Journal of Cardiovascular Pharmacology and Therapeutics
Volume 13 Number 2
June 2008 120-129

10.1177/1074248407313821
http://cpt.sagepub.com
hosted at
http://online.sagepub.com

The Protective Effect of Bergamot Oil Extract on Lecitine-like OxyLDL Receptor-1 Expression in Balloon Injury-related Neointima Formation

Vincenzo Mollace, MD, Salvatore Ragusa, PhD, Iolanda Sacco, PhD, Carolina Muscoli, PhD, Francesca Sculco, Valeria Visalli, Ernesto Palma, MD, Saverio Muscoli, MD, Luigi Mondello, PhD, Paola Dugo, PhD, Domenicantonio Rotiroti, MD, and Francesco Romeo, MD

Lectin-like oxyLDL receptor-1 (LOX-1) has recently been suggested to be involved in smooth muscle cell (SMC) proliferation and neointima formation in injured blood vessels. This study evaluates the effect of the nonvolatile fraction (NVF), the antioxidant component of bergamot essential oil (BEO), on LOX-1 expression and free radical generation in a model of rat angioplasty. Common carotid arteries injured by balloon angioplasty were removed after 14 days for histopathological, biochemical, and immunohistochemical studies. Balloon injury led to a significant restenosis with SMC proliferation and neointima formation, accompanied by increased expression of LOX-1 receptor, malondialdehyde and superoxide formation, and nitrotyrosine staining. Pretreatment of rats with BEO-NVF reduced the neointima proliferation together with free radical formation and LOX-1 expression in a dose-dependent manner. These results suggest that natural antioxidants may be relevant in the treatment of vascular disorders in which proliferation of SMCs and oxyLDL-related endothelial cell dysfunction are involved.

Keywords: balloon injury; oxidative stress; oxyLDL; bergamot extract

Evidence suggests that vascular injury due to catheter-directed arterial interventional procedures can lead to proliferation of subendothelial vascular smooth muscle cells (SMCs) and neointima formation leading to vascular occlusion and restenosis.[1-9] Many factors have been shown to stimulate the proliferation of SMCs subsequent to vascular injury. These include the disruption of endothelial cell layer,[10,11] the release of growth factors via activation of circulating leukocytes and macrophages,[12-15] and the overproduction of reactive oxygen species (ROS) which activate redox-sensitive signalling pathways,[16] an effect which still remains to be elucidated.

ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\cdot$) can directly cause cell damage, induce the expression of proinflammatory genes, and has been shown to enhance the catabolism of nitric oxide (NO) via the formation of peroxynitrite ($ONOO^-$). On the other hand, peroxynitrite generation after balloon injured arterial vessels leads to neointima formation that has been shown to be prevented by pretreatment of rats with novel antioxidant molecules.[16] Despite the available evidence, however, the underlying

From the University "Magna Graecia" of Catanzaro, Italy (VM, SR, IS, CM, FS, VV, EP, DR); San Raffaele Pisana IRCCS, Rome, Italy (VM, CM); Centro di Ricerca Salus, Bruzzano Zeffirio (Rc), Italy (VM); Institute Mondino-Tor Vergata, Rome, Italy (VM, CM); University of Messina, Italy (LM, PD); University of Rome "Tor Vergata", Rome, Italy (SM, FR).

Address correspondence to: Prof. Vincenzo Mollace, Faculty of Pharmacy, University "Magna Graecia" of Catanzaro, Complesso Nini' Barbieri, Roccelletta di Borgia, 88100, Catanzaro, Italy; e-mail: mollace@libero.it.

molecular and cellular mechanisms of the oxidative process of injured vessels remain unclear.

Recently, it has been shown that oxidative stress accelerates the oxidative modification of low-density lipoprotein (LDL).[16] The increased concentration of oxyLDL is followed by an inflammatory response in vascular tissues which is accompanied by overexpression of the lectin-like oxyLDL receptor-1 (LOX-1),[17-24] an effect which is accounted to trigger SMC proliferation. The enhanced expression of LOX-1 can be inhibited by antioxidants, indicating that restoring the oxidant balance into vascular tissues may be relevant in the maintenance of vascular homeostasis.[25]

In order to allow the vascular tissues protection from oxidative insults, conventional peptidyl free radical scavengers were used for in vivo settings showing limited effect, perhaps due to their short half life and their very low penetration in vascular tissues. As a result of the increased interest in biologically active compounds in food, many research studies have investigated the detection and quantification of natural nonpeptidyl antioxidants.[25,26] Among them, citrus plants have shown a variety of antioxidant compounds including flavonoids, lemonoids, and coumarines.[27,28]

Recently, evidence has been collected indicating that the nonvolatile fraction of bergamot essential oil (*Citrus Bergamia Risso* and *Poiteau*) is rich in natural antioxidants such as furocoumarins (bergapten and bergamottin, in particular), coumarins, and flavonoids,[27-30] which have been suggested to exert a cardioprotective effect in vivo.[27] Beside these preliminary results, however, no clear evidence has been provided on the potential benefits of bergamot oil extract administration in the treatment of vascular injury disease. This study has been designated to evaluate the potential protective effect of nonvolatile fraction of bergamot essential oil (BEO-NVF) on neointima formation and LOX-1 overexpression in balloon-injured common carotid arteries and its correlation with the antioxidant properties of this natural bergamot oil extract of vascular injuries in vivo.

Materials and Methods

Male Wistar rats (350–400 g; Charles River Italia, Calco, Italy) were used for these studies. All rats were housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Catanzaro "Magna Graecia" in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 11692) as well as with European Economic Community regulations (86/609/EEC) and National Institutes of Health guidelines on laboratory animal welfare. All rats were maintained under identical conditions of temperature (21 ± 1°C), humidity (60 ± 5%), and light-dark cycle, and chow and water were available ad libitum.

