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 mechanisms 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 (NQO)1 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 G_1/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 G_1 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.[1] 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.[2–4] Several therapeutic trials have been conducted based on these studies[5–7]; however, the underlying molecular mechanisms are not completely understood.

AMP-activated protein kinase (AMPK), which is an important cellular fuel sensor,[8] has been suggested to participate in the regulation of cell polarity and mitosis under the control of tumor suppressors[9,10]; therefore, it is suggested that AMPK is an important therapeutic target to prevent or treat vascular proliferative diseases, as well as cancer.[11] AMPK activation has been shown to cause cell cycle arrest in human aortic smooth muscle cells (SMCs) and rabbit aortic strips.[12] 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.[13] 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 G_0 phase). The mechanism of cell cycle arrest associated with AMPK activation is mediated mainly by the upregulation of the expression and phosphor-

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ylation of the tumor suppressor p53, which in turn leads to an increase in the expression of $p21^{CIP}$, 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)1 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 (AMPKKs) 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 kinase β (CaMKKβ)[18,19] (which is activated by cellular Ca^{2+} concentration). However, the relative importance of LKB1 and CaMKK β 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 β-lapachone (βL) by using NAD(P)H as an electron donor.[20,21] Accordingly, the cells treated with βL shows the accelerated NAD(P)H oxidation by NQO1 and this pharmacological effect is related to inhibitory roles of βL 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 βL on VSMC proliferation. Here, we have shown for the first time that βL stimulation of NQO1 suppressed VSMCs proliferation in vitro and arterial neointimal formation in vivo through the activation of AMPK.

Materials and Methods

Materials

βL (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2b]pyran-5,6-dione) was chemically synthesized by Mazence (Suwon, Korea). ES936, platelet-derived growth factor (PDGF), 5-aminoimidazole-4-carboxamide 1-β-ribofuranoside (AICAR), and BAPTA/AM [1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester] were purchased from Sigma Chemical Co (St Louis, Mo). Dicoumarol, compound C (Comp C), and STO609 were purchased from Calbiochem (San Diego, Calif). The adenoviral expression vector of dominant-negative AMPK (Ad-DN-AMPK)[22] and Ad-DN-LKB1[23] were described previously. Human aortic smooth muscle cells (HASMCs) 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

βL 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 βL-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, βL treatment significantly reduced the Ki67 expression induced by BI (Figure 1B). The number of Ki670-positive cells in the neointimal region of BI-βL group was significantly lower than that of BI group (Figure 1C).

