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Mechanisms of nebivolol-mediated effects on bFGF-induced vascular smooth muscle cell proliferation and migration

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A B S T R A C T

Background: Nebivolol is a β-adrenergic receptor antagonist that has intrinsic activity on β_3-adrenergic receptors (β_3-ARs). Previous studies suggest that nebivolol inhibits bFGF-induced vascular smooth muscle cell (VSMC) proliferation and migration and vascular injury-induced neointima formation through activation of β_3-ARs. However, our recently published data shown that activation of β_3-ARs produced the opposite results, suggesting that the mechanisms of nebivolol-mediated effects are not fully understood. The current project was to study the mechanisms of nebivolol's effects on bFGF-induced VSMC proliferation and migration by comparing to the selective β_3-AR agonist, CL316,243.

Methods: VSMCs isolated from Sprague Dawley rat aortas were pretreated with nebivolol or CL316,243 followed by stimulation with bFGF. Cell proliferation and migration and phosphorylation of ERK and AKT were measured.

Results: We found that pretreatment of VSMCs with nebivolol produced biphasic effects on bFGF-induced VSMC proliferation, manifested as potentiation at lower concentrations and inhibition at the higher concentration. The effects of low concentrations of nebivolol on bFGF-induced VSMC proliferation was blocked by the selective β_3-AR antagonist, SR59230A. Nebivolol inhibited bFGF-induced cell migration at all concentrations tested. In addition, only higher concentrations of nebivolol significantly inhibited bFGF-induced AKT phosphorylation but not ERK phosphorylation whereas CL316,243 at all concentrations tested significantly enhanced bFGF-induced VSMC proliferation and migration and higher concentrations of CL316,243 not only enhanced bFGF-induced AKT phosphorylation but also ERK phosphorylation.

Conclusion: Our data suggest that the effect of nebivolol on bFGF-induced cell proliferation is concentration-dependent. The enhancement on bFGF-induced cell proliferation at lower concentrations appears to be mainly mediated by activation of β_3-ARs but the inhibitory effects on bFGF-mediated cell proliferation as well as migration may occur through different mechanisms. AKT signaling is only involved in high concentrations of nebivolol-mediated effects.

1. Introduction

Coronary angioplasty is a least invasive procedure that is used to re-open a blocked coronary artery caused by atherosclerosis. A major drawback of the procedure is development of restenosis (Popma et al., 1991), which is mainly caused by upregulation of growth factors and inflammatory mediators, leading to migration and proliferation of vascular smooth muscle cells (VSMC) and subsequent neointima thickening (Ross, 1993; Lai et al., 1996). Basic fibroblast growth factor (bFGF) is one of the major growth factors involved in this process by stimulating downstream mitogen-activated protein (MAP) kinases and Protein kinase B (AKT) pathways (Chang et al., 2022a).

bFGF produces its effects by binding to its specific receptors (FGF receptors) (Beenken et al., 2009), triggering receptor phosphorylation and further binding to the adaptor proteins. The interaction activates MAPK and AKT pathways (Wong et al., 2002; Lax et al., 2002). The activated MAPK and/or AKT translocate to the nucleus, stimulating the expression of genes associated with cell proliferation and migration. This process induces smooth muscle cell phenotype switch from contractile to proliferative and changes of cell polarity, leading to cell proliferation and migration (Lax et al., 2002; Dailey et al., 2005; Muto et al., 2007). Previous studies revealed that blockade of MAPK and AKT pathways inhibits bFGF-induced vascular smooth muscle cell proliferation and migration (Chang et al., 2022a) as well as vascular injury-induced intimal hyperplasia (Pan et al., 2003), suggesting that bFGF-induced activation of MAPK and AKT pathway is involved in vascular injury-induced vascular restenosis.

β_3-adrenergic receptors (β_3-ARs) are part of the G protein-couple receptor (GPCR) family that are found in many systems and tissues, such as adipose tissue and SMC of the detrusor muscle (Granneman et al., 1992; Lelias et al., 1993; Strosberg et al., 1996; Coman et al., 2009). In cardiovascular system, β_3-ARs have significant roles in heart

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function, vascular tone, and endothelial function – especially in cases of heart failure (Gauthier et al., 2007). β_3-ARs are expressed at low levels in healthy myocardial tissue, but are upregulated in heart failure. Activation of β_3-ARs in the myocardial tissues of heart failure has a negative inotropic effect that involves NO-dependent pathway. This increased activity of β_3-ARs serves as a protective mechanism in the case of heart failure where sympathetic activity is high (Gauthier et al., 2007). Notably, β_3-ARs can also reduce vascular smooth muscle tone and induce vasodilation through eNOS/GMP signaling (Michel et al., 2020; Moens et al., 2010). Our previous studies found that β_3-ARs are highly expressed in neointima following vascular injury and activation of β_3-ARs with the selective receptor agonist, CL316,243, potentiated bFGF-mediated VSMC migration and proliferation (Chang et al., 2022a), suggesting that β_3-ARs may play a role in vascular injury-induced neointima formation and subsequent restenosis.

Nebivolol is a β_1-selective antagonist with β_3-AR agonistic effects. Previous studies found that nebivolol inhibited proliferation of human coronary artery smooth muscle cells both alone and in the presence of mitogens and long-term use of nebivolol reduced vascular injury-induced neointima formation. Since this inhibitory effect was not observed with metoprolol or bisoprolol (β_1-selective antagonists) alone, nor was it affected by a non-selective β-AR antagonist, suggesting that β_3-AR agonism was involved (Brehm et al., 2001). Because the results are conflicted with the one that we observed with the selective β3-AR agonist CL316,243, it indicates that mechanisms of nebivolol's effects on VSMC proliferation and migration and neointima formation are not well defined and may be more complicated.

