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Rosuvastatin displays anti-atherothrombotic and anti-inflammatory properties in apoE-deficient mice

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Abstract

Inflammation contributes importantly to all stages of atherosclerosis, including the onset of acute thrombotic complications. In clinical trials, statins are beneficial in the primary and secondary prevention of coronary heart disease. Moreover, statins have been shown to possess several pleiotropic properties independent of cholesterol lowering in experimental settings. Based on these premises, we investigated the anti-inflammatory and anti-atherothrombotic properties of rosuvastatin *in vivo*, testing its effect on cholesterol and monocyte accumulation, and on adhesion molecules and tissue factor (TF) expression. ApoE-deficient female mice were fed a cholesterol-rich diet containing rosuvastatin (0, 1, 2 or 10 mg kg^{-1} d^{-1}) for 12 weeks. Treatment with rosuvastatin did not significantly affect either body weight gain or plasma total cholesterol (C) and triglyceride levels. However, rosuvastatin treatment dose-dependently reduced ICAM-1 expression in the aortic valves (V) (up to 40% inhibition, $p < 0.05$) and in the proximal segment of the ascending aorta (AA) (−50%, $p < 0.001$). Similarly, rosuvastatin inhibited VCAM-1 expression in the V (−40%) and in the AA (−35%, $p < 0.05$). Moreover, there was a reduced accumulation of macrophages in the V in a dose-dependent and statistically significant manner (−45%, $p < 0.01$). These anti-inflammatory effects were reflected in a reduction of cholesterol deposition in the entire aorta, both in the free and in the esterified form. Finally, the expression of tissue factor, the most potent pro-thrombogenic agent, was consistently reduced in AA by rosuvastatin treatment (−71%, $p < 0.001$). Altogether, these data demonstrate that rosuvastatin has anti-inflammatory and anti-atherothrombotic activities in apoE-deficient mice that could translate in a beneficial effect on atherogenesis.

Keywords: Apolipoprotein E; Atherosclerosis; Inflammation; Rosuvastatin; Thrombosis

1. Introduction

Atherosclerosis is an inflammatory process that triggers and promotes lesion development up to the point of acute thrombotic complications and clinical events [1]. Inflammation appears to be crucial in all stages of atherosclerosis, from the very early steps of initiation, through the progression and finally to clinical complications [2]. Distinct adhesion molecules regulate different stages of leukocyte immigration at inflammatory sites in a multistep process [3]. Leukocyte recruitment occurs in lesion-prone areas of the arterial tree and results in sub endothelial accumulation of lymphocytes and monocytes that differentiate into macrophages [3]. Abundant macrophages characterize plaques that are vulnerable to fatal disruption in humans [4,5]. Inflammation, with an accumulation of monocytes/macrophages, may also contribute to thrombosis after plaque disruption by producing and exposing tissue factor (TF) in the lesion. TF is the most potent pro-thrombotic agent known; it plays a prominent role as the initiator of the extrinsic coagulation pathway and it has been localized in lipid-enriched macrophages present in human atherosclerotic plaques [6].

The inhibitors of 3-hydroxy-3-methyl-glutaryl Coenzyme A (HMG-CoA) reductase (statins) exert their biological effects by blocking the conversion of HMG-CoA to mevalonate in the hepatic cholesterol biosynthesis pathway [7]. The subsequent lipid-lowering effect is correlated with a decreased risk of coronary and cerebrovascular events, and results in increased survival rates in patients with coronary artery disease [8,9]. More recently, very high-intensity statin therapy using rosuvastatin 40 mg d^{-1} achieved a mean reduction of 53.2% of LDL-C and increased HDL-C by 14.7%, resulting in significant regression

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of atherosclerosis in patients [10]. However, numerous studies have shown that statins may also exert effects beyond their lipid-lowering properties [11–16]. Rosuvastatin has been shown to have anti-atherosclerotic effects and to protect from cerebral ischemia, independently of changes in cholesterol levels, in mice [17–20].

The most widely used animal model of induction of the atherogenic process is the apolipoprotein E (apoE)-deficient (apoE$^{-/-}$) mouse [21]. ApoE$^{-/-}$ mice develop pronounced hypercholesterolemia on a normal chow diet, with foam cell-rich fatty lesions in the aortic sinus and proximal aorta by 3 months of age [22,23], and fibrous lesions after 8–9 months [22,24]. The atherosclerotic lesions in these mice have features characteristic of those seen in humans and other species [22,24,25], they contain oxidation-specific epitopes [26], and the expression of adhesion molecules VCAM-1 and ICAM-1, as well as of TF is upregulated in this mouse strain [27,28]. The apoE$^{-/-}$ mice respond appropriately to a human-like Western type (cholesterol-rich) diet and to pharmacological treatment [29,30]. On the Western type diet, adhesion of monocytes and advanced lesions develop at a significantly earlier age and lesion size is increased compared to normal chow-fed mice. However, in contrast to humans, the effect of statins on plasma cholesterol levels in these mice is quite variable [30–32], and it has been postulated that therapeutic effects of statin may depend on the presence of a functional apolipoprotein E [32].