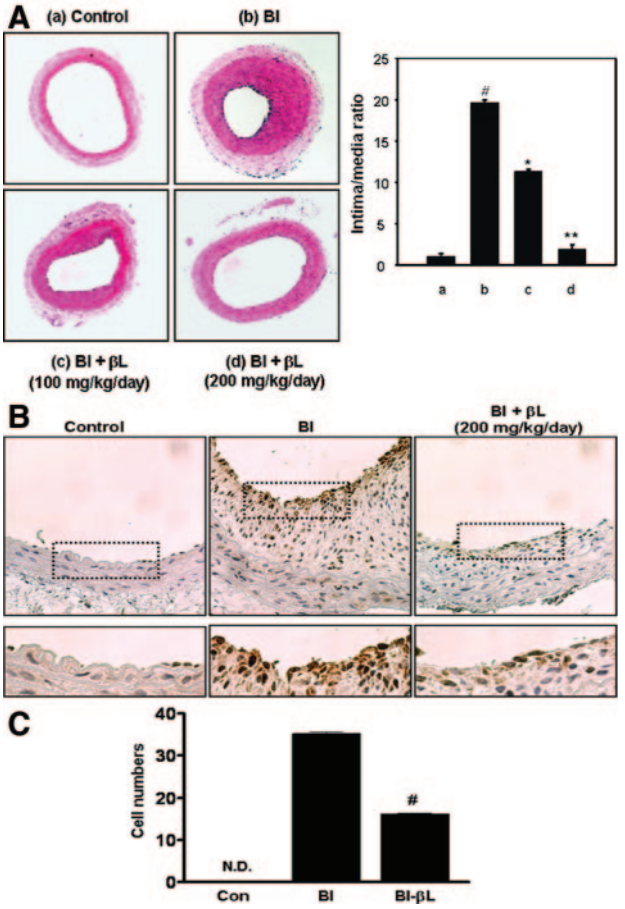

Figure 1. Effect of βL on neointimal formation after BI of rat carotid artery. A, Representative cross-sections of the left common carotid arteries obtained from control rats without BI (a), BI rats (b), BI rats treated with 100 mg/kg per day of βL (c), and BI rats treated with 200 mg/kg per day of βL (d). Arteries were obtained from rats 14 days after BI and were stained with hematoxylin/eosin. Pictures are shown at ×25 magnification. For morphometric analysis, the width of the medial and intimal layers was measured. Neointimal formation was analyzed by the ratio of intima/media. Values represent means±SEM (n=4), with statistical significance determined by Students *t* test as #$P<0.005$ vs control, and *$P<0.05$ and **$P<0.005$ when compared with BI rats. B, Immunohistochemistry staining for Ki67. The left common carotid arteries were isolated from control rats, BI rats, and BI rats treated with βL (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 numbers 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 Ki670-positive cells/μm^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-βL group vs BI group.

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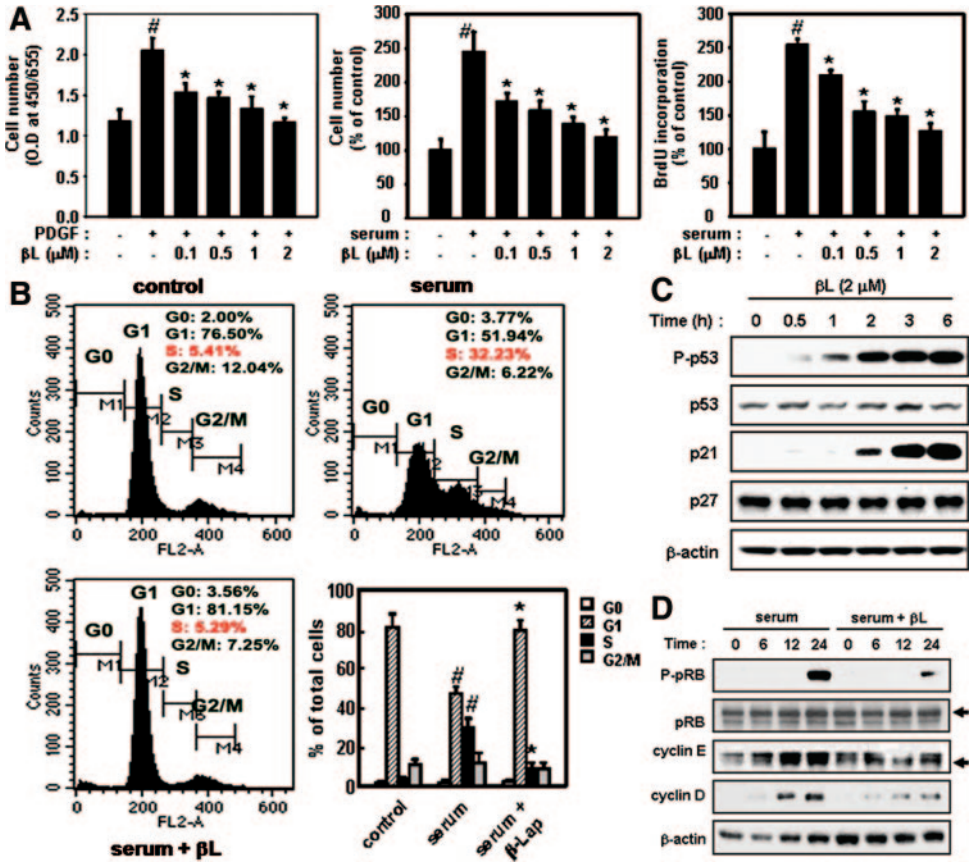