The purpose of this study was to study the mechanism of nebivolol in affecting vascular smooth muscle cell proliferation and migration and possible involvement of β_3-ARs by comparing to the selective β_3-AR agonist, CL316,243.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), DMEM/F12, penicillin/streptomycin, and amphotericin B were purchased from Thermofisher (Waltham, MA). Porcine pancreatic collagenase type I and elastase were purchased from Worthington (Lakewood, NJ). 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). Male Sprague-Dawley rats were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). CL316,243, nebivolol, and SA59230A were purchased from Cayman Chemicals (Ann Arbor, MI). All other reagents were obtained from Sigma Aldrich (St. Louis, MO).

2.2. Cell culture

VSMCs were previously isolated from the thoracic aortae of 100–125 g male Sprague-Dawley rats by enzymatic dissociation following the published procedure (Brown et al., 1999). The rationale of using VSMCs isolated from male Sprague-Dawley rats was based on the following findings: 1) the prevalence of coronary atherosclerosis is higher in men than that in women (Benjamin et al., 2019), 2) β_3-ARs are upregulated in male carotid artery injury model and activation of β_3-ARs potentiates bFGF-induced VSMC (isolated from male Sprague-Dawley rats) proliferation and migration (Chang et al., 2022b). Cells were grown in DMEM/F12 containing 10% of fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in a cell culture humidified incubator with 37 °C, 95% air, and 5% CO_2. The experiments were carried out with cells from passages 10–15 to mimic the morphological and functional phenotype that occur in neointima after vascular injury (Schwartz et al., 1995; Bochaton-Piallat et al., 1996; Chamley-Campbell et al., 1981; Hedin et al., 1991; Majesky et al., 1992). The aortic smooth muscle cell isolation 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. Cell migration assay

Cell migration was measured by a wound healing assay (Chang et al., 2006). The initial cell-free area or "wound" was created by a scratch with a 100 μl sterile pipette tip, and the pictures of cell-free area were captured before and after 24-h treatment with bFGF using a microscope connected to a digital camera (Scion Corporation, Frederick, MD). The cell-free areas were measured using Image J software (NIH). Cell migration is expressed as percentage changes of the cell-free area after 24-h treatment with bFGF. 5 μM of hydroxyurea was used during the 24-h treatment with bFGF to prohibit cell proliferation (Rodriguez et al., 2005).

2.4. Cell proliferation

Cell proliferation was measured by using resazurin, an indirect method to measure cell proliferation by monitoring viable cell number (Chang et al., 2022a; Riss et al., 2004; Wolf et al., 2012; Ivanov et al., 2014; Ren et al., 2015). Resazurin is reduced to resorufin, a fluorescent product, by live cells. The intensity of fluorescence reflects the number of live cells. Briefly, following the treatment, cells were incubated with medium containing 0.5% FBS and 15 μg/ml resazurin for 2 h and the fluorescence intensity was measured at Excitation of 530/30 nm and Emission of 590/20 nm.

2.5. ERK and AKT phosphorylation

ERK and AKT phosphorylation were measured by Western blot analysis. Briefly, 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). Proteins were separated with 10% polyacrylamide gel electrophoresis (PAGE) and probed with antibodies against phospho-ERK or AKT (Ser 473). Membranes were stripped and reprobed with antibodies directed against total ERK or AKT respectively. 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 respectively. 1:1000 dilutions of antibodies were used to detect phospho-ERK, total ERK, and total AKT and 1:500 dilution of the antibody was used to detect phospho-AKT.

2.6. Statistical analysis

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

3. Results

3.1. Pre-treatment of VSMCs with nebivolol produced biphasic effects on bFGF-induced cell proliferation

Five concentrations (0.1, 0.3, 1, 3, and 10 μM) of nebivolol were utilized to test the dose-response effects on bFGF-induced smooth muscle cell proliferation. The cells were pretreated with different concentrations of nebivolol for 24 h in DMEM/F12 medium containing 0.5% FBS followed by stimulation with 5 ng/ml of bFGF for 24 h in the presence or absence of the same concentrations of nebivolol. Our preliminary data (not shown here) revealed that bFGF induced cell proliferation dose-dependently. 5 ng/mL was chosen for subsequent

experiments since this concentration induced the response significantly, but not so much that it would veil other treatments' effects. Treatment with nebivolol alone at low concentrations (0.1 μM and 0.3 μM) for 24 h significantly increased cell proliferation compared to the control (25%). Pretreatment with low concentrations of nebivolol increased bFGF-induced cell proliferation in an additive manner, whereas higher concentrations of nebivolol, most notably, 3 and 10 μM decreased bFGF-induced cell proliferation (manifested as a 17% decrease for 3 μM and a 33% decrease for 10 μM respectively when compared to bFGF alone) (Fig. 1).

3.2. Pre-treatment of VSMCs with CL316,243, a selective β_3-AR agonist, potentiated bFGF-induced cell proliferation

Five concentrations of CL316,243 were utilized: 0.1, 0.3, 1, 3, and 10 μM. Alone, treatment with CL316,243 slightly but not significantly increased cell proliferation compared to the control. When cells were pre-treated with CL316,243 at all concentrations tested, the proliferation was significantly potentiated over bFGF alone. No inhibition of cell proliferation was seen, even at the highest concentration of CL316,243 tested (Fig. 2). The biphasic results seen with nebivolol were not observed with CL316,243. This suggests that nebivolol may be acting through different mechanisms than CL316,243 in affecting bFGF-induced cell proliferation, at least partly at higher concentrations.

