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A Novel function of Nebivolol: Stimulation of Adipose-derived Stem Cell Proliferation and Inhibition of Differentiation

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Abstract

Tissue engineering is limited by the time of culture expansion of cells needed for scaffold seeding. Thus, a simple means of accelerated stem cell proliferation could represent a significant advance. Here, Nebivolol was investigated for its effect on the replicative capacity of adipose-derived stem cells (ASCs). This study indicates that the number of ASCs with Nebivolol treatment showed a significant population increase of 51.5% compared to untreated cells ($p<0.01$). Cell cycle analysis showed a significant decrease in the percentage of ASCs in G1 phase with Nebivolol treatment compared to untreated cells ($p<0.01$), suggesting that Nebivolol shortens the G1 phase of ASCs, resulting in a faster proliferative rate. Furthermore, our results showed that Nebivolol significantly increased colony-forming units of ASCs ($p<0.01$). Despite increasing ASC proliferative potential, we showed that Nebivolol has an inhibitory effect on adipogenic and osteogenic differentiation potential as indicated by significantly reduced expression of CCAAT Enhancer Binding Protein alpha ($P<0.01$) and lipoprotein lipase ($P<0.01$) and inhibited activity of alkaline phosphatase ($P<0.01$), respectively. Taken together, these results showed that Nebivolol accelerated ASC proliferation through shortening G1 phase, while inhibiting both adipogenic and osteogenic potentials of ASCs. These data identify a novel and simple approach to accelerate stem cell expansion *in vitro* before cell differentiation.

Key Words: Nebivolol, Adipose-derived stem cells, Cell proliferation, Differentiation, Adipogenesis, Osteogenesis.

Introduction

Beta-adrenergic receptor blockers are widely used to treat cardiovascular diseases[1, 2]. In particular, Nebivolol is a third-generation betablocker with unique pharmacological properties[3-6]. Nebivolol is a highly selective β1-blocker that shows more favorable characteristics than other β-blockers for cardiovascular disease treatment. Unlike Atenolol and Propranolol, for example, Nebivolol acutely reduced systemic blood pressure without causing a negative inotropic response[7-9]. Nebivolol also dilates arteries through mechanisms involving cyclic GMP and nitric oxide (NO), which is attributed largely to activation of endothelial NO synthase in vascular endothelial cells[10-14]. One of the many physiological functions of NO is to modulate cell proliferation[15]. There is some evidence that NO stimulates cell proliferation under certain conditions[16] although most reports demonstrate that NO inhibits cell growth[15]. Other studies showed that Nebivolol inhibits vascular smooth muscle cell proliferation in a concentration- and time-dependent manner by a mechanism involving NO, while other β-blockers such as Propranolol, Metoprolol and Bisoprolol had no effect on cell proliferation[17-19]. Cardiovascular and neural tissue injuries, such as myocardial infarction (MI) and spinal cord injury, are pathological events for which there has been no satisfactory treatment to date[20-22]. In particular, cardiovascular disease is a leading cause of morbidity and mortality worldwide. Despite significant advances in recent years in medical and interventional therapy, the treatment of heart failure resulting from the death of myocardial cells and subsequent tissue remodeling, is still a challenging problem[23] and has stimulated an intense search for new therapeutic agents. Stem cells have been recognized for their potential for treatment of cardiovascular diseases, since their multipotential capacity can be used to regenerate structurally and functionally damaged tissues. Adipose-derived stem cells (ASCs) have gained substantial attention since they are abundantly present in adipose tissue, which is highly vascularized and contains significant presence of stem cells. Adipose tissue can be easily harvested using small surgical interventions such as liposuction. For medical and research applications, ASCs have advantages compared to other types of stem cells including: 1) embryonic stem cells, which limit the clinical use because of ethical concerns and their risk of teratoma formation, 2) induced pluripotent stem cells, which are compromised by concerns for cancer formation, and 3) bone marrow-derived mesenchymal stem cells due to the limitation in obtaining large quantities[24, 25]. The universal early multipotent stem cells reside in adipose tissue and comprise up to 10% of all cells of the tissue. Several studies have demonstrated that ASCs can differentiate into all three germ layers under the guidance of the respective microenvironment[26-32]. In this study we examined whether and how Nebivolol exerts its effects on the proliferation and differentiation potential of ASCs.

