Full Paper

Anti-atherogenic Effects of a New Thienylacylhydrazone Derivative, LASSBio-788, in Rats Fed a Hypercholesterolemic Diet

Nadia Alice Vieira da Motta[1], Arthur Eugen Kümmerle[2], Elisabeth Marostica[1],
Caroline Fernandes dos Santos[1], Carlos Alberto Manssour Fraga[3], Eliezer Jesus Barreiro[3],
Ana Luisa Palhares de Miranda[3], and Fernanda Carla Ferreira de Brito[1],*

[1]*Laboratory of Experimental Pharmacology (LAFE), Department of Physiology and Pharmacology, Biomedical Institute,
Fluminense Federal University (UFF), Room 204-A, 24420-210 Niterói, RJ, Brazil*
[2]*Department of Chemistry, Institute of Exact Sciences, Federal Rural University of Rio de Janeiro (UFRRJ),
23890-000 Seropedica, RJ, Brazil*
[3]*Laboratory of Evaluation and Synthesis of Bioactive Substances (LASSBio®), Federal University of Rio de Janeiro (UFRJ),
Center for Health Sciences (CCS), P.O. Box 68024, 21944-971 Rio de Janeiro, RJ, Brazil*

Abstract. The compound LASSBio-788 (*N*-Allyl (2-thienylidene) 3,4-methylenedioxybenzoyl-hydrazine) is a thienylacylhydrazone derivative shown to have antiplatelet, vasodilatory, and anti-inflammatory properties in vitro. We hypothesize that LASSBio-788 may exert beneficial effects on atherosclerosis. Male wistar rats were divided into 4 groups: Control group received standard rat chow, hypercholesterolemic group (HC) and HC+788 (compound LASSBio-788 group) received hypercholesterolemic diet for 45 days. HC+788 group received compound LASSBio-788 (100 μmol/kg) once daily in the last 15 days. LASSBio-788 reduced the levels of total cholesterol (109.1 ± 4.3 vs. 361.0 ± 12.8 mg/dl), triglycerides (66.1 ± 1.1 vs. 186.9 ± 17.7 mg/dl), LDLc (63.2 ± 6.1 vs. 330.9 ± 9.7 mg/dl), VLDLc (9.8 ± 1.1 vs. 45.0 ± 4.6 mg/dl) and malondialdehyde (4.8 ± 0.3 vs. 9.4 ± 0.5 nmol/ml) compared to the HC group. LASSBio-788 presented antiplatelet properties and decreased inflammatory markers levels. LASSBio-788 promoted a decrease in contractile response to phenylephrine and an improvement in endothelium-dependent vasorelaxant response by increasing two-fold the expression of nitric oxide synthase (eNOS). Our results suggest that the compound LASSBio-788 represents a new multi-targeted drug candidate for the treatment of atherosclerosis.

Keywords: atherosclerosis, anti-platelet, anti-inflammatory, lipid-lowering,
thienylacylhydrazone derivative

Introduction

Atherosclerosis is a chronic inflammatory process that results from interactions among oxidized low-density lipoproteins (Ox-LDL), macrophages, lymphocytes, and other cellular elements of the arterial wall. Numerous studies have shown that atherogenesis is closely related to the inflammatory and endothelial responses after injury (1, 2).

Platelets play a central role in haemostasis, inflammation, and thrombosis. Platelets mediate plaque formation before actual vessel occlusion at the site of inflammation. Therefore, platelets act as a link among diverse processes, which culminate in atherogenesis (3). Due to the close association between inflammation, atherosclerosis, and thrombosis, it is possible that effective and sustained platelet inhibition may provide both an antithrombotic and anti-inflammatory effect (4). Thus, the development of antiplatelet agents that exert pleiotropic effects is relevant, especially because the available therapeutic arsenal is restricted and unreliable (5).

The compound LASSBio-788 is a thienylacylhydrazone derivative, synthesized from natural safrole. In vitro studies have demonstrated that LASSBio-788 exerts relevant antiplatelet activities and reduces phosphodiesterase (PDE) 2 and PDE5 activities (6). Silva et al. (7) have demonstrated that LASSBio-788 has relevant vasodilatory properties through the modulation of cyclic nucleotides. LASSBio-788 was rationally designed as a PDE inhibitor and contains the *N*-acylhydrazone subunit as the pharmacophore group (8 – 10).

Several studies have indicated that the *N*-acylhydrazone subunit is a pharmacophore group with analgesic, anti-inflammatory, and antiplatelet properties (11). Therefore, we hypothesized that the compound LASSBio-788 may exert beneficial effects on atherosclerosis, which is a disease that closely associates with inflammation in the cardiovascular system. The present study was designed to investigate the pharmacological properties of the thienylacylhydrazone LASSBio-788 derivative in an experimental atherosclerosis model. To evaluate its in vivo properties, LASSBio-788 was chronically administered to Wistar rats fed a hypercholesterolemic diet (HCD).

Materials and Methods

Materials

General experimental procedures using LASSBio-788 [Patent Appl. WO 02/098856] have been previously reported (7). The analytical results for carbon (C), hydrogen (H), and nitrogen (N) for LASSBio-788 were within ± 0.4% of the theoretical values. All of the reagents used were purchased from Sigma Chem, Co. (St. Louis, MO, USA). Rabbit anti-eNOS, goat anti-NF-κB, and rabbit anti-β-actin primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibodies, horseradish peroxidase–conjugated goat anti-rabbit antibody and horseradish peroxidase–conjugated rabbit anti-goat antibody, were purchased from Bio-Rad (Hercules, CA, USA). Cyclophilin B was purchased from Thermo Scientific (San Jose, CA, USA). TNF-α, ICAM-1, IL-1, and IL-6 ELISA immunoassay kits were purchased from R&D Systems (Minneapolis, MN, USA). Total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL), very low-density lipoprotein cholesterol (VLDL), and high-density lipoprotein cholesterol (HDL) standard assay kits were purchased from Labtest (Minas Gerais, Brazil).

Experimental design

The animal protocols were approved by the Ethics Committee for Experimental Research of the Federal Fluminense University (CEPA/UFF-00116/09). Male Wistar rat strains (150 – 200 g) were housed under conditions of controlled temperature (25°C ± 2°C) with a 12-h day / 12-h night cycle. The rats had free access to food and water ad libitum.