Vascular Injury Induced by Balloon Angioplasty

The rats were anesthetized with intramuscular 100 mg/kg ketamine (Sigma Chimica, Milan, Italy) and 5 mg/kg xylazine (Sigma Chimica, Milan, Italy). The carotid artery was injured using a balloon embolectomy catheter, as previously described and validated.[2] In brief, the balloon catheter (2F Fogarty, Baxter Corporation, Santa Ana, California, USA) was introduced through the left external carotid artery into the carotid artery and the balloon was inflated at 1.5 atmospheric pressure using a calibration device (Indeflator Plus 20, Advanced Cardiovascular System, Inc., Temecula, California) and pulled 3 times. To keep the duration of the injury that might influence the vascular SMC proliferation constant, we maintained the time of balloon inflation to 18 seconds. In an additional group of rats (sham, n = 10), the effects of the anaesthesia and the surgical procedure (without the balloon injury) were also assessed.

Drug Dosage and Administration

NVF of bergamot essential oil (BEO-NVF) was obtained as previously described.[29] BEO-NVF (5–30 μL/kg) or saline was given daily intraperitoneally (250μL) for 14 days after balloon injury. The same drug administration protocol was performed in sham rats. No toxic effects were observed after BEO-NVF administration over the 14 days of treatment.

Morphology in Evaluation of the Carotid Artery

At the indicated time, rats were anesthetized with an intramuscular injection of 100 mg/kg ketamine and 5 mg/kg xylazine, and the carotid arteries were fixed by transcardial perfusion at 120 mm Hg with 100 ml of phosphate-buffered saline (pH 7.2), followed by 150 ml of 4% paraformaldehyde (pH 7.2). The carotid arteries were removed and 6 cross sections

were cut (each 6 mm in thickness) from the approximate midportion of the artery. Three sections were stained with hematoxylin-eosin to demarcate cell types; the remaining 3 sections were stained with aldehyde fuchsin and counterstained with Van Gieson's solution to demarcate the internal elastic lamina. The sections were photographed under low power, videodigitized, and stored in the image analysis system (Mipron, Kontron Electronics, Eching, Germany) in a 512×512 matrix with an 8-bit gray scale and a 12-field view. The media, neointima, and vessel wall were traced carefully, and the ratios between the neointima and media were calculated as shown previously.[2] All histological studies were performed in a blinded fashion.

Malondialdehyde Determinations

Malondialdehyde (MDA) used as a biochemical marker for lipid peroxidation was measured 14 days after induction of balloon injury in the carotid artery of either untreated or BEO-NVF-treated rats. Injured carotid artery of rat was surgically identified, removed, frozen in liquid nitrogen, then homogenized in potassium chloride (1.15%). Chloroform (2 mL) was then added to each homogenate and spun for 30 minutes. The organic layer of the sample was removed and dried under nitrogen gas and reconstituted with 100 μL of saline. MDA generation was evaluated by the assay of thiobarbituric acid (TBA)-reacting compounds. The addition of a solution of 20 μL of sodium dodecyl sulphate (SDS; 8.1%), 150 μL of 20% acetic acid solution (pH3.5), 150 μL of 0.8% TBA, and 400 μL of distilled water produced a chromogenic product which was extracted in n-butanol and pyridine. Next, the organic layer was removed and MDA levels were read at 532 nm and expressed as nmol MDA/g wet tissue.

Immunohistochemistry

After transcardiacal perfusion, the carotid arteries were fixed in 4% paraformaldehyde. Cryosections (each 6 μm in thickness) were blocked for 1 hour with 100% normal horse serum (Vector Laboratories, Burlingame, California), followed by application of the primary antinitrotyrosine antibody (1:2000, Cayman Chemicals, Michigan, USA) overnight at 4°C; then sections were washed with PBS and incubated with secondary biotinylated goat antirabbit IgG antibody (Chemicon, Billerica, USA) for 1 hour at room temperature (RT). Sections were washed as before and treatment with peroxidase-conjugated avidin–biotin-complexes (Vectastain Elite ABC peroxidase kit; Vector Laboratories, Burlingame, CA) and the metal enhanced DAB kit (Pierce Chemical, Rockford, IL) was performed by manufacturer's instructions.

Detection of In Situ Generation of Reactive Oxygen Species (ROS)

To detect in situ generation of ROS in the carotid artery specimens, fluorescence microphotography with dihydroethidium (DHE) was performed. Fourteen days after induction of balloon injury in carotid artery of either untreated or BEO-NVF-treated rats, injured carotid arteries of rat was surgically identified, removed, and then frozen in liquid nitrogen. Frozen samples were cut into 6 μm-thick sections and placed on glass slides. Sections were washed with PBS (2×10 minutes) and then incubated with DHE (10 μM) in a light-protected chamber for 40 minutes. After washing with PBS, sections were examined for the hydroethidine oxidation product, ethidium accumulation, by a laser scanning confocal imaging system (excitation 510 nm; emission 580 nm).

Western Blotting Analysis for LOX-1 Protein

Carotid artery lysates from each experiment (30 μg/lane) were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After incubation in blocking solution (4% dry nonfat milk, Sigma-Aldrich, St. Louis, MO), membranes were incubated with anti-LOX-1 (1:10 000, gift from Professor T. Sawamura, Osaka, Japan) overnight at 4°C. Membranes were rinsed and then incubated with antimouse IgG antibody (GE Healthcare, Buckinghamshire, England) for 1 hour at room temperature. The specific complex was detected by an enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, England) and relative intensities of protein bands were analyzed by MSF-300G scanner (Microtek International, Inc., Hsinchu, Taiwan). Membranes were stripped with restore western blot stripping reagent following the manufacturer's instructions (Pierce Chemical, Rockford, IL) and then blocked (1 hour at room temperature) with blocking solution and incubated with mouse monoclonal anti-β actin (2 hours at room temperature, 1:3000 dilution; Sigma). After rinsing, the membranes were incubated

