmannin has been widely used to study the PI3K/AKT signaling transduction pathway (Arcaro and Wymann, 1993; Adi et al., 2001).

Cells were pretreated with different concentrations of U0126 (0.1, 1, or 10 μM) or wortmannin (0.1, 0.3, or 1 μM) for 1 h followed by stimulation with 15 ng/ml of bFGF for 5 min. As shown in Figs. 7 and 8, U0126 or wortmannin produced dose-dependent inhibition on bFGF-induced ERK phosphorylation or AKT phosphorylation respectively, with 1 μM of U0126 or 10 μM of wortmannin completely eliminated bFGF-induced phosphorylation of ERK or AKT respectively. Therefore, in the following experiments, we chose 1 μM of U0126 to block ERK signaling and 10 μM of wortmannin to block AKT signaling. The pretreatment time and

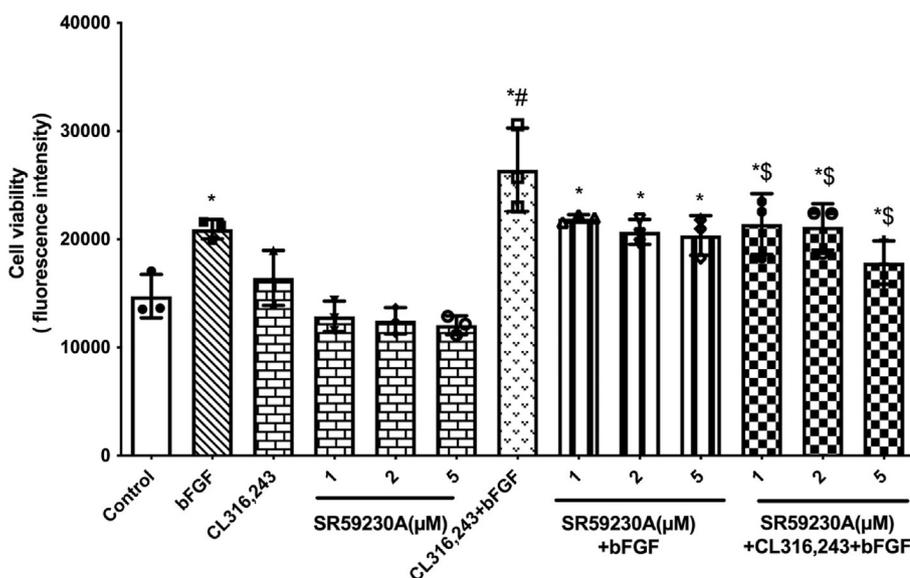


Fig. 3. SR59230A blocked the effect of CL316,243 on bFGF-mediated increase in cell viability. VSMCs were pretreated with 5 μM of SR59230A for 1 h followed by the treatment with 5 μM of CL316,243 (CL) in serum-free medium containing vehicle or SR59230A. 24 h later, the cells were stimulated with 15 ng/ml of bFGF for an additional 24 h in the presence and absence of CL316,243 and/or SR59230A. Cell viability was measured using resazurin and expressed as intensity of fluorescence (mean ± SD). Results are from three independent experiments. Data were analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. *p < 0.05, compared to control, #p < 0.05 compared to bFGF alone, §p < 0.05 compared to CL316,243+bFGF.