Figure 2. Effects of βL on the proliferation of primary RASMCs. A, Effect of βL on the proliferation and BrdUrd incorporation of primary RASMCs. Cells incubated with serum-starved medium for 24 hours were stimulated with 20 ng/mL PDGF (left) or 15% serum (middle), together with or without the indicated concentrations of βL. Cells were harvested after 48 hours of incubation, and cell density was determined using the trypan blue staining method. For the ELISA-based BrdUrd incorporation assay, quiescent cells grown on 96-well plates were stimulated with 15% serum in the presence or absence of βL. After 20 hours of incubation, the BrdUrd assay was performed following the instructions of the manufacturer. Values represent means±SEM from at least 3 independent experiments. #$P<0.01$ vs control and *$P<0.05$ vs PDGF- or serum-stimulated. B, Cell cycle analysis by flow cytometry. Quiescent cells were pretreated with or without 2 μmol/L βL for 2 hours and were then stimulated with serum for 20 hours. Cells were harvested and fixed with 4% paraformaldehyde, permeabilized, and then stained with propidium iodide. Cells (200 000 cells) were separated by flow cytometer. Data are the percentage of cells in each cell cycle vs the total number of cells. Values represent means±SEM obtained from 3 independent experiments. C, Quiescent cells were incubated with βL for the indicated period of time and were then harvested. Western blot analyses were performed using P-p53, p53, p21, and p27 antibodies, following the instructions of the manufacturer. D, Quiescent cells were pretreated with or without βL for 2 hours and were then stimulated with serum for the indicated time periods. Western blot analyses were performed using antibodies for P-pRb, pRb, cyclin D, and cyclin E. The arrow represents the specific protein band. For C and D, 20 μg of cell extracts were separated on a 10% SDS-PAGE and transferred to a poly(vinylidene fluoride) membrane. β-Actin was used as a protein loading control.

βL Inhibits the PDGF- or Serum-Induced Proliferation of VSMCs and DNA Synthesis in VSMCs

To examine whether βL modulates SMC proliferation in vitro, we investigated the proliferation and the DNA synthesis of primary isolated RASMCs in the presence of βL. In our pharmacokinetic studies in vivo, administration of βL at a dose of 100 mg/kg body weight PO in rats gave a peak (maximum) plasma concentration of 3.2±1.3 μmol/L in 4 hours (supplemental Table I). Therefore, we choose 2 μmol/L βL in our in vitro study because 2 μmol/L βL 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 βL 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 βL (Figure 2A, right). These data suggest that the inhibitory effect of βL on proliferation of RASMCs may not be attributable to apoptosis or necrosis but to cell cycle arrest.

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

βL 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 βL 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 $p53^{Ser18}$ was significantly increased by βL treatment at 1 hour and lasted for at least 6 hours (Figure 2C). The expression of p21 was significantly increased by βL, whereas the levels of p27 were not changed (Figure 2C). We also examined the effect of βL on the phosphorylation of pRb, which is a cell cycle regulator protein that acts between the G_1 and S phases. The phosphorylation of pRb (P-pRb) induced by incubation with serum was significantly suppressed by βL treatment (Figure 2D).