In the present study, we investigated the anti-inflammatory and anti-atherothrombotic properties of rosuvastatin *in vivo* in 10-week-old apoE$^{-/-}$ mice fed a cholesterol-rich diet containing the drug for 3 months, testing its effect on cholesterol and monocyte accumulation, and on adhesion molecules and TF expression.

2. Methods

2.1. Animals

Female apoE$^{-/-}$ mice (Charles River Italy, Calco, Italy; 8–9 mice per group, 10 weeks old) were fed for 12 weeks with a cholesterol rich diet (21% fat by weight, 0.15% by weight cholesterol and 19.5% by weight casein without sodium cholate) [24] containing or not rosuvastatin at 3 dose levels: 1, 2 or 10 mg kg^{-1} d^{-1}. Females were chosen since we have previously shown that female apoE$^{-/-}$ mice have more extensive atherosclerotic lesions than male littermates [33].

2.2. Plasma drug levels

Blood was collected into lithium heparin (1.5 mg ml blood^{-1}) containing tubes 1 h after the last dose of rosuvastatin. Plasma was isolated at 4 °C, diluted into an equal volume of acetate buffer (0.1 M pH 4), and immediately frozen at −70 °C until analysed. Rosuvastatin levels were measured by reverse phase liquid chromatography with electrospray ionisation tandem mass spectrometric detection [34]. All sample preparation was done by robotic liquid handling. Quality control samples, standards and plasma samples were transferred to a 96-well filter plate. Plasma samples were precipitated with ethanol containing an internal rosuvastatin standard. The samples were vacuum filtered and the filtrate transferred to a 96-well collection plate, the filtrate was evaporated to dryness and reconstituted with an acetonitrile:water solution. The extract was injected onto a Polar RP analytical column in line to the mass spectrometer. The anylates were detected by multiple reaction monitoring (MRM) via positive electrospray ionisation tandem mass spectrometry. The lower limit of quantization (LLOQ) for rosuvastatin was 0.05 ng ml^{-1} from a 200 μl plasma sample. The upper limit of quantization for undiluted samples was 100 ng ml^{-1}. Dilutions of 1:10 were validated to extend the working range for quantization to 1000 ng ml^{-1}.

2.3. Lipids

Blood was drawn, after overnight fasting, at baseline, and after 4, 8, and 12 weeks of treatment (at sacrifice) to perform lipid analysis. Total cholesterol and triglyceride levels were determined enzymatically (Clonital, Verona, Italy).

2.4. Lesion area

At the time of sacrifice, after overnight fasting, mice were anaesthetized and perfused with PBS and then with 4% paraformaldehyde (PFA). Heart and thoracic aorta were removed and processed, as previously described [31,33,35], for histological or lipids analysis. The top half of the heart, with 1 mm of proximal aorta attached containing the aortic root, was stored briefly on ice in PBS, then overnight in 4% PFA +15% of sucrose, and the day after frozen in OCT (Optimal Cutting Temperature-BDH Laboratory Supplies) embedding medium over liquid nitrogen-isopentane. The fresh frozen hearts were used to examine the morphology of lesions in the aortic root area. Sequential 20 μm sections were cut until the aortic valve appeared. From this point on, serial 10 μm sections were collected on gelatinized slides. Lipids in heart valves were stained with Oil Red O (Sigma), counterstained with haematoxylin and lesion quantified using a computerized system.

2.5. Cholesterol accumulation in the aorta

The remaining part of the aorta was washed in cold PBS, blotted dry, weighed, minced and extracted with chloroform/methanol (2:1) as described [31]. Total and free cholesterol levels in the aortic extracts were measured using an enzymatic colorimetric assay. The cholesteryl ester content in each sample was calculated by subtracting the value of free cholesterol from that of total cholesterol. All values are expressed as nmol/mg wet tissue.

2.6. Immunohistochemical detection of adhesion molecule and inflammatory markers

For immunohistochemical detection of adhesion molecules and inflammatory markers, tissue sections, obtained from the aortic valves and the first portion of the ascending aorta, immedi-

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ately distal to the valves, were washed in PBS, treated for 20 min in 0.3% H_2O_2 and blocked 20 min with 1.5% normal horse serum (Vector Laboratories). Tissue sections were incubated for 1 h at room temperature with the goat polyclonal antibody against the carboxy terminus of the mouse ICAM-1 (*sc*-1511) or VCAM-1 (*sc*-1504) (Santa Cruz Biotechnology, Inc.) diluted 1:20 in PBS. The slides were washed and then exposed for 30 min to biotinylated horse anti-goat IgG (Vector Laboratories) diluted 1:200 in PBS containing 1.5% normal horse serum.

