Journal of Cardiology (2009) **54**, 375—385

ELSEVIER

JOURNAL of CARDIOLOGY
Official Journal of the Japanese College of Cardiology
www.elsevier.com/locate/jjcc

ORIGINAL ARTICLE

The effect of alpha lipoic acid in a porcine in-stent restenosis model

Sang Yup Lim (MD)[a], Eun Hui Bae (MD)[b], Myung Ho Jeong (MD)[b,*], Ju Han Kim (MD)[b], Young Joon Hong (MD)[b], Doo Sun Sim (MD)[b], Yong Sook Kim (PhD)[b], In Kyu Park (PhD)[b], Youngkeun Ahn (MD)[b], Sun-Jung Song (PhD)[c], Dong Lyun Cho (PhD)[c], Kyoung Seok Kim (MS)[c], Jung Chaee Kang (MD)[b]

[a] *The Cardiovascular Center of Korea University Ansan Hospital, Seoul, Republic of Korea*
[b] *The Heart Center of Chonnam National University Hospital, Gwang Ju, Republic of Korea*
[c] *Center for Functional Nano Fine Chemicals of Chonnam National University, Gwangju, Republic of Korea*

Received 10 April 2009; received in revised form 4 June 2009; accepted 12 June 2009
Available online 23 July 2009

KEYWORDS
Stents;
Restenosis;
Inflammation;
Endothelium

Summary
Background: The aim of this study was to investigate the effect of alpha lipoic acid (α-LA) on a porcine in-stent restenosis (ISR) model.
Methods: In protocol 1, porcine vascular smooth muscle cells (PVSMC) were stimulated by granulocyte-colony stimulating factor (G-CSF) in the presence or absence of α-LA. MTT (3-[4,5-dimethylthiazole-2-yl] 2,5-diphenyl tetrazolium bromide) assay and western blotting were used to determine the cell growth inhibitory rate and anti-inflammatory effect associated with nuclear factor-κb (NF-κb) and extracellular signal-regulated kinase (ERK). In protocol 2, 28 days after balloon overdilation injuries, 24 bare metal stents were placed in coronary artery of 12 pigs. The pigs were randomly divided to receive control diet with or without α-LA (100 mg/kg). In protocol 3, 8 control stents and 8 α-LA coated stents were randomly implanted in 2 coronary arteries of 8 pigs and follow-up coronary angiogram and histopathologic assessment were performed 4 weeks after stenting.
Results: Protocol 1. The proliferation of PVSMC was inhibited and protein expression of NF-κb and ERK were attenuated by α-LA pretreatment. Protocol 2. On histopathologic analysis, the neointimal area ($4.0 \pm 1.0\,mm^2$ vs. $1.5 \pm 0.7\,mm^2$, $p < 0.001$) and histopathologic area of stenosis ($66.7 \pm 10.7\%$ vs. $24.2 \pm 9.7\%$, $p < 0.001$) were reduced in the α-LA feeding group compared to controls. Protocol 3. On histopathologic analysis, the neointimal area ($3.9 \pm 0.8\,mm^2$ vs. $1.0 \pm 0.4\,mm^2$, $p < 0.001$), and

* Corresponding author at: Cardiovascular Research Institute of Chonnam National University, The Heart Research Center of Chonnam National University Hospital, 8 Hak-dong, Dong-gu, Gwangju 501-757, Republic of Korea. Tel.: +82 62 220 6243; fax: +82 62 228 7174.
E-mail address: myungho@chollian.net (M.H. Jeong).

0914-5087/$ — see front matter
doi:10.1016/j.jjcc.2009.06.005

the histopathologic area of stenosis (67.1 ± 8.8% vs. 17.4 ± 10.0%, $p < 0.001$) were reduced in the α-LA coated stent group compared to the control stent group.
Conclusions: α-LA feeding and α-LA coated stents inhibit neointimal hyperplasia in porcine ISR, possibly through inhibiting the activation of NF-κb pathway and proliferation of PVSMC.

Introduction

Despite improvements in coronary interventions, in-stent restenosis (ISR) remains a major problem [1—3]. Drug-eluting stents (DES) have been considered the most successful tool in preventing ISR. Unfortunately, it has been reported that DES may result in delayed arterial healing and cause late stent thrombosis when compared with bare metal stent implantation [4,5]. In addition, the US Food and Drug Administration has warned that DES may be a cause of systemic and intrastent hypersensitivity reactions, late thrombosis, and death [6]. Therefore, a new generation of DES to overcome the inflammation, delayed endothelial healing, and late stent thrombosis is needed.

α-Lipoic acid (α-LA) is a potent antioxidant, and acts as a cofactor of key mitochondrial enzymes such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase [7]. α-LA exists endogenously and improves diabetic-induced endothelial dysfunction, probably due to antioxidant effects and direct free-radical scavenging properties [8]. Moreover, α-LA inhibits the inflammatory pathway and prevents neointimal hyperplasia after stenting in the carotid artery [9,10].

The present study aimed to investigate whether α-LA feeding and α-LA coated stents prevent ISR in the porcine coronary artery restenosis model.

Methods

Protocol 1

We pretreated porcine aorta smooth muscle cells (PASMCs; Modern Cell & Tissue Technologies, Seoul, Korea) with α-LA (100, 250, and 500 mM) 24 h before stimulation by granulocyte-colony stimulating factor (G-CSF) (100 ng/ml, CJ, Ichon, Korea). After 24 h of G-CSF stimulation, we collected cell extracts and performed immunoblotting for phospho-p65 (Cell Signaling, Danvers, MA, USA), extracellular signal-regulated kinases (ERK) (Cell Signaling), phosphorylated ERK (Cell Signaling), signal transducers and activators of transcription (STAT)-3 (Cell Signaling), phosphorylated STAT-3 (Cell Signaling), and β-actin (Sigma, St Louis, MO, USA).

Next, we performed the proliferation rate assay. We pretreated α-LA (100 mM) with PASMCs 2 h before stimulation by G-CSF (100 ng/ml). After 48 h of G-CSF stimulation, MTT (3-[4,5-dimethylthiazole-2-yl] 2,5-diphenyl tetrazolium bromide, 5 mg/ml in PBS, Sigma, Seoul, Korea) was added to cells, and they were incubated further for 4 h at 37 °C. Supernatants were removed by aspiration, and then dimethyl sulfoxide (DMSO) was added to solubilize the precipitated dyes. Absorbance was measured at a wavelength of 570 nm.