Materials and Methods

Drug

Nebivolol (Berlin-Chemie Berlin, Germany) is a lipophilic substance that was dissolved in 100% methanol as 1mM stock solution and stored in -20°C. The working concentration of Nebivolol in growth

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medium was 1uM (1/1000 of stock solution), while the final methanol concentration in the experiments was below 0.1% and corresponds to a typical clinical dosage in patients.

Isolation and expansion of ASCs

Adipose tissues were obtained based on the approved protocol by the Institutional Review Board of the Tulane University Health Sciences Center. ASCs were isolated from gross specimens from each donor using previously described methods[33]. Briefly, 50g of tissue was minced and digested with collagenase Type I (Invitrogen Corp., Carlsbad, CA, USA) for 60min at 37°C. After being treated with red blood cell lysis buffer (BioWhittaker, Walkersville, MD, USA), the cells were plated in low glucose DMEM medium, supplemented with 20% fetal bovine serum (Atlanta Biological, Atlanta, GA) and 1% Penicillin/Streptomycin (Cellgro, Herndon, VA, USA) at 37°C with a 5% CO_2. Upon reaching 70% confluency, the cells were passaged further.

Nebivolol effect on cell replication

ASCs were plated at a density of 2 X 10^4 cells/well in 6-well plate after initial passage with or without addition of Nebivolol. The medium was changed daily with or without Nebivolol treatment. ASCs were counted after 72h using cell counting chambers. This experiment was performed in triplicate.

Cell cycle analysis

ASCs were plated at a density of 3 X 10^4 cells/well in a 6-well plate with or without Nebivolol treatment. The medium was changed daily with or without addition of Nebivolol. Cell cycle analysis was performed after 72h with propidium iodide using a COULTER DNA PREP Reagents Kit (Beckman Coulter, Inc.) and Beckman Coulter EPICS Flow Cytometer. This experiment was performed in triplicate.

Colony-forming unit assay

ASCs were plated at densities of 225 and 112.5 cells/well in a 6-well plate with or without Nebivolol treatment. The medium was changed with or without daily addition of Nebivolol. After 12 days, cultures were fixed and stained with 1% crystal violet in 100% methanol. Colonies were counted.

Multilineage potential of ASCs

For adipogenic differentiation, ASCs were seeded at 2×10^5 cells/well in a 6-well tissue culture plate containing the normal growth medium. The cells were prepared in triplicate. After 24 hours, the induction was started by replacing the culture medium with adipogenic differentiation medium with or without Nebivolol treatment. The medium was changed daily with or without Nebivolol treatment. After 16 days the adipogenic potential was evaluated by realtime polymerase chain reaction (PCR) analyses of lineagespecific genes and Oil Red O staining (Diagnostic BioSystems). For the osteogenic differentiation assay, ASCs were seeded at 0.5×10^5 cells/well in a 6-well tissue culture plate containing normal growth medium. After 24 hours, the induction was started by replacing culture medium with osteogenic differentiation medium with or without Nebivolol treatment. The medium was changed daily with or without Nebivolol treatment. After 15 days the osteogenic differentiation potential was quantified for alkaline phosphatase (ALP) activity using the SensoLyte_ pNPP Alkaline Phosphatase Assay Kit (AnaSpec). The experiments were performed in triplicate.

Quantitative real-time polymerase chain reaction analysis

Total cellular RNA was isolated from ASC cultures treated with or without Nebivolol after culturing for 72 hours. cDNA was obtained by the High Capacity Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The following primers were used:

GAPDH
5' CGAGATCCCTCCAAAATCAA 3'
5' GGTGCTAAGCAGTTGGTGGT 3';

CEBPA
5′- CTAGGAACACGAAGCACGAT-3′
5′-ATGGTGGTTTAGCAGAGACG-3′;

LPL,
5'-TTGCATATTTCCAGTCACATAAGCA-3'
5'-AGCCACGGACTCTGCTACTC-3'.