For immunohistochemical detection of macrophage accumulation, tissue sections were washed in PBS, treated with pepsin (Sigma–Aldrich) 0.05% in HCl 20 μM for 20 min at 37 °C, incubated with H_2O_2 0.3% for 20 min and then with 1.5% normal rabbit serum (Vector Laboratories) for 30 min. The specific rat anti-mouse MOMA-2 antibody (MCA519G Serotec), recognising an intracellular antigen of mouse macrophages, was diluted 1:25 in PBS and added for 1 h at room temperature. The slides were washed and then exposed for 30 min to biotinylated rabbit anti-rat IgG diluted 1:200 in PBS containing 1.5% normal rabbit serum.

The reactivity of the antibodies for ICAM-1, VCAM-1, MOMA-2 was amplified using the ABC kit (Vectors Laboratories) for 30 min and detected using AEC complex (Sigma) as substrate. The slides were counterstained with hematoxylin and then mounted with glycerol.

2.7. Immunohistochemical detection of tissue factor

For immunohistochemical detection of TF, cryosections (10 μm) obtained from the aortic valves and the first portion of the ascending aorta, immediately distal to valves, were incubated for 60 min with H_2O_2 to saturate endogenous peroxidase and then treated with Avidin-Biotin Blocking Kit (Vector Laboratories Inc.) to avoid non-specific signals. Slides were then incubated for 60 min at 37 °C with a specific goat anti-human tissue factor antibody (AD 4501, American Diagnostica) with 2% horse serum, then rinsed with PBS and incubated for 30 min with a biotinylated horse anti-goat IgG secondary antibody in PBS containing 2% horse serum. Labeling was performed with avidin-biotin-peroxydase kit (Vectastain ABC Elite, Vector Laboratories Inc.) followed by 3,3-diaminobenzidine (DAB, Vector Laboratories Inc.). Images were taken of at least two sections for each animal and the TF-positive area in each section was measured by computer-assisted image analysis (KS300, Carl Zeiss).

2.8. Quantitative analysis of proteins expression and lesion area

The extent of TF-positive and lesion areas was measured using a computer-assisted color image analysis (OPTIMAS 6.2), and employing standard image processing methods. The intensity of the staining for ICAM, VCAM and MOMA was assessed by five independent observers using an arbitrary score. To ensure an unbiased result, morphometric data were collected in a blinded fashion. The data represent the mean ± S.D. of 18 samples analyzed for each treatment group ($n = 9$, two sections for each animals).

2.9. Statistics

The data have been expressed as mean ± S.D. (± S.E.M. in some Figures). Differences between groups have been evaluated by Kruskal–Wallis test followed by the Dunns test. Statistical significance has been assigned at the 95% confidence level ($p < 0.05$).

3. Results

In preliminary dose-finding studies the addition of rosuvastatin (at 1, 2 or 10 mg kg^{-1} d^{-1}) to the hypercholesterolic diet resulted in drug plasma levels of up to 100 ng ml^{-1}, a value similar to those measured in patients in clinic at the highest registered dose of 40 mg d^{-1} (22 ng ml^{-1}; equivalent to 44 nM). A previous report showed a dose-dependent decrease in body weight in mice treated with statins [31]. In our experiments, rosuvastatin did not affect mice body weight at any dosage used (Table 1).

3.1. Rosuvastatin does not affect plasma lipids levels

To evaluate the effect of rosuvastatin on plasma lipids levels, analysis was performed at baseline, and after 4, 8, and 12 weeks of treatment (at sacrifice). The table reports the plasma total cholesterol and triglyceride levels at the time of sacrifice. The data clearly show that, as expected, the western type diet significantly increased the total cholesterol levels compared to baseline values, while triglycerides levels were unaffected. Treatment with rosuvastatin at all doses tested did not alter either plasma cholesterol or triglyceride levels compared to control animals (Table 1).

Table 1
Effect of rosuvastatin on body weight, plasma total cholesterol and triglyceride levels, and lesion size in the aortic valves of apoE$^{-/-}$ mice at the end of 12 weeks of treatment

Treatment (mg kg^{-1} d^{-1})	Animals per group	Animal weight (g)	Total cholesterol (mg dl^{-1})	Triglycerides (mg dl^{-1})	Lesion area (μm^2 section^{-1})
Baseline	9	19.65 ± 0.82*	298 ± 62*	87 ± 29	9554 ± 1012*
HC diet (Control)	8	28.46 ± 4.44	1055 ± 376	93 ± 30	428489 ± 36898
HC + rosuvastatin (1)	8	27.62 ± 0.84	848 ± 249	97 ± 29	369648 ± 111709
HC + rosuvastatin (2)	9	28.72 ± 1.44	1060 ± 201	88 ± 12	359841 ± 104085
HC + rosuvastatin (10)	9	26.56 ± 2.75	975 ± 109	93 ± 10	435334 ± 133058

*$p < 0.001$ vs. control animals; Baseline = mice were sacrificed at time 0 (before starting the HC diet); HC = hypercholesterolic diet; N.D. = not determined. Data are the mean ± S.D.

