A nitric oxide-donor pravastatin hybrid drug exerts antiplatelet and antiatherogenic activity in mice

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ABSTRACT

Aim of the present study was to compare the lipid-lowering, antithrombotic and antiatherogenic properties of NCX-6550, nitropravastatin, a nitric-oxide donating derivative of pravastatin, with those of pravastatin in hypercholesterolemic mice. LDL receptor-deficient mice (LDLR$^{-/-}$) on a normal diet (ND) showed enhanced cholesterol levels as compared to wild type (WT) mice (6.8±1.2 mmol/L and 2.8±0.82 mmol/L, respectively). High fat diet (HFD) induced a large enhancement of cholesterolemia in LDLR$^{-/-}$ mice (23.7±5.7 mmol/L, p<0.0001 *vs* LDLR$^{-/-}$ ND and WT mice. Treatment with NCX 6550 (48 mg/kg), but not with equimolar pravastatin, reduced cholesterol in LDLR$^{-/-}$HFD. Platelet adhesion to collagen under high shear rate (3000 sec^{-1}) was significantly higher in LDLR$^{-/-}$ than in normal mice, and further enhanced in LDLR$^{-/-}$HFD (-27%, p<0.0001 *vs* untreated). NCX 6550 (48 mg/kg), but not pravastatin, reduced platelet adhesion, especially in LDLR$^{-/-}$HFD. U46619-induced platelet aggregation *ex vivo* was also inhibited by NCX 6550 (48 mg/kg) but not by the parent compound. Finally, photochemically-induced acute (1 hr) femoral artery thrombosis and delayed (21 days) intimal thickening was assessed. Thrombus size was larger in LDLR$^{-/-}$ on HFD than in normocholesterolemic mice (0.46±0.04 *vs* 0.18±0.08 mg) and it was reduced by NCX 6550 (48 mg/kg) (0.08±0.02 mg, p<0.0001), but not by pravastatin (0.4±0.01 mg p=NS). Intimal thickening was greater in hypercholesterolemic than in normal mice (I/M normal=0.53±0.16, LDLR$^{-/-}$=1.1±0.15, LDLR$^{-/-}$HFD=1.75 ±0.25). Both NCX 6550 and pravastatin reduced intimal thickening in normal (-95% and -74.5%, respectively) and LDLR$^{-/-}$ mice (-98% and -91%), while in strongly hyperlipidemic animals (LDLR$^{-/-}$HFD) NCX 6550 was more effective than pravastatin (-98% *vs* -65%, p<0.0001). NCX 6550 shows greater antithrombotic and antiatherogenic activity than pravastatin in highly hypercholesterolemic mice.

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INTRODUCTION

Atherosclerosis is a chronic inflammatory process and inflammation plays an important role in the development of cardiovascular (CV) disease.[1] In mice, atherogenesis may be triggered by raising plasma cholesterol levels and to this end LDL receptor-deficient mice (LDLR$^{-/-}$) fed a high fat/high cholesterol diet represent a commonly used model of atherosclerosis.[2] Indeed, young LDLR$^{-/-}$ mice on a normal diet are characterized by an only slightly elevated plasma cholesterol, a phenomenon attributable to the presence of an alternative ApoB-II pathway for LDL clearance in the mouse. Male LDLR$^{-/-}$ mice fed a western-type diet additioned with 1% cholesterol, without cholate, instead display a strong elevation of LDL cholesterol and total cholesterol that reaches ~25 mmol/L and extensive atherosclerosis[3] and represent thus a good model to study lipid lowering agents and drugs potentially preventing atherosclerosis progression.

Hyperlipidemia in humans is characterized by enhanced oxidative stress, generation of biologically active oxidized lipids and platelet hyperreactivity which contribute to the enhanced CV risk.[4] LDLR$^{-/-}$ mice on a high-fat diet have been shown to recapitulate human hyperlipidemia findings with the accumulation of oxidized lipids and in particular of the atherogenic oxidized choline glycerophospholipids ($oxPC_{CD36}$), in serum, increased platelet reactivity through the interaction between $oxPC_{CD36}$ and platelet CD36 and the development of a prothrombotic phenotype.[5]

Inflammation is important in the pathogenesis of atherosclerotic disease since atherosclerotic plaques are strongly infiltrated by activated immune cells and atherosclerosis associates with enhanced circulating levels of inflammatory biomarkers, such as interleukin-6 (IL-6) or tumor necrosis α (TNF-α).[6] IL-6 is a multifunctional cytokine with endocrine and paracrine effects, which mediates several functions in host defense, but also promotes atherogenesis, dyslipidemia, hypertension and insulin resistance through activated macrophages and lymphocytes. It is also an active signaling molecule synthesized in adipose tissue.[7]

Statins are widely used in the treatment of cardiovascular disease associated with atherogenic dyslipidemia. In addition to their potent action in cholesterol-rich lipoproteins, statins have pleiotropic properties that may contribute to their beneficial effects on cardiovascular diseases.[8] Many of the beneficial pleiotropic effects of statins occur as a result of improved endothelial function and reduced inflammatory processes.[9] Despite their therapeutic efficacy, monotherapy with statins alone is often insufficient to achieve therapeutic goals in patients with atherosclerosis.

Hypercholesterolemia-induced vascular disease is characterized by the loss of the physiological properties of endothelium, known as endothelial dysfunction.[10,11] Endothelial dysfunction is an early event in the pathogenesis of atherosclerosis and is essentially due to diminished production/availability of nitric oxide (NO), decreased eNOS expression, eNOS uncoupling and enhanced NO degradation.[12] Endothelial- and platelet-derived NO inhibits inflammatory cell activation thus exerting a potential antiatherogenic activity.[13] Statins, despite their ability to enhance NO-production and bioavailability by increasing eNOS mRNA stability blocking the geranylgeranylation of the GTPase Rho,[14] may not be able to normalize endothelial function in severe hypercholesterolemia or in acute coronary syndromes. The activity of statins may be potentiated by their combination with molecules of proven anti-inflammatory activity, such as cilostazol, a phosphodiesterase inhibitor, or nitric oxide donors.[15,16]

Moreover, in the last few years, a growing body of evidence has highlighted a 10-12% increase in new-onset diabetes mellitus in hypercholesterolemic patients on statin therapy.[17] Therefore, in the last years, a new class of hybrid drugs combining a statin with a NO-donating moiety has been developed and shown to exert additional properties *in vitro* and *in vivo* as compared to the parent compounds, such as enhanced anti-inflammatory and antiproliferative activities.[18,19] antiplatelet and antithrombotic activities[20] and a pro-healing effect related to potentiation of vascular regeneration in a mouse model of limb ischemia.[21] Moreover, several previous observations show that NO-donating agents, such as the NO-donating aspirin NCX 4016 reduce the onset of diabetes by combining antioxidant, anti-platelet activation and endothelial protective properties.[22–24]

We have previously shown that the addition of a NO-donating moiety to atorvastatin, a lipophilic statin, enhances its effectiveness in models of *in vivo* peroxidation and strong atherosclerosis-related inflammation.[16] Considering that the hydrophilic pravastatin was reported to be less diabetogenic compared with other statins[25] and that its anti-inflammatory and anti-atherogenic effect is magnified when combined with an antiplatelet drug,[15] we focused our work on a new hybrid drug combining NO with pravastatin (NCX 6550) with the aim of characterizing its antiplatelet, antithrombotic and anti-atherosclerotic effects.