Animal preparation

The animal study was approved by the Ethics Committee of Chonnam National University Medical School and Chonnam National University Hospital, and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Study animals were female swine weighing 25—35 kg. To prevent acute thrombosis after stenting, premedication with aspirin 100 mg and clopidogrel 75 mg per day was given for 7 days before the procedure. On the procedure day, pigs were anesthetized with ketamine (20 mg/kg intramuscularly) and xylazine (2 mg/kg intramuscularly). They received intramuscular ketamine every 30 min and supplemental oxygen continuously through oxygen mask. Subcutaneous 2% lidocaine at the cut-down site was administered, left carotid artery was surgically exposed, and a 7 French sheath was inserted. Continuous hemodynamic and surface electrocardiographic monitoring was maintained throughout the procedure. Then 5000 units heparin was administered intravenously as a bolus prior to the procedure, the target coronary artery was engaged using standard 7 F guide catheters and control angiograms of both coronary arteries were performed using nonionic contrast agent in two orthogonal views. The stent was deployed by inflating the balloon and the resulting stent-

to-artery ratio was 1.1:1. Coronary angiograms were obtained immediately after stent implantation. Then, all equipment was removed and the carotid artery was ligated.

Protocol 2

Balloon overdilatation injuries were performed in the proximal left anterior descending artery and proximal left circumflex by alternative manner for 12 pigs. Four weeks after the balloon overdilatation injury, 24 bare metal stents (3.0 mm × 17 mm MAC; AMG, Raesfeld-Erle, Germany) were placed for 24 injured coronary arteries. The pigs were randomly divided to receive control diet (aspirin and clopidogrel) with or without α-LA (100 mg/kg) during 4 weeks.

Protocol 3

The 8 bare metal (3.0 mm × 17 mm MAC) stents and 8 α-LA coating (3.0 mm × 17 mm α-LA coated MAC) stents were implanted in the proximal left anterior descending artery and proximal left circumflex artery by alternative manner for 8 pigs. All animals received 100 mg of aspirin and 75 mg of clopidogrel daily until death.

Four weeks after stenting, the animals underwent follow-up angiography in the same orthogonal views before death with 20 ml of potassium chloride intracoronary injection. The hearts were removed, and the coronary arteries were pressure-perfusion fixed at 110 mmHg in 10% neutral buffered formalin overnight. Arteries were step-sectioned, processed routinely for light microscopy, and stained for histological analysis.

α-LA coating method

A plasma polymerization reaction was performed to attach amine radical to MAC stent (AMG) surface. Before the surface modification, the MAC stent is cleaned by conventional cleaning method to remove macro-scale contaminants and with oxygen, argon, or argon and hydrogen plasma for further cleaning. A stent was fixed in tubular reactor, which was made by pyrex glass tube, and then the pressure was dropped to less than 5 mTorr by vacuum pump.

For attachment of amine radical to stent surface, diaminocyclohexane monomer was drifted to tubular reactor as constant dose (0.96 SCCM) and plasma was generated using radiofrequency power generator. The power for polymerization of plasma was 100 W for 5 min and then 60 W for 15 min. A 7 cm × 1 cm glass tube was boiled in 100 °C water for 5 min and then taken out and allowed to dry in an incubator. For the coating of α-LA, the modified stent is immersed in 2 ml of α-LA solution for 5—80 min at temperatures between 25 and 50 °C. The carboxy radical of α-LA was introduced to amine radical attached to stent to achieve covalent bond and improved attachment power between stent and α-LA. This reaction was performed for 1 h and then stent was taken out and allowed to dry for more than 24 h. After the coating, the stent was rinsed with deionized water for 1 min to remove weakly bound α-LA. After grafting reaction, the remaining solution was taken by UV—vis spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) at 330 nm to determine the grafted amount. For the release kinetics of α-LA from the stent, the stent was placed in a glass vial and immersed in 100 ml of phosphate-buffered saline. The released amount of α-LA to the buffer solution was measured using an absorbance test for ultraviolet at 278 nm [11].

Histopathological analysis

For immunohistochemistry, the stent was removed carefully from the coronary artery, and the segments embedded in paraffin-blocks for immunohistochemical studies and methyl methacrylate. Sections were cut with the low speed diamond wafer mounted to the Buehler Isomet saw (Buehler Ltd., Lake Bluff, IL, USA), leaving the stent wires intact in the cross-sections to minimize potential artifacts from removal of stent wires. Sections were obtained and stained with hematoxylin—eosin stain for histological analysis. The number of stained cells in the intima was quantified by counting 20 high-power fields using light microscopy. Immunohistochemical studies were performed using primary antibodies against STAT-3 (Cell Signaling), phosphorylated STAT-3 (Cell Signaling), Akt, phosphorylated Akt, and vascular endothelial growth factor (VEGF, Santa Cruz, Santa Cruz, CA, USA). Stain for proliferating cell nuclear antigen (PCNA, 1:2000, Dako, Glostrup, Denmark) also was done.

Measurements of the histopathologic sections were performed using a calibrated microscope, digital video imaging system, and microcomputer program (Visus 2000 Visual Image Analysis System, IMT Tech, San Diego, CA, USA). Borders were manually traced for lumen area, area circumscribed by the internal elastic lamina, and the innermost border of the external elastic lamina (external elastic lamina area). Morphometric analysis of neointimal area for a given vessel was calculated as the measured internal elastic lamina area minus lumen area.

Arterial injury at each strut site was determined by the anatomic structures penetrated by each strut. A numeric value was assigned, as previously described by Schwartz et al. [12]: 0 = no injury; 1 = break in the internal elastic membrane; 2 = perforation of the media; 3 = perforation of the external elastic membrane to the adventitia. The average injury score for each segment was calculated by dividing the sum of injury scores by the total number of struts at the examined section. Histopathologic stenosis was calculated as $100 \times (1 - [\text{lesion lumen area}/\text{lesion internal elastic lamina area}])$.

With regard to the inflammatory score for each individual strut, the grading is as follows: 0 = no inflammatory cells surrounding the strut; 1 = light, non-circumferential lymphohistiocytic infiltrate surrounding strut; 2 = localized, moderate to dense cellular aggregate surrounding the strut non-circumferentially; and 3 = circumferential dense lymphohistiocytic cell infiltration of the strut. The inflammatory score for each cross-section was calculated by dividing the sum of the individual inflammatory scores by the total number of struts at the examined section. The stent endothelialization score was defined as the extent of the circumference of the arterial lumen covered by endothelium and was scored from 1 to 3: 1, less than 25%; 2, 25% to 75%; 3, more than 75%.