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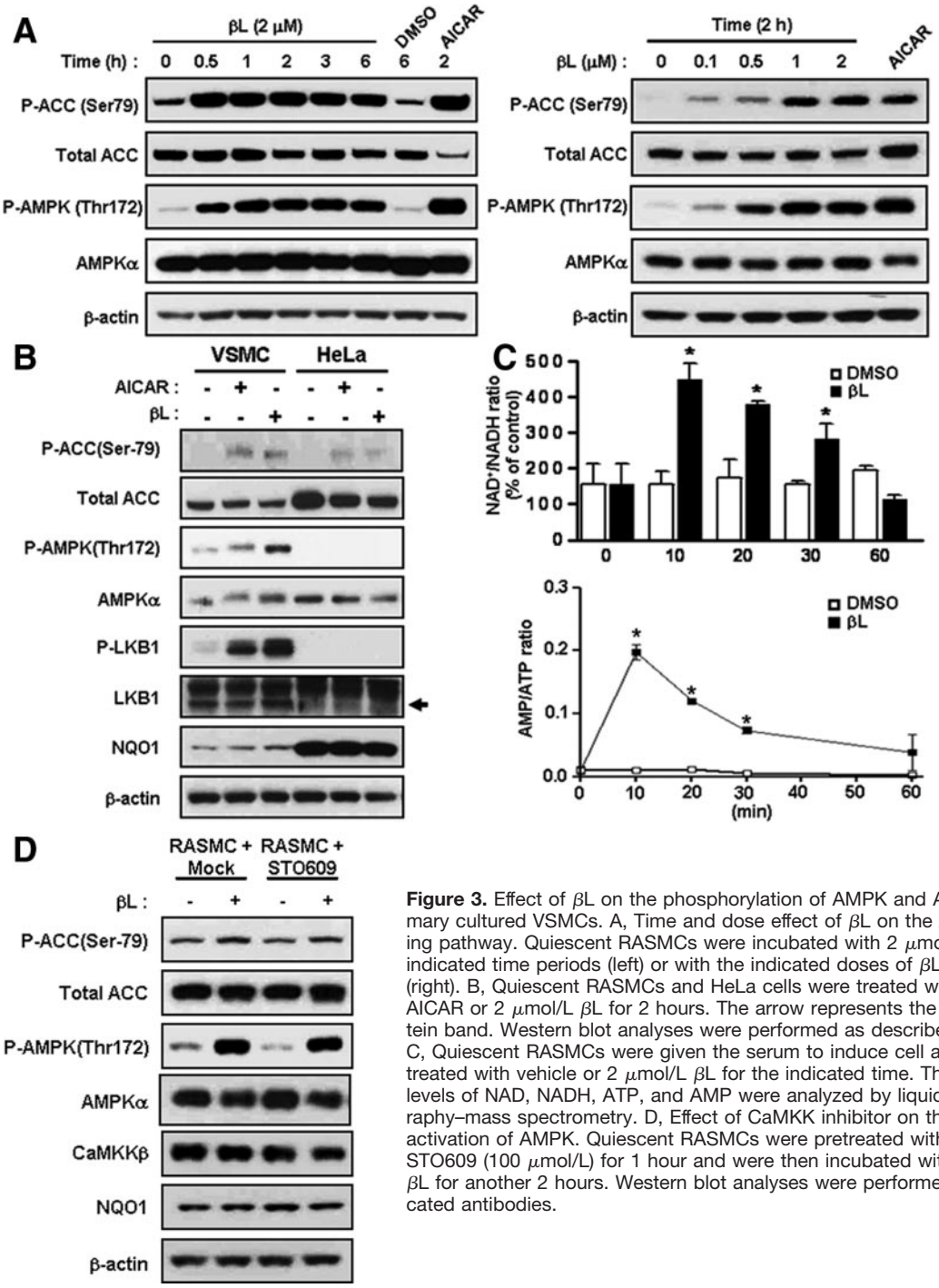

Figure 3. Effect of βL on the phosphorylation of AMPK and ACC1 in primary cultured VSMCs. A, Time and dose effect of βL on the AMPK signaling pathway. Quiescent RASMCs were incubated with 2 μmol/L βL for the indicated time periods (left) or with the indicated doses of βL for 2 hours (right). B, Quiescent RASMCs and HeLa cells were treated with 1 mmol/L AICAR or 2 μmol/L βL for 2 hours. The arrow represents the specific protein band. Western blot analyses were performed as described in Figure 2. C, Quiescent RASMCs were given the serum to induce cell activation and treated with vehicle or 2 μmol/L βL for the indicated time. The intracellular levels of NAD, NADH, ATP, and AMP were analyzed by liquid chromatography–mass spectrometry. D, Effect of CaMKK inhibitor on the βL-induced activation of AMPK. Quiescent RASMCs were pretreated with or without STO609 (100 μmol/L) for 1 hour and were then incubated with 2 μmol/L βL for another 2 hours. Western blot analyses were performed using indicated antibodies.

