Heme Oxygenase-1 Increases Endothelial Progenitor Cells

Ben J. Wu, Robyn G. Midwinter, Carlos Cassano, Konstanze Beck, Yutang Wang, Dechaboon Changsiri, Jennifer R. Gamble, Roland Stocker

Objectives—Induction of heme oxygenase-1 (HO-1) protects against atherosclerotic disease in part by promoting reendothelialization. As endothelial progenitor cells (EPCs) contribute to reendothelialization, we examined the role of HO-1 on bone marrow and circulating EPCs.

Methods and Results—In a rabbit model of aortic balloon injury, pharmacological induction of HO-1 enhanced reendothelialization at sites with and without adjacent blood vessels, the latter indicative of a contribution by EPCs. Coinciding with maximal HO-1 induction in the injured vessel, plasma concentrations of bilirubin and the numbers of circulating progenitor cells were elevated. Both processes were abolished by cotreatment of the animals with an inhibitor of HO-1. Inducers of HO-1 promoted bone marrow cells to form progenitor cell colonies, and Flk-1⁺/Sca-1⁺-cells to adhere to the luminal surface of the injured vessel. In noninjured mice, HO-1 inducers also increased bone marrow and circulating EPCs, and the ability of these cells to differentiate and form colonies. Compared to wild-type mice, bone marrow cells from HO-1⁻/⁻ mice generated fewer endothelial colony-forming cells, and HO-1 inducers failed to promote CFU-Hill colony formation.

Conclusions—These findings suggest that HO-1 contributes to vascular repair by increasing circulating EPCs derived from the bone marrow. (Arterioscler Thromb Vasc Biol. 2009;29:1537-1542.)

Key Words: bilirubin ■ carbon monoxide ■ endothelial cells ■ succinobucol ■ vascular injury

Atherosclerotic disease is characterized by heightened inflammation and oxidative stress. As a result, there is considerable interest in compounds with antiinflammatory and antioxidant properties as potential novel drugs against the disease. Recently, a class of sulfur-containing phenols, structurally based on protubol, has been shown to inhibit atherosclerotic vascular diseases by inducing heme oxygenase-1 (HO-1) in arterial cells.

HO-1 is the inducible form of heme oxygenases that metabolize heme to carbon monoxide (CO), iron, and biliverdin, which is converted to bilirubin by biliverdin reductase. Mounting evidence suggests that upregulation of HO-1 protects against vascular diseases, including atherosclerosis and intimal hyperplasia. HO-1 is thought to protect via promoting reendothelialization, inducing antiinflammatory activities, inhibiting smooth muscle cell proliferation, regulating vascular tone, and by increasing cellular antioxidant activities. Of these beneficial activities, promotion of reendothelialization could conceivably play a primary protective role. This is because the functional endothelium regulates vascular homeostasis, by controlling endothelial adhesion of inflammatory cells and proliferation of smooth muscle cells. Reendothelialization of a denuded arterial surface is achieved by the migration and proliferation of endothelial cells from the injury border zone, or from adjacent branching blood vessels. In addition, bone marrow–derived endothelial progenitor cells (EPCs) can contribute to reendothelialization.

Whether increased recruitment of EPCs is involved in the promotion of reendothelialization by HO-1 is presently not known. We therefore investigated this possibility, using probucol and its derivative succinobucol as pharmacological inducers of HO-1, and mice deficient in HO-1 (HO-1⁻/⁻) as a molecular tool. The results obtained show that HO-1 increases the numbers of circulating and bone marrow early and late outgrowth progenitor cells, and enhances the maturation of bone marrow–derived progenitor cells. These novel activities likely contribute to the ability of HO-1 to enhance reendothelialization and hence the repair of injured blood vessels.

Materials and Methods

An expanded Materials and Methods section is described in the supplemental materials (available online at http://atvb.ahajournals.org).

Animal Models

Rabbits fed 1% probucol (Medichem) or 0.02% succinobucol (Chenaphor) underwent aortic balloon injury. Intimal hyperplasia and reendothelialization were evaluated by immunohistochemistry. Damaged and undamaged aorta were also stained for Sca-1⁺, Flk-1⁺, CD31⁺, and assessed for heme oxygenase mRNA and activity. Age- and gender-matched heme oxygenase-1 knock out (HO-1⁻/⁻) and appropriate wild-type (HO-1⁺/⁺) mice were fed normal chow ±0.02% succinobucol (wt/wt) for 2 weeks before bone marrow or blood was collected.

Flow Cytometry

Cells obtained from peripheral blood collected from rabbits 4 days after balloon injury, or blood and bone marrow from noninjured mice, were analyzed in a FACS Caliber flow cytometer (Becton Dickinson). Details are provided in supplemental materials.
Probucol and succinobucol inhibit intimal hyperplasia after balloon injury in the rabbit. Aortic cross sections A (bar=500 μm) and intima-to-media ratio (B) of control (Ctrl), probucol (PB), and succinobucol (SB)-treated animals 6 weeks after injury. C and D, Plasma and aortic cholesterol concentration. *P<0.05 vs control.

