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α-Lipoic Acid Exerts a Primary Prevention for the Neointimal Hyperplasia in Balloon-Injured Rat Carotid Arteries

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Abstract

Background: It has been reported that α-lipoic acid is an anti-oxidative and anti-inflammatory agent, and exhibits beneficial effects in experimental disease models such as hypertension and acute kidney injury. In the present study, we examined effects of α-lipoic acid on neointimal hyperplasia in balloon-injured carotid arteries.

Methods: Male rats underwent balloon injury of the right carotid artery with a 2F Fogarty balloon catheter. α-Lipoic acid was administrated to balloon-injured rats in the following treatment schedules; (1) days -3 to 14, (2) days -3 to 3, (3) days 7 to 14 and (4) vehicle. We evaluated the neointimal formation, NADPH oxidase-dependent superoxide production, nitrotyrosine expression and p50 subunit of nuclear factor kappa B (NF-κB p50) expression in balloon-injured and uninjured carotid arteries.

Results: In vehicle-treated rats, a significant neointimal formation was observed at 14 days after balloon injury in the carotid artery, and NADPH oxidase-dependent superoxide production, nitrotyrosine expression and NF-κB p50 expression in injured right carotid arteries significantly increased at 2 days after balloon injury as compared with uninjured left carotid arteries. The long-term treatment (initiated 3 days before surgery and continued for 14 days after balloon injury; days -3 to 14) with α-lipoic acid (100 mg/kg/day) markedly reduced the neointimal formation, NADPH oxidase-mediated superoxide production, nitrotyrosine and NF-κB p50 expression. The superoxide production, nitrotyroeine and NF-κB p50 expression at 2 days after balloon injury were significantly inhibited by the short-term treatment with α-lipoic acid (days -3 to 2). The short-term treatment (days -3 to 3) also could suppress the neointimal formation at 14 days after balloon injury.

Conclusion: The present study suggests that the treatment with α-lipoic acid leads to a reduction of neointimal formation, probably by inhibiting enhanced superoxide production, nitrotyrosine and NF-κB p50 activation in an early phase after balloon injury.

Keywords: α-Lipoic acid; Balloon-injury; NADPH oxidase-dependent superoxide production; nuclear factor kappa B; Neointimal hyperplasia

Introduction

The incidence of restenosis after percutaneous transluminal coronary angioplasty (PTCA) is decreasing gradually with improvement of the skill of angioplasty and the invention of stent. However, neointimal hyperplasia and restenosis after PTCA remain at high risk [1]. It has been reported that the increase of reactive oxygen species on the vessel wall is involved in the pathogenesis of restenosis after PTCA [2,3]. The mechanisms of neointimal hyperplasia after PTCA are similar to arteriosclerosis process. Increase in reactive oxygen species production leads to develop the migration and proliferation of vascular smooth muscle cells, and this development induces arteriosclerosis and restenosis after PTCA.

α-Lipoic acid, a dithiol compound, is a protein-bound cofactor for mitochondrial α-keto acid and thus serves a critical role in mitochondrial energy metabolism. In addition, α-lipoic acid has been attracted attention for a powerful antioxidant: this compound exerts a scavenging ability on hydroxyl radicals, singlet oxygen, hydrogen peroxide, peroxynitrite and nitric oxide [4] and is also able to chelate metal ions such as iron, copper and zinc [5]. Furthermore, α-lipoic acid induces the recycling of endogenous glutathione which acts as an electron donor [6]. Thus, α-lipoic acid can not only decrease the generation of reactive oxygen species, but also activate the endogenous antioxidative factors. It has also been reported that α-lipoic acid treatment exerts the beneficial effects in some clinical trials such as diabetic nephropathy and Alzheimer's disease [7,8].

We have reported that in rat balloon injury model, suppression of reactive oxygen species production after PTCA was important for the prevention of restenosis after PTCA in an early phase [9,10]. This led us to examine the effects of α-lipoic acid on neointimal hyperplasia after balloon injury and mechanisms of its action. Moreover, we evaluated whether therapeutic efficacy of α-lipoic acid is influenced by the time of initiation of therapy and its term, after balloon injury.