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 βL treatment (Figure 2D). These results indicate that βL inhibited the proliferation of VSMCs by inhibition of the G_1/S phase cell cycle transition, which is induced by activation of p53 and induction of p21.

βL 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 βL on the phosphorylation of AMPK and ACC1, which is a target of AMPK. βL 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). βL also increased the phosphorylation of LKB1, which is an upstream kinase of AMPK. Activation of LKB1 by either βL or AICAR, using as a positive control, induced the phosphorylation of AMPK and ACC1 in RASMCs but not in HeLa cells that lack LKB1 expression (Figure 3B).[19] The phosphorylation of AMPK and ACC1 induced by βL treatment was inhibited by adenovirus-

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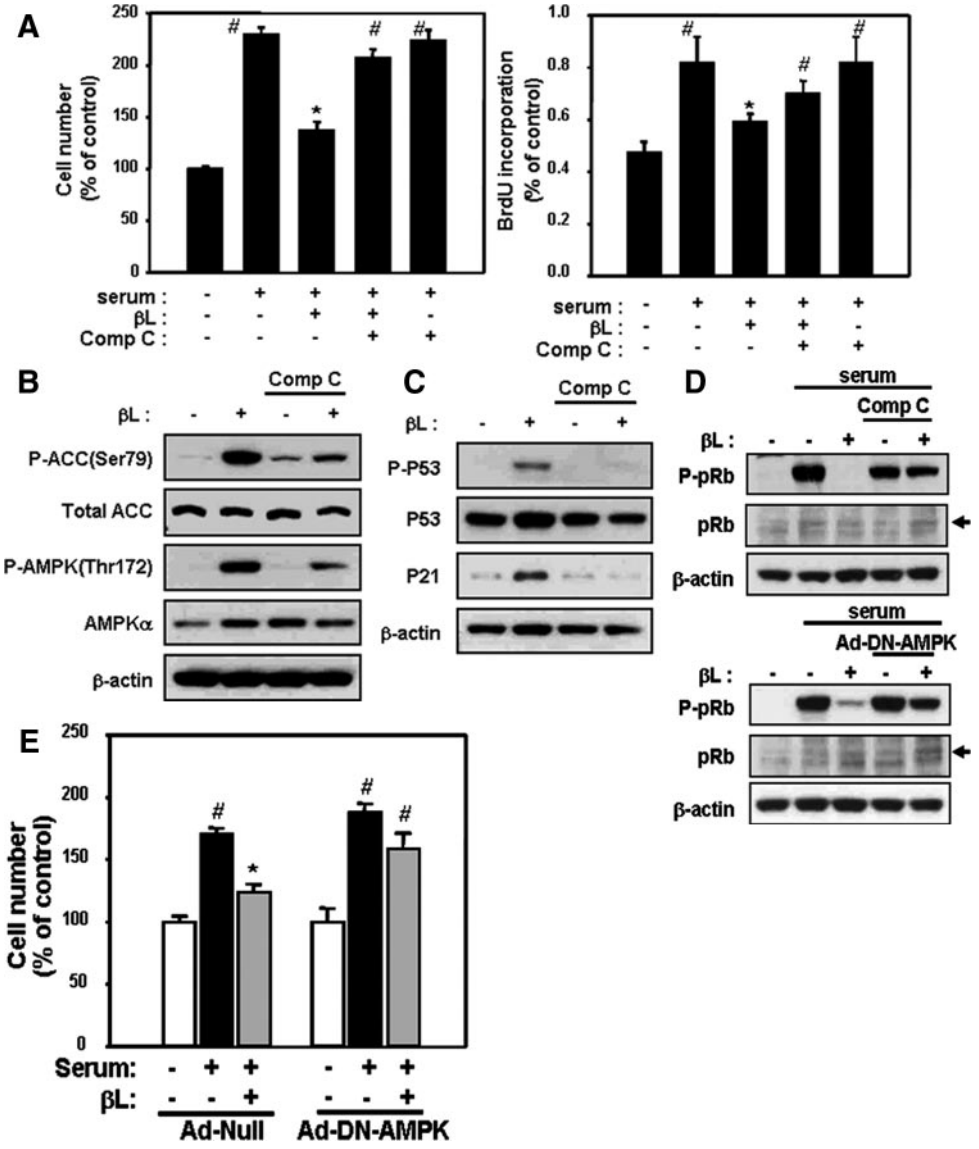