3.3. Pre-treatment of VSMCs with nebivolol inhibited bFGF-induced cell migration; pre-treatment of VSMCs with CL316,243 potentiated bFGF-induced cell migration

A concentration of 0.3 μM was used for both nebivolol and CL316,243 for the cell migration experiments. This concentration was chosen for both nebivolol and CL316,243 since they were shown to increase bFGF-induced cell proliferation in a moderate manner mentioned above.

As shown in Fig. 3, 5 ng/mL of bFGF alone was shown to significantly increase cell migration. Both nebivolol and CL316,243 alone at the concentration of 0.3 μM showed no significant effect on cell migration. Pretreatment of VSMCs with 0.3 μM nebivolol significantly inhibited bFGF-induced cell migration, manifested as a 38% reduction when compared to bFGF treatment alone. In contrast to the results observed from nebivolol, pretreatment of VSMCs with 0.3 μM CL316,243 potentiated bFGF-induced cell migration, manifested as a 13 % increase when compared to bFGF treatment alone. These findings suggest that nebivolol may be acting through different mechanisms when compared to CL316,243 in affecting bFGF-induced cell migration.

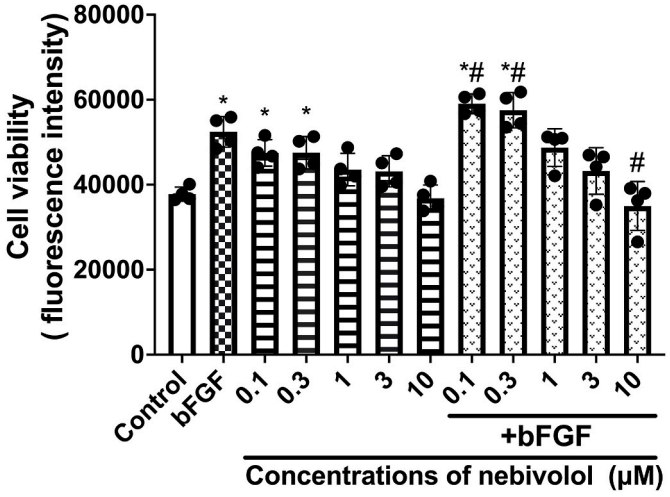

Fig. 1. Nebivolol produced biphasic effects on bFGF-induced vascular smooth muscle cell proliferation. VSMCs were pre-treated with the indicated concentration of nebivolol (0.1, 0.3, 1, 3, or 10 μM) in DMEM/F12 containing 0.5% FBS for 24 h, followed by stimulation with bFGF (5 ng/mL) for 24 h in the presence or absence of the same concentrations of nebivolol. A resazurin assay was used to measure cell proliferation, and results are reported as intensity of fluorescence (mean ± SD). Data are from 4 independent experiments and were analyzed using one-way ANOVA followed by Turkey Multiple Comparison Test. *p < 0.05; compared to control; #p < 0.05, compared to bFGF alone.

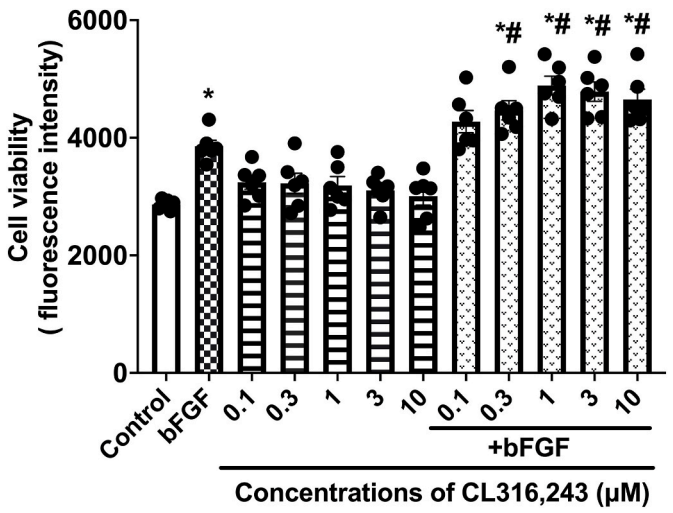

Fig. 2. CL316,243 potentiated bFGF-induced cell proliferation. VSMCs were pre-treated with the indicated concentration of CL316,243 (0.1, 0.3, 1, 3, or 10 μM) in DMEM/F12 containing 0.5% FBS for 24 h, followed by stimulation with bFGF (5 ng/mL) for 24 h in the presence or absence of the same concentrations of CL316,243. A resazurin assay was used to measure cell proliferation, and results are reported as intensity of fluorescence (mean ± SD). Data are from 6 independent experiments and were analyzed using one-way ANOVA followed by Turkey Multiple Comparison Test. *p < 0.05; compared to control, #p < 0.05, compared to bFGF alone.

3.4. Lower concentrations of nebivolol and CL316,243 failed to alter bFGF-induced phosphorylation of ERK and AKT

Previous studies shown that 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). Our published data suggest that activation of ERK and AKT signaling pathway is involved in high concentrations (2 and 5 μM) of CL316,243-mediated effects on bFGF-induced VSMC proliferation and migration (Chang et al., 2022a). To understand if low concentrations of nebivolol and CL316,243 activates the same pathways in increasing bFGF-induced cell proliferation, we tested the effects of low concentrations of nebivolol and CL316,243 on bFGF-induced ERK and AKT phosphorylation. Cells were pretreated with 0.3 μM of nebivolol or CL316,243 for 24 h followed by a stimulation with 5 ng/ml of bFGF for 10 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 and lasted until 30 min. As shown in Fig. 4A and B, neither nebivolol nor CL316,243 at the concentration tested showed any effects on bFGF-induced phosphorylation of ERK or AKT, suggesting that both signaling pathways may not be involved in the cellular functional changes caused by low concentrations of nebivolol and CL316,243, although both nebivolol and CL316,243 at the concentration tested shown an increase in bFGF-induced cell proliferation.