Materials and Methods

Animals and experimental design

Ten-week old male Sprague–Dawley rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). All animals were maintained at the departmental animal care facility of Osaka University of Pharmaceutical Sciences in accordance with the guidelines of the Recommendations from the Declaration of Helsinki. They were housed in a light controlled room with a 12-hour light/dark cycle and were allowed ad libitum access to food and water. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences.

Several experiments were carried out according to the protocol indicated below. In the 1st experiments, animals were divided following 4 groups; one group was treated with vehicle and others were treated α-lipoic acid (10, 30 or 100 mg/kg/day, s.c.). α-Lipoic acid treatment was initiated 3 days before and continued until 14 days after balloon injury (days -3 to 14). We evaluated the neointimal formation at 14 days after balloon injury. In the 2nd experiment, we measured NADPH oxidase-dependent superoxide production in balloon-injured and uninjured carotid arteries at 2 days after balloon injury. In the 3rd experiment, we carried out immunohistochemistry of nitrotyrosine and NF-κB p50 in balloon-injured and uninjured carotid arteries after 2 days. In the 4th experiments, animals were divided into 4 groups in the following α-lipoic acid- treatment schedules; (1) days -3 to 14, (2) days -3 to 3, (3) days 7 to 14 and (4) vehicle.

Balloon injury procedure

Rats were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) and the right carotid artery was injured with a 2F Fogarty balloon catheter (Baxter International, Deerfield, IL, USA) as described previously [9]. The left carotid artery was not damaged (uninjured artery).

Morphometric analysis

Two weeks after balloon injury of the right carotid artery, rats were killed with overdose of sodium pentobarbital (75 mg/kg), and then the vascular system was perfused with 10 % formalin to fix the arteries. Excised carotid arteries were embedded in paraffin, serially sectioned (4 μm) and stained with Elastica van Gieson stain. Morphometric analysis of each arterial segment was performed with a computer-based Motic Image Plus 2.0 Morphometric system (Shimadzu, Kyoto). The degree of neointimal formation of the injured carotid artery was expressed as the absolute area of neointima and intima/media ratios as described previously [10].

Measurement of NADPH oxidase-dependent superoxide production

We measured superoxide production depended on NADPH oxidase in both injured and uninjured common carotid arteries. Superoxide production was measured by lucigenin chemiluminescence in the presence of 100 μM NADPH and 100 μM lucigenin, as described previously [10]. Chemiluminescence was expressed as relative light units per minute per milligram vessel dry weight (RLU/min/mg).

Immunohistochemistry

Immunohistochemistry was used to evaluate the expression of the nitrotyrosine and the nuclear localization sequence (NLS) of the NF-κB p50 subunit which indicate nitrosative-oxidative stress and inflammation, respectively. After transcardial perfusion, the carotid arteries were fixed in 10% formaldehyde and embedded in paraffin. After deparaffinization in xylene and rehydration in graded ethanol, 4 μm sections immersed in a vessel filled with 10 mmol/L citrate buffer, pH 6.0, were heated with microwave for 15 minutes. After cooling at room temperature for 30 minutes, these slides were treated with 3% hydrogen peroxide for 15 minutes to block endogenous peroxide activity, and then rinsed briefly in PBS. Nonspecific binding was blocked by incubating the slides with a blocking solution (5% skim milk in PBS) for 30 minutes. The sections were incubated overnight at 4°C with primary anti-nitrotyrosine (1:100; Cayman Chemical, Inc., Ann Arbor, MI, USA) and primary anti-NF-κB p50 (NLS) (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) then reacted with Histofine Simple-Stain MAX-PO (Nichirei Corporation, Tokyo, Japan) for 1 hour at room temperature. The sections were developed with diaminobenzidine using a Simple stain DAB buffer (Nichirei Corporation).

Drugs

α-Lipoic acid, purchased from Nacalai Tesque (Kyoto, Japan), was dissolved in a solution consisting 20% ethanol and 80% corn oil. Other chemicals were obtained Nacalai Tesque and Wako Pure Chemical Industries (Osaka, Japan).

Statistical Analysis

All values were expressed as mean ± S.E.M. For statistical analysis, we used the one-way analysis of variance combined with Dunnett's or Bonferroni's multiple range tests for multiple comparisons. Differences were considered significant at a value of $P<0.05$.

