

Antioxidants protect from atherosclerosis by a heme oxygenase-1 pathway that is independent of free radical scavenging

Ben J. Wu,¹ Krishna Kathir,^{1,2} Paul K. Witting,¹ Konstanze Beck,¹ Katherine Choy,¹ Cheng Li,¹ Kevin D. Croft,⁴ Trevor A. Mori,⁴ David Tanous,¹ Mark R. Adams,³ Antony K. Lau,² and Roland Stocker¹

¹Centre for Vascular Research, School of Medical Sciences, University of New South Wales, and Department of Haematology, Prince of Wales Hospital, Sydney NSW 2052, Australia

²Eastern Heart Clinic, Prince of Wales Hospital, Sydney NSW 2031, Australia

³Department of Medicine, University of Sydney, and Department of Cardiology, Royal Prince Alfred Hospital, Sydney NSW 2050, Australia

⁴School of Medicine and Pharmacology, Royal Perth Hospital Unit, University of Western Australia, Perth WA 6001, Australia

Oxidative stress is implicated in atherogenesis, yet most clinical trials with antioxidants, particularly vitamin E, have failed to protect against atherosclerotic diseases. A striking exception is probucol, which retards atherosclerosis in carotid arteries and restenosis of coronary arteries after angioplasty. Because probucol has in vitro cellular-protective effects independent of inhibiting lipid oxidation, we investigated the mode of action of probucol in vivo. We used three models of vascular disease: apolipoprotein E-deficient mice, a model of atherosclerosis; rabbit aortic balloon injury, a model of restenosis; and carotid injury in obese Zucker rats, a model of type 2 diabetes. Unexpectedly, we observed that the phenol moieties of probucol were insufficient, whereas its sulphur atoms were required for protection. Probuco and its sulphur-containing metabolite, but not a sulphur-free phenolic analogue, protected via cell-specific effects on inhibiting macrophage accumulation, stimulating reendothelialization, and inhibiting vascular smooth muscle cell proliferation. These processes were mediated via induction of heme oxygenase-1 (HO-1), an activity not shared by vitamin E. Our findings identify HO-1 as the molecular target of probucol. They indicate 2-electron rather than radical (1-electron) oxidants as important contributors to atherogenesis, and point to novel lead compounds for therapeutic intervention against atherosclerotic diseases.

CORRESPONDENCE

Roland Stocker:
r.stocker@unsw.edu.au

Abbreviations used: *ApoE*^{-/-}, apolipoprotein E-deficient; ARE, antioxidant response elements; HO-1, heme oxygenase-1; Nrf2, NF-E2-related factor 2; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

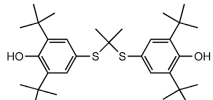
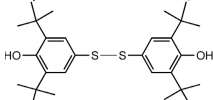
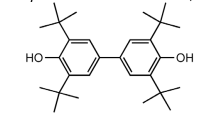
Atherosclerosis and related cardiovascular diseases represent a state of inflammation and heightened oxidative stress characterized by the accumulation of macrophages and products of lipid and protein oxidation in affected blood vessels. Abundant data support the oxidative modification hypothesis of atherosclerosis, according to which oxidation of low-density lipoprotein is an early event in and contributes to atherogenesis (1). However, this hypothesis has been challenged recently by the failure of antioxidants, in particular vitamin E, to reduce disease progression and clinical events in patients at risk of or with established atherosclerosis (2).

Various reasons may contribute to this failure. For example, there is little direct evidence that vitamin E acts as an antioxidant in human blood vessels (2, 3), and this antioxidant does not effectively scavenge oxidants like hypochlorite that have been implicated in causing oxidative damage relevant to atherogenesis (2–4). Also, the subjects enrolled in the clinical trials of vitamin E were not selected for evidence of increased oxidative stress, and this could impact on the effectiveness of antioxidants (5).

An exception to the failure of antioxidants to reduce atherosclerotic disease is probucol, a rarely used cholesterol-lowering drug composed of a diphenol linked by an isopropylidene dithio “bridge” (Table I). In humans, probucol inhibits atherosclerosis in the carotid artery (6) and neointimal proliferation after coronary

K. Kathir's present address is Vascular Biology Group, ANZAC Research Institute, Concord Hospital, Concord NSW 2139, Australia.

Table I. Structure of probucol and its analogues used in this study

Compound	Structure	One-electron potential (mV)
Probucol		545
Probucol dithiobisphenol		417
Probucol bisphenol		307

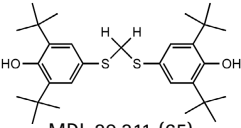
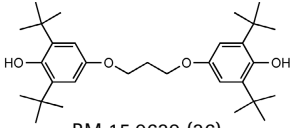
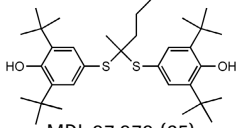
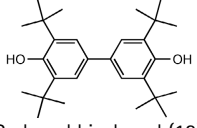
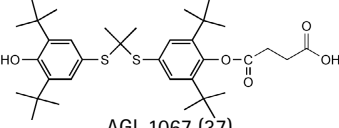
Redox potentials for the one-electron oxidation of the compounds listed were determined for analytically pure samples of probucol, probucol dithiobisphenol, and probucol bisphenol by cyclic voltammetry using a BAS electrochemical analyzer as described previously (reference 55). In brief, each compound (final concentration, 1 mM) was dissolved in deoxygenated, redistilled dimethylsulfoxide (Sigma-Aldrich), containing 200 mM tetrabutylammonium tetrafluoroborate (Sigma-Aldrich) as the supporting electrolyte. Cyclic voltammetry was performed with the scan rate set to 100 mVs⁻¹ sweeping the scan range 0 to 1.5 V at 20 °C.

angioplasty with (7) and without (8) stent deployment. Similarly, probucol retards atherosclerosis (9) and intimal thickening after balloon injury in animals (10). Several previous studies have addressed the anti-atherosclerotic activity of probucol-like diphenols (Table II). We interpret the outcome of these studies as evidence for the notion that the sulfur atoms are required, whereas the two phenol groups may not be sufficient for protection. In the present study, we tested this hypothesis by comparing the ability of diphenols that contain (i.e., probucol and probucol dithiobisphenol) or do not contain the sulfur atoms (probucol bisphenol; Table I) to protect against atherosclerotic vascular disease.

RESULTS

In apolipoprotein E-deficient (*Apoe*^{-/-}) mice, a commonly used model of hypercholesterolemia-induced atherosclerosis (11), probucol strongly inhibits disease in the descending aorta (9, 12). To determine the structural requirements underlying this activity, we compared the anti-atherosclerotic effect of dietary supplementation with 1% (wt/wt) probucol with that of 0.02% probucol dithiobisphenol or 0.02% probucol bisphenol (Table I) in *Apoe*^{-/-} mice fed a high-fat diet for 5 mo. These compounds were chosen because we wished to test the hypothesis that the sulfur atoms (present in probucol and probucol dithiobisphenol, but absent in probucol bisphenol) are required, whereas the two phenol groups (present in all three compounds) may not be sufficient for anti-atherosclerotic activity (Table II). The dosages used were chosen based on a previous study (13). They resulted in comparable total aortic concentration of the respective drug in the probucol and probucol dithiobisphenol groups (21.8 ± 8.4 and 7.1 ± 1.4 nmol/mg protein, respectively; P = 0.109). Drug levels were somewhat higher in the probucol bisphenol group (92.8 ± 18

Table II. Chemical structure of probucol analogues used in previous atherosclerosis intervention studies

Active	Inactive
 MDL 29,311 (65)	 BM 15.0639 (36)
 MDL 27,272 (65)	 Probucol bisphenol (13)
 AGI-1067 (37)	

The compounds shown are separated into antioxidants that inhibit (active) and those that do not inhibit atherosclerosis (inactive). Anti-atherosclerotic activity was tested in Watanabe heritable hyperlipidemic rabbits, except for AGI-1067, which was used in *Apoe*^{-/-} mice and in low-density lipoprotein receptor-null mice. Note that probucol analogues without sulphur atoms are inactive, whereas analogues with altered "dithio-bridge" or blocked phenolic group retain anti-atherosclerotic activity.

nmol/mg protein; P = 0.023 and 0.009 for probucol bisphenol vs. probucol and probucol dithiobisphenol, respectively; n = 3 pools of five aortas per pool for each treatment).