Cultured of ECFCs and CFU-Hill
Mouse endothelial colony-forming cells (ECFCs) were prepared from bone marrow mononuclear cells isolated from uninjured mice. Bone marrow cells isolated from femurs of uninjured mice or rabbits 4 days post injury were used to culture CFU-Hill (see supplemental materials).

Statistical Analyses
Data are expressed as means±SEM and analyzed as described in the supplemental materials.

Results
Succinobucol Inhibits Intimal Hyperplasia, Promotes Reendothelialization, and Induces HO-1
Rabbits matched for baseline plasma cholesterol were fed a limited amount (100 g/d) of standard chow ±1% probucol (positive control) or 0.02% (wt/wt) succinobucol for 9 weeks, with endothelial denudation of the abdominal aorta performed at the end of week 3. The dose of the drugs used, based on previous studies, resulted in drug concentrations formed at the end of week 3. The dose of the drugs used, based on previous studies,9,10 resulted in drug concentrations limited amount (100 g/d) of standard chow ±1% probucol (positive control) or 0.02% (wt/wt) succinobucol for 9 weeks, with endothelial denudation of the abdominal aorta per-

Figure 1. Probucol and succinobucol induce HO-1 in injured vessel of rabbits. Aortic HO-1 mRNA (n=13 to 19; A), HO activity (B), and plasma bilirubin (C) in animals 4 days after injury (n=5 to 6). Rabbits received control chow (Ctrl), probucol (PB), or succinobucol (SB) without (closed bars) and with (open bars) cotreatment with SnPP. *P<0.05 vs control.

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Figure 2. Probucol and succinobucol induce HO-1 in injured vessel of rabbits. Aortic HO-1 mRNA (n=13 to 19; A), HO activity (B), and plasma bilirubin (C) in animals 4 days after injury (n=5 to 6). Rabbits received control chow (Ctrl), probucol (PB), or succinobucol (SB) without (closed bars) and with (open bars) cotreatment with SnPP. *P<0.05 vs control.

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Re-endothelialized (%)

Figure 3. Probucol and succinobucol promote reendothelialization at branched and nonbranched sites of the injured rabbit aorta. Reendothelialization at branched (A and C) and non-branched (B and D) sites was assessed as described in supplemental materials. Animals received control chow (Ctrl), probucol (PB), or succinobucol (SB) without (A and B) and with SnPP cotreatment (C and D). n=6, *P<0.05 vs control.

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homing to sites of endothelial disruption and incorporating into nascent endothelium. These progenitor cells commonly express the stem cell antigen, Sca-1, a well-recognized marker of hematopoietic stem cells. Sca-1 cells adhered to the denuded vessel surface 4 days postinjury, and probucol and succinobucol significantly increased the number of these cells (Figure 4A). Some of the endothelial-like cells also stained positive for Flk1, another endothelial marker (Figure 4B). The observed probucol-mediated increase in Sca-1 and Flk1 cells was abolished by cotreatment of the animals with SnPP (Figure 4C and 4D), suggesting that it was dependent on HO activity. In contrast, at this time point none of the endothelial-like cells stained positive for CD31, RAM-11, or F4/F80 (not shown). Also, probucol and succinobucol did not increase the number of T and B cells, as assessed by CD3 and CD19 staining, respectively (supplemental Figure III), suggesting that these cells are not derived from CFU-Hill colonies, which are known to be composed of T- and B-cells, as well as myeloid cells. This excludes the possibility that an increase in these immune cells was responsible for the increase in Sca1 cells seen in animals treated with the HO-1 inducers. Together, these results suggest that the promotion of reendothelialization by probucol and succinobucol is HO-1-dependent and involves the participation of progenitor cells.

Progenitor cells that attach to the surface of injured blood vessels can originate from a subpopulation of circulating, bone marrow-derived cells. We therefore collected peripheral blood from rabbits 4 days after aortic injury and assessed mononuclear cells for the presence of Sca-1 and Flk1. This time point was chosen because HO-1 is maximally expressed in the rabbit vessel wall 4 days after injury, and this coincides with maximal number of circulating progenitor cells (Supplemental Figure IV). Compared to control, rabbits treated with probucol or succinobucol had significantly increased numbers of Sca-1 and Flk1 cells (Figure 5A and 5B), with isotype controls showing minimal staining (Supplemental Figure V). Importantly, there was no significant difference in the total white cell count between control and drug-treated animals (Supplemental Table).

We next cultured isolated blood mononuclear cells for 7 days under conditions that promote growth of progenitor cells, defined here as adherent cells positive for Dil-acLDL and isolectin staining, respectively (supplemental Figure III), suggesting that these cells are not derived from CFU-Hill colonies, which are known to be composed of T- and B-cells, as well as myeloid cells. This excludes the possibility that an increase in these immune cells was responsible for the increase in Sca1 cells seen in animals treated with the HO-1 inducers. Together, these results suggest that the promotion of reendothelialization by probucol and succinobucol is HO-1-dependent and involves the participation of progenitor cells.

