The Nitric Oxide-Donating Pravastatin Derivative, NCX 6550 [(1S-[1αβS*,δS*],2α,6α,8β-(R*),8αa]-1,2,6,7,8,8a-Hexahydro-β,δ,6-tri hydroxy-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

G. Dever, C. M. Spickett, S. Kennedy, C. Rush, G. Tennant, A. Monopoli, and C. L. Wainwright

Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom (G.D., C.M.S., S.K., C.R., G.T.); Nicox Research Institute, Milan, Italy (A.M.); and School of Pharmacy, Robert Gordon University, Aberdeen, Scotland, United Kingdom (C.L.W.)

Received June 12, 2006; accepted September 26, 2006

ABSTRACT

Statins possess anti-inflammatory effects that may contribute to their ability to slow atherogenesis, whereas nitric oxide (NO) also influences inflammatory cell adhesion. This study aimed to determine whether a novel NO-donating pravastatin derivative, NCX 6550 [(1S-[1αβS*,δS*],2α,6α,8β-(R*),8αa]-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], has greater anti-inflammatory properties compared with pravastatin in normal and atherosclerotic apolipoprotein E receptor knockout (ApoE−/−) mice. NCX 6550 (48.5 mg/kg) or pravastatin (8.8 mg/kg) was administered to C57BL/6 mice (8.8 ± 1.9% versus 16.6 ± 6.7% adhesion; P < 0.05) and ApoE−/− mice (9.3 ± 2.9% versus 23.4 ± 4.6% adhesion; P < 0.05), concomitant with an inhibition of endothelial intercellular adhesion molecule-1 expression. NCX 6550 also significantly reduced phorbol 12-myristate 13-acetate-induced ROS production that was enhanced in isolated ApoE−/− splenocytes. Conversely, pravastatin had no significant effects on adhesion in normal or ApoE−/− mice but reduced the enhanced ROS production from ApoE−/− splenocytes. In separate groups of ApoE−/− mice, NCX 6550 significantly enhanced endothelium-dependent relaxation to carbachol in aortic segments precontracted with phenylephrine (−logEC50, 5.81 ± 0.15; P < 0.001) and pravastatin-treated (−logEC50, 5.57 ± 0.45; P < 0.005) mice. NCX 6550 also significantly reduced plasma monocyte chemoattractant protein-1 levels (648.8 pg/ml) compared with both vehicle (1191.1 pg/ml; P < 0.001) and pravastatin (847 ± 71.0 pg/ml; P < 0.005) treatment. These data show that NCX 6550 exerts superior anti-inflammatory actions compared with pravastatin, possibly through NO-related mechanisms.

Atherosclerosis is now generally acknowledged to be an inflammatory disease where inflammation develops at certain predislocation sites in response to endothelial injury. Attachment of leukocytes to atherosclerotic blood vessels (Ramos et al., 1999), coupled with up-regulation of the vascular adhesion molecules vascular cell adhesion molecule-1 (VCAM-1, ICAM-1) (Nakashima et al., 1998), is a fundamental step in the development of atherosclerosis. Recently, adhesion of cultured murine monocytoid WEH1 78/24 cells to artery segments of ApoE−/− mice has been demonstrated (Li et al., 2005), supporting the notion that a hyperinflammatory state exists in developing atherosclerosis. This has been attributed to increased levels of a number of cytokines, which act to elevate adhesion molecule expression. Thrombin, in addition to playing a role in the coagulation cascade, is also involved.

ABBREVIATIONS: ICAM, intercellular adhesion molecule; ApoE−/−, apolipoprotein E receptor knockout; ROS, reactive oxygen species; NO, nitric oxide; NCX 6550, [(1S-[1αβS*,δS*],2α,6α,8β-(R*),8αa]-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); MCP, monocyte chemoattractant protein; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; ANOVA, analysis of variance; CL, chemiluminescence.
in the regulation of inflammation and has been shown to induce monocyte adhesion to endothelial cells (human umbilical vein endothelial cells) through induction of ICAM-1 and increased expression of vascular cell adhesion molecule-1, P-selectin, and E-selectin (Kaplanski et al., 1998) due to an action at protease activated receptor-1. Consequently, thrombin has been implicated in atherogenesis (Coughlin, 2005). However, there has been no direct demonstration of a hyperinflammatory response to thrombin in atherosclerotic blood vessels. The first aim of the current study was therefore to compare adhesion of splenocyte preparations, commonly used as a source of immune cells, with thrombin-challenged arterial tissue from normal (C57BL/6) and atherosclerotic (ApoE−/−) mice.