MATERIALS AND METHODS

Mice

Wild-type C57BL/6J male mice were purchased from Charles River Laboratories (Lecco, Italy). LDLR$^{-/-}$ mice were purchased from The Jackson Laboratories.[26] LDLR$^{-/-}$ mice had been backcrossed 10 generations onto the C57BK6/J background. Groups of male mice were placed on the different diets after weaning at 4 weeks of age.

LDLR$^{-/-}$ mice were genotyped by using three primer sets that specifically amplified the wild-type or the knockout LDLR allele (forward: 5'- CCA TAT GCA TCC CCA GTC TT-3'; reverse: 5'- GCG ATG GAT ACA CTC ACT G C-3'; primer sequence used for NEO detection: 5'- AAT CCA TCT TGT TCA ATG GCC GAT C-3').

The study was approved by the Committee on Ethics of Animal Experiments of the University of Perugia and by the Italian Ministry of Public Health (Authorization n° 49/2004-B).

Diet

C57BL6/J male mice were maintained under normal diet (ND) (Mucedola, Milan, Italy). LDLR$^{-/-}$ male mice were maintained under normal chow until 4 weeks of age and then they were randomly divided in two groups: one maintained under normal chow, and one switched to a high fat diet (HFD) (Tekland Custom Diet, Wisconsin, diet #TD 95286: 21% [wt/wt] fat, 1% cholesterol, 19.5% casein, and no sodium cholate) for 20 weeks. Drug treat-

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ment was initiated starting from the 16th week of the different diet regimens.

Drugs

Mice were administered pravastatin (40 mg/kg), a pravastatin hybrid donor-NO compound (NCX 6550) (48 mg/kg) or their vehicle by oral route (gavage) once a day for 3 or 4 weeks, depending on the end point of the experiment.

NCX 6550 and pravastatin were dissolved in DMSO (2%) and then diluted in PEG 400 for oral gavage. The concentration of DMSO in the administered mixture never exceeded 1%. The doses of the drugs used were equivalent on the basis of the respective molecular weights (NCX 6550/pravastatin: 1.2/1) and contained the same amount of pravastatin.

Platelet aggregation

Blood was collected by cardiac puncture in 4% trisodium citrate (1:10, v/v) from anesthetized LDLR$^{-/-}$ mice (blood was pooled from at least 5 mice for each treatment group) 1h after the last administration of NCX 6550 (48 mg/kg, o.i.d.), pravastatin (40 mg/kg, o.i.d.) or vehicle. Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 150×g for 15 min at room temperature. Light transmission aggregometry was carried out using a four-channel aggregometer (Aggregocorder, Menarini, Florence, Italy), using the thromboxane (TxA_2) analogue U46619 (3 μM) (MM3), collagen (2 μg/ml) and ADP (3 μM) as aggregating agents (n=3 experiments, 8 animal per group) as previously described.[27]

Platelet adhesion to collagen under flow

Platelet adhesion to a collagen-coated surface under flow conditions was studied according to published protocols.[16,28] Briefly, citrated blood samples (0.5 ml) were passed through a rectangular parallel plate perfusion chamber over a plastic coverslip sprayed with collagen from equine tendon (~ 30 μg/cm^2), at a wall shear rate of 3,000 s^{-1}. The chamber was then perfused with 0.1 % bovine serum albumin in physiologic solution, pH 7.3, to remove all residual blood and then the coverslip was harvested, gently washed with 10 mM HEPES, pH 7.3, and fixed with 0.25 % glutaric-dialdehyde in PBS, pH 7.4. Adhering platelets were stained with May-Grünwald/Giemsa and observed under an optical microscope. The area covered by platelets was measured with a computerized image analyzer (NIH Scion Image, Frederick, Maryland).[16,28]

Serum lipids

Blood from mice fasted overnight was collected by cardiac puncture into glass tubes without anticoagulant. After one hour at 37°C serum was retrieved by centrifugation at 1000×g for 15 min at room temperature. Total, LDL, HDL cholesterol and triglycerides were measured by a commercial colorimetric method (Menarini Pharmaceuticals, Firenze, Italy).[16] LDL cholesterol was calculated from total and HDL cholesterol and triglyceride levels.

Photochemical injury-induced femoral artery thrombosis

Mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and a butterfly 25G needle was inserted in one of the tail veins for rose Bengal infusion. The right femoral artery was surgically exposed and transilluminated with green light (wavelength 540 nm), by a xenon lamp with a heat-absorbing filter, through an optic fiber positioned 5 mm away from the arterial segment (Hamamatsu Photonics, Shizuoka, Japan). Green light irradiation was protracted for 25 min; the infusion of rose Bengal (20 mg/kg) started 5 min after the beginning of irradiation and lasted for 5 min. Blood flow was recorded by a perivascular flow probe (0.5 Transonic System) connected to a flowmeter (Transonic System Inc, model T402, Ithaca, NY, USA) and interpreted by a computerized data acquisition program (Biopac System Inc, Santa Barbara, CA, USA). Time to occlusion of the femoral artery was measured. One hour after the end of irradiation, the femoral artery was excised and the thrombus was weighed after rinsing, blotting on filter paper and drying overnight at 60°C.[16,29–31]