Figure 4. The effect of βL on the suppression of VSMC proliferation is dependent on AMPK. A, Quiescent VSMCs grown in 96-well plates were pretreated with 10 μmol/L Comp C for 2 hours. Cells were then stimulated with serum in the presence or absence of βL, cell density was determined using the trypan blue counting assay (left), and cell proliferation was determined by BrdUrd assay (right). #$P<0.01$ vs control and *$P<0.05$ vs serum-stimulated. B and C, Quiescent VSMCs were pretreated with Comp C for 2 hours and were then stimulated with serum in the presence or absence of βL. Western blot analyses were performed using the indicated antibodies. D, Quiescent cells were pretreated with Comp C (top) or infected with Ad-DN-AMPK (bottom) and were then stimulated by serum in the presence or absence of βL. Western blot analyses were performed using the indicated antibodies. Twenty micrograms of protein were used in each lane, and β-actin was used to confirm equal loading. Representative blots of 3 independent experiments are shown. The arrow represents the specific protein band. E, Quiescent VSMCs grown in 6-well plates were infected with adenoviral vectors expressing Ad-null or Ad-DN-AMPK and were stimulated with serum for 20 hours in the presence or absence of βL. Cell density was determined using the trypan blue cell counting assay. #$P<0.01$ vs control and *$P<0.05$ vs serum-stimulated.

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,[26] 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).

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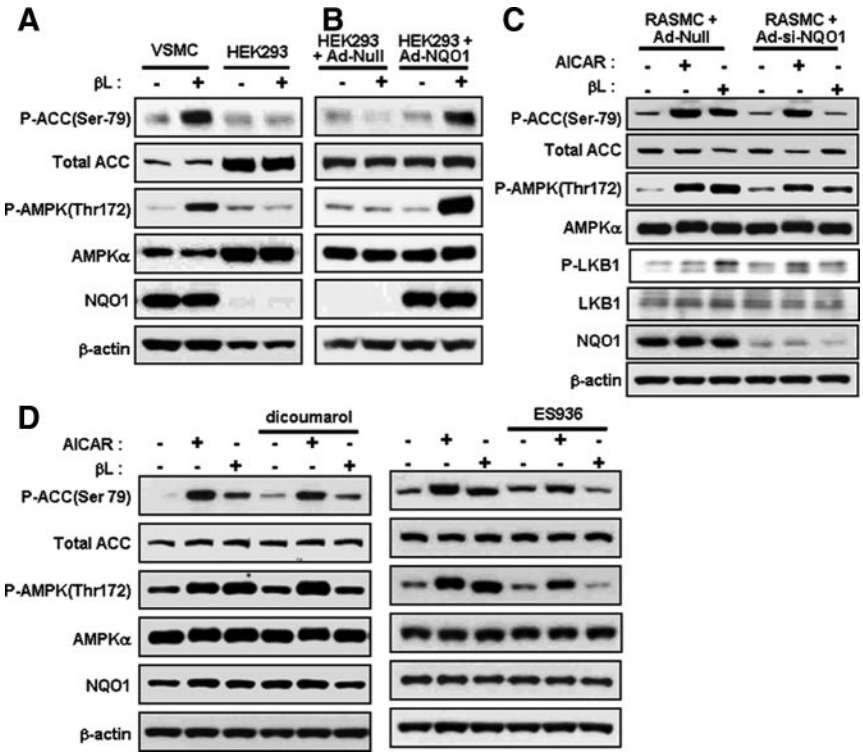