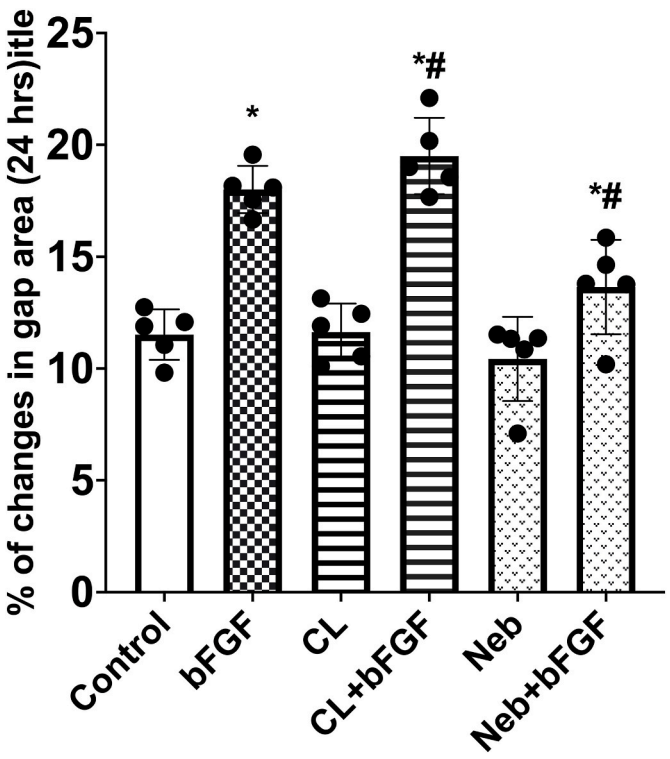

Fig. 3. Nebivolol inhibited bFGF-induced vascular smooth muscle cell migration, whereas CL316,243 potentiated bFGF-induced vascular smooth muscle cell migration. VSMCs were pretreated with 0.3 μM of nebivolol (Neb) or CL316,243 (CL) in DMEM/F12 containing 0.5% FBS for 24 h. A cell-free area was created using a 100 μl pipette tip. Cells were stimulated with bFGF (5 ng/mL) in the presence or absence of the same concentrations of nebivolol or CL316,243 for additional 24 h. Photos of the cell-free areas were taken before and after bFGF treatment, and a cell free area was measured. Results are expressed in % change of cell-free area (mean ± SD) after 24 h of bFGF treatment. Data are from 5 independent experiments and were analyzed using one-way ANOVA followed by Turkey Multiple Comparison Test. *$p < 0.05$, compared to control; #$p < 0.05$, compared to bFGF alone.

3.5. SR59230A, a selective β_3-AR antagonist, blocked low concentrations of nebivolol-mediated effect in cell proliferation

Since low concentrations of nebivolol produced the similar effects on bFGF-induced cell proliferation as CL316,243, our next experiment was designed to test if the results observed from the low concentrations of nebivolol occurs through activation of β_3-ARs. We pretreated the VSMCs with different concentrations of the selective β_3-AR antagonist, SR59230A in the presence or absence of nebivolol followed by stimulation with bFGF, to test if SR59230A blocks the effect of low concentration of nebivolol on bFGF-induced cell proliferation. As shown in Fig. 5, SR59230A alone did not have significant effect on cell proliferation but blocked the effect of nebivolol on bFGF-induced cell proliferation at the concentrations of 1 and 3 μM, suggesting that activation of β_3-ARs is involved in the effect of low concentrations of nebivolol on bFGF-induced cell proliferation.

3.6. bFGF-induced cell migration was inhibited by high concentrations of nebivolol but enhanced by high concentrations of CL316,243

Since the lower concentrations of nebivolol produced opposite effects on bFGF-induced cell migration as compared to CL316,243, our next experiments were to test if the same trend occurs to higher concentrations of both agents. As shown in Fig. 6A and B, nebivolol at 3 μM alone did not affect VSMC migration whereas 10 μM of nebivolol inhibited VSMC migration. Nebivolol at both concentrations suppressed bFGF-induced VSMC migration, which is similar to the results observed at lower concentration (0.3 μM). In contrast to the data obtained from nebivolol, high concentrations of CL316,243 produced opposite effect on bFGF-induced cell migration, manifested as potentiation of bFGF-induced cell migration, which is consistent with the results observed from low concentration of CL316,243 (0.3 μM). Together, these data suggest that the effect of nebivolol, at both low or high concentrations, on bFGF-induced cell migration may not occur through activation of β_3-ARs.

3.7. Higher concentrations of nebivolol inhibited bFGF-induced AKT phosphorylation but not ERK phosphorylation, whereas higher concentrations of CL316,243 enhanced bFGF-induced phosphorylation of both ERK and AKT

To understand the mechanisms of higher concentrations of nebivolol's effects on bFGF-induced cellular functional changes, we tested if ERK and AKT signaling pathways are involved. As shown in Figs. 7A and 8A, high concentrations of nebivolol (3 and 10 μM) inhibited bFGF-induced AKT phosphorylation but not ERK phosphorylation, suggesting the only AKT pathway may be involved. Consistent with our previous findings, higher concentrations of CL316,243 enhanced bFGF-induced phosphorylation of both ERK and AKT (Figs. 7B and 8B). Together, these data suggest that the mechanisms of high concentrations of nebivolol on bFGF-induced cellular functional changes are more complicated, possibly through AKT but not ERK pathway.