Femoral arteries atherogenesis

To evaluate femoral artery intima hyperplasia triggered by photochemical injury, the wound was closed and animals were returned to their cages. Drugs were administered once a day for 21 days by gavage starting one hour before the induction of femoral artery damage. One hour after the last drug administration mice were anesthetized, the chest and abdominal cavities were opened and a catheter was inserted into the left ventricle and circulation was first washed with saline and then with a solution of 2% glutaraldehyde and 1% paraformaldehyde (PFA) in 0.1 mM PBS, pH 7.4, at physiologic pressure (90 to 100 mmHg) for 10 min. The femoral artery was then removed and fixed overnight in 4% PFA in 0.1 mM PBS. Femoral artery segments were embedded in paraffin and cut consecutively in 5μm thick sections. Sections taken 500 μm apart, were stained with hematoxylin and eosin for morphometric analysis. Media and intima area were measured using a specific software (NIH Scion Image, Bethesda, MD, USA). Sections were assessed blindly as regards to treatment.[16,31]

Quantification of atherosclerosis in the aorta

Atherosclerotic lesions were quantified by *en face* analysis of the whole aorta and expressed as percent of

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the entire surface area of the aorta covered by plaques. For *en face* preparations, a cannula was inserted into the left ventricle and the aortic tree was fixed by perfusion with ice-cold PBS containing 4% PFA, 5% sucrose and 2 µM EDTA for 10 min.[16] The aorta was then opened longitudinally, from the heart to the iliac arteries, while still attached to the heart and major branching arteries. The primary incision followed the ventral side of the aorta and the inner curvature of the arch. To obtain a flat preparation for imaging, a second incision was made along the outer curvature of the arch. The aorta (from the heart to the iliac bifurcation) was then removed and "pinned out" on a black wax surface in a dissecting pan using stainless steel pins 0.15 mm in diameter. Aortas were briefly rinsed in 70% ethanol; immersed for 6 min in a filtered solution containing 0.5% Sudan IV, 35% ethanol, and 50% acetone, and destained for 5 min in 80% ethanol. The Sudan IV–stained aortas were photographed for quantification of atherosclerotic lesions.[16,32]

Circulating tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) levels

TNFα and IL-6 were assessed in serum using commercial ELISA kits purchased from R&D System (Minneapolis, MN, USA) (Quantikine Mouse TNFα Immunoassay, cat. #MTA00 and Mouse IL-6 Quantikine ELISA Kit, cat. #M6000B).[16]

Plasmatic nitrites and nitrates

Blood was collected in trisodium citrate (4%) and plasma was obtained by centrifugation at 1,000×g for 10 min and nitrites and nitrates (NOx) levels were measured by a colorimetric, non-enzymatic method (Oxford Biomedical Research, Rochester Hills, MI, USA), as reported elsewhere.[16,33]

Plasma cGMP

Blood was collected in trisodium citrate (4%) and plasma was obtained by centrifugation at 1,000×g for 10 min and plasma cGMP levels were measured by ELISA (GE Healthcare, Milan, Italy), as reported elsewhere.[16,33]

Statistical analysis

Data were analyzed by one way ANOVA, followed by the Newman-Keuls multiple comparison test between all groups. The correlation between different parameters was assessed by the Spearman's test. All analyses were performed by the GraphPad Prism 4.00 for Windows software (GraphPad Software, San Diego, CA, USA). Data are expressed as arithmetic means ± SEM. A p value of less than 0.05 was considered as statistically significant.

RESULTS

NCX 6550 and pravastatin reduce serum cholesterol

Serum cholesterol was enhanced in wild-type mice under HFD as compared to wild-type mice on a normal diet (5.95±0.25 *vs* 2.87±0.82 mmol/L, respectively, p<0.05). LDLR$^{-/-}$ mice kept under HFD had strikingly higher serum cholesterol as compared with LDLR$^{-/-}$ mice on a normal diet (23.74±5.7 and 6.77±1.23 mmol/L, respectively, p<0.05) (Figure 1a). The lipid lowering effect of NCX 6550 in conditions of stronger hypercholesterolemia was not enhanced as compared to equimolar pravastatin. (Figure 1b).

Hypercholesterolemia induces platelet hyperreactivity

Platelet aggregation

In LDLR-HFD mice platelet aggregation was significantly enhanced as compared to normocholesterolemic mice using all agonists (Figure 2a). In conditions of strong cholesterolemia (LDLR$^{-/-}$HFD), *ex vivo* platelet aggregation was significantly inhibited by NCX 6550 treatment but not by pravastatin with all the agonists used (Figure 2b).

Platelet adhesion to a collagen-coated surface ex vivo

Platelet adhesion to collagen was significantly enhanced in strongly hypercholesterolemic mice (Figure 2c).

Treatment with NCX 6550, but not pravastatin, significantly reduced platelet adhesion to collagen in normocholesterolemic wild-type (7.4%) as well as in LDLR-ND mice (23%); the reduction of platelet adhesion induced by

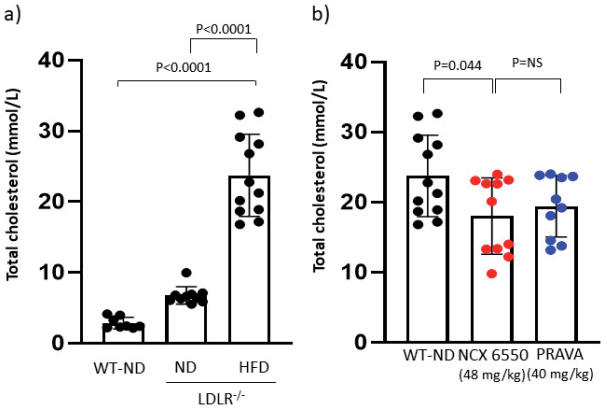

Figure 1. a) Sixteen weeks of high fat diet (HFD) strikingly enhance total serum cholesterol in LDLR$^{-/-}$ mice. b) NCX 6550 48 mg/kg significantly reduced total cholesterol levels, more than equimolar pravastatin (n=10-12 mice per group). Statistical analysis: ANOVA followed by the Newman-Keuls multiple comparisons test.

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NCX 6550 was even more marked in strongly hypercholesterolemic LDLR$^{-/-}$HFD mice (36.4%) (Figure 2d).

Effects on thrombosis

Femoral artery thrombosis

Thrombus weight of wild-type mice was 0.18±0.08 mg. In vehicle-treated LDLR$^{-/-}$ mice on ND thrombus weight was 0.26±0.018 mg and it was further, significantly, enhanced when these mice were fed a HFD for 16 weeks (0.46±0.08 mg) (Figure 3a). In LDLR$^{-/-}$ mice on HFD NCX 6550 (48 mg/kg) but not pravastatin, significantly reduced thrombus weight (0.08±0.02 and 0.38±0.08, respectively, n=5) (Figure 3b).