The value of introducing the lipid-lowering statins into the management of patients with coronary artery disease has been illustrated through the significant benefit of these drugs in primary (Shepherd et al., 1995) and secondary prevention of symptomatic coronary heart disease (Scandinavian Simvastatin Survival Study (4S), 1994). Detailed analyses of data from these trials, however, suggest that lipid-lowering by statins does not solely account for the significant clinical outcomes, and that statins possess additional (pleiotropic) effects beyond their lipid-lowering capacity (Downs et al., 1998). Among the reported pleiotropic effects of statins, demonstrations of their anti-inflammatory and anti-adhesive effects are abundant (Stalker et al., 2001; Fischetti et al., 2004). Furthermore, fluvastatin (Bando et al., 2003), and other statins have been shown to inhibit formation of reactive oxygen species (ROS) by inflammatory cells. However, although these effects are readily demonstrated following acute challenge with supratherapeutic concentrations in vitro, the effects in vivo often require prolonged administration before they are observed. Although nitric oxide (NO) was originally identified as a key mediator in the maintenance of vascular tone, it also exerts anti-inflammatory effects. NO, either generated endogenously or released from NO-donating molecules, inhibits leukocyte adhesion through a reduction in endothelial expression of adhesion molecules such as P-selectin (Davenpeck et al., 1994) and ICAM-1 (Berendji-Grun et al., 2001). Recent studies have reported superior anti-inflammatory properties of novel NO-releasing statins (nitrostatins) over the respective native statins in RAW 264.7 murine macrophage cells (Ongini et al., 2004; Rossiello et al., 2005). The NO-donating moiety of nitrostatins is similar to other nitro compounds such as nitro aspirin, which yields NO through metabolic hydrolysis, resulting in relatively long-lasting plasma levels of NO (Muscara et al., 2001). Furthermore, studies conducted with the NO-releasing derivative of pravastatin, NCX 6550, showed that the compound given to hypercholesterolemic CD1 mice is equally effective as equivalent doses of the native statin at lowering cholesterol (S. Momi, G. Guglielmini, A. Monopoli, E. Ongini, and P. Gresele, personal communication). Thus, NO released by these molecules may provide a more rapid anti-inflammatory action than can be achieved with a native statin, while still affording a reduction in cholesterol levels. Thus, the aim of the present study was to compare the effects of short-term (5 days) in vivo administration of NCX 6550 and native pravastatin on ex vivo splenocyte adhesion to arterial segments, splenocyte ROS production, and endothelial ICAM-1 expression in tissues from normal (C57BL/6) and atherosclerotic (ApoE−/−) mice. In addition, we determined the effects of these interventions on endothelium-dependent vasorelaxant function and plasma MCP-1 levels in ApoE−/− mice.

Although many studies that investigated leukocyte-endothelial adhesion have used in vitro cell models, such as myeloid cell adhesion to human umbilical vein endothelial cell monolayers (McGettrick et al., 2006), we chose to use a more physiological model involving in vivo dosing with statins followed by ex vivo measurement of isolated splenocyte adhesion to arterial tissue. Although this approach has the limitation of being a static model of vascular adhesion, it has the advantage of allowing atherosclerosis-susceptible arterioles to be studied, in contrast to intravital microscopy, which is a dynamic model that allows the detection of adhesion in the presence of shear stress but which involves visualizing microvascular beds that are not generally susceptible to atherosclerotic plaque development, such as the mesenteric bed.

Materials and Methods

Materials. C57BL/6 (Harlan, UK Ltd) and ApoE−/− (Charles River, Margate, Kent, UK) mice were bred in house at the University of Strathclyde (Glasgow, UK). NCX 6550 was synthesized at Nicox (Bresso, Milan, Italy). All chemicals were purchased from Sigma-Aldrich (Dorset, Poole, UK), unless otherwise stated.