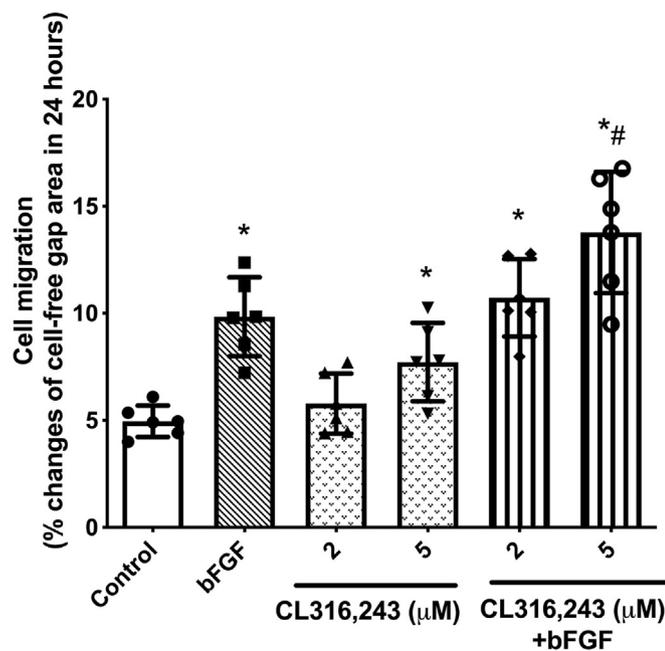


Fig. 4. CL316,243 enhanced bFGF-induced cell migration. VSMCs were pretreated with different concentrations of CL316,243 in serum-free medium for 24 h followed by a stimulation with 15 ng/ml of bFGF for 24 h in the presence and absence of the same concentrations of CL316,243. A cell-free area was created by using a 10 μ l pipette tip before the treatment with bFGF. The cell-free area was measured before and after the bFGF treatment. Cell migration is expressed as percentage changes of cell-free area in 24 h (mean \pm SD). Results are from six independent experiments. Data were analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. * $p < 0.05$, compared to control, # $p < 0.05$ compared to bFGF alone.

concentrations with U0126 and wortmannin was determined by following the published protocols (Nagayama et al., 2015) and manufacturer's recommendations.

3.6. Pretreatment of VSMCs with U0126 or wortmannin inhibited bFGF-induced VSMC migration

As shown in Fig. 9, bFGF alone induced more than a 50% increase in cell migration. Neither 1 μ M of U0126 nor 10 μ M of wortmannin alone had any effect on baseline cell migration. However, pretreatment of VSMCs with the same concentrations of U0126 or wortmannin completely blocked bFGF-induced cell migration.

3.7. Pretreatment of VSMCs with wortmannin inhibited bFGF-induced VSMC proliferation

As shown in Fig. 10, bFGF induced a drastic increase in cell proliferation. Wortmannin alone at the concentration of 10 μ M did not affect baseline cell proliferation. However, the agent at the same concentration significantly inhibited bFGF-mediated cell proliferation. In contrast, pretreatment of the VSMCs with 1 μ M of U0126 failed to alter bFGF-induced cell proliferation.

4. Discussion

The novel findings in our current study include: 1) β_3 -ARs were significantly upregulated in the neointima following vascular injury; 2) pretreatment of VSMC with CL316,243 significantly potentiated bFGF-induced cell migration and proliferation as well as phosphorylation of ERK and AKT; 3) inhibition of ERK signaling blocked bFGF-induced cell migration but not proliferation; 4) inhibition of AKT signaling