Different levels of cholesterolemia modulated not only thrombus size but also the time to occlusion. To a reduced thrombus size corresponded a longer time to occlusion after photochemically-induced damage to the femoral artery: time to occlusion in wild-type, LDLR$^{-/-}$ on ND and LDLR$^{-/-}$ on HFD was progressively shorter (Figure 3c). Besides a reduced thrombus weight observed in LDLR$^{-/-}$ mice on HFD upon NCX 6550 administration, a significant enhancement of time to occlusion was achieved. Pravastatin slightly prolonged time to occlusion (Figure 3d).

Effects on atherosclerosis

Photochemical injury-induced intimal thickening of femoral arteries

Neointima hyperplasia was enhanced in LDLR$^{-/-}$ mice on ND as compared with wild-type mice (I/M=1.077±0.15 *vs* 0.53±0.16, respectively) and even more strongly in LDLR$^{-/-}$ mice on HFD (I/M=1.75±0.25, p<0.001 *vs* wild-type mice) (Figure 4a).

Both drugs, NCX 6550 and pravastatin, were able to re-

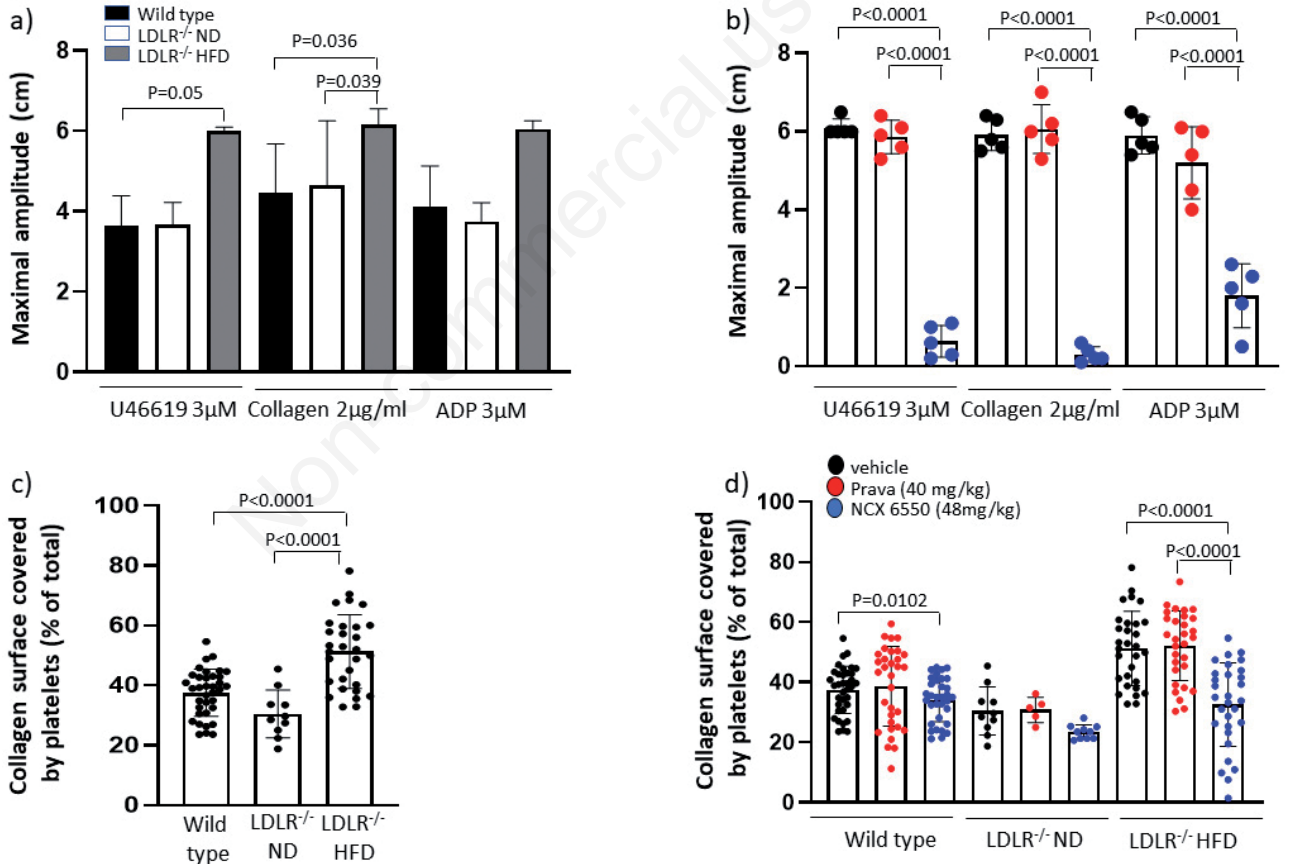

Figure 2. a) *Ex vivo* U46619-, collagen- and ADP-induced platelet aggregation in wild-type mice kept on standard diet (SD), LDLR$^{-/-}$ mice on SD, and LDLR$^{-/-}$ mice on high fat diet (HFD). Five mice per group were used. Data are expressed as mean ± SEM of 3 experiments. b) Effect of NCX 6550 (48 mg/kg, blue circles) and pravastatin (40 mg/kg, red circles) on U46619-, collagen and ADP-induced platelet aggregation in LDLR$^{-/-}$ mice on HFD. Platelet aggregation was evaluated with platelet rich plasma. Black circles: platelets from vehicle-treated mice. c) *Ex*-vivo platelet adhesion to a collagen-coated surface under flow was carried out in wild-type mice kept on SD, LDLR$^{-/-}$ on SD, and LDLR$^{-/-}$ mice on HFD. d) Effect of 4-weeks treatment with NCX 6550 and pravastatin on platelet adhesion under shear in HFD-fed in LDLR$^{-/-}$ mice. Statistical analysis: ANOVA followed by the Newman-Keuls multiple comparisons test (n=6-9 per group).

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duce intimal hyperplasia in wild type and in LDLR-ND mice. Interestingly, in strongly hypercholesterolemic mice, while NCX 6550 maintained its antiproliferative activity, pravastatin lost a significant part of its effect (Figure 4b).

Aortic atherosclerosis

Extensive atherosclerotic lesions were observed throughout the aortas in LDLR$^{-/-}$ mice on HFD (40.1±1.1 % of surface covered by plaques). Treatment with NCX 6550 (48 mg/kg for 3 weeks) reduced the lesion area by 65% (14.04±3.3 % of surface covered by plaques, n=5, p<0.0001 *vs* control), significantly more than pravastatin (40 mg/kg for 3 weeks) (24.3±2.1 % of surface covered by plaques, 39% reduction, n=4, p<0.001 *vs* control) (Figure 4c,d).