Drugs and statistical analysis

Drugs were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless stated otherwise. Statistical analysis was performed with the aid of the commercially available software (SPSS Version 11, SPSS Inc., Chicago, IL, USA). The data are presented as rates or mean value $\pm$ SEM. Probability values are two-sided from the Student's *t*-test for continuous variables and the Fisher exact test for categorical variables. To examine the correlations between the measured histologic variables, regression analysis was applied for each set of measured variables. *p*-Values <0.05 were considered significant.

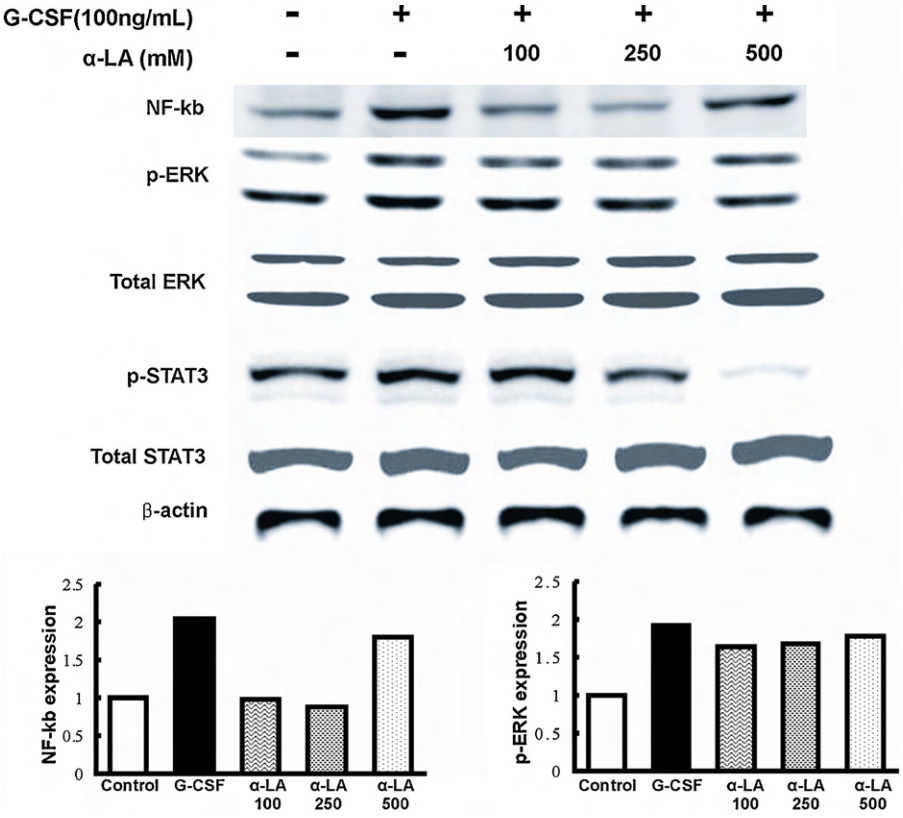

Figure 1 Semiquantitative immunoblotting of phospho-p65, phosphorylated extracellular signal-regulated kinase (p-ERK), and phosphorylated signal transducers and activators of transcription (p-STAT)3 in porcine aortic smooth muscle cells. Densitometric analysis revealed increased protein expression of phospho-p65 and p-ERK after granulocyte-colony stimulating factor (G-CSF) stimulation, which was attenuated by pretreatment with α-lipoic acid (LA) in a dose-dependent manner. The protein expression of nuclear factor (NF)-κb and p-ERK were also attenuated by α-LA pretreatment.

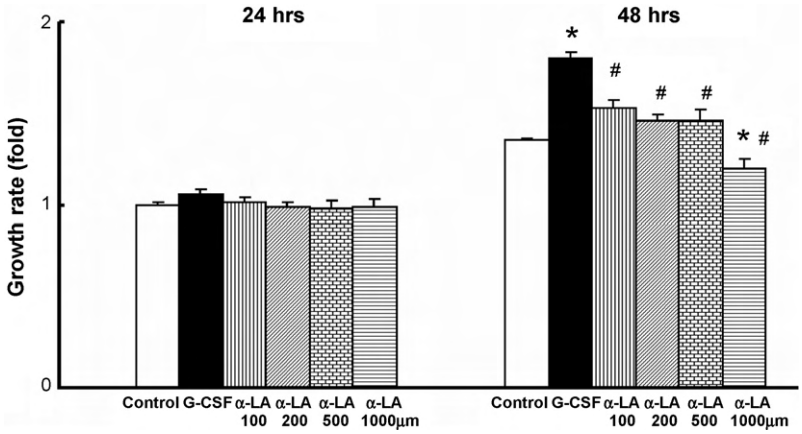

Figure 2 Columns show cell growth rate with or without pretreatment of α-lipoic acid (LA) on granulocyte-colony stimulating factor (G-CSF) induced porcine aortic smooth muscle cell proliferation by MTT (3-[4,5-dimethylthiazole-2-yl] 2,5-diphenyl tetrazolium bromide) assay. The growth rate was accelerated by G-CSF and attenuated by α-LA pretreatment. $^{*}p < 0.05$ compared with control cells, $^{\#}p < 0.05$ compared with G-CSF stimulated cells.

Results

Cellular behavior and signaling molecules of PASMCs

The protein expression of phospho-p65, phosphorylated ERK, and phosphorylated STAT-3 were increased on G-CSF stimulation in PASMCs, which was attenuated by pretreatment with α-LA dose dependently. And the protein expression of nuclear factor (NF)-κb and phosphorylated ERK were also attenuated by α-LA pretreatment (Fig. 1).

To determine whether α-LA prevents G-CSF-induced neointimal hyperplasia in PASMCs, cellular growth rate was determined by the MTT dye method. It shows a gradual increased proliferation of PASMCs treated with G-CSF compared to the control, while it was attenuated by pretreatment with α-LA at 48 h after G-CSF stimulation (Fig. 2).