The experimental protocol consisted of 45 days of experimentation. The rats were randomly divided into four groups, each of which contained 10 animals. The control group (C) received standard rat chow for 30 days and was then administered gum arabic solution by i.p. injection once a day for 15 days; the hypercholesterolemic group (HC) received HCD for 30 days and was then administered gum arabic solution by i.p. injection once a day for 15 days; the compound LASSBio-788 group (HC+788) received HCD for 30 days and was then administered the compound LASSBio-788 (100 μmol/kg) by i.p. injection once a day for 15 days; In some experiments we have included the LASSBio-788 group, positive control group (C+788), that received standard rat chow for 30 days and was then administered LASSBio-788 (100 μmol/kg) by i.p. injection once a day for 15 days. This dose was employed, based on previous reports (6). The HCD was prepared following the method of Ramesh et al. (12) by mixing equal quantities of a commercial standard chow (Nuvilab®, Brazil) and hypercholesterolemic constituents. The final form of the HCD contained 5% cholesterol, 20% sucrose, 20% hydrogenated vegetable oil, 2% sodium cholate, 20% lactose, 0.4% choline chloride, and 0.15% thiouracil and was mixed with an equal quantity (w/w) of the commercial standard chow.

At the end of the experimental period, all of the animals were euthanized by cervical decapitation under anesthesia (thiopental sodium, 80 mg/kg). The blood samples were collected from each animal, and the aortas were excised. No significant difference in body weights of the three experimental groups was observed throughput the experimental period (data not shown).

Biochemical analysis

The serum lipid profiles [the levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL), very low-density lipoprotein cholesterol (VLDL), and high-density lipoprotein cholesterol (HDL)] were determined using standard assay kits (Labtest). The units were expressed in mg/dl.

Determination of lipid peroxidation in serum samples

The mean concentration of malondialdehyde (MDA), a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid–reacting substances (TBARS) following the method of Ohkawa et al. (13).

Platelet aggregation studies

The rat blood was removed by cardiac puncture and was collected into tubes containing a 3.8% trisodium citrate (9:1 v/v) solution. The rat platelet-rich plasma was prepared by centrifugation at $250 \times g$ for 10 min at room temperature. The platelet-poor plasma was prepared by centrifugation of the pellet at $1500 \times g$ for 10 min at room temperature.

Platelet aggregation was monitored by the turbidimetric method described by Born and Cross (14), using a platelet lumi-aggregometer (Model 560CA; Chrono-log Corporation, Havertown, PA, USA). The platelet-rich plasma (400 μl) was incubated at 37°C for 1 min with continuous stirring at 1200 rpm. The aggregation of the platelet-rich plasma was induced by ADP (0.1, 0.5, 1, 5, 10, and 20 μM) and collagen (0.1, 0.5, 1, 5, 10, and 20 μg/ml). Platelet aggregation was expressed as percentage of aggregation in response to ADP or collagen.

Vascular reactivity studies

The thoracic aortas were; dissected and prepared for isometric tension recording. The aortas were placed in freshly prepared Krebs solution containing 119.0 mM NaCl, 14.9 mM NaHCO$_3$, 11.5 mM glucose, 4.7 mM KCl, 4.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, and 1.6 mM CaCl$_2$. The aortas were cleaned of all adherent tissue and cut into rings of approximately 3 mm. Each ring was suspended between two wire hooks, mounted in 4-ml organ chambers with Krebs solution at 37°C, pH 7.4 and continuously aerated with a mixture of 95% O$_2$ and 5% CO$_2$ under a resting tension of 1 g. The isometric tension of the tissues was recorded by a force-displacement transducer (Ugo Basile, Comerio, VA, Italy) connected to the software (Data Capsule Digital Recorder 17400, Ugo Basile).

After a stabilization period of 1 h, the intact endothelium aortic rings were pre-contracted with phenylephrine (1 μM). The cumulative concentration–response curves to acetylcholine (ACh, $10^{-9} – 10^{-4}$ M) were obtained for the aortic rings. The relaxation curves were expressed as the percent change from the contraction induced by phenylephrine. The cumulative concentration–response curves to phenylephrine were also determined ($10^{-9} – 10^{-4}$ M).

TNF-α, ICAM-1, IL-1, and IL-6 measurements

The serum levels of TNF-α, ICAM-1, IL-1, and IL-6 were assessed using the commercially available Quantikine rat TNF-α, ICAM-1, IL-1, and IL-6 Immunoassay. The levels of TNF-α, ICAM-1, IL-1, and IL-6 in the serum were assessed by measuring the absorbance at 450 nm using an ELISA reader (TP reader, Thermoplate®) and extrapolating from a standard curve.

Western blot analysis

The descending aortas were obtained from the different experimental groups, and the protein extracts were prepared as previously described (15). The extracts were then subjected to western blot analysis (7.5% SDS-PAGE, 50 μg protein per lane) using a primary antibody: rabbit anti-rat eNOS (1:500) or goat anti-rat NF-κB (1:1500) and a secondary antibody: horseradish peroxidase–conjugated goat anti-rabbit antibody (1:1000) or horseradish peroxidase–conjugated donkey anti-goat (1:2000); blots were incubated with the primary or secondary antibody for 1 h. Cyclophilin B and β-actin were used as a control. The proteins were visualized by a chemiluminescent method (GE Healthcare, Amersham Place, Little Chalfont, UK). The values were expressed in arbitrary units after densitometric analysis with Scion Image software (2000; Scion Corp., Frederick, MD, USA).

Histological analysis

The aortas were fixed in 10% buffered formalin. The aortas were cut transversely at the arch and the mid-thoracic level. They were fixed in Bouin's fluid for 24 h and were passed through graded alcohol solutions to process them for routine histopathological examination. The sections (6 – 8-μm-thick) were obtained with a microtome, mounted on glass slides, and processed for histological studies with haematoxylin/eosin staining. The vessel wall and vascular smooth muscle fibers (micrometer) was then quantified by computer-assisted morphometry (NIS – Elements® v. 3.01).

Random fragments were obtained from all liver lobes in order to evaluate the presence of liver injury such as lipid accumulation within hepatocytes (steatosis) as well as liver inflammation and fibrosis. Liver samples followed the routine histological procedures similar to aorta rings.

Statistical analyses

All variables are expressed as the means ± S.E.M. The group differences of variables were compared by using a one-way ANOVA followed by a post-hoc Bonferroni Multiple Comparison Test. For all analyses, a value of $P < 0.05$ was considered to be statistically significant. All analyses were performed using the Graph Pad Prism 5.0 software (GraphPad, San Diego, CA, USA).