Circulating inflammatory biomarkers

Consistent with the inflammatory condition associated with atherosclerosis, circulating levels of the inflammatory markers TNF-α and IL-6 were significantly increased in hypercholesterolemic conditions (Figure 5a,c). In LDLR$^{-/-}$ mice on HFD 3 weeks treatment with NCX 6550 reduced IL-6 and TNF-α levels. Pravastatin reduced them also (Figure 5b,d).

Nitric oxide and its effects

The administration of NCX 6550 for 3 weeks, but not of pravastatin, enhanced significantly plasmatic NO_2/NO_3 in strong hypercholesterolemic LDLR$^{-/-}$ mice (Figure 6a). Similarly, in LDLR$^{-/-}$ mice treated with a single administration of NCX 6550 (48 mg/kg, p.o.) a marked increase of plasmatic cGMP was found (control: 6.094±4.4; NCX 6550: 20.52±6.2 pmol/ml, n=5, p=0.0214 *vs* vehicle). Pravastatin did not change plasmatic cGMP (Figure 6b).

DISCUSSION

An increased platelet reactivity and, therefore of their thrombogenic potential, is a feature of a number of pathophysiological conditions associated with cardiovascular risk factors, including dyslipidemia, diabetes and the metabolic syndrome.[4] Indeed, it has been suggested that the CV risk associated with dyslipidemia may be due to effects on both thrombogenesis and atherogenesis;[34] thus, the control of platelet reactivity is regarded to be critical for the prevention of coronary artery disease in hyperlipidemic patients.[35]

In this paper we show that increasing levels of cholesterolemia translate in progressively greater platelet hyper-

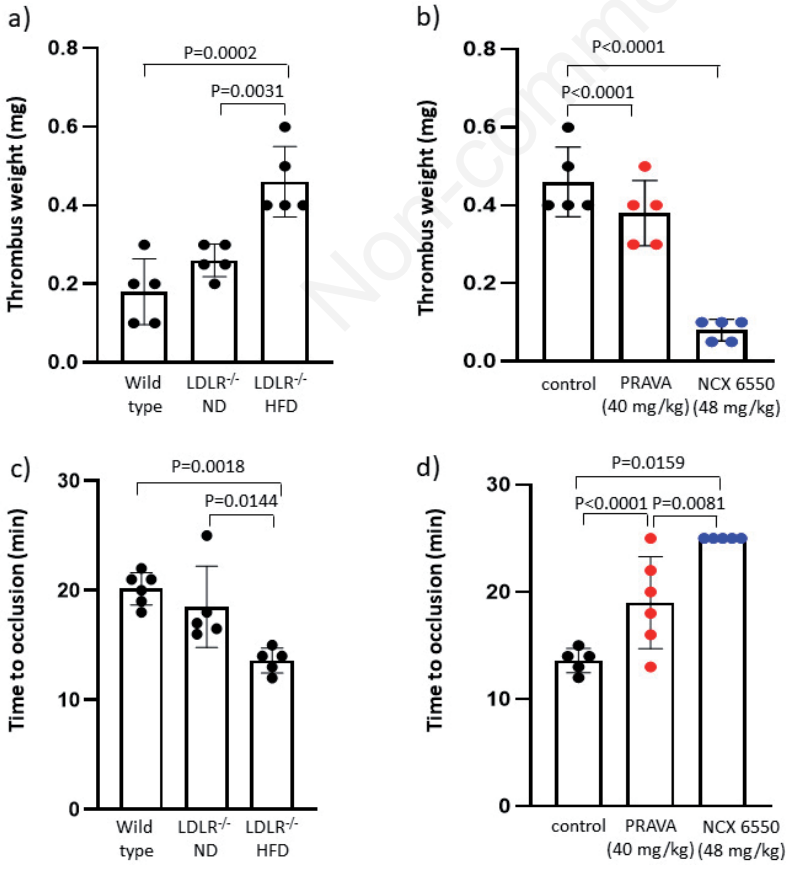

Figure 3. Effect of cholesterolemia on photochemically-induced thrombosis in mice kept on SD, LDLR$^{-/-}$ on SD, and LDLR$^{-/-}$ mice on HFD on thrombus weight (a) and on time to occlusion (c). Pravastatin and, even more, NCX 6550, reduced thrombus weight (b) and time to occlusion (d) in LDLR$^{-/-}$ mice on high fat diet (HFD) (n=5 per treatment). Statistical analysis: ANOVA followed by the Newman-Keuls multiple comparisons test (n=6-9 per group).

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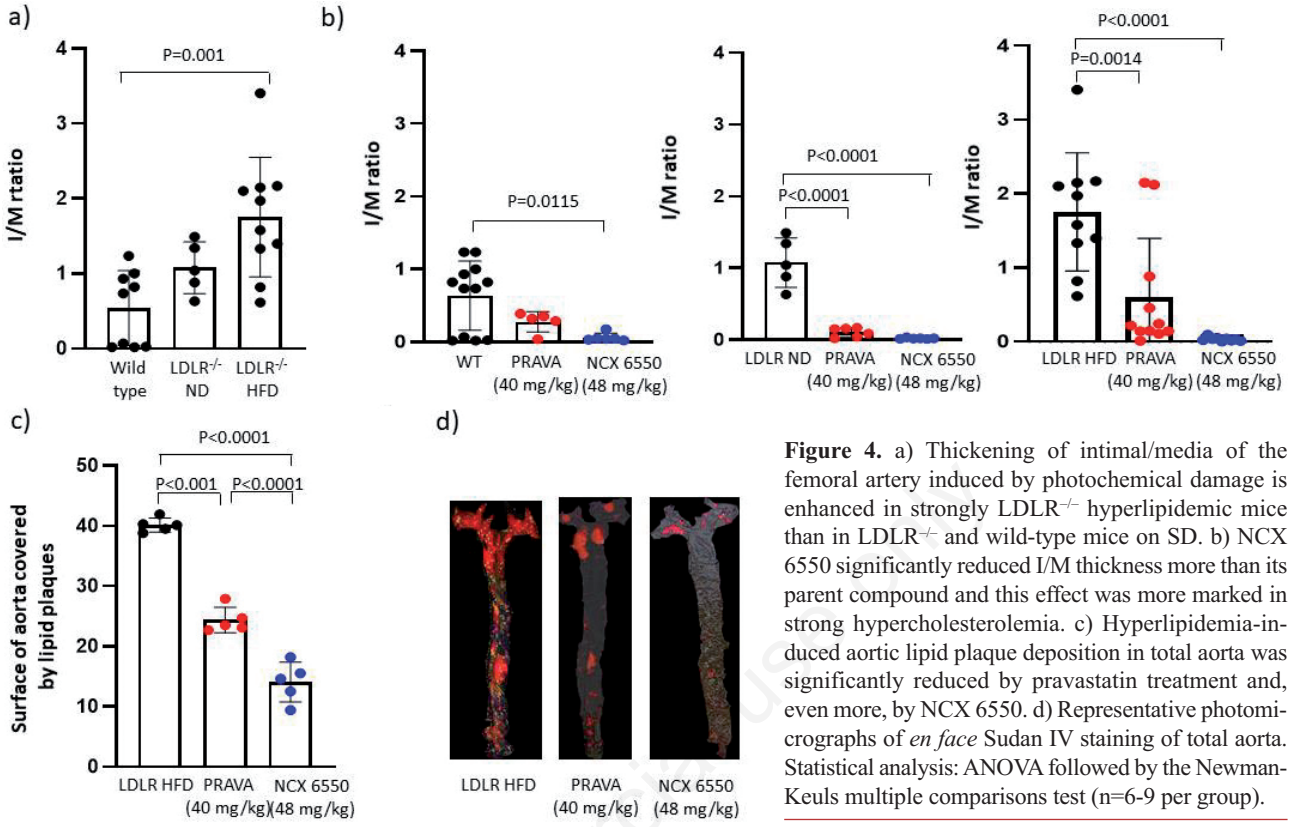