Results

Response of rat carotid arteries to balloon injury

Representative cross-sections of the injured and uninjured carotid arteries in rats treated with vehicle or α-lipoic acid (100 mg/kg/day) are shown in Figure1. No neointimal formation was observed in the uninjured artery. In the injured arteries treated with vehicle, the intima/media ratios were 1.38 ± 0.12 (Figure1). Although α-lipoic acid at 10 mg/kg/day did not reduce neointimal formation, higher doses (30 and 100 mg/kg/day) significantly reduced neointimal formation by 38.1% and 49.4%, respectively (Figure1). On the other hand, α-lipoic acid treatment did not affect the media area (data not shown).

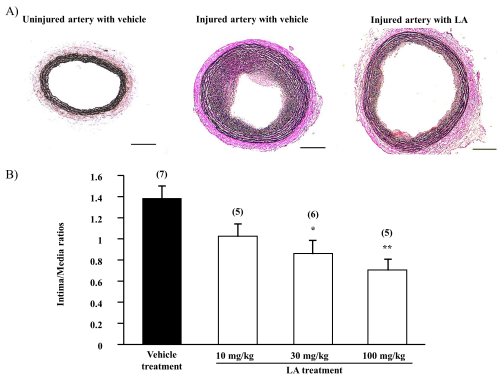

Figure 1: Morphometric analysis of carotid arterial cross-sections at 14 days after balloon injury. Light micrographs of the neointimal formation in balloon-injured carotid arteries at 14 days after balloon injury (A). All figures are depicted at 100×magnification. The scale represents 0.2 mm. Dose-response with α-lipoic acid for intima to media ratios of carotid arteries at 14 days after balloon injury (B). Closed bar; vehicle treatment, and open bar; LA treatment. Each value represents the mean ± S.E.M. of number of animals in parentheses. *P<0.05, **P<0.01, compared with vehicle. LA, α-lipoic acid.

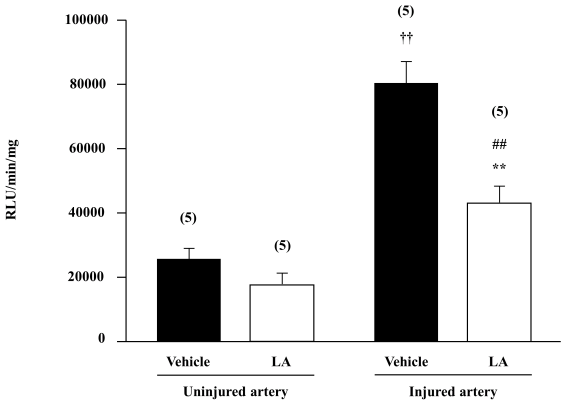

Figure 2: The level of NADPH oxidase-dependent superoxide production on uninjured and injured carotid arteries at 2 days after balloon injury. Closed bar; vehicle treatment, and open bar; LA treatment. Each value represents the mean ± S.E.M. †† P<0.01, compared with uninjured artery with vehicle. Each value represents the mean ± S.E.M. of number of animals in parentheses. **P<0.01, compared with injured artery with vehicle. $^{\#\#}$P<0.01, compared with uninjured artery with LA treatment. LA, α-lipoic acid.

Effect of α-lpoic acid on NADPH oxidase-dependent superoxide production in injured and uninjured carotid arteries

In the previous study [10], we found that NADPH oxidase-dependent superoxide production in injured carotid arteries began to increase at day 2; this increase continued through day 7, and then returned to the level of uninjured arteries at day 14. As shown in Figure2, NADPH oxidase-dependent superoxide production was markedly increased in the carotid arteries at day 2 after balloon injury, and this increment was significantly decreased by α-lipoic acid treatment (100 mg/kg/day) (80134 ± 6655 vs 42553 ± 5038 RLU/min/mg).

Effect of α-lipoic acid on nitrotyrosine expression in balloon-injured carotid arteries

In vehicle treated group, the nitrotyrosine expression prominently increased in the media at day 2 as compared with uninjured carotid arteries.α-Lipoic acid (100 mg/kg/day) treatment from days -3 to 2 suppressed markedly the expression (Figure 3).