Results

Effects of LASSBio-788 on the lipid profile and lipid peroxidation in serum

The levels of total cholesterol, triglycerides, LDL-cholesterol, and VLDL-cholesterol were significantly

Table 1. Effect of compound LASSBio-788 on serum levels of lipids and MDA

Parameters evaluated	Experimental groups			
	Control	HC	HC+788	C+788
Total cholesterol (mg/dl)	51.0 ± 1.2	$361.0 \pm 12.8^{**}$	$109.1 \pm 4.3^{\#\#}$	$62.0 \pm 4.4^{\#\#}$
Triglycerides (mg/dl)	72.0 ± 6.4	$186.9 \pm 17.7^{**}$	$66.1 \pm 1.1^{\#\#}$	$82.9 \pm 5.2^{\#\#}$
cLDL (mg/dl)	15.6 ± 2.2	$330.9 \pm 9.7^{**}$	$63.2 \pm 6.1^{\#\#}$	$11.1 \pm 1.1^{\#\#}$
cVLDL (mg/dl)	14.1 ± 1.2	$45.0 \pm 4.6^{**}$	$9.8 \pm 1.1^{\#\#}$	$17.6 \pm 0.5^{\#\#}$
cHDL (mg/dl)	32.4 ± 2.6	$25.5 \pm 0.8^{*}$	$35.5 \pm 2.1^{\#}$	$45.5 \pm 2.2^{\#\#}$
Atherogenic index[†]	0.9 ± 0.06	$12.2 \pm 0.8^{*}$	$2.3 \pm 0.1^{\#\#}$	$1.3 \pm 0.07^{\#\#}$
MDA (nmol/ml)	4.3 ± 0.5	$9.4 \pm 0.5^{*}$	$4.8 \pm 0.3^{\#}$	$4.9 \pm 0.4^{\#}$

Rats were treated and serum levels of lipids were measured as described in Materials and Methods. [†]: (Total cholesterol − HDL-cholesterol) / HDL-cholesterol (Mean ± S.E.M., n = 8). *$P < 0.05$ and **$P < 0.001$, when compared with C group; [#]$P < 0.05$ and [##]$P < 0.001$, when compared with HC group.
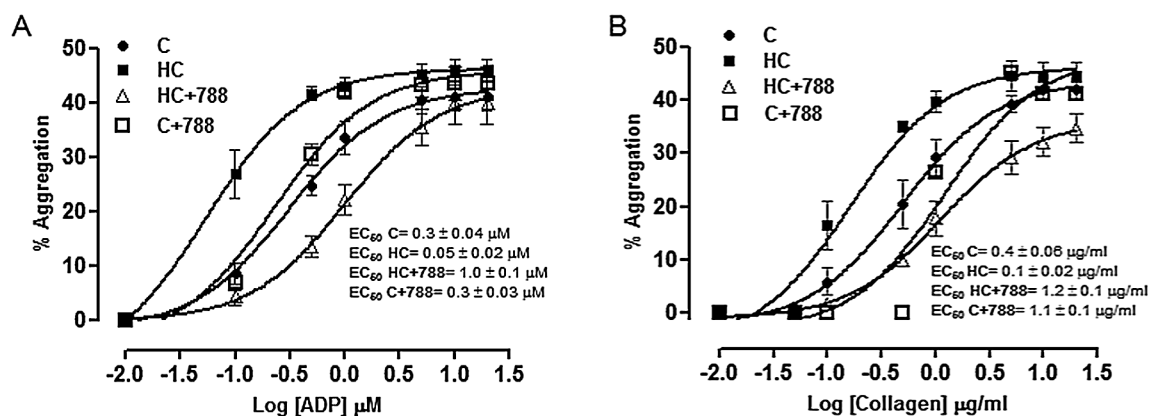

Fig. 1. LASSBio-788 inhibited platelet aggregation induced by ADP and collagen. Effects of LASSBio-788 on concentration–response curves to A) ADP (0.1, 0.5, 1, 5, 10, 20 μM) and B) collagen (0.1, 0.5, 1, 5, 10, 20 μg/ml) in rat PRP. The platelet aggregation was monitored by the turbidimetric method using a platelet lumi-aggregometer. Values are means ± S.E.M. of 5 independent experiments in duplicate.

elevated in the HC group compared to those in the C group, whereas the HDL level was significantly lower in the HC group compared to that in the C group. Administration of LASSBio-788 promoted a decrease in the serum levels of total cholesterol, triglycerides, LDL-cholesterol, and VLDL-cholesterol and increased the serum level of HDL-cholesterol. The increased atherogenic index in the HC group was markedly decreased by the treatment with LASSBio-788. The concentration of MDA in the serum samples of the HC group was higher than that in the C group, but the treatment with LASSBio-788 promoted a marked decrease in MDA concentrations in the serum. In this assay there were no statistical differences between the C, HC+788, and C+788 groups (Table 1).

Effects of LASSBio-788 on platelet aggregation induced by ADP and collagen

The platelet aggregation induced by ADP was increased in the HC group (EC$_{50}$ = 0.05 ± 0.02 μM) compared to the C group (EC$_{50}$ = 0.3 ± 0.04 μM). These results indicate that the platelet aggregation increased in response to HCD. On the other hand, the group that received chronic treatment with LASSBio-788 showed a decreased response to ADP with an EC$_{50}$ of 1.0 ± 0.1 μM. Furthermore, LASSBio-788 promoted a significant decrease in the maximum platelet aggregation percentage at different ADP concentrations (0.1, 0.5, and 1.0 μM) compared with the HC group (Fig. 1A).

When we employed collagen as an agonist, the HC group also showed an increased platelet aggregation response (EC$_{50}$ = 0.1 ± 0.02 μg/ml) when compared to the C group (EC$_{50}$ = 0.4 ± 0.06 μg/ml). The HC+788 group showed a decreased response to collagen (EC$_{50}$ =
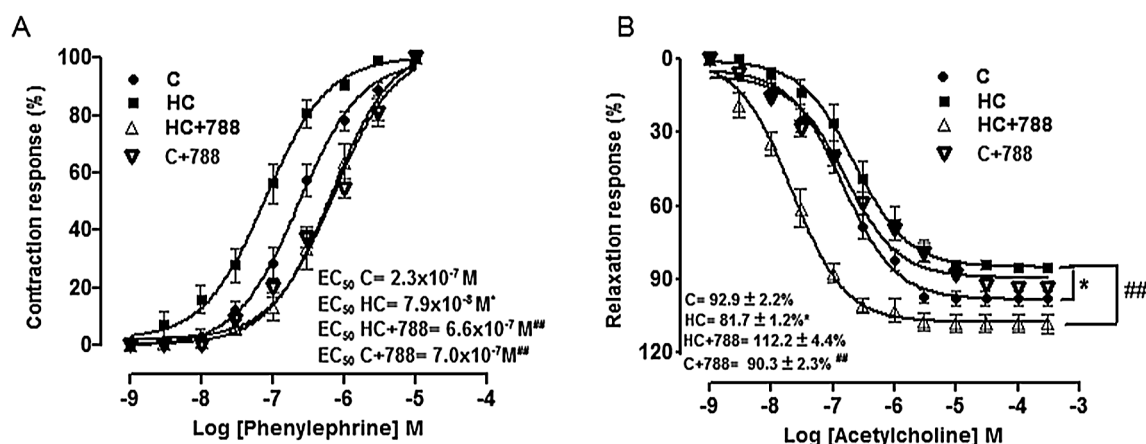
Fig. 2. Effects of LASSBio-788 on concentration–response curves to phenylephrine (A) and acetylcholine (B) in aorta rings. The thoracic aortas were dissected and prepared for isometric tension recording as described in Materials and Methods. Mean ± S.E.M., n = 12 rings, 6 animals each group; *$P < 0.05$, compared with the C group; ##$P < 0.001$, when compared with the HC group.