Experimental Design. Forty C57BL/6 (18–24 g) and 52 ApoE−/− (26–35 g) age-matched mice of either sex were employed in the study, under a project license issued under the UK Animals (Scientific Procedures) Act 1986. The ApoE−/− mice were fed an atherogenic diet (1% cholesterol, 5% lard) for 12 weeks postweaning, and control normocholesterolemic C57BL/6 mice were fed normal laboratory chow. The mice were then employed for the following studies. A group of C57BL/6 (n = 10) and ApoE−/− (n = 10) mice were used in preliminary experiments to determine any difference between the strains with respect to the adhesive response to thrombin, ICAM-1 expression, and splenocyte ROS generation. Three groups (n = 10 per group) of mice of each strain were administered vehicle [dimethyl sulfoxide/castor oil/polyethylene glycol 400/water, 1:2:7:10 (v/v/v)], native pravastatin (40 mg/kg), or NCX 6550 (equimolar dose, 48.5 mg/kg) by oral gavage every day (at 10:00 AM) for 5 days. One hour after the final dose, the mice were euthanized by CO2 asphyxiation. Heparinized blood was obtained by cardiac puncture immediately following euthanasia for subsequent measurement of plasma cholesterol levels using standard assay kits (R-Biopharm, Darmstadt, Germany). Three groups (n = 4 per group) of ApoE−/− mice were administered vehicle, pravastatin, or NCX 6550 (all as above) for 5 days. Immediately following euthanasia, blood was collected by cardiac puncture into heparinized tubes for measurement of plasma MCP-1 using an enzyme-linked immunosorbent assay kit (Insight Bioscience, Wembley, UK), and the aorta were harvested for determination of blood vessel function.

Splenocyte Isolation and Radiolabeling. Splenocyte suspensions were prepared by disrupting the spleens over a 200-μm mesh (Caddis Precision Meshes Limited, London, UK) into 3 ml of RPMI 1640 medium (Dutch modification; Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (Invitrogen). The resulting cell suspensions were passed through a 200-μm mesh and centrifuged at 125g for 10 min. The supernatants were removed, and the pellets containing the cells were resuspended by 4 ml of distilled H2O for 30 s (to lyse erythrocytes), followed by the addition of 4 ml of 1.8% NaCl to restore isotonicity. The splenocyte suspensions were filtered through a 200-μm mesh, centrifuged, and the resulting cell pellets were resuspended in 2 ml of RPMI. Cell density was determined using a hemocytometer, and the suspension was diluted as necessary to achieve a final density of 1 × 106 cells/ml. The resuspended leukocytes (1 ml) were labeled for 1 h at 37°C in a humidified chamber with 185
kBq of $^{51}$Cr (GE Healthcare, Little Chalfont, Buckinghamshire, UK); the cells were agitated every 15 min to minimize cell sedimentation. The cells were washed twice with RPMI 1640 and resuspended in RPMI 1640 to $1 \times 10^6$ cells/ml.

**Splenocyte Characterization.** Splenocyte preparations were characterized in samples from three separate C57BL/6 and three ApoE/–/– mice using flow cytometry. In brief, cell suspensions were incubated with leukocyte Fc receptor blocking buffer (anti-CD 16/32 hybridoma supernatant, 10% mouse serum, and 0.1% azide) for 5 min to prevent binding of antibody to cells via Fc regions. The cell suspensions were then incubated with a mixture of cell lineage antibodies for 30 min at 4°C. B lymphocytes were identified using fluorescein isothiocyanate-conjugated anti-CD45RB/220 (clone RA3–6B2; Pharmingen, Oxford, UK), CD4+ T lymphocytes using peridinin chlorophyll protein-cyanin 5.5-conjugated anti-CD4 (clone GKL1.5; Pharmingen), and myeloid cells using phycoerythrin-conjugated anti-CD11b (clone M1/70; Pharmingen). The cells were washed in fluorescence-activated cell sorting buffer [phosphate-buffered saline (PBS), 2% fetal calf serum, and 0.1% azide] prior to acquisition using a BD FACSCanto flow cytometer with FACSDiva software (BD Biosciences, San Jose, CA). FlowJo software (Tree Star Inc., Ashland, OR) was used for three color analysis. To ascertain which cell types were adhering to the artery surface, cells were added to pinned-out segments of aorta for 30 min and adherent cells were harvested by addition of ice-cold PBS solution. The suspensions of adherent cells were incubated with the same three antibodies and run through the flow cytometer.