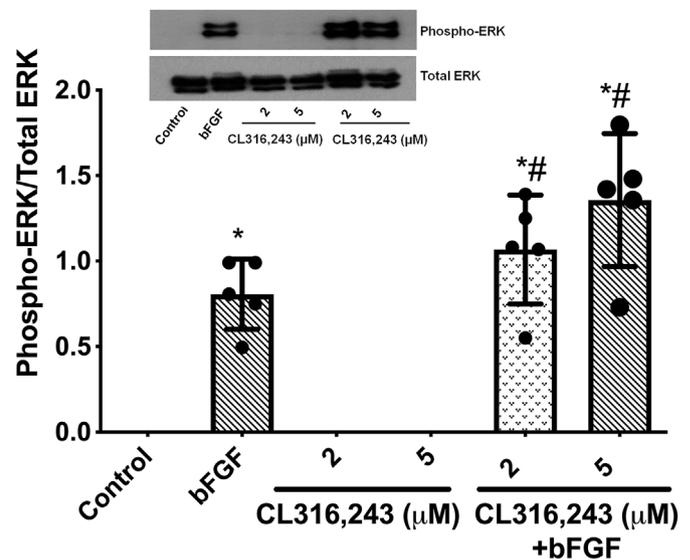


Fig. 5. CL316,243 potentiated bFGF-induced ERK phosphorylation in VSMCs. Cells were pretreated with different concentrations of CL316,243 in serum-free medium for 24 h followed by a stimulation with 15 ng/ml of bFGF for 5 min in the presence of the same concentrations of CL316,243. The levels of ERK phosphorylation were determined by Western blot analyses. The membranes were probed for total ERK, stripped, and re-probed for phospho-ERK. The top blot shows phospho-ERK and the bottom blot is the total ERK. Images were scanned and quantified with Image J software. Graphs show the summary of five independent experiments. Results are expressed as the ratio of phospho-ERK to total ERK levels (means \pm SD). Data were analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. * $p < 0.05$, compared to control, # $p < 0.05$ compared to bFGF alone.

significantly reduced bFGF-induced cell migration and proliferation.

Although it is well known that β_3 -ARs are expressed in the endothelial, vascular smooth muscle, and adventitia cells (Gauthier et al., 1996; Moniotte et al., 2001; Leblanc and Tabrizchi, 2018), the function of β_3 -ARs in vascular injury-induced neointima formation is not well understood. Previous studies revealed that nebivolol, a β_1 - and β_2 -AR antagonist with the properties of β_3 -AR receptor agonist modifies several vascular injury-related events, including inhibiting growth and inducing apoptosis of VSMCs, improving endothelial function, suppressing expression of inflammatory genes, and reducing vascular injury-induced neointima formation (Brehm et al., 2001; Ignarro et al., 2002; Wolf et al., 2007). Since these effects were not observed with selective β_1 - and β_2 -AR antagonists, it was concluded that they may be mediated by activation of β_3 -ARs and activation of β_3 -ARs may play a negative role in vascular injury-induced neointima formation. Others have argued that nebivolol is unlikely to significantly activate β_3 -ARs *in vivo* since it has a very low affinity for β_3 -ARs (about 500–1000 times weaker than that for β_1 -ARs and β_2 -ARs) and $\sim 98\%$ is bound to proteins in the plasma (Pauwels et al., 1988; Frazier et al., 2011). Moreover, since nebivolol is not a selective β_3 -AR agonist, its effects may not reflect the actual function of β_3 -ARs. In contrast to the findings about nebivolol, Kou et al. showed that a selective β_3 -AR antagonist, SR59230A completely abolishes endothelial cell migration induced by epinephrine (Kou and Michel, 2007), indicating an undefined role of β_3 -ARs in vascular injury-induced neointima formation. Our experiments revealed a significant upregulation of β_3 -AR expression in the newly formed intima following vascular injury, especially in the area close to the lumen, suggesting that β_3 -ARs may play a role in vascular injury-induced neointima formation.

To understand the role of the upregulated β_3 -ARs in vascular injury-induced neointima formation, we first tested the effects of CL316,243, a highly selective β_3 -AR agonist on bFGF-induced VSMC migration and proliferation, the two key components that contribute to vascular injury-induced neointima formation (Dartsch et al., 1990; Casscells, 1992;