Probucol strongly decreased atherosclerosis at all three aortic sites examined (Fig. 1, A and B). Probucol dithiobisphenol showed comparable efficacy, whereas probucol bisphenol had no significant effect (Fig. 1, A and B). Unlike probucol, however, probucol dithiobisphenol did not lower plasma cholesterol (Fig. 1 C) or high-density lipoprotein (Fig. 1 D), an undesirable side effect of probucol (7) and the reason why the drug was withdrawn from the pharmaceutical market in the U.S. Consistent with their anti-atherosclerotic activity, probucol and probucol dithiobisphenol, but not probucol bisphenol, decreased the aortic content of neutral lipids (cholesterylesters plus triglycerides; Fig. 1 E), but the hydroperoxides of these lipids were not affected (Fig. 1 F). Inhibition of atherosclerosis was associated with fewer macrophages (Fig. 1, B and G) and proliferating cells (Fig. 1 H).

We next performed a similar structure-function study in a rabbit model of intimal hyperplasia in response to injury, using the same drug dosage regime. Animals were matched for baseline plasma cholesterol and fed a limited amount (100 g/day) of standard diet ± the test drug to control for probucol's cholesterol-lowering effect. Rabbits treated for 9 wk with control chow supplemented with 1% (wt/wt) probucol, 0.02% probucol dithiobisphenol, or 0.02% probucol bisphenol had indistinguishable concentrations of the respective drug in the injured aorta. Thus, the amounts of total drug in the vessel wall were 663 ± 114, 235 ± 64, and 614 ± 258

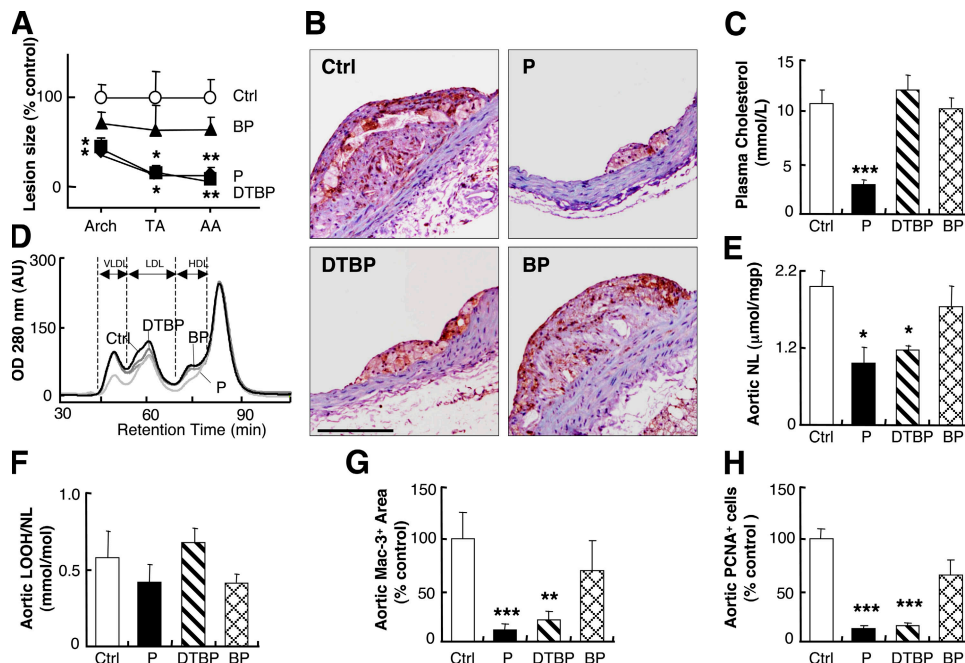


Figure 1. Probucol and probucol dithiobisphenol, but not probucol bisphenol, inhibit atherosclerosis in *ApoE*^{-/-} mice. (A) Lesion area in animals treated with probucol (P, ◆), probucol dithiobisphenol (DTBP, ■), or probucol bisphenol (BP, ▲) compared with control (Ctrl, ○). *n* = 10 for each site, each using two, six, and three serial sections for arch, thoracic (TA), and abdominal aorta (AA), respectively. (B) Representative cross sections of abdominal aorta from control and the three treatment groups stained for macrophages indicating respective lesion size. Bar, 25 μm. (C) Plasma cholesterol, *n* = 10. (D) FPLC chromatograms of lipoproteins

from pooled plasma (*n* = 10) of control, probucol, probucol dithiobisphenol, and probucol bisphenol animals. (E) Neutral lipids (NL, comprised of cholesterylesters and triglycerides) and (F) their hydroperoxides (LOOH) standardized to NL. *n* = 3 pools of five aortas per pool. (G) Average lesion area covered by Mac-3⁺ cells (i.e., macrophages) and (H) PCNA⁺ (i.e., proliferating) cells in the arch, thoracic, and abdominal aorta as compared with corresponding control. *n* = 3 for each site (three serial sections/site). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 compared with control.

pmol per mg protein for probucol, probucol dithiobisphenol, and probucol bisphenol, respectively (*n* = 6 per group, *P* > 0.05 for all comparisons). Similar to the mouse atherosclerosis study, rabbits receiving probucol or probucol dithiobisphenol,

but not probucol bisphenol, were protected from disease, as assessed by the intima-to-media ratio (Fig. 2, A and B). In this model, the vessel contents of both nonoxidized (Fig. 2 C) and oxidized neutral lipids (Fig. 2 D) remained unaltered,

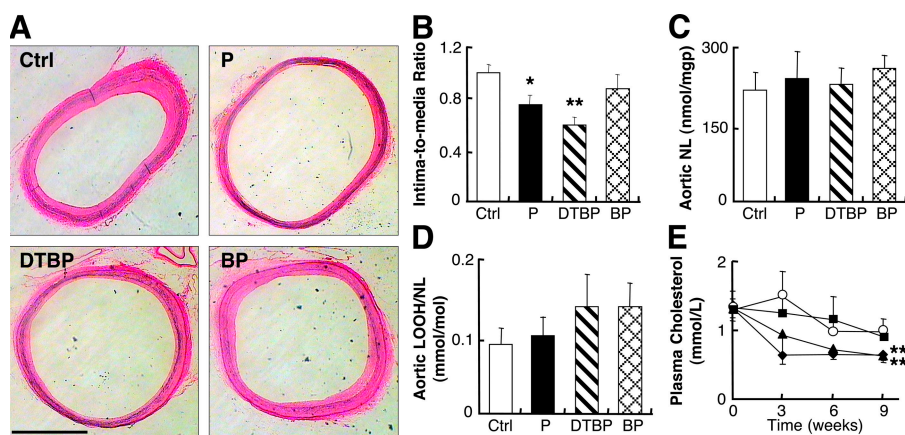


Figure 2. Probucol and probucol dithiobisphenol, but not probucol bisphenol, inhibit intimal hyperplasia in rabbits in response to vessel injury. (A) Representative Verhoeff's hematoxylin-stained cross sections. Bar, 1,000 μm. (B) Intima-to-media ratio of vessels from control and drug-treated rabbits 6 wk after aortic balloon injury (eight serial sections

per aortic segment, 100 μm apart). (C) Neutral lipids and (D) their hydroperoxides. (E) Time-dependent changes in plasma cholesterol of control rabbits or animals treated with probucol, probucol dithiobisphenol, or probucol bisphenol. All results are from six rabbits per group. Symbols and abbreviations are as described in the legend to Fig. 1.

and probucol and probucol bisphenol, but not probucol dithiobisphenol, significantly decreased plasma cholesterol (Fig. 2 E). Thus, results from the two animal models indicate that the protective activity seen with probucol and probucol dithiobisphenol is independent of both cholesterol lowering and inhibition of lipid oxidation.

Reendothelialization is a key repair process in response to arterial injury, and it is promoted by probucol (14). 6 wk after injury, the injured aortic surface is partially covered by endothelium regenerating from branch orifices (Fig. 3 A). Compared with control, probucol and probucol dithiobisphenol, but not probucol bisphenol, significantly enhanced the extent of this reendothelialization (Fig. 3 B) and decreased the intima-to-media ratio significantly at these reendothelialized sites (Fig. 3 C). Functional studies using aortic rings taken adjacent to branches showed that probucol and probucol dithiobisphenol, but not probucol bisphenol, enhanced endothelium-dependent relaxation (Fig. 3 D). This was associated with increased aortic content of guanosine 3',5'-cyclic monophosphate in response to acetylcholine (Fig. 3 E), indicating enhanced nitric oxide synthase activity. None of the compounds affected endothe-

lium-independent relaxation induced by sodium nitroprusside (Fig. 3 F). Thus, probucol dithiobisphenol, but not probucol bisphenol, mimics the effect of probucol on promoting the regeneration of functional endothelium.