Table 2. Effects of compound LASSBio-788 on TNF-α, ICAM-1, IL-1, and IL-6 production in serum

Inflammatory markers (pg/ml)	Experimental groups		
	Control	HC	HC+788
TNF-α	34.7 ± 3.1	52.2 ± 4.4**	33.3 ± 2.6##
ICAM-1	396.9 ± 34.9	1080.0 ± 76.2**	531.9 ± 57.5##
IL-1	27.8 ± 1.8	43.3 ± 3.5**	28.2 ± 2.7##
IL-6	139.0 ± 6.1	170.9 ± 2.2**	140.4 ± 9.3##

The serum levels of inflammatory markers were measured by ELISA. Values are expressed as the mean ± S.E.M., n = 8 in each group. *$P < 0.05$ and **$P < 0.001$, when compared with the C group; #$P < 0.05$ and ##$P < 0.001$, when compared with the HC group.

4.2 ± 0.8 μg/ml) when compared with the HC group. The maximum platelet aggregation response to collagen was reduced by the LASSBio-788 treatment at all of the collagen concentrations (0.1 – 20.0 μg/ml) when compared to the HC group (Fig. 1B). In the C+788 group we did not observe any statistical difference from the C group in both platelet aggregation induced by ADP or by collagen (Fig. 1: A and B).

Effects of LASSBio-788 on vascular reactivity in aortic rings

The HC group showed a significant increase in the contraction response to phenylephrine compared with the C group (7.9×10^{-8} M vs. 2.3×10^{-7} M). Nevertheless, the HC+788 group demonstrated a decrease in the contraction response promoted by HCD. A similar response was observed in the C+788 group, demonstrating a significant effect of LASSBio-788 on contractile response to phenylephrine (Fig. 2A). HCD promoted a significant decrease in the endothelium-dependent

relaxation induced by increasing doses of acetylcholine ($10^{-9} – 10^{-4}$ M). Indeed, we found that the relaxation of the aortic rings was significantly lower in the HC group compared to the C group (81.7% ± 1.2% vs. 92.9% ± 2.2%, respectively). However, chronic treatment with LASSBio-788 produced a significant increase in the maximal responses to acetylcholine (112.2% ± 4.4%), compared with the HC group. There were no statistical differences between the C+788 group and the C group (Fig. 2B).

Effects of the compound LASSBio-788 on TNF-α, ICAM-1, IL-1, and IL-6 in serum levels

In our study, we observed that HCD promoted a significant increase of TNF-α, ICAM-1, IL-1, and IL-6 levels. However, chronic treatment with LASSBio-788 promoted a decrease of TNF-α, ICAM-1, IL-1, and IL-6 levels when compared with the HC group, indicating an important anti-inflammatory effect (Table 2).

(A)

(B)

(C)

(D)
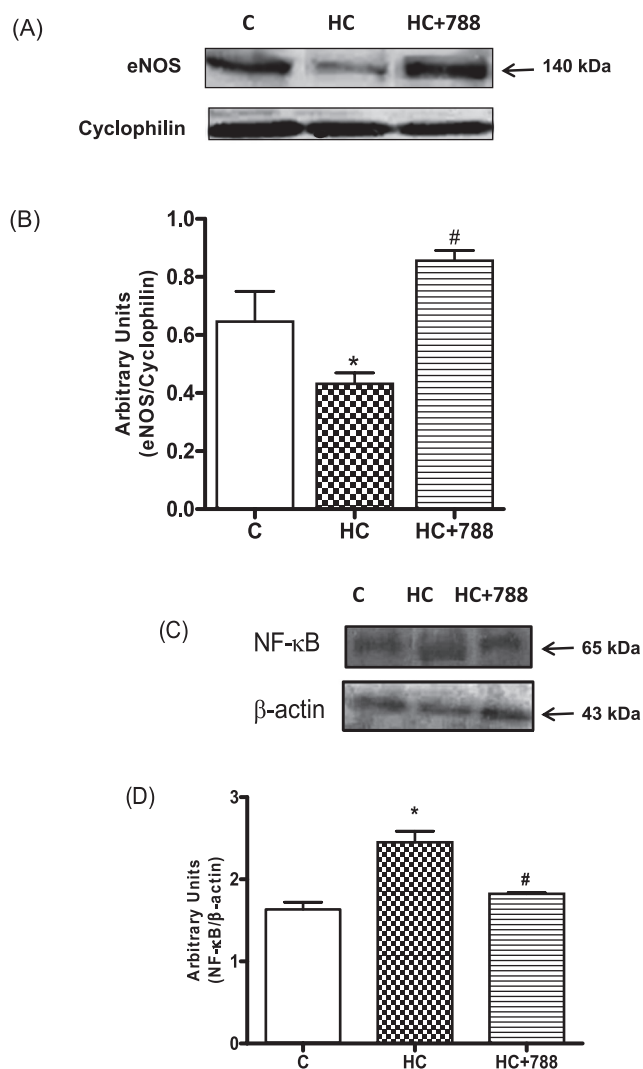
Fig. 3. LASSBio-788 increased the eNOS and decreased NF-κB protein expression in aorta extracts. The extracts were subjected to western blot analysis using a rabbit anti-rat eNOS and goat anti-rat NF-κB primary antibody and a secondary goat anti-rabbit and donkey anti-goat horseradish peroxidase–conjugated antibody for 1 h each. A and C) Representative conjugated immunoblot results of eNOs and NF-κB expression protein from the control group (C), hypercholesterolemic group (HC), hypercholesterolemic treated with LASSBio-788 group (HC+788). B and D) Densitometric analysis data from different experimental groups. The protein levels of each group were standardized for cyclophilin and β-actin levels, respectively. Values are the mean ± S.E.M. from three independent experiments. *$P < 0.05$ vs. C, #$P < 0.05$ vs. HC.

Effects of LASSBio-788 on eNOS and NF-κB protein expression

According to the densitometric analysis from the western blot assay, the eNOS protein expression was decreased by more than 30% in the HC group when compared to the C group. The treatment with the LASSBio-788 compound reversed this effect, and the

levels of the eNOS protein were increased twofold in the aorta of the HC+788 group, as shown in Fig. 3, A and B. In the NF-κB protein expression assay, HCD promoted an increase of protein expression in aortas. The chronic treatment with LASSBio-788 was able to decrease the NF-κB protein expression (Fig. 3: C and D). Equal loading of protein onto the gel was confirmed by immunodetection of the cyclophilin and β-actin control.

