Anti-atherogenic mechanisms of berberine

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Anti-atherogenic mechanisms of berberine

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Seahyoung Lee

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Abstract

Anti-atherogenic mechanisms of berberine

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Berberine, an alkaloid isolated from plants, has been used in traditional Chinese medicine. To date, berberine's lipid lowering effect by up-regulating LDLR has the most clinical impact than others regarding cardiovascular system. However, rare attempts have been made to test possible anti-atherogenic effect of berberine other than LDLR up-regulation. Thus, in the present study, we tried to elucidate various effects of berberine on cardiovascular system. Our data show that berberine effectively inhibited rVSMC proliferation and migration, and this was also confirmed in vivo by showing berberine treatment improved neointima formation after carotid balloon injury in rat. We also newly discovered that berberine induced LDLR up-regulation also involves JNK pathway besides ERK pathway that has been already reported. Another new finding from the present study is that berberine inhibits adipocyte differentiation involves Foxo-1 activation and this possibly

results in PPARγ down-regulation which is known to be an important factor of adipocyte differentiation. We also examined the effect of beberine on LDLR -/- mouse fed western diet to determine possible effects of berberine other than LDLR up-regulation that might have influence on lipid metabolism. According to our data berberine not just failed to improve lipid profile of LDLR -/- mouse but significantly worsened it. This observation indicates there might be a feedback system involves berberine, lipoprotein production, and up-regulation of LDLR.

In the present study, we demonstrated that berberine can be beneficial to cardiovascular system by suppressing restenosis, inhibiting adipocyte differentiation, and decreasing immune response. In examining such effect of berberine, we found new transcriptional mechanism of berberine induced LDLR up-regulation and new potential target of berberine in adipocyte differentiation. However, we also found that berberine might have serious adverse effect under the circumstances where functional LDLR is missing. This observation can be clinically important because it implies precaution should be taken in using berberine as a therapeutic agent for treating patient without functional LDLR such as Familial Hypercholesterolemia.

Key words: berberine, restenosis, Foxo-1, LDLR, and hypercholesterolemia

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I. Introduction

As our society becomes more accustomed to Western life style and diet, the risk of cardiovascular diseases (CVD) such as atherosclerosis is ever increasing. As a consequence, the demand for developing drugs to cure or prevent CVD is also increasing more than ever. To meet this end, although synthesizing completely new drug substances can be one way, there has been a great effort to screen drug substances among already existing natural substances that can be used for such purpose. In search of such drug compounds, many researchers have been testing natural compounds extracted from plants. These compounds might have their own advantage because it is

most likely that they have been used in folk remedy for centuries, making their safety to be proven to some extent, and berberine is one of such compounds.

Berberine, an alkaloid isolated from rhizomes, roots, and stem bulk of the plants such as the Berberidaceae, Ranunculaceae families, and Chinese herb Huanglian (Figure 1), has long been known for its anti-microbial activity in treatment of infectious diarrhea and dysentery in traditional Chinese medicine[1-4]. Furthermore, during the last decade, studies have shown that it also has other various effects including anti-cancer activity[5-7] and, most interesting to us, various beneficial effects on cardiovascular system. Among these effects, berberine's lipid lowering effect by up-regulating low density lipoprotein receptor (LDLR)[8,9] has the most clinical impact than others considering hypercholesterolemia or dyslipidemia can develop serious health problems such as atherosclerosis. LDLR mediated hepatic uptake is responsible for the removal of more than 70 % of human low density lipoprotein cholesterol (LDL-c), which is thought to be the major risk factor for CVD[10]. Thus regulation of LDLR expression by therapeutic agents can be a powerful strategy to directly influence plasma cholesterol levels.

However, since an observable end result of certain chemical agents is usually the result of many different, but interconnected, biological processes,

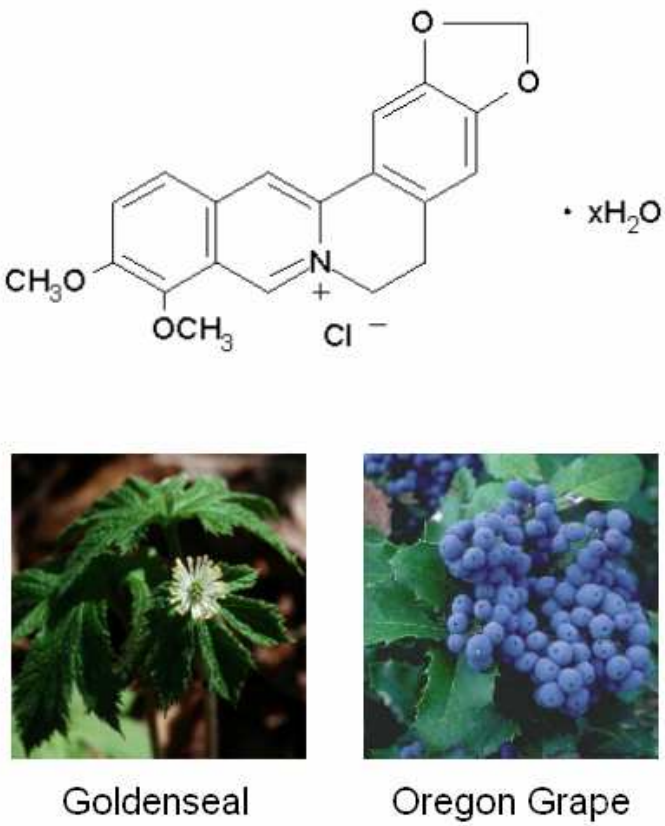


Figure 1. Chemical structure of berberine and representative plants containing berberine

caused by that particular agents, it is highly possible that there exist mechanisms other than LDLR up-regulation contribute to the lipid lowering effect of berberine. Nevertheless, rare attempts have been made to find such mechanism of berberine. Thus we first tested the hypothesis that berberine could inhibit vascular smooth muscle cell (VSMC) proliferation and migration, processes known to play important roles in various pathogenic vascular conditions including restenosis. We also evaluated the effect of berberine in vivo using rat carotid balloon injury model.

Furthermore, as briefly mentioned above, berberine has been reported to improve lipid profile of hyperlipidemic hamsters by up-regulating LDLR expression via extracellular signal-induced kinase (ERK) pathway[9]. In this study, Kong and colleagues have reported that berberine stabilizes LDLR mRNA through post-transcriptional mechanism rather than promoting transcription, and they claimed that ERK pathway is the single most important pathway in that mechanism. However, it is not convincing enough that only ERK pathway is involved in berberine induced LDLR up-regulation and it also has not been studied to what extend berberine can improve, if it can at all, lipid profile of the animal model without functional LDLR. To examine the effect of berberine on LDLR -/- mouse can be clinically important because this animal model can reflect familial hypercholesterolemia, a clinically

important lipid metabolism related pathologic condition[11].

To answer these questions we first screened other possible signal pathways that might be involved in berberine induced LDLR up-regulation using different signal pathway inhibitors. Our preliminary result showed that c-jun N-termial kinase (JNK)/c-jun pathway is also involved in berberine induced LDLR up-regulation. Thus, we additionally performed LDLR promoter assay using luciferase reporter vector containing putative c-jun binding site. In addition, we examined the effect of berberine on LDLR -/- mice fed western diet to see whether berberine possesses different lipid lowering mechanism other than up-regulating hepatic LDLR.

Another effect of berberine though to be beneficial to cardiovascular system is its inhibitory effect on adipocyte differentiation. Berberine has been reported to suppress adipocyte differentiation using peroxisome proliferator activated receptor gamma (PPARγ) dependent pathway[12]. Another study also reported that berberine treatment decreased adipogenic factors such as sterol regulatory element binding protein 1c (SREBP-1c), CCAAT/enhancer binding protein alpha (C/EBP-1), PPAR-γ, fatty acid synthase, and lipoprotein lipase (LPL) in 3T3-L1 cells[13]. To better understand the role of berberine in adipocyte differentiation, we first confirmed its inhibitory effect on adipocyte differentiation and further tried to find an unkonwn mechanism of berberine

in inhibition of adipocyte differentiation using 3T3 L-1 pre-adipocytes.

For this part of the study, we hypothesized berberine can affect the expression and activity of forkhead transcription factor 1 (foxo-1) which has been reported to be involved in adipocyte differentiation[14]. Foxo-1 is the most abundant isoform of foxo family in adipocyte and its active form inhibits the differentiation of pre-adipocytes. Active form of foxo-1 mostly resides in the nucleus in basal condition, but is being excluded as PI3K-Akt pathway is activated. Furthermore, since active form of dephosphorylated foxo-1 has been known to suppress PPARγ transcriptional activity[15], we thought it was worth to examine the effect of berberine on foxo-1 expression and activation during adipocyte differentiation. Our data indicates berberine caused activation of foxo-1 during adipocyte differentiation and this might be one of the mechanisms that berberine suppresses adipocyte diffrerentiation with.

In the present study we tried to elucidate the effect of berberine on cardiovascular system by taking various approaches and integrating results from those approaches. The result of this study will help us better understand the role of berberine on cardiovascular system so that we can make a more informed decision in case of berberine being used as a therapeutic drug in clinical settings.

II. Materials and Methods

1. Culture of rat vascular smooth muscle cell

Rat VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats weighing 200~250 g (ORIENT-Charles River Technology, Seoul, Korea) by using the method described previously[16]. More than 95 % of the cells were positive for α-actin and the exhibited the typical hill-and-valley morphology of SMCs. In this study, passages between 5 and 10 were used for the experiments. The cells were maintained in DMEM containing 10 % FBS.

2. In vitro treatment of berberine and growth factors

Cells were starved in DMEM containing 0.1 % FBS for 24 hours before the treatment of chemicals. For the in vitro experiments, 100 nM of angiotensin II (AngII) (Sigma-Aldrich, St. Louis, MO, USA.), 10 μM of berberine-chloride (Sigma), 1 ng/ml of heparin binding epidermal growth factor (HB-EGF, R&D systems, Minneapolis, MN, USA.), 1 ng/ml of basic fibroblast growth factor (bFGF, R&D systems), and 1 ng/ml of epidermal growth factor (EGF, R&D systems) were used. For the berberine pretreatment, one day starved cells were treated with berberine for 30 minutes before the treatment with other growth factors.

3. MTT assay and [^{3}H]- thymidine incorporation assay

For MTT assays, cells were seeded in 96-well plates at a density of $5x10^3$ cells/well and the assays were done using Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA) as recommended by the manufacturer. DNA synthesis assays were performed for cells plated in 96-well plates at a density of 3.5 x 10^3 cells/well. Cells were starved in DMEM with 0.1 % FBS for 24 hours, then preincubated with or without Berberine before the treatment with other growth factors. Twenty four hours after the growth factor treatments, 2 μCi/ml of [^{3}H]-thymidine was added and the cells were incubated for additional 4 hours before transferred to a filtermat (Perkin Elmer, Waltharn, MA, USA) using a Tomtec havester 96 (Tomtec, Hamden, CT, USA). The amount of [^{3}H]-thymidine incorporated was measured by a Wallac microbeta Trilux 1450 counter (PerkinElmer).