Figure 4. a) Thickening of intimal/media of the femoral artery induced by photochemical damage is enhanced in strongly LDLR$^{-/-}$ hyperlipidemic mice than in LDLR$^{-/-}$ and wild-type mice on SD. b) NCX 6550 significantly reduced I/M thickness more than its parent compound and this effect was more marked in strong hypercholesterolemia. c) Hyperlipidemia-induced aortic lipid plaque deposition in total aorta was significantly reduced by pravastatin treatment and, even more, by NCX 6550. d) Representative photomicrographs of *en face* Sudan IV staining of total aorta. Statistical analysis: ANOVA followed by the Newman-Keuls multiple comparisons test (n=6-9 per group).

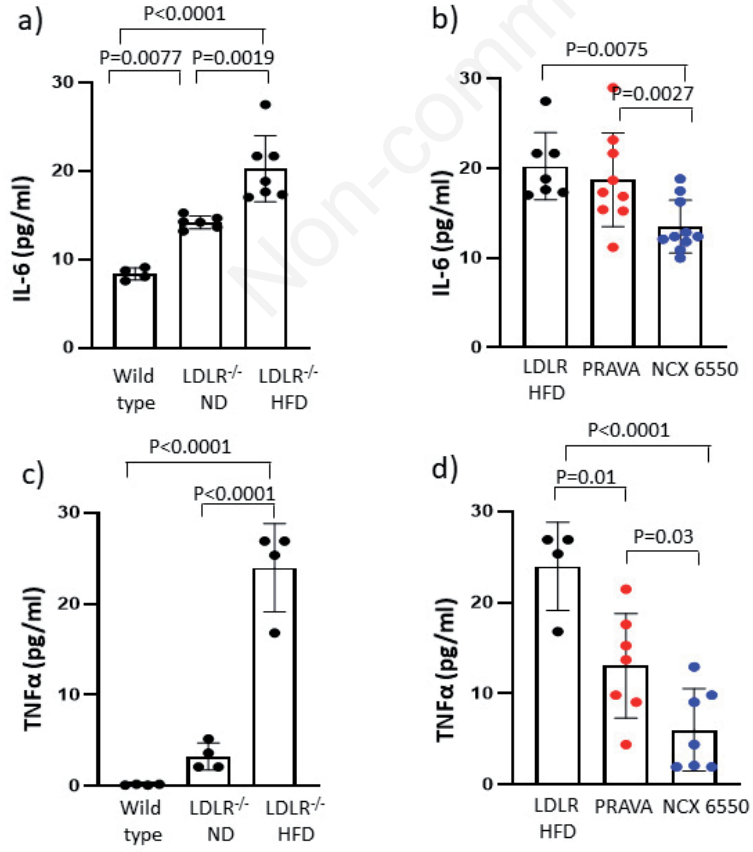

Figure 5. IL-6 and TNFα levels in wild-type mice kept on a standard diet (SD), LDLR$^{-/-}$ mice on a SD, and LDLR$^{-/-}$ mice on a high fat diet (HFD) (a,c). Effect of pravastatin or NCX 6550 on the levels of serum IL-6 (b) and TNFα (d) of LDLR$^{-/-}$ mice kept on a HFD. Statistical analysis: ANOVA followed by the Newman-Keuls multiple comparisons test (n=6-9 per group).

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reactivity in mice which associates with a marked prothrombotic phenotype. The response of platelets from strongly hypercholesterolemic mice to platelet agonists was enhanced as compared to platelets from normocholesterolemic or mildly hypercholesterolemic LDLR$^{-/-}$ mice. Moreover, platelet adhesion to collagen under high shear rate, a model that reproduces the conditions occurring within stenotic coronary arteries,[36] was also enhanced in strong hypercholesterolemic mice. Interestingly, the NO-donor pravastatin hybrid drug NCX 6550, but not pravastatin, was able to inhibit both platelet aggregation and platelet adhesion to collagen in strongly hypercholesterolemic mice, an observation of particular interest considering that aspirin, the conventional antiplatelet agent used for CV prevention, is not able to prevent platelet adhesion in this model.[33]

NCX 6550 and pravastatin displayed a similar lipid-lowering activity, but only NCX 6550 was able to exert an antiatherogenic and anti-inflammatory activity, likely due to the released nitric oxide. The anti-inflammatory activity of NCX 6550 was previously shown also in human monocyte/macrophages where NCX 6550 was more effective than pravastatin in inhibiting PMA-evoked release of TNF-α and IL-6.[37]

Indeed, the administration of NCX 6550, but not of pravastatin, strongly enhanced the levels in plasma of NO-degradation products and of cGMP, confirming the *in vivo* release of biologically relevant amounts of NO.

Photochemical injury is a relatively non-invasive method for inducing endothelial injury which is particularly useful for small animals, such as mice.[38] Endothelial injury elicited by rose Bengal is mediated by the local generation of superoxide anion,[38] a type of injury that may occur in humans during the progression of atherosclerosis and which may also contribute to plaque disruption.[36] These processes are strongly exacerbated by hypercholesterolemia. In fact, in hypercholesterolemic animals the time to thrombus formation was significantly shortened and thrombus size enhanced. Interestingly, NCX 6550 significantly prevented thrombosis in these conditions, while pravastatin was ineffective.