Effects of LASSBio-788 on histological parameters

A thickening of the vessel wall was observed in the segments of the thoracic aorta obtained from the HC group (142.5 ± 1.5 μM) when compared with the C group (100.0 ± 2.0 μM). However, treatment with LASSBio-788 ameliorated these pathological changes promoted by HCD (100.0 ± 1.8 μM). The same pattern was observed in the C+788 group (102.0 ± 2.7 μM) (Fig. 4: A – D).

Figure 5 shows three representative images of the liver from the C group (Fig. 5A), hypercholesterolemic rats (HC) (Fig. 5B), and rats treated with LASBio-788 (Fig. 5C). In control rats the liver morphology is characteristic of a healthy liver, but HCD diet induced lipid accumulation in the cytoplasm of almost all hepatocytes (microsteatosis). The arrows point to areas of connective tissue proliferation within liver parenchyma and around the portal space, which would be an indication of tissue inflammation. In a low magnification, it is also clear that there is development of a diffuse lobular pattern, which is usually not characteristic of a rat liver, indicating the development of liver cirrhosis. On the other hand, treatment with the LASSBio-788 protected the liver from injury, since its morphology closely resembles that of the C group. Liver morphology also suggests that LASSBio-788 is not hepatotoxic (Fig. 5D).

Discussion

Atherosclerosis is a chronic inflammatory disease that can lead to acute cardiovascular events (1). Atherosclerosis is caused by the deposition of lipoproteins in the arterial wall. The lipoproteins interact with other systems to promote platelet aggregation, immune responses, and endothelial damage (16). Because hyperlipidemia, inflammation, and immune modulation play key roles in the development of atherosclerosis, the use of lipid-lowering, anti-inflammatory, and anti-platelet agents has been the basis of atherosclerosis treatment. In our experimental study, an animal model of hyperlipidemia was employed to assess the physiological changes promoted by HCD after 45 days of administration and the possible in vivo effects of LASSBio-788 in this model.

Hypercholesterolemia is one of the most important
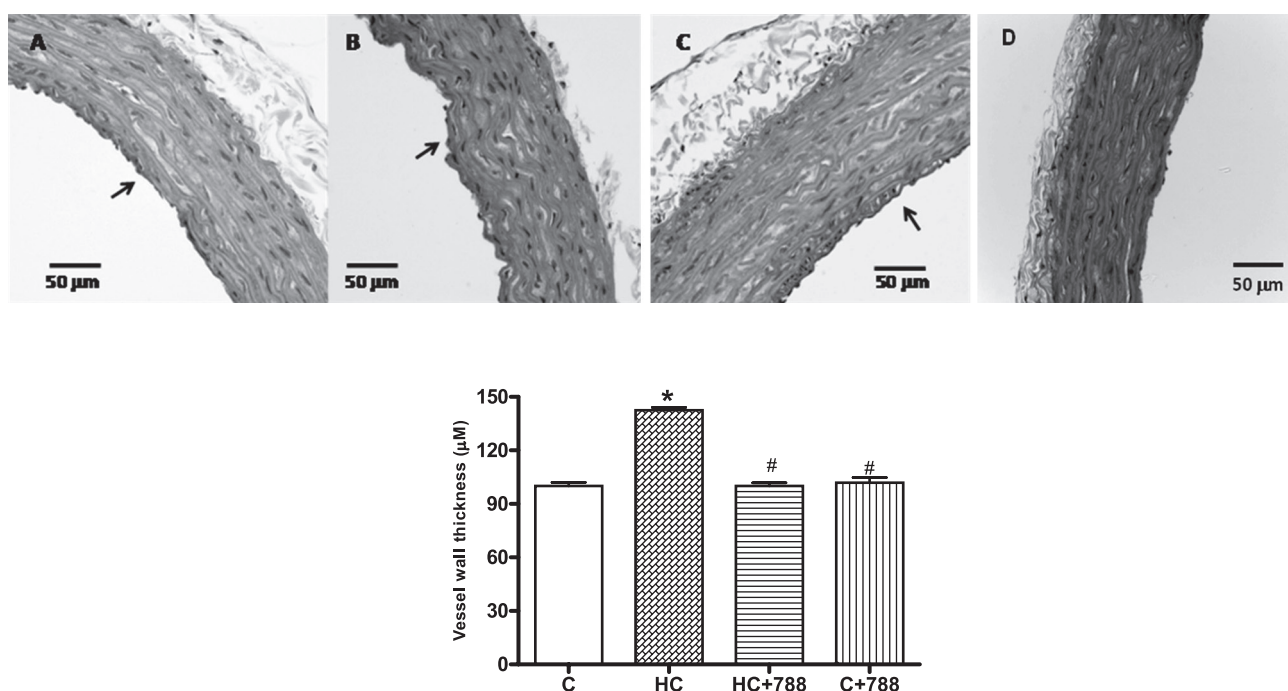
Fig. 4. LASSBio-788 reduced the thickening of the vessel wall. The aortas were fixed in 10% buffered formalin. The aortas were cut transversely at the arch and the mid-thoracic level. The cross-sections of thoracic aorta were stained with hemotoxylin–eosin. A) C group, B) HC group, C) HC+788 group, and D) C+788 group. E) The vessel wall thickness was then quantified by computer-assisted morphometry. *$P < 0.05$ vs. C group, #$P < 0.05$ vs. HC group.
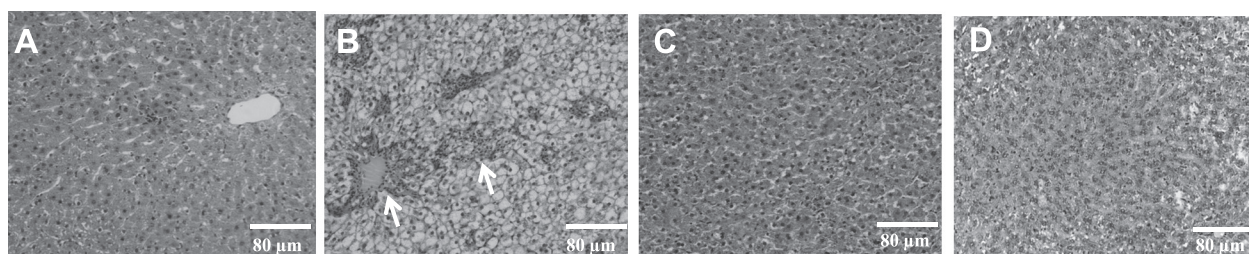
Fig. 5. Photomicrographs of the liver from control rats, C group (A); rats fed with the hypercholesterolemic diet, HC group (B); rats fed with hypercholesterolemic diet + LASSBio-788, HC+788 group (C); and rats fed with standard rat chow + LASSBio-788, C+788 group (D). The arrows point to areas of connective tissue proliferation, which is an indication of tissue inflammation. The tissues were fixed in 10% buffered formalin. The cross-sections of liver samples were stained with hemotoxylin–eosin.