4. Migration assay

Wound healing assay was done as previously described[17]. Briefly, cells were grown to near confluence (< 85 %) in 35 mm dishes then starved in DMEM containing 0.1% FBS for 24 hours before any treatment. The wounds were produced by scratching the bottom of dish using a 200 μl pipette tip and the cells were washed with PBS. For the berberine pretreatment group, the

cells were incubated with berberine for 30 minutes before any other treatments. The cellular migration was induced by either AngII or HB-EGF. The cells were cultured additional 4 more days before measuring the distance migrated. VSMC migration was also assessed using a modified Boyden's chamber method[18]. Cells (5×10^4) were seeded onto the upper surface of an 8-μm pore size chamber (Costar. Corning, NY, USA), and medium containing either 100 nM or 1 μM of AngII was placed into the lower chamber. These cells were allowed to migrate for 12 hours then the cells in the upper chamber were fixed with 4 % paraformaldehyde. The cells were stained with crystal violet dye. The migrated cells were counted under a light microscopy (400 ×). The number of cells was recorded from at least 5 fields per each well.

5. Western blot analysis

Cells were plated in 6 cm culture dishes (5×10^5 cells/well) and starved for 24 hours in DMEM containing 0.1 % FBS before any treatment. After appropriate treatment, cell lysates were prepared and equal amount of proteins (20 μg) were subjected to Western blot analysis. Blots were blocked with 5 % dry milk and incubated with proper primary antibodies. Anti p-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA.), anti ERK1/2 (Santa Cruz Biotechnology), anti p-Akt (Santa Cruz Biothechnology), anti p-

p70S6 kinase (Cell Signaling Technology, Danvers, MA, USA.), anti p-EGFR (Cell Signaling Technology) anti-β-actin (Sigma), anti Foxo-1 (Cell Signaling Technology), anti p-Foxo-1 (Cell Signaling Technology), and anti CD36 (Santa Cruz Biotechnology) primary antibodies were used. Peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology) and anti-mouse antibody (Santa Cruz Biotechnology) were used as secondary antibodies. Immuno-positive bands were visualized by ECL (enhanced chemifluorescent labeling) kit (Amersham, Buckingham-shire, England). Each experiment was triplicated at least.

6. Cell cycle analysis

Cells were detached with trypsin/EDTA then fixed with 100 % ethanol at 4 °C for 1 hour. Fixed cells were washed with PBS twice then stained with propodium iodide at room temperature for 30 minutes. Cell cycle analysis was done using FACS Caliber system (Becton Dickinson, Franklin lakes, NJ, USA).

7. Rat carotid artery injury model

The rats were maintained according to the ethical guidelines of our institution, and the Committee on Animal Investigation of the Yonsei

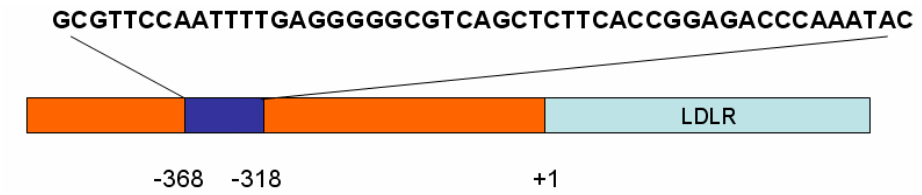
University approved the experimental protocol. All procedures were done in accordance with institutional guidelines for animal research. Balloon injury was performed on male Sprague-Dawley rats (ORIENT-Charles River Technology). Rats were separated into two groups: the vehicle (DMSO) treated group (n = 8) and the Berberine treated group (n = 8). Either DMSO or Berberine in DMSO (100 μg/kg/d) was administered to rats by Alzet mini osmotic pump 2004 (Durect Corporation, Cupertino, CA, USA) for 14 days before the balloon injury and additional 14 days after the balloon injury. For balloon injury, rats were anesthetized with ketamin and xylazine mixture (150 mg/kg and 10 mg/kg respectively; IP), and the endothelium of the left common carotid artery was denuded by three passages of a Fogarty 2F balloon catheter (Edwards Lifesciences, Irvine, CA, USA) as previously described[19]. The neointimal and medial areas were measured 14 days after the injury (28 days after the initial berberine treatment), and the neointimal formation was expressed as the ratio of the neointimal to the medial area.

8. Construction of luciferase vector

Using web based program (wwwmgs.bionet.nsc.ru), putative c-jun binding site in the 3,000 base pairs from the transcriptional start point of human LDLR promoter was selected (Figure 2A). Fragments containing

selected c-jun binding site (-368~ -318) were produced by PCR using *pfu* polymerase then cloned into PGL3 luciferase reporter vector (Promega) (Figure 2B). Luciferase vectors with/without putative c-jun binding site were transfected into 293 cells using lipofectamin (Invitorgen, Carlsbad, CA, USA). Cells were treated with berberine with or without JNK inhibitor SP600125 pretreatment. Luciferase activity was measured using MicroLumatPlus LB96V (Berthold technologies, Oak Ridge, TN, USA).

A



B

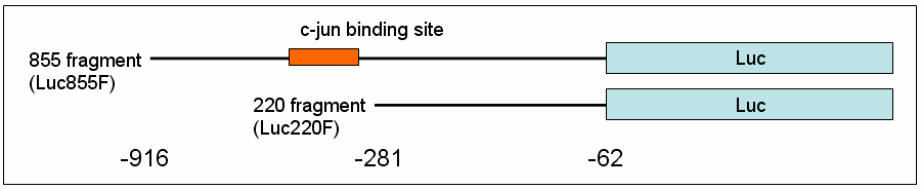


Figure 2. Construction of luciferase reporter vector with putative c-jun binding site. (A) Putative c-jun binding site was determined through analysis of human LDLR promoter. (B) Schematic representation of two luciferase vectors with/without putative c-jun binding site.

9. Berberine treatment of LDLR -/- mouse

For the evaluation of berberine effect other than LDLR up-regulation, 7~9 week old male B6;129S-*Ldlr*^{tm1Her} mice were used. LDLR-/- mice were divided into 3 groups; vehicle (DMSO), BBR5 (5 mg/kg/day), and BBR10 (10 mg/kg/day). Animals were received berberine via daily i.p. injection (200 μl) for 8 weeks and fed on western diet throughout the experiment (Table 1). Body weight was measured every weeks and blood was withdrawn using eye bleeding method with capillary tube every 4 weeks for serum lipid profile analysis. At the end of experiment, animals were sacrificed and organs were either fixed in formalin or frozen at –70 °C for further analysis.

10. Serum lipid profile analysis

Approximately 150~200 μl of blood per animal was collected using non-heparinized capillary tube. Collected blood samples were settled at room temperature for 30 minutes. Supernatant was obtained by centrifugation at 3,000 rpm for 10 minutes. Analysis of blood lipid profile was done at KRIBB (the Korean Research Institute of Biosciences and Biotechnology).

Table 1. Composition of western diet

Ingredient	g/kg
Casein	195
DL-Methionine	3
Cornstarch	150
Sucrose	341.46
Cellulose	50
Milk Fat	210
Salt Mix #200000	35
Vitamin Mix #310035	10
Calcium Carbonate	4
Cholesterol	1.5
Ethoxyquin	0.04

11. Measurement of epididymal adipocyte size

At the end of experiment, mice were sacrificed and epididymal adipo tissues were obtained. A line was drawn on the image of the slide section of adididymal adipo tissue and the size of adipocytes crossed by the line was measured using NIH provided Image J software (http://rsb.info.nih.gov/ij).

12. Oil Red O staining of thoracic aorta

Thoracic aorta was fixed over night in 4% paraformaldehyde. After fixation, thoracic aorta was washed with 1x PBS 3 times, and then fixed on a plate with pins. Fixed thoracic aorta was washed with DW, and then incubated in absolute propylene glycol for 2 minutes. After 16 hours of Oil red O staining, thoracic aorta was immersed in 85% propylene glycol for 1 minute, then washed with DW 3 times.

13. Assessment of lipid accumulation in aortic valve lesion

Hearts were frozen in embedding media and the frozen blocks were sectioned at a thickness of 20 μm at -17°C using CM3050 cryostat (Leica, Wetzlar, Germany). Sectioned slices were attached to microscope slides (Fisher scientific, USA). Slides were immersed in DW for 2 minutes, then immersed in 100 % 1,2-propranediol for 2 minutes. Oil red O solution was

applied to the slides then incubated for 16 hours at room temperature. After Oil-red-O staining, slides were washed 3 times in 85 % 1,2-propranediol for 2 minutes and DW for 1 minute. Background was reduced using 1 % HCl in EtOH and slides were washed in DW for 1 minute. Slides were counterstained with 0.5 % cresyl violet. For microscopic examination, Nikon E600 upright light microscope (Melville, NY, USA) was used and the image was taken using Nikon Coolpix 4300 digital camera.

14. Immunohistochemistry- MOMA staining

Aortic valve slides were immersed in 0.1 M PBS for 10 minutes. For tissue preoxidase inactivation, slides were pretreated with 1 % H_2O2 in 50 % MtOH for 30 minutes. Slides were then immersed in 0.1 % triton X-100 in 0.1 M PBS for 1 hour. Biotin conjugated anti-mouse metallophilic macrophages monoclonal rat antibody (Bachem, San Carlos, CA, USA) was applied dropwise then incubated at room temperature overnight. Slides were washed with 0.1 % triton X-100 in 0.1 M PBS for 10 minutes, then with 0.1 M PBS for 10 minutes twice. Streptavidin-HRP conjugate (Dako, Glostrup, Denmark) was applied dropwise then incubated at room temperature for 1 hour. Slides were washed with 1 % triton X-100 in 0.1 M PBS and 0.1 M PBS as mentioned above. Sigmafast DAB with metal enhancer (Sigma) was applied

dropwise and slides were washed with DW followed by dehydration step and mounting.

15. Statistical analysis

For all quantification analysis, at least three independent experiments were performed. Results are shown as mean ± SEM. The significance of differences for paired data was determined using Student's *t*-test. *P* value less than 0.05 was considered significant.

III. Results

1. Cytotoxicity of berberine

To test the cytotoxicity of berberine, we treated rVSMCs with various concentrations of berberine (10, 50, 100, 200, and 300 μM) for 24 hours. The result of MTT assay shows that berberine did not show significant cytotoxicity up to 50 μM (Figure 3A). Based on the reports that berberine can inhibit proliferation of cancer cell, we tested the hypothesis that berberine treatment would also inhibit the proliferation of vascular cells such as endothelial cells and, especially vascular smooth muscle cells which play an important role in the formation of fibrous cap or restenosis. Growth arrested rVSMCs were treated HB-EGF (1 ng/ml), EGF (1 ng/ml), and bFGF (1 ng/ml) with or without 30 minutes of berberine pretreatment, and proliferations was measured by a MTT assay. In every set, berberine pretreatment significantly inhibited growth factor induced rVSMCs proliferation (Figure 3B).