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Fig. 1. Representative cryosections of the aortic root from control or rosuvastatin-treated mice stained with Oil Red O as described in the Section 2.

3.2. Rosuvastatin reduces cholesterol accumulation in the aorta

The extent of rosuvastatin effect on atherosclerotic lesions in the apoE$^{-/-}$ mice was quantified by measuring the area with lipid deposition in aortic valve (V) sections stained with Oil Red O. The analysis of the data indicates that the treatment with rosuvastatin did not affect the lipid content of the lesion area in the valves (Fig. 1, and Table 1). However, when we measured the effect of rosuvastatin on cholesterol accumulation in the entire aorta, a parameter previously used to quantify atherosclerosis in mice [31,36], we observed a statistically significant reduction of cholesterol accumulation following rosuvastatin treatment, both in the free and in the esterified form (Fig. 2).

3.3. Rosuvastatin reduces the expression of adhesion molecules in the aortic valves

Since VCAM-1, together with P- and E-selectin, is involved in the initial steps of leukocyte infiltration into inflammatory sites [27], we analyzed the effect of rosuvastatin on the expression of this adhesion molecule in the aortic valves. We observed a dose-dependent inhibitory effect of rosuvastatin on VCAM-1 expression by up to 40% (Fig. 3, panel A). We also analyzed the effect of rosuvastatin on the expression of ICAM-1, an adhesion molecule which is thought to be involved in the firm adhesion step in leukocyte infiltration [27]. The data shown in Fig. 3 (panel B) demonstrate that rosuvastatin treatment reduced ICAM-1 expression in the aortic valves (up to 40% inhibition). The results are statistically significant at the 10 mg kg^{-1} d^{-1} dose ($p < 0.05$).

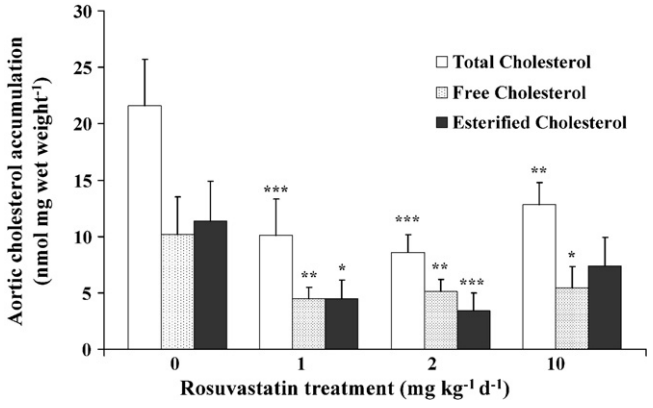

Fig. 2. Effect of rosuvastatin on cholesterol accumulation in the aorta of apoE$^{-/-}$ mice. Animals were fed a cholesterol-rich diet containing rosuvastatin (0, 1, 2 or 10 mg kg^{-1} d^{-1}) for 12 weeks. Cholesterol content was measured in lipid extracts as described in the Section 2. Data represented are the mean ± S.D. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

3.4. Rosuvastatin reduces macrophage accumulation in the aortic valves

To assess whether the reduction of adhesion molecule expression was reflected in a reduced macrophage infiltration, we analyzed the accumulation of macrophages in the aortic valves of mice treated with rosuvastatin. Tissue sections were incubated with the specific antibodies for murine macrophages (MOMA-2). The data shown in Fig. 4 demonstrate that rosuvastatin treatment reduced the accumulation of MOMA-2 positive cells in the aortic valves by 45% ($p < 0.01$) at the 10 mg kg^{-1} dose.

Rosuvastatin did not affect macrophage accumulation in the ascending aorta (data not shown).

3.5. Rosuvastatin reduces the expression of adhesion molecules in the ascending aorta

To further characterize the anti-inflammatory potential of rosuvastatin, we evaluated its effect on VCAM-1 and ICAM-1 expression in the first part of the ascending aorta of statin-treated mice. The data reported in Fig. 5 show that rosuvastatin treatment significantly and dose-dependently reduced the expression of both adhesion molecules up to 50%.