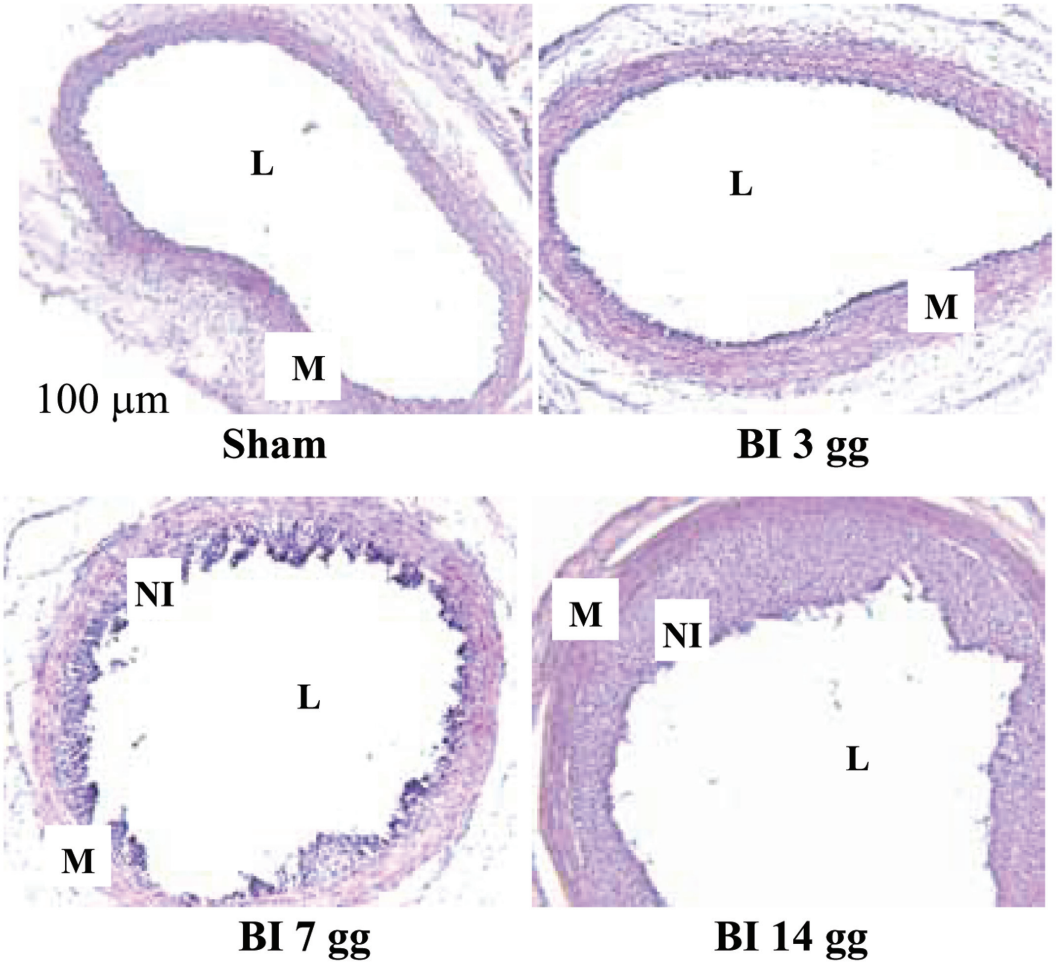

Figure 1. Balloon injury (BI) is accompanied by neointima formation of the carotid artery, compared to sham-operated rats, in a time-dependent manner as shown by representative histological examination. Neointimal formation (NI) is increased in injured vessels as shown by representative histological examination.
M, media; L, lumen.

with antimouse horseradish peroxidase-conjugated secondary antibody (1:20 000 dilution; GE Healthcare, Buckinghamshire, England) and the specific complex was detected by an enhanced chemiluminescence detection system and relative intensities of protein bands were analyzed by MSF-300G scanner. No difference for β-actin was detected among the lanes.

Statistical analysis

Results are shown as mean ± SEM for number of animals. Unless specified, statistical analysis was made using ANOVA followed by post hoc Tukey's test. A P value <.05 was considered significant.

Results

In rats undergoing balloon injury of the left common carotid artery, a significant proliferation of subendothelial vascular SMCs occurred compared with sham-operated animals ($P < .05$ and $P < .001$ for 7 days and 14 days after balloon injury, respectively; Figures 1 and 2). Indeed, the mechanical injury was accompanied by disruption of the EC layer of injured blood vessels with active proliferation of SMCs and subsequent neointima formation (Figure 1). In particular, the cross-sectional areas of vessel walls increased significantly 7 days after injury (n = 20, $P < .05$; Figure 2B), reaching maximum peaks 14 days after balloon

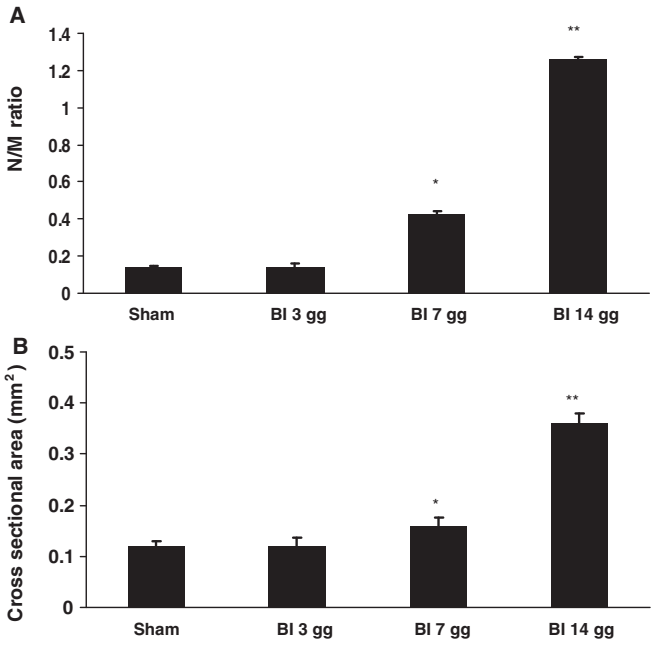

Figure 2. Neointima/media ratio (A) and cross sectional area (B) are increased in injured common carotid after balloon injury (BI) in a time-dependent manner.
$^{*}P < .05$, $^{**}P < .001$ when compared to sham.

angioplasty (n = 20, $P < .001$; Figure 2B), an effect accompanied by similar changes occurring in the neointima/media ratio (n = 20, $P < .05$ and $P < .001$ for 7 and 14 days, respectively, when compared to sham; Figure 2A).