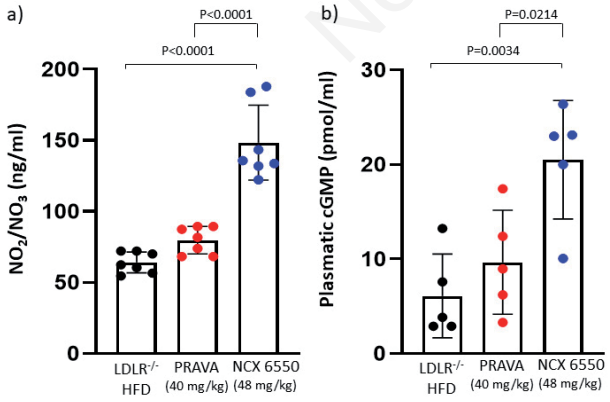

Figure 6. Effect of pravastatin or NCX 6550 on (a) plasma NO_2/NO_3 production (n=7 mice per group), expressed as ng/ml and (b) plasma levels of cGMP (n=5 mice per group), as measured by ELISA in LDLR$^{-/-}$ mice fed an HFD. Statistical analysis: ANOVA followed by the Newman-Keuls multiple comparisons test.

Hypercholesterolemia is associated with a low-grade inflammatory state;[35] this is partly due to the generation of reactive oxygen species that induce lipid peroxidation of cell-membrane phospholipids and of circulating LDL and these may participate in potentiating platelet activation,[39] thus contributing to platelet hyperreactivity. Moreover, the enhanced release of reactive oxygen species contributes to the scavenging of nitric oxide and to the reduction of its bioavailability.[40] In this model NCX 6550 successfully prevented atherosclerosis and inflammation while pravastatin was significantly less effective. This suggests that in conditions of severe endothelial injury associated with the generation of oxygen radicals the direct supply of NO allows to attain a significant anti-atherosclerotic effect by enhancing the vascular protective actions of the statin, and to prevent platelet hyperresponsiveness. The photochemical generation of oxygen radicals in the femoral artery produced systemic inflammation too, as shown by the rise of circulating IL-6 and TNFα, which were significantly more reduced by NCX 6560 than by pravastatin.

Statin therapy is associated with a small but significant absolute increase in new onset diabetes, more marked in people with atherosclerosis and the metabolic syndrome.[41] The diabetogenic risk appears to be lower with pravastatin.[42] Pravastatin was reported to inhibit TNFα generation *in vivo* and indeed circulating TNFα levels of hypercholesterolemic patients were also significantly reduced after 8 weeks of pravastatin treatment.[43,44] Since TNFα plays a key role in the pathogenesis of insulin resistance and type 2 diabetes,[45] the slow and sustained release of NO from NCX 6550,[46] producing a reduction of circulating TNFα (as we show here) may potentially contribute to the prevention of diabetes. Moreover, the release of NO *in vivo* in amount sufficient to inhibit the platelet hyperactivity induced by acute hyperglycemia in diabetic patients stimulates glucose transport in adipocytes without negatively affecting insulin sensitivity and it could therefore help in better controlling glycemia in type 2 diabetes.[47]

CONCLUSIONS

In conclusion, our study shows that in a model of accelerated atherosclerosis and increased thrombotic tendency a NO-donating pravastatin hybrid drug has a strong and prompt antithrombotic, anti-inflammatory and anti-

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atherosclerotic effect. The addition of a NO-donating molecule to pravastatin may enhance its effectiveness in disease conditions of *in vivo* peroxidation and strong atherosclerosis-related inflammation, such as those found in high-risk patients with acute coronary syndromes, potentially reducing adverse events.