All reactions were run using a Bio-Rad iCycler (Bio-Rad Laboratories).

Statistical Analysis

Error bars in the figures represent the mean and SD of three biological samples. Student's t test was performed to evaluate whether the difference between two conditions was significant. Significant differences were marked with *$p < 0.01$.

Results

Effect of Nebivolol on cell replication

Flow Cytometry analysis of untreated and Nebivolol treated ASCs showed their positive expression for CD44, CD90, CD105, and CD73. This analysis indicates that ASCs were negative for the expression of CD34, CD11b, CD45 and HLA DR, as previously reported[33]. In addition, these data suggest that Nebivolol treatment exerts no effect on the expression of these surface markers. To analyze the effect of Nebivolol treatment on the expansion ability of ASCs, cells were seeded ($2X10^4$ cells/well) in cell culture media with or without Nebivolol (1μM). Counting the cells in different time point after Nebivolol treatment showed that the total ASCs number is significantly higher than control untreated ASCs after 72h (Nebivolol treated ASCs: 9.6, 10.9 and $10.3X10^4$ cells vs. control untreated ASCs 6.8, 6.7 and $7.0X10^4$ cells; $p<0.01$) (Table 1). Also, these data suggest that Nebivolol treatment affects the population doubling time (PDT) of ASCs ($PDT = [T * \log 2] / \log [N_f / N_i]$, where N_f = the number of cells at time t and N_i = the initial number of cells and T = time period). The results showed that the PDT is about 30 h in Nebivolol-treated ASCs compared to 40h in untreated control ASCs. The median cell numbers are $10.3X10^4$ of Nebivolol treated versus $6.8X10^4$ of untreated control ASCs representing a 51.5% increase in cell number of Nebivolol-treated ASCs. These results suggest that Nebivolol effectively accelerates ASC replication.

Effect of Nebivolol on cell cycle

Since the S- and G2/M-phases of mammalian cell cycle are relatively invariant in length with changing interdivision time[34-37], faster cell growth rate/shorter interdivision time with Nebivolol treatment is due to shorter G1-phase. In order to confirm this, we performed cell cycle analysis since the duration of each phase can be estimated from

Table 1. Cell counting analysis for ASCs treated with/without Nebivolol. Cells were seeded at a density of 2 X 10^4 cells/well in a 6-well plate in the presence or absence of Nebivolol. The numbers of cells were counted after 72h.

Treatment	The numbers of cells (triplicates)	mean	SD	P value
Control	6.8 X 10^4, 6.7 X 10^4, 7.0 X 10^4	6.8 X 10^4	0.15	< 0.01
Nebivolol	9.6 X 10^4, 10.9 X 10^4, 10.3 X 10^4	10.3 X 10^4	0.65	