Treatment of rats with BEO-NVF (5–30 μL/kg given i.p. daily after balloon injury) antagonized balloon-induced neointima formation (n = 10 for each dose; Figure 3). Indeed, both cross-sectional area of injured carotid artery and neointima/media ratio were reduced dose-dependently by daily administration of the BEO-NVF ($P < .05$ for 5μL/kg and $P < .001$ for 15 and 30 μL/kg when compared to balloon injured carotids; Figure 4A, B).

Balloon injury was also associated with free radical formation as measured by malondialdehyde (n = 10, $P < .001$ for 14 days after balloon injury when compared to sham; Figure 5), nitrotyrosine staining in vascular tissues of injured rats (Figure 6), and formation of reactive oxygen species as measured by the expression of the oxidation of hydroethidine (Figure 7), indicating overproduction of ROS in balloon-injured carotid arteries. Similarly, LOX-1 expression was raised in the injured carotid arteries after angioplasty as shown by

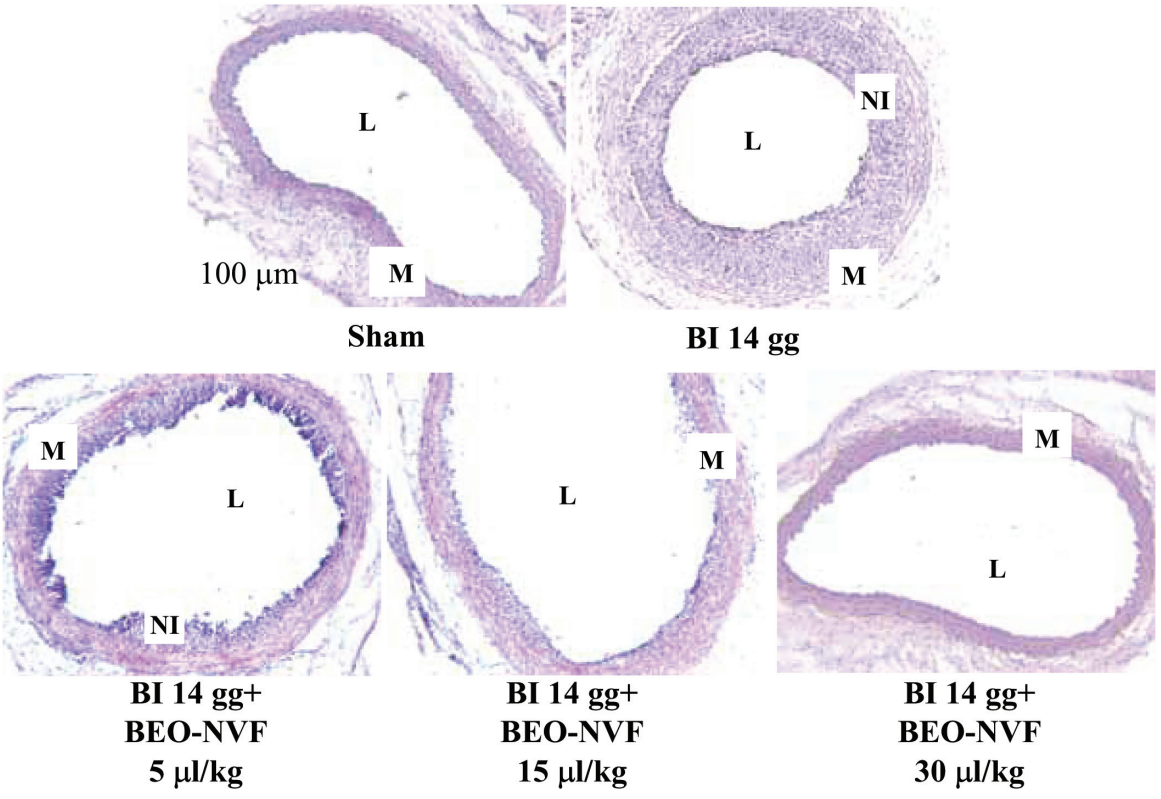

Figure 3. Bergamot essential oil nonvolatile fraction (BEO-NVF) (5–30 μL/kg given i.p. daily during the postangioplasty period up to 14 days) significantly antagonized balloon-induced restenosis dose-dependently.

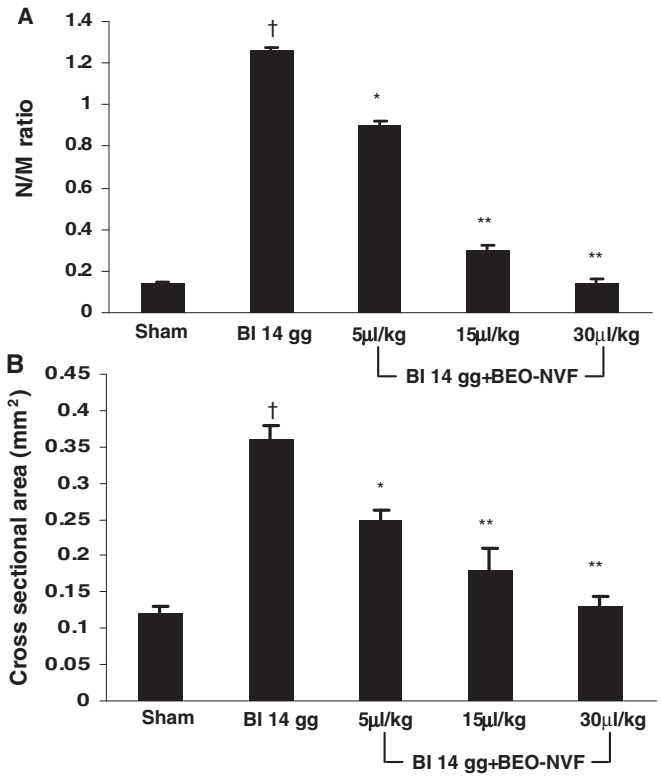

Figure 4. The effect of balloon injury (BI) on neointima/media ratio (A) and cross sectional area (B) of carotid arteries is dose-dependently reversed by bergamot essential oil nonvolatile fraction (BEO-NVF) (5–30 µL/kg given i.p. daily for 14 days). $^{\dagger}P < .001$ when compared to sham; $^{*}P < .05$, $^{**}P < .001$ for treated vs. BEO-NVF-untreated rats.

