Activation of NAD(P)H:Quinone Oxidoreductase 1 Prevents Arterial Restenosis by Suppressing Vascular Smooth Muscle Cell Proliferation

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Abstract—Abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) are important pathogenic factors in atherosclerosis and restenosis after vascular injury. In this study, we investigated the effects of β-lapachone (βL) (3,4-Dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione), which is a potent antitumor agent that stimulates NAD(P)H:quinone oxidoreductase (NQO1) activity, on neointimal formation in animals given vascular injury and on the proliferation of VSMCs cultured in vitro. βL significantly reduced the neointimal formation induced by balloon injury. βL also dose-dependently inhibited the FCS- or platelet-derived growth factor–induced proliferation of VSMCs by inhibiting G1/S phase transition. βL increased the phosphorylation of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase 1 in rat and human VSMCs. Chemical inhibitors of AMPK or dominant-negative AMPK blocked the βL-induced suppression of cell proliferation and the G1 cell cycle arrest, in vitro and in vivo. The activation of AMPK in VSMCs by βL is mediated by LKB1 in the presence of NQO1. Taken together, these results show that βL inhibits VSMCs proliferation via the NQO1 and LKB1-dependent activation of AMPK. These observations provide the molecular basis that pharmacological stimulation of NQO1 activity is a new therapy for the treatment of vascular restenosis and/or atherosclerosis which are caused by proliferation of VSMCs. (Circ Res. 2009; 104:842-850.)

Key Words: vascular smooth muscle cell  β-lapachone  AMPK  NQO1  restenosis

Increased proliferation and migration of vascular smooth muscle cells (VSMCs) are important pathogenic factors in development of atherosclerosis and restenosis after percutaneous transluminal coronary angioplasty. Several signaling regulators, including mitogen-activated protein kinase (MAPKs) and nitric oxide, have been implicated in arterial restenosis and neointimal hyperplasia by increasing the proliferation and migration of VSMCs. Several therapeutic trials have been conducted based on these studies; however, the underlying molecular mechanisms are not completely understood.

AMP-activated protein kinase (AMPK), which is an important cellular fuel sensor, has been suggested to participate in the regulation of cell polarity and mitosis under the control of tumor suppressors; therefore, it is suggested that AMPK is an important therapeutic target to prevent or treat vascular proliferative diseases, as well as cancer. AMPK activation has been shown to cause cell cycle arrest in human aortic smooth muscle cells (SMCs) and rabbit aortic strips. In addition, the well-known AMPK activator, AICAR, inhibits angiotensin II–stimulated thymidine incorporation in SMCs, and administration of AICAR prevents neointimal formation in the rat balloon injury model. These observations suggest that AMPK activation can inhibit proliferative signaling of VSMCs from a variety of stimuli, including growth factors produced by macrophages and platelets as well as vascular injury, thus resulting in the maintenance of VSMCs in a quiescent state (similar to the G0 phase). The mechanism of cell cycle arrest associated with AMPK activation is mediated mainly by the upregulation of the expression and phosphor-
ylation of the tumor suppressor p53, which in turn leads to an increase in the expression of p21CIP, a cyclin-dependent kinase inhibitor (CDKI), via a transcriptional mechanism.\(^{11,12}\) In addition, AMPK activation phosphorylates and inactivates a number of metabolic enzymes that mediate ATP-consuming cellular events; these enzymes include acetyl-CoA carboxylase (ACC)I and HMG-CoA reductase, which are involved in synthesis of fatty acids and cholesterol, respectively. AMPK activation also activates ATP-generating processes, including the uptake and oxidation of glucose and fatty acids.\(^{14,15}\) At least two AMPK upstream kinases (AMPKK\(s\)) are expressed in mammals. The phosphorylation of the Thr172 site of AMPK is mediated by LKB1\(^{14-16,17}\) (which is activated by increase in AMP/ATP ratio) and by Ca\(^{2+}\)/calmodulin-dependent kinase \(\beta\) (CaMKK\(\beta\))\(^{18,19}\) (which is activated by cellular Ca\(^{2+}\) concentration). However, the relative importance of LKB1 and CaMKK \(\beta\) as a regulator of VSMC proliferation in vivo has not yet been determined.