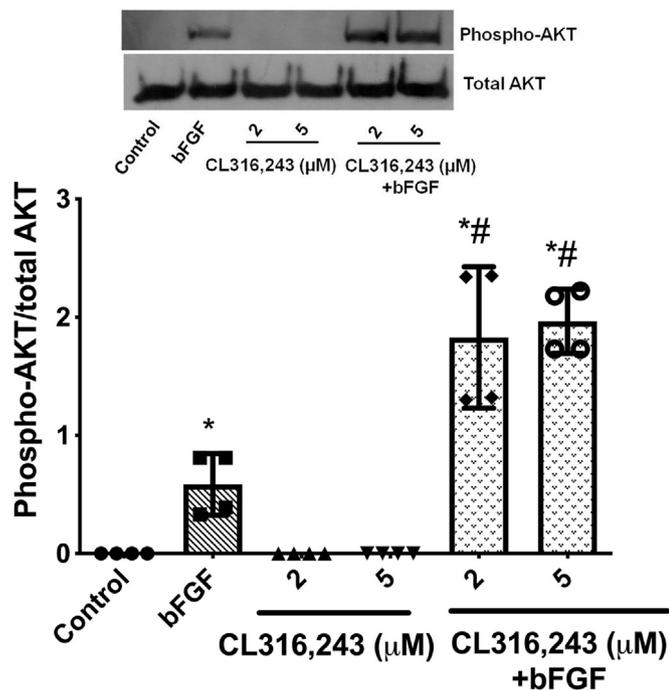


Fig. 6. CL316,243 enhanced bFGF-induced AKT phosphorylation in VSMCs. Cells were pretreated with different concentrations of CL316,243 in serum-free medium for 24 h followed by the stimulation with 15 ng/ml of bFGF for 5 min in the presence or absence of the same concentrations of CL316,243. The levels of AKT phosphorylation were determined by Western blot analyses. The membranes were probed with antibody directed against phospho-AKT, stripped, and reprobated for total AKT. The top blot shows phospho-AKT and the bottom blot is the total AKT. Images were scanned and quantified with Image J software. Graphs show the summary of four independent experiments. Results are expressed as the ratio of phospho-AKT to total AKT levels (means \pm SD). Data were analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. * $p < 0.05$, compared to control, # $p < 0.05$ compared to bFGF alone.

Owens et al., 2004; Kawai-Kowase and Owens, 2007; Marx et al., 2011). We found that pretreatment of VSMCs with CL316,243 substantially potentiated bFGF-induced cell proliferation and migration, suggesting that CL316,243 produces its effects through interaction with a bFGF-activated pathway. To confirm if these effects are in fact mediated by activation of β_3 -ARs, we tested if blocking β_3 -ARs with a selective β_3 -AR antagonist, SR59230A, eliminates the effects of CL316,243 on bFGF-induced cell proliferation. SR59230A has ~10-fold higher selectivity for β_3 -ARs compared to that for other β -ARs (Nisoli et al., 1996). Several studies revealed that SR59230A antagonizes the β_3 -AR-mediated cAMP accumulation, glycerol release, gene expression in rat brown fat cells, and reduces lipid mobilizing factor-induced lipolytic response in mouse white adipocytes (Nisoli et al., 1996; Russell et al., 2002). Here, we have shown that the effect of CL316,243 on bFGF-induced cell proliferation was completely abolished by SR59230A (Fig. 3), indicating that CL316,243 potentiates bFGF-mediated effects through activation of β_3 -ARs. The data provide the evidence of an existing interaction between β_3 -ARs and bFGF for the first time. Our results suggest that upregulation of β_3 -ARs may participate in the pathogenesis of vascular injury-induced neointima formation by potentiating bFGF-stimulated VSMC proliferation and migration. These findings are opposite to the conclusion from nebulivolol's effects on VSMCs and vascular injury-induced neointima formation documented by Wolf et al. (Wolf et al., 2007) but consistent with the endothelial response to the β_3 -AR antagonist reported by Kou et al. (Kou and Michel, 2007). The identification of agonistic effects of β_3 -ARs on bFGF provides new insights into the pathogenesis of vascular injury-induced neointima formation.