4. Discussion

The purpose of this project was to gain a deeper understanding of the mechanism of Nebivolol and its potential β_3-AR-mediated effects on VSMC migration and proliferation. This was accomplished by comparing the effects of nebivolol with CL316,243, a selective β3-AR agonist, in the presence and absence of bFGF. The main findings include: 1) Pretreatment with nebivolol produced dose-dependent biphasic effects on bFGF-induced cell proliferation, manifested as potentiation at low concentrations and inhibition at high concentrations, 2) nebivolol at all concentrations tested inhibited bFGF-induced cell migration, 3) higher concentrations of nebivolol inhibited bFGF-induced AKT phosphorylation but not ERK phosphorylation.

Understanding the mechanism leading to restenosis after angioplasty, or other vascular injury, is vital to increase the chance of prevention and ensure long-term success of vascular intervention procedures. The main risk factor for restenosis after angioplasty is increased proliferation and migration of vascular smooth muscle cells caused by the vascular injury-induced inflammatory response and upregulation of growth factors (Lindner et al., 1991; Gori, 2022).

Previous studies suggest that nebivolol has inhibitory effects on VSMC proliferation via β_3-AR agonism. Brehm et al. showed that nebivolol inhibits proliferation of human coronary artery smooth muscle cells both alone and in the presence of mitogens. This inhibitory effect was not seen with metoprolol or bisoprolol (β_1-AR selective antagonists) alone, nor was it affected by a non-selective β-AR antagonist, implying possible β_3-AR agonism involvement (Brehm et al., 2001). Others found that pretreatment with nebivolol significantly reduces vascular injury-induced neointima formation in rat carotid injury model and decreases the expression of growth factors in human coronary artery smooth muscle cell and endothelial cells (Wolf et al., 2007). However, our published data shown that vascular injury upregulated β_3-ARs in neointima and treatment of VSMCs with CL316,243, a selective β_3-AR, potentiated bFGF-induced proliferation and migration, suggesting that β_3-AR activation may play a role in potentiating vascular injury-induced neointima formation (Chang et al., 2022a) and the inhibitory effects of nebivolol on VSMC proliferation and migration observed by the others may not occur through β_3-AR agonism.

Our current study found that nebivolol's effects on VSMC

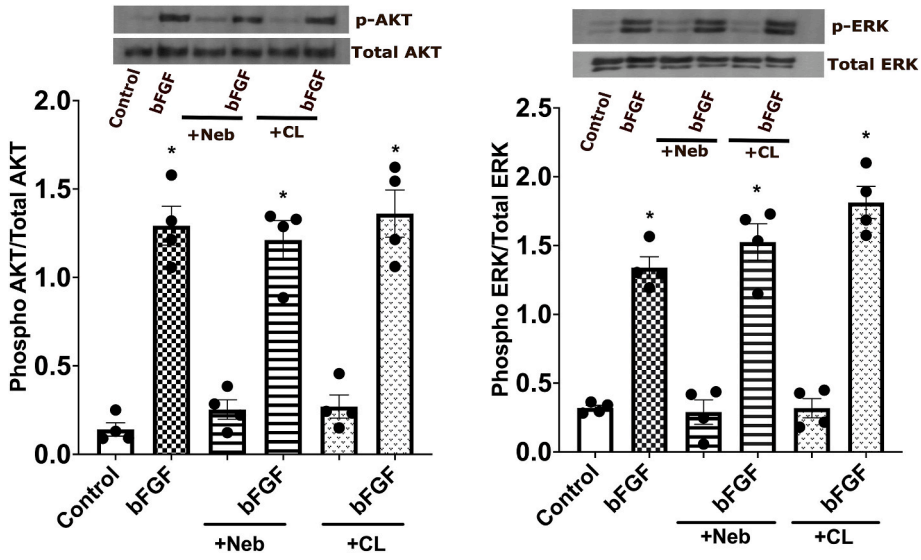

Fig. 4. Lower concentrations of nebivolol and CL316,243 failed to alter bFGF-induced ERK and AKT phosphorylation. Cells were pretreated with 0.3 μM of nebivolol (Neb) or CL316,243 (CL) in DMEM/F12 containing 0.5% FBS for 24 h followed by a stimulation with 5 ng/ml of bFGF for 10 min in the presence or absence of the same concentrations of nebivolol or CL316,243. The levels of ERK or AKT phosphorylation were determined by Western blot analyses. The membranes were stripped and reprobed for total ERK or AKT. The top blot shows phospho-ERK (p-ERK) or AKT (p-AKT) and the bottom blot is the total ERK or 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-ERK (AKT) to total ERK (AKT) levels (means ± SD). Data are from 4 independent experiments and were analyzed by using one-way ANOVA followed by Turkey Multiple Comparison Test. *$p < 0.05$, compared to control.