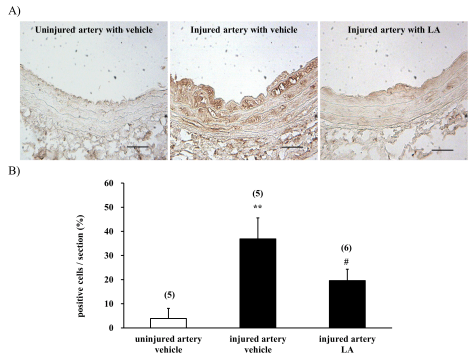

Figure 3: Representative immunohistochemical staining showing nitrotyrosine expression in balloon-injured carotid arteries at 2 days after balloon injury. All figures are depicted at 400×magnification (A). The scale represents 0.05 mm. Semi-quantification of nitrotyrosine expression in the medial region (B). Each value represents the mean ± S.E.M. of number of animals in parentheses. **P<0.01, compared with uninjured artery with vehicle. $^{\#}$P<0.05, compared with injured artery with vehicle. LA, α-lipoic acid.

Effect of α-lipoic acid on NF-κB p50 expression in balloon-injured carotid arteries

In vehicle treated group, the NF-κB p50 expression prominently increased in the media at day 2. α-Lipoic acid (100 mg/kg/day) treatment from days -3 to 2 suppressed markedly the expression (Figure 4).

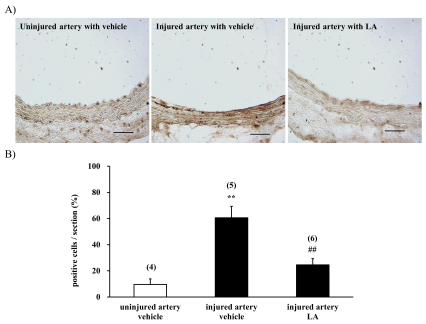

Figure 4: Representative immunohistochemical staining showing NF-κB p50 expression in balloon-injured carotid arteries at 2 days after balloon injury. All figures are depicted at 400×magnification (A). The scale represents 0.05 mm. Semi-quantification of NF-κB p50 expression in the medial region (B). Each value represents the mean ± S.E.M. of number of animals in parentheses. ** P<0.01, compared with uninjured artery with vehicle. ## P<0.01, compared with injured artery with vehicle. NF-κB, nuclear factor kappa B.

Time-window response of the balloon-injured carotid arteries

Neointimal formation was reduced by α-lipoic acid (100 mg/kg/day) treatment from days -3 to 14 compared with vehicle group (intima/media ratios; 0.70 ± 0.12 vs 1.38 ± 0.12). Seven days treatment from days -3 to 3 also suppressed neointimal formation at day 14 after balloon injury (0.81 ± 0.12), to the same extent as days -3 to 14 group. However, seven days treatment from days 7 to 14 failed to suppress neointimal formation (1.62 ± 0.13) (Figure 5).

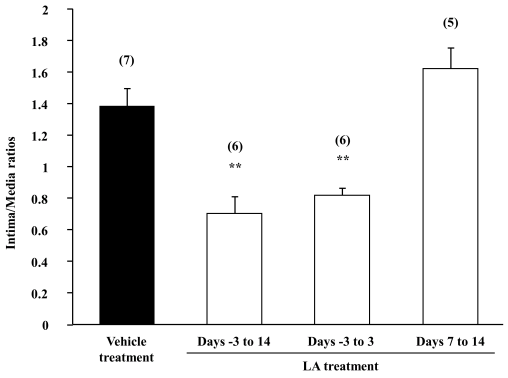

Figure 5: Time-window response of intima to media ratios in the balloon-injured carotid arteries. Each value represents the mean ± S.E.M. of number of animals in parentheses. ** P< 0.01, compared with vehicle.

Discussion

The current study showed that the treatment with α-lipoic acid attenuated neointimal formation after balloon injury in the right common carotid arteries of rats. We found that the therapeutic efficacy of α-lipoic acid was accompanied by decreasing NADPH oxidase-dependent superoxide production, and the expression of nitrotyrosine and NF-κB p50 in balloon-injured carotid arteries. A more interesting observation was that the short-term α-lipoic acid treatment from day -3 to day 3 was also able to reduce the neointimal formation to a level comparable to that seen with continuous α-lipoic acid given from day -3 to day 14 after balloon injury. Furthermore, the vascular oxidative stress, nitrosative stress and inflammatory response were augmented at 2 days after balloon injury, and α-lipoic acid was able to inhibit these augmentations. Thus, it seems likely that α-lipoic acid treatment ameliorated neointimal formation in the balloon-injured carotid arteries by suppressing oxidative stress, peroxinitration and inflammatory factor on vascular smooth muscle cells in an early phase after vascular injury.