3.6. Rosuvastatin reduces tissue factor expression in the ascending aorta

Finally, to assess the anti-thrombotic potential of rosuvastatin, we evaluated its effect on TF expression in the aorta of statin-treated mice. TF expression in sections of the aortic valves was not affected by the Western type diet nor by treatment with rosuvastatin (data not shown). In contrast, a significant increase (+62%, as compared to baseline values) in the TF positive area induced by the Western type diet was observed in sections from the first portion of the ascending aorta (Fig. 6). Rosuvastatin significantly reduced TF expression with the highest inhibitory effect observed at the lowest dosage used (71% reduction compared to control, $p < 0.001$; Fig. 6).

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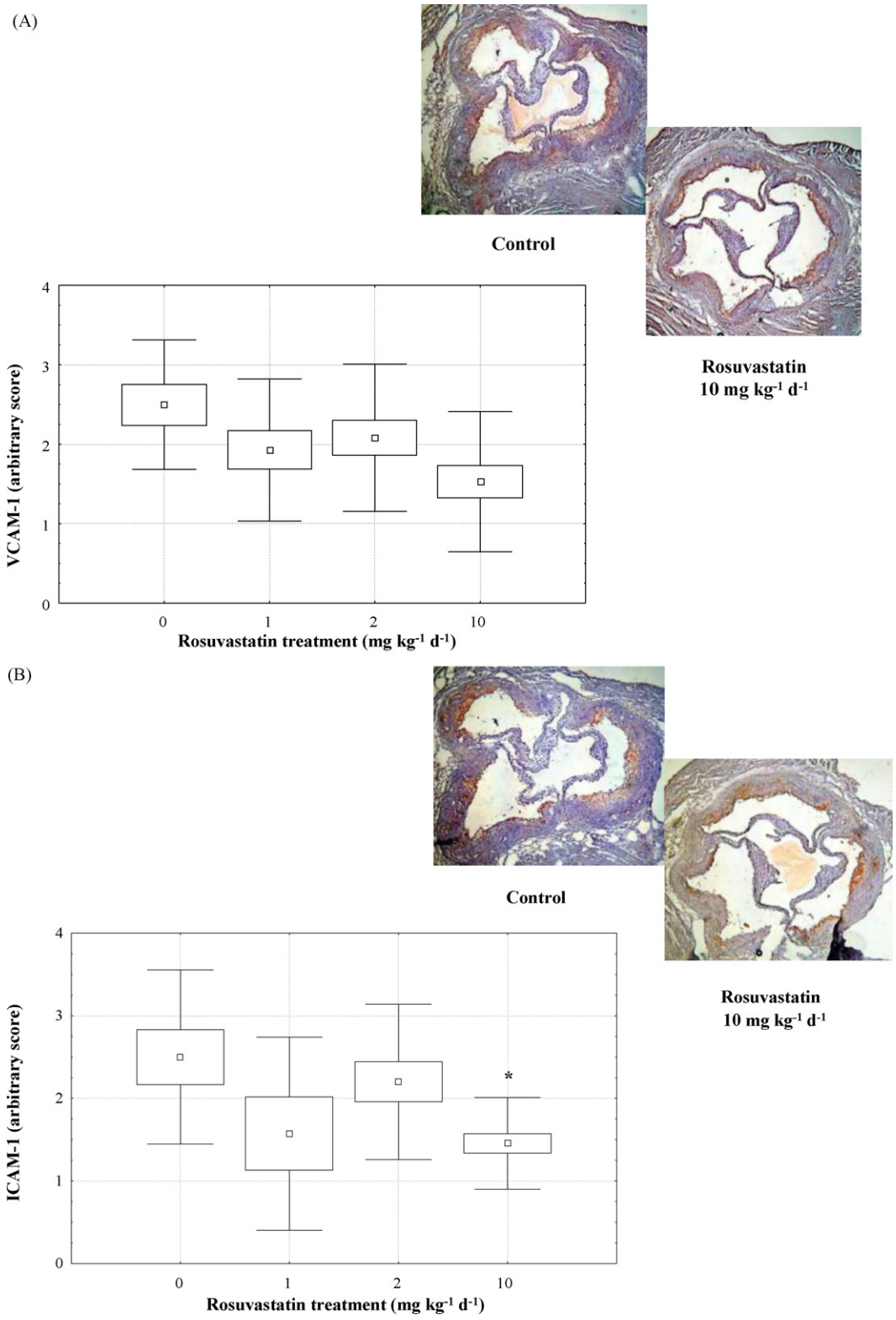

Fig. 3. Effect of rosuvastatin treatment on VCAM-1 (A) and ICAM-1 (B) expression in aortic valves of apoE$^{-/-}$ mice. Cryosections of the aortic valves from control or rosuvastatin-treated mice were stained for VCAM-1 or ICAM-1 expression as described in the Section 2. Boxplots represent the mean ± S.D. ±S.E.M. $^{*}p < 0.05$ vs. control. Insets: representative pictures of the effect of rosuvastatin on adhesion molecules expression.