**Assessment of Splenocyte Adhesion to Aortic Segments.** The method employed was a modification of a method using rabbit tissue developed in our laboratory to determine the effects of vascular injury on inflammatory cell adhesion (Kennedy et al., 2000). Homologous aortic lengths were removed and cut into two segments (aortic arch and thoracic aorta), which were pinned out luminal-side up onto Sylgard blocks (Dow Corning, Midland, MI). The artery segments were incubated with 10 μl of 10 U/ml thrombin for 10 min in a humidified chamber (37°C), washed, and then incubated with a 5-μl aliquot of the labeled leukocytes for a further 30 min. The segments were then washed with RPMI 1640, transferred into microtubes, and assayed for $^{51}$Cr in a gamma counter (Cobra Auto-gamma; Canberra Industries, Meriden, CT). Aliquots (5 μl) of labeled and unlabeled cells were also counted to allow calculation of leukocyte adhesion using the following equation:

$$\text{% Adhesion} = \frac{\gamma_{\text{artery}} - \text{background}}{\gamma_{\text{splenocytes}} - \text{background}} \times 100 \quad (1)$$

where $\gamma_{\text{artery}}$ is the count from the artery and $\gamma_{\text{splenocytes}}$ is the count from a 5-μl aliquot of labeled splenocytes.

**Measurement of Splenocyte ROS Generation.** A 450-μl aliquot of unlabeled splenocyte suspension ($1 \times 10^6$ cells/ml) was diluted 1:1 with PBS in a plastic cuvette containing a stir bar, which was then placed in the prewarming chamber of a chemiluminometer (Chrono-log Corporation, Havertown, PA) for 2 min. One hundred microliters of 400 μg/ml luminol (5-amino-2,3-dihydro-1,4-phenylalazine-dione) were added prior to the cuvette being transferred to the photomultiplier compartment. After 1 min, the cells were stimulated with 5 to 100 ng/ml (final concentrations) of phorbol 12-myristate 13-acetate (PMA), and a cumulative chemiluminescent signal was measured for 15 min.

**Immunocytochemistry.** Upon completion of the adhesion assay, artery segments were fixed in formalin solution (4% formaldehyde) for 48 h and stored in PBS at 4°C. The tissues were subsequently processed and embedded in blocks of paraffin wax. Four-micrometer transverse sections were cut and mounted on 3-aminopropyl triethoxysilane-treated slides and oven-dried for 1 h at 60°C. Sections were then stained for ICAM-1 with goat anti-human/anti-mouse ICAM-1 as the primary polyclonal antibody (1:50; R&D Systems, Minneapolis, MN) and biotinylated rabbit anti-secondary antibody (1:400; DakoCytoImation Ltd., Ely, Cambridgeshire, UK) using the streptavidin-horse radish peroxidase (Vector Laboratories, Peterborough, UK) method as described previously in detail (Kennedy et al., 2000). The sections were then counterstained with hematoxylin prior to mounting in DPX (VWR, West Chester, PA). Sections were subsequently subjected to semiquantitative scoring analysis by an investigator (C.W.) blinded to the treatment. Sections were scored from 0 to 3: 0, none/background; 0.5, focal staining of the endothelium; 1, mild circumferential positivity on the endothelium; 2, moderate circumferential positivity on the endothelium; and 3, intense circumferential positivity on the endothelium.

**Assessment of Endothelial Function.** Endothelial function was measured using a small artery wire myograph (Danish Myo Technology, Aarhus, Denmark). In brief, 2-mm segments of thoracic aorta were mounted on two 40-μm stainless steel wires and normalized using a previously published method. Arteries were bathed in Krebs' solution, maintained at 37°C, and aerated with 95% O$_2$ and 5% CO$_2$ throughout. Following 30 min of equilibration, all arteries were constricted by the addition of a previously determined EC$_{50}$ of phenylephrine (0.2 μM). Once contraction had stabilized, endothelium-dependent relaxation was measured by cumulative addition of carbachol (10$^{-9}$ to 10$^{-6}$ M). Maximal relaxation ($E_{max}$) and $-\log$EC$_{50}$ for each group of mice were calculated by fitting sigmoidal curves to each complete data set using GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA).