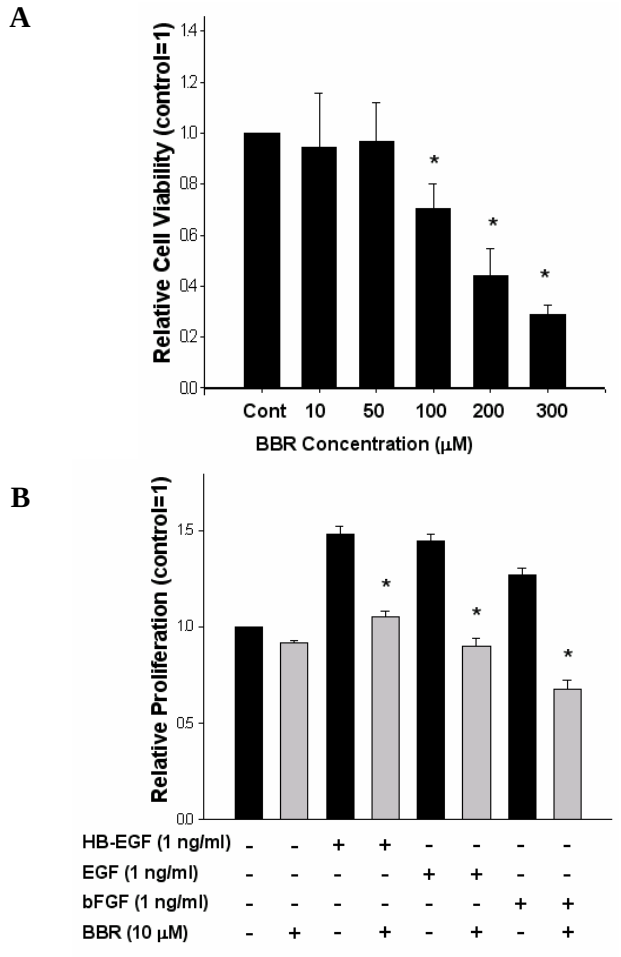


Figure 3. Effect of berberine on rVSMC viability and proliferation. (A) rVSMCs were incubated with various concentrations of berberine for 24 hours. Relative cell viability was measured by MTT assay. (B) rVSMCs were incubated with different growth factors for 24 hours with/without berberine in the media. Results are means ± SEM of at least 3 experiments. ∗ indicates $p<0.05$.

2. Effect of berberine on rVSMC proliferation

To assess the effect of berberine on cardiovascular system, we employed two interconnected growth factors AngII and HB-EGF, which are known to play important roles in pathogenesis of cardiovascular system. For the MTT assay, the cells were starved for 24 hours before any treatment. For the berberine pretreatment group, cells were treated with 10 μM of berberine for 30 minutes preceding 24 hours of AngII or HB-EGF treatment. Compared to control group, both AngII (100 nM) and HB-EGF (1 ng/ml) treatment increased cellular proliferation by approximately 20%, but berberine pretreatment significantly inhibited the increase in cellular proliferation induced by both growth factor (Figure 4A). We also tested the effect of berberine on DNA synthesis by [^{3}H]-Thymidine incorporation assay. The result was essentially identical to that of MTT assay. Both AngII and HB-EGF treatment increased DNA synthesis about 50~60 %, but the berberine pretreatment effectively blocked this increase in DNA synthesis (Figure 4B).

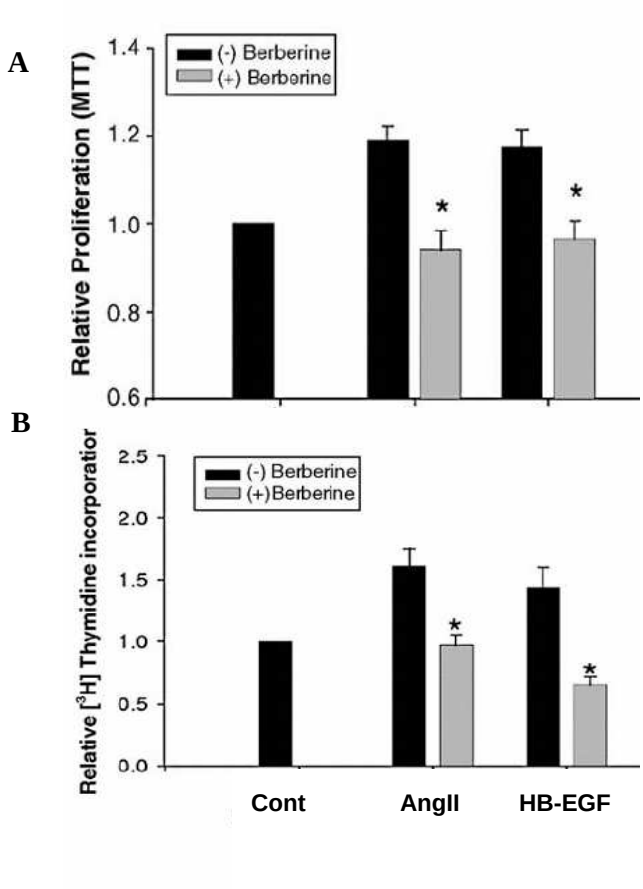


Figure 4. Berberine inhibits AngII/HB-EGF induced rVSMC proliferation. rVSMCs were incubated with AngII (100 nM) or HB-EGF (1 ng/ml) for 24 hours with/without berberine in the media. (A) Result of MTT assay. (B) Result of [^{3}H]-thymidine incorporation assay. Results are means ± SEM of at least 3 experiments.∗ indicates $p<0.05$.

3. Effect of berberine on rVSMC migration

Our data shows AngII or HB-EGF treatment increased the distance migrated about 25 % compared to control group (Figure 5). However, when pretreated with berberine, the migratory effect of AngII and HB-EGF was significantly suppressed so that the distance migrated remained similar to that of control group (Figure 6A). We also evaluated the effect of berberine on AngII induced cellular migration using a modified Boyden's chamber method. When the migration was induced by 100 nM of AngII, berberine pretreatment significantly inhibited cellular migration. However, when 1 μM of AngII was used to induce migration, inhibitory effect of berberine was not statistically significant (Figure 6B).

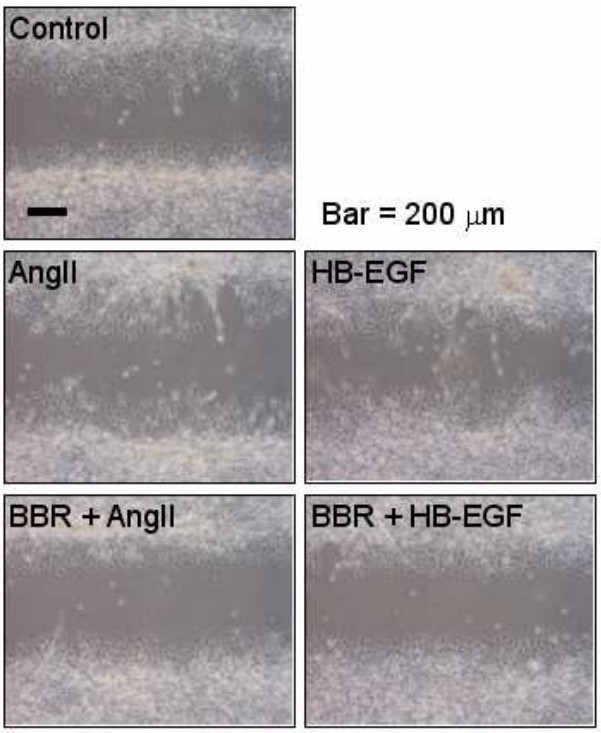


Figure 5. Berberine inhibits AngII/HB-EGF induced rVSMC migration. After the wound was made by using yellow pipette tip, rVSMC migration was induced by AngII (100 nM) or HB-EGF (1 ng/ml) with/without berberine (10 μM) in the media. Picture was taken 4 days after the treatment.

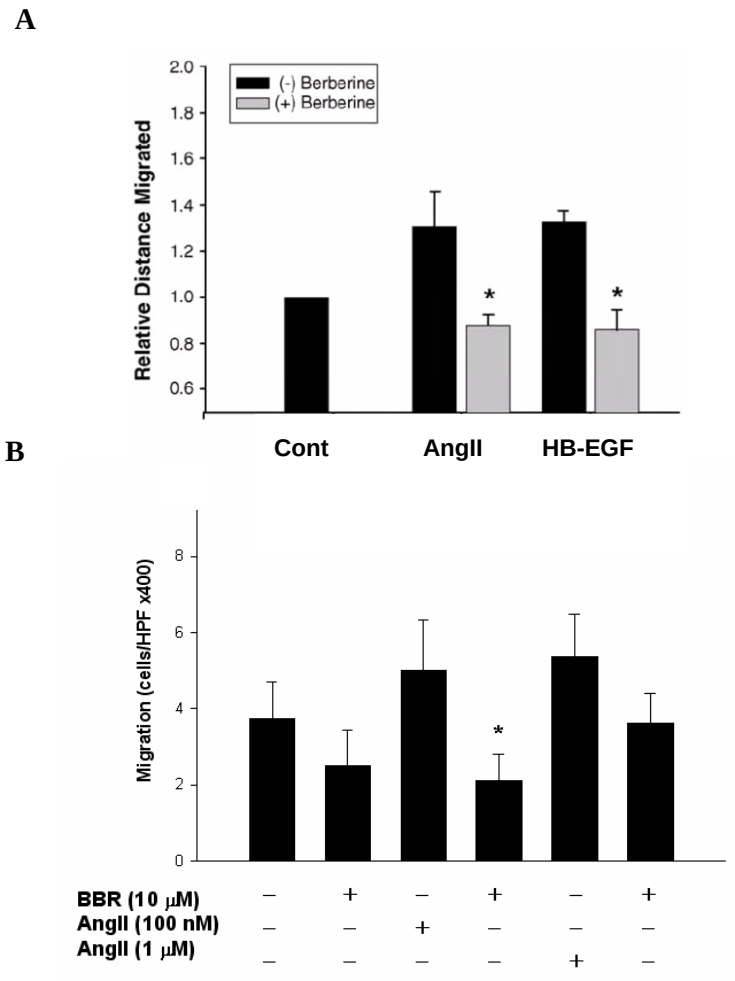


Figure 6. Anti-migratory effect of berberine measured. (A) The distance migrated in the wound healing assay was quantified. Migration was induced by AngII (100 nM) or HB-EGF (1 ng/ml). (B) Effect of berberine on rVSMC migration was also evaluated using Transwell assay. Results are means ± SEM of at least 3 experiments.∗ indicates $p<0.05$.

4. Effect of berberine on MAPK and Akt signal pathway

When cells were treated with HB-EGF, phosphorylation of ERK1/2 was induced as early as 5 minutes after the treatment and lasted up to 60 minutes, then diminished thereafter. This expression pattern of p-ERK1/2 was weakened with the berberine pretreatment but the change was not significant. In addition, HB-EGF treatment induced phosphorylation of EGFR although it only lasted first 30 to 60 minutes when treated with HB-EGF or AngII, respectively. Again, the p-EGFR expression pattern was not significantly changed by berberine pretreatment. However, for the Akt and its downstream factor p70S6K, berberine pretreatment delayed and decreased phosphorylation of these molecules (Figure 7). This effect of berberine on Akt pathway was also observed when AngII was used in the place of HB-EGF. AngII-induced Akt phosphorylation and subsequent p70S6K phosphorylation were delayed and partially inhibited by berberine pretreatment, while berberine pretretment did not affect the phosphorylation of ERK1/2. Although the previous studies showed that AngII could induce phosphorylation of EGFR as early as 3 minutes after the treatment using immunoprecipitaion with p-Tyr antibody, we could not detect p-EGFR expression by Western blot with p-EGFR antibody.