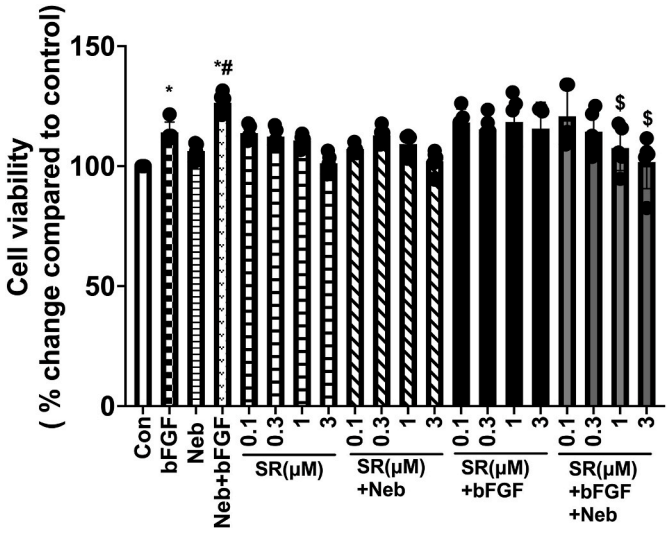

Fig. 5. SR59230A blocked the effect of low concentration of nebivolol on bFGF-mediated increase in cell viability. VSMCs were pretreated with indicated concentrations of SR59230A (SR) for 1 h followed by the treatment with or without 0.3 μM of nebivolol (Neb) in DMEM/F12 containing 0.5% FBS in the presence or absence of same concentrations of SR59230A for 24 h. The cells were then stimulated with 5 ng/ml of bFGF for an additional 24 h in the presence and absence of same concentrations of nebivolol and/or SR59230A. A resazurin assay was used to measure cell proliferation and results are expressed as intensity of fluorescence (mean ± SD). Data are from 4 independent experiments and were analyzed by using one-way ANOVA followed by Turkey Multiple Comparison Test. *$p < 0.05$, compared to control, #$p < 0.05$ compared to bFGF alone, $$p < 0.05$ compared to the combination of nebivolol and bFGF.

proliferation are concentration-dependent, producing biphasic effects on bFGF-induced cell proliferation. At lower concentrations, nebivolol increased bFGF-induced VSMC proliferation, which is consistent with the results observed in cells treated with CL316,243, and blocked by a selective β_3-AR antagonist, SR52390A, suggesting that it occurs through activation of β_3-ARs. In contrast to the results observed with lower concentrations of nebivolol, higher concentrations of nebivolol significantly inhibited bFGF-induced cell proliferation, which is opposite to the results observed from CL316,243, indicating that different mechanism(s) may be involved.

Consistent with the findings from the others (Brehm et al., 2001), we found that nebivolol inhibited bFGF-induced migration at all concentrations tested. In contrast, CL316,243 at all concentrations tested showed significant potentiation on bFGF-induced cell migration, suggesting that inhibitory effects of nebivolol on bFGF-induced proliferation and migration may occur through a different mechanism other than β_3-AR agonism.

It is well-known that the ERK/MAPK and PI3K/AKT pathways are involved in bFGF-mediated effects (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). Our published data shown that both pathways are involved higher concentrations (2 and 5 μM) of CL316,243-mediated effects on bFGF-induced cell proliferation and migration (Chang et al., 2022a). Our current experiments tested the involvement of ERK and AKT signaling in lower concentrations of nebivolol and CL316,243 on bFGF-induced cell proliferation and migration. We found that although bFGF induced significant increase in phosphorylation of both ERK and AKT, neither nebivolol or CL316,243 at lower concentrations produced any effects on bFGF-induced ERK and AKT phosphorylation. In contrast, higher concentrations of nebivolol inhibited bFGF-induced AKT phosphorylation but not ERK phosphorylation, whereas higher concentrations of CL316,243 enhanced bFGF-induced phosphorylation of both ERK and AKT. These data suggest that ERK and AKT signaling are not involved in lower concentrations of nebivolol and CL316,243-mediated effects, only AKT signaling is involved in higher concentration of nebivolol-mediated effects, and both pathways are involved in higher concentrations of CL316,243-mediated effects.

Our findings indicate that the mechanisms of nebivolol-mediated effects on bFGF-induced VSMC proliferation and migration may involve different pathways. The dose-related and functional-driven responses reflect that more than one mechanism that might be involved.

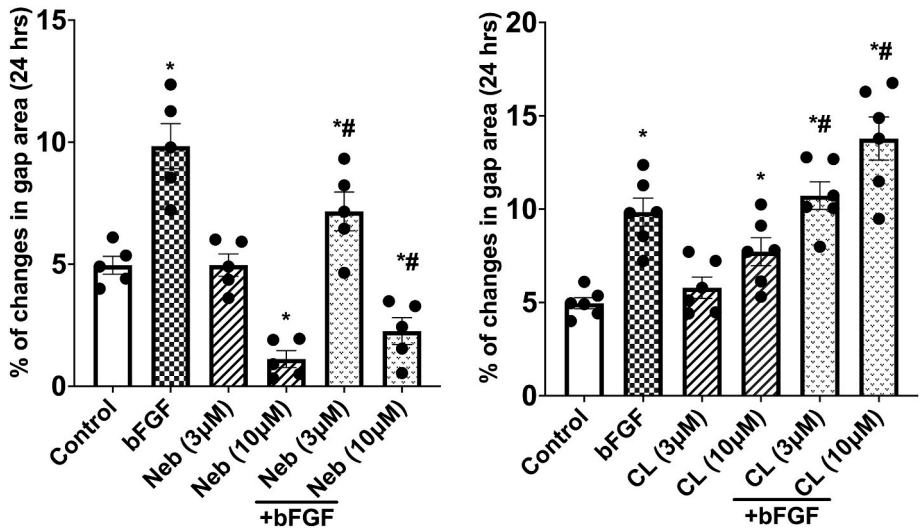

Fig. 6. High concentrations of nebivolol suppressed bFGF-induced VSMC migration, whereas high concentrations of CL316,243 potentiated bFGF-induced VSMC migration. VSMCs were pretreated with higher concentrations (3 or 10 μM) of nebivolol (Neb, 6A) or CL316,243 (CL, 6B) in DMEM/F12 containing 0.5% FBS for 24 h. A cell-free area was created using a 100 μl pipette tip and cells were then stimulated with 5 ng/mL of bFGF in the presence or absence of the same concentrations of Nebivolol or CL316,243 for additional 24 h. Photos of the cell-free areas were taken before and after bFGF treatment and a cell free area was measured. Results are expressed in % change of cell-free area (mean ± SD) after 24 h of bFGF treatment. Data are from 6 independent experiments and were analyzed using one-way ANOVA followed by Turkey Multiple Comparison Test. *p < 0.05, compared to control; #p < 0.05, compared to bFGF alone.