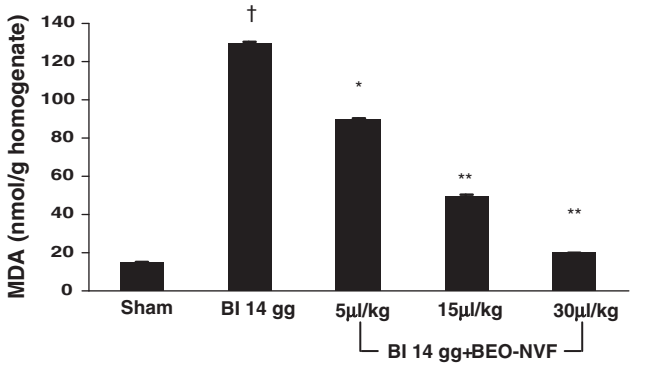

Figure 5. Malondialdehyde (MDA) level increased following balloon injury. Treatment with bergamot essential oil nonvolatile fraction (BEO-NVF) (5–30 µL/kg given i.p. daily for 14 days) antagonized MDA overproduction dose-dependently. $^{\dagger}P < .001$ when compared to control; $^{*}P < .05$ and $^{**}P < .001$ when compared to BEO-NVF-untreated rats.

western blot analysis (Figure 8). We further analyzed the specimens 14 days after balloon injury. Treatment with BEO-NVF (5–30 µL/kg given i.p. daily for 14 days) antagonized MDA overproduction dose-dependently ($P < .05$ for 5µL/kg and $P < .001$ for 15 and 30 µL/kg when compared to balloon injured carotids; Figure 5). Analysis of nitrotyrosine staining and in situ ROS generation detected by DHE revealed that the ROS level generated from the BEO-NVF treated rats was markedly reduced compared with injured carotids (Figures 6 and 7). In addition, the daily treatment of rats with BEO-NVF during the postinjury period significantly reduced LOX-1 expression dose-dependently in vascular tissue (5–30 µL/kg given i.p. daily after balloon injury; Figure 8).

Discussion

The data presented here show, for the first time, that bergamot oil extract given i.p. daily for 14 consecutive days, is able to antagonize SMC proliferation and neointima formation into rat carotid artery subsequent to balloon injury. In addition, such a treatment was clearly related to the antioxidant activity of bergamot oil components, as shown by the significant reduction of nitrotyrosine staining into injured blood vessels, an effect due to the reduced generation of peroxynitrite, a powerful oxidant free radical. Finally, NVF of bergamot oil prevented balloon injury-related overexpression of LOX-1, the receptor for oxyLDL, an effect which we have previously shown to underlie the imbalance of redox status of arterial blood vessels,[25] thereby leading to SMC proliferation. As previously documented, peroxynitrite generation is a crucial step in activating proliferation of subintimal SMCs which follows vascular injury; also, LOX-1 expression is involved in this process, which leads to the reactive neointima formation.[25] Furthermore, it has recently been shown that treatment with BEO is able to reduce neuronal damage following excitotoxicity and protect against N-methyl-D-aspartic acid (NMDA)-induced cell death by affecting different death pathways.[31] In particular, BEO (0.01%) prevented the accumulation of intracellular damage caused by 1 mM NMDA.[31]

Production of ROS that leads to activation of specific signaling pathways, induction of redox-sensitive genes, and impairment of endothelium-dependent relaxation,[16] is regulated by hormone-sensitive enzymes such as the vascular NAD(P)H oxidases, and their metabolism is coordinated by antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. ROS serve as second messengers to activate multiple intracellular proteins and enzymes,