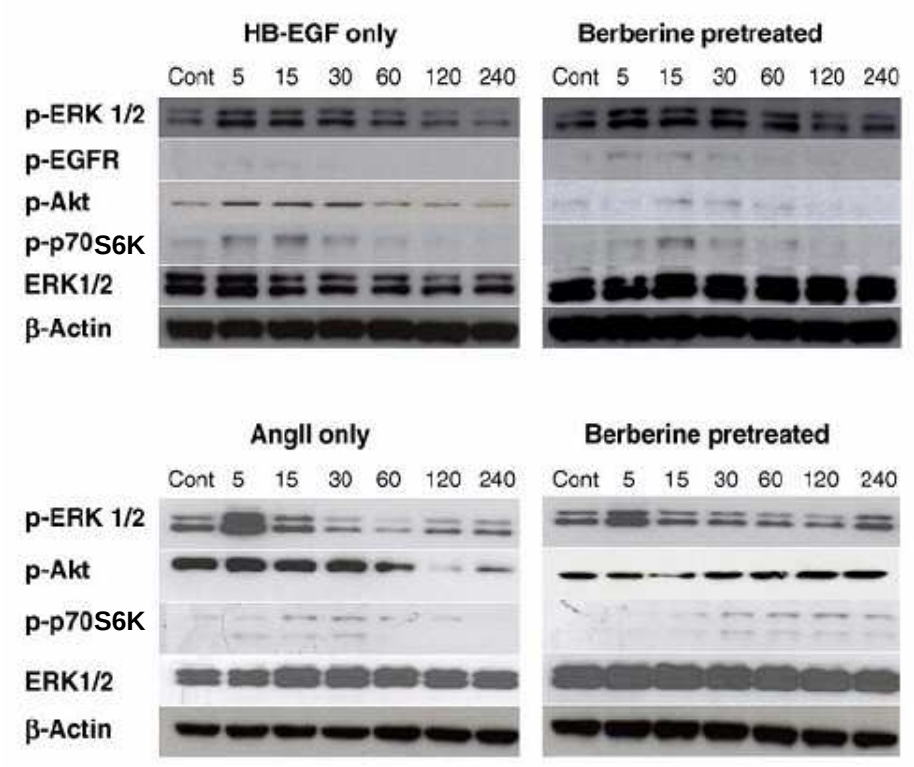


Figure 7. Berberine mainly inhibits/delays Akt pathway rather than ERK pathway. ERK and Akt pathway activation with berberine treatment was examined by Western blot. One day starved rVSMCs were treated with berberine cell lysates were prepared at indicated times (minutes).

5. Effect of berberine on cell cycle

HB-EGF and AngII treatment induced phosphorylation of pRb. Although HB-EGF induced phosphorylation of pRb lasted up to 6 hours, AngII induced phosphorylation of pRb lasted relatively short period of time. Inhibitory effect of berberine on pRb phosphorylation was most obvious at 6 hour (Figure 8A). The result of cell cycle analysis using FACS indicated that berberine treatment decreased the percentage of cells in S phase in both AngII and HB-EGF treated cells. However, the decrease was not statistically significant in either case (Figure 8B).

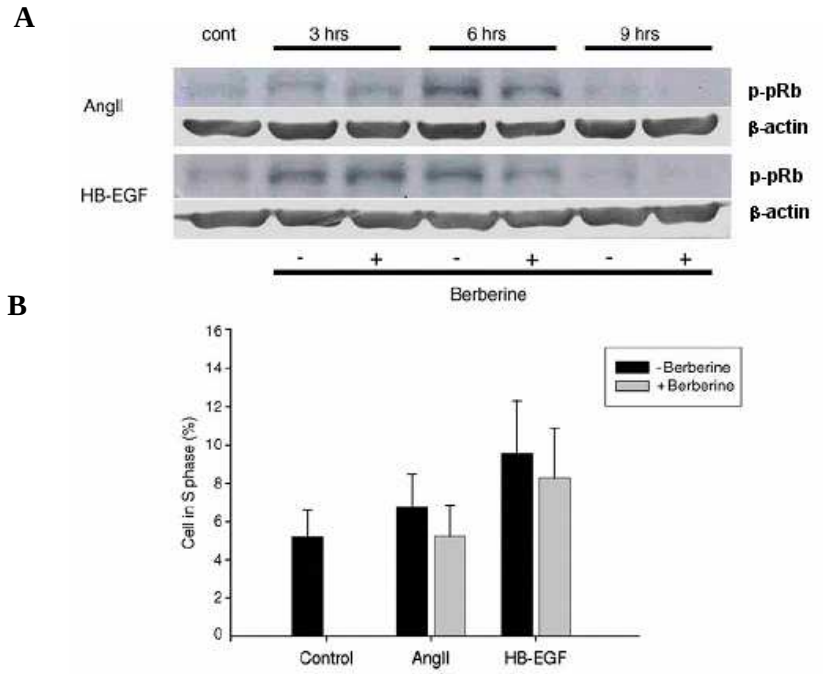


Figure 8. Effect of berberine on cell cycle. (A) Protein samples were collected at 3, 6, and 9 hours after either AngII (100 nM) or HB-EGF (1 ng/mL) treatment, with or without berberine treatment. Expression of pRb was examined byWestern blot using an anti-pRb antibody. (B) Cell cycle analysis was done by FACS. Cells were fixed for 24 hours after either treatment (100 nM of AngII or 1 ng/mL of HB-EGF) then stained with propodium iodide. The percentage of the cells in S phase was used as the parameter. Results are means ± SEM of at least 3 experiments.

6. Effect of berberine on neointima formation

We employed well established rat carotid injury model to evaluate the anti-proliferative effect of berberine in vivo. The animals were subjected to either a chronic berberine treatment (100 μg/kg/d) or vehicle (DMSO) treatment for 14 days before the injury by using an osmotic pump. Each group included 8 animals. The animals had lived for additional 14 days before the injury by using an osmotic pump. Each group included 8 animals. The animals had lived for additional 14 days before they were sacrificed, and the pumps were in place and working during that post injury period. The mean intimal and medial areas were measured 14 days. After the injury, the neointima/media ratio was 1.14 ± 0.11 in DMSO treated group and 0.85 ± 0.06 in berberine treated group (Figure 9D, $P<0.05$). The reduction was approximately 25 % and this neointima formation inhibitory effect of berberine was moderate but statistically significant. Representative carotid sections are shown in Figure 9A, B, and C.

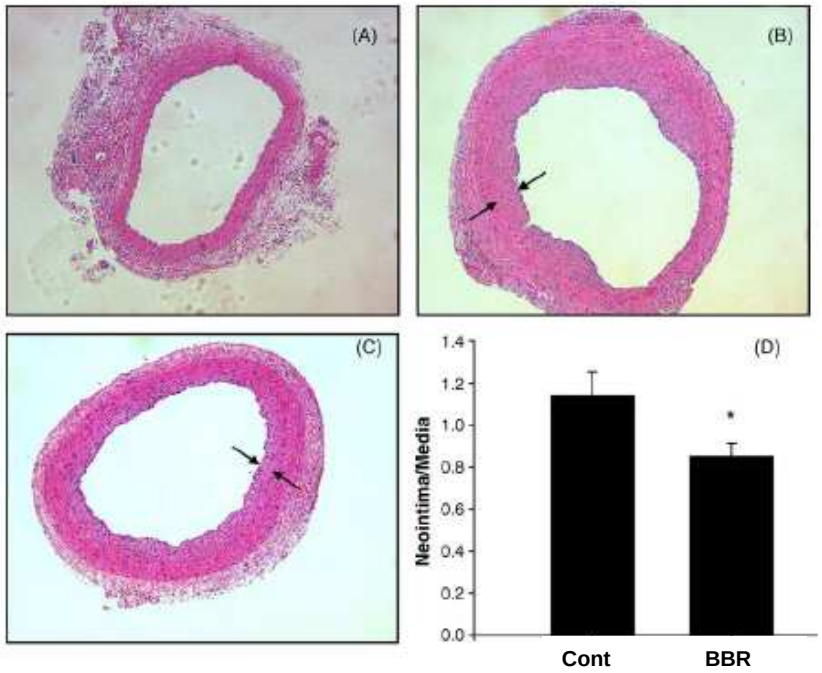


Figure 9. Berberine pretreatment improves neointima formation. Rats were either received chronic DMSO (control) or berberine (100 μg/kg/d) treatment by osmotic pump implants for 28 days. (A) Normal rat carotid artery. (B) DMSO treated carotid artery. (C) Berberine treated carotid artery. (D) Neointima/media ratio is calculated. Arrow heads indicate neointima formed. Results are means ± SEM (n = 8 for each group). * indicates $p<0.05$.

7. Effect of berberine on JNK pathway in LDLR up-regulation

Berberine treatment increased hepatic LDLR mRNA expression as early as 30 minutes and this increase was still observed at 8 hours (Figure 10A). This effect of berberine was dose dependent (Figure 10B). To examine whether berberine induced LDLR up-regulation only involves ERK pathway as reported, hepatocytes were pretreated with various signal pathway inhibitor before berberine treatment. According to our data, ERK pathway inhibitors PD98065 and c-raf pretreatment decreased berberine induced LDLR mRNA expression. Furthermore, JNK pathway inhibitor SP600125 also decreased LDLR mRNA expression (Figure 11A). Berberine dose dependently increased LDLR mRNA expression and this was inhibited by SP600125 pretreatment (Figure 11B).

To confirm the involvement of JNK pathway in berberine induced LDLR up-regulation, activation of JNK pathway after berberine treatment was evaluated using Western blot. Four hours of berberine treatment increased phosphorylation of both JNK and its downstream molecule c-jun (Figure 12). Since phosphorylated c-jun translocates into nucleus where it acts as a transcription factor by formaing AP-1 complex[20], phosphorylation of c-jun was evaluated using nuclear fraction.

To examine whether the activation of JNK pathway by berberine is

directly involved in LDLR up-regulation, promoter assay using luciferase reporter vectors with putative c-jun binding site was performed. Four hours of berberine treatment significantly increased luciferase activity in the group transfected with luciferase vector with c-jun binding site. However, this berberine induced luciferase activity was not observed in the group transfected with luciferase vector without c-jun binding site. Furthermore, this increase of luciferase activity by berberine treatment was significantly inhibited by SP600125 pretreatment (Figure 13).