NQO1 is a cytosolic antioxidant flavoprotein that catalyzes the reduction of the natural compound \(\beta\)-lapachone (\(\beta\)L) by using NAD(P)\(\text{H}\) as an electron donor.\(^{20,21}\) Accordingly, the cells treated with \(\beta\)L shows the accelerated NAD(P)\(\text{H}\) oxidation by NQO1 and this pharmacological effect is related to inhibitory roles of \(\beta\)L in cell proliferation, particularly in cancer cells which usually express high levels of NQO1. Interestingly, the expression of NQO1 is strongly induced by oxidative and inflammatory stresses, suggesting it is a useful pharmacological target in arterial restenosis and atherosclerosis. Based on these ideas,\(^{12,13}\) we investigated the potential effects of \(\beta\)L on VSMC proliferation. Here, we have shown for the first time that \(\beta\)L stimulation of NQO1 suppressed VSMCs proliferation in vitro and arterial neointima formation in vivo through the activation of AMPK.

Materials and Methods

**Materials**

\(\beta\)L (3,4-dihydro-2,2-dimethyl-2\(\text{H}\)-naphtho[1,2\(b\)]pyran-5,6-dione) was chemically synthesized by Mazence (Suwon, Korea). ES936, platelet-derived growth factor (PDGF), 5-aminoimidazole-4-carboxamide 1-\(\beta\)-ribofuranoside (AICAR), and BAPTA/AM [1,2-bis-(2-aminophenoxy)ethane-\(N,N',N''\)-tetraacetic acid acetoxymethyl ester] were purchased from Sigma Chemical Co (St Louis, Mo). Dicoumarol, compound C (Comp C), and ST0609 were purchased from Calbiochem (San Diego, Calif). The adenoviral expression vector of dominant-negative AMPK (Ad-DN-AMPK)\(^{23}\) and Ad-DN-LKB1\(^{24}\) were described previously. Human aortic smooth muscle cells (HASM\(c\)) were isolated from the thoracic aorta of kidney transplantation donors using the explant method, as described previously.\(^{24}\) Tissue collection was approved by the Ethics Committee of our Institution. Cells were cultured in DMEM (Gibco BRL, Frederick, Md) containing 20% FBS.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Results**

**\(\beta\)L Reduces Neointimal Hyperplasia in Balloon-Injured Rat Carotid Arteries**

The formation of the neointima was significantly increased in the BI group when compared with the control group. The neointimal hyperplasia induced by BI was significantly diminished in the \(\beta\)L-treated groups, in a dose-dependent manner (Figure 1A). The expression of Ki67, an index of cell proliferation,\(^{25}\) was upregulated in the neointimal region of the BI group; however, \(\beta\)L treatment significantly reduced the Ki67 expression induced by BI (Figure 1B). The number of Ki67-positive cells in the neointimal region of BI-\(\beta\)L group was significantly lower than that of BI group (Figure 1C).

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Effect of \(\beta\)L on neointimal formation after BI of rat carotid artery. A, Representative cross-sections of the left common carotid arteries obtained from control rats without BI (a), BI rats (b), BI rats treated with 100 mg/kg per day of \(\beta\)L (c), and BI rats treated with 200 mg/kg per day of \(\beta\)L (d). Arteries were obtained from rats 14 days after BI and were stained with hematoxylin/eosin. Pictures are shown at ×25 magnification.

Effects on Ki67 expression were measured in arterial sections of rats. B, Immunohistochemistry staining for Ki67. C, Cell number analysis of immunohistochemistry staining of Ki67. The left common carotid arteries were isolated from control rats, BI rats, and BI rats treated with \(\beta\)L (200 mg/kg per day) at day 7 after BI, and immunohistochemistry was carried out using Ki67 antibody following the instructions of the manufacturer. C, The analysis of immunohistochemistry staining of Ki67. The Ki67-positive cell number of samples in the section of Figure 1B were counted under microscope. The area of neointimal region was calculated by computer-based morphometric analyzer. The values are Ki67-positive cells/\(\mu\)m\(^2\); means±SEM obtained from 4 rats per group. N.D. indicates not determined because there is no neointimal formation in control group. *\(P<0.01\), BI-\(\beta\)L group vs BI group.
\(\beta L\) Inhibits the PDGF- or Serum-Induced Proliferation of VSMCs and DNA Synthesis in VSMCs