Figure 5. βL-induced AMPK activation requires NQO1. A, Quiescent VSMCs or HEK293 cells were treated with 2 μmol/L βL for 2 hours. B, HEK293 cells were infected with adenoviral vectors expressing null or NQO1 and were incubated for 24 hours. The cells were then treated with 2 μmol/L βL for 2 hours. C, Quiescent VSMCs were infected with adenoviral vectors expressing null or siRNA of NQO1 and were incubated for 24 hours. The cells were then treated with 2 μmol/L βL or 1 mmol/L AICAR for 2 hours. D, Quiescent VSMCs pretreated with 100 μmol/L dicoumarol (left) for 30 minutes or 250 nmol/L ES936 (right) for 1 hour were treated with 2 μmol/L βL or 1 mmol/L AICAR for 2 hours. Western blot analyses were performed using the indicated antibodies. Twenty micrograms of proteins were loaded in each lane and β-actin was used to confirm equal loading. Representative blots of 3 independent experiments are shown.

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 G_1/S cell cycle transition (Figure 6B). We also examined the effect of dicoumarol on the phosphorylation of proteins involved in cell cycle control, ie, 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 with 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 showed 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]

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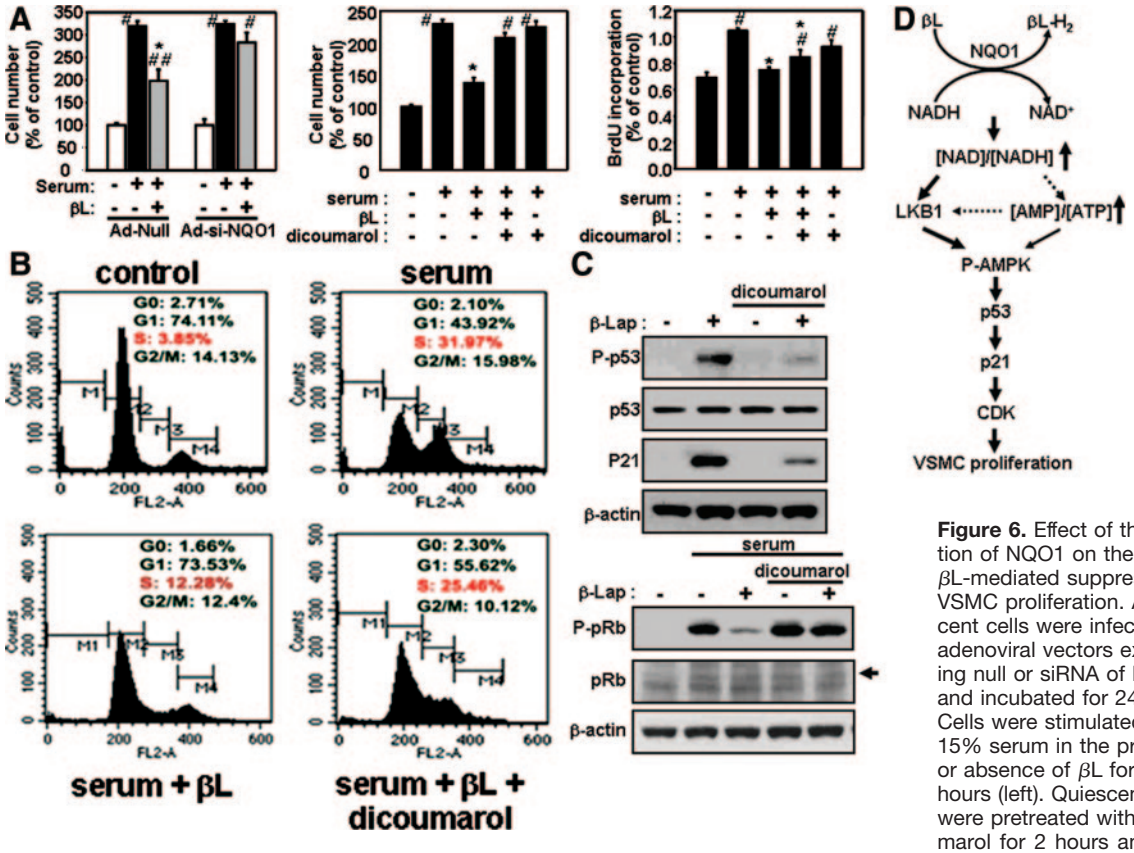