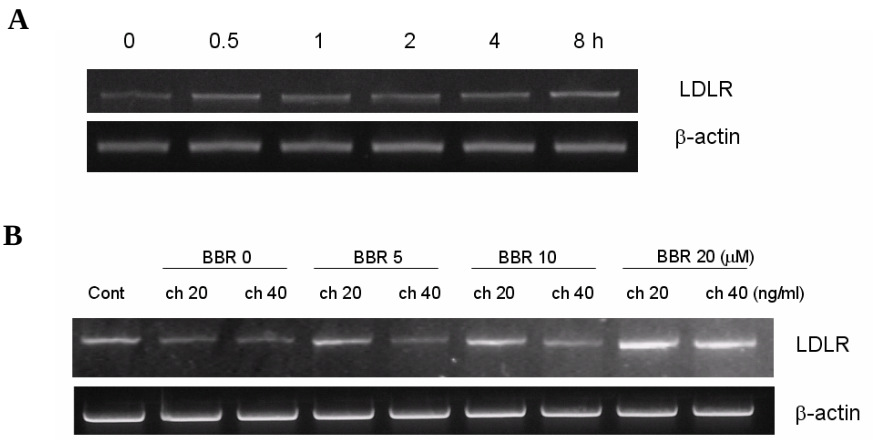


Figure 10. Berberine up-regulates hepatic LDLR mRNA expression. (A) One day starved hepatocytes were treated with berberine (10 μM) and samples were collected at times indicated. (B) Hepatocytes were pretreated with cholesterol for 30 minutes to decrease basal LDLR mRNA level, and then treated with increasing concentrations of berberine for 4 hours. ch: cholesterol.

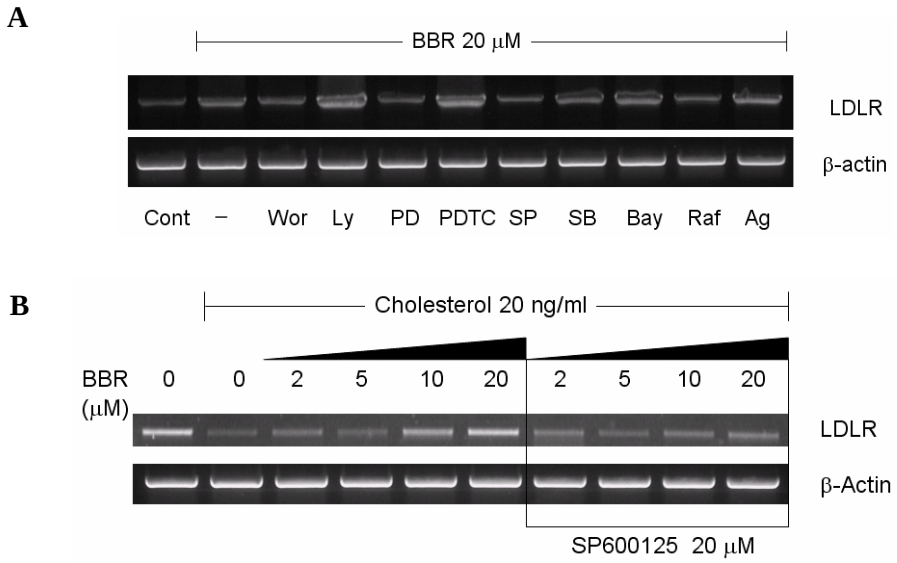


Figure 11. Berberine induced hepatic LDLR mRNA up-regulation involves JNK pathway. (A) Hepatocytes were treated with various signal pathway inhibiters 30 minutes before berberine treatment. Hepatocytes were collected 4 hours after berberine treatment and LDLR mRNA expression was detected using PCR. (B) Hepatocytes were treated with increasing concentrations of berberine with/without JNK pathway inhibitor SP600125 pretreatment. Wor: wortmannin, Akt inhibitor (100 nM), Ly: ly294002, Akt inhibitor (10 μM), PD: PD98059, ERK inhibitor (10 μM), PDTC: NF-kB inhibitor (50 μM), SP: SP600125, JNK inhibitor (20 μM), SB: SB203580, p38 inhibitor (10 μM), Bay: Bay117082, NF-kB inhibitor (10 μM), Raf: c-raf inhibitor (10 μM), and Ag: Ag490, JAK-STAT inhibitor (10 μM).

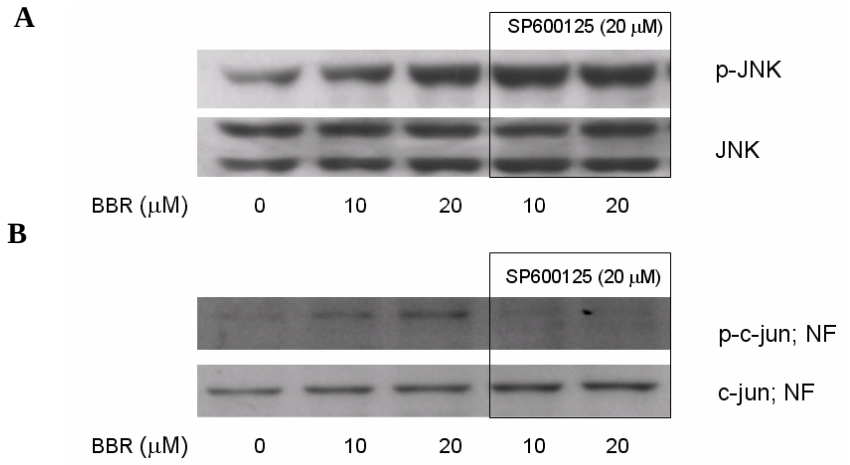


Figure 12. Berberine activates JNK pathway in hepatocyte. (A) Hepatocytes were treated with berberine for 4 hours then phosphorylation of JNK was detected using cytosolic protein. (B) Phosphorylation of c-jun was detected using nuclear protein.

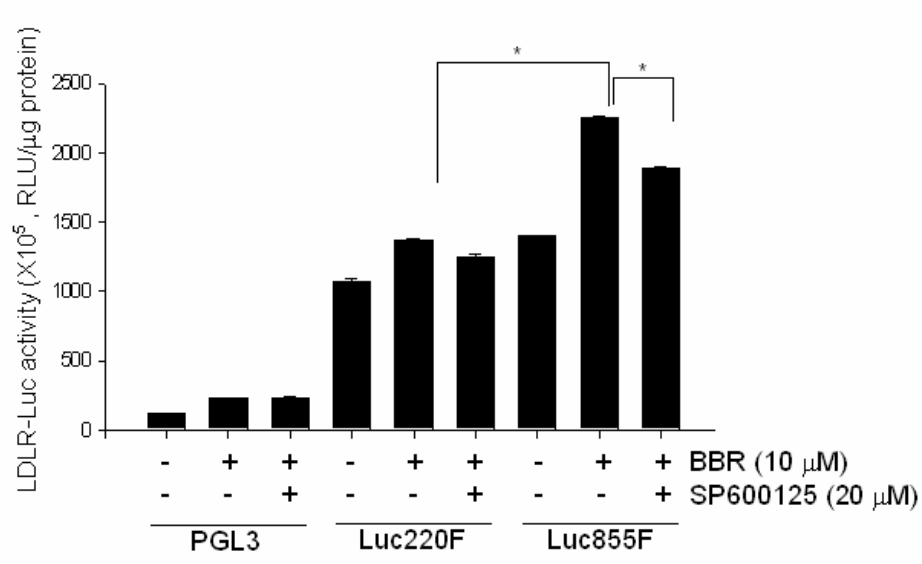


Figure 13. JNK pathway is directly involved in berberine induced LDLR transcription. Luciferase reporter vectors with/without putative c-junbinding site were transfected into 293 cells. Transfected cells were treated with berberine (10 μM) with/without JNK inhibitor SP600125 pretreatment (20 μM). Luc220F: luciferase reporter vector without putative c-jun binding site, Luc855F: luciferase reporter vector with putative c-jun binding site. Results are means ± SEM of at least 3 experiments. ∗ indicates $p<0.05$.

8. Effect of berberine on iNOS expression and NO production in macrophage

According to our data, LPS treatment increased iNOS expression in macrophage. However, this LPS induced iNOS expression was decreased by berberine treatment in a dose dependent manner. Furthermore, this effect of berberine seemed to be p38 and JNK dependent as evidenced by Western blot (Figure 14A). To see whether berberine induced iNOS inhibition resulted in decrease of NO production, LPS induced NO production with/without berberine pretreatment. Both LPS from *E.Coli* and *Streptococcus* increased NO production in macrophage, however, this increase was inhibited by berberine pretreatment (Figure 14B).

9. Effect of berberine on COX-1 and CD36 expression in macrophage.

LPS treatment increased COX-2 protein expression in macrophage, but no significant increase of COX-1 protein was observed. With berberine pretreatment, COX-1 protein expression was decreased while berberine failed to decrease COX-2 protein expression (Figure 15A). LPS treatment, which has been known to decrease CD36 expression, had no significant effect. However, berberine treatment synergistically decreased CD36 mRNA expression with LPS treatment (Figure 15B).

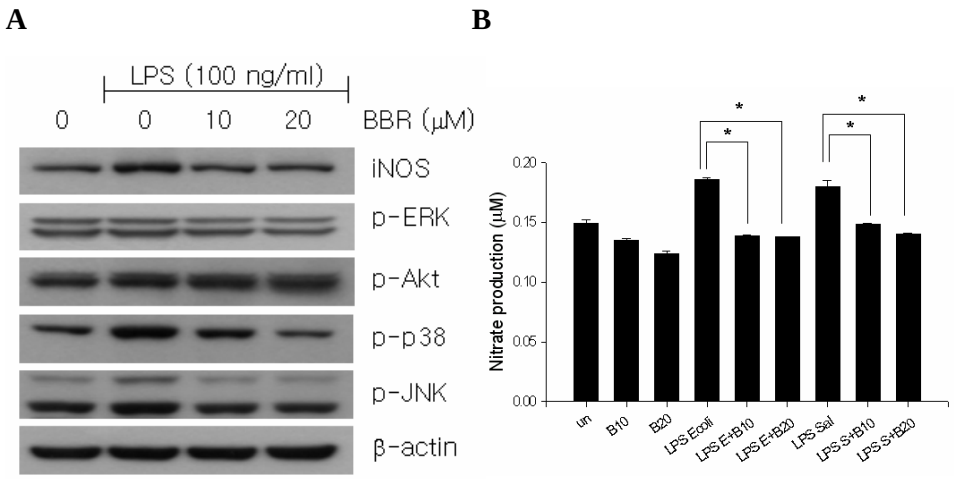


Figure 14. Effect of berberine on iNOS expression in macrophage. (A) Raw 264.7 cells were pretreated with berberine then iNOS expression was induced by LPS (100 ng/ml) for 24 hours. Cells then collected and iNOS expression was detected by Western blot. (B) Raw cells were pretreated with berberine then activated by LPS (100 ng/ml) for 24 hours. Production of NO was measured using Griess reagent. LPS E.coli: LPS originated from E. Coli, LPS St: LPS originated from Streptococcus. Results are means ± SEM of at least 3 experiments. ∗ indicates $p<0.05$.