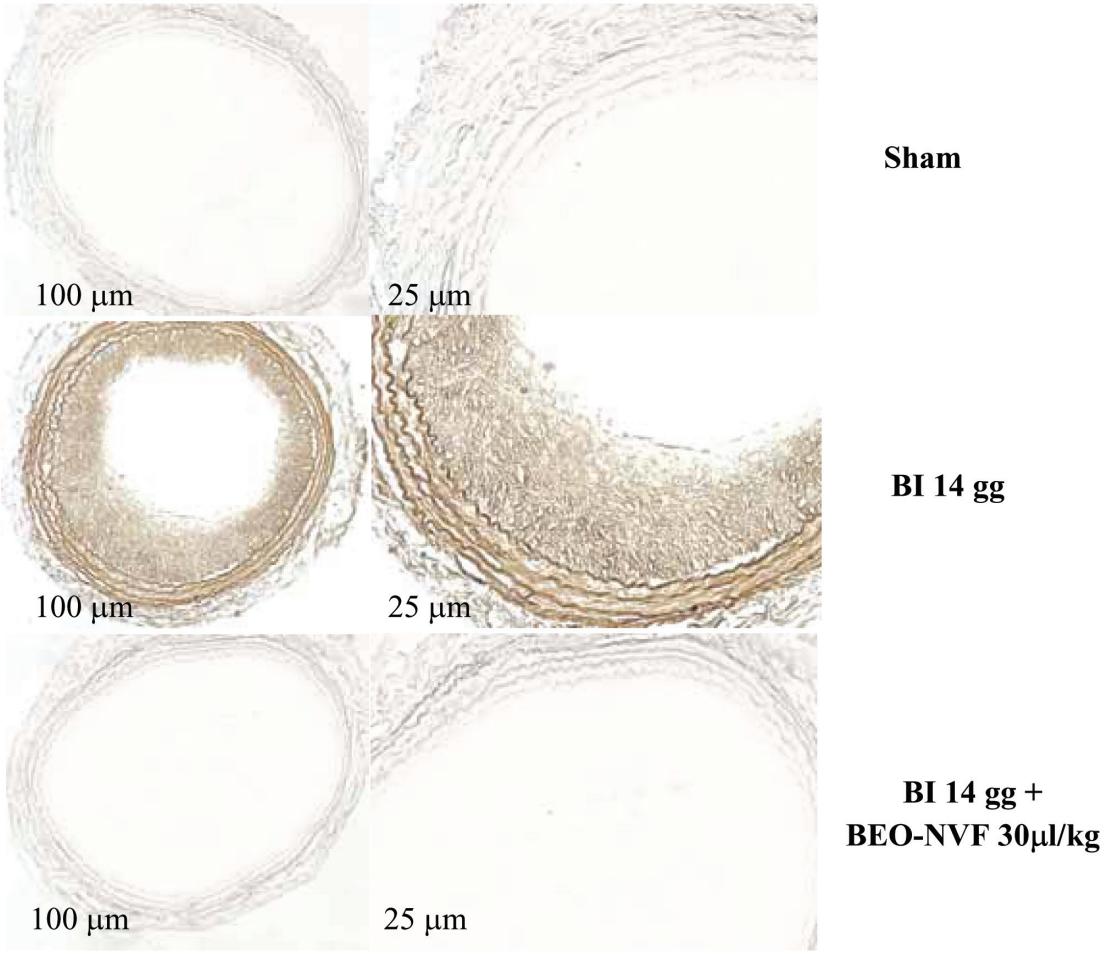

Figure 6. Balloon injury (BI) was accompanied by nitrotyrosine staining in carotid artery 14 days after injury, compared to sham operated rats. Bergamot essential oil nonvolatile fraction (BEO-NVF) (30 μL/kg given i.p. daily for 14 days) reversed this effect as shown by representative immunohistochemical analysis of 5 separate experiments.

including the epidermal growth factor receptor, c-Src, p38 mitogen-activated protein kinase, Ras, and Akt/protein kinase B. Activation of these signaling cascades and redox-sensitive transcription factors leads to induction of many genes with important functional roles in the pathophysiology of vascular cells.[16] Thus, ROS participate in vascular SMC growth and migration, modulation of EC function, expression of a proinflammatory phenotype, and modification of the extracellular matrix. All these events play important roles in vascular diseases, such as hypertension and atherosclerosis, suggesting that ROS and the associated signaling pathways may represent important therapeutic targets.

Our data indicate that LOX-1 may be a crucial link between ROS generation and activation of redox-sensitive genes involved in SMC proliferation. Previous in vitro studies have indicated that LOX-1 expression is upregulated by a host of stimuli including inflammatory cytokines, mechanical forces, and oxyLDL.[32-35] All these lead to ROS production, which has been shown to upregulate LOX-1 in the reperfused and injured tissues.[25,36,37] In addition, evidence exists to suggest that ROS can activate redox-sensitive transcription factors such as NF-kB and AP-1[35] and that the ROS-related activation of NF-kB and AP-1 leads to activation of the promoter region of the LOX-1 gene.[38] Finally, it has recently been reported that ROS are generated upon stimulation of LOX-1 by oxyLDL, which subsequently activate the transcription factor NF-kB, which in turn upregulate the Ang II type 1 receptor with subsequent overexpression of LOX-1 receptor in a positive feedback fashion.[39] Thus, it can