HO-1 Increases Bone Marrow EPC Numbers and Maturation

Two different populations of cells are thought to contribute to the repair of damaged blood vessels. CFU-Hill enhance angiogenesis in a paracrine manner, whereas late outgrowth endothelial cells (or ECFCs) directly participate in

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Figure 4. Probucol or succinobucol increase Sca-1 and Flk1 cells on the luminal side of injured rabbit vessels in a HO-dependent manner. Cross-sections, taken 4 days after injury, show Sca-1 (A) and Flk1 (B) cells in Ctrl, PB-, and SB-treated animals (bar=10 μm). C and D, Number of Sca-1 and Flk1 cells per section, respectively. Animal treatments, symbols, and abbreviations are as described in the legend to Figure 2. n=5, *P<0.05 vs control.

Figure 5. Probucol and succinobucol increase circulating EPCs via HO-1 in rabbits. A, Representative FACS analysis of Sca-1 and Flk1 cells. B, Images of Dil-acLDL plus isolectin (yellow) cells. C, Percentage of Sca-1 and Flk1 cells. D, Numbers of Dil-acLDL plus isolectin cells in peripheral blood. E, CFU-Hill derived from bone marrow (n=6–9) 4 days after injury. Animal treatments, symbols, and abbreviations are as described in the legend to Figure 2. n=5, *P<0.05 vs control.
vasculogenesis.\textsuperscript{19} Rabbit bone marrow was collected 4 days after aortic injury, and cells were cultured for 6 days under endothelial cell growth conditions. The typical CFU formed presented as a central cord of cells with elongated spindle-shaped cells sprouting at the periphery of the colony typical of CFU-Hill (not shown). Initial characterization revealed that the majority of CFU cells expressed the endothelial cell markers Flk1 and von Willebrand factor, with less than half of the cells showing weak staining for CD45 (a monocytic marker), and no cells staining for RAM11 (a macrophage marker; supplemental Figure VI). In addition, these cells stained positive for isocitritin (not shown). Collectively, these data suggest that the majority of cells in these CFU are CFU-Hill, as demonstrated previously.\textsuperscript{20} Importantly, treatment of rabbits with either of the 2 HO-1 inducers significantly increased the number of CFU compared to control (Figure 5E).

We next investigated the involvement of HO-1 in the formation of CFU-Hill and ECFCs in noninjured wild-type (HO-1\textsuperscript{+/+}) and HO-1\textsuperscript{+/−} mice.\textsuperscript{2} succinobucol supplementation for 2 weeks. CFU-Hill were characterized as cells possessing nonspecific esterase activity, expressing Flk1, CD34, CD45 and CD115, and not being able to form tubules when plated on matrigel, whereas ECFCs formed secondary colonies on replating, lacked nonspecific esterase activity, expressing Flk1, CD34, CD45 and CD115, and not being able to form tubules possessing nonspecific esterase activity. As blockade of HO activity also prevents HO-1 induction of CFU-Hill from (C) HO-1\textsuperscript{+/+} mice (n=5) and (D) HO-1\textsuperscript{−/−} mice (n=5) treated with or without succinobucol. E, Bone marrow-derived ECFCs from HO-1\textsuperscript{+/−} mice\textsuperscript{2} succinobucol (n=4). FACS analysis of CD34\textsuperscript{+}/Flk1\textsuperscript{+}/CD45\textsuperscript{−} (F) and CD34\textsuperscript{+}/Flk1\textsuperscript{+}/CD45\textsuperscript{−} lin\textsuperscript{−} (H) blood cells (n=4) of control (HO-1\textsuperscript{+/+}) mice\textsuperscript{2} succinobucol. \textsuperscript{*P}<0.05 vs control. (C) \textsuperscript{*P}<0.01 vs control.

![Figure 6A](http://atvb.ahajournals.org/)

**Figure 6.** Effect of HO-1 on progenitor cells in mice. Number of CFU-Hill (A, n=8 to 12) and ECFCs (B, n=4) from bone marrow of HO-1\textsuperscript{+/+} and HO-1\textsuperscript{−/−} mice. C and D, Time-dependent formation of CFU-Hill from (C) HO-1\textsuperscript{−/−} (n=5) and (D) HO-1\textsuperscript{+/−} mice (n=5) treated with or without succinobucol. E, Bone marrow-derived ECFCs from HO-1\textsuperscript{−/−} mice\textsuperscript{2} succinobucol (n=4). FACS analysis of CD34\textsuperscript{+}/Flk1\textsuperscript{+}/CD45\textsuperscript{−} (F) and CD34\textsuperscript{+}/Flk1\textsuperscript{+}/CD45\textsuperscript{−} lin\textsuperscript{−} (H) blood cells (n=4) of control (HO-1\textsuperscript{+/+}) mice\textsuperscript{2} succinobucol. \textsuperscript{*P}<0.05 vs control. (C) \textsuperscript{*P}<0.01 vs control.

of the present study is that pharmacological inducers of HO-1 also effectively promote reendothelialization in areas distant to intact endothelium, suggesting that HO-1 aids the repair process by increasing EPCs. It is now increasingly appreciated that EPCs contribute to reendothelialization.\textsuperscript{24} What is new, however, is that HO-1 increases the number of bone marrow and circulating progenitor cells.

The evidence to support a role for HO-1 in increasing both CFU-Hill and ECFCs capable of vascular repair is based on pharmacological and molecular studies. Thus, the increase in circulating progenitor cells was blocked when rabbits were treated with a HO inhibitor, indicating a requirement for enzyme activity. As blockade of HO activity also prevents HO-1 inducers from inhibiting intimal hyperplasia,\textsuperscript{2} the present results directly link the HO-1–dependent increase in circulating progenitor cells to enhanced vascular repair. This interpretation is supported by the observation that HO-1 inducers increased the number of Flk1\textsuperscript{+} and Sca-1\textsuperscript{−}-cells in the injured aorta, and that this increase was dependent on HO activity. Although we are unaware of previous studies describing Sca-1 staining of rabbit tissue, we consistently observed such staining in rabbit aortas and circulating cells. Our studies in noninjured mice show that HO-1 increases bone marrow and circulating ECFCs. Pharmacological HO-1 inducers failed to enhance bone marrow CFU-Hill in HO-1\textsuperscript{−/−} mice, and these animals also showed a decreased ability to form ECFCs compared to wild-type controls. Together, these results indicate an intrinsic role for HO-1 in regulating bone marrow ECFCs and CFU-Hill. Whether this is attributable to}
HO-1 directly increasing the mobilization of progenitor cells remains to be established, although others have reported HO-1 to be important in the mobilization of progenitor cells from the bone marrow after ischemic hind limb injury.25 To clarify this issue further, bone marrow transplant experiments tracking specifically labeled pools of cells will need to be carried out.

The number of circulating EPCs inversely correlates with the risk for atherosclerosis26 and the extent of ischemia after stroke or myocardial infarction.27 Several compounds, including erythropoietin28 and statins,29 promote EPC mobilization and increase circulating EPCs. Interestingly, erythropoietin30 and statins31 can induce HO-1 expression, raising the possibility that their ability to mobilize EPCs is mediated at least in part via HO-1 induction. However, the ability of HO-1 to increase ECFCs reported here is unlikely mediated by erythropoietin, because HO-1−/− mice have increased circulating erythropoietin compared to HO-1+/+ animals, and succinobucol decreased circulating erythropoietin (data not shown).

The ability of HO-1 to promote reendothelialization is reminiscent of the ability of the enzyme to impact on angiogenesis. For example, adenoviral delivery of HO-1 improves blood flow to ischemic limbs, an effect abolished by HO inhibition.32 Vascular endothelial growth factor increases cellular HO-1 activity, and HO-1 inhibition abrogates angiogenesis driven by vascular endothelial growth factor.33 This suggests that HO-1 may act as an amplifying factor for angiogenesis. Deshanen et al34 reported inhibition of HO-1 to prevent in vitro endothelial tube formation induced by SDF-1, a chemokine that contributes to neovascularization and plays a major role in the migration, recruitment, and retention of EPCs to sites of injury. These authors also reported the migratory potential of EPCs from HO-1−/− mice to be impaired, and HO-1 to be required for EPC recruitment and wound healing in a retinal vascular repair model.34 Interestingly, mechanistic studies pointed to an important role for SDF-1–mediated induction of HO-1 and CO, yet SDF-1 expression was similar in HO-1−/− and HO-1+/+ mice,34 a finding confirmed by us (data not shown). However, a recent study reported HO-1 overexpression to increase, and HO-1 deficiency to decrease, SDF-1 in ischemic hearts.35 These observations suggest that HO-1–inducing drugs may promote reendothelialization via changing SDF-1 expression. Together, these findings indicate a role for HO-1 at several steps in EPC-mediated vascular repair, ie, the growth and maturation of bone marrow EPCs, as well as their homing to and local migration at sites of injury.

Kong and coworkers previously reported reendothelialization to be enhanced and intimal thickening to be inhibited in injured vessels treated in situ with exogenous EPCs.20 In that study, no additional reduction in hyperplasia was seen in vessels transplanted with HO-1–overexpressing compared to control EPCs.20 In contrast, we observed a clear benefit of HO-1 and HO-1 inducers on endogenous EPCs, including increased adherence of progenitor cells to the damaged artery early after injury, and increased reendothelialization of non-branched areas. A benefit may have been masked in the previous study where comparatively (to endogenous EPCs) large numbers of EPCs were added locally, causing a massive (ie, 300%) increase in reendothelialization.20 Indeed, the work of Deshanen et al shows that HO-1 is important in EPC homing to and migration at the injured blood vessel.34

This is the first study to demonstrate that succinobucol induced HO-1 in an injured artery, and that this increases EPC numbers and contributes to the reendothelialization of the damaged vessel. In light of our previous studies,2 these findings suggest that induction of HO-1 is a major mode of action of succinobucol. Succinobucol is an orally active and metabolically stable derivative of probucol, currently undergoing clinical trial as a novel antidiabetic agent.36 The drug reduces atherosclerotic lesions in different experimental models.10 In humans, succinobucol improves coronary artery lumen size after percutaneous coronary intervention,37 and it significantly reduces atheroma compared to baseline (but not placebo).38 Although the drug missed its primary composite end point in the recently completed Phase III trial, succinobucol, in conjunction with current standard of care, did show benefit in the “hard” cardiovascular end points, such as death, heart attack, and stroke, and it substantially decreased new onset of type 2 diabetes.38 The ability to induce HO-1 can explain the beneficial activities of succinobucol against atherosclerosis and restenosis.2 Similarly, it may help elucidate the potential antidiabetic action of succinobucol and related compounds.39 Diabetes is associated with a decrease in circulating EPCs and EPC function40 that may contribute to vascular disease.41 Also, HO-1 induction is known to attenuate inflammation and oxidative stress4 thought to contribute to diabetes.42

It remains unclear how HO-1 increases ECFCs, although enzyme activity is required (Figure 5). Product(s) of HO-1 action may be involved. In this context, addition of CO-releasing molecules to endothelial cells or arterial rings in vitro promotes capillary sprouting, even under conditions where endogenous HO-1 is absent or inhibited.34,43 The CO-mediated promotion of an angiogenic response may occur via synthesis of vascular endothelial growth factor.43 In contrast to CO, bilirubin does not share the angiogenesis promoting activity.34 Also, preliminary data suggest that bilirubin or CO-releasing molecule-2 do not affect the formation of CFU-Hill from bone marrow cells (not shown).

In conclusion, reendothelialization after vascular injury occurs via a HO-1–dependent pathway that involves ECFCs and can be enhanced by HO-1 inducers, such as succinobucol and related compounds. Our results suggest that targeting HO-1 is a therapeutically feasible approach to enhance the repair of damaged blood vessels and possibly other tissues.

Acknowledgments

We thank Dr Cacang Suarna for help with the HPLC analysis of succinobucol and probucol, Jie Liu for help with bone marrow cell experiments, and Dr Sabine Wimmer-Kleikamp with the help of immunofluorescence analysis.

Sources of Funding

This work was supported by Program Grant 455395 from Australian National Health & Medical Research Council (NHMRC). R.S. is supported by a NHMRC Senior Principal Research Fellowship and a University of Sydney Professorial Fellowship. J.G. and R.S. are University of Sydney Medical Foundation Fellows.

Disclosures

None.
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Arterioscler Thromb Vasc Biol. 2009;29:1537-1542; originally published online June 18, 2009;
doi: 10.1161/ATVBAHA.109.184713
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplement Material

Heme oxygenase-1 increases endothelial progenitor cells

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Materials and Methods

Animals

Three groups of male New Zealand White rabbits (1.8-2.2 kg, Merunga Farm, Coffs Harbour, Australia), matched for body weight and baseline plasma cholesterol, were fed 100 g per day of normal chow ± 1% probucol (Medichem, Girona, Spain) or 0.02% succinobucol (wt/wt) for up to nine weeks with water provided ad libitum. Succinobucol (purity >99%) was custom synthesized from probucol by Chemaphor Inc (Ottawa, Canada). Aortic balloon-injury was carried out at the end of week three, resulting in complete endothelial denudation. Harvesting of aortas was done after a further 4 days (n=13-19/group) or 6 weeks (n=6/group). Three separate groups of rabbits (n=5-6 per group) on normal chow ± 1% probucol or 0.02% succinobucol received intra-peritoneal injection of tin (IV) protoporphyrin IX dichloride (Frontier Scientific, 7.5 mg/kg) every other day for 25 or 63 days.

All BALB/c mice derived from HO-1−/− breeders generously provided by Dr. Miguel Soares (Instituto Gulbenkian de Ciencia, Oeiras, Portugal) and originally derived from the colony of Dr Yet (Brigham and Women’s Hospital and Harvard Medical School, Boston, Mass.). Genotypes were verified by genomic PCR using the following primers: Hmox1 (5'-ATGCCCCACTCTACTTCCCTG-3' and 5'-AGGCGGTCTTAGCCTCTTCTG-3') and neomycin (5'-CTGGGCACAACAGACAATCGG-3' and 5'-AAGCACGAGGAAGCGGTCAG-3'). Four groups of 7-16 week old male and female sex- and age-matched HO-1−/− and their control littermates (HO-1+/+) were fed normal chow ± 0.02% succinobucol (wt/wt) for two weeks before mice were euthanized and the bone marrow collected.

The University of Sydney Animal Care and Ethics Committee approved all procedures reported here.