To examine whether \(\beta L\) modulates SMC proliferation in vitro, we investigated the proliferation and the DNA synthesis of primary isolated RASMCs in the presence of \(\beta L\). In our pharmacokinetic studies in vivo, administration of \(\beta L\) at a dose of 100 mg/kg body weight PO in rats gave a peak (maximum) plasma concentration of 3.2±1.3 \(\mu\)mol/L in 4 hours (supplemental Table I). Therefore, we chose 2 \(\mu\)mol/L \(\beta L\) in our in vitro study because 2 \(\mu\)mol/L \(\beta L\) could induce the AMPK phosphorylation without the cytotoxic effect in the VSMCs. The number of RASMCs was remarkably increased by PDGF or serum compared with that of the control (Figure 2A). Treatment with \(\beta L\) significantly reduced the number of RASMCs increased by either PDGF or serum (Figure 2A, left and middle, respectively). DNA synthesis rate, as measured by 5-bromodeoxyuridine (BrdUrd) incorporation assay, was significantly increased by serum treatment. Both cell number and DNA synthesis rate were dose dependently reduced in the presence of \(\beta L\) (Figure 2A, right). These data suggest that the inhibitory effect of \(\beta L\) on proliferation of RASMCs may not be attributable to apoptosis or necrosis but to cell cycle arrest.

Flow cytometric analysis showed that \(\beta L\) inhibited the \(G_1\)/S phase transition of RASMCs (Figure 2B). The S phase of RASMCs was significantly reduced in the presence of \(\beta L\) when compared with that of VSMCs treated with serum (5.24±2.99% versus 32.23±2.50%, respectively).

\(\beta L\) Activates p53 and p21 but Inhibits Phosphorylation of pRb

The p53 tumor suppressor is a key regulator of cell cycle. We examined the effect of \(\beta L\) on the activation of p53 protein and on the expression of p21 and p27, which are 2 key regulator proteins downstream of p53. Phosphorylation of p53\(^{Ser^{15}}\) was significantly increased by \(\beta L\) treatment at 1 hour and lasted for at least 6 hours (Figure 2C). The expression of p21 was significantly increased by \(\beta L\), whereas the levels of p27 were not changed (Figure 2C). We also examined the effect of \(\beta L\) on the phosphorylation of pRb, which is a cell cycle regulator protein that acts between the G1 and S phases. The phosphorylation of pRb (P-pRb) induced by incubation with serum was significantly suppressed by \(\beta L\) treatment (Figure 2D).
Other proteins involved in cell cycle regulation, ie, cyclin D and cyclin E, were also induced by incubation with serum, whereas this effect was significantly inhibited by \( \beta \text{L} \) treatment (Figure 2D). These results indicate that \( \beta \text{L} \) inhibited the proliferation of VSMCs by inhibition of the G1/S phase cell cycle transition, which is induced by activation of p53 and induction of p21.

**\( \beta \text{L} \) Activates AMPK and LKB1 in VSMCs**

As AMPK is a major regulator of intracellular energy balance and cell proliferation,\(^{12}\) we examined the effect of \( \beta \text{L} \) on the phosphorylation of AMPK and ACC1, which is a target of AMPK. \( \beta \text{L} \) increased the phosphorylation of AMPK and ACC1 in a time- and dose-dependent manner in primary isolated RASMCs (Figure 3A) and HASMCs (Figure I, A and B, in the online data supplement). \( \beta \text{L} \) also increased the phosphorylation of LKB1, which is an upstream kinase of AMPK. Activation of LKB1 by either \( \beta \text{L} \) or AICAR, using as a positive control, induced the phosphorylation of AMPK, Quescent RASMCs were pretreated with or without ST0609 (100 \( \mu \text{mol/L} \)) for 1 hour and were then incubated with 2 \( \mu \text{mol/L} \) \( \beta \text{L} \) for another 2 hours. Western blot analyses were performed using indicated antibodies.
mediated overexpression of the DN-LKB1 in RASMCs (supplemental Figure II).

We next examined the effect of βL on the cellular levels of ATP, AMP, NAD, and NADH in the RASMCs. As shown in Figure 3C, the ratios of [NAD]/[NADH] (top) and [AMP]/[ATP] (bottom) were significantly increased by βL treatment. These data indicated that βL-induced cellular energy depletion results in activation of LKB1-AMPK signaling pathway, which may lead to growth arrest of VSMCs. In addition, βL treatment induced the phosphorylation of AMPK and LKB1 in carotid arteries in vivo compared with those of the BI group (supplemental Figure III).

We further tested the effect of CaMKKβ on the βL-mediated activation of AMPK in RASMCs. The phosphorylation of AMPK and ACC1 by βL was not inhibited by a specific CaMKKβ inhibitor, STO609 (Figure 3D). These data suggest that activation of AMPK by βL is mediated by LKB1 but not by CaMKKβ.