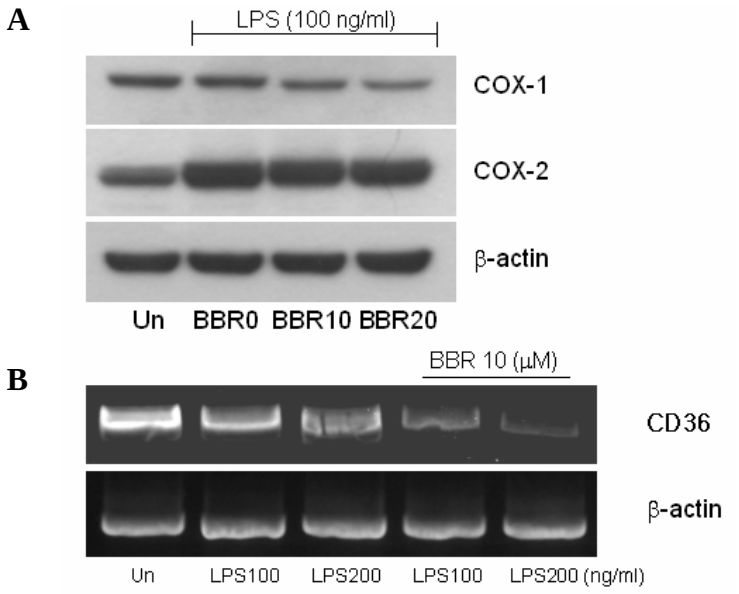


Figure 15. Berberine inhibits LPS induced COX-1 and CD36 expression in macrophages. (A) Raw cells were pretreated with berberine (10 μM) then activated by LPS (100 ng/ml). COX-1 and 2 expressions were detected by Western blot. (B) Raw cells were pretreated with berberine then activated with LPS. CD36 expression was detected by RT-PCR.

10. Effect of berberine on adipocyte differentiation and Foxo-1 activation

As evidenced by Oil red O staining, berberine treatment inhibited adipocyte differentiation in a dose dependent manner (Figure 16). To examine the underlying mechanism of this effect of berberine, activation status of Akt and foxo-1 was evaluated by Western blot. According to our data, berberine dose dependently decreased Akt phosphorylation and Foxo-1 phosphorylation. Furthermore, berberine also inhibited cell cycle inhibitor p27 expression (Figure 17A). We also observed that berberine inhibited PPARγ mRNA expression in a dose dependent manner (Figure 17B).

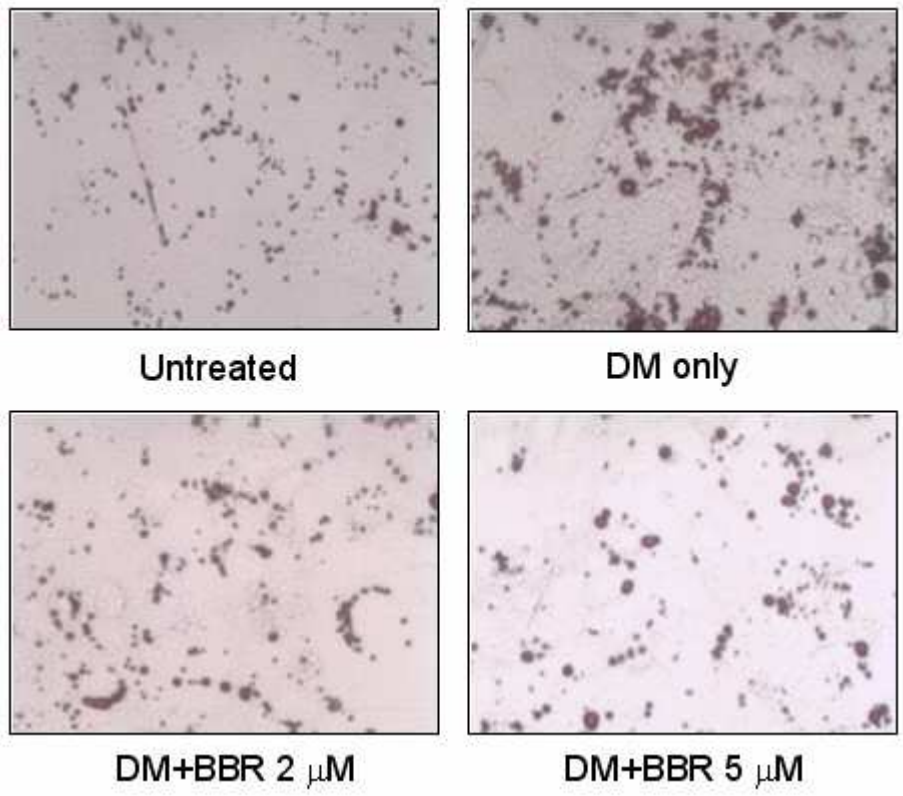


Figure 16. Berberine inhibits adipocyte differentiation in a dose dependent manner. Differentiation of 3T3 pre-adipocytes were induced by differentiation media for 5 days with or without berberine in the media. Adipocyte differentiation was evaluated by Oil red O staining. DM: 10% DMEM supplemented with insulin (1 μg/ml), isobutylmethylxanthine (0.25 μM), and dexamethasone (0.25 μM).

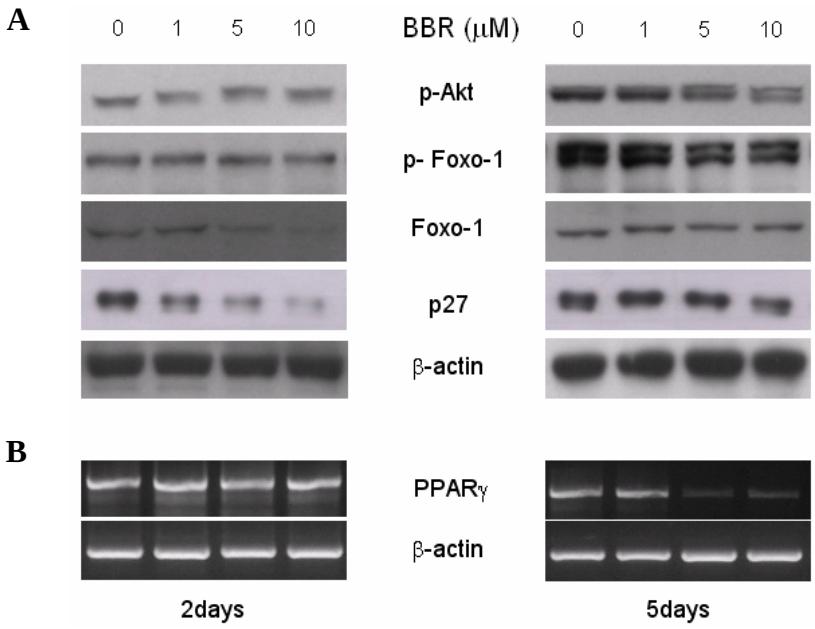


Figure 17. Berberine inhibits adipocyte differentiation possibly through PPARγ suppression by activating Foxo-1. (A) Pre-adipocytes treated with DM with/without berberine for 5 days. Cells were collected at 2 and 5 days, and expressions of p-Akt, p-Foxo1, and p27 were detected by Western blot. (B) Effect of berberine on PPARγ mRNA expression was evaluated by RT-PCR.

11. Effect of berberine on LDLR -/- mice fed western diet.

To evaluate the effect of berberine which dose not involve LDLR up-regulation, LDLR -/- mice were received berberine and western diet for 8 weeks. Serum lipid profile analysis result showed that berberine did not have lipid lowering effect without functional LDLR. On the contrary, berberine treatment significantly increased total cholesterol, TG, and LDL in LDLR -/- animals (Table 2). With berberine treatment, liver weight was slightly decreased while brown adipose tissue weight was slightly increased. However, these changes were not statistically significant. No change of white adipose tissue weight was observed (Figure 18). Berberine treatment also failed to decrease epididymal adipocyte size (Figure 19).

To examine the effect of berberine on lipid accumulation in blood vessel, thoracic aorta was stained with Oil red O. No significant effect of berberine on lipid accumulation of thoracic aorta was observed with naked eye examination (Figure 20). Berberine treatment failed to significantly inhibit lesion formation of aortic valve. However, lipid accumulation in the lesion was significantly decreased with berberine treatment (Figure 21). Furthermore, presence of macrophage in the lesion was also decreased with berberine treatment as evidenced by MOMA staining (Figure 22).

Table 2. Effect of berberine on the lipid profile of LDLR -/- mice fed western diet.

	0 weeks	4 weeks	8 weeks
Body Weight (g)			
Vehicle (n=6)	24.67 ± 1.54	26.45 ± 1.06	26.08 ± 1.39
Berberine 5mg (n=7)	22.98 ± 0.88	25.24 ± 0.96	24.98 ± 0.94
Berberine 10mg (n=7)	23.91 ± 0.93	25.96 ± 0.66	25.18 ± 0.86
Total Cholesterol (mg/dl)			
Vehicle (n=6)	319.00 ± 28.25	1001.00 ± 198.72	832.00 ± 131.70
Berberine 5mg (n=7)	376.29 ± 21.98	1814.57 ± 142.49	2166.86 ± 201.31
Berberine 10mg (n=7)	296.57 ± 30.62	2278.29 ± 155.70	2214.29 ± 230.72
Triglycerides (mg/dl)			
Vehicle (n=6)	215.00 ± 19.31	358.00 ± 49.98	635.33 ± 148.68
Berberine 5mg (n=7)	249.43 ± 22.48	558.86 ± 77.52	2018.29 ± 193.35
Berberine 10mg (n=7)	246.00 ± 28.45	625.71 ± 170.90	961.14 ± 294.40
HDL (mg/dl)			
Vehicle (n=6)	129.00 ± 10.25	89.00 ± 5.46	96.67 ± 5.10
Berberine 5mg (n=7)	144.86 ± 7.68	121.71 ± 6.10	112.57 ± 7.22
Berberine 10mg (n=7)	110.57 ± 14.57	135.43 ± 9.34	90.86 ± 6.15
LDL (mg/dl)			
Vehicle (n=6)	81.00 ± 7.86	319.00 ± 76.20	196.00 ± 44.08
Berberine 5mg (n=7)	81.43 ± 8.16	689.14 ± 42.82	581.14 ± 68.41
Berberine 10mg (n=7)	65.14 ± 4.62	816.86 ± 41.78	601.71 ± 82.77

Vehicle=DMSO

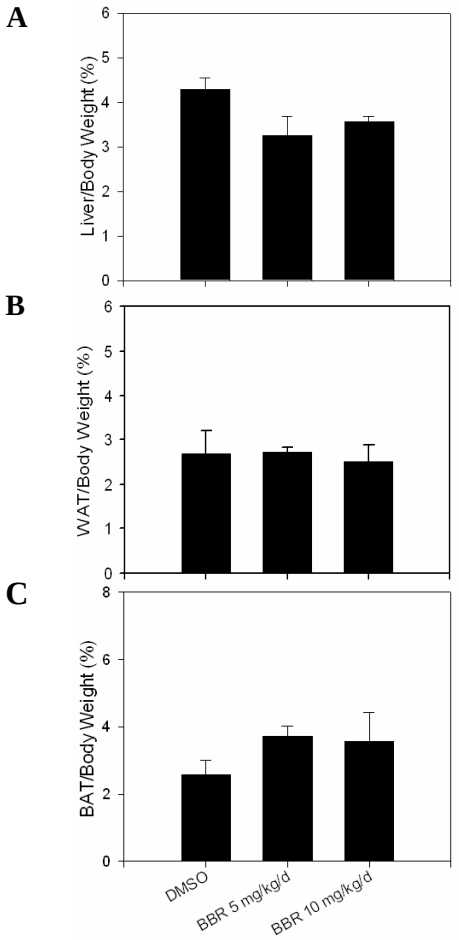


Figure 18. Effect of berberine on liver, white adipose tissue, and brown adipose tissue weight of LDLR -/- mice. LDLR -/- mice were treated with berberine as indicated for 8 weeks under western diet. At the end of experiment, animals were sacrificed and the weight of (A) liver, (B) white adipose tissue, and (C) brown adipose tissue were measured. Results are means ± SEM of at least 3 experiments.