Biochemical analyses
Rabbit blood was drawn into EDTA or heparin vacutainer tubes (Becton Dickinson), and then stored at –80°C until used. Aortas were collected as described, and also stored at –80°C until used. Plasma bilirubin was determined by HPLC. Aortas were homogenized and, where required, extracted. Hemoglobin and blood cell analysis was carried out using routine clinical biochemistry tests. Cholesterol was determined by HPLC after organic extraction of individual plasma and aortic samples as described. For determination of probucol, plasma and aortic homogenates were extracted as described previously. For determination of succinobucol, an aliquot of plasma or aortic homogenate was added to an equal volume of 5% metaphosphoric acid to precipitate protein. Following centrifugation, the resulting supernate was extracted with methanol/hexane, and succinobucol analyzed in both phases. The same HPLC assay was used for probucol and succinobucol determination, consisting of a 250 x 4.6 mm SUPELCOSIL LC-18 column with guard column, and eluted at 1.5 mL/min with methanol/acetonitrile/water/acetic acid (10/20/3/0.1, vol/vol/vol/vol) and monitored at 270 nm. Under these conditions the retention times for succinobucol and probucol were 9.1 and 13.4 min, respectively. All compounds were quantified by area comparison using authentic standards. Heme oxygenase activity was determined in microsomes prepared from homogenized aortic tissue and assessed by HPLC as described.  

**Lesion assessment, reendothelialization and immunohistochemistry**

Aortic lesion assessment was performed at the third pair of lumbar arteries as described, using 8 serial sections 100 µm apart per segment. For immunohistochemistry, aortic sections were incubated with anti-CD31 (DAKO, 1:50 dilution), anti-Sca-1 (BD Biosciences, 1:100), anti-Flk1 (Santa Cruz, 1:50), anti-OKT3 (Beckman, 1:50), anti-CD19 (Chemicon, 1:50), anti-RAM11 (Thermo, 1:100), or anti-F4/80 antibody (Caltag Lab., 1:100), and using avidin-biotin-horseradish peroxidase for detection (Vectorstain Elite ABC Kit, Vector Laboratories).
Digital images were taken for quantitative morphometry. Intimal and medial areas were determined using Adobe Photoshop V6.0 by tracing.

Reendothelialization at branched sites was determined in longitudinal sections as the distance of CD31\(^{+}\)-cells from the branch orifice. At non-branched sites, reendothelialization was determined using cross sections taken at sites ~10 mm away from branching points and injury zones, and expressed as the proportion of the luminal circumference that stained positive for CD31 using Scion Image Software. Total Flk1\(^{+}\), Sca-1\(^{+}\), OKT3\(^{+}\) and CD19\(^{+}\) cells were counted manually at high magnification (40 x objective). The extent of intimal hyperplasia was expressed as the intima-to-media ratio. A single operator using coded samples performed all analyses blinded.

**RT-PCR**

Total RNA was isolated with TRIzol (Invitrogen) from RNAlater (Ambion) treated frozen tissues, leukocytes, CFU-Hill, and ECFCs. Complementary DNA was prepared using the Superscript III first strand synthesis kit using oligo (dT) primers (Invitrogen). Real-time quantitative PCR was performed on ABI PRISM 7700 Sequence Detection System and Rotor-Gene 3000 (Corbett Research) using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA.) The amount of mRNA was determined relative to HMBS mRNA using the comparative C\(_T\) method described in the ABI 7700 Sequence Detector User Bulletin 2. PCR products were verified by melting curve and sequence analysis. The PCR primers used were: HMBS forward, 5'-GAGTGATTCCGCTGGGTACC-3'; HMBS reverse, 5'-GGCTCCGATGGTAAGGCTCC-3'; HO-1 forward, 5'-TGGAGCTGGACATGGCCTTC-3'; HO-1 reverse, 5'-TCTGGGAGGTTCAAGG-3'; CD115 forward 5'-TGTCATCGAGCCTAGTGGC-3'; CD115 reverse 5'-GGCTCCGATGGTAAGGCTCC-3'; CD14 forward 5'-CTCTGTCTTAAAGCGGCTCTAC-3'; CD14 reverse 5'-GTTGCGGAGGTCAAGATGTT-3'.
**EPC culture from rabbit blood**

Total mononuclear cells were isolated by density gradient centrifugation with Histopaque-1077 (Sigma) from 5 mL peripheral blood obtained from rabbits 4 days post injury. Immediately after isolation, 5 x 10^6 cells were seeded per well in a 6-well fibronectin (Sigma)-coated plate and maintained in M199 medium (Invitrogen) supplemented with 20% fetal bovine serum (Invitrogen), EGM-2 Single Quots (Clonetics) and antibiotics. Media was changed every 3 days. After 7 days culture, cells were washed with medium and incubated with 10 µg/mL 1,1'-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (Dil-acLDL; Invitrogen) for 4 h. Cells were then fixed in 2% paraformaldehyde and counterstained with 5 µg/mL FITC-conjugated isolectin (Invitrogen) for 30 min. Cells showing Dil-acLDL uptake and FITC-isolectin staining were identified by fluorescent microscopy by an investigator blinded to treatment, with 10 randomly selected 10x power fields counted for each well. Cells staining positive for both antigens were judged to be progenitor cells.\(^\text{10}\)

**Culture of ECFC**

ECFC were prepared from bone marrow mononuclear cells isolated from 6-8 week old male and female uninjured mice, separated using histopaque (1083, Sigma) gradient centrifugation and were cultured in DMEM, 10% fetal bovine serum (FBS, Invitrogen) and 10 ng of mouse vascular endothelial growth factor (Invitrogen) on plates coated with fibronectin (Sigma). After seven days, adherent cells were trypsinized and re-plated at 100,000 cells per well of 24-well fibronectin-coated plates in DMEM/FBS/VEGF media or where indicated EPC media.\(^\text{11}\) Colonies were counted on day 20.

**Culture of CFU-Hill**

Bone marrow cells, isolated from femurs of uninjured mice (7-16 weeks old), and rabbits 4 days post injury, were seeded at 5 x 10^6 cells per well in 6-well plates coated with fibronectin
and maintained in M199 medium (Invitrogen) supplemented with 20% FBS, EGM-2 Single Quots (Clonetics) and antibiotics. Media was changed every 3 days. Colonies, defined as a central cord of mass cells with elongated spindle-shaped cells sprouting at the periphery of the colony, were counted manually by an investigator blinded to treatment.

**Fluorescence-activated cell sorting**

Peripheral blood (5 mL) was collected from rabbits 4 days post injury. After lysis of red cells, mononuclear cells were analyzed for the expression of FITC-conjugated Sea-1 (BD Biosciences) and Flk1 (Santa Cruz) conjugated with the corresponding phycoerythrin-labeled secondary antibody (Sigma). Respective isotype antibodies served as negative controls. Immunofluorescence-labeled cells were fixed with 2% paraformaldehyde and analyzed by quantitative flow cytometry using a FACStar flow cytometer (Becton Dickinson) and Cell Quest Software and counting 10,000 events per sample. EPC cultured for 6 days were detached with EDTA and the cell pellet blocked with PBS containing 0.1% FCS for 30 min at 4°C. Cells were incubated with phycoerythrin-labeled anti-mouse VE Cadherin (BD Bioscience) or Flk1 (Santa Cruz) conjugated with the corresponding phycoerythrin-labeled secondary antibody (Sigma). Cells were analyzed using FACSCalibur.

Peripheral blood was pooled from 3-4 mice and total mononuclear cells isolated by density gradient centrifugation using Histopaque-1083 (Sigma). Cells were lineage-depleted (Miltenyi Biotech) and labeled with the indicated antibodies. Cells were then re-suspended in FACS buffer (PBS containing 0.5% BSA and 2 mM EDTA, pH 7.2), 10⁶ cells counted in a FACS Canto flow cytometer (Becton Dickinson), and the acquired data analyzed for the expression of various CD antigens using the FlowJo software.

Bone marrow was collected from control and succinobucol (0.02%) fed mice after 2 weeks. Whole bone marrow cells were obtained by flushing femurs and tibiae with 1 x PBS. Bone marrow cells were stained with phycoerythrin-conjugated CD34 (clone MEC14.7,
Biolegend), FITC-conjugated CD45 (clone 30F11, Miltenyi Biotech) and APC-conjugated Flk1 (BD Biosciences), for the detection of CFU-Hill and ECFC. Respective isotype antibodies (rat IgG2a-PE, Rat IgG2b-FITC and rat IgG2a-APC, BD Biosciences) were used as negative controls. The cells were re-suspended in FACS buffer, 10^6 cells counted in a FACS Canto flow cytometer (Becton Dickinson), and the acquired data analyzed for the expression of various CD antigens using the FlowJo software.

**Immunofluorescence**

Cells were stained as described previously.\textsuperscript{14,15} Briefly, cells grown on fibronectin-coated cover slides were fixed for 30 min with 4% paraformaldehyde and then permeabilized for 10 min with 0.2% Triton X-100, except for cells stained for CD14 that were fixed in acetone. Following blocking for 1 h with 0.2% BSA in PBS-T, cells were incubated with 1:50 dilution of anti-human von Willebrand factor, anti-human CD31, anti-human CD14 (DAKO), anti-mouse Flk1 (Santa Cruz) or anti-rabbit RAM11 antibody (Thermo Scientific). Cells were then stained with 1:500 dilution of Alexa Fluor 546 goat anti-mouse or Alexa Fluor 486 goat anti-rabbit IgG conjugate (Molecular Probes).

**Non-specific esterase activity**

Non-specific esterase activity was determined using the α-naphthyl acetate esterase kit (Sigma) and following the manufacturer’s instructions.

**Matrigel Assay**

Geltrex (Invitrogen) was added to the well of a 96-well plate for 30 min as per Manufacturer’s instructions. Cells (5 x 10^4 cells/mL) were then added, followed by culture for 24 hr at 37 °C, and tube formation assessed by phase contrast microscopy.