REFERENCES

1. Ross R. Atherosclerosis -- an inflammatory disease. N Engl J Med 1999;340:115-26.
2. Getz GS, Reardon CA. Diet and murine atherosclerosis. Arterioscler Thromb Vasc Biol 2006;26:242-9.
3. Carmeliet P, Moons L, Collen D. Mouse models of angiogenesis, arterial stenosis, atherosclerosis and homeostasis. Cardiovasc Res 1998;39:8-33.
4. Davì G, Gresele P, Violi F, et al. Diabetes mellitus, hypercholesterolemia, and hypertension but not vascular disease per se are associated with persistent platelet activation in vivo. Evidence derived from the study of peripheral arterial disease. Circulation 1997;96:69-75.
5. Podrez EA, Byzova TV, Febbraio M, et al. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. Nat Med 2007;13:1086-95.
6. Hansson GK. Immune mechanisms in atherosclerosis. Arterioscler Thromb Vasc Biol 2001;21:1876–90.
7. Papanicolaou DA, Vgontzas AN. Editorial. Interleukin-6: the endocrine cytokine. J Clin Endocrinol Metab 2000;85:1331–2.
8. Wierzbicki AS, Poston R, Ferro A. The lipid and non-lipid effects of statins. Pharmacol Ther 2003;99:95-112.
9. Blum A, Shamburek R. The pleiotropic effects of statins on endothelial function, vascular inflammation, immunomodulation and thrombogenesis. Atherosclerosis 2009;203:325-30.
10. Feron O, Dessy C, Moniotte S, et al. Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. J Clin Invest 1999;103:897-905.
11. Maxwell AJ. Mechanism of dysfunction of the nitric oxide pathway in vascular disease. Nitric oxide: Biol Chem 2002;6:101-24.
12. Gresele P, Momi S, Migliacci R. Endothelium, venous thromboembolism and ischemic cardiovascular events. Thromb Haemost 2010;103:56-61.
13. Greenwood J, Mason JC. Statins and the vascular endothelial inflammatory response. Trends Immunol 2007;28:88-98.
14. Laufs U, Liao JK. Post-trascriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. J Biol Chem 1998;273:24266-71.
15. Park KY, Heo TH. Combination therapy with cilostazol and pravastatin improves antiatherogenic effects in low-density lipoprotein receptor knockout mice. Cardiovasc Ther 2018;36:e12476.
16. Momi S, Monopoli A, Alberti PF, et al. Nitric oxide enhances the anti-inflammatory and anti-atherogenic activity of atorvastatin in a mouse model of accelerated atherosclerosis. Cardiovasc Res 2012;94: 428-38.
17. Ridker PM, Pradhan A, MacFadyen JG, et al. Cardiovascular benefts and diabetes risks of statin therapy in primary prevention: an analysis from the JUPITER trial. Lancet 2012;380:565-71.
18. Ongini E, Impagnatiello F, Bonazzi A, et al. Nitric oxide (NO)-releasing statin derivatives, a class of drugs showing enhanced antiproliferative and antiinflammatory properties. Proc Natl Acad Sci USA 2004;101:8497-502.
19. Dever G, Spickett CM, Kennedy S, et al. The nitric oxide-donating pravastatin derivative, NCX 6550 [(1S-[1α(βS*,δS*),2α,6α,8β-(R*),(8aα]]-1,2,6,7,8,8a-Hexahydro β,δ,6-trihydroxy-2-methyl-8-(2-methyl-1-oxobutoxy-1-naphtalene-heptanoic Acid 4-(Nitrooxy)butyl Ester)], reduces splenocyte adhesion and reactive oxygen species generation in normal and atherosclerotic mice. J Pharmacol Exp Ther 2007;320:419-26.
20. Rossiello MR, Momi S, Caracchini R, et al. A novel nitric oxide-releasing statin derivative exerts an antiplatelet/antithrombotic activity and inhibits tissue factor expression. J Thromb Haemost 2005;3:2554-62.
21. Emanueli C, Monopoli A, Kraenkel N, et al. Nitropravastatin stimulates reparative neovascularization and improves recovery from limb ischemia in type-1 diabetic mice. Brit J Pharmacol 2007;150: 873-82.
22. Kaddai V, Gonzalez T, Bolla M, et al. The nitric oxide-donating derivative of acetylsalicylic acid, NCX 4016, stimulates glucose transport and glucose transporters translocation in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 2008;295:E162-9.
23. Pieper GM, Siebeneich W, Olds CL, et al. Vascular protective actions of a nitric oxide aspirin analog in both in vitro and in vivo models of diabetes mellitus. Free Radic Biol Med 2002;32:1143-56.
24. Pretorius M, Brown NJ. Endogenous nitric oxide contributes to bradykinin-stimulated glucose uptake but attenuates vascular tissue-type plasminogen activator release. J Pharmacol Exp Ther 2010;332:291-7.
25. Freeman DJ, Norrie J, Sattar N, et al. Pravastatin and the development of diabetes mellitus: evidence for a protective treatment effect in the West of Scotland Coronary Prevention Study. Circulation 2001;103:357-62.
26. Ishibashi S, Brown MS, Goldstein JL, et al. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest 1993;92:883-93.
27. Momi S, Pitchford SC, Alberti PF, et al. Nitroaspirin plus clopidogrel versus aspirin plus clopidogrel against platelet thromboembolism and intimal thickening in mice. Thromb Haemost 2005;93:535-43.
28. Sixma JJ, de Groot PG, van Zanten H, Ijsseldijk M. A new perfusion chamber to detect platelet adhesion using a small volume of blood. Thromb Res 1988;92:43-6.
29. Kikuchi S, Umemura K, Kondo K, et al. Photochemically induced endothelial injury in the mouse as a screening model for inhibitors of vascular intimal thickening. Arterioscler Thromb Vasc Biol 1998;18:1069-78.
30. Momi S, Falcinelli E, Giannini S, et al. Loss of matrix metalloproteinase 2 in platelets reduces arterial thrombosis in vivo. J Exp Med 2009;206:2365-79.
31. Momi S, Caracchini R, Falcinelli E, et al. Stimulation of platelet nitric oxide production by nebivolol prevents thrombosis. Arterioscler Thromb Vasc Biol 2014;34:820-9.

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32. Huo Y, Schober A, Forlow SB, et al. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. Nat Med 2003;9:61-7.
33. Momi S, Impagnatiello F, Guzzetta M, et al. NCX 6560, a nitric oxide-releasing derivative of atorvastatin, inhibits cholesterol biosynthesis and shows anti-inflammatory and antithrombotic properties. Eur J Pharmacol 2007;570:115-24.
34. Davì G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med 2007;357:2482-94.
35. Braunwald E, Angiolillo D, Bates E, et al. The problem of persistent platelet activation in acute coronary syndromes and following percutaneous coronary intervention. Clin Cardiol 2008;31:117-20.
36. Vilahur D, Duran X, Juan-Babot O, et al. Antithrombotic effects of saratin on human atherosclerotic plaques. Thromb Haemost 2004;92:191-200.
37. Amoruso A, Bardelli C, Fresu LG, et al. The nitric oxide-donating pravastatin, NCX 6550, inhibits cytokine release and NF-κB activation while enhancing PPARγ expression in human monocyte/macrophages. Pharmacol Res 2010;62:391-9.
38. Vandeplassche G, Bernier M, Kusama BM. Siglet oxigen and myocardial injury: ultrastructural, cytochemical and electrocardiographic consequences of photoactivation of rose Bengal. J Mol Cell Cardiol 1990;22:287-301.
39. Gresele P, Falcinelli E, Momi S. Potentiation and priming of platelet activation: a potential target for antiplatelet therapy. Trends Pharmacol Sci 2008;29:352-60.
40. Krotz F, Sohn HY, Pohl U. Reactive oxygen species: players in the platelet game. Aterioscler Thromb Vasc Biol 2004;24:1988-96.
41. Carmena R, Betteridge DJ. Diabetogenic Action of Statins: Mechanisms. Curr Atheroscler Rep 2019;21:23.
42. Chen YH, Yang YC, Chen W, et al. Risk of macrovascular complications in statin-treated patients developing diabetes. Diabetes Res Clin Pract 2019;157:107870.
43. Wang HM, Gao JH, Lu JL. Pravastatin improves atherosclerosis in mice with hyperlipidemia by inhibiting TREM-1/DAP12. Eur Rev Med Pharmacol Sci 2018;22:4995-5003.
44. Solheim S, Seljeflot I, Arnesen H, et al. Reduced levels of TNF alpha in hypercholesterolemic individuals after treatment with pravastatin for 8 weeks. Atherosclerosis 2001;157:411-5.
45. Akash MSH, Rehman K, Liaqat A. Tumor Necrosis Factor-Alpha: Role in Development of Insulin Resistance and Pathogenesis of Type 2 Diabetes Mellitus. J Cell Biochem 2018;119:105-10.
46. Muscara MN, Lovren F, McKnight W, et al. Vasorelaxant effects of a nitric oxide-releasing aspirin derivative in normotensive and hypotensive rats. Br J Pharmacol 2001;133:1314-22.
47. Gresele P, Marzotti S, Guglielmini G, et al. Hyperglycemia-induced platelet activation in type 2 diabetes is resistant to aspirin but not to a nitric oxide-donating agent. Diabetes Care 2010;33:1262-8.

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