Effects of alpha-LA feeding on ISR

Mortality for this study was zero. There was no significant difference in stent-to-artery ratio between two groups. On histopathologic analysis, injury score, internal elastic lamina areas were not significantly different between the two groups (Table 1). The neointimal area was $4.0 \pm 1.0\,mm^2$ in the control group and $1.5 \pm 0.7\,mm^2$ in the α-LA feeding group ($p < 0.001$), and the histopathologic area of stenosis was $66.7 \pm 10.7\%$ in the control group and $24.2 \pm 9.7\%$ in the α-LA feeding group ($p < 0.001$, Table 2, Fig. 3A and B). The inflammatory score was 2.8 ± 1.5 in the control group and 1.3 ± 0.4 in the α-LA feeding group ($p < 0.05$, Table 2, Fig. 3C and D). The endothelialization score showed no

Table 1 Quantitative coronary angiographic findings of porcine coronary arteries in α-lipoic acid (LA) feeding study.

	Control group ($n = 12$)	α-LA feeding group ($n = 12$)
Baseline (2)		
Proximal diameter	3.1 ± 0.6	3.0 ± 1.1
Distal diameter	2.7 ± 0.4	2.6 ± 0.9
Reference diameter	2.9 ± 1.1	2.9 ± 0.1
Post-stenting diameter	3.2 ± 0.9	3.2 ± 0.7
4 weeks after stenting (mm)		
Proximal diameter	3.1 ± 0.7	3.1 ± 0.7
Distal diameter	2.8 ± 0.2	2.6 ± 0.2
Target reference diameter	2.9 ± 0.1	2.8 ± 0.2
Minimal luminal diameter	1.7 ± 0.3	3.0 ± 0.2*

Values are expressed as mean ± SEM.
* $p < 0.05$, when compared with control group.

Table 2 Histopathologic assessment of porcine coronary arteries in α-lipoic acid (LA) feeding study.

	Control group ($n = 12$)	α-LA feeding group ($n = 12$)
Injury score	1.7 ± 0.7	1.3 ± 0.5
Lumen area (mm^2)	1.9 ± 0.4	4.6 ± 0.7*
IEL area (mm^2)	5.9 ± 0.8	6.1 ± 0.7
Neointima area (mm^2)	4.0 ± 1.0	1.5 ± 0.7*
Area stenosis (%)	66.7 ± 10.7	24.2 ± 9.7*
Inflammation score	2.8 ± 1.5	1.3 ± 0.4*
Endothelialization score	2.9 ± 0.8	2.9 ± 0.9

Values are expressed as mean ± SEM. IEL, internal elastic lamina.
* $p < 0.05$, when compared with control group.

significant difference between the two groups (Table 2).

Effects of alpha-LA coated stent on ISR

The median thickness of coating was 1 μm and the amount of α-LA coating on the surface of the stent was 200 μg/stent. The grafted amount of ALA was calculated from the UV standard calibration curve and the grafted amount onto a stent surface was about 200 μg. The α-LA coating on the surface of the stent was confirmed by scanning electron microscopy. Release kinetics demonstrated stable release of α-LA from the stent surface over 35 days [12]. The mortality rate was also zero. There was no significant difference in stent-to-artery ratio

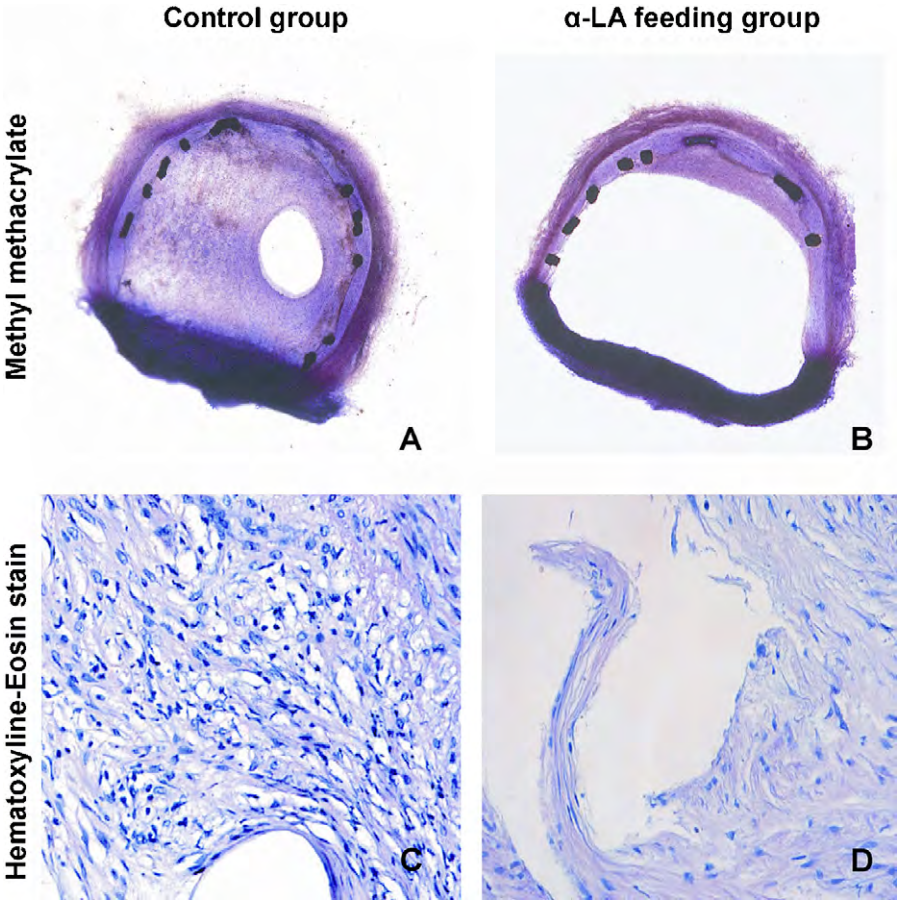

Figure 3 Methyl methacrylate (A and B) and hematoxylin and eosin (C and D) stain in the α-lipoic acid (LA) feeding study. In-stent neointimal area was smaller in the α-LA feeding group compared with the control group. (A) Control group; (B) α-LA feeding group. Magnification: 10×. α-LA feeding group shows lower inflammatory cell infiltration compared with control group. (C) Control group; (D) α-LA feeding group. Magnification: 200×.

Table 3 Quantitative coronary angiographic findings of porcine coronary arteries in α-lipoic acid (LA) coated stent study.