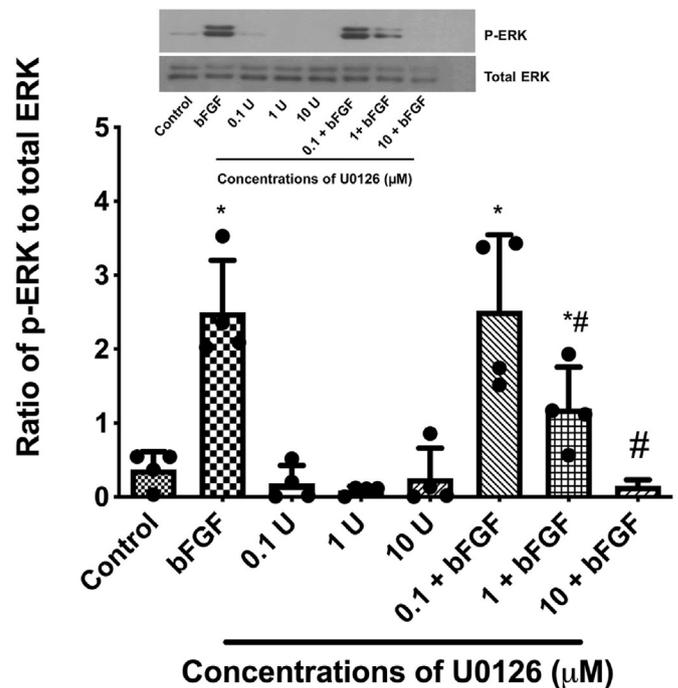


Fig. 7. U0126 inhibited bFGF-induced ERK phosphorylation in VSMCs in a dose-dependent manner. Cells were pretreated with different concentrations of U0126 for 1 h followed by a stimulation with 15 ng/ml of bFGF for 5 min in the presence or absence of the same concentrations of U0126. The levels of ERK phosphorylation were determined by Western blot analyses. The top blot shows phospho-ERK and the bottom blot is the total ERK. Images were scanned and quantified with Image J software. Graphs show the summary of four independent experiments. Results are expressed as the ratio of phospho-ERK to total ERK levels (means \pm SD). Data were analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. * $p < 0.05$, compared to control, # $p < 0.05$ compared to bFGF alone.

Although our results revealed that pretreatment of VSMCs with CL316,243 significantly enhanced bFGF-induced ERK and AKT phosphorylation, the two major pathways that are involved in bFGF-mediated effects, it is not clear if ERK and AKT phosphorylation are essential for bFGF-induced cell migration and proliferation and augmentation of bFGF-induced cell migration and proliferation by activation of β_3 -ARs is the consequence of enhanced ERK and/or AKT phosphorylation. By using selective ERK and AKT inhibitors, we discovered that blockade of ERK or AKT phosphorylation significantly inhibited bFGF-induced VSMC migration, suggesting that both pathways are involved in bFGF-induced cell migration and that activation of β_3 -ARs enhances bFGF-induced cell migration by potentiating bFGF-induced ERK and AKT phosphorylation. Our data show that only blocking phosphorylation of AKT but not ERK inhibited bFGF-induced cell proliferation, indicating that AKT pathway may be the major contributor to bFGF-induced cell proliferation and that activation of β_3 -AR enhances bFGF-induced cell proliferation by augmenting bFGF-induced AKT phosphorylation. Our results do not support the involvement of ERK pathway in bFGF-induced cell proliferation as previously thought, at least in our model.

5. Conclusions

To our knowledge, this is the first evidence showing that β_3 -ARs are upregulated in injured blood vessels and that activation of β_3 -ARs potentiates bFGF-induced vascular smooth muscle cell migration and proliferation by interacting with bFGF signaling transduction pathways. Our data suggest that upregulation of β_3 -ARs in injured blood vessels may participate in vascular injury-induced neointimal thickening by