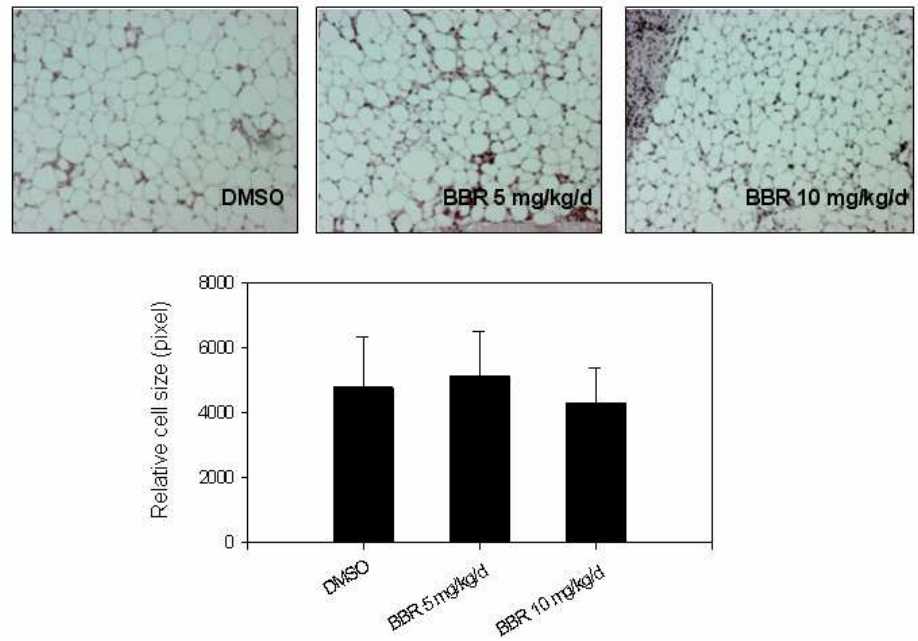


Figure 19. Effect of berberine on epididymal adipocyte size of LDLR -/- mice. LDLR -/- mice were treated with berberine as indicated for 8 weeks under western diet. At the end of experiment, animals were sacrificed and the size of epididymal adipocyte was measured. Results are means ± SEM of at least 3 experiments.

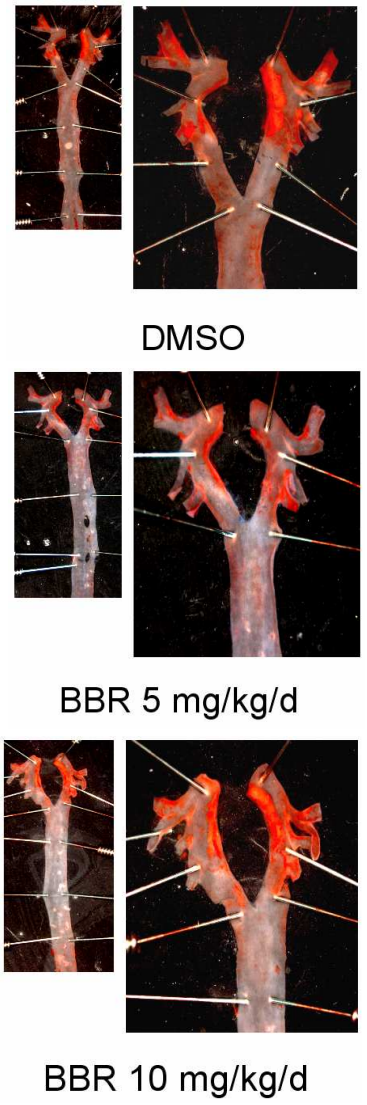


Figure 20. Effect of berberine on lipid accumulation of thoracic aorta. LDLR -/- mice were treated with berberine as indicated for 8 weeks under western diet. At the end of experiment, animals were sacrificed and the lipid accumulation of thoracic aorta was evaluated by Oil red O staining.

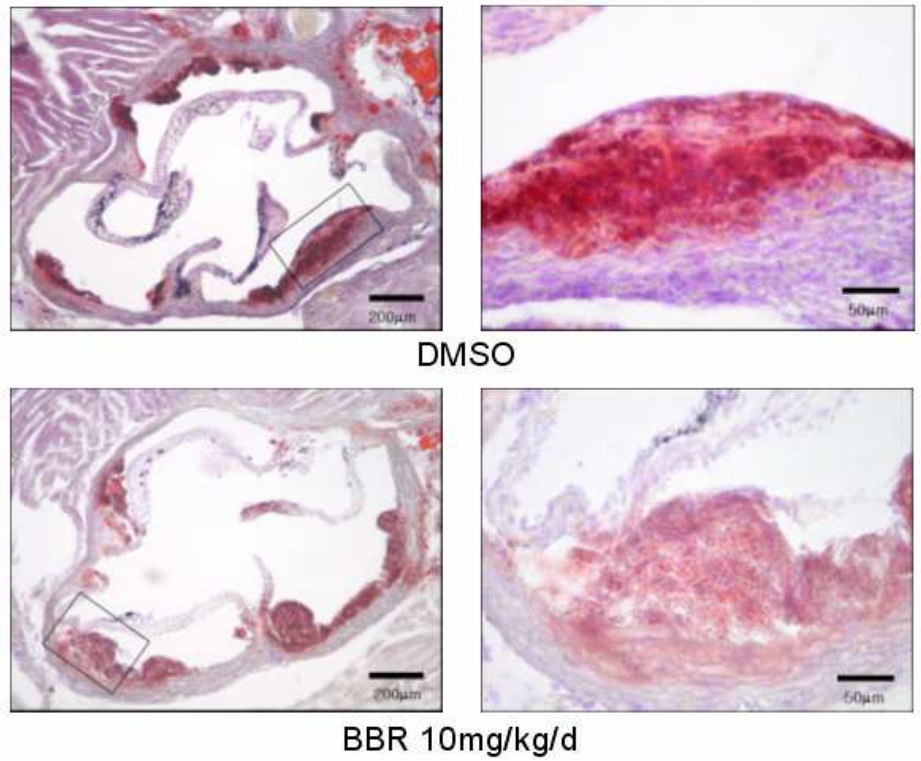


Figure 21. Effect of berberine on lipid accumulation in aortic valve atherosclerotic lesion. LDLR -/- mice were treated with berberine as indicated for 8 weeks under western diet. At the end of experiment, animals were sacrificed and the lipid accumulation of aortic valve was evaluated by Oil red O staining.

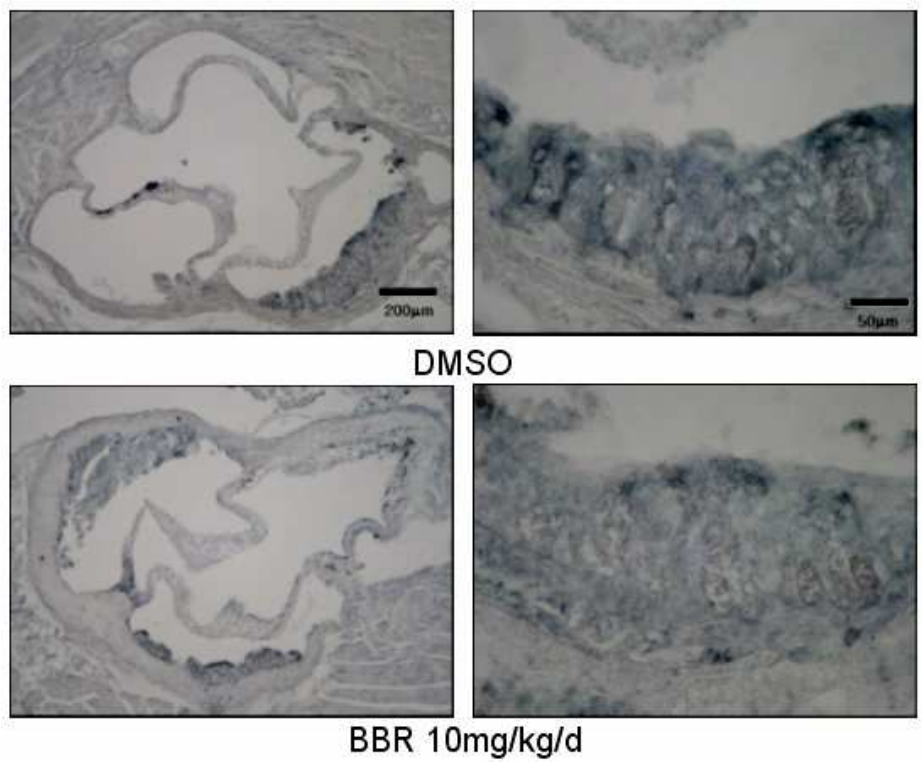


Figure 22. Effect of berberine on macrophage recruitment in aortic valve atherosclerotic lesion. LDLR -/- mice were treated with berberine as indicated for 8 weeks under western diet. At the end of experiment, animals were sacrificed and the presence of macrophage in atherosclerotic lesion of aortic valve was detected by MOMA staining.

VI. Discussion

Recent studies have shown that berberine exerts cardio-protective effects in animal models[21] and humans[22], has anti-hyperglycemic activity[23,24], exerts protective effects against hypertrophy[25,26] and ischemia-reperfusion injury[22,27], inhibits adipocyte differentiation[12,28,29], and lipid lowering effect[8,9]. Besides, it has been also known that berberine has positive inotropic, negative chronotropic, anti-arrhythmic, and vasodilator effects[30]. Despite these positive end results, its mode of action in terms of cellular signal transduction is still largely unknown. In the present study, based on the previous reports that berberine inhibited cancer cell proliferation[5,6] and endothelial cells[31], we first tested the hypothesis that berberine could also inhibit rVSMC proliferation which is one of the key events in the process of atherosclerosis[32]. To minimize cytotoxicity of berberine treatment, we used 10 μM of berberine, a minimal concentration which displayed no significant cytotoxicity in MTT assay (Figure 3A). Our results show 30 minutes of berberine pretreatment significantly inhibited rVSMC proliferation regardless of the type of growth factors (Figure 3B) indicating that there is growth factor type independent mechanism among the possible anti-proliferative mechanisms of berberine. One of the possible explanations for this general anti-proliferative effect of

berberine might come from the fact that berberine acts as a K^+ channel blocker. Although this K^+ channel blocking effect of berberine has been associated mostly with its anti- arrhythmic effect[33], it is possible that this K^+ channel blocking effect of berberine might also play an important role in inhibition of cellular proliferation since it has been reported that K^+ channel blocker inhibited cellular proliferation by increasing cellular volume[34].

To further characterize the effect of berberine, specifically on pathogenic cardiovascular conditions such as restenosis, we focused on its effect against AngII and HB-EGF. AngII is a well known pathogenic factor in atherosclerosis, hypertension, and restenosis[35], and it has been reported that AngII transactivates EGFR by using HB-EGF as a mediator[36]. Thus, we used these two factors to induce VSMC proliferation and migration. When rVSMCs were pretreated with berberine, AngII or HB-EGF induced cellular proliferation and DNA synthesis were significantly suppressed (Figure 4). For cellular migration, berberine also inhibited AngII or HB-EGF induced rVSMC migration measured by wound healing assay (Figure 5 and 6A). We also performed a Transwell assay using two different concentrations of AngII. Although, berberine pretreatment failed to inhibit cellular migration with statistical significance when high concentration of AngII was used to induce migration, the result essentially similar to the result of wound healing assay

(Figure 6B). These data indicate berberine effectively inhibits both VSMC proliferation and migration which are clinically important in the process of atherosclerosis.