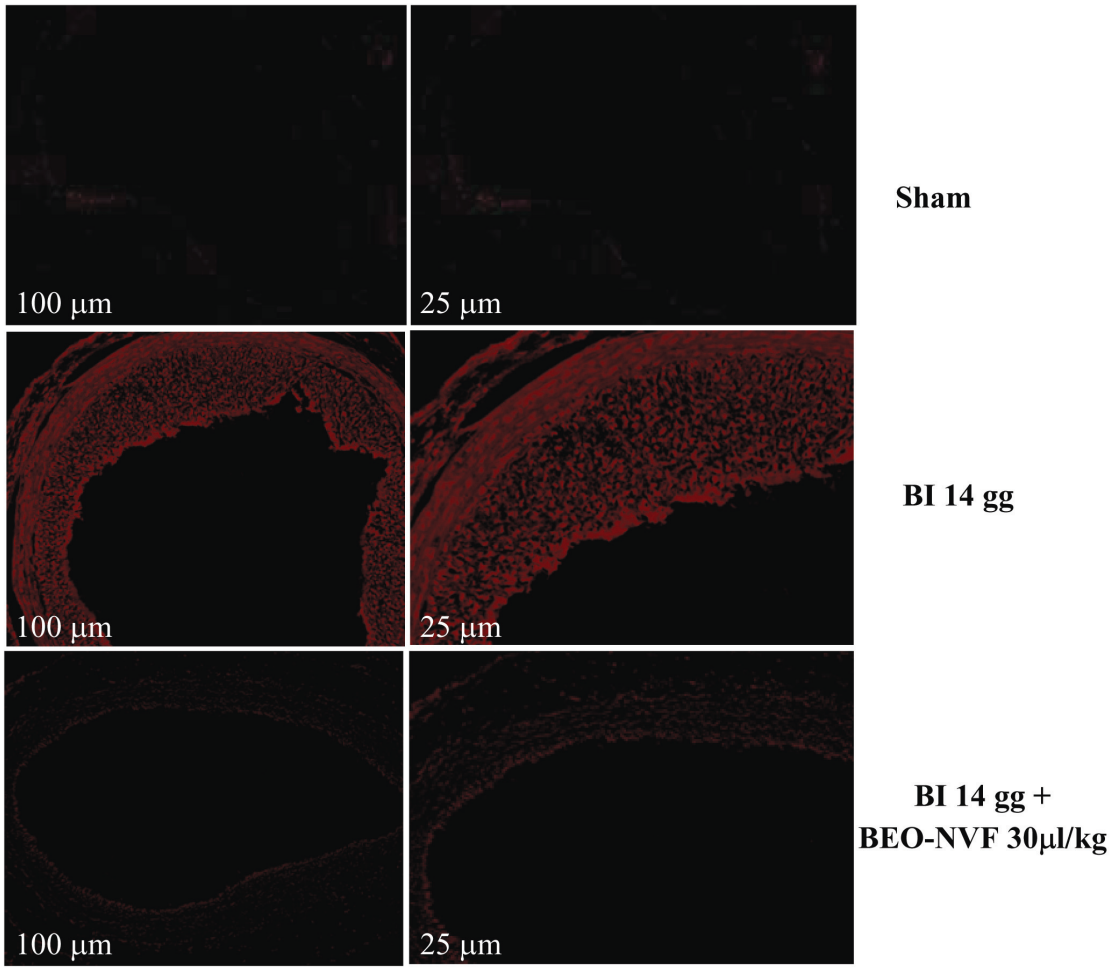

Figure 7. Reactive oxygen species (ROS) generation was monitored by the oxidation of dihydroethidium (DHE) on the sections from the injured arteries at day 14 whether treated or not with bergamot essential oil nonvolatile fraction (BEO-NVF) (30 μL/kg given i.p. daily for 14 days). Images are representative of 5 separate experiments.

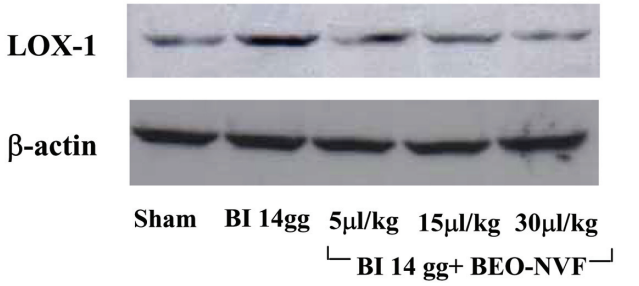

Figure 8. Neointima formation in carotid artery 14 days after balloon injury is accompanied by intense expression of lectin-like oxyLDL receptor-1 (LOX-1) receptor as shown by western blotting analysis. Bergamot essential oil nonvolatile fraction (BEO-NVF) (5–30 μL/kg given i.p. daily for 14 days) reversed this effect in a dose-dependent fashion. No difference for β-actin was detected among the lanes. Gels are representative of 5 separate experiments.

be suggested that the resultant ROS would further upregulate LOX-1 expression, leading to the formation of a feedback loop.

These effects are antagonised by the antioxidant properties shown by the nonvolatile fraction of bergamot essential oil. Indeed, both generation of ROS, as shown by the nitrotyrosine staining, and LOX-1 expression, which occurred earlier than the neointima formation,[25] were counteracted significantly by the NVF-bergamot essential oil treatment. All these effects occurred by day 3 (data not shown), suggesting that oxidative stress and LOX-1 expression are early events in the biochemical changes that can be found in vascular tissue after induction of injury, and that restoring antioxidant status by treating rats with BEO-NVF reduces restenosis of injured arterial vessels by counteracting free radical formation and

LOX-1 expression. This indicates that oxidative stress triggers the cascade of events which generates, via inflammation of the vascular wall and LOX-1 expression, the proliferation of SMC accompanying balloon injury and that restoring the antioxidant status of blood vessels by means of exogenous antioxidant molecules may exert a protective effect.

In conclusion, our studies suggest that NVF of bergamot essential oil, a natural antioxidant rich fraction of this citrus, inhibits oxidative stress which occurs in injured arteries and modulates both LOX-1 expression and neointima formation. This may be relevant as an alternative approach to conventional antiatherogenic compounds in the treatment of vascular disorders in which proliferation of vascular SMCs and oxyLDL-related EC dysfunction occur.

Acknowledgements

The authors thank Mr Giovanni Politi (University "Magna Graecia", Catanzaro, Italy) for excellent technical support.