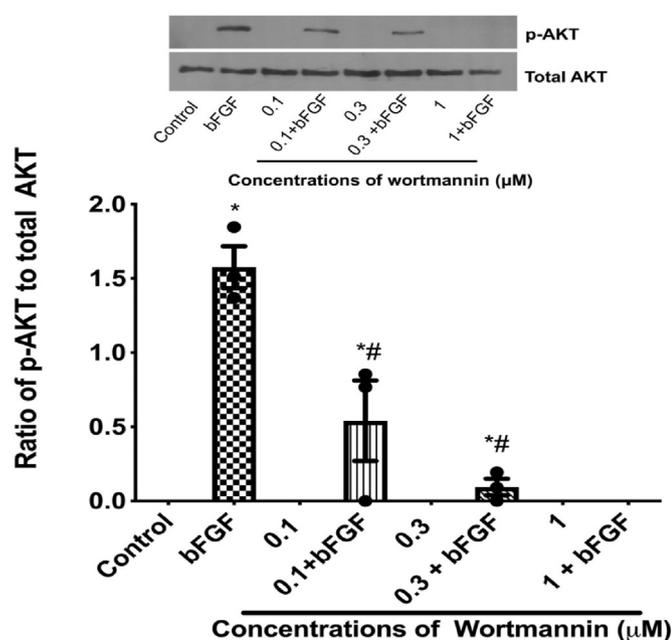


Fig. 8. Wortmannin inhibited bFGF-induced AKT phosphorylation in VSMCs in a dose-dependent manner. Cells were pretreated with different concentrations of wortmannin for 1 h followed by a stimulation with 15 ng/ml of bFGF for 5 min in the presence or absence of the same concentrations of wortmannin. The levels of AKT phosphorylation were determined by Western blot analyses. The top blot shows phospho-AKT and the bottom blot is the total AKT. Images were scanned and quantified with Image J software. Graphs show the summary of three independent experiments. Results are expressed as the ratio of phospho-AKT to total AKT levels (means \pm SD). Data were analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. * $p < 0.05$, compared to control, # $p < 0.05$ compared to bFGF alone.

potentiating bFGF-induced cell migration and proliferation via enhancing the phosphorylation of ERK and AKT, the two major signaling transduction pathways involved in bFGF-related effects.

Exactly how β_3 -AR activation potentiates bFGF-induced phosphorylation of ERK and AKT is not understood. One possibility is through modulating the expression or activity of phosphatases that are involved in the dephosphorylation of ERK and AKT. It is known that PI3K/AKT is negatively regulated by the lipid phosphatase called phosphatase and tensin homolog (PTEN). PTEN dephosphorylates phosphatidylinositol 3, 4, 5-triphosphate, reduces the activity of PI3K, and downregulates the signal of its downstream target, AKT (Berchuck and Boyd, 1995; Mae-hama and Dixon, 1998; Stambolic et al., 1998; Di Cristofano and Pandolfi, 2000; Kanamori et al., 2001). ERK-mediated signaling is negatively regulated by an ERK-specific phosphatase, named MAP kinase phosphatase 3 (MKP₃) (Muda et al., 1996; Camps et al., 1998; Farooq and Zhou, 2004; Tsang and Dawid, 2004; Buffet et al., 2017). MKP₃ dephosphorylates the phosphorylated ERK and subsequently inhibits ERK-mediated effects (Keyse, 2000). Future studies investigating the mechanisms by which β_3 -AR activation potentiates bFGF-induced effects on vascular smooth muscle cells are warranted.

Although our current results indicate that β_3 -ARs likely plays a role in vascular injury-induced intima thickening, there is no direct *in vivo* evidence to validate the results. Further studies exploring the role of β_3 -ARs in vascular injury-induced intima thickening via using a selective β_3 -AR antagonist or a selective β_3 -AR agonist are required. If blockade of β_3 -ARs reduces or activation of β_3 -ARs enhances vascular injury-induced intima thickening, it would validate our *in vitro* data and provide evidence that