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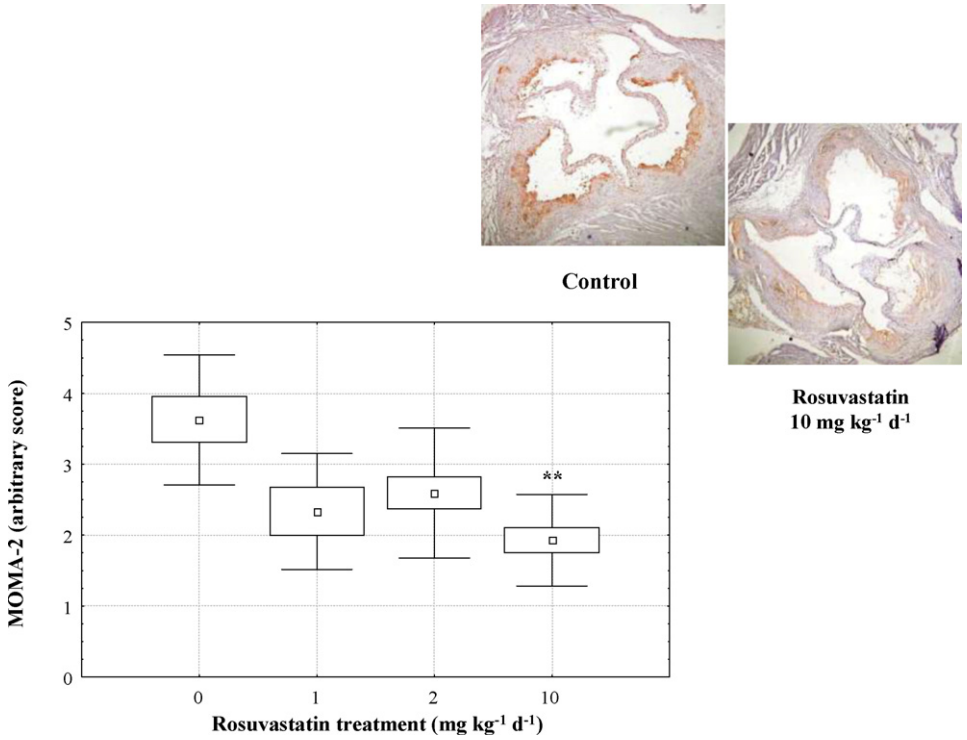

Fig. 4. Effect of rosuvastatin treatment on macrophage accumulation in aortic valves of apoE$^{-/-}$ mice. Cryosections of the aorta from control or rosuvastatin-treated mice were stained for MOMA-2 positive cells as described in the Section 2. Boxplots represent the mean ± S.D. ±S.E.M $^{**}p < 0.01$ vs. control. Insets: representative pictures of the effect of rosuvastatin on macrophage accumulation.

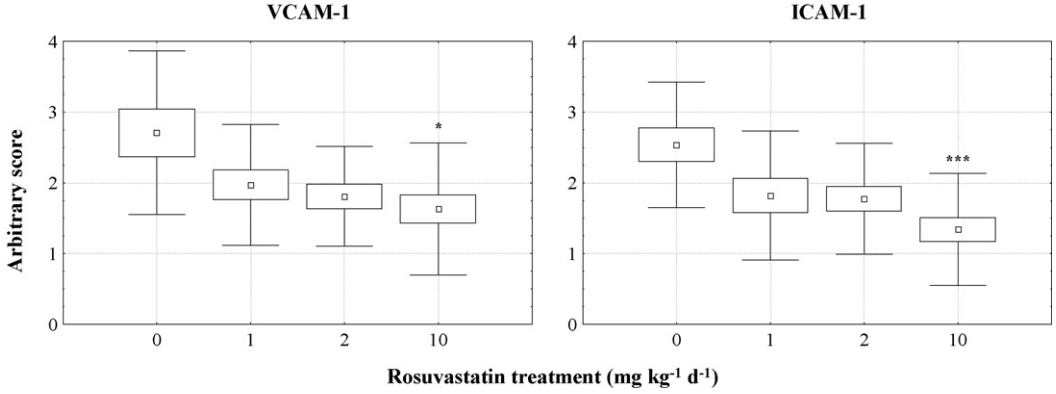

Fig. 5. Effect of rosuvastatin treatment on VCAM-1 and ICAM-1 expression in the ascending portion of the aorta of apoE$^{-/-}$ mice. Cryosections of the aorta from control or rosuvastatin-treated mice were stained for VCAM-1 or ICAM-1 expression as described in the Section 2. Boxplots represent the mean ± S.D. ±S.E.M $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control.