**Inhibition of AMPK Activity Blocks the βL-Induced Suppression of VSMC Proliferation**

To further demonstrate that AMPK activation is necessary for the βL-induced suppression of VSMCs proliferation, we examined the effects of pharmacological or molecular AMPK inhibitors on VSMCs proliferation. Comp C (10 μmol/L), a specific inhibitor of AMPK,28 or DN-AMPK blocked the βL-induced suppression of VSMC proliferation in vitro (Figure 4A and 4E) and neointimal formation in vivo (supplemental Figure IV). The βL-induced phosphorylation of AMPK and ACC1 in RASMCs was significantly inhibited by pretreatment of Comp C (Figure 4B) or Ad-DN-AMPK (supplemental Figure VA). In addition, phosphorylation of p53 and expression of p21 induced by βL were blocked by pretreatment of Comp C (Figure 4C) or Ad-DN-AMPK (supplemental Figure VB), leading to the suppression of the βL-induced inhibition of pRB phosphorylation (Figure 4D).
**βL-Stimulated AMPK Activation Is Dependent on NQO1 Activity**

We next examined whether the βL-induced AMPK activation in VSMCs requires NQO1. βL-induced AMPK phosphorylation in RASMCs but not in NQO1-deficient HEK293 cells (Figure 5A). In contrast, the adenoviral overexpression of NQO1 in HEK293 cells led to the βL-mediated phosphorylation of AMPK (Figure 5B). Knockdown of NQO1 in RASMCs by small interfering (si)RNA inhibited the activation of LKB1-AMPK signaling pathway, leading to the inhibition of phosphorylation of ACC1 by βL (Figure 5C). The pharmacological inhibitors of NQO1, dicoumarol and ES936, prevented phosphorylation of AMPK and ACC1 by βL but not by AICAR (Figure 5D).

To further demonstrate these findings in relation to intracellular energy state, we examined the effect of NQO1 and/or βL on intracellular ATP levels. As shown in supplemental Figure VI, NQO1 overexpression alone did not change the cellular ATP level in HEK293 cells, but βL treatment in these cells led to a significant decrease in ATP level. In addition, βL-induced decrease in ATP level in RASMCs was blocked by Ad-si-NQO1 infection. Collectively, these results indicate that NQO1 is required for the βL-induced activation of AMPK.

**Inhibition of NQO1 Prevents the βL-Induced Suppression of VSMC Proliferation**

We examined whether βL regulates VSMC proliferation in an NQO1-dependent manner. Inhibition of NQO1 using siRNA or dicoumarol abrogated the βL-induced inhibition of cell proliferation and DNA synthesis (Figure 6A). Flow cytometric analysis showed that dicoumarol restored the βL-induced inhibition of the G1/S cell cycle transition (Figure 6B). We also examined the effect of dicoumarol on the phosphorylation of proteins involved in cell cycle control, i.e., p53 and pRb. The phosphorylation of p53 induced by βL was significantly suppressed by dicoumarol and led to the down-regulation of p21. The decrease in pRb phosphorylation mediated by βL was also restored by dicoumarol treatment (Figure 6C).

To clarify, whether the βL-induced cell growth arrest is related to other kinase activation, we further examined the effect of βL on phosphorylation of Erk. As shown in supplemental Figure VII, phosphorylation of Erk was not altered by βL treatment at given time compared to that of vehicle treated control.

**Toxicity Analysis of βL**

We next evaluated side effects of βL treatment in vivo. As shown in supplemental Table II, βL-treated rats did not show any significant abnormalities in serum GOT, GPT, creatinine, and albumin, but BUN was slightly increased in βL-treated group compared with vehicle group. βL treatment reduced food intake in rats in early time of experiment (first 4 days after BI), but the food intake came back to normal during last 9 days of experiment (supplemental Figure VIII). However, the body weight at the end of experiment in βL-treated rats is slightly lower than BI group (250±4 versus 282±5, respectively). These observations suggest that βL did not show any toxic effects in vivo. The difference of body weight between 2 groups may be a beneficial effect of βL because of AMPK activation and increase of energy expenditure.²⁶
Discussion

In the present study, we demonstrated for the first time that \( \beta \)L prevents the neointimal formation in vivo induced by vascular injury and inhibits the serum- or PDGF-induced proliferation of human or rat VSMCs in vitro. The expression of Ki67,\(^{25} \) a proliferation marker, was increased in the neointima of the BI group, but not in the \( \beta \)L-treated group, indicating that \( \beta \)L suppresses neointimal formation by decreasing cell proliferation in vivo. As suggested in Figure 6D, \( \beta \)L increased the [AMP]/[ATP] ratio (Figure 3C), which is presumably induced by enhanced oxidation of NADH. The activation of LKB1 mediated by an increase of [AMP]/[ATP] ratio and/or of [NAD]/[NADH] ratio induces phosphorylation of AMPK and leads to the activation of p53 and p21 (a cyclin-dependent kinase inhibitor). Activated p21 inhibits the activity of cyclins/CDKs and the phosphorylation of pRb, thus leading to cell cycle arrest in VSMCs.