pathogenic factors for atherosclerosis because it alters endothelial cell function and increases the permeability of LDL-cholesterol (17). In this study, HCD administration increased the levels of total cholesterol, triglycerides, LDL-cholesterol, and VLDL-cholesterol and decreased the HDL-cholesterol level. These evidences demonstrate that hypercholesterolemia contributes to atherogenesis development in this rat model. These findings are in agreement with previous reports (12, 18, 19), which showed that elevated total cholesterol and LDL-cholesterol levels promote oxidative damage and inflam-

mation and impair endothelial function. The treatment with LASSBio-788 promoted a significant decrease in the lipid parameters evaluated and promoted an increase of HDL-cholesterol (Table 1).

In vitro studies have shown that LASSBio-788 increases cGMP levels in platelets through the modulation of PDE (6). Cilostazol and dipyridamole are PDE inhibitors that present antiplatelet and vasodilatory properties, and they exert pleiotropic effects, including antioxidant (20), anti-inflammatory (2), and lipid-lowering properties (21). The exact mechanisms involved in the ability of

LASSBio-788 to lower lipoprotein in serum are still unknown. More studies are necessary for a better understanding of its real mechanism. The anti-hypercholesterolemic effects of LASSBio-788 that we have observed appear to be related to PDE modulation.

Oxidative stress results from the disruption of the delicate balance between oxidative (pro-oxidative) and anti-oxidative processes. Oxidative stress is postulated to play an important role in the pathogenesis of atherosclerosis. Many authors have described the direct relationship between lipid peroxidation and hypercholesterolemia in animal models (22). In the present study, we observed an increase of lipid peroxidation in the HC group as evidenced by the increased serum levels of MDA. However, our results showed that there was a significant reduction of the MDA serum levels in the HC+788 group. These results demonstrate a potential antioxidant effect for LASSBio-788, which minimizes the oxidative damage caused by HCD administration. This is in agreement with previous studies that have demonstrated that dipyridamole exerts a powerful antioxidant effect through the inhibition of LDL oxidation (23).

Platelets take part not only in hemostasis and thrombus formation but also in the development of atherosclerosis, which is the main cause of cardiovascular disease and stroke (24). High plasma levels of cholesterol induce vascular oxidative stress and profoundly change the reactivity of both blood vessels and platelets (25). In the present study, we demonstrated that the administration of HCD evoked a significant decrease in the EC_{50} values for both ADP and collagen, suggesting an increase in the platelet response evoked by these agonists. Nevertheless, the LASSBio-788 treatment promoted a significant inhibition of platelet aggregation, which was reflected in the decreased maximum response and potency to collagen and ADP. These results evidence an important antiplatelet effect of LASSBio-788 in vivo (Fig. 1: A and B). Our findings are in line with previous reports that have shown that LASSBio-788 in vitro is able to inhibit collagen and ADP-induced platelet aggregation in human and rabbit platelet-rich plasma (6). These authors have described that LASSBio-788 presented antiplatelet actions through the enhancement of cGMP production and the inhibition of thromboxane A_2 production associated with PDE inhibition.

The normal physiologic function of the arterial endothelium is important in the maintenance of vascular tone and the regulation of blood pressure and blood flow to organs and tissues (26). However, when the endothelial function is impaired, the ability to maintain the vascular tone is impaired, which leads to a decrease in the endothelium-dependent vasodilator responses, a decrease in the ROS production, disruption of the eNOS, and a decrease in NO bioavailability (27). Furthermore, studies have shown that ingestion of a high fat diet provokes endothelial dysfunction, leading to the impairment of vascular relaxing responses and an increased response to vasoconstrictor agonists in experimental animals (26, 28). Thus, both the pharmacologic reduction of plasma lipid levels and the antioxidant therapy have been associated with an improvement of the endothelial function. In the present study, HCD administration in the HC group led to an increase in the contraction response to phenylephrine in aortic rings and promoted an impairment of acetylcholine-induced endothelium-dependent relaxation, which confirms previous studies. Silva et al. (7) have demonstrated that LASSBio-788 exerts a potential in vitro vasodilatory effect in the aortic rings. Similarly, we have found that chronic administration of LASSBio-788 suppresses the contraction response caused by HCD and promotes an enhancement of endothelium-dependent vasodilatation. In a similar way in the C+788 group, we have observed that LASSBio-788 administration to animals fed a regular diet produced a reduction in the contractile response to phenylephrine, but there were no statistical differences between the C+788 group and C group in the relaxation induced by acetylcholine. This result highlights the ability of LASSBio-788 to promote an improvement of vascular function impaired by HCD administration.

At the cellular level, TNF-mediates activation of different pathways leading to the activation of transcription factors such as NF-κB. This transcription factor plays a central role in the development of inflammation through further regulation of genes encoding not only pro-inflammatory cytokines, but also adhesion molecules such as E-selectin, VCAM-1 and ICAM-1, chemokines, growth factors, and inducible enzymes such as cyclooxygenase-2 (COX2) and inducible nitric oxide synthase (iNOS) (29). Nitric oxide (NO) synthesis and release blocks the expression of NF-κB–regulated inflammatory molecules and adhesion molecules (ICAM-1, VCAM-1), prevents platelet activation, and induces vasodilation. Furthermore, high plasma levels of atherogenic lipoproteins leads to decreased NO bioavailability (30), besides impairing eNOS activation by acetylcholine (31, 32).

In western blotting assay studies, LASSBio-788 increased eNOS and decreased NF-κB protein expression in aortas. According to the results obtained in this assay, we can suggest that the beneficial effect promoted by LASSBio-788 on vascular bed may be associated with the inhibition of transcription factor NF-κB and the increase of NO bioavailability.

Several studies have shown that TNF-α, ICAM-1,

IL-1, and IL-6 are important mediators of inflammation and contribute to the development of atherosclerosis. TNF-α promotes the impairment of endothelium-dependent vasorelaxation in a variety of vascular beds and decreases the release of NO, while increasing the generation of arterial ROS (27). Remnant lipoprotein particles derived from VLDL and chylomicrons play a role as atherogenic factors, and they are able to induce superoxide formation and the secretion of TNF-α in human endothelial cells (20). Previous reports have demonstrated that TNF-α increases PDE2 activity in HUVECs, leading to a decrease in cyclic nucleotide accumulation. However, high cyclic nucleotide levels were able to prevent a rise in endothelial permeability promoted by TNF-α (33, 34). High levels of cAMP/cGMP can be achieved either by activation of the adenylyl/guanylyl cyclases or by inhibition of PDEs. Thus, cyclic nucleotide modulators may play an important role in PDE inhibition, improving the endothelial permeability.