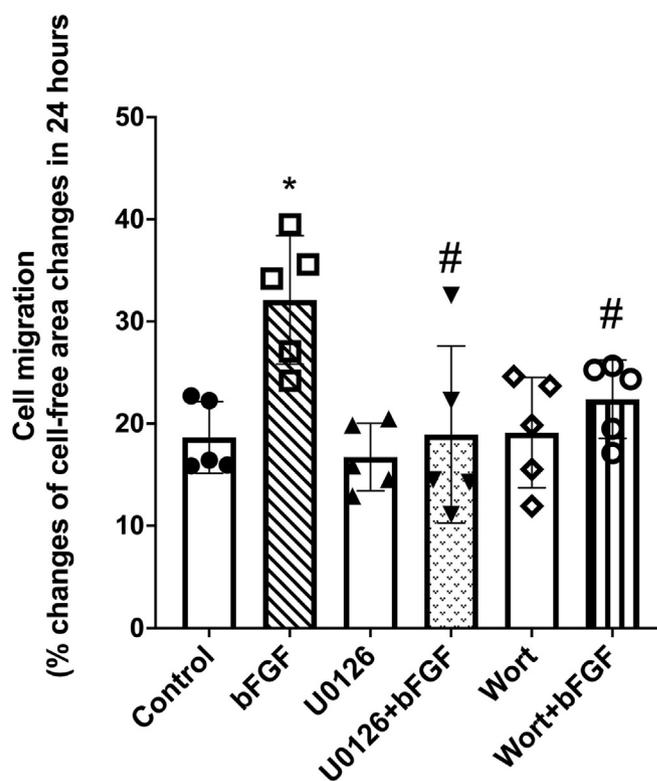


Fig. 9. Pretreatment VSMCs with U0126 and wortmannin blocked bFGF-induced cell migration. VSMCs were pretreated with 1 μ M of U0126 or 10 μ M of wortmannin (wort) in serum-free medium for 1 h followed by a stimulation with 15 ng/ml of bFGF for 24 h in the presence or absence of the same concentrations of U0126 or wortmannin. A cell-free area was created by using a 10 μ l pipette tip before the treatment with bFGF. The cell-free area was measured before and after the bFGF treatment. Cell migration is expressed as percentage changes of cell-free area in 24 h (mean \pm SD). Graphs show the summary from five independent experiments. Data were analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. * $p < 0.05$, compared to control, # $p < 0.05$ compared to bFGF alone.

selective β_3 -AR antagonists may help to slow or prevent intima thickening and, thus, vascular restenosis in patients who underwent angioplasty.

Conflict of interest

There are no conflicts of interest.

CRediT authorship contribution statement

Yingzi Chang: Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Validation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Lei Alena Dagat:** Data curation, Validation. **Aisha Yusuf:** Data curation, Validation. **Yusuf Zahriya:** Data curation, Validation. **Kotryna Stapu-tyte:** Data curation, Validation. **Emma Worley:** Data curation, Validation. **Alex Holt:** Data curation, Validation. **Natalie Canuteson:** Data curation, Validation. **Vereena Messieha:** Data curation, Validation. **Kasey Halila:** Data curation, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial

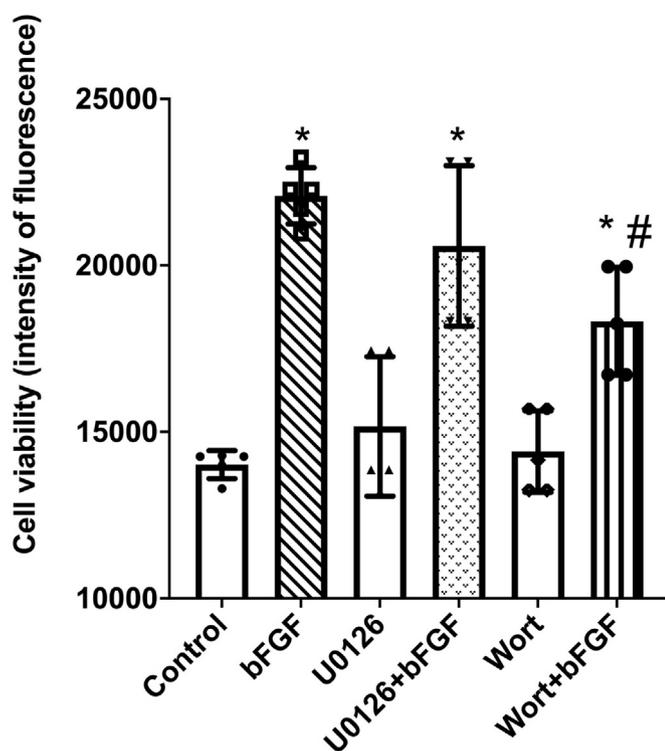


Fig. 10. Pretreatment VSMCs with wortmannin but not U0126 reduced on bFGF-mediated cell proliferation. VSMCs were pretreated with 1 μ M of U0126 or 10 μ M of wortmannin (Wort) for 1 h followed by a treatment with 15 ng/ml of bFGF for 24 h in the presence or absence of U0126 or wortmannin. Cell viability was measured using resazurin and expressed as intensity of fluorescence (mean \pm SD). Results are from five independent experiments. Data were analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. * p < 0.05, compared to control, # p < 0.05 compared to bFGF alone.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Mr. Michael Cramberg and Dr. Byunghee Han for their technical assistance and Dr. Keith Elmslie for his manuscript editing. This study was funded by the A.T. Still University Warner/Ferumatturo Grant.

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