Probucol Protects Against Smooth Muscle Cell Proliferation by Upregulating Heme Oxygenase-1

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Background—Evidence suggests that induction of heme oxygenase-1 (HO-1) inhibits proliferation of vascular smooth muscle cells and intimal thickening after arterial injury, and therapeutic molecules induce HO-1. Probucol is the only oral drug that inhibits restenosis in humans and intimal thickening in animals, although its underlying mechanism remains unclear.

Methods and Results—Aortas were harvested from New Zealand White rabbits fed normal or 0.75% (wt/wt) probucol-fortified chow, with or without endothelial denudation of the abdominal aorta on day 21, and analyzed for heme oxygenase and apoptosis. Uninjured aortas were harvested on day 21 and balloon-injured aortas on days 22 and 25. Probucol significantly increased mRNA of HO-1 assessed by real-time PCR and HO activity in aortas at all time points. Probucol also enhanced apoptosis of medial cells in the injured aorta, as evidenced by the TUNEL assay. Furthermore, probucol (100 μmol/L) increased HO-1 mRNA and HO activity when added to rabbit aortic smooth muscle cells (RASMCs) cultured in serum-free medium for 24 hours. Induction of HO-1 mRNA was inhibited by actinomycin D and was associated with inhibition of RASMC proliferation. This probucol-induced increase in HO-1 mRNA and inhibition of RASMC proliferation was prevented by the HO inhibitor Sn(IV) protoporphyrin or transfection with small interference RNA (siRNA) to knockdown HO-1, but not by inactive Cu(II) protoporphyrin or scrambled siRNA.

Conclusions—Probucol induces HO-1, and this contributes to the inhibition of vascular SMC proliferation. This novel finding may explain how probucol inhibits restenosis and highlights HO-1 as a target for therapeutic intervention against occlusive vascular disease. (Circulation. 2004;110:1855-1860.)

Key Words: angioplasty ■ antioxidants ■ atherosclerosis ■ endothelium ■ restenosis

Angioplasty has revolutionized the treatment of advanced atherosclerosis. The advent of drug-eluting stents to locally deliver agents that inhibit cell proliferation has almost completely prevented in-stent restenosis.1,2 However, there remain concerns with drug-eluting stents, such as late thrombosis3 possibly due to inhibition of reendothelialization,4 localized hypersensitivity reactions,5 decreased efficacy in diabetic patients,6 and that the underlying atherosclerotic process is not targeted.

Probucol, a rarely used cholesterol-lowering drug with antioxidant properties, is the only agