Cholesteryl Ester Transfer Protein Inhibition Enhances Endothelial Repair and Improves Endothelial Function in the Rabbit

Ben J. Wu, Sudichhya Shrestha, Kwok L. Ong, Douglas Johns, Liming Hou, Philip J. Barter, Kerry-Anne Rye

Objective—High-density lipoproteins (HDLs) can potentially protect against atherosclerosis by multiple mechanisms, including enhancement of endothelial repair and improvement of endothelial function. This study asks if increasing HDL levels by inhibiting cholesteryl ester transfer protein activity with the anacetrapib analog, des-fluoro-anacetrapib, enhances endothelial repair and improves endothelial function in New Zealand White rabbits with balloon injury of the abdominal aorta.

Approach and Results—New Zealand White rabbits received chow or chow supplemented with 0.07% or 0.14% (wt/wt) des-fluoro-anacetrapib for 8 weeks. Endothelial denudation of the abdominal aorta was carried out after 2 weeks. The animals were euthanized 6 weeks postinjury. Treatment with 0.07% and 0.14% des-fluoro-anacetrapib reduced cholesteryl ester transfer protein activity by 81±4.9% and 92±12%, increased plasma apolipoprotein A–I levels by 1.4±0.1-fold and 1.5±0.1-fold, increased plasma HDL-cholesterol levels by 1.8±0.2-fold and 1.9±0.1-fold, reduced intimal hyperplasia by 37±11% and 51±10%, and inhibited vascular cell proliferation by 25±6.1% and 35±6.7%, respectively. Re-endothelialization of the injured aorta increased from 43±6.7% (control) to 69±6.6% and 76±7.7% in the 0.07% and 0.14% des-fluoro-anacetrapib-treated animals, respectively. Aortic ring relaxation and guanosine 3′,5′-cyclic monophosphate production in response to acetylcholine were also improved. Incubation of HDLs from the des-fluoro-anacetrapib-treated animals with human coronary artery endothelial cells increased cell proliferation and migration relative to control. These effects were abolished by knockdown of scavenger receptor-B1 and PDZ domain-containing protein 1 and by pharmacological inhibition of phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt.

Conclusions—Increasing HDL levels by inhibiting cholesteryl ester transfer protein reduces intimal thickening and regenerates functional endothelium in damaged New Zealand White rabbit aortas in an scavenger receptor-B1-dependent and phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt-dependent manner. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBHA.114.304747.)

Key Words: endothelium

Population studies have consistently shown that plasma high-density lipoprotein cholesterol (HDL-C) levels correlate inversely with the risk of having a cardiovascular event.1 HDLs have several potentially cardioprotective properties, the best known of which relates to the removal of cholesterol from macrophages in the artery wall in the first step of reverse cholesterol transport.2 HDLs also inhibit vascular inflammation,3 reduce oxidative stress in macrophages,4 prevent oxidation of low-density lipoproteins (LDLs),5 reduce thrombosis,6 enhance endothelial function,7 increase angiogenesis,8 and improve pancreatic β-cell function.9

We have previously reported that discoidal reconstituted HDLs comprising apolipoprotein (apo) A–I, the main HDL apolipoprotein, complexed with phospholipid, (A–I)rdHDLs, inhibit adhesion molecule expression in cytokine-activated human umbilical vein endothelial cells and human coronary artery endothelial cells (HCAECs).10,11 We also have reported that intravenous infusions of lipid-free apoA–I and (A–I)rdHDLs inhibit acute vascular inflammation in normocholesterolemic New Zealand White (NZW) rabbits12,13 and reduce atherosclerosis in cholesterole-fed NZW rabbits.14 This suggests that therapies that increase endogenous HDL levels may attenuate atherosclerotic lesion development and progression.
Cholesteryl ester transfer protein (CETP) transfers cholesteryl esters from HDLs to LDLs and triglyceride-rich lipoproteins. Inhibition of CETP activity increases HDL-C and apoA-I levels and has been proposed as a strategy for reducing cardiovascular events. Genetic studies have also indicated that CETP inhibition is potentially cardioprotective. Furthermore, increasing HDL-C levels with the CETP inhibitor torcetrapib and JTT-705 inhibits atherosclerotic lesion development in rabbits, which have naturally high levels of CETP activity, but has so far not reduced cardiovascular events in humans.

As endothelial injury is a key early event in atherosclerotic lesion development, and we have reported previously that increasing circulating HDL levels with (A–I)rHDL infusions enhances the repair of damaged endothelium in mice, we ask in this study if increasing HDL levels by inhibiting CETP transfer activity also increases HDL-phospholipid levels in NZW rabbits with balloon injury of the abdominal aorta. This question has been addressed using des-fluoro-anacetrapib, an analog of the CETP inhibitor, anacetrapib (Figure I in the online-only Data Supplement), to treat NZW rabbits with endothelial denudation of the abdominal aorta 2 weeks after study commencement and were euthanized at 6 weeks postinjury. At euthanasia, the plasma concentration in the animals treated with 0.07% or 0.14% (wt/wt) des-fluoro-anacetrapib was 0.34±0.13 μmol/L and 0.65±0.34 μmol/L, respectively.

At euthanasia, plasma CETP activity (expressed as the % cholesteryl esters transferred from HDL₃ to LDLs) in the animals treated with 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib was 2.9±0.8% and 1.1±1.8%, respectively, compared with 15.7±1.7% in the control animals (P<0.05; Table). Plasma total cholesterol levels increased from 0.56±0.16 mmol/L for the control animals to 0.98±0.12 and 1.01±0.13 mmol/L for the 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib-treated animals, respectively (P<0.05). HDL total cholesterol levels increased from 0.49±0.03 mmol/L (control) to 0.75±0.11 and 0.78±0.05 mmol/L, and HDL unesterified cholesterol levels increased from 0.11±0.01 mmol/L in the animals treated with 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib-treated animals (P<0.05). HDL cholesteryl ester levels increased from 0.38±0.03 mmol/L (control) to 0.58±0.08 and 0.60±0.07 mmol/L, respectively, in the animals treated with 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib (P<0.05). Treatment with 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib also increased HDL-phospholipid levels from 0.81±0.02 mmol/L (control) to 0.97±0.07 and 1.03±0.08 mmol/L (P<0.05) while apoA-I levels increased from 0.43±0.06 mg/mL to 0.58±0.05 and 0.61±0.05 mg/mL (Table).

Gel permeation chromatography of plasma samples from the control and des-fluoro-anacetrapib-treated animals established that HDL total cholesterol levels were increased and for 8 weeks. The remaining animals consumed chow supplemented with 0.07% or 0.14% (wt/wt) des-fluoro-anacetrapib for 8 weeks. All the animals were subjected to endothelial denudation of the abdominal aorta 2 weeks after study commencement and were euthanized at 6 weeks postinjury. Results are expressed as mean±SEM (n=6). ApoA-I indicates apolipoprotein A-I; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; TC, total cholesterol; and UC, unesterified cholesterol. *P<0.05 vs control.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Des-fluoro-anacetrapib Treatment Increases HDL Size and HDL-C Levels in NZW Rabbits

Three groups of NZW rabbits (n=6/group) were used for the study. The control animals were maintained on normal chow diet supplemented with 0.07% or 0.14% (wt/wt) with 0.07% or 0.14% (wt/wt) des-fluoro-anacetrapib for 8 weeks as described in the legend of Figure 1. Plasma CETP activity was determined as the transfer of cholesteryl esters from HDL₃ to low-density lipoprotein. HDL lipid and apoA-I concentrations were determined as described in Materials and Methods section. Results are expressed as mean±SEM (n=6). ApoA-I indicates apolipoprotein A-I; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; TC, total cholesterol; and UC, unesterified cholesterol. *P<0.05 vs control.
NZW rabbits were treated with 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib, intimal hyperplasia decreased by 35±5.9% and 56±8.2%, respectively, compared with control (Figure 2A and 2B; \( P<0.05 \) for both). Aortic media area was not affected (Figure 2C). The aortic intima/media ratio decreased by 37±11% and 51±10% in the animals treated with 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib, respectively (Figure 2D; \( P<0.05 \) for both). Treatment with 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib also inhibited cell proliferation within the injured aortas, with the number of PCNA\(^\text{+}\) cells decreasing by 25±6.1% and 35±6.7% (wt/wt), respectively, relative to control (Figure 2E; \( P<0.05 \) for both).

Des-fluoro-anacetrapib Treatment Promotes Functional Re-Endothelialization of Balloon-Injured NZW Rabbit Aortas

Re-endothelialization is a key repair process in response to arterial injury.\(^{28}\) At 6 weeks postinjury, the damaged aortic surface in the balloon-injured animals was partially covered by CD31\(^+\) endothelial cells that had regenerated from branch orifices (Figure 3A). In the control animals, 43±6.7% of the endothelial surface was CD31\(^+\) compared with 69±6.6% and 76±7.7% for the 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib-treated animals, respectively (Figure 3B; \( P<0.05 \) for both).

The increased re-endothelialization in the des-fluoro-anacetrapib-treated animals was associated with improved endothelial function. Treatment with 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib enhanced maximal endothelium-dependent relaxation in response to acetylcholine in pre-constricted aortic rings by 1.8±0.3-fold and 2.2±0.5-fold, respectively, relative to control (Figure 3C; \( P<0.05 \) for both). Assessment of endothelium-independent relaxation with sodium nitroprusside was, by contrast, comparable for the control and des-fluoro-anacetrapib-treated animals (Figure 3D). Aortic guanosine 3′,5′-cyclic monophosphate levels in response to acetylcholine, as a measure of nitric oxide synthase activity, increased from 151±18 pmol/g wet wt (control) to 215±20 and 261±34 pmol/g wet wt in the 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib-treated animals, respectively (Figure 3E; \( P<0.05 \) for both).

We have reported previously that endothelial progenitor cells (EPCs) contribute to endothelial repair in NZW rabbits, with a maximal response being apparent at 4 days post balloon injury.\(^{28}\) We have also reported that (A–I)rHDL infusions increase EPC (stem cell antigen-1\(^+\)-cells) recruitment to damaged endothelium in mice.\(^{7}\) To determine if des-fluoro-anacetrapib treatment enhances re-endothelialization of balloon-injured NZW rabbit aortas by recruiting EPCs to areas of damage, 3 additional groups of rabbits (n=6/group) were maintained on chow (control) or chow supplemented with 0.07% or 0.14% (wt/wt) des-fluoro-anacetrapib for 18 days. Endothelial denudation of the abdominal aorta was carried out after 14 days of dietary des-fluoro-anacetrapib supplementation, and the animals were euthanized 4 days later.

At euthanasia, circulating fetal liver kinase 1/stem cell antigen-1\(^+\)-EPCs (Figure IIA in the online-only Data Supplement) were increased in the des-fluoro-anacetrapib-treated animals compared with the control animals (Figure 1A). This indicates that des-fluoro-anacetrapib treatment increases HDL particle size and HDL-C levels. The increase in HDL size was confirmed by subjecting plasma to 2D gel electrophoresis and immunoblotting for apoA-I (Figure 1B). The apoA-I−containing particles in the control and des-fluoro-anacetrapib-treated animals migrated to an α-position when subjected to agarose gel electrophoresis. The absence of pre-β-migrating particles in these samples is consistent with what we have reported previously.\(^{27}\) Populations of large, α-migrating HDL particles were apparent in the des-fluoro-anacetrapib-treated, but not in the control, animals.

Des-fluoro-anacetrapib Treatment Inhibits Intimal Hyperplasia in NZW Rabbits in Response to Aortic Balloon Injury

We have reported previously that balloon injury of the abdominal aorta induces intimal hyperplasia and increases vascular smooth muscle cell proliferation.\(^{28}\) When balloon-injured
Supplement) and the number of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated LDL/FITC-lectin/4′,6-diamidino-2-phenylindole+ cells (Figure IIB in the online-only Data Supplement) in cultured monocytes from the treated and control animals were comparable. Recruitment of stem cell antigen-1+-cells to the damaged endothelium was minimal in the control animals and not enhanced in the des-fluor-anacetrapib-treated animals (Figure IIC in the online-only Data Supplement).

When taken together, these results indicate that treatment with des-fluoro-anacetrapib increases the regeneration of functional endothelium in NZW rabbits in response to aortic balloon injury by enhancing the proliferation and migration of endothelial cells from branching blood vessels adjacent to the site of vascular injury.

HDLs From Des-fluoro-anacetrapib-Treated NZW Rabbits Increase HCAEC Proliferation and Migration

To determine if the enhanced re-endothelialization in des-fluoro-anacetrapib-treated, balloon-injured NZW rabbits was due to improved HDL particle function or an increase in plasma HDL levels, HDLs were isolated from control- and des-fluoro-anacetrapib-treated animals and incubated with HCAECs at identical apoA–I concentrations or at apoA–I concentrations that reflected their on-treatment plasma levels at euthanasia.

The effect of des-fluoro-anacetrapib treatment on HCAEC proliferation was assessed by trypan blue exclusion. Incubation of HCAECs with HDLs from control and des-fluoro-anacetrapib-treated rabbits at concentrations comparable to their plasma apoA–I levels (0.43 mg/mL for the control animals, 0.58 mg/mL for the animals treated with 0.07% [wt/wt] des-fluoro-anacetrapib, and 0.61 mg/mL for the animals that received 0.14% [wt/wt] des-fluoro anacetrapib) increased the number of viable HCAECs from 14±1.8×10⁴ cells (control) to 20±1.8×10⁴ and 25±3.0×10⁴ cells, respectively (Figure 4A; P<0.05 for both). Cell proliferation was further verified using the xCELLigence system. The cell index, which is a quantitative measure of the number of cells in each well, was 0.59±0.06 for the control samples and 0.87±0.04 and 0.89±0.03 for the animals that received 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib, respectively (P<0.05 for both). Cell proliferation was further verified using the xCELLigence system. The cell index, which is a quantitative measure of the number of cells in each well, was 0.59±0.06 for the control samples and 0.87±0.04 and 0.89±0.03 for the animals that received 0.07% and 0.14% (wt/wt) des-fluoro-anacetrapib, respectively (P<0.05 for both).

![Figure 2](attachment://image.png)
When HDLs from the des-fluoro-anacetrapib-treated, balloon-injured NZW rabbits were incubated with HCAECs at equivalent apoA–I concentrations, cell proliferation (Figure IVA in the online-only Data Supplement) and migration (Figure IVB in the online-only Data Supplement) was comparable to that of the control animals. This indicates that des-fluoro-anacetrapib treatment enhances endothelial repair in balloon-injured NZW rabbits by increasing plasma HDL levels and not by increasing HDL particle function.

**HDLs From Des-fluoro-anacetrapib-Treated NZW Rabbits Increase HCAEC Proliferation and Migration in an SR-B1-, PDZ Domain-Containing Protein 1-, and PI3K/Akt-Dependent Manner**

As HDLs promote endothelial cell migration by activating the PI3K/Akt signal transduction pathway in a scavenger receptor class B-type 1 (SR-B1)- and PDZ1-dependent manner, we also asked if this pathway explained why HDLs from des-fluoro-anacetrapib-treated, balloon-injured NZW rabbits increased endothelial cell proliferation and migration.

Transfection of HCAECs with SR-B1 small interfering RNA (siRNA), PDZ domain-containing protein 1 (PDZK1) siRNA, or scrambled siRNA (siControl) decreased SR-B1 and PDZK1 protein levels by 73±9.5% and 85±7.1%, respectively (Figure V A and VB in the online-only Data Supplement; \( P < 0.05 \) for both). The transfected cells were incubated in the absence or presence of HDLs from rabbits treated for 8 weeks with 0.14% (wt/wt) des-fluoro-anacetrapib. The concentration of apoA–I in the incubations (0.61 mg/mL) was identical to the plasma apoA–I level at euthanasia.

Incubation of the transfected HCAECs in the absence of HDLs did not affect endothelial cell proliferation (Figure 5A and 5C) or migration (Figure 5B and 5D). Incubation of scrambled siRNA-transfected HCAECs (siControl) with HDLs from the des-fluoro-anacetrapib-treated rabbits increased the number of endothelial cells from 11±1.5×10⁴ to 25±2.7×10⁴ (Figure 5A and 5C, open bars), while endothelial cell migration increased from 21±2.7 to 61±7.6 cells/area (Figure 5B and 5D, open bars; \( P < 0.05 \) for all). The capacity of the HDLs to enhance endothelial cell proliferation and migration in HCAECs transfected with SR-B1 siRNA was inhibited.
by 46±7.2% (Figure 5A, closed bar) and 50±6.3% (Figure 5B, closed bar), respectively (P<0.05 for both). Similarly, incubation with HDLs inhibited proliferation and migration by 47±11% (Figure 5C, closed bar) and 59±7.8% (Figure 5D, closed bar), respectively (P<0.05 for both), in HCAECs transfected with PDZK1 siRNA.

Involvement of the PI3K/Akt pathway in HCAEC proliferation and migration was also assessed. Incubation of nontransfected HCAECs with HDLs increased cell proliferation by 2.2±0.2-fold (from 12±2.1×10^4 cells to 26±1.8×10^4 cells; Figure 5E, open bars) and migration from 20±6.5 to 59±4.8 cells/area (Figure 5F, open bars), respectively (P<0.05 for both). Preincubation of the HCAECs with the PI3K/Akt inhibitor, LY294002, prior to incubation with HDLs, reduced endothelial cell proliferation and migration by 32±5.5% (Figure 5E, closed bar) and 43±10% (Figure 5F, closed bar), respectively (P<0.05 for both versus control).

Activation of PI3K/Akt by HDLs from des-fluoro-anacetrapib-treated rabbits was also investigated. HCAECs were incubated for ≤6 hours with HDLs from rabbits treated for 8 weeks with 0.14% (wt/wt) des-fluoro-anacetrapib. The concentration of apoA-I in these incubations was identical to the plasma apoA-I level at the time of euthanasia. Relative to cells incubated in the absence of HDLs, Akt phosphorylation (p-Akt) increased 6.7±0.8-fold (P<0.05), 4.6±0.7-fold (P<0.05),...
2.7±0.5-fold (P<0.05), and 1.6±0.5-fold (ns) in HCAECs incubated with HDLs for 1, 2, 4, and 6 hours, respectively (Figure VIA in the online-only Data Supplement).

To ascertain if activation of PI3K/Akt by HDLs was dependent on SR-B1, HCAECs were transfected with SR-B1 siRNA or scrambled siRNA (siControl), then incubated for 1 hour in the absence or presence of HDLs from rabbits treated for 8 weeks with 0.14% (wt/wt) des-fluoro-anacetrapib. The concentration of apoA-I in the incubations was identical to the plasma apoA-I level at the time of euthanasia. Under these conditions, p-Akt protein levels increased 5.2±0.5-fold (P<0.05) in the scrambled siRNA-transfected HCAECs (Figure VIB in the online-only Data Supplement, open bars), whereas p-Akt levels were reduced by 45±7.7% in the SR-B1 siRNA-transfected HCAECs (P<0.05; Figure VIB in the online-only Data Supplement, closed bar).

Collectively, these results indicate that HDLs from rabbits treated with 0.14% (wt/wt) des-fluoro-anacetrapib promote endothelial cell proliferation and migration by activating the PI3K/Akt signal transduction pathway in an SR-B1-dependent manner.

To determine if the HDL-mediated enhancement of HCAEC proliferation and migration was dependent on the ATP-binding cassette transporters, ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1), cells were transfected with ABCA1 siRNA, ABCG1 siRNA, or scrambled siRNA (siControl). ABCA1 and ABCG1 protein levels were reduced by 80±7.5% and 74±4.2%, respectively, in the transfected cells (Figure VC and VD in the online-only Data Supplement; P<0.05 for both). Incubation of the ABCA1 siRNA- and ABCG1 siRNA-transfected cells with or without HDLs from des-fluoro-anacetrapib-treated rabbits at a final apoA-I concentration of 0.61 mg/mL did not affect endothelial cell proliferation (Figure VIIA in the online-only Data Supplement) or migration (Figure VIB in the online-only Data Supplement). This indicates that HDLs enhance endothelial cell proliferation and migration independent of ABCA1 and ABCG1.

Overall, these results indicate that HDLs from des-fluoro-anacetrapib-treated rabbits enhance endothelial cell proliferation and migration via an SR-B1/PDZK1/PI3K/Akt-dependent pathway.

Discussion

This study establishes that increasing HDL and apoA–I levels by inhibiting CETP activity with des-fluoro-anacetrapib protects against intimal hyperplasia (Figure 2) and promotes functional re-endothelialization (Figure 3) in normocholesterolemic NZW rabbits with endothelial denudation of the abdominal aorta. We further demonstrate that HDLs isolated from des-fluoro-anacetrapib-treated NZW rabbits increase endothelial cell proliferation and migration in vitro in an SR-B1/PDZK1- and PI3K/Akt-dependent manner (Figures 4 and 5).

The results show that treatment of NZW rabbits with des-fluoro-anacetrapib increases HDL and apoA–I levels, and HDL particle size is in agreement with what has been reported for humans and NZW rabbits treated with the CETP inhibitor, torcetrapib. One of the key questions arising from these observations is whether the cardiovascular benefit of increasing circulating HDL levels by inhibiting CETP activity improves HDL particle function or whether it is related to the increase in circulating HDL levels. The present results indicate that the reduced intimal hyperplasia, enhanced re-endothelialization, and improved vascular reactivity that was observed in the des-fluoro-anacetrapib-treated, balloon-injured NZW rabbits are due to the increase in HDL levels and not due to an improvement of HDL particle function.

HDLs have been shown to enhance endothelial cell migration in a nitric oxide–dependent manner in processes that involve SR-B1 as well as Rac GTPase, Src kinases, phosphatidylinositol 3-kinase, and p44/42 mitogen-activated protein kinase activation. HDLs also increase endothelial cell proliferation via a cell surface F(1)-ATPase. On the contrary, HDLs reduce myeloid cell proliferation by increasing cholesterol efflux via the ATP-binding cassette transporters, ABCA1 and ABCG1. These opposing effects of HDLs on endothelial cell and myeloid cell proliferation highlight the functional diversity of the HDL fraction.

One of the most interesting outcomes of the current study is that the HDL-mediated increase in endothelial cell proliferation and migration in the des-fluoro-anacetrapib-treated animals was dependent on SR-B1, but not ABCA1 or ABCG1. Several of the cardioprotective and anti diabetic effects of HDLs are mediated by binding to SR-B1. For example, HDL-induced endothelial nitric oxide synthase activation, the promotion of endothelial cell migration and re-endothelialization after endothelial injury, and increased glucose uptake by adipocytes and glycogen synthesis in muscle all involve HDL-SR-B1 interactions. The ability of HDLs to improve endothelial cell migration has also been reported to involve PI3K/Akt signal transduction, SR-B1, and PDZK1.

Circulating EPCs play an important role in vascular endothelial cell repair and are related to plasma HDL levels. We have shown previously that EPCs contribute to endothelial repair in NZW rabbits and that (A–I)rHDL infusions increase EPC recruitment to damaged endothelium in mice. However, EPCs did not contribute to the functional aortic re-endothelialization in balloon-injured, des-fluoro-anacetrapib-treated NZW rabbits in the present study (Figure II in the online-only Data Supplement). This may be because large HDL particles impair EPC function, and moderate to high concentrations of HDLs enhance EPC senescence. This suggests that increasing HDL particle size by inhibiting CETP activity may selectively attenuate EPC function without altering the ability of individual HDL particles to increase the proliferation and migration of pre-existing endothelial cells.

A potential limitation of this study was the use of normocholesterolemic NZW rabbits that have low plasma LDL levels. Although it may have been more physiologically relevant to use cholesterol-fed rabbits, which have elevated LDL levels, this would have confounded the results because des-fluoro-anacetrapib treatment increases HDL levels and decreases LDL levels. As such it would not have been possible to attribute the outcome of the study unequivocally to an increase in plasma HDL levels. This issue was circumvented by treating humans and NZW rabbits treated with the CETP inhibitor, torcetrapib.
normocholesterolemic NZW rabbits with des-fluoro-anoacetrapib, so that the reduction in LDL levels was minor relative to the increase in HDL levels.

In conclusion, this study establishes that treatment with des-fluoro-anoacetrapib protects against intimal hyperplasia and promotes functional re-endothelialization in NZW rabbits with endothelial denudation of the abdominal aorta. It remains to be seen if these beneficial effects are also apparent in humans and whether they translate into a reduction in cardiovascular events in people treated with CETP inhibitors.

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**Disclosures**

D. Johns is an employee of Merck & Co. The other authors report no conflicts.

**References**


This study establishes that elevating plasma high-density lipoprotein and apolipoprotein A-I levels by inhibiting cholesteryl ester transfer protein activity in New Zealand White rabbits enhances endothelial repair by increasing endothelial cell proliferation and the migration of endothelial cells to areas of damage. Inhibition of cholesteryl ester transfer protein also improves endothelial function and reduces intimal hyperplasia. These findings establish that cholesteryl ester transfer protein inhibition improves several of the key cardioprotective functions of high-density lipoproteins. Further studies are required to ascertain if these beneficial effects of cholesteryl ester transfer protein inhibition are also apparent in humans.

Significance

This study establishes that elevating plasma high-density lipoprotein and apolipoprotein A-I levels by inhibiting cholesteryl ester transfer protein activity in New Zealand White rabbits enhances endothelial repair by increasing endothelial cell proliferation and the migration of endothelial cells to areas of damage. Inhibition of cholesteryl ester transfer protein also improves endothelial function and reduces intimal hyperplasia. These findings establish that cholesteryl ester transfer protein inhibition improves several of the key cardioprotective functions of high-density lipoproteins. Further studies are required to ascertain if these beneficial effects of cholesteryl ester transfer protein inhibition are also apparent in humans.
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SUPPLEMENTAL MATERIAL

Cholesteryl Ester Transfer Protein Inhibition Enhances Endothelial Repair and Improves Endothelial Function in the Rabbit

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Supplemental Fig. I. Structure of des-fluoro-anacetrapib and anacetrapib.

X=H: Anacetrapib
X=F: Des-fluoro-anacetrapib
Supplemental Fig. II. Des-fluoro-anacetrapib treatment does not increase circulating EPC levels or enhance recruitment of EPCs to the denuded endothelial surface in balloon-injured NZW rabbit aortas. NZW rabbits (n=6/group) received regular chow (Ctrl) or chow supplemented with 0.07% (wt/wt) or 0.14% (wt/wt) des-fluoro-anacetrapib for 18 days. Endothelial denudation of the abdominal aorta was carried out with a balloon catheter following 14 days of dietary des-fluoro-anacetrapib supplementation. The animals were sacrificed 4 days after aortic balloon injury. Panel A: Percentage of Sca-1⁺ and Flk1⁺-cells, and Panel B: Number of Dil-acLDL⁺/lectin⁺/DAPI⁺ cells in peripheral blood measured 4 days after injury. Panel C: The number of Sca-1⁺-cells in abdominal aortic cross-sections at 4 days after injury. Data are expressed as mean±SEM.
Supplemental Fig. III. HDLs from des-fluoro-anacetrapib-treated NZW rabbits increase HCAEC proliferation. HCAECs were seeded in a 96-well xCELLigence E-plate and incubated in serum-free EBM-2 for 24 h with HDLs isolated by ultracentrifugation from plasma of NZW rabbits that received chow (Ctrl) or chow supplemented with either 0.07% or 0.14% (wt/wt) des-fluoro-anacetrapib for 8 weeks. The final apoA-I concentrations in the incubations were comparable to the mean plasma apoA-I level: 0.43 mg/mL for control [Ctrl], 0.58 mg/mL for 0.07% des-fluoro-anacetrapib and 0.61 mg/ml for 0.14% des-fluoro anacetrapib. The cell index was determined using the xCELLigence system. Data are expressed as mean±SEM of three independent experiments. *<0.05 vs Ctrl.
Supplemental Fig. IV. Des-fluoro-anacetrapib treatment does not affect the capacity of HDLs to promote endothelial cell proliferation and migration. HDLs were isolated from plasma of NZW rabbits that received chow (Ctrl) or chow supplemented with either 0.07% or 0.14% des-fluoro-anacetrapib for 8 weeks. The HDLs were then incubated for 24 h with HCAECs (final apoA-I concentrations 0.25, 0.5 and 1.0 mg/mL). Panel A: HCAEC proliferation as assessed by Trypan blue exclusion. Panel B: HCAEC migration assessed as the number of cells migrating past the scratch-wound edge. Data are expressed as mean±SEM of three independent experiments. *p<0.05 vs relative HDLs at 0.5 mg/mL.
Supplemental Fig. V. Knock down of SR-B1, PDZK1, ABCA1 and ABCG1 in HCAECs. HCAECs were transfected with scrambled siRNA (siControl, open bars), SR-B1 siRNA (siSR-B1), PDZK1 siRNA (siPDZK1), ABCA1 siRNA (siABCA1) and ABCG1 siRNA (siABCG1) (closed bars) as described in Materials and Methods. Transfection efficiency was monitored by western blotting with anti-SR-B1 (Panel A), anti-PDZK1 (Panel B), anti-ABCA1 (Panel C) and anti-ABCG1 (Panel D) antibodies using β-actin as a loading control. Data represent the mean±SEM (n=3). *p<0.05 vs scrambled siRNA.
Supplemental Fig. VI. HDLs from des-fluoro-anacetrapib-treated NZW rabbits activate Akt in an SR-B1-dependent manner. HCAECs were incubated for 0-6 h with HDLs isolated from NZW rabbits treated for 8 weeks with 0.14% (wt/wt) des-fluoro-anacetrapib (final apoA-I concentration 0.61 mg/mL). (Panel A) The cells were lysed, immunoblotted for phosphorylated Akt (p-Akt) and total Akt and the p-Akt/total Akt ratio was quantified. (Panel B) HCAECs were transfected for 48 h with scrambled siRNA (siControl) and SR-B1 siRNA (siSR-B1), then incubated for a further 1 h in the absence or presence of isolated HDLs (final apoA-I concentration 0.61 mg/mL) from NZW rabbits treated for 8 weeks with 0.14% (wt/wt) des-fluoro-anacetrapib The cells were lysed, immunoblotted for phosphorylated Akt (p-Akt) and total Akt and the p-Akt/total Akt ratio was quantified. Data are expressed as mean±SEM of three independent experiments. *p<0.05 compared to 0 h. #p<0.05 siControl versus siSR-B1.
Supplemental Fig. VII. HDLs from des-fluoro-anacetrapib-treated NZW rabbits increase HCAEC proliferation and migration independent of ABCA1 and ABCG1. HCAECs were transfected for 48 h with ABCA1 siRNA, ABCG1 siRNA or scrambled siRNA, then incubated for a further 24 h in the absence (open bars) or presence (closed bars) of HDLs (final apoA-I concentration 0.61 mg/mL) isolated from NZW rabbits treated for 8 weeks with chow supplemented with 0.14% (wt/wt) des-fluoro-anacetrapib. Panel A: HCAEC proliferation assessed by Trypan Blue exclusion. Panel B: HCAEC migration assessed as the number of cells migrating past the scratch wound edge. Data are expressed as the mean±SEM of three independent experiments. #p<0.05.
MATERIALS AND METHODS