**Measurement of Plasma MCP-1 Levels.** Plasma MCP-1 levels were measured using a mouse CCL2 enzyme-linked immunosorbent assay kit (Insight Bioscience). In brief, NUNC Maxisorp flat-bottom, high-protein binding capacity 96-well plates (Nalge Nunc International, Naperville, IL) were coated overnight at 4°C with 100 μl/well purified anti-mouse MCP-1 capture antibody (1:250 in coating buffer; Insight Bioscience), after which they were aspirated and washed three times with 300 μl/well wash buffer (0.05% Tween 20 in PBS). Nonspecific binding was blocked by incubation with 200 μl/well assay diluent (10% fetal bovine serum in PBS) for 1 h at room temperature, the wells were washed, and standards (0–200 pg/ml recombinant mouse MCP-1) and plasma samples (100 μl/well) were added. The plate was then incubated at room temperature for 2 h, aspirated and washed, and 100 μl/well detection antibody (1:250 in assay buffer) was added, followed by incubation at room temperature for 1 h. After a further aspiration and wash, 100 μl of avidin-horseradish peroxidase (1:250 in assay buffer) was added to each well, the plate was incubated at room temperature for 30 min, after which each plate was subjected to seven cycles of aspirate and wash. One hundred microliters of 60 μg/ml tetramethylbenzidine substrate solution was added to each well, the plate was incubated at room temperature for 15 min, and the reaction was stopped by the addition of 50 μl/well stop solution (1 M H$_2$PO$_4$). The plate was read on a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA). MCP-1 levels were determined by subtracting the absorbance values at 570 nm from those at 450 nm and extrapolating values from the standard curve.

**Statistics.** Values shown are mean ± S.E.M. Multiple group comparisons for percent splenocyte adhesion, ROS generation, plasma cholesterol, MCP-1 levels, and immunostaining scores were performed using one-way ANOVA and Dunnett's post hoc test. For the functional studies, complete dose response curves were compared by two-way ANOVA, whereas $E_{max}$ and $-\log$EC$_{50}$ values were compared by one-way ANOVA and Tukey post hoc test.

**Results**

**Characterization of Splenocyte Preparations.** Splenocytes isolated from the C57BL/6 mice all contained similar proportions of CD4$^+$ T lymphocytes, B-220$^+$ B lymphocytes, and CD11b$^+$ myeloid cells. Together, these ac-
counted for approximately 80% of the cell population. Analysis of the cell types adhering after 30-min contact with arterial segments revealed that all three cell types were present in approximately the same proportion as was found in the splenocyte isolates (Fig. 1). In ApoE−/− mice, both the isolated splenocytes and the adherent cells exhibited a greater proportion of B lymphocytes than in the C57BL/6 mice, although the proportion of CD4+ T lymphocytes and CD11b+ myeloid cells was similar in both strains (Fig. 1). The remaining 10 to 20% of cells in both strains were most probably CD8+ and γδ T lymphocytes.

**Influence of Atherosclerosis on Adhesive Response to Thrombin.** In a preliminary series of experiments performed in a separate group of mice that did not receive any form of oral drug administration, a significant difference was observed in the adhesive response to thrombin in both the aortic arch and the thoracic aorta from ApoE−/− mice compared with normal mice (Fig. 2A). Furthermore, splenocytes isolated from ApoE−/− mice generated substantially more ROS on challenge with PMA than splenocytes from C57BL/6 mice (Fig. 2B).