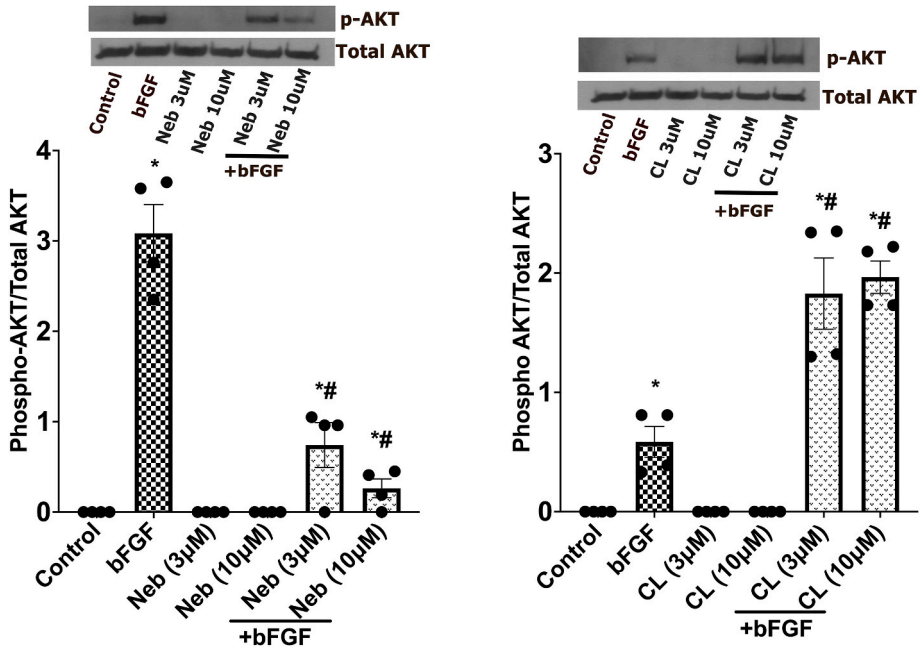

Fig. 7. High concentrations of nebivolol reduced bFGF-induced AKT phosphorylation, whereas high concentrations of CL316,243 enhanced bFGF-induced AKT phosphorylation. Cells were pretreated with 3 μM or 10 μM of nebivolol (neb, 7A) or CL316,243 (CL, 7B) in DMEM/F12 containing 0.5% FBS for 24 h followed by a stimulation with 5 ng/ml of bFGF for 10 min in the presence or absence of the same concentrations of nebivolol or CL316,243. The levels of AKT phosphorylation were determined by Western blot analyses via probing with antibody directed against phospho-AKT. The membranes were then stripped and reprobed 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 4 independent experiments. Results are expressed as the ratio of phospho-AKT to total AKT levels (means ± SD). Data were analyzed by using one-way ANOVA followed by Turkey Multiple Comparison Test. *p < 0.05, compared to control; #p < 0.05, compared to bFGF alone.

One of the major mechanisms that are involved nebivolol-mediated effects could be its antioxidant effects. It has been reported that vascular injury-induced restenosis involves production of reactive oxygen species (ROS) and reduction of the expression and activity of enzymes involved in nitric oxide (NO) production and degradation, both can reduce the level of NO (Omar et al., 1991). Several studies indicate that nebivolol possesses antioxidant property (Cominacini et al., 2003; Janssen et al., 1999). Nebivolol reduces intracellular formation of ROS and prevents the depletion of intracellular NO, which are believed to be due to its free radical scavenging property that is not observed with propranolol, a non-selective β receptor antagonist (Janssen et al., 1999), nor metoprolol, a selective β_1 receptor antagonist (de Groot et al., 2004), indicating that nebivolol possesses ROS scavenging activity.