	Control stent group ($n = 8$)	α-LA coated stent group ($n = 8$)
Baseline (2)		
Proximal diameter	3.1 ± 00.6	3.0 ± 0.8
Distal diameter	2.7 ± 0.7	2.7 ± 0.9
Reference diameter	2.9 ± 0.2	2.8 ± 0.7
Post-stenting diameter	3.2 ± 0.9	3.1 ± 0.7
4 weeks after stenting (mm)		
Proximal diameter	3.1 ± 0.7	3.0 ± 0.9
Distal diameter	2.8 ± 0.2	2.6 ± 0.2
Target reference diameter	2.9 ± 00.1	2.9 ± 0.2
Minimal luminal diameter	1.6 ± 0.4	3.1 ± 0.2*

Values are expressed as mean ± SEM.
* $p < 0.05$, when compared with control group.

Table 4 Histopathologic assessment of porcine coronary arteries in α-lipoic acid (LA) coated stent study.

	Control stent group ($n = 8$)	α-LA coated stent group ($n = 8$)
Injury score	1.7 ± 0.7	1.3 ± 0.5
Lumen area (mm^2)	1.9 ± 0.4	4.9 ± 1.2*
IEL area (mm^2)	5.8 ± 0.7	5.9 ± 0.9
Neointima area (mm^2)	3.9 ± 0.8	1.0 ± 0.4*
Area stenosis (%)	67.1 ± 8.8	17.4 ± 10.0*
Inflammation score	2.4 ± 0.7	1.1 ± 0.6*
Endothelialization score	2.9 ± 0.8	2.9 ± 0.7

Values are expressed as mean ± SEM. IEL, internal elastic lamina.
* $p < 0.05$, when compared with control group.

between the two groups. The injury score and internal elastic lamina area were not significantly different between the two groups (Table 3). The neointimal area was $3.9 \pm 0.8\,mm^2$ in the control stent group and $1.0 \pm 0.4\,mm^2$ in the α-LA coated stent group ($p < 0.001$), and the histopathologic area of stenosis was 67.1 ± 8.8% in the control stent group and 17.4 ± 10.0% in the α-LA coated stent group ($p < 0.001$, Table 4, Fig. 4A and B). The inflammatory score was 2.4 ± 0.7 in the control group and 1.1 ± 0.6 in the α-LA coated stent group ($p < 0.05$, Table 4, Fig. 4C and D). The endothelialization score showed no significant difference between the two groups (Table 4).

Immunohistochemical analysis

Fig. 5 shows the immunohistochemical staining for p-Akt (Fig. 5A and B) and VEGF (Fig. 5C and D). The stent-based α-LA delivery inhibits p-Akt and VEGF expression in PASMCs effectively. Fig. 6 shows the immunohistochemical staining for p-STAT-3 (Fig. 6A and B) and PCNA (Fig. 6C and D). The stent-based α-LA delivery inhibits p-STAT-3 expression and cell proliferation effectively.

Discussion

This study demonstrates that both oral administration of α-LA and α-LA coated stents inhibit neointimal formation in the porcine coronary restenosis model. The profound reduction in neointimal formation with oral α-LA and α-LA coated stent is associated with an inhibition of activation of NF-κb, ERK, STAT-3, and proliferation of vascular smooth muscle cells. Our findings document the feasibility of oral administration of α-LA and α-LA coated stent, and the efficacy data support the notion that stent-based α-LA delivery is a promising approach for the prevention of restenosis.

ISR due to neointimal hyperplasia remains the major limitation of coronary stent implantation. The development of neointimal hyperplasia plays an important role in the mechanism of restenosis [13]. Because of the role of inflammation in restenosis, the inflammatory cells seemed to be an optimal target in the fight against restenosis. The pathological process of ISR is characterized by an inflammatory healing response after stretch and damage of the vessel wall [14,15]. Initially, platelets are activated and attach around the stent

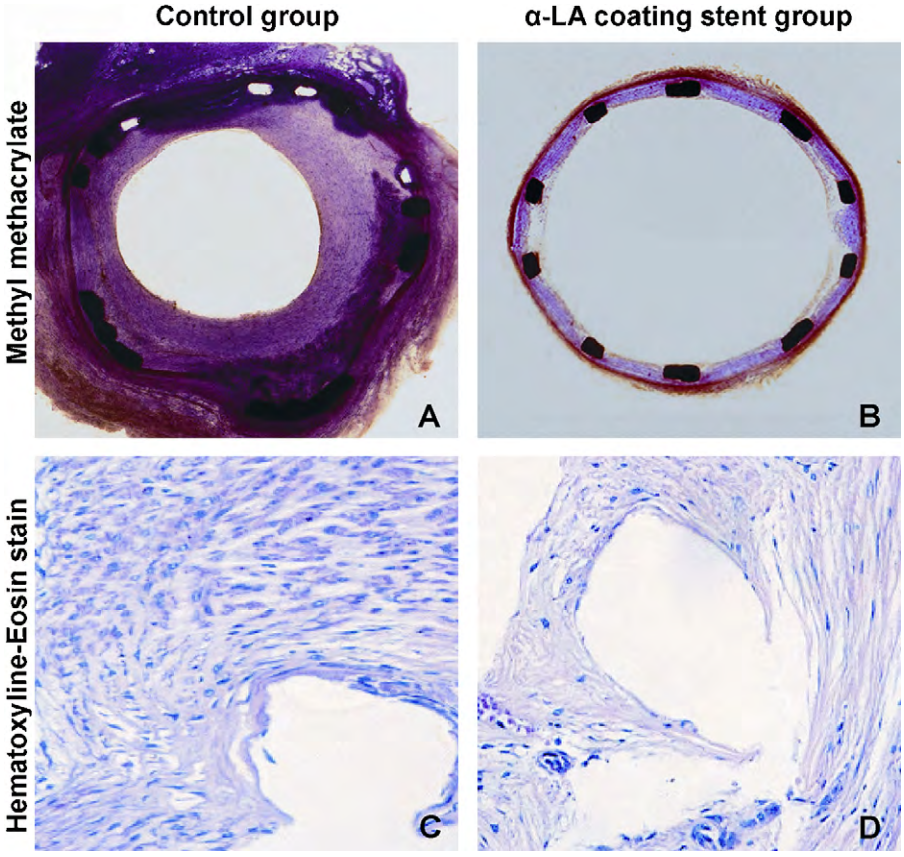

Figure 4 Methyl methacrylate (A and B) and hematoxylin and eosin (C and D) stain in the α-lipoic acid (LA) coated stent study. In-stent neointimal area was smaller in the α-LA coated stent group compared with the control group. (A) Control group; (B) α-LA coating stent group. Magnification: 10×. The α-LA feeding group showed lower inflammatory cell infiltration compared with control group. (C) Control group; (D) α-LA coated stent group. Magnification: 200×.

struts followed by adhesion of inflammatory cells. Cytokines and growth factors are released, leading to migration and proliferation of vascular smooth muscle cell [16].