4. Discussion

In this study, we showed that rosuvastatin possesses anti-inflammatory and anti-atherothrombotic properties in hypercholesterolemic apoE$^{-/-}$ mice, in the absence of a cholesterol-lowering effect. Indeed, under our experimental conditions rosuvastatin treatment did not affect either plasma lipids (total cholesterol and triglycerides) or the overall exposure to cholesterol during the treatment. A similar lack of effect has been observed by Sparrow et al. [31]. Statins have been reported to be ineffective in lowering cholesterol levels in both wild-type and apoE$^{-/-}$ mice, probably due to the lack of one of the ligands for the LDL receptor (apoE) and/or to the very strong compensatory increase in HMG-CoA reductase that occurs in this species [31,37]. The absence of a strong cholesterol-lowering activity by rosuvastatin allowed us to assess its anti-inflammatory and anti-thrombotic effects and to consider them independently from the hypocholesterolemic properties of the drug. As measured by immunohistochemistry, the drug inhibited ICAM-1 and VCAM-1 expression in the aortic valves and in the first portion of the ascending aorta in a dose-dependent manner. These adhesion molecules are involved in all three steps of leukocyte recruitment during the initial phases of the atherogenic process [27]. VCAM-1, together with P- and E-selectin, is involved in the first step of tethering and rolling of monocytes and lymphocytes and in second-stage arrest and firm adhesion;

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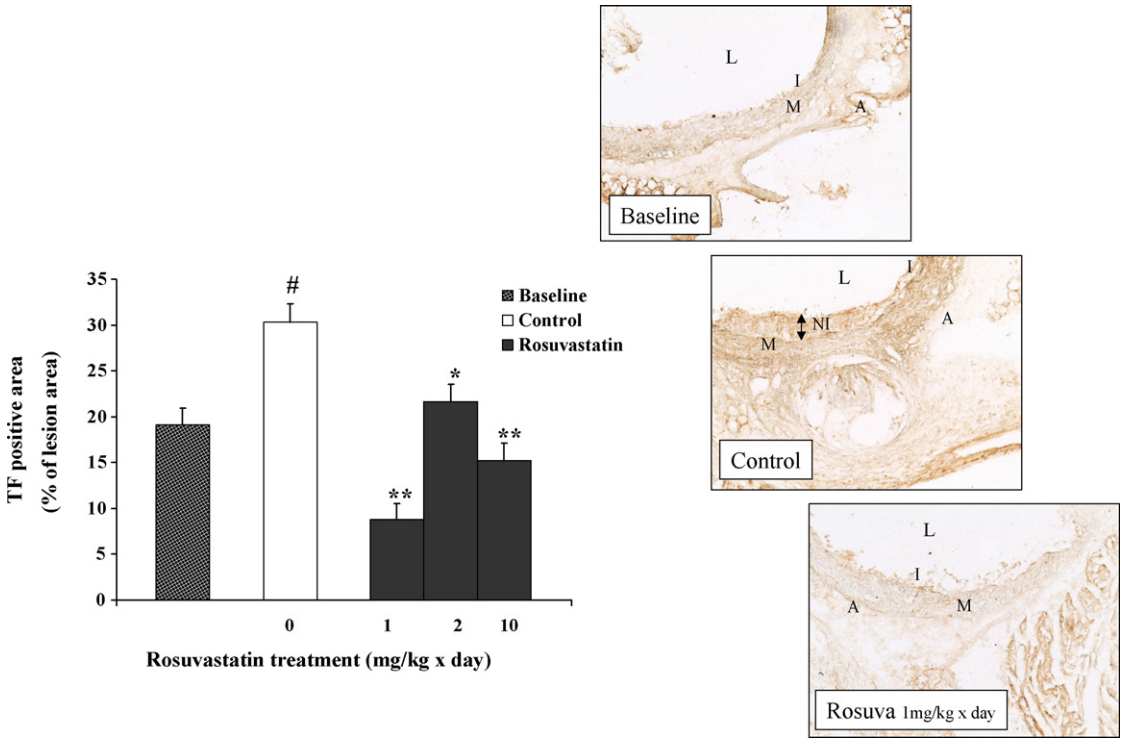

Fig. 6. Effect of rosuvastatin on tissue factor expression in the ascending portion of the aorta of apo $E^{-/-}$ mice. Cryosections of the aorta from control or rosuvastatin-treated mice were stained for TF positive cells as described in the Section 2. Data represent the mean ± S.D. $^{\#}p < 0.01$ vs. baseline; $^{*}p < 0.05$, $^{**}p < 0.001$ vs. control. L = lumen, I = intima, M = media, A = adventitia, NI = neointima.