Increasing evidence points to a key role of heme oxygenase-1 (HO-1) in the control of intimal hyperplasia (15). Previous studies (15) established that vascular injury transiently induces HO-1, and in our rabbit balloon injury model, HO-1 mRNA and heme oxygenase activity are induced maximally on day 4 after injury, with HO-1 mRNA and enzyme activity returning to baseline level on day 7 (16). Immunohistochemistry performed on aortas 4 d after injury showed induction of HO-1 in the media close to the luminal side in animals treated with probucol or probucol dithiobisphenol, but not probucol bisphenol (Fig. 4 A). Enhanced HO-1 expression was accompanied by significant increases in tissue levels of HO-1 mRNA (Fig. 4 B) and heme oxygenase activity (Fig. 4 C).

Induction of HO-1 in vascular smooth muscle cells is linked to enhanced apoptosis resulting in decreased proliferation (17). Indeed, probucol and probucol dithiobisphenol, but not probucol bisphenol, significantly enhanced early apoptosis,

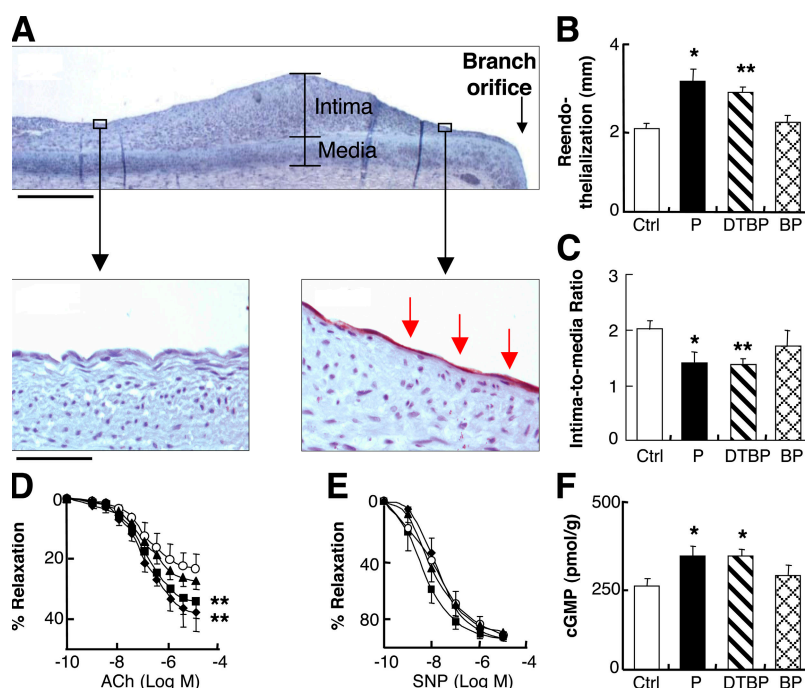


Figure 3. Probucol and probucol dithiobisphenol, but not probucol bisphenol, promote functional reendothelialization in rabbit aortas after injury. (A) Representative longitudinal section with branch orifice (bar, 500 μ m) showing denuded and CD-31⁺ reendothelialized aortic surface (red staining) distant and close to the branch orifice, respectively (bar, 25 μ m), 6 wk after injury. (B) Reendothelialization assessed by the length (mm) of section covered by CD-31⁺ cells from the branch orifice for each group 6 wk after injury (three to six serial sections per segment, 100 μ m apart). (C) Intima-to-media ratio determined by dividing the intimal over the medial area covered by an intact endothelium distal to the branch of the third lumbar artery (three to six serial

sections per segment, 100 μ m apart). (D–F) Aortic rings obtained proximally to the second lumbar arteries of the four groups were used for vessel ring studies and examined for relaxation after precontraction in response to acetylcholine (ACh, D) and sodium nitroprusside (SNP, E), with results expressed as percentage of maximal relaxation. (F) Content of guanosine 3',5'-cyclic monophosphate (cGMP) in aortic rings (obtained distally to the second lumbar arteries of the four groups) exposed to acetylcholine after preincubation in the presence of 3-isobutyl-1-methylxanthine and inhibitor of phosphodiesterases. All results are from six rabbits per group. Symbols and abbreviations are as described in the legend to Fig. 1.

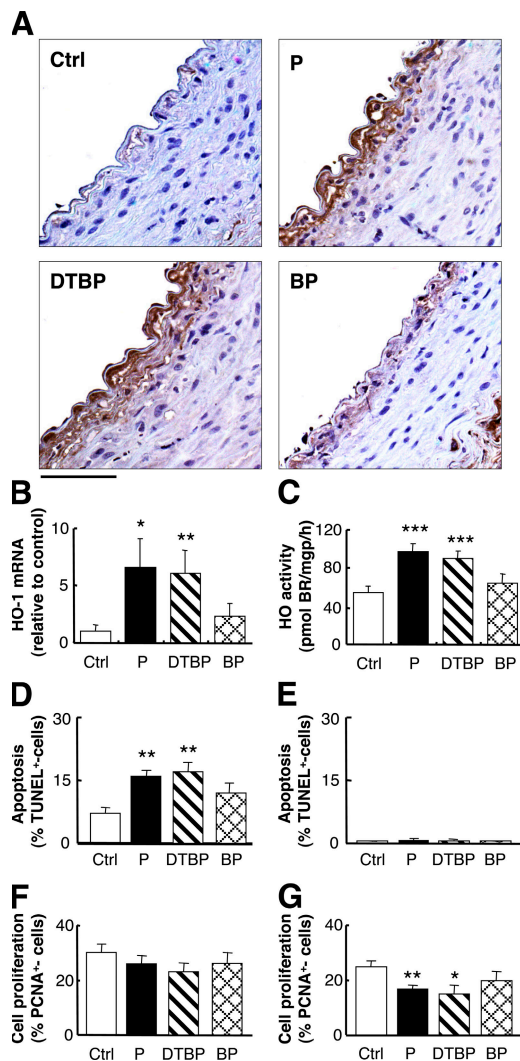


Figure 4. Probucol and probucol dithiobisphenol, but not probucol bisphenol, induce HO-1 in balloon-injured rabbit aortas, and this is related to subsequent apoptosis and vascular smooth muscle cell proliferation. (A) Representative cross sections taken from control and treated rabbits 4 d after aortic balloon injury and stained for HO-1. Bar, 25 μ m. $n = 4$ for each treatment (three serial sections/aorta). (B) HO-1 mRNA and (C) heme oxygenase activity in aortas taken from control and treated rabbits 4 d after injury. $n = 8$ per treatment. (D–G) Apoptosis and cell proliferation were assessed by the proportion of cells in corresponding aortic sections ($n = 3$ per aorta) obtained from differently treated animals ($n = 4$ –6 per group) staining positive for TUNEL and PCNA, respectively, as described in Materials and methods. (D) Apoptosis 4 d after injury. (E) Apoptosis 42 d after injury. (F) Proliferation 4 d after injury. (G) Proliferation 42 d after injury. Symbols and abbreviations are as described in the legend to Fig. 1.

i.e., at day 4 (Fig. 4 D) but not day 42 after balloon injury (Fig. 4 E) as assessed by the number of vascular cells positive for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL). Increased apoptosis was associated with a significant late decrease in cell proliferation, i.e., at day 42 (Fig. 4 G) but not day 4 after injury

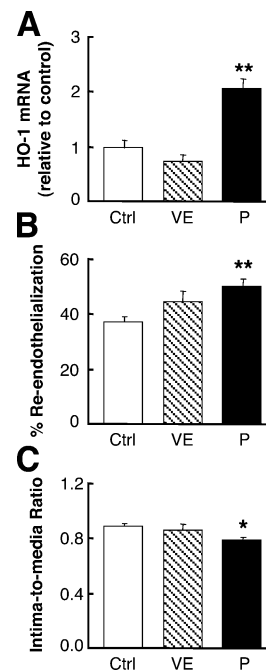


Figure 5. Vitamin E fails to inhibit intimal hyperplasia and does not promote reendothelialization or induce HO-1. (A) HO-1 mRNA assessed by real-time RT-PCR in rabbit aortic smooth muscle cells cultured for 24 h in the presence of vehicle (control), 50 μ M probucol, or 50 μ M α -tocopherol (VE). (B) Reendothelialization assessed by Evans blue staining for the three groups of rabbits shown in A 3 wk after injury. (C) Intima-to-media ratio of vessels from control rabbits and animals treated with probucol or α -tocopheryl acetate (VE) ($n = 6$ per group) 3 wk after aortic balloon injury (five serial sections per aortic segment, 100 μ m apart). Results show mean \pm SEM of a triplicate experiment performed twice with similar results obtained in both experiments. Symbols and abbreviations are as described in the legend to Fig. 1.

(Fig. 4 F) as assessed by the proportion of cells positive for proliferating cell nuclear antigen (PCNA). Immunostaining for HO-1 was no longer detected in damaged vessels 42 d after injury (not depicted).

The oxidative modification hypothesis of atherosclerosis has been challenged recently by the failure of vitamin E, either alone or in combination with vitamin C, selenium, or β -carotene, to reduce disease progression and clinical events in patients at risk of or with established atherosclerosis (2). As vitamin E administration blocks rather than induces HO-1 in vivo (18–20), we therefore next compared probucol with vitamin E in the rabbit aortic balloon injury model. Unlike probucol, vitamin E failed to induce HO-1 in vascular smooth muscle cells in vitro (Fig. 5 A). This was reflected by a lack of ability of the vitamin to promote reendothelialization (Fig. 5 B) and to inhibit intimal hyperplasia (Fig. 5 C). These results contrast to the cellular effects of vitamin E and probucol.

To directly link HO-1 induction with inhibition of intimal hyperplasia, we repeated the intervention with probucol and probucol dithiobisphenol in the rabbit aortic balloon injury model with animals treated with tin protoporphyrin-IX, an

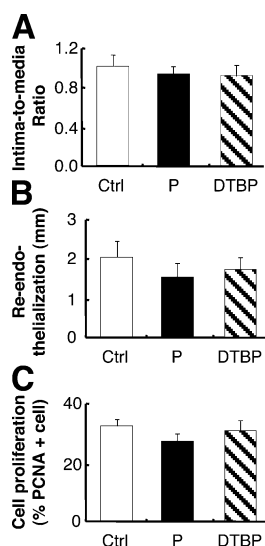


Figure 6. HO-1 is the molecular target for probucol and probucol dithiobisphenol. All animals ($n = 6$ per group) received tin(IV) protoporphyrin IX dichloride to block heme oxygenase. (A) Intima-to-media ratio of vessels 6 wk after aortic balloon injury (eight serial sections per segment, $100 \mu\text{m}$ apart). (B) Reendothelialization assessed by the length (mm) of section covered by CD-31⁺ cells from the branch orifice (three to six serial sections per segment, $100 \mu\text{m}$ apart), and (C) cell proliferation is indicated by PCNA⁺ cells 6 wk after injury. Symbols and abbreviations are as described in the legend to Fig. 1.

established inhibitor of heme oxygenase (21). In these rabbits, probucol and probucol dithiobisphenol no longer attenuated intimal hyperplasia when compared with control animals that received tin protoporphyrin only (Fig. 6 A). Similarly, inhibition of heme oxygenase abolished the ability of probucol and probucol dithiobisphenol to promote reendothelialization (Fig. 6 B) and inhibit smooth muscle cell proliferation (Fig. 6 C). Collectively, these findings suggest that HO-1 is a molecular target of the vascular-protective effects of the redox-active compound probucol and its active metabolite probucol dithiobisphenol.

To exclude the possibility that HO-1 induction as the underlying mode of probucol's protection is limited to rabbits, we examined the effect of heme oxygenase blockade on the action of probucol in a second species. We used carotid balloon injury (22) in obese Zucker rats as an animal model of type 2 diabetic vascular restenosis. The obese Zucker rat has an autosomal-recessive inheritance resulting in hyperglycemia, insulin resistance, and hyperlipidemia (23), and thus simulates human type 2 diabetes. Also, in analogy to the increased restenosis rate in humans with type 2 diabetes (24), the intimal area of carotid arteries 2 wk after injury was significantly greater in obese compared with lean Zucker rats that are phenotypically normal and act as control (Table III). There was also a comparatively smaller but significant increase in medial area in obese rats. As a result, the intima-to-media ratio was increased substantially in obese compared with lean rats (Table III).

Probucol (1%, wt/wt) treatment had no significant effect on body weight, fasting blood glucose, and hemoglobin glycosylation, or plasma triglycerides and nonesterified cholesterol (not depicted). Probucol also had no effect on plasma concentrations of F₂-isoprostanes, a commonly used marker of in vivo lipid oxidation (25), whether expressed in absolute terms (2.05 ± 0.17 vs. 1.96 ± 0.27 ng/ml for obese vs. obese plus probucol, respectively) or corrected for the parent lipid, arachidonic acid (8.0 ± 4.4 vs. 4.3 ± 0.6 pg/ μg for obese vs. obese plus probucol, respectively). These results suggest that in this model, probucol did not act as an inhibitor of lipid oxidation. Despite this, however, probucol significantly inhibited intimal hyperplasia as measured by the decrease in both intimal area and intima-to-media ratio (Table III). This protective effect was not due to vascular remodeling, as probucol did not affect medial area (Table III) and the length of the external elastic lamina (not depicted). Rather, probucol significantly increased reendothelialization (Table III), as observed in the rabbit model. Lean rats at the same time point were completely reendothelialized. Enhancement of reendothelialization of injured arteries by probucol was associated with increased expression of HO-1, as seen with immunohistochemistry (not depicted), as well as a decrease in total and proliferating cells in the neointima (Table III). Finally, and similar to the rabbit restenosis model, the heme oxygenase inhibitor, tin protoporphyrin, significantly attenuated the ability of probucol to decrease intimal area and the intima-to-media ratio, and this was associated with a decrease in the extent of reendothelialization (Table IV). These results confirm that blockade of heme oxygenase prevents the beneficial activities of probucol independently of the animal model used.

DISCUSSION

Here we identify a novel structural element and mode of action by which the redox-active compound probucol inhibits

Table III. Morphometry of carotid artery after balloon injury of lean and obese Zucker rats

	Lean ($n = 9$)	Obese ($n = 9$)	Obese + probucol ($n = 9$)
Intimal area (mm ²)	0.01 ± 0.0	0.12 ± 0.01^a	0.05 ± 0.01^b
Medial area (mm ²)	0.13 ± 0.01	0.16 ± 0.0^a	0.15 ± 0.01
Intima-to-media ratio	0.08 ± 0.03	0.74 ± 0.05^a	0.28 ± 0.06^b
Reendothelialization (%)	100 ± 0	42 ± 8^a	80 ± 13^b
Total intimal cells (number per high powered field)	ND	237 ± 13	78 ± 28^b
PCNA ⁺ -cells in intima	ND	50 ± 17	11 ± 7^b

Probucol (1%, wt/wt) was administered for 4 wk, with carotid balloon injury performed after 2 wk and carotid artery morphometry, reendothelialization, and intimal cell proliferation assessed at the end of the 4-wk intervention as described in Materials and methods. Total and PCNA⁺ cells were assessed as the number of cells per high power field (average of three fields). Values are mean \pm SEM. ND, not detected.

^a $P < 0.05$ versus lean.

^b $P < 0.05$ versus obese.

Table IV. Blocking heme oxygenase attenuates protection by probucol against intimal hyperplasia in obese Zucker rats

	Probucol –SnPP-IX (n = 6)	Probucol +SnPP-IX (n = 6)
Intimal area (mm ²)	0.003 ± 0.002	0.03 ± 0.01 ^a
Medial area (mm ²)	0.14 ± 0.0	0.15 ± 0
Intima-to-media ratio	0.03 ± 0.01	0.24 ± 0.06 ^a
Reendothelialization (%)	93 ± 7	61 ± 7 ^a

Experimental conditions were essentially as described in the legend to Table III, except that in these animals, 10 mg tin protoporphyrin-IX was applied topically around the injured artery at the time of surgery, using a pluronic gel (+SnPP-IX) or gel alone (–SnPP-IX) as described previously (reference 57). The smaller lesions seen here in probucol-treated obese rats –SnPP-IX compared to that shown in Table III may be due to different batches of animals used in the two separate intervention studies and/or to effects of the gel alone. Values are mean ± SEM. ^aP < 0.05 versus +SnPP-IX.

atherosclerotic vascular disease in three different models of vascular injury: the *Apoe*^{–/–} mouse model of atherosclerosis, the rabbit arterial injury model of restenosis, and the obese Zucker rat model of type 2 diabetes. We demonstrate for the first time that the sulfur moiety is critical for in vivo protection and identify HO-1 as a molecular target for active redox compounds.

Our results may help explain why phenolic antioxidants like vitamin E have failed to protect against cardiovascular disease (2). Thus, the observation that probucol dithiobisphenol, but not probucol bisphenol, inhibited disease in the models used here lends support for the notion that the sulfur rather than the phenol moieties are critical for protection against atherosclerotic vascular disease by redox-active compounds. This notion is consistent with the finding that in the rabbit balloon injury model, vitamin E failed to inhibit intimal hyperplasia, and it did not promote reendothelialization or induce HO-1 in smooth muscle cells. Sulfur atoms commonly engage in 2e-oxidation reactions (26), against which phenolic radical (i.e., 1e-oxidant) scavengers like probucol bisphenol and vitamin E offer little protection (2, 27). This implies that 2e-redox reactions may be more important than radical reactions in the pathogenesis of atherosclerotic disease. Indeed, 2e-oxidant-mediated oxidation of cysteine residues in the thiolate form is increasingly linked to the regulation of key enzymes (e.g., thioredoxin, Ras GTPases, tyrosine kinases, phosphatases, and transcription factors) involved in processes central to atherogenesis, such as cell proliferation (28), endothelial function (29), and cell signaling (30). Also, oxidation of probucol's sulfur atoms by 2e-oxidants (27) may relate to both the extent to which this antioxidant protects against atherosclerosis in *Apoe*^{–/–} mice (12) and the observed dependency of its vascular protection on induction of HO-1.

Compared with inducing HO-1, lowering plasma cholesterol and inhibiting arterial lipid oxidation appear to be of lesser importance for probucol's vascular protection because both probucol and probucol dithiobisphenol inhibited lesion formation in the models, yet only probucol decreased plasma cholesterol. Also, probucol bisphenol lowered plasma cholesterol yet failed to protect in the rabbit restenosis model,

consistent with the failure of lipid-lowering drugs to decrease restenosis after coronary angioplasty in humans (31). Thus, hypocholesterolemia can be dissociated from, and is not required for, the in vivo cardioprotective effect of probucol, as shown previously in a rabbit atherosclerosis study (32). The observed lack of a high-density cholesterol-lowering effect of probucol dithiobisphenol is consistent with a previous report (33). It indicates that this analogue does not share this undesirable side effect of the parent drug.

In both *Apoe*^{–/–} mice and injured rabbits, the proportion of aortic lipids present as lipid hydroperoxides (primary products of lipid peroxidation) was unaffected by any of the interventions. Similarly, in the rat model of type 2 diabetes, probucol did not significantly alter circulating concentrations of F₂-isoprostanes, a commonly used marker of (secondary) lipid oxidation (25). Thus, inhibition of lipid peroxidation is not likely to explain the protective activity of probucol (and probucol dithiobisphenol) in the animal models used here, consistent with previous studies (9, 12, 14, 34). Because protection was independent of lipid oxidation, our present results do not support the idea that lipoprotein lipid oxidation importantly contributes to atherosclerotic vascular disease. Indeed, if lipid oxidation were important in causing disease, we would have expected protection to be greatest with probucol bisphenol and smallest with probucol. This is because lipid peroxidation is a radical mediated chain reaction expected to be inhibited in the order probucol bisphenol > probucol dithiobisphenol > probucol, given their respective 1e-oxidation potentials (Table I). In this context, it is important to note that probucol bisphenol accumulated in the aorta to concentrations comparable to, if not exceeding those of, probucol and probucol dithiobisphenol. Thus, limited accessibility (compared with probucol) cannot explain the failure of probucol bisphenol to protect against atherosclerotic disease.

The apparent unimportance of inhibiting aortic lipid peroxidation combined with the inability of probucol bisphenol to inhibit atherosclerosis and intimal hyperplasia suggests that the phenolic moieties are not sufficient for vascular protection by antioxidants. If so, it helps explain why other phenolic antioxidants, such as α-tocopherol (vitamin E; reference 35) and the propane diether analogue of probucol (Table II; reference 36) do not generally protect against atherosclerosis. This interpretation is supported by the fact that AGI-1067 (i.e., probucol with one of its two phenol groups blocked by esterification to succinate; Table II) inhibits atherosclerosis in *Apoe*^{–/–} and in low-density lipoprotein receptor-deficient mice (37), and restenosis after coronary angioplasty in humans (38).

As indicated, our observation that the sulfur rather than phenolic moieties of probucol are important for protection suggests that 2e-redox reactions (that sulfur atoms typically engage with) are likely important in atherosclerotic diseases. There is recent evidence that probucol and probucol dithiobisphenol can effectively intercept 2e-oxidant-mediated processes. Thus, probucol and probucol dithiobisphenol, but not probucol bisphenol, protect against endothelial dysfunction induced by the 2e-oxidant hypochlorite (27). Metabolism of

probulcol to probucol bisphenol and probucol diphenoquinone occurs in diseased arteries *in vivo*. Interestingly, such metabolism *in vitro* is mediated via sulfur oxidation and recapitulated best by 2*e*-oxidants such as hypochlorite and peroxynitrite (27) that are implicated in atherogenesis (2). Furthermore, the anti-atherosclerotic activity of probucol in aortas of *Apoe*^{-/-} mice parallels the extent to which the drug is metabolized to probucol bisphenol and its diphenoquinone (12). Collectively, these findings suggest that for efficient *in vivo* protection, redox-active compounds require an ability to intercept 2*e*-redox reactions that take place during atherosclerotic vascular disease and target sulfur atoms, such as protein cysteine residues. Importantly, the present and previous results (12, 27) do not establish that probucol and probucol dithiobisphenol themselves intercept relevant 2*e*-redox reactions *in vivo*. Indeed, probucol and probucol dithiobisphenol react with hypochlorite at rates of $\sim 10^2 \text{ M}^{-1} \text{ s}^{-1}$ (27); i.e., their ability to scavenge this 2*e*-oxidant is at best comparable to that of other antioxidants, such as ascorbate and vitamin E (39, 40). It is therefore most likely that *in vivo* 2*e*-redox reactions “activate” probucol and probucol dithiobisphenol to some important metabolite that then intercepts 2*e*-redox reactions and thus protects against atherosclerotic vascular disease. The precise identity of the putative metabolite remains to be established, although it is clear that probucol bisphenol, a known metabolite of probucol, is not generally active in protecting against vascular disease.

The proposed ability of probucol's sulfur atoms to undergo 2*e*-redox reactions in the artery wall may also be linked to the observed dependency of vascular protection on induction of HO-1. HO-1 is a redox-sensitive enzyme responsive to several stress stimulants, including heme, nitric oxide, hypoxia, oxidized low-density lipoprotein, heavy metals, and UV light (41). Perhaps most notable, the promoter region of the HO-1 gene contains multiple copies of antioxidant response elements (ARE) that are critical for enzyme induction (42) and that are tightly regulated by the redox-sensitive transcription factor NF-E2-related factor 2 (Nrf2). Under resting conditions, Nrf2 is sequestered in the cytosol by the binding of Keap1. Upon stimulation, Nrf2 dissociates from Keap1 and translocates to the nucleus where it induces HO-1 transcription. Interestingly, Nrf2 liberation from Keap1 is regulated by redox changes to specific cysteine residues on Keap1 (43, 44) so that the putative active probucol metabolite formed by 2*e*-redox reactions may induce HO-1 via ARE. Preliminary data obtained using a reporter construct indicate that probucol and probucol dithiobisphenol do not directly induce ARE-driven luciferase *in vitro* (unpublished data). Furthermore, treatment of rabbits with either of the two compounds does not induce hepatic glutathione *S*-transferase, a phase II enzyme known to be under the control of ARE, and they do not elevate plasma levels of liver enzymes (unpublished data). These findings indicate that although induction of HO-1 is of central importance for the protective effects of probucol and probucol dithiobisphenol, the precise mechanism underlying this induction remains to be elucidated, although it does not appear

to represent a global cellular protective mechanism involving a general phase II response.

Independently of the mode of its induction, HO-1 is a protective enzyme, variously manifested in different vascular cells (41). The protection offered by HO-1 induction may be based on cellular increases in the concentration of antioxidant bile pigments (45) or carbon monoxide (46) and/or a decrease in redox-available iron (47). In vascular smooth muscle cells, induction of HO-1 stimulates apoptosis (17) and inhibits proliferation and migration (15). In contrast, overexpression of HO-1 in endothelial cells protects against apoptosis (17) and promotes proliferation (48). These cellular activities of increased HO-1 activity translate into protection against atherosclerotic vascular disease. Thus, in animals, increasing HO-1 activity attenuates atherosclerosis (49, 50), whereas inhibiting HO-1 augments disease (51). Also, HO-1 gene delivery inhibits neointima formation after vascular injury (15, 52). Therefore, the differential effects of probucol (and probucol dithiobisphenol) in the rabbit restenosis and rat type 2 diabetes models used here are all reminiscent of the actions of HO-1 in different animal models of atherosclerotic vascular disease.

The ability to completely block the *in vivo* protective cellular activities of probucol in two different animal models by inhibiting heme oxygenase activity highlights HO-1 as a potential therapeutic target in atherosclerotic diseases. Also, it suggests that modulation of HO-1 induction in vascular cells may be a suitable surrogate *in vitro* assay to screen for novel anti-atherosclerotic agents. In addition, our observations provide novel and plausible explanations for both the anti-atherosclerotic and anti-restenotic actions of probucol, as well as the apparent failure of vitamin E. Finally, our findings highlight sulfur moieties as critical for the design of efficient redox-active compounds against atherosclerotic vascular disease and indicate probucol dithiobisphenol as a lead compound for a novel class of therapeutic drugs.

MATERIALS AND METHODS

Materials. Chemicals were obtained from Sigma-Aldrich, except for probucol (4',4'-(isopropylidene dithio)bis(2,6-di-*tert*-butyl)phenol), which was provided by AstraZeneca, Sweden, and probucol dithiobisphenol (4,4'-dithiobis(2,6-di-*tert*-butyl-phenol) and probucol bisphenol (3,3',5,5'-tetra-*tert*-butyl-4,4'-bisphenol), which were obtained from Polysciences. Hydroperoxides of cholesteryllinoleate were prepared as described previously (53). 3,3',5,5'-tetra-*tert*-butyl-4,4'-diphenoquinone (diphenoquinone) was prepared from probucol bisphenol (54).

Determination of redox potentials. Redox potentials were determined for analytical samples of probucol, probucol dithiobisphenol, and probucol dithiobisphenol (Polysciences) by cyclic voltammetry using a BAS electrochemical analyzer as described previously (55).

Animal models. Four groups of 8–10-wk-old male *Apoe*^{-/-} mice (Animal Resources Centre, Perth, Australia) were fed a high-fat diet based on Harlan Teklad diet TD88137 (9) \pm 1% (wt/wt) probucol, 0.02% probucol dithiobisphenol, or 0.02% probucol bisphenol for 5 mo. Tissue harvesting and analyses were performed as described previously (9, 12), using 15 and 10 mice in each group for biochemical and histological analyses, respectively.

For the rabbit aortic balloon injury model, four groups of male New Zealand White rabbits (1.8–2.2 kg; Merunga Farm) were matched for body weight and baseline plasma cholesterol, and fed 100 g per day of normal

chow \pm 1% (wt/wt) probucol, 0.02% probucol dithiobisphenol, or 0.02% probucol bisphenol (wt/wt) for up to 9 wk with water provided ad libitum. Experiments comparing probucol and vitamin E used three groups of rabbits fed normal chow \pm 1% (wt/wt) α -tocopheryl acetate or 1% probucol (wt/wt) for 6 wk. Aortic balloon injury to achieve complete endothelial denudation was performed at the end of week 3 (14). The harvesting of aortas (14) was performed after an additional 4 d ($n = 8$ per group) or 6 wk ($n = 6$ per group). Three separate groups of rabbits ($n = 6$ per group) on normal chow \pm 1% probucol or 0.02% probucol dithiobisphenol received intraperitoneal injection of tin(IV) protoporphyrin-IX dichloride (7.5 mg/kg; Frontier Scientific) every other day (56) for the entire 9-wk period. None of the treatments affected the body weight of the animals.

For the type 2 diabetes model, 11–13-wk-old lean and obese Zucker rats ($n = 6$ –9 per group) were obtained from Monash Animal Services. Animals were fed chow with or without added probucol 1% (wt/wt) for 2 wk before balloon injury of the left common carotid artery (22). Complete endothelial denudation was confirmed for both lean and obese rats with Evan's Blue staining immediately after balloon injury in a pilot study. For in vivo inhibition of HO-1, 10 mg tin(IV) protoporphyrin-IX (Porphyrin Products) was applied topically around the injured artery at the time of surgery, using a pluronic gel (BASF) or gel alone (controls), as described previously (57). After injury, the external carotid artery was ligated, and animals were fed their respective diet for another 2 wk. The University of New South Wales Animal Care & Ethics Committee approved all animal studies reported here.

Lesion assessment and immunohistochemistry. In *Apoe*^{-/-} mice, lesion areas were determined in the aortic arch (50), descending thoracic, and abdominal aorta (12). In rabbits, aortic lesion assessment was performed at the third pair of lumbar arteries as described previously (14, 16). In Zucker rats, 5- μ m-thick transverse cross sections were obtained from the mid-third of the common carotid artery ($n = 3$ per animal). Immediately adjacent sections were used for immunohistochemistry, using Mac-3 (macrophages, dilution 1:200; DakoCytomation), PCNA (cell proliferation, dilution 1:500; DakoCytomation), von Willebrand Factor or CD31 (endothelium, dilution 1:200 or 1:50; DakoCytomation), anti-rat HO-1 monoclonal (dilution 1:50; Santa Cruz Biotechnology, Inc.), and polyclonal (dilution 1:2,000; Stress-Gen Biotechnologies) antibodies with avidin-biotin-horseradish peroxidase for signal detection (Vectorstain Elite ABC kit; Vector Laboratories). Apoptosis was assessed using the TUNEL assay kit (Roche) according to the manufacturer's instructions. Digital images were taken for quantitative morphometric analysis. Intimal, medial, and Mac-3⁺ areas were determined using Adobe Photoshop V6.0 by tracing (9, 51). Reendothelialization was determined in longitudinal sections as the distance of CD31⁺ cells from the branch orifice and in cross sections as the percentage of cells positive for von Willebrand Factor from the circumference of lumen using Scion Image Software. For experiments with vitamin E, Evans blue was injected intravenously at week 6 just before aorta harvesting, and reendothelialization was determined by the extent of denuded (blue) versus reendothelialized (white) areas from photographs of the en face aortas (14). Total cell profiles, TUNEL⁺ cells, and PCNA⁺ cells were counted manually at a high magnification (40 \times objective). The extent of intimal hyperplasia was expressed as the intima-to-media ratio. A single operator using coded samples performed all analyses blinded.

Biochemical analyses. Fresh whole blood obtained when animals were killed was used to measure blood glucose and glycosylated hemoglobin using a standardized portable glucometer (Roche) and the Hemoglobin A_{1c} Reagent kit (DCA 2000), respectively. Plasma was obtained by centrifuging the EDTA blood (1,000 g at 4°C for 10 min) and was then stored at -80°C until used. The profile of lipoproteins was determined by FPLC (58) from pooled plasma of 10 mice of each group. Plasma triglycerides were quantified using an Infinity Triglycerides kit (Sigma-Aldrich). Plasma F₂-isoprostanes and arachidonic acid were determined by stable isotope dilution gas chromatography-mass spectrometry (59) and gas chromatography (60), respectively, as described previously (59). Plasma cholesterol was determined

by HPLC after organic extraction of individual plasma samples, as described previously (12).

For aortic materials, entire aortas from five mice or individual 2-cm segments of the abdominal aorta at the celiac artery from rabbits were used. The aortic segments were homogenized and extracted (61), and the organic phase was analyzed by HPLC with UV (for cholesterol and nonoxidized neutral lipids, cholesterylestes plus triglycerides) and postcolumn chemiluminescence detection (for hydroperoxides of the neutral lipids; references 62 and 63). Probucol, probucol dithiobisphenol, probucol bisphenol, and probucol diphenoquinone were analyzed by gradient RP-HPLC (53). All compounds were quantified by area comparison using authentic standards.

Heme oxygenase. For HO-1 expression, rabbit aortic smooth muscle cells (Cell Applications) (passages 6–11) were cultured in 12-well plates until 60–70% confluent as described previously (16). Cells were then incubated for 24 h in DMEM containing 0.2% fetal calf serum and 0.3% BSA with or without added probucol (50- μ M final concentration) or α -tocopherol (vitamin E, 50 μ M). Total RNA was extracted by directly adding TRIzol reagent (Invitrogen) to the cells according to the manufacturer's instructions. For tissue, total RNA was isolated with TRIzol as described previously (16). Corresponding cDNA was prepared using the Superscript III first strand synthesis kit (Invitrogen). Real-time PCR was performed using an ABI PRISM 7700 Sequence Detection System with the SYBR Green PCR Master Mix (Applied Biosystems). Hydroxymethylbilane synthase (HMBS) was used to normalize RNA quantity, using the following PCR primers: HMBS forward, 5'-GAGTGATTCGCGTGGGTACC-3'; HMBS reverse, 5'-GGCTCCGATGGTGAAGCC-3'. The primers for HO-1 were: forward, 5'-TGGAGCTGGACATGGCCTTC-3'; reverse, 5'-TCTGGGC-GATCTTCTTAAGG-3'. The amount of HO-1 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 sequence analysis. Heme oxygenase activity was determined in microsomes prepared from homogenized aortic tissue and assessed by HPLC as described previously (16).

Vascular reactivity. 3-mm segments of the abdominal aorta at the second pair of lumbar arteries from rabbits were used for isometric tension experiments, with relaxation of precontracted segments (to 80% maximal contraction) monitored (14). Segments extending proximally were analyzed for guanosine 3',5'-cyclic monophosphate content as an index of nitric oxide synthase activity (14, 64).

Statistics. Data are expressed as mean \pm SEM. One-way ANOVA and the student-Newman-Keul's test were used to evaluate differences between groups, whereas acetylcholine and sodium nitroprusside dose responses curves were compared by two-way ANOVA for repeated measures, with $P < 0.05$ considered significant.

We thank J.Y. Hou, G. McKenzie, F. Png, B. Rayner, M. Sarris, and D. Sullivan for assistance; and C. Chesterman, B. Halliwell, J. Heinecke, N. Hunt, W. Jessup, and J. Keane for critically reading the manuscript.

The National Health & Medical Research Council of Australia (grants to R. Stocker) and Australian Research Council (Fellowship to P.K. Witting) provided financial support.

The authors have no conflicting financial interests.

Submitted: 18 November 2005

Accepted: 15 March 2006

REFERENCES

- Steinberg, D., S. Parthasarathy, T.E. Carew, J.C. Khoo, and J.L. Witztum. 1989. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* 320:915–924.
- Stocker, R., and J.F. Keane Jr. 2004. Role of oxidative modifications in atherosclerosis. *Physiol. Rev.* 84:1381–1478.

3. Upston, J.M., L. Kritharides, and R. Stocker. 2003. The role of vitamin E in atherosclerosis. *Prog. Lipid Res.* 42:405–422.
4. Hazell, L.J., and R. Stocker. 1997. α -Tocopherol does not inhibit hypochlorite-induced oxidation of apolipoprotein B-100 of low-density lipoprotein. *FEBS Lett.* 414:541–544.
5. Meagher, E.A., O.P. Barry, J.A. Lawson, J. Rokach, and G. FitzGerald. 2001. Effects of vitamin E on lipid peroxidation in healthy persons. *JAMA.* 285:1178–1182.
6. Sawayama, Y., C. Shimizu, N. Maeda, M. Tatsukawa, N. Kinukawa, S. Koyanagi, S. Kashiwagi, and J. Hayashi. 2002. Effects of probucol and pravastatin on common carotid atherosclerosis in patients with asymptomatic hypercholesterolemia. Fukuoka Atherosclerosis Trial (FAST). *J. Am. Coll. Cardiol.* 39:610–616.
7. Tardif, J.C., J. Gregoire, L. Schwartz, L. Title, L. Laramée, F. Reeves, J. Lesperance, M.G. Bourassa, P.L. L'Allier, M. Glass, et al. 2003. Effects of AGI-1067 and probucol after percutaneous coronary interventions. *Circulation.* 107:552–558.
8. Tardif, J.-C., G. Côté, J. Lesperance, M. Bourassa, J. Lambert, S. Doucet, L. Bilodeau, S. Nattel, and P. de Guise. 1997. Probuco and multivitamins in the prevention of restenosis after coronary angioplasty. *N. Engl. J. Med.* 337:365–372.
9. Witting, P.K., K. Pettersson, J. Letters, and R. Stocker. 2000. Site-specific anti-atherogenic effect of probucol in apolipoprotein E deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 20:e26–e33.
10. Schneider, J.E., B.C. Berk, M.B. Gravanis, E.C. Santoian, G.D. Cipolla, N. Tarazona, B. Lassegue, and S.B. King III. 1993. Probuco decreases neointimal formation in a swine model of coronary artery balloon injury. A possible role for antioxidants in restenosis. *Circulation.* 88:628–637.
11. Zhang, S.H., R.L. Reddick, J.A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* 258:468–471.
12. Choy, K., K. Beck, F.Y. Png, B.J. Wu, S.B. Leichtweis, S.R. Thomas, J.Y. Hou, K.D. Croft, T.A. Mori, and R. Stocker. 2005. Processes involved in the site-specific effect of probucol on atherosclerosis in apolipoprotein E gene knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 25:1684–1690.
13. Witting, P., K. Pettersson, A.-M. Östlund-Lindqvist, C. Westerlund, M. Wågberg, and R. Stocker. 1999. Dissociation of atherosclerosis from aortic accumulation of lipid hydro(pero)xides in Watanabe heritable hyperlipidemic rabbits. *J. Clin. Invest.* 104:213–220.
14. Lau, A.K., S.B. Leichtweis, P. Hume, R. Mashima, J.Y. Hou, X. Chaufour, B. Wilkinson, N.H. Hunt, D.S. Celermajer, and R. Stocker. 2003. Probuco promotes functional reendothelialization in balloon-injured rabbit aortas. *Circulation.* 107:2031–2036.
15. Duckers, H.J., M. Boehm, A.L. True, S.-F. Yet, H. San, J.L. Park, R.C. Webb, M.-E. Lee, G.J. Nable, and E.G. Nabel. 2001. Heme oxygenase-1 protects against vascular constriction and proliferation. *Nat. Med.* 7:693–698.
16. Deng, Y.M., B.J. Wu, P.K. Witting, and R. Stocker. 2004. Probuco protects against smooth muscle cell proliferation by upregulating heme oxygenase-1. *Circulation.* 110:1855–1860.
17. Liu, X.-M., G.B. Chapman, H. Wang, and W. Durante. 2002. Adenovirus-mediated heme oxygenase-1 gene expression stimulates apoptosis in vascular smooth muscle cells. *Circulation.* 105:79–84.
18. Offord, E.A., J.C. Gautier, O. Avanti, C. Scaletta, F. Runge, K. Kramer, and L.A. Applegate. 2002. Photoprotective potential of lycopene, beta-carotene, vitamin E, vitamin C and carnosic acid in UVA-irradiated human skin fibroblasts. *Free Radic. Biol. Med.* 32:1293–1303.
19. Noriega, G.O., S. Gonzales, M.L. Tomaro, and A.M. Batlle. 2002. Paraquat-generated oxidative stress in rat liver induces heme oxygenase-1 and aminolevulinic acid synthase. *Free Radic. Res.* 36:633–639.
20. Calvisi, D.F., S. Ladu, K. Hironaka, V.M. Factor, and S.S. Thorgeirsson. 2004. Vitamin E down-modulates iNOS and NADPH oxidase in c-Myc/TGF- α transgenic mouse model of liver cancer. *J. Hepatol.* 41:815–822.
21. Drummond, G.S., and A. Kappas. 1981. Prevention of neonatal hyperbilirubinemia by tin protoporphyrin IX, a potent competitive inhibitor of heme oxygenase. *Proc. Natl. Acad. Sci. USA.* 78:6466–6470.
22. Clowes, A.W., M.A. Reidy, and M.M. Clowes. 1983. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab. Invest.* 49:327–333.
23. Haudenschild, C.C., W. Van Sickle, and A.V. Chobanian. 1981. Response of the aorta of the obese Zucker rat to injury. *Arteriosclerosis.* 1:186–191.
24. Kornowski, R., G.S. Mintz, K.M. Kent, A.D. Pichard, L.F. Satler, T.A. Bucher, M.K. Hong, J.J. Popma, and M.B. Leon. 1997. Increased restenosis in diabetes mellitus after coronary interventions is due to exaggerated intimal hyperplasia. A serial intravascular ultrasound study. *Circulation.* 95:1366–1369.
25. Morrow, J.D., and L.J. Roberts. 1997. The isoprostanes: unique bioactive products of lipid peroxidation. *Prog. Lipid Res.* 36:1–21.
26. March, J. 1977. *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure.* McGraw-Hill, New York. 1328 pp.
27. Witting, P.K., B.J. Wu, M. Raftery, P. Southwell-Keely, and R. Stocker. 2005. Probuco protects against hypochlorite-induced endothelial dysfunction. Identification of a novel pathway of probuco oxidation to a biologically active intermediate. *J. Biol. Chem.* 280:15612–15618.
28. Sundaresan, M., Z.X. Yu, V.J. Ferrans, K. Irani, and T. Finkel. 1995. Requirement for generation of H₂O₂ for platelet-derived growth factor signal transduction. *Science.* 270:296–299.
29. Zou, M.H., C. Shi, and R.A. Cohen. 2002. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J. Clin. Invest.* 109:817–826.
30. Forman, H.J., J.M. Fukuto, and M. Torres. 2004. Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *Am. J. Physiol. Cell Physiol.* 287:C246–C256.
31. Bult, H. 2000. Restenosis: a challenge for pharmacology. *Trends Pharmacol. Sci.* 21:274–279.
32. Carew, T.E., D.C. Schwenke, and D. Steinberg. 1987. Antiatherogenic effect of probuco unrelated to its hypocholesterolemic effect: evidence that antioxidants *in vivo* can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. USA.* 84:7725–7729.
33. Barnhart, J.W., J.A. Sefranka, and D.D. McIntosh. 1970. Synthesis of hypocholesterolemic activity of alkylidenedithio bisphenols. *J. Med. Chem.* 13:722–725.
34. O'Brien, K., Y. Nagano, A. Gown, T. Kita, and A. Chait. 1991. Probuco treatment affects the cellular composition but not anti-oxidized low density lipoprotein immunoreactivity of plaques from Watanabe heritable hyperlipidemic rabbits. *Arterioscler. Thromb.* 11:751–759.
35. Stocker, R. 1999. The ambivalence of vitamin E in atherosclerosis. *Trends Biochem. Sci.* 24:219–223.
36. Fruebis, J., D. Steinberg, H.A. Dresel, and T.A. Carew. 1994. A comparison of the antiatherogenic effects of probuco and a structural analogue of probuco in low density lipoprotein receptor-deficient rabbits. *J. Clin. Invest.* 94:392–398.
37. Sundell, C.L., P.K. Somers, C.Q. Meng, L.K. Hoong, K.L. Suen, R.R. Hill, L.K. Landers, A. Chapman, D. Butteiger, M. Jones, et al. 2003. AGI-1067: a multifunctional phenolic antioxidant, lipid modulator, anti-inflammatory and antiatherosclerotic agent. *J. Pharmacol. Exp. Ther.* 305:1116–1123.
38. Tardif, J.C. 2003. Clinical results with AGI-1067: a novel antioxidant vascular protectant. *Am. J. Cardiol.* 91:41A–49A.
39. Prütz, W.A. 1996. Hypochlorous acid interactions with thiols, nucleotides, DNA, and other biological substrates. *Arch. Biochem. Biophys.* 332:110–120.
40. Yan, L.J., M.G. Traber, H. Kobuchi, S. Matsugo, H.J. Tritschler, and L. Packer. 1996. Efficacy of hypochlorous acid scavengers in the prevention of protein carbonyl formation. *Arch. Biochem. Biophys.* 327:330–334.
41. Otterbein, L.E., M.P. Soares, K. Yamashita, and F.H. Bach. 2003. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol.* 24:449–455.
42. Alam, J., D. Stewart, C. Touchard, S. Boinapally, A.M. Choi, and J.L. Cook. 1999. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J. Biol. Chem.* 274:26071–26078.