Figure 6. Effect of the inhibition of NQO1 on the βL-mediated suppression of VSMC proliferation. A, Quiescent cells were infected with adenoviral vectors expressing null or siRNA of NQO1 and incubated for 24 hours. Cells were stimulated with 15% serum in the presence or absence of βL for 48 hours (left). Quiescent cells were pretreated with dicoumarol for 2 hours and stimulated with 15% serum in the presence or absence of βL for 48 hours (middle). Cell density was determined using the trypan blue counting assay. For the BrdUrd incorporation assay, cells were prepared as described above and then incubated with BrdUrd for 24 hours, and the BrdUrd incorporation assay was performed using the instructions of the manufacturer. #$P<0.01$ and ##$P<0.05$ vs control, and *$P<0.05$ vs serum-stimulated. B, Quiescent cells pretreated with 100 μmol/L dicoumarol for 30 minutes were stimulated with serum in the presence or absence of βL for 20 hours. The percentage of hypodiploid cells was then determined using FACS analysis. C, Quiescent VSMCs pretreated with 100 μmol/L dicoumarol for 30 minutes were treated with 2 μmol/L βL for 2 hours. Western blot analyses were performed using the indicated antibodies. Twenty micrograms of protein were loaded in each lane and β-actin was used to confirm equal loading. Representative blots of 3 independent experiments are shown. The arrow represents the specific protein band. D, Schematic model for the inhibitory mechanism of βL on the cell cycle arrest in VSMCs.

Discussion

In the present study, we demonstrated for the first time that βL 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 βL-treated group, indicating that βL suppresses neointimal formation by decreasing cell proliferation in vivo. As suggested in Figure 6D, βL 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 βL 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 βL 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 βL 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 a antioxidant flavoenzyme that uses NAD(P)H as an electron donor to the catalyze the reduction of substrates. Previous studies suggest that βL 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 βL in NQO1-expressing VSMCs increases phosphorylation and activation of AMPK. The higher [NAD]/[NADH] and [AMP]/[ATP] ratios in βL-treated VSMCs led to the activation of AMPK (Figure 3C). Several pieces of evidence support that NQO1 is required for βL-induced AMPK activation. Phosphorylation of AMPK by βL treatment was demonstrated only in NQO1-expressing cells including VSMCs. However, NQO1-deficent HEK293 cells and RASMCs infected with Ad-si-NQO1 did not show AMPK activation by βL 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 βL treatment.

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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.[14,18,19,30] 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, or angiotensin II.[12,13] Igata et al showed that AMPK activated by AICAR induces phosphorylation of p53 and upregulates p21, but not p27, in VSMCs, which results in cell growth arrest.[12] Overexpression of p21 but not p27 prevents proliferation of human glioma cells.[31] These results indicate that p21 may be a major regulator of p53-induced cell growth arrest in VSMCs, as well as in certain cancer cells. The observations gleaned from the present study indicate that the upregulation of p21 mediated by activated p53 results in the suppression of cyclin E and cyclin D (Figure 2D), which leads to the inhibition of the formation of cyclin D/E and cyclin-dependent kinase (CDK) complexes. The cyclin D and cyclin E suppressive mechanisms are not clearly established. 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.[32–34] 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 prevented 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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Activation of NAD(P)H:Quinone Oxidoreductase 1 Prevents Arterial Restenosis by Suppressing Vascular Smooth Muscle Cell Proliferation

Sun-Yee Kim, Nam Ho Jeoung, Chang Joo Oh, Young-Keun Choi, Hyo-Jeong Lee, Han-Jong Kim, Joon-Young Kim, Jung Hwan Hwang, Surendar Tadi, Yong-Hyeon Yim, Ki-Up Lee, Keun-Gyu Park, Seung Huh, Ki-Nam Min, Kyeong-Hoon Jeong, Myoung Gyu Park, Tae Hwan Kwak, Gi Ryang Kweon, Kouichi Inukai, Minho Shong and In-Kyu Lee

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