ICAM-1 is required to mediate the arrest and firm adhesion of lymphocytes, monocytes, and neutrophils at the level of the vascular endothelium. VCAM-1 and ICAM-1 expression is upregulated at atherosclerosis-prone sites in the endothelium of apo$E^{-/-}$ mice [27]. Our data are consistent with the data by Stalker et al. [18] who showed that rosuvastatin exerts important anti-inflammatory effects by inhibiting the expression of the endothelial cell adhesion molecule P-selectin. The inhibitory effect on adhesion molecule expression may explain the fact that rosuvastatin treatment also reduced MOMA-2 positive macrophage accumulation in aortic valves of apo$E^{-/-}$ mice. Similar data have been obtained with statins in mice [19,38], and in rabbits [39]. Moreover, statins directly block the LFA-1–intercellular adhesion molecule-1 interaction that occurs during leukocyte extravasation to sites of inflammation [40]. Alternatively, by blocking Rho A activation, statins may inhibit actin polymerization in monocytic cells [31], thus potentially disturbing integrin-dependent leukocyte adhesion, a process known to be modulated by cytoskeletal organization.

Kleeman et al. [19] showed that rosuvastatin reduces the expression of proatherogenic cytokines, such as MCP-1 and TNF-α, in the vessel wall, and also of the two general inflammatory markers serum amyloid A and fibrinogen. An anti-inflammatory action has been described also for simvastatin [31]. All these experimental data are in agreement with the anti-inflammatory effects already described in pre-clinical and clinical studies with statins [18,19,41–43].

These anti-inflammatory effects, and the reduction of macrophage accumulation, may have resulted in the reduced accumulation of cholesterol in the thoracic aorta of hypercholesterolemic apo$E^{-/-}$ mice. It has been shown that there is a positive correlation between aortic cholesterol content and atherosclerosis measured according to other criteria [31]. However, we did not observe any effect of rosuvastatin treatment on Oil Red O staining in the aortic valves. This discrepancy could be explained by the fact that the area available for lesion development in the valves saturates easily and therefore there is a smaller window in which to detect changes [31]. Aortic cholesterol content is less likely to saturate [44], and usually pharmacological intervention shows larger effects in the thoracic aorta than in the arch both in rabbits and in mice [45,46].

Inflammation is associated with numerous metabolic diseases, such as obesity and insulin resistance [47], and atherosclerosis [3]. An accumulation of activated mononuclear cells may contribute to thrombosis after plaque disruption by producing TF in the lesion [48]. TF is the most potent prothrombotic agent known; it plays a prominent role as the initiator of the extrinsic coagulation pathway and it has been localized in lipid-enriched macrophages of human atherosclerotic plaques [19]. Recent evidence suggests that inhibition of tissue factor or elements in the tissue factor pathway (i.e., factors VIIa and Xa, or thrombin) has the potential to further improve outcomes in atherothrombosis [49]. Lipophilic statins (simvastatin, fluvastatin) have been shown to decrease TF expression and activity in cultured human monocyte-derived macrophages [50], and significantly reduced TF in carotid lesions of cholesterol-fed rabbits [39]. Here, we show that rosuvastatin also inhibited the area positive for TF expression in the aorta of hypercholes-

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terolemic apoE$^{-/-}$ mice. These data are in agreement with Schäfer's who has demonstrated that rosuvastatin reduces the thrombotic potential in apoE$^{-/-}$ mice. Although TF was not measured, fibrin deposition in the vessel wall was decreased [51]. In addition, Steiner has recently shown that simvastatin blunts the increase of LPS-induced TF expression in monocytes *in vivo* [52]. The fact that in our experimental condition rosuvastatin did not exert a dose-dependent reduction of TF expression is consistent with other studies performed in animal models where reduction of several parameters did not follow a dose-dependent fashion [53–55].

Overall our data show anti-inflammatory and anti-atherothrombotic effects of rosuvastatin in the absence of any effect on plasma lipid levels. However, these potentially protective effects are not paralleled by a consistent reduction in the aortic lipid lesion area. The paper by Kleemann showed an anti-inflammatory effect of rosuvastatin, together with a reduction of plasma lipid levels and of lesion area [27]. The partial discrepancy of our data could be due to the different animal model used: apoE$^{-/-}$ versus apoE3 Leiden mice. It has been reported that the anti-atherosclerotic effects of statins depend on the presence of apoE [32]. Therefore, the apoE3 Leiden circulating in the plasma of the transgenic mice, although a dysfunctional apoE3 mutation, could have been sufficient to allow the anti-atherogenic effects of rosuvastatin to be expressed, since apoE3 Leiden demonstrates only a mild reduction in binding efficiency to the LDL receptor versus normal apoE [56]. On the contrary, in the apoE$^{-/-}$ mice the absence of the apolipoprotein could have impaired the statin hypocholesterolemic effect.

In conclusion, our data demonstrate that rosuvastatin may reduce cholesterol accumulation and attenuate the inflammatory and thrombogenic potential of atherosclerotic lesions in the aorta of hypercholesterolemic apoE$^{-/-}$ mice independently of its lipid-lowering effect, thus providing further insights on the complex mode of action of statins.

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