**Cholesteryl Ester Transfer Protein Inhibition Enhances Endothelial Repair and Improves Endothelial Function in the Rabbit**

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MATERIALS AND METHODS

Animal studies

Six groups of male New Zealand White (NZW) rabbits (n=6/group) weighing approximately 2.2 kg (Institute of Medical and Veterinary Science, South Australia) received either regular chow (Groups 1 and 4) or chow supplemented with 0.07% (wt/wt) (Groups 2 and 5) or 0.14% (wt/wt) (Groups 3 and 6) des-fluoro-anacetrapib (Merck & Co., Inc. Kenilworth, NJ, USA) for up to 8 weeks. Endothelial denudation of the abdominal aorta was carried out with a balloon catheter as described previously under general anaesthesia (inhaled isoflurane, 4-5% for induction and 1.5-2% for maintenance) after 2 weeks of dietary des-fluoro-anacetrapib supplementation1. 2. Briefly, a 3F Fogarty embolectomy catheter (Baxter, Old Toongabbie, NSW, Australia, Catalogue Number: 120803F) was inserted into the right femoral artery, advanced 25 cm proximally, and then withdrawn to the origin with the balloon inflated with 0.2 mL saline. This step was repeated twice. The animals were sacrificed 6 weeks (Groups 1, 2, and 3) or 4 days (Groups 4, 5, and 6) after aortic balloon injury. All the procedures were approved by the Sydney Local Health Network Animal Welfare Committee.

Biochemical analyses

Blood was collected into EDTA vacutainer tubes (BD Pharmingen, North Ryde, NSW, Australia, Catalogue Number: 367873) at the time of euthanasia (Groups 1, 2, and 3). Plasma was isolated by centrifugation (1,000 x g at 4 °C for 10 min). Plasma apolipoprotein (apo) A-I concentrations were determined immuno turbidometrically using sheep anti-rabbit apoA-I polyclonal antibodies. Total cholesterol, unesterified cholesterol and phospholipid concentrations were determined enzymatically. Cholesteryl ester concentrations were determined by subtracting the concentration of unesterified cholesterol from the total cholesterol concentration. Plasma HDL total cholesterol, unesterified cholesterol and phospholipid levels were determined after precipitating apoB-containing lipoproteins with polyethylene glycol 6000. All analyses were performed on an AU480 Auto-Analyzer (Beckman Coulter, Fullerton, CA).

Cholesterol profiles were determined by loading plasma (200 µL) onto two Superdex 200 columns (GE Healthcare, Little Chalfont, UK) connected in series and attached to a fast protein liquid chromatography system (GE Healthcare). Very low density lipoproteins, LDLs and HDLs were resolved at a flow rate of 0.3 mL/min. Fractions were collected at 1 min intervals. The plasma distribution of apoA-I was assessed by 2-D gel electrophoresis and immunoblotting with a sheep anti-rabbit apoA-I polyclonal antibody.

Cholesteryl ester transfer protein (CETP) assay

HDLs and LDLs were isolated by ultracentrifugation from pooled samples of normal human plasma. Activity of CETP was assessed as the transfer of [3H]-labelled cholesteryl esters from high density lipoproteins (HDLs) to low density lipoproteins (LDLs) as described. Briefly, an aliquot of plasma was incubated with [3H] cholesteryl ester-labelled HDL3 and unlabelled LDLs at 37 °C for 3 h. After precipitation of LDLs with heparin (5,000 IU/mL):MnCl2 (2 mol/L) (1:1), the radioactivity in the supernatant was determined by liquid scintillation counting. CETP activity was calculated as the percentage of radiolabelled cholesteryl esters transferred from HDL3 to LDLs.

Des-fluoro-anacetrapib analysis

For quantitation of plasma des-fluoro-anacetrapib levels, plasma samples were rendered basic by addition of carbonate buffer (pH 9.8), extracted with 80/20 (v/v) hexane/isopropanol and dried under nitrogen. Following reconstitution in 50/50/0.1 (v/v/v) acetonitrile/water/formic acid, the samples were injected onto a Waters Acquity UPLC® BEH Shield RP18 2.1 x 50 mm (1.7 µm particle size) column (Water’s Acquity HPLC/MS/MS system). The des-fluoro-anacetrapib was eluted isocratically with 80/20/0.1 (v/v/v) acetonitrile/water/formic acid at a flow rate of 0.6 mL/min and detected with a Sciex API 4000/5000/5500 triple quadrupole MS/MS system equipped with a TurboIonSpray™
ionization source operated in the positive ion mode. Peak areas were integrated using the Sciex program Analyst® (AB Sciex, Ontario, Canada). Peak area ratios of analyte to internal standard were plotted and standard curves constructed using linear (weighted 1/x²) regression. Des-fluoro-anacetrapib concentrations in quality control and rabbit plasma samples were derived from the standard curve.