43. Wakabayashi, N., A.T. Dinkova-Kostova, W.D. Holtzclaw, M.I. Kang, A. Kobayashi, M. Yamamoto, T.W. Kensler, and P. Talalay. 2004. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc. Natl. Acad. Sci. USA*. 101:2040–2045.
44. Eggler, A.L., G. Liu, J.M. Pezzuto, R.B. van Breemen, and A.D. Mesecar. 2005. Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh 2. *Proc. Natl. Acad. Sci. USA*. 102:10070–10075.
45. Stocker, R., Y. Yamamoto, A.F. McDonagh, A.N. Glazer, and B.N. Ames. 1987. Bilirubin is an antioxidant of possible physiological importance. *Science*. 235:1043–1046.
46. Abraham, N.G., and A. Kappas. 2005. Heme oxygenase and the cardiovascular-renal system. *Free Radic. Biol. Med.* 39:1–25.
47. Vile, G.F., and R.M. Tyrrell. 1993. Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. *J. Biol. Chem.* 268:14678–14681.
48. Deramandt, B.M., S. Braunstein, P. Remy, and N.G. Abraham. 1998. Gene transfer of human heme oxygenase into coronary endothelial cells potentially promotes angiogenesis. *J. Cell. Biochem.* 68:121–127.
49. Ishikawa, K., D. Sugawara, X. Wang, K. Suzuki, H. Itabe, Y. Maruyama, and A.J. Lusis. 2001. Heme oxygenase-1 inhibits atherosclerotic lesion formation in ldl-receptor knockout mice. *Circ. Res.* 88:506–512.
50. Juan, S.H., T.S. Lee, K.W. Tseng, J.Y. Liou, S.K. Shyue, K.K. Wu, and L.Y. Chau. 2001. Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 104:1519–1525.
51. Ishikawa, K., D. Sugawara, J. Goto, Y. Watanabe, K. Kawamura, M. Shiomi, H. Itabe, and Y. Maruyama. 2001. Heme oxygenase-1 inhibits atherogenesis in Watanabe heritable hyperlipidemic rabbits. *Circulation*. 104:1831–1836.
52. Tulis, D.A., W. Durante, X. Liu, A.J. Evans, K.J. Peyton, and A.I. Schafer. 2001. Adenovirus-mediated heme oxygenase-1 gene delivery inhibits injury-induced vascular neointima formation. *Circulation*. 104:2710–2715.
53. Witting, P.K., K. Pettersson, A.-M. Östlund-Lindqvist, C. Westerlund, A. Westin Eriksson, and R. Stocker. 1999. Inhibition by a co-antioxidant of aortic lipoprotein lipid peroxidation and atherosclerosis in apolipoprotein E and low density lipoprotein receptor gene double knockout mice. *FASEB J.* 13:667–675.
54. Pelter, A., and S. Elgendy. 1988. Phenolic oxidation with (diacetoxyiodo) benzene. *Tetrahedron Lett.* 29:677–680.
55. Lay, P.A., R.K. Norris, and P.K. Witting. 1996. Cyclic voltammetric and electron paramagnetic studies of 9-substituted and 9,10-disubstituted nitroethanoanthracenes. *Aust. J. Chem.* 49:1279–1286.
56. Togane, Y., T. Morita, M. Suematsu, Y. Ishimura, J.I. Yamazaki, and S. Katayama. 2000. Protective roles of endogenous carbon monoxide in neointimal development elicited by arterial injury. *Am. J. Physiol. Heart Circ. Physiol.* 278:H623–H632.
57. Aizawa, T., N. Ishizaka, J. Taguchi, S. Kimura, K. Kurokawa, and M. Ohno. 1999. Balloon injury does not induce heme oxygenase-1 expression, but administration of hemein inhibits neointimal formation in balloon-injured rat carotid artery. *Biochem. Biophys. Res. Commun.* 261:302–307.
58. Choy, K.J., Y.M. Deng, J.Y. Hou, B. Wu, A.K. Lau, P.K. Witting, and R. Stocker. 2003. Coenzyme Q₁₀ supplementation inhibits aortic lipid oxidation but fails to attenuate intimal thickening in balloon-injured New Zealand White rabbits. *Free Radic. Biol. Med.* 35:300–309.
59. Mori, T.A., K.D. Croft, I.B. Puddey, and L.J. Beilin. 1999. An improved method for the measurement of urinary and plasma F₂-isoprostanes using gas chromatography-mass spectrometry. *Anal. Biochem.* 268:117–125.
60. Woodman, R.J., T.A. Mori, V. Burke, I.B. Puddey, G.F. Watts, and L.J. Beilin. 2002. Effects of purified eicosapentaenoic and docosahexaenoic acids on glycemic control, blood pressure, and serum lipids in type 2 diabetic patients with treated hypertension. *Am. J. Clin. Nutr.* 76:1007–1015.
61. Letters, J.M., P.K. Witting, J.K. Christison, A. Westin Eriksson, K. Pettersson, and R. Stocker. 1999. Changes to lipids and antioxidants in plasma and aortae of apoE-deficient mice. *J. Lipid Res.* 40:1104–1112.
62. Sattler, W., D. Mohr, and R. Stocker. 1994. Rapid isolation of lipoproteins and assessment of their peroxidation by HPLC postcolumn chemiluminescence. *Methods Enzymol.* 233:469–489.
63. Witting, P.K., D. Mohr, and R. Stocker. 1999. Assessment of pro- and anti-oxidant activity of vitamin E in human low density lipoprotein and plasma. *Methods Enzymol.* 299:362–375.
64. Hayashi, T., M. Jayachandran, D. Sumi, N.K. Thakur, T. Esaki, E. Muto, H. Kano, Y. Asai, and A. Iguchi. 2000. Physiological concentration of 17 β -estradiol retards the progression of severe atherosclerosis induced by a high-cholesterol diet plus balloon catheter injury: role of NO. *Arterioscler. Thromb. Vasc. Biol.* 20:1613–1621.
65. Mao, S.J., M.T. Yates, A.E. Rechten, R.L. Jackson, and W.A. VanSickle. 1991. Antioxidant activity of probucol and its analogues in hypercholesterolemic Watanabe rabbits. *J. Med. Chem.* 34:298–302.