Additionally, it has been shown that nebivolol has anti-inflammatory

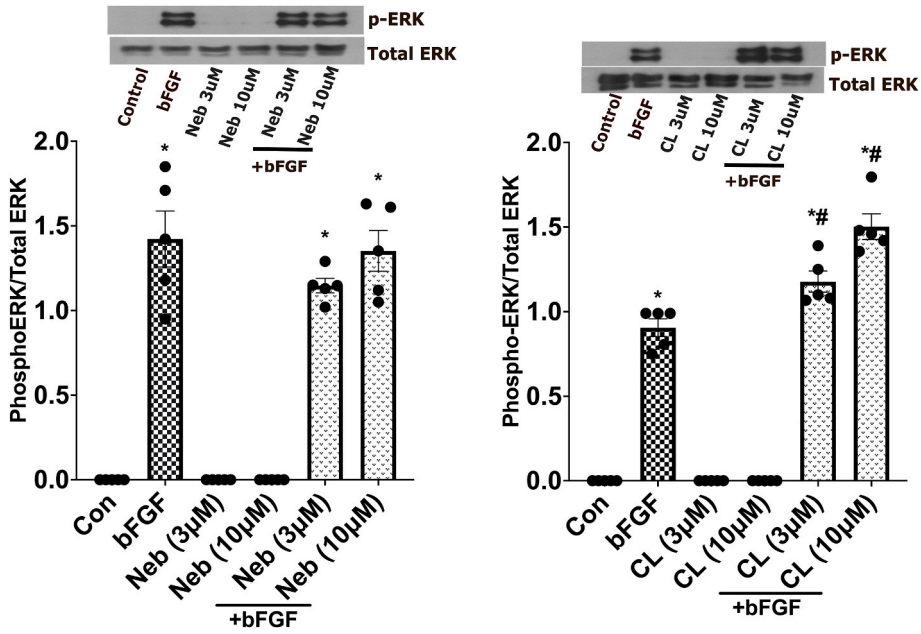

Fig. 8. High concentrations of nebivolol had no effect on bFGF-induced ERK phosphorylation, whereas high concentrations of CL316,243 potentiated bFGF-induced ERK phosphorylation. Cells were pretreated with 3 μM or 10 μM of nebivolol (Neb) or CL316,243 (CL) in DMEM/F12 containing 0.5% FBS for 24 h followed by a stimulation with 5 ng/ml of bFGF for 10 min in the presence of the same concentrations of nebivolol or CL316,243. ERK phosphorylation were determined by Western blot analyses via probing with antibody directed against phospho-ERK. The membranes were then stripped and reprobed for total 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 5 independent experiments. Results are expressed as the ratio of phospho-ERK to total ERK levels (means ± SD). Data were analyzed by using one-way ANOVA followed by Turkey Multiple Comparison Test. *$p < 0.05$, compared to control; #$p < 0.05$, compared to bFGF alone.

properties. Wolf et al. revealed that treatment of human coronary artery SMCs with nebivolol downregulates genes involved in inflammation, including inerleukin-1α (IL-1α), tumor necrosis factor (TNG)- α, and growth-related oncogenes 2 and 3; and an upregulation of genes involved in oxidative stress protection including seladin-1 and vasoactive intestinal peptide receptor 1 (VIPR1). These effects were not observed with Metoprolol of the same concentration (Wolf et al., 2007). It is well established that inflammatory response is the initial step in vascular injury-induced restenosis. Increased inflammatory molecule expression and responses have been found following vascular injury (Tanaka et al., 1993; Rogers et al., 1996; Welt et al., 2000). The anti-inflammatory properties of nebivolol may contribute to the anti-mitogenic and anti-motogenic effects in VSMCs as well as its ability to reduce vascular injury-induced neointima formation observed by the others (Wolf et al., 2007).

Finally, Brehm et al. showed that Nebivolol also blocks entry into cell cycle phase G2 (rapid cell growth and protein synthesis preparing for mitosis) and dose-dependently induces VSMC apoptosis (Brehm et al., 2001), suggesting that this may be one of the contributors that are involved in nebivolol-mediated effects on VSMC proliferation.

Based on our data, the signaling transduction pathways that are involved in high concentrations of nebivolol-mediated effects appear to be at least partially due to inhibition of AKT phosphorylation but not ERK phosphorylation, which is opposite to the results observed with the β_3-AR agonist, CL316,243. This further confirmed our hypothesis that the inhibitory effects of nebivolol on bFGF-induced VSMC proliferation and migration may not occur through β_3-AR agonism possibly through antioxidant, anti-inflammatory, and/or preventing cells from entering phase G2. The mechanism of differential effects of nebivolol on AKT and ERK signal pathways at high concentrations is unclear. While both AKT and MAPK pathways have been implicated in VSMC proliferation and migration (Zhu et al., 2015; Yang et al., 2013; Ouyang et al., 2014; Xue et al., 2016), the exact difference among their downstream targets is not fully understood. Zhang et al. found that VSMC phenotype switching is regulated by a balance between AKT and MAPK signaling during aging and hypertension, characterized as reduced AKT signaling but increased MAPK signaling, suggesting that AKT and MAPK pathways may respond differently to various stimuli under specific conditions (Hayashi et al., 1999; Zhang et al., 2018).

In conclusion, our findings suggest that nebivolol is likely acting at least in part/at lower concentrations, via β_3-AR agonism in terms of increasing bFGF-induced VSMC proliferation. Other mechanisms, such as antioxidant and/or anti-inflammatory properties, may be involved in nebivolol-mediated effects on bFGF-induced VSMC migration as well as high concentrations of nebivolol-mediated effects on bFGF-induced VSMC proliferation. Future studies will be needed to delve into the potential mechanism that nebivolol is acting through.

The present study expanded our understanding of complicated mechanisms underlying the effects of nebivolol on VSMC proliferation and migration. Our findings may contribute to developing a potential therapeutic approach that utilizes higher concentrations of nebivolol following angioplasty to reduce the risk of the procedure-associated vascular restenosis.

CRediT authorship contribution statement

Elaina Seemann: Conceptualization, Methodology, Data curation, Formal analysis, Validation, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Trevor Beeler:** Data curation, Validation, Writing – review & editing. **Mohammed Alfarra:** Data curation, Validation, Writing – review & editing. **Mark Cosio:** Data curation, Validation, Writing – review & editing. **Charles Chan:** Data curation, Validation, Writing – review & editing. **Peyton Grant:** Data curation, Validation, Writing – review & editing. **Yingzi Chang:** Conceptualization, Methodology, Data curation, Formal analysis, Validation, Writing – review & editing, Visualization, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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