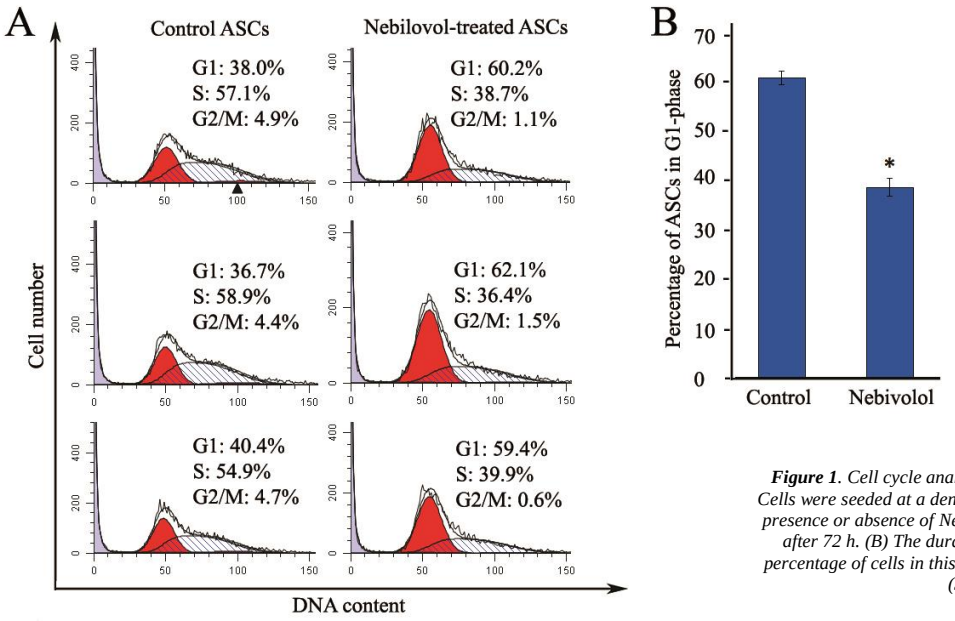

Figure 1. *Cell cycle analysis for ASCs treated with/without Nebivolol. Cells were seeded at a density of 3 X 10^4 cells/well in a 6-well plate in the presence or absence of Nebivolol. (A) Cell cycle analysis was performed after 72 h. (B) The duration of G1-phase can be estimated from the percentage of cells in this phase. Error bars indicate standard deviation (SD, n =3; *P < 0.01).*

the percentage of cells in that phase. The results showed that the cell number of ASCs in fraction of G1-phase with Nebivolol treatment after 72h is significantly lower than control (Nebivolol: 38%, 36.74%, 40.42% vs. control: 60.2%, 62.08%, 59.44%) (Figure 1A). The median percentage of cells in G1-phase is 38.4% in Nebivolol treated cells versus 60.6% in control ASCs corresponding to a 36.6% (38.4% vs. 60.6%) decrease in percentage of cells in G1-phase of Nebivolol-treated ASCs (Figure 1B).

Effect of Nebivolol on colony-forming unit (CFU) of ASCs

The colony-forming potential of ASCs was investigated in Nebivolol treated cells and compared to control ASCs. The aim was to analyze whether Nebivolol treated cells maintained the ability to give rise to fibroblast-like colonies. The CFU colony forming ability of ASCs with Nebivolol treatment after 12 days was significantly higher than

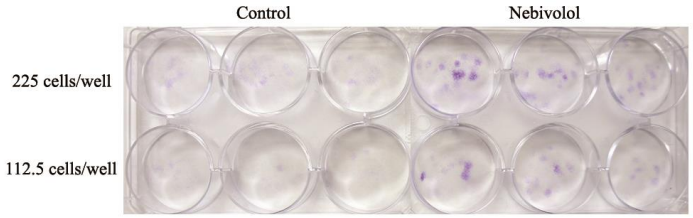

Figure 2. *Effect of Nebivolol on colony-forming unit of ASCs. ASCs were seeded at a density of 225 and 112.5 cells/well in a 6-well plate with or without Nebivolol treatment. The medium was supplemented with Nebivolol daily. After 12 days, cultures were fixed and stained with 1% crystal violet in 100% methanol. All the colonies size 3mm and larger were counted.*

control (p<0.01) (Fig. 2 and Table 2). The median CFU frequencies are 19 and 9.3 of Nebivolol treated cells vs 10.7 and 5.7 of control in the cell seeding density of 225 cells/well and 112.5 cells/well, respectively, in a 6-well plate. There was a 77% (19 vs. 10.7) and 63% (9.3 vs. 5.7) increase in colonies of Nebivolol treated ASCs in the cell seeding density of 225 cells/well and 112.5 cells/well, respectively, in comparison with control. Taken together, these results suggest that ASCs with Nebivolol treatment can effectively promote the ability of CFU as an indirect indicator of stemness.