In our study, the HCD administration induced an increase of the serum levels of TNF-α, ICAM-1, IL-1, and IL-6 in the HC group, corroborating previous reports (2). Omi et al. (35) described that cilostazol inhibits the expression of adhesion molecules, which promotes a reduction in neutrophil migration. In addition, Shin et al. (36) have reported that cilostazol exerts anti-inflammatory effects by reducing superoxide production and inhibiting the release of cytokines, including TNF-α and IL-1, from HUVECs. We have demonstrated that LASSBio-788 reduces the levels of TNF-α, ICAM-1, IL-1, and IL-6, which results in a significant anti-

inflammatory response.

Several studies have reported anti-inflammatory properties associated with cyclic nucleotide modulator drugs such as cilostazol. Therefore, we propose that the anti-atherogenic effect of LASSBio-788 can be explained by decreasing of TNF-α, which leads to a reduction in ROS and pro-inflammatory cytokines. These effects improve the endothelial function and platelet activity, leading to an increase in vasorelaxant response. Our hypothesis was confirmed through the e-NOS and NF-κB western blotting experiment. In Fig. 3, we demonstrated a reduction of e-NOS expression in the HC group, whereas the expression was augmented in the HC+788 group. On the other hand, there was an augmentation of NF-κB expression in the HC group, whereas the expression was diminished in the HC+788 group.

In accordance with the functional changes observed in the aortic segments from the HC group, histological examinations revealed a thickening of the aortic wall as well as vascular smooth muscle fibers. These results are compatible with the process of atherosclerosis development. As theorized, these morphological changes were prevented by the treatment with LASSBio-788 (Fig. 4: A – D). Figure 5A shows the normal gross appearance of the liver morphology in the C group, but HC (Fig. 5B) have microvesicular steatosis with some degree of connective tissue proliferation in the portal space. However, livers from rats treated with LASSBio-788 (Fig. 5C) closely resemble those of the C group. Also, we did not find any binucleated hepatocytes, an indication of liver injury, in LASSBio-788–treated rats.

We evaluated the effects of LASSBio-788 admin-
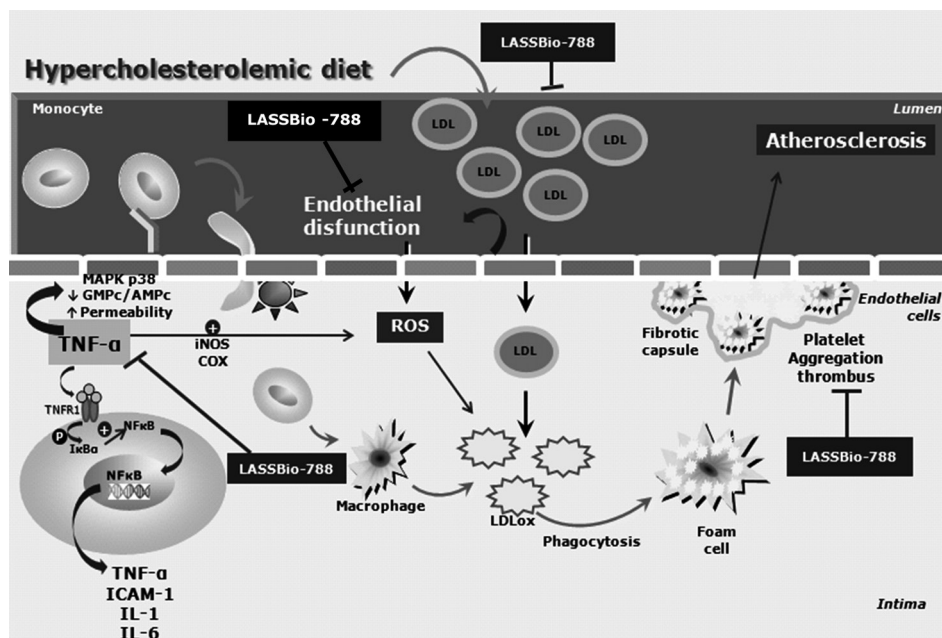
Fig. 6. Schematic LASSBio-788 pharmacological actions related to its anti-atherogenic effect. LASSBio-788 exerts anti-atherogenic action through its lipid-lowering, antioxidant, anti-platelet, vasorelaxant, and anti-inflammatory properties. The possible mechanism of action of this compound is associated with increased cyclic nucleotide level, which leads to the inhibition of TNF-α and consequently reducing the activation of NF-κB. These effects lead to decreased production of inflammatory mediators such as IL-1, IL-6, and ICAM-1. Their inhibition result in decreased oxidative stress and improvement of endothelial function.

istered chronically in animals fed a commercial standard rat chow and we have observed that chronic treatment with this compound did not show any significant difference in lipid profile, oxidative stress, platelet aggregation, and histological parameters when compared to the C group, except for the contraction response study, consistent with the observations of Silva et al (7). According to these results, we can suggest that the compound LASSBio-788 acts only on inflammatory events, not exerting any important effects in healthy animals.

In summary, our results show that the chronic treatment with LASSBio-788 promotes anti-atherogenic effects in vivo and presents lipid-lowering, antioxidant, antiplatelet, vasorelaxant, and anti-inflammatory properties. The possible mechanism of action of this compound is associated with an increase in cyclic nucleotide production, which leads to the inhibition of TNF-α production and other pro-inflammatory cytokines (Fig. 6). These effects lead to a decrease of oxidative stress and an improvement of the vasodilatory response. Taken together, we conclude that the compound LASSBio-788 represents a new multi-targeted drug candidate for atherosclerosis treatment.

References

1 Ross R. Atherosclerosis – An inflammatory disease. NEJM. 1999;340:115–126.

2 Lee JH, Oh GT, Park SY, Choi JH, Park JG, Kim CD, et al. Cilostazol reduces atherosclerosis by inhibition of superoxide and tumor necrosis factor-α formation in low-density lipoprotein receptor-null mice fed high cholesterol. JPET. 2005;313:502–509.

3 Jennings LK. Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherotrombosis. Thromb Haemost. 2009;102:248–257.

4 Muhlestein JB. Effect of antiplatelet therapy on inflammatory markers in atherothrombotic patients. Thromb Haemost. 2010;103:71–82.

5 Tendera M, Wojakowski W. Role of antiplatelet drugs in the prevention of cardiovascular events. Thromb Res. 2003;110:355–359.