**Thrombin-Stimulated Splenocyte Adhesion and ICAM-1 Expression.** Figure 3A illustrates splenocyte adhesion to arterial segments from vehicle- and drug-treated C57BL/6 mice following 5 days of pretreatment. Native pravastatin had no effect, whereas NCX 6550 caused a reduction in splenocyte adhesion that, in the aortic and thoracic segments, was statistically significant compared with both the vehicle and pravastatin groups (P < 0.05). NCX 6550 also inhibited adhesion in the thoracic segment in the ApoE−/− mice, whereas pravastatin did not (Fig. 3B); no significant reduction in adhesion to the aortic segment with NCX 6550 was observed, presumably due to the greater variability within this group (P = 0.17). There was no significant difference in the extent of ICAM-1 expression in thrombin-treated arterial segments from vehicle controls for either strain of mouse (Fig. 4). NCX 6550 significantly reduced the extent of ICAM-1 staining observed in the segments from C57BL/6 and ApoE−/− groups compared with vehicle-treated mice. Pravastatin, on the other hand, only reduced ICAM-1 expression in thrombin-treated segments from C57BL/6 mice and not the ApoE−/− mice.

**Splenocyte Reactive Oxygen Species Generation.** Treatment with pravastatin had no effect on the extent of ROS generation from C57BL/6 splenocytes, whereas NCX 6550 significantly reduced ROS generation in response to the highest concentration of PMA tested (Fig. 5A). In contrast, both pravastatin and NCX 6550 significantly attenuated the enhancement in ROS production in the ApoE−/− splenocytes to a similar degree (Fig. 5B).

**Endothelial Function.** Treatment with NCX 6550 significantly enhanced endothelium-dependent relaxation in response to carbachol (Fig. 6A) in ApoE−/− mice by causing a
significant shift to the left of the dose-response curve (EC$_{50}$, 0.43 ± 0.37 μM compared with 1.55 ± 0.15 μM in vehicle-treated mice; $P < 0.05$) and an increase in $E_{\text{max}}$ (67.55 ± 9.4% relaxation) compared with vehicle-treated mice ($E_{\text{max}}$, 39.1 ± 3.6%; $P < 0.05$). Pravastatin did not cause any shift in the dose-response curve (EC$_{50}$, 2.73 ± 0.45 μM), but the maximal relaxant response was increased ($E_{\text{max}}$, 63.4 ± 16.9%; $P < 0.05$ compared with vehicle).

**Plasma MCP-1.** Both pravastatin and NCX 6550 reduced circulating MCP-1 levels (847.2 ± 71.0 pg/ml, $P < 0.01$ and 648.8 ± 47.4 pg/ml, $P < 0.001$, respectively) compared with vehicle control (1191.1 ± 176.1 pg/ml). MCP-1 levels in NCX 6550-treated mice were significantly ($P < 0.05$) lower than levels measured in mice treated with pravastatin.

**Plasma Cholesterol Levels.** Plasma cholesterol levels in vehicle-treated ApoE$^{-/-}$ mice (1835 ± 345 μg/ml) were significantly higher ($P < 0.01$) than in vehicle-treated C57BL/6 mice (550 ± 83 μg/ml). Plasma cholesterol levels were not affected by 5-day treatment with either pravastatin (428 ± 153 and 2526 ± 350 μg/ml in C57BL/6 and ApoE$^{-/-}$, respec-
tively) or NCX 6550 (305 ± 47 and 1628 ± 392 μg/ml in C57BL/6 and ApoE−/−, respectively).

**Discussion**

**Hypercholesterolemia Increases Thrombin-Stimulated Splenocyte Adhesion and ROS Generation.** In the present study, we have shown that in vitro thrombin-induced adhesion of splenocytes to autologous arterial segments is enhanced in ApoE−/− mice compared with wild-type controls. Although there was a higher proportion of B lymphocytes in the splenocyte preparations from ApoE−/− mice (51.4 ± 0.5% of the total cell population) compared with C57BL/6 mice (33.6 ± 4.2%), our finding that for each strain the adherent cell populations were made up of similar ratios of these three cell types implies that the increased adhesion is not due to the increased B lymphocyte adhesion alone. Regarding the molecular basis of this enhanced adhesion, ICAM-1 is known to play a key role in firm adhesion of monocytes and lymphocytes in response to thrombin (Nie et al., 1997). However, our immunocytochemical determination of ICAM-1 expression does not support the notion that increased ICAM-1 expression is responsible for the increased adhesion in ApoE−/− arteries, although ROS-induced increases in binding affinity of ICAM-1 (Sellak et al., 1994) may play a role. Alternatively, we have found that expression of the thrombin receptor protease-activated receptor-1 is increased in arteries from ApoE−/− mice (K. Pugh, C. L. Wainwright, R. M. Wadsworth, J. Waller, and A. M. Miller, unpublished data), which might explain the enhanced response to thrombin.