Based on observations that ISR is a consequence of inflammation, smooth muscle cell proliferation, and migration, several immunosuppressive and antiproliferative therapies have been investigated to inhibit these processes [17—19]. Recent successes in early clinical trials with DES using antiproliferative agents such as sirolimus and paclitaxel have been quite promising [20—22]. Sirolimus inhibits smooth muscle cell proliferation by blocking the cell cycle in the G1 (growth) to S (DNA synthesis) phase, thus preventing cell replication. Paclitaxel works by binding to polymerized tubulin, thereby stabilizing it against disassembly, thus inhibiting cell mitosis (M-phase of the cell cycle). However, current DES result in delayed arterial healing and may cause late stent thrombosis and even cause death, and many clinical cases have been reported [4—6].

α-LA has been used as therapy for many diseases, especially diabetes. α-LA supplementation was reported to increase unbound lipoic acid levels, which can reduce oxidative stress and act as a potent antioxidant [23]. α-LA is readily converted to its reduced form dihydrolipoic acid (DHLA) in various tissues, which increases intracellular levels of coenzyme NADPH, and NADH via increased glutathione availability. Both α-LA and DHLA have recently gained some recognition as useful biological antioxidants. They influence a number of cell processes including direct radical scavenging, and modulating transcription factor activity, especially that of NF-κb [24,25]. The present in vivo data show the protein expression of NF-κb and phosphorylated ERK was attenuated by α-LA pretreatment and suggests that α-LA has anti-inflammatory effects as well as a previous known antioxidant effect.

The cellular proliferation induced by G-CSF was inhibited by α-LA pretreatment in MTT assay. In addition, in porcine experiment, staining for PCNA showed a lower ratio of PCNA positive cells in the α-

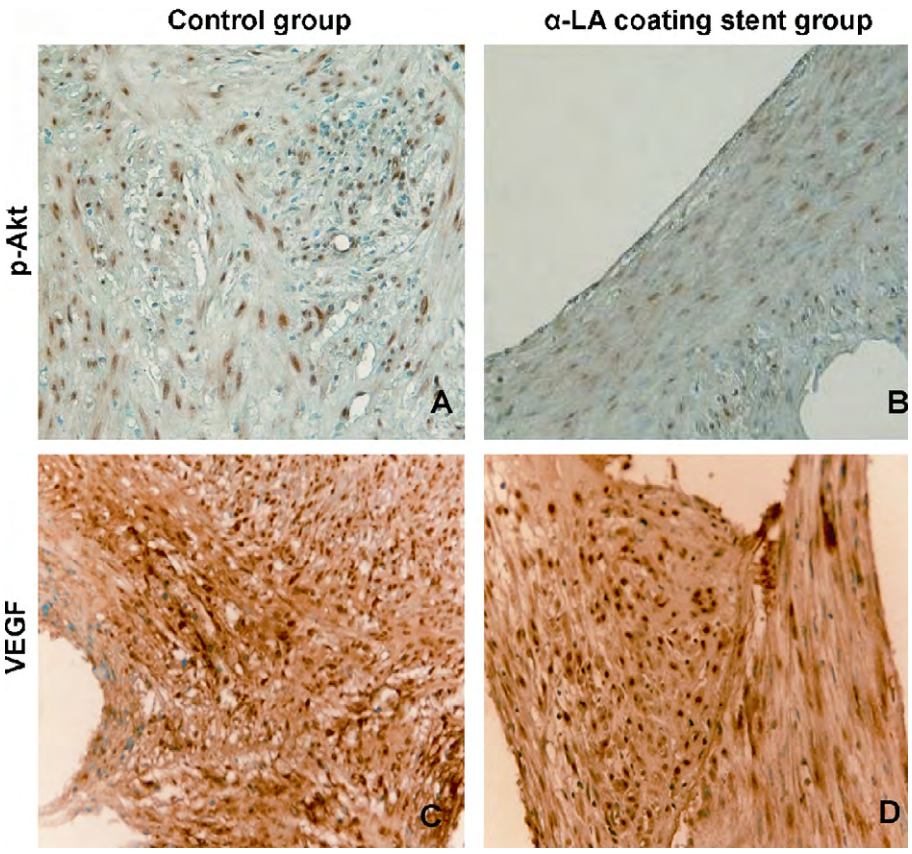

Figure 5 Immunohistochemical staining for phosphorylated Akt and vascular endothelial growth factor (VEGF). (A) and (C) Control stent (bare metal stent); (B) and (D) α-lipoic acid (LA) coated stent. α-LA coated stent attenuated immunolabeling of p-Akt effectively after 28 days of stenting. Magnification: 200×.

LA coated stent group. These data suggest that α-LA may attenuate cell proliferation. Moreover, a significant reduction in stent-associated inflammation and neointimal hyperplasia was observed at 28 days for both administration of oral α-LA and stent-based local delivery of α-LA and suggests that both α-LA and its reduced form, DHLA, have similar antiproliferatory and anti-inflammatory effects on vascular smooth muscle cells.

In our data, endothelialization score showed no significant difference after α-LA treatment compared with bare metal stent. We thought that is the difference between this and preexisting DES, such as sirolimus- or paclitaxel-eluting stents. If the same result could be reproduced in human studies, the α-LA coating stent may be an ideal stent that inhibits neointimal proliferation effectively and does not interfere with re-endothelialization.

We used antibodies for STAT-3 and ERK in western blotting and Akt, VEGF, and STAT-3 for immunohistochemical staining. There have been several reports suggesting that STAT-3 phosphorylation promotes smooth muscle cell growth [26]. VEGF plays an important role in neointimal development in vessel wall injury [27,28]. In our porcine model, VEGF was expressed at high levels in the neointima of the bare metal stent group. Activation of the Akt pathway triggered by mechanical stretch may also be a contributory factor to ISR formation [29]. Activation of ERK and c-Jun N-terminal kinase (JNK) triggers smooth muscle cell proliferation, leading to neointimal formation [30,31].