α-Lipoic acid is known to have therapeutic potential as a powerful antioxidant [11], and its therapeutic usefulness has been noted in disease animal models such as hypertension and ischemia/reperfusion-induced acute kidney injury[12-14]. In balloon-injury animal models, there is evidence that prominent oxidative stress is produced in balloon-injured carotid arteries of rats [15,16], and the treatment with antioxidant such as N-acetyl-cysteine exhibits a beneficial effect for the prevention of neointimal formation in balloon-injured carotid arteries[17,18]. In the present study, we demonstrated that α-lipoic acid reduced neointimal formation accompanied by vascular endothelial lesion. The present results further supported our previous findings showing that the NADPH oxidase-dependent superoxide production and the expression of nitrotyrosine in injured vessels was markedly elevated within 3 days after balloon injury [10]. It has been known that nitrotyrosine is a footprint of peroxinitrite and also reporter that systemic levels of nitrotyrosine associated with the prevalence of coronary artery disease [19]. The formation of nitrotyrosine is the consequence of an imbalance between superoxide and nitric oxide formation in diseased artery. In the present study, we

have demonstrated that the expression of nitrotyrosine in the media of injured carotid arteries were markedly increased at 2 days after balloon injury. The increased nitrotyrosine expression was suppressed by α-lipoic acid treatment. Taken together with the findings that α-lipoic acid suppressed increases in the NADPH oxidase-dependent superoxide production and nitrotyrosine expression in the injured carotid arteries at 2 days after balloon injury, it is conceivable that α-lipoic acid decreased neointimal formation by correcting the imbalance between superoxide and nitric oxide formation in an early phase after balloon injury.

It has been well shown that the expression of several genes involved in inflammatory and proliferative responses of cells are regulated by NF-κB [20,21]. The remarkable activation of NF-κB was reported in balloon-injured carotid arteries and its activation was related to neointimal formation after balloon injury [22-25]. Liu et al. [25] also demonstrated that the expression of NF-κB in injured carotid arteries increased at 3 days after balloon injury. We also noted that the expression of NF-κB p50, in addition to superoxide production, was significantly increased at 2 days after balloon injury, and these increases were suppressed by the treatment with α-lipoic acid. Suzuki et al. [26] have reported that α-lipoic acid treatment inhibits NF-κB activation in cultured T cells or endotherial cells. Taken together, it seems likely that the inhibition of neointimal formation by α-lipoic acid treatment is due to the suppression of increases in NADPH oxidase-dependent superoxide production, nitrosative stress and NF-κB p50 expression in early phase after balloon injury.

In the present study, we examined how the timing of initiation of α-lipoic acid treatment influenced the therapeutic efficacy. Our data clearly indicated that early initiation of α-lipoic acid treatment prior to the balloon injury is very important to suppress the neointimal formation. On the other hand, we noted that the delayed α-lipoic acid treatment after the balloon injury was ineffective in suppressing the neointimal formation. We also demonstrated that the short-term (7 days) α-lipoic acid treatment from days -3 to 3 prevented neointimal formation. However, the delayed α-lipoic acid treatment (days 7 to 14) could not prevent neointimal hyperplasia, even though the duration of treatment was same. We reported that NADPH oxidase-mediated superoxide production was markedly enhanced in the carotid arteries at 2 days after balloon injury and kept high level until day 7, but thereafter decreased to basal level [10]. These time-course changes in the superoxide production after balloon injury may be closely related to the fact that the early but not delayed α-lipoic acid treatment is efficient in suppressing the neointimal formation.

Conclusion

α-Lipoic acid treatment leads to a reduction of neointimal formation by inhibiting enhanced superoxide production and NF-κB p50 expression in an early phase after balloon injury. Furthermore, short-term α-lipoic acid treatment started prior to the vascular injury was effective in preventing neointimal formation. This short-term α-lipoic acid treatment also could inhibit NADPH oxidase-dependent superoxide production, nitrotyrosine and NF-κB p50 expression in an early phase after balloon injury. Our results suggest that antioxidant agents such as α-lipoic acid may exert a primary prevention for the incidence of cardiovascular events and restenosis after PTCA.

Conflict of interest

The authors declare that they have no conflict of interest.

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