6 Brito FCF, Kummerle AE, Lugnier C, Fraga CAM, Barreiro EJ, Miranda ALP. Novel thienylacylhydrazone derivatives inhibit platelet aggregation through cyclic nucleotides modulation and thromboxane A$_2$ synthesis inhibition. Eur J Pharmacol. 2010;638:5–12.

7 Silva AG, Zapata-Sudo G, Kummerle AE, Fraga CAM, Barreiro EJ, Sudo RT. Synthesis and vasodilatory activity of new N-acylhydrazone derivatives, designed as LASSBio-294 analogues. Bioorg Med Chem. 2005;13:3434–3437.

8 Silva CL, Noel F, Barreiro EJ. Cyclic GMP-dependent vasodilatory properties of LASSBio-294 in rat aorta. Br J Pharmacol. 2002;135:293–298.

9 Barreiro EJ, Fraga CAM, Miranda ALP. [Medicinal chemistry of n-acylhydrazones: novel lead-compounds of analgesic, anti-inflammatory and antithrombotic drugs]. Química Nova. 2002;

25:129–148. (text in Portuguese with English abstract)

10 Sudo RT, Zapata-Sudo G, Barreiro EJ. The new compound, LASSBio-294, increases the contractility of intact and saponin-skinned cardiac muscle from Wistar rats. Br J Pharmacol. 2001;134:603–613.

11 Frag CAM, Barreiro EJ. Medicinal chemistry of N-acylhydrazones: new lead-compounds of analgesic, anti-inflammatory and antithrombotic drugs. Curr Med Chem. 2006;13:167–198.

12 Ramesh E, Elanchezhian R, Sakthivel M, Jayakumar T, Senthilkumarb RS, Geraldine P, et al. Epigallocatechin gallate improves serum lipid profile and erythrocyte and cardiac tissue antioxidant parameters in Wistar rats fed an atherogenic diet. Fundam Clin Pharmacol. 2008;22:275–284.

13 Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;85:351–358.

14 Born GVR, Cross MJ. The aggregation of blood platelets. J Physiol. 1963;168:178–195.

15 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–685.

16 Steinberg D. Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. Nature. 2002;8:1211–1217.

17 Maxfield FR, Tabas I. Role of cholesterol and lipid organization in disease. Nature. 2005;438:612–621.

18 Ratheesh M, Shyni GL, Sindhu G, Helen A. Inhibitory effect of Ruta graveolens L. on oxidative damage, inflammation and aortic pathology in hypercholesteromic rats. Exp Toxicol Pathol. 2011;63:285–290.

19 Wu Y, Li J, Wang J, Si Q, Zhang J, Jiang Y, et al. Anti-atherogenic effects of centipede acidic protein in rats fed an atherogenic diet. J Ethnopharmacol. 2009;122:509–516.

20 Park SY, Lee JH, Kim YK, Kim CD, Rhim BY, Lee WS, et al. Cilostazol prevents remnant lipoprotein particle-induced monocyte adhesion to endothelial cells by suppression of adhesion molecules and monocyte chemoattractant protein-1 expression via lectin-like receptor for oxidized low-density lipoprotein receptor activation. JPET. 2005;312:1241–1248.

21 Elam MB, Heckman J, Crouse JR, Hunninghake DB, Herd JA, Davidson M, et al. Effect of the novel antiplatelet agent cilostazol on plasma lipoproteins in patients with intermittent claudication. Arterioscler Thromb Vasc Biol. 1998;18:1942–1947.

22 Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. J Clin Invest. 1991;88:1785–1792.

23 Kusmic C, Picano E, Busceti CL, Petersen C, Barsacchi R. The antioxidant drug dipyridamole spares the vitamin E and thiols in red blood cells after oxidative stress. Cardiov Res. 2000;47:510–514.

24 Alexandru N, Popov D, Dragan E, Andrei E, Georgescu A. Platelet activation in hypertension associated with hypercholesterolemia: effects of irbesartan. J Thromb Haem. 2011;9:173–184.

25 Sengupta A, Ghosh M. Modulation of platelet aggregation, haematological and histological parameters by structured lipids on hypercholesterolaemic rats. Lipids. 2010;45:393–400.

26 Naderali EK, Brown MJ, Pickavance LC, Wilding JPH, Doyle PJ, Williams G. Dietary obesity in the rat induces endothelial dysfunction without causing insulin resistance: a possible role for triacylglycerols. Clin Sci. 2001;101:499–506.

27 Woodman CR, Thompson MA, Turk JR, Laughlin MH.

Endurance exercise training improves endothelium-dependent relaxation in brachial arteries from hypercholesterolemic male pigs. J Appl Physiol. 2005;99:1412–1421.

28 Kang KK, Yu JY, Yoo M, Kwon JW. The effect of DA-8159, a novel PDE5 inhibitor, on erectile function in the rat model of hypercholesterolemic erectile dysfunction. Int J Impot Res. 2005;17:409–416.

29 Bond M, Chase AJ, Baker AH, Newby AC. Inhibition of transcription factor NF-kappaB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. Cardiovasc Res. 2001;50:556–565.

30 Vidal F, Colome C, Martinez-Gonzalez J, Badimon L. Atherogenic concentrations of native low-density lipoproteins down-regulate nitric-oxide-synthase mRNA and protein levels in endothelial cells. Eur J Biochem. 1998;252:378–384.

31 Liao JK, Shin WS, Lee WY, Clark SL. Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. J Biol Chem. 1995;270:319–324.

32 Blair A, Shaul PW, Yuhanna IS, Conrad PA, Smart EJ. Oxidized low density lipoprotein displaces endothelial nitric-oxide syn-thase (eNOS) from plasmalemmal caveolae and impairs eNOS activation. J Biol Chem. 1999;274:32512–32519.

33 Suttorp N, Weber U, Welsch T, Schudt C. Role of phosphodies-terases in the regulation of endothelial permeability in vitro. J Clin Invest. 1993;91:1421–1428.

34 Lum H, Jaffe HA, Schulz IT, Masood A, RayChaudhury A, Green RD. Expression of PKA inhibitor (PKI) gene abolishes cAMP-mediated protection to endothelial barrier dysfunction. Am J Physiol. 1999;277:C580–C588.

35 Omi H, Okayama N, Shimizu M, Fukutomi T, Nakamura A, Imaeda K, et al. Cilostazol inhibits high glucose-mediated endothelial-neutrophil adhesion by decreasing adhesion molecule expression via NO production. Microvasc Res. 2004;68:119–125.

36 Shin HK, Kim YK, Kim KY, Lee JH, Hong KW. Remnant lipoprotein particles induce apoptosis in endothelial cells by NAD(P)H oxidase-mediated production of superoxide and cyto-kines via lectin-like oxidized low-density lipoprotein receptor-1 activation: prevention by cilostazol. Circulation. 2004;109:1022–1028.