To identify the underlying signal transduction pathway of this anti-proliferation and migration effect of berberine we examined the phosphorylation of Akt and ERK, two major molecules known to be involved in cellular proliferation. Our data shows that berberine pretreatment slightly decreased HB-EGF induced ERK phosphorylation but the effect was not significant (Figure 7). In addition, also the phosphorylation of EGFR, a receptor in the upstream of ERK1/2 pathway, was not significantly altered by berberine pretreatment in HB-EGF stimulated groups. On the contrary, berbernine pretreatment delayed and partially inhibited Akt phosphorylation and subsequent phosphorylation of p70S6K, a downstream effecter of Akt. This Akt pathway selective inhibitory effect was also observed when AngII were used in the place of HB-EGF (Figure 7). Although the previous studies have shown that AngII phosphorylates EGFR as early as few minutes after the stimulation using[37], we were not able to detect phosphoryltion of EGFR by AngII with standard Western blot using 20 μg of protein.

These data indicate that activation of EGFR by AngII or HB-EGF triggers two separate cellular proliferative signaling pathways Akt and ERK

1/2, in line with the previous study reported that MAPK and PI3K pathways are the two major downstream pathways of activated EGFR[38]. However, we were not able to elucidate how berberine selectively inhibits Akt phosphorylation without significantly affecting ERK 1/2 phosphorylation in the present study. At this moment, we can only speculate that berberine exerts its affect on upstream effectors of Akt pathway such as SHIP, PTEN, and PDK1/2.

In relation to these anti-proliferative and anti-migratory effects of berberine, its effect on cell cycle was also evaluated. Although the temporal patterns of AngII and HB-EGF induced phosphorylation of pRb were different, berberine suppressed phosphorylation of pRb at 6 hour in both cases (Figure 8A). Also cell cycle analysis indicated that berberine decreased the number of cells entering S phase (Figure 8B). However, such trend was not statistically significant. The discrepancy of temporal expression pattern of Rb phosphorylation might be explained by the fact that AngII requires activation of metalloprotease to transactivate EGFR utilizing HB-EGF[36]. Furthermore, throughout our experiment and at the given concentrations, AngII was relatively inferior to induce mitotic effect compared to HB-EGF. This suggests, at least in activating EGFR and its downstream cascade of events, AngII initiated signal cascade via angiotensin receptors might not be 100%

channeled to transactivate EGFR. This is probably why we observed relatively prompt and potent activity of HB-EGF compared to AngII.

In *in vivo* experiment, chronic berberine treatment moderately but significantly improved neointima formation after balloon injury (Figure 9). Since restenosis is a major limitation of balloon angioplasty in clinics and various drug eluting stents are currently being clinically tested but they also have certain limitations such as relatively short duration of drug release, difficulty in implanting stents in some cases, and their high costs[39], this result might be clinically significant because it suggests possibility of berberien as a orally administrable restenosis controlling agent. Considering the benefits berberine will bring if its inhibitory effect on neointima formation is once validated in a human trial, it would be worth while to further test berberine as a strong candidate agent to control restenosis.

We then investigated whether bereberine induced LDLR up-regulation solely dependent on ERK pathway as previously reported[9]. According to our data, berberine induced LDLR up-regulation was not only inhibited by ERK pathway inhibitors, also by JNK pathway inhibitor (Figure 11). We further confirmed that berberine treatment activated by phosphorylating JNK and its downstream molecule c-jun. This berberine induced activation of JNK pathway was also inhibited by JNK inhibitor

SP600125 (Figure 12). Since phosphorylated c-jun translocates into nucleus and acts as a transcription factor, we constructed luciferase reporter vectors with/without putative c-jun binding site of human LDLR promoter. According to the result of luciferase assay, berberine treatment significantly increased luciferase activity only in reporter vector with c-jun binding site and this increase was abolished JNK pathway inhibitor pretreatment (Figure 13). These results suggest berberine induced hepatic LDLR up-regulation also involves JNK pathway as well as ERK pathway.

We also examined the effect of berberine on macrophage using Raw 264.7 cells. According to our data, berberine treatment inhibited LPS induced iNOS expression and NO production. The result of Western blot indicates this effect of berberine possibly involves p38 and JNK pathway (Figure 14). Furthermore, berberine also inhibited COX-1 protein expression and CD36 mRNA expression (Figure 15). These data suggest that berberine can be a potential anti-inflammatory agent.

Regarding adipocyte differentiation, it has been reported that berberine inhibits adipocyte differentiation by down regulating PPARγ which is known to play an important role in adipocyte differentiation[12,13]. In search of new target of berberine in adipocyte differentiation, we found that Foxo-1 has been reported to be involved in adipocyte differentiation[14] and also down

regulates PPARγ expression[15]. Thus, in this study, we examined the effect of berberine on Foxo-1 activation during adipocyte differentiation. First we confirmed berberine treatment inhibits adipocyte differentiation (Figure 16). We also confirmed PPARγ mRNA expression was inhibited by berberine treatment as previously reported. According to our Western blot data, berberine treatment decreased phosphorylation of both Akt and Foxo-1 at day 5 (Figure17A). Since activated (dephoshporylated) Foxo-1 translocates into nucleus and acts as suppressor of PPARγ transcription[15], this result well agreed with decreased PPARγ expression (Figure 17B). In addition, berberine treatment also decreased cell cycle inhibitor p27 expression. It has been reported that p27 expression is increased during terminal differentiation[40]. Thus, it is plausible that berberine, in part, inhibits adipocyte differentiation by decreasing p27 expression.

Lastly, we investigated the effect of berberine on LDLR -/- mice to evaluate the effect of berberine other than LDLR up-regulation. Quite contrary to what we expected, 8 weeks of berberine treatment failed to improve lipid profile of LDLR -/- mice fed western diet (Table 2). As a matter of fact, berberine treatment worsened lipid profile. This result implies there might be a feedback mechanism involving berberine induced lipoprotein production and subsequent LDLR up-regulation. Otherwise, lipid profile of

berberine treated animals should have remained similar level as that of vehicle treated animals even without function LDLR present. We also examined the changes in the weight of liver, white adipose tissue or brown adipose tissue (Figure 18). Although berberine treatment slightly decreased the weight of liver while increasing the weight of brown adipose tissue, these changes were not statistically significant. Likewise, berberine treatment did not cause significant change of the epididymal adipocyte size (Figure 19) or lipid accumulation in thoracic aorta (Figure 20). Nevertheless, berberine treatment did decrease lipid accumulation in the aortic valve lesion (Figure 21) and this seems to be linked with decreased macrophage presence in the region (Figure 22).

These results regarding the effect of berberine on LDLR -/- can be clinically important because they indicate berberine might produce serious side effects in patients with disease caused by non-functional LDLR such as familial hypercholesterolemia. Thus, it is important to further evaluate the effect of berberine in various animal models such as LDLR -/+ animal to fully credit its bio safety before developing it as a therapeutic drug.

V. Conclusion

In the present study, we demonstrated that berberine can be beneficial to cardiovascular system by suppressing restenosis, inhibiting adipocyte differentiation, and decreasing immune response. In examining such effect of berberine, we found new transcriptional mechanism of berberine induced LDLR up-regulation and new potential target of berberine in adipocyte differentiation. However, we also found that berberine might have serious negative effect under the circumstances where functional LDLR is missing. This observation can be clinically important because it implies precaution should be taken in using berberine as a therapeutic agent for treating patient without functional LDLR such as Familial Hypercholesterolemia.

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Abstract (in Korean)

버베린의 항동맥경화 기전에 관한 연구

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중국에서 오랜 기간 배탈, 설사 등의 민간 치료에 널리 사용되어 온 버베린은 황백나무 껍질 등에서 추출되는 식물성 알칼리 물질로 현재까지의 연구 결과에 따르면 간의 LDLR 발현을 증가시켜 혈중 지질을 개선시키는 효과가 있는 것으로 알려져 있다. 그러나 이러한 연구 결과 이외의 버베린이 심혈관계에 미칠 수 영향을 전반적으로 살펴본 연구는 현재까지 없는 실정이다. 따라서 본 연구에서는 다양한 접근 방법과 실험을 통해 버베린이 심혈관계에 미치는 영향을 알아보고자 하였다.

먼저 본 연구를 통해 버베린이 동맥경화 또는 혈관 재협착 과정에서 중요한 역할을 하는 혈관평활근 세포의 증식과 이동을 효과적으로 감소시키는 것을 시험관 내에서 확인하였으며 이러한 효과를 백서의 경동맥 재협착 모델을 통해 생체 내에서도 확인하였다. 또한 기존의 연구결과와는 달리 ERK 신호 기전 이외에 JNK 신호 기전 역시 버베린에 의한 LDLR 발현 증가 과정에 관여하는 것을

확인하였다. 지방 세포의 분화에 미치는 영향과 관련해서는 버베린의 의한 지방 세포 분화 억제 기전에 Foxo-1이 관여하는 것을 발견하였으며 아마도 이러한 기전을 통해 지방 세포의 분화에 중요한 역할을 하는 것으로 알려진 PPARγ의 억제를 가져오는 것으로 생각된다.

또한 버베린이 지질 대사에 있어 LDLR 발현 증가 이외에 미치는 영향을 알아보기 위해 LDLR -/- 생쥐를 이용해 실험한 결과 버베린은 고지방식을 섭취한 이들 동물에서는 지질 개선의 효과가 없으며 오히려 악화시킨다는 것을 확인하였다. 버베린 처리는 대동맥 판막의 동맥경화반 형성 자체는 억제하지 못하였으나 이 경우에 있어 동맥경화반 내의 지방축적은 감소시키는 효과가 있음을 확인하였으며 이러한 효과는 아마도 동맥경화반으로의 대식세포의 유입 억제와 관련이 있는 것으로 생각된다.

본 연구를 통해 버베린은 재협착을 억제하고 지방 세포의 분화를 억제하며 염증 반응을 감소시키는 등의 기전을 통해 심혈관계에 긍정적인 효과를 갖는다는 것을 확인하였으며 이러한 연구 과정에서 버베린에 의한 LDLR 발현 증가 기전에 JNK 기전 역시 관여한다는 것과 지방세포 분화 억제 효과에는 Foxo-1이 관여한다는 것을 발견하였다. 또한 버베린은 LDLR -/- 동물 실험을 통해 LDLR의 기능이 손상된 경우 버베린에 의해 심각한 역효과가 생길

수 있다는 가능성 역시 확인하였다. 이 결과는 버베린이 임상적 약물로 사용되는 경우 LDLR의 기능이 손상된 가족성 고콜레스테롤혈증 환자들의 경우에는 그 사용에 있어 각별한 주의가 필요하다는 것을 시사한다.

핵심단어: 버베린, 재협착, Foxo-1, LDLR, 가족성 고콜레스테롤혈증

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