References

1. Carter AJ, Laird JR, Farb A, et al. Morphologic characteristics of lesion formation and time course of SMC proliferation in a porcine proliferative restenosis model. *J Am Coll Cardiol.* 1994;24:1398-405.
2. Indolfi C, Esposito G, Di Lorenzo E, et al. SMC proliferation is proportional to the degree of balloon injury in a rat model of angioplasty. *Circulation.* 1995;92:1230-1235.
3. Hoffmann R, Mintz GS, Dussaillant GR, et al. Patterns and mechanisms of in-stent restenosis: a serial intravascular ultrasound study. *Circulation.* 1996;94:1247-1254.
4. Roubin GS, Cannon AD, Agrawal SK, et al. Intracoronary stenting for acute or threatened closure complicating percutaneous transluminal coronary angioplasty. *Circulation.* 1992;85:916-927.
5. Indolfi C, Stabile E, Perrino C, Chiariello M. Mechanisms of restenosis after angioplasty and approach to therapy. *Int J Mol Med.* 1998;2:143-148.
6. Holmes DR Jr, Vliestra RE, Smith HC, et al. Restenosis after percutaneous transluminal coronary angioplasty: a report from the PTCA registry of the National Heart, Lung and Blood Institute. *Am J Cardiol.* 1984;53:77C-81C.
7. McBride W, Lange RA, Hillis LD. Restenosis after successful coronary angioplasty. *N Engl J Med.* 1988; 318:1734-1737.
8. Leimgruber PP, Roubin GS, Hollman J, et al. Restenosis after successful coronary angioplasty in patients with single-vessel disease. *Circulation.* 1986;73:710-717.
9. Guiteras Val P, Bourassa MG, David PR, et al. Restenosis after successful percutaneous transluminal coronary angioplasty: the Montreal Heart Institute experience. *Am J Cardiol.* 1987;60 (suppl):50B-55B.
10. Asahara T, Bauters C, Pastore C, et al. Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation.* 1995; 91:2793-2801.
11. Harker LA, Slichter SJ, Scott CR, et al. Homocystinemia: vascular injury and arterial thrombosis. *N Engl J Med.* 1974;291:537-543.
12. Serrano C, Ramires J, Venturinelli M, et al. Coronary angioplasty results in leukocyte and platelet activation with adhesion molecules expression: evidence of inflammatory responses in coronary angioplasty. *J Am Coll Cardiol.* 1997;29:1276-1283.
13. Kastrati A, Koch W, Berger PB, et al. Protective role against restenosis from an interleukin-1 receptor antagonist gene polymorphism in patients treated with coronary stenting. *J Am Coll Cardiol.* 2000;36:2168-2173.
14. Danenberg HD, Welt FGP, Walker M III, et al. Systemic inflammation induced by lipopolysaccharide increases neointimal formation after balloon and stent injury in rabbits. *Circulation.* 2002;105:2917-2922.
15. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med.* 1999;340:115-126.
16. Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscl Thromb Vasc Biol.* 2000;20:2175-2183.
17. Brown MR, Miller FJ Jr, Li WG, et al. Overexpression of human catalase inhibits proliferation and promotes apoptosis in vascular SMCs. *Circ Res.* 1999;85:524-533.
18. Nishio E, Watanabe Y. The involvement of reactive oxygen species and arachidonic acid in α1-adrenoceptor-induced SMC proliferation and migration. *Br J Pharmacol.* 1997; 121:665-670.
19. Cushing SD, Berliner JA, Valente AJ, et al. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and SMCs. *Proc Natl Acad Sci USA.* 1990;87:5134-5138.
20. Li D, Mehta JL. Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. *Circulation.* 2000;101:2889-2895.
21. Sawamura T, Kume N, Aoyama T, et al. An endothelial receptor for oxidized low density lipoprotein. *Nature.* 1997;386:73-77.
22. Kume N, Kita T. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in atherogenesis. *Trends Cardiovasc Med.* 2001;11:22-25.
23. Mehta JL, Li D. Identification, regulation and function of a novel lectin-like oxidized low-density lipoprotein receptor. *J Am Coll Cardiol.* 2002;39:1429-1435.
24. Nagase M, Ando K, Nagase T, Kaname S, Sawamura T, Fujita T. Redox-sensitive regulation of lox-1 gene expression

in vascular endothelium. *Biochem Biophys Res Commun.* 2001;281:720-725.
25. Muscoli C, Sacco I, Alecce W, et al. The protective effect of superoxide dismutase mimetic M40401 on balloon injury-related neointima formation: role of lectin-like oxidized low-density lipoprotein receptor-1. *J Pharmacol Exp Ther.* 2004;311:44-50.
26. Fylaktakidou KC, Hadjipavlou-Litina DJ, Litinas KE, Nicolaides DN. Natural and synthetic coumarin derivatives with anti-inflammatory/antioxidant activities. *Curr Pharm Des.* 2004;10:3813-3833.
27. Kris-Etherton PM, Hecker KD, Bonanome A, et al. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am J Med.* 2002;113(suppl)9B:71S-88S.
28. Gattuso G, Barreca D, Caristi C, Gargiulli C, Leuzzi U. Distribution of flavonoids and furocoumarins in juices from cultivars of *Citrus Bergamia Risso*. *J Agric Food Chem.* 2007;55(24):9921-9927.
29. Dugo P, Mondello L, Dugo L, et al. LC-MS for the identification of oxygen heterocyclic compounds in citrus essential oils. *J Pharm Biomed Anal.* 2000;24:147-154.
30. Dugo P, Presti ML, Ohman M, et al. Determination of flavonoids in citrus juices by micro-HPLC-ESI/MS. *J Sep Sci.* 2005;28:1149-1156.
31. Corasaniti MT, Maiuolo J, Maida S, et al. Cell signaling pathways in the mechanisms of neuroprotection afforded by bergamot essential oil against NMDA-induced cell death in vitro. *Br J Pharmacol.* 2007;151:518-529.
32. Nagase M, Abe J, Takahashi K, et al. Genomic organization and regulation of expression of the lectin-like oxidized low-density lipoprotein receptor (LOX-1) gene. *J Biol Chem.* 1998;273:33702-33707.
33. Kume N, Murase T, Moriwaki H, et al. Inducible expression of lectin-like oxidized LDL receptor-1 in vascular endothelial cells. *Circ Res.* 1998;83:322-327.
34. Aoyama T, Fujiwara H, Masaki T, Sawamura T. Induction of lectin-like oxidized LDL receptor by oxidized LDL and lysophosphatidylcholine in cultured endothelial cells. *J Mol Cell Cardiol.* 1999;31:2101-2114.
35. Kunsch C, Medford RM. Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res.* 1999; 85:753-766.
36. Hinagata J, Kakutani M, Fujii M, et al. Oxidized LDL receptor LOX-1 is involved in neointimal hyperplasia after balloon arterial injury in a rat model. *Cardiovas Res.* 2006;69:263-271.
37. Li D, Williams V, Chen H, Sawamura T, et al. Expression of lectin-like oxidized low-density lipoprotein receptors during ischemia-reperfusion and its role in determination of apoptosis and left ventricular dysfunction. *J Am Coll Cardiol.* 2003;41(6):1048-1055.
38. Cominacini L, Pasini AF, Garbin U, et al. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. *J Biol Chem.* 2000;275:12633-12638.
39. Li D, Romeo F, Mehta JL. Oxidized LDL upregulates angiotensin II type 1 receptor expression in cultured human coronary artery endothelial cells: the potential role of transcription factor NF-kappaB. *Circulation.* 2000;102: 1970-1976.