Effect of Nebivolol on cell differentiation potential

In order to study the multilineage differentiation capacity of ASCs, the cells were differentiated toward the adipogenic and osteogenic lineages using appropriate differentiation media and supplements. For induction of adipogenesis, accumulation of lipid droplets could be seen with the bright-field microscope after just 4 to 5 days of differentiation and after 14 days multiple lipid droplets were clearly visible and stained by Oil Red-O. Our results showed less lipid accumulation in cells treated with Nebivolol than untreated control cells (Figure 3A), suggesting that Nebivolol inhibits adipogenic differentiation. To verify the observed effects of Nebivolol on adipogenic differentiation, the expression of adipocyte-specific markers CEBPA and LPL which are highly expressed in mature adipocytes[38, 39] were assessed. As shown in Figure 3B, both CEBPA and LPL expression were markedly inhibited in Nebivolol treated cells comparted with control ASCs (P < 0.01), suggesting that Nebivolol has negative effect on adipogenic potential of ASCs. For osteogenic differentiated cells, alkaline phosphatase (ALP) activity, an indicator of osteogenic differentiation potential of ASCs, was measured by an enzyme assay after cells were cultured in osteogenic

Table 2. Colony-forming unit (CFU) assay for ASCs cell treated with/without Nebivolol. Cells were seeded at a density of 225 and 112.5 cells/well in a 6-well plate in the presence or absence of Nebivolol. All the colonies size 3mm and larger were counted (SD, n =3; *P < 0.01).

Cell density	Treatment	Numbers of colonies (triplicates)	Mean	SD	P value
225 cells/well	Control	10, 10, 12	10.7	1.2	< 0.01
	Nebivolol	18, 18, 21	19	1.7	
112.5 cells/well	Control	5, 6, 6	5.6	0.6	< 0.01
	Nebivolol	9, 9, 10	9.3	0.6	

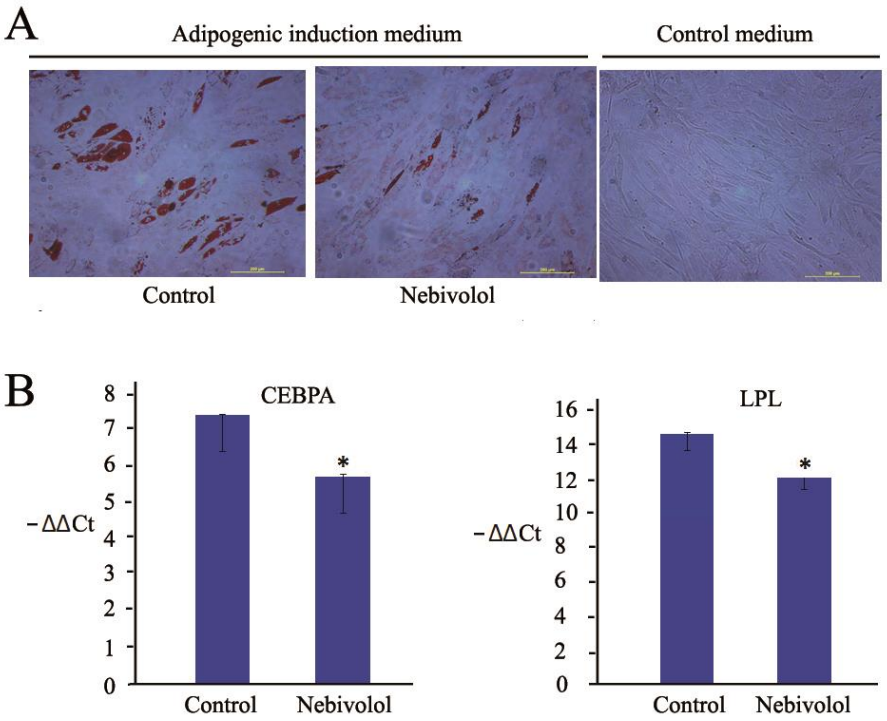

Figure 3. *Effect of Nebivolol on adipogenic differentiation potential of ASC. ASCs were induced to undergo differentiation with or without Nebivolol treatment for 16 days.* ***(A)*** *Cells were stained with Oil Red O for lipid droplets.* ***(B)*** *RNAs were prepared after 16 days of differentiation. Gene expression of the adipogenic markers CEBPA and LPL were analyzed by reverse transcription real time PCR. Data are expressed as -ΔΔCt relative to GAPDH expression. Error bars indicate standard deviation (SD, n =3). *P < 0.01.*