Prevention of ISR appears to require such a multipronged attack to block all the processes. In our study, α-LA blocked multiple cytokines such as Akt, ERK, VEGF, and STAT-3 and inhibited the proliferation of vascular smooth muscle cells.

Study limitations

It remains to be established whether our findings in normal non-atherosclerotic porcine coronary arteries stimulated with oversized balloons and stents for neointimal proliferation could be extrapolated to human clinical scenarios with preexisting

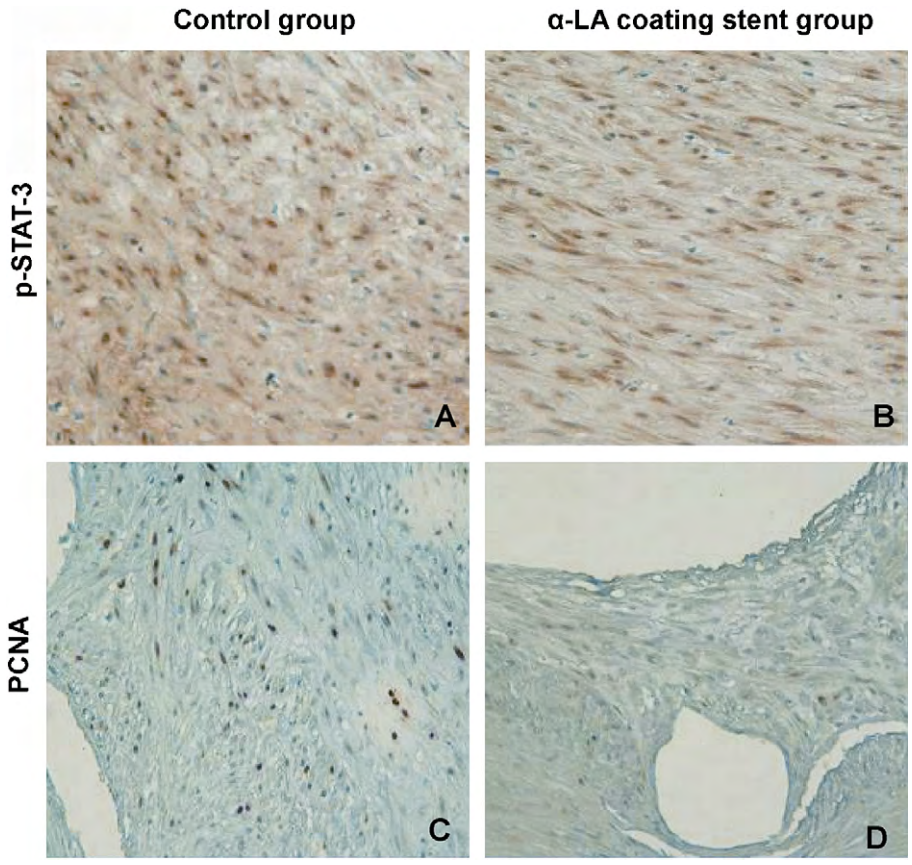

Figure 6 Immunohistochemical staining for phosphorylated signal transducers and activators of transcription (p-STAT)-3 and proliferating cell nuclear antigen (PCNA). (A) and (C) Control stent (bare metal stent); (B) and (D) α-lipoic acid (LA) coated stent. α-LA coated stent attenuated immunolabeling of p-STAT-3 and PCNA effectively after 28 days of stenting. Magnification: 200$\times$.

atherosclerosis. Second, the observed efficacy at 28 days may not be sustained after the drug concentration wanes to a subtherapeutic level and the dose—response effects for this α-LA coated stent are incompletely characterized.

Conclusions

This is the first experimental study to demonstrate that administration of oral α-LA and α-LA coated stents are feasible and effectively reduce in-stent neointimal formation. Stent-based local delivery of α-LA profoundly suppresses neointimal hyperplasia in the porcine ISR model by inhibition of ERK, STAT-3, and proliferation of vascular smooth muscle cells.

Acknowledgments

This study was funded by grants from the Korea Healthcare Technology R&D Project (A084869), Ministry for Health, Welfare and Family Affairs, Republic of Korea, and the Cardiovascular Research Foundation, Asia.

References

[1] Faxon DP, Spiro TE, Minor S, Cote G, Douglas J, Gottlieb R, Califf R, Dorosti K, Topol E, Gordon JB, Ohmen M, ERA investigators. Low molecular weight heparin in prevention of restenosis after angioplasty. Results of Enoxaparin Restenosis (ERA) Trial. Circulation 1994;90:908—14.

[2] Hamm CW, Reimers J, Ischinger T, Rupprecht HJ, Berger J, Bleifeld W. A randomized study of coronary angioplasty compared with bypass surgery in patients with symptomatic multivessel coronary disease. German Angioplasty Bypass Surgery Investigation (GABI). N Engl J Med 1994;331:1037—43.

[3] Kent KM, Bentivoglio LG, Block PC, Bourassa MG, Cowley MJ, Dorros G, Detre KM, Gosselin AJ, Gruentzig AR, Kelsey SF. Long-term efficacy of percutaneous transluminal coronary angioplasty (PTCA): report from the National Heart, Lung, and Blood Institute PTCA Registry. Am J Cardiol 1984;53:27C—31C.

[4] Joner M, Finn AV, Farb A, Mont EK, Kolodgie FD, Ladich E, Kutys R, Skorija K, Gold HK, Virmani R. Pathology of