Bey et al reported that \( \beta \)L induces apoptosis in non–small cell lung cancer cells, which is mediated by production of reactive oxygen species (ROS), induction of DNA damage by ROS, and depletion of ATP.\(^{20} \) In contrast, the suppressive mechanism of VSMC proliferation by \( \beta \)L appeared to be cell cycle arrest, rather than apoptosis, because the cell population of the G\(_1\) phase but not G\(_0\) phase was significantly increased by \( \beta \)L when compared with that of serum-induced cells. This result is in good agreement with the observation that AMPK activated by AICAR has antiapoptotic and antiproliferative effects in HASMCs.\(^{12} \)

NQO1 is an antioxidant flavoenzyme that uses NAD(P)H as an electron donor to catalyze the reduction of substrates. Previous studies suggest that \( \beta \)L is a specific and high-affinity substrate of NQO1 in vitro and in vivo.\(^{27–29} \) In this study, we have shown that the enhanced NAD(P)H oxidation induced by \( \beta \)L in NQO1-expressing VSMCs increases phosphorylation and activation of AMPK. The higher [NAD]/[NADH] and [AMP]/[ATP] ratios in \( \beta \)L-treated VSMCs led to the activation of AMPK (Figure 3C). Several pieces of evidence support that NQO1 is required for \( \beta \)L-induced AMPK activation. Phosphorylation of AMPK by \( \beta \)L treatment was demonstrated only in NQO1-expressing cells including VSMCs. However, NQO1-deficient HEK293 cells and RASMCs infected with Ad-si-NQO1 did not show AMPK activation by \( \beta \)L although AMPK was still activated by AICAR treatment in these cells (Figure 5C). Conversely, reexpression of NQO1 in NQO1-deficient HEK293 cells gains ability to activate AMPK in response to \( \beta \)L treatment.
Although NQO1 is an effective enzyme to catalyze the exogenous chemical substrates, the endogenous substrates are obscure and remain to be identified. In this situation, overexpression of catalytically active NQO1 in VSMCs or other cells is not enough to change the level of cytosolic NAD and NADH. Therefore, overexpression of NQO1 in cells without addition of exogenous substrates did not show AMPK activation and cell cycle arrest. Consistent with these findings, we have shown that NQO1-deficient and -expressing VSMCs show no differences in basal and serum stimulated cell proliferation (Figure 6A).

Depletion of cellular ATP levels induces activation of LKB1 and CaMKKβ caused by influx of Ca$^{2+}$, which ultimately results in the activation of AMPK. In this study, we examined the roles of LKB1 and CaMKKβ on the βL-induced activation of AMPK (Figure 3B and 3D). The βL-induced AMPK activation was presumably mediated by LKB1 but not CaMKKβ because AMPK was activated by βL in LKB1-expressing cells but not in LKB1 deficient cells and Ad-DN-LKB1-infected VSMCs (supplemental Figure II). Furthermore, activation of AMPK by βL was not inhibited by a CaMKKβ inhibitor (Figure 3D); however, we could not completely exclude the involvement of CaMKKβ in the signaling pathway.

Progression of the cell cycle is a tightly controlled process that is regulated positively by cyclins and cyclin-dependent kinases, and negatively by CDK inhibitors, p21, p27, and tumor suppressors. Previous reports suggest that activation of AMPK induces cell cycle arrest and suppresses proliferation of VSMCs and of rabbit aortic strips induced by FCS, PDGF, LPS, and VEGF. 


It has been reported that cyclin D is unstable and has a short half-life and that its degradation is mediated by the 26S proteasome in an ubiquitin-dependent manner. Further studies are required to elucidate the effect of βL on the stability of cyclins in VSMCs.

In summary, we demonstrated that βL-induced cellular NADH depletion mediated by NQO1 prevents the proliferation of VSMCs induced by serum in vitro and balloon injury in vivo, through cell cycle arrest. Our observations indicate that NQO1 may be a new drug target for the treatment of atherosclerosis and restenosis after percutaneous transluminal coronary angioplasty.

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**Disclosures**

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**References**


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