Blood vessels from atherosclerotic animals (Stokes et al., 2002) and humans with coronary artery disease (Spiekermann et al., 2003) generate elevated levels of ROS. Our observation that splenocytes from ApoE−/− mice similarly generate substantially greater amounts of ROS is, to our knowledge, the first demonstration of this phenomenon, although the relative contribution of each cell type in the splenocyte suspensions to the total CL signal is unknown. Elevated ROS generation from leukocytes has been described in obese (Dandonna et al., 2001) and type II diabetic (Orie et al., 2000) patients, but data from patients with atherosclerosis are conflicting (Araujo et al., 1995; Eid et al., 2002). ROS generation by monocytes and neutrophils in disease models is well established, but evidence is now mounting that T lymphocytes also generate ROS (Williams and Kwon, 2004), for example, by the lipoxygenase pathway (Los et al., 1995), mitochondrial electron transport chain (Griendling et al., 2000), or NOX-based NAD(P)H oxidases (Lambeth, 2004), although there is no information regarding the effect of hypercholesterolemia on these systems. The present use of luminol as a chemiluminescent probe measures total ROS production by the splenocyte preparations, which precludes determining whether the enhanced CL signal from ApoE−/− splenocytes is due to overproduction of superoxide or other ROS. We have found previously (Lim et al., 2006) that a combination of superoxide dismutase, catalase, and sodium azide cannot completely inhibit CL generated by mouse splenocytes, implying that superoxide, hydrogen peroxide, and myeloperoxidase products do not account for all of the ROS generated and that other ROS (such as hydroxyl radical, singlet oxygen, lipid peroxide, and nitric oxide) may also contribute to the CL signal.

The NO-Donating Pravastatin Derivative (NCX 6550), but Not Pravastatin, Inhibits Thrombin-Induced Splenocyte Adhesion in ApoE−/− Mice. The key finding of this study was that NCX 6550 significantly reduced splenocyte adhesion to arterial tissue from both normocholesterolemic and hypercholesterolemic mice and inhibited ICAM-1 expression in arterial segments challenged with thrombin. In contrast, native pravastatin did not attenuate thrombin-stimulated adhesion, although it did reduce the expression of ICAM-1 in C57BL/6, but not in ApoE−/− mice. This apparent dissociation between an antiadhesive response and inhibition of ICAM-1 expression supports the findings in untreated animals. The failure of pravastatin to inhibit splenocyte adhesion ex vivo deviates from the growing body of evidence that statins, including pravastatin, exert some of their effects through anti-inflammatory mechanisms that are unrelated to lipid lowering (Schonbeck and Libby, 2004). However, most studies that demonstrate an anti-inflammatory effect of statins employed treatment periods longer than the 5-day period in the present study, suggesting that long-term treatment is required to observe an anti-inflammatory effect. For example, 2- and 4-week treatment with rosuvastatin was required to attenuate adhesion of a monocyte cell line to aortic segments and vascular ROS production in ApoE−/− mice (Li et al., 2005). Interestingly, in that study, cholesterol levels were reduced by rosuvastatin, whereas in the present study, no observable effects of either pravastatin or NCX 6550 were detected (presumably due to the different duration of drug treatment). Moreover, most in vitro studies demonstrating an anti-inflammatory effect of statins have employed concentrations in the micromolar range, whereas in vivo data suggest that plasma concentrations in the nanomolar range may have an effect on leukocyte trafficking and recruitment. This has led to the notion that cell adhesion and migration in vivo, which is a dynamic environment, may be more sensitive to inhibition by statins than adhesion under static in vitro conditions. Although in the present study pravastatin was administered in vivo, the adhesion measurements were performed ex vivo under static conditions, which could explain the lack of effect of pravastatin.