Assessment of aortic morphology and re-endothelialisation

Sections (~3 mm) were taken from the abdominal aorta at the third pair of lumbar arteries, fixed in 4% (v/v) cold paraformaldehyde, stored in 70% (v/v) ethanol, embedded in paraffin and sectioned (5 µm) for morphologic assessment by staining with Verhoeff's hematoxylin. Immediately adjacent sections were used for immunohistochemical analysis using mouse monoclonal antibodies against proliferating cell nuclear antigen (PCNA) (dilution 1:200; Dako, Glostrup, Denmark, Catalogue Number: M0879) and CD31 (dilution 1:50; Dako, Catalogue Number: M0823), and rat monoclonal antibodies against stem cell antigen-1 (Sca-1) (1:100 dilution; BD Pharmingen, North Ryde, NSW, Australia, Catalogue Number: 557404). Polyclonal goat anti-mouse and rabbit anti-rat IgG-HRP (dilution 1:200) (Dako, Catalogue Number: P0447 and P0450) were used as secondary antibodies. Staining was visualized using the Horse Radish Peroxidase (HRP)-3,3’ Diaminobenzidine (DAB) system (Envision Mouse Kit, Dako, Catalogue Number: K4007), followed by counter staining with haematoxylin. The sections were imaged using an upright light microscope (Zeiss, Germany). Planimetry (Adobe Photoshop V6.0) was performed by tracing the intimal and medial areas. The results were expressed as the total pixel numbers. Intimal hyperplasia was determined as the intima/media ratio. Total cell profiles, PCNA+ cells and Sca-1+ cells were quantified manually (40x magnification). Re-endothelialization was determined from the lumen circumference as the percentage of the endothelium staining positive for CD31 using ImageJ software (http://rsb.info.nih.gov/ij/). A single operator using coded samples who was blinded to the treatment performed all of the analyses.

Assessment of endothelial function ex vivo

Isometric tension experiments were carried out within 4 h of sacrifice using aortic rings isolated from the abdominal aorta proximally at the second pair of lumbar arteries (~3 mm in length). The rings were placed in cold Krebs buffer solution (Sigma-Aldrich, St Louis, MO, Catalogue Number: K3753) aerated with 95% O₂/5% CO₂ and mounted in a Myobath system (World Precision Instruments, Sarasota, FL, USA) containing 20 mL of Krebs buffer solution aerated at 37 °C with 95% O₂/5% CO₂. Ring viability was confirmed by incremental constriction (2.5 g load) in response to phenylephrine (Sigma-Aldrich, Catalogue Number: P1240000). After pre-constriction to 50% maximal response, endothelium-dependent vasodilation was determined by exposing the rings to incremental doses (0.001 to 10 µmol/L) of acetylcholine (Sigma-Aldrich, Catalogue Number: A6625), and then to sodium nitroprusside (Sigma-Aldrich, Catalogue Number: PHR1423) to assess endothelium-independent vasodilation.

Segments (~3 mm in length) isolated from the abdominal aorta distally at the second pair of lumbar arteries were analyzed for guanosine 3’,5’-cyclic monophosphate (cGMP) content to assess nitric oxide synthase activity. The aortic rings were incubated for 15 min at 37 °C in a 5% CO₂ incubator in Krebs buffer solution supplemented with 200 µmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich, Catalogue Number: I5879) to inhibit phosphodiesterases. The rings were then exposed to 1 µmol/L acetylcholine for 1 min at 37 °C, snap-frozen, and stored at -80 °C. Trichloroacetic acid 5% (w/v) was added to the frozen samples, which were homogenized as described⁹ and then acetylated with 4% (v/v) acetic anhydride in a 0.64 mol/L KOH solution. The cGMP content of the samples was determined using an enzyme immunoassay kit according to the manufacturer’s instructions (Cayman Chemicals, Ann Arbor, MI, Catalogue Number: 581021).
Flow cytometry
Blood (5 mL) was collected at the time of euthanasia (Groups 4, 5, and 6) and the red cells were lysed. Mononuclear cells were analyzed for expression of FITC-conjugated Sca-1 (BD Biosciences, North Ryde, NSW, Australia, Catalogue Number: 557405) and PE-conjugated fetal liver kinase 1 (Flk1) (BD Biosciences, Catalogue Number: 555308). Isotype antibodies served as negative controls (BD Biosciences, Catalogue Number: 553929 and 553930). The immunofluorescently labelled cells were fixed with 2% (v/v) paraformaldehyde and analyzed by quantitative flow cytometry using a FACSVantage flow cytometer (BD Biosciences, North Ryde, NSW, Australia) and Cell Quest Software. Typically, 10,000 cells were analyzed based on forward versus side scatter gating.

Endothelial progenitor cell culture
Mononuclear cells were isolated by density gradient centrifugation with Lymphoprep (Axis-Shield, Oslo, Norway, Catalogue Number: 1114545) from 10 mL of blood obtained at the time of euthanasia (Groups 4, 5, and 6). Immediately after isolation, the cells were seeded in 6-well fibronectin-coated plates (1 x 10^7 /well) (BD Biosciences, Catalogue Number: 354402) and maintained in EGM-2 medium (Cell Applications, San Diego, CA, USA, Catalogue Number: CC-3162) supplemented with 20% fetal bovine serum (Cell Applications, Catalogue Number: 026-100) and antibiotics. The medium was changed every day for 4 days, at which time the cells were washed and incubated with fresh medium for 4 h with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (Dil-acLDL, 4 µg/mL, Invitrogen, Carlsbad, CA, USA, Catalogue Number: L3484). The cells were then fixed in ice-cold 70% (v/v) ethanol and counterstained with 1% FITC-conjugated Ulex-lectin (Sigma-Aldrich, Catalogue Number: L9006) and Vectamount-4',6-Diamidino-2-Phenyldinole (DAPI) (Vector Labs, Burlingame, CA, USA, Catalogue Number: H-1200). Cellular Dil-acLDL uptake and FITC-lectin and DAPI staining were determined by fluorescent microscopy. Cells that had ingested Dil-acLDL and stained positively for all three antigens were judged to be endothelial progenitor cells.