**Statistical analyses**
Data are expressed as mean ± SEM. The Wilcoxon 2-Sample Test was used to evaluate differences, except for differences in CFU-Hill colony growth between treatments and the effects of succinobucol treatment (one-way ANOVA followed by the student-Newman-Keul’s test), and mRNA data (unpaired student’s t-test). P≤0.05 was considered significant.
References


**Supplemental Table.** Hemoglobin and blood cell analysis of plasma from control probucol and succinobucol treated rabbits, four days after aortic balloon injury.

<table>
<thead>
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<th></th>
<th>Control</th>
<th>Probucol</th>
<th>Succinobucol</th>
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<tr>
<td>Hemoglobin (g/L)</td>
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<td>120 ± 4</td>
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<td>Packed cell volume (%)</td>
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<td>White cell count (x10^9/L)</td>
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<td>8.2 ± 1.2</td>
<td>6.3 ± 0.9</td>
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Data are expressed as mean ± SEM, n= 4-5.
Supplemental Figure I. Effect of probucol and succinobucol on HO-1 mRNA in rabbit leukocytes (A) and bone marrow (B). Rabbits were fed chow without (Ctrl) or with probucol (PB) or succinobucol (SB) for two weeks, before aortic balloon injury was performed. Four days after injury, peripheral blood leukocytes (A) and bone marrow cells were harvested and RNA isolated. Expression of HO-1 mRNA was then determined as described in the Materials and Methods section. Data are expressed as mean ± SEM with n=3-5.
Supplemental Figure II. Probucol and succinobucol promote reendothelialization at branched and non-branched sites. (A) Representative longitudinal sections of damaged aortas from control and probucol or succinobucol treated animals (top, bar = 500 µm). The branch orifice is located on the right hand side (black arrow). Representative higher power pictures show denuded (left, bar = 25 µm) and reendothelialized aortic surface (right, brown stain for CD31 expression, bar = 25 µm) distant and close to the branch orifice, respectively. (B) Representative IgG isotype control for (A). (C) Representative cross-section of damaged aorta taken from a control animal ~10 mm away from any branch orifice or injury zone (left, bar = 500 µm). Higher power fields (right) show two sections of CD31+ cells (bar = 10 µm). (D) IgG isotype control for (C).
Supplemental Figure III. Number of T-cells (OKT3\(^+\)) and B-cells (CD19\(^+\)) present on the luminal side of the injured vessel wall in control (Ctrl), probucol (PB) and succinobucol (SB) treated rabbits 4 days after aortic balloon injury (n=5, three serial sections per segment). Data are expressed as mean ± SEM. *P<0.05 compared with control.
Supplemental Figure IV. Kinetic of circulating EPC following aortic balloon injury. Quantitative FACS analysis of the percentage of Sca-1 and Flk1$^+$ cells in peripheral blood from two rabbits after 0, 4 and 7 days of aortic balloon injury.
Supplemental Figure V. Representative control FACS analysis of circulating EPC stained with phycoerythrin-labelled anti-mouse IgG1 (IgG1 PE) and FITC-labelled anti-rat IgG1. Representative examples of gating strategy (indicating forward to side scatter, top) and a result from FACS analysis (bottom, indicating minimal non-specific staining) are shown.
Supplemental Figure VI. Characterization of rabbit bone marrow-derived EPC. Representative images of CFU-Hill after 6 days of culture and following staining for (A) Flk1, (B) von Willebrand factor, (C) CD14, (D) VE-cadherin, (E) RAM-11 and (F) negative control), as described in the Materials and Methods section.
Supplemental Figure VII. Characterization of mouse CFU-Hill and ECFC from bone marrow. Representative photographs of (A) CFU-Hill and (B) ECFC colonies indicating that only ECFC are able to form secondary colonies after re-plating (right hand side images). (C) Formation of capillary-like tubules from ECFC (right) but not CFU-Hill colonies (left) these cells were grown in EPC media as described in the Materials and Methods section. (D) Non-specific esterase activity seen in CFU-Hill colonies (left) but not in ECFC (right). (E) RT-PCR analysis of ECFC (E), MNC (M), CFU-Hill (H1) and CFU-Hill (H2) for CD115, CD14, CD45 and actin. Results are representative of two independent experiments, each performed with cells isolated from three different mice. (F, G) Representative FACS analyses, indicating the expression of endothelial antigens on (F) CFU-Hill and (G) ECFC, with isotype controls overlaid in black.
Supplemental Figure VIII. Phenotypic analysis of CD34\(^+\)/CD45\(^+\) peripheral blood mononuclear cells. Peripheral blood mononuclear cells were isolated from wild type mice, gated for CD34\(^+\)/CD45\(^+\)lin\(^-\)-cells as described in the legend to Figure 6H, before the additional phenotyping shown was carried out. Isotype controls (red) are overlaid on each histogram for each antigen tested (blue). The experiment was performed once.
Supplemental Figure IX. Percentage Flk1⁺- or VE-cadherin⁺-cells in the CFU-Hill obtained from bone marrow cells isolated from HO-1⁺/⁺ (filled bars) and HO-1⁻/⁻ mice (open bars) (n=5-8) after 6 days of culture. Data are expressed as mean±SEM. *P<0.05 compared to respective HO-1⁺/⁺ data.