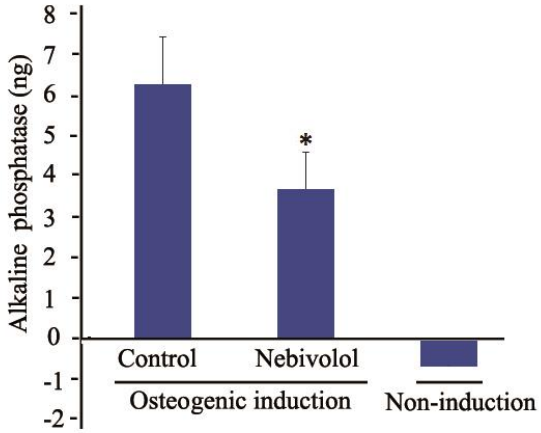

Figure 4. *Effect of Nebivolol on osteogenic differentiation potential of ASCs. ASCs were induced to undergo differentiation with or without Nebivolol treatment for 15 days. Osteogenic differentiation potential was quantified by monitoring alkaline phosphatase activity using the SensoLyte_ pNPP Alkaline Phosphatase Assay Kit (AnaSpec). The experiments were performed in triplicates. Error bars indicate standard deviation (SD, n =3). *P < 0.01.*

medium for 15 days with and without Nebivolol treatment. As shown in Figure 4, ALP activity in cells treated with Nebivolol was markedly reduced when compared to the control cells (P<0.01), suggesting that Nebivolol inhibits the osteogenic potential of ASCs.

Discussion

Clinical studies indicated that β-blockers have protective effects in patients with cardiovascular disease[40]. Unlike other classic β-blockers such as Atenolol or Propranolol, the highly selective β1-blocker Nebivolol showed the ability to inhibit vascular smooth muscle cell proliferation[17-19]. This study for the first time demonstrates that Nebivolol accelerates the proliferation of human ASCs and a cell counting assay indicated that the number of ASCs with Nebivolol treatment after 72h was increased by 51.5%. Further analysis showed that the percentage of ASCs in G1-phase with Nebivolol treatment after 72h decreased from 60.6% (control) to 38.4%, suggesting that Nebivolol treatment shortens the G1-phase of ASCs, resulting in faster cell replication. Moreover, CFU assay showed the colonies of ASCs with Nebivolol treatment after 12 days increased by 77% in the cells seeding density of 225 cells/well and by 63% in density of 112.5 cells/well, respectively, in comparison with control, which is consistent with the results from the cell counting assay. Differentiation studies demonstrated that Nebivolol treatment inhibited both adipogenic and osteogenic differentiation of ASCs. Even though stem cells have emerged as promising agents for the treatment of many diseases as well as tissue injury, a number of important challenges remain. For example, the process of producing a sufficient number of ASCs by culturing and expansions for tissue engineering or allogenic cell usage and hematopoietic reconstitution is critical.

Although a great deal of research has been performed to develop advanced techniques for cell culture and expansion, no effective means of accelerating stem cell proliferation has been described to date. Thus, a simple means by which the rate of stem cell proliferation would be enhanced is significant. This study indicated that Nebivolol in a dose corresponding to a typical

plasma concentration in a patient significantly promotes ASC proliferation *in vitro*.

Conclusion

The data in this study indicate that Nebivolol increases ASC proliferation. Cell cycle analysis showed a shorter G1 phase in ASCs treated with Nebivolol. However, Nebivolol treatment decreased both adipogenic and osteogenic potentials of ASCs. These data provide a novel and effective approach to accelerate stem cell expansion *in vitro*, and yield a clinical implication of Nebivolol on stem cell biology.

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Abbreviations

ASCs	: Adipose-derived stem cells
NO	: Nitric oxide
CCAAT	: Cytosine-cytosine-adenosine-adenosine-thymidine
CEBPA	: CCAAT-enhancer-binding protein alpha
MI	: Myocardial infarction
GMP	: Guanosine monophosphate
PCR	: Polymerase chain reaction
ALP	: Aalkaline phosphatase
CFU	: Colony-forming unit
LPL	: Lipoprotein lipase
GAPDH	: Glyceraldehyde 3-phosphate dehydrogenase

Potential Conflicts of Interests

None

Sponsors / Grants

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