The superior ability of NCX 6550 over native pravastatin to reduce splenocyte adhesion ex vivo, to improve endothelial function, and to reduce MCP-1 levels suggests that it is the NO moiety on this compound that is responsible for its antiadhesive effect. Using spectroscopy to measure nitrosyl hemoglobin in rat whole blood, Ongini et al. (2004) have demonstrated a linear, time-dependent increase in NO release from NCX 6550 that was consistent with slow NO release kinetics. The quantity of NO released was suggested to parallel that produced by endothelial nitric-oxide synthase under physiological conditions. The increase in local NO levels resulting from NCX 6550 treatment could result in an anti-inflammatory effect since NO is known to interfere with the release of a number of inflammatory mediators (such as caveolin-1 and nuclear factor κB) and the expression of adhesion molecules (Guzik et al., 2003). An alternative explanation for the superior effect of NCX 6550, however, could be attributed to a physicochemical, rather than a pharmacological, difference between the two compounds, since pravastatin is known to be weakly effective in vitro because of its low.
lipophilicity, whereas the different physicochemical properties of NCX 6550 make it more lipophilic and consequently increase its penetration into cells (Ongini et al., 2004).

Both NCX 6550 and Pravastatin Inhibit Splenocyte ROS Production. In contrast to the findings with splenocyte adhesion and ICAM-1 expression, both pravastatin and NCX 6550 attenuated the increase in ROS generation from ApoE−/− splenocytes. As would be expected after such a short period of administration (5 days), neither drug reduced plasma cholesterol; therefore, this effect is unlikely to be mediated through a lipid-lowering effect. However, this shared property does imply that it is an effect mediated through the statin molecule. It has been demonstrated that statins can reduce ROS generation from vascular tissue by reducing levels of p22phox mRNA (Wassmann et al., 2001) and can act directly as ROS scavengers (Bandoh et al., 2003).

The present novel finding that statins can also inhibit ROS generation from inflammatory cells that are intimately involved in atherogenesis and plaque rupture offers a further mechanism by which these compounds exert their highly beneficial effect. Interestingly, NCX 6550 also reduced ROS generation in response to the highest concentration of PMA tested in splenocytes from normcholesterolemic mice. This action may be related to the NO moiety since we have previously shown that NO-donating drugs can scavenge ROS generated by inflammatory cells (Demiryurek et al., 1997).

NCX 6550 and Pravastatin on Endothelial Function. Among the proposed pleiotropic effects of statins is an improvement in endothelial function through an increased bioavailability of nitric oxide, a reduction in oxidative stress, and the promotion of re-endothelialization (Wolfrum et al., 2003). Our present studies support this notion through the demonstration that pravastatin increased the maximal response to carbachol. However, under the same conditions, treatment with NCX 6550 exerted a superior effect on endothelial function by increasing the sensitivity to an endothelium-dependent vasodilator (as demonstrated by the leftward shift in EC50) as well as increasing maximal response. This is consistent with previous observations that NCX 6550 improves endothelial function in hypertensive rats (Presotto et al., 2005) and is probably due to the enhanced NO availability provided by the molecule.

NCX 6550 and Pravastatin on MCP-1 Levels. MCP-1 is known to mediate monocyte recruitment into vessel walls at sites of atherosclerosis, and raised MCP-1 levels have been associated with cardiovascular disease risk factors. The recent demonstration that NCX 6550 attenuated the increase in ROS generation from ApoE−/− splenocytes (Bandoh et al., 2003) was consistent with numerous studies demonstrating the ability of statins to reduce MCP-1 (for review, see Ballantyne and Nambi, 2005) and is probably due to the enhanced NO availability provided by the molecule.

Conclusions

Taken together, our findings suggest that NCX 6550, which retains the properties of the parent statin compound while having the added asset of slow nitric oxide release, is significantly more effective than native pravastatin in relation to several inflammatory markers. Clinically, this may be important in terms of maintaining plaque stability and endothelial function that is compromised by raised circulating cholesterol levels, and the provision of these beneficial effects early on in drug treatment (i.e., within days rather than months) certainly warrants further investigation.

References


Address correspondence to: Cherry L. Wainwright, School of Pharmacy, The Robert Gordon University, Schoolhill, Aberdeen AB10 1FR, UK. E-mail: c.wainwright@rgu.ac.uk