Cell culture and transfection
Human coronary artery endothelial cells (HCAECs) (Cell Applications, San Diego, CA, Catalogue Number: 300-05a) were cultured at 37 °C in MesoEndo Cell Growth Medium (Cell Applications, Catalogue Number: 212-500) in a 5% CO2 incubator. The ATP binding cassette transporters ABCA1, ABCG1 and scavenger receptor-B1 (SR-B1) were knocked down by transfecting the HCAECs at 37 °C for 48 h with 200 pmol of specific ABCA1, ABCG1 and SR-B1 siRNAs, respectively (SMARTpool, mixture of four different target-specific sequences), or scrambled siRNA (control) (Thermo Scientific, Lafayette, CO, Catalogue Numbers: L-008615-00-005, L-004128-00-0005, L-010592-00-0005 and D-001810-10-15, respectively) using the Opti-MEM/Lipofectamine system (Invitrogen, Carlsbad, CA, Catalogue Number: 772661). The adaptor PDZ domain-containing protein 1 (PDZK1) was silenced by transfecting HCAECs at 37 °C for 24 h with 60 pmol of specific siRNAs against PDZK1 (siRNA transfection reagent system, Santa-Cruz Biotechnology, Catalogue Number: sc-106840). Control cells were transfected with scrambled siRNA (Santa-Cruz Biotechnology, Catalogue Number: sc-37007). Knockdown of ABCA1, ABCG1, SR-B1 and PDZK1 in total cell lysates was confirmed by Western blotting. For cell signalling experiments, HCAECs were pre-incubated for 1 h with LY294004 (final concentration 10 µmol/L) (Merck, Australia, Catalogue Number: 440204), then incubated for a further 24 h with ultracentrifugally isolated HDLs (1.063 < d < 1.25 g/ml) at a final apoA-I concentration comparable to the mean plasma apoA-I level: 0.43 mg/mL for the control animals [Ctrl], 0.58 mg/mL for the animals that received chow supplemented with 0.07% (wt/wt) des-fluorooracetrapib and 0.61 mg/mL for the animals that received chow supplemented with 0.14% (wt/wt) des-fluorooracetrapib.

For activation of PI3K/Akt, non-transfected and siRNA-transfected HCAECs were grown to confluence then incubated with serum-free Endothelial Cell Basal Medium-2 (EBM-2) (Lonza, Sydney, Australia, Catalogue Number: cc-3156) for 6 h. The HCAECs were then
incubated for up to 6 h with HDLs (final apoA-I concentration 0.61 mg/mL) isolated from NZW rabbits treated for 8 weeks with 0.14% (w/w) des-fluoro-anacetrapib.

**Cell proliferation**

HCAECs were grown to 50% confluence. The culture medium was replaced with serum-free EBM-2 (Lonza), and the cells were incubated for a further 6 h. The HCAECs were then incubated for 24 h with ultracentrifugally isolated HDLs from plasma of NZW rabbits that received chow with or without des-fluoro-anacetrapib at a final apoA-I concentration of 0.25, 0.5 and 1.0 mg/mL or at a concentration comparable to the mean plasma apoA-I level: 0.43 mg/mL for the control animals [Ctrl], 0.58 mg/mL for the animals that received chow supplemented with 0.07% (wt/wt) des-fluoro-anacetrapib and 0.61 mg/mL for the animals that received chow supplemented with 0.14% (wt/wt) des-fluoro-anacetrapib. Cell proliferation was assessed by Trypan blue exclusion and manual counting of total HCAECs using a hemocytometer.

Cell proliferation was also quantified by seeding HCAECs in a 96-well xCELLigence E-plate (John Morris Scientific, Sydney, Australia, Catalogue Number: 05232368001). The cells were incubated with serum-free EBM-2 (Lonza) for 24 h with HDLs isolated from plasma of NZW rabbits that received chow with or without des-fluoro-anacetrapib at a concentration comparable to the mean plasma apoA-I level as described above. The cell index was determined as the electronic impedance of sensor electrodes using the xCELLigence system (ACEA Biosciences, San Diego, CA).

**Cell migration**

HCAECs were grown to confluence then incubated with serum-free EBM-2 (Lonza) for 6 h. A defined region of HCAECs was removed with a cell scraper (“scratch”). The cells were then incubated for 24 h with HDLs isolated from plasma of NZW rabbits that received chow with or without des-fluoro-anacetrapib at a final apoA-I concentration of 0.25, 0.5 and 1.0 mg/mL or at a concentration comparable to the mean plasma apoA-I level as described above. The number of cells that migrated across the wound edge was counted manually.

**Western Blotting**

HCAECs were washed with ice cold PBS and lysed in 20 mmol/L Tris buffer (pH 7.5) containing 0.5 mmol/L EDTA-Na2, 0.5 mmol/L EGTA-Na2 and protease inhibitors. The cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, Catalogue Number: NP0321) and incubated overnight with mouse anti-human ABCA1 (1:500) (Abcam, Cambridge, UK, Catalogue Number: ab18180), rabbit anti-human ABCG1 (1:500) (Abcam, Catalogue Number: ab52617), rabbit anti-human SR-B1 (1:200) (Epitomics, Catalogue Number: 1971-1), and mouse anti-human PDZK1 (1:200) (Santa-Cruz Biotechnology, Catalogue Number: sc-100337) monoclonal antibodies, rabbit anti-human Akt (1:1000) and rabbit anti-human p-Akt (1:500) (Cell Signaling, Danvers, MA, Catalogue Numbers: 9272 and 4058). A mouse anti-human monoclonal antibody against β-actin (1:3000) (Sigma-Aldrich, Catalogue Number: A1978) was used as the loading control. Secondary antibodies were polyclonal goal anti-rabbit and anti-mouse IgG-HRP (Santa-Cruz Biotechnology, Catalogue Number: sc-2004). Immunoreactive proteins were detected by ECL and analysed with Quantity One 1-D Analysis Software (Bio-Rad, Gladesville, NSW, Australia).

**Statistics**

Data are expressed as mean ± SEM. One-way ANOVA and the Newman-Keul’s post-hoc test were used to evaluate differences between groups. Acetylcholine and sodium nitroprusside dose response curves were compared between groups by one-way ANOVA for repeated-measures with Bonferroni corrections. All statistical analyses were carried out using GraphPad Prism software version 4.03 (GraphPad Software, Inc. San Diego, CA). A value of p<0.05 was considered significant.
REFERENCES