drug-eluting stents in humans: delayed healing and late thrombotic risk. J Am Coll Cardiol 2006;48:193—202.
[5] Virmani R, Farb A, Guagliumi G, Kolodgie FD. Drug-eluting stents: caution and concerns for long-term outcome. Coron Artery Dis 2004;15:313—8.
[6] Nebeker JR, Virmani R, Bennett CL, Hoffman JM, Samore MH, Alvarez J, Davidson CJ, McKoy JM, Raisch DW, Whisenant BK, Yarnold PR, Belknap SM, West DP, Gage JE, Morse RE, et al. Hypersensitivity cases associated with drug-eluting coronary stents: a review of available cases from the Research on Adverse Drug Events and Reports (RADAR) project. J Am Coll Cardiol 2006;47:175—81.
[7] Packer L, Roy S, Sen CK. Alpha-lipoic acid: a metabolic antioxidant and potential redox modulator of transcription. Adv Pharmacol 1997;38:79—101.
[8] Ziegler D, Hanefeld M, Ruhnau KJ, Hasche H, Lobisch M, Schutte K, Kerum G, Malessa R. Treatment of symptomatic diabetic polyneuropathy with the antioxidant alpha-lipoic acid: a 7-month multicenter randomized controlled trial (ALADIN III Study). ALADIN III Study Group. Alpha-Lipoic Acid in Diabetic Neuropathy. Diabetes Care 1999;22:1296—301.
[9] Sung MJ, Kim W, Ahn SY, Cho CH, Koh GY, Moon SO, Kim DH, Lee S, Kang KP, Jang KY, Park SK. Protective effect of alpha-lipoic acid in lipopolysaccharide-induced endothelial fractalkine expression. Circ Res 2005;97:880—90.
[10] Lee KM, Park KG, Kim YD, Lee HJ, Kim HT, Cho WH, Kim HS, Han SW, Koh GY, Park JY, Lee KU, Kim JG, Lee IK. Alpha-lipoic acid inhibits fractalkine expression and prevents neointimal hyperplasia after balloon injury in rat carotid artery. Atherosclerosis 2006;189:106—14.
[11] Song SJ, Kim SK, Kim KH, Li HJ, Lim GT, Jeong MH, Kim JH, Kim BH, Ko YM, Cho DL. Preparation of biocompatible stent surface by plasma polymeration followed by chemical grafting of drug compounds. J Mater Chem 2009;19:3248—52.
[12] Schwartz RS, Huber KC, Murphy JG, Edwards WD, Camrud AR, Vlietstra RE, Holmes DR. Restenosis and the proportional neointimal response to coronary artery injury: results in a porcine model. J Am Coll Cardiol 1992;19:267—74.
[13] Kornowski R, Hong MK, Tio FO, Bramwell O, Wu H, Leon MB. In-stent restenosis: contributions of inflammatory responses and arterial injury to neointimal hyperplasia. J Am Coll Cardiol 1998;31:224—30.
[14] Farb A, Sangiorgi G, Carter AJ, Walley VM, Edwards WD, Schwartz RS, Virmani R. Pathology of acute and chronic coronary stenting in humans. Circulation 1999;99:44—52.
[15] Grewe PH, Deneke T, Machraoui A, Barmeyer J, Muller KM. Acute and chronic tissue response to coronary stent implantation: pathologic findings in human specimen. J Am Coll Cardiol 2000;35:157—63.
[16] Brasen JH, Kivela A, Roser K, Rissanen TT, Niemi M, Luft FC, Donath K, Yla-Herttuala S. Angiogenesis, vascular endothelial growth factor and platelet-derived growth factor-BB expression, iron deposition, and oxidation-specific epitopes in stented human coronary arteries. Arterioscler Thromb Vasc Biol 2001;21:1720—6.
[17] Faxon DP. Systemic drug therapy for restenosis: ''deja vu all over again''. Circulation 2002;106:2296—8.
[18] Lincoff AM, Topol EJ, Ellis SG. Local drug delivery for the prevention of restenosis. Fact, fancy, and future. Circulation 1994;90:2070—84.
[19] Versaci F, Gaspardone A, Tomai F, Ribichini F, Russo P, Proietti I, Ghini AS, Ferrero V, Chiariello L, Gioffre PA, Romeo F, Crea F. Immunosuppressive therapy for the prevention of restenosis after coronary artery stent implantation (IMPRESS Study). J Am Coll Cardiol 2002;40:1935—42.
[20] O'Neill WW, Leon MB. Drug-eluting stents: costs versus clinical benefit. Circulation 2003;107:3008—11.
[21] Lemos PA, Serruys PW, Sousa JE. Drug-eluting stents: cost versus clinical benefit. Circulation 2003;107:3003—7.
[22] Kereiakes DJ. Hippocrates revisited: the evidence for drug-eluting stents. Circulation 2003;107:3012—4.
[23] Suzuki YJ, Tsuchiya M, Packer L. Thioctic acid and dihydrolipoic acid are novel antioxidants which interact with reactive oxygen species. Free Radic Res Commun 1991;15:255—63.
[24] Packer L. Alpha-lipoic acid: a metabolic antioxidant which regulates NF-kappa B signal transduction and protects against oxidative injury. Drug Metab Rev 1998;30:245—75.
[25] Scott BC, Aruoma OI, Evans PJ, O'Neill C, Van der Vliet A, Cross CE, Tritschler H, Halliwell B. Lipoic and dihydrolipoic acids as antioxidants. A critical evaluation. Free Radic Res 1994;20:119—33.
[26] Seki Y, Kai H, Shibata R, Nagata T, Yasukawa H, Yoshimura A, Imaizumi T. Role of the JAK/STAT pathway in rat carotid artery remodeling after vascular injury. Circ Res 2000;87:12—8.
[27] Lemstrom KB, Krebs R, Nykanen AI, Tikkanen JM, Sihvola RK, Aaltola EM, Hayry PJ, Wood J, Alitalo K, Yla-Herttuala S, Koskinen PK. Vascular endothelial growth factor enhances cardiac allograft arteriosclerosis. Circulation 2002;105:2524—30.
[28] Asahara T, Chen D, Tsurumi Y, Kearney M, Rossow S, Passeri J, Symes JF, Isner JM. Accelerated restitution of endothelial integrity and endothelium-dependent function after phVEGF165 gene transfer. Circulation 1996;94:3291—302.
[29] Mitra AK, Agrawal DK. In stent restenosis: bane of the stent era. J Clin Pathol 2006;59:232—9.
[30] Squadrito F, Minutoli L, Esposito M, Bitto A, Marini H, Seminara P, Crisafulli A, Passaniti M, Adamo EB, Marini R, Guarini S, Altavilla D. Lipid peroxidation triggers both c-Jun N-terminal kinase (JNK) and extracellular-regulated kinase (ERK) activation and neointimal hyperplasia induced by cessation of blood flow in the mouse carotid artery. Atherosclerosis 2005;178:295—302.
[31] Izumi Y, Kim S, Namba M, Yasumoto H, Miyazaki H, Hoshiga M, Kaneda Y, Morishita R, Zhan Y, Iwao H. Gene transfer of dominant-negative mutants of extracellular signal-regulated kinase and c-Jun NH2-terminal kinase prevents neointimal formation in balloon-injured rat artery. Circ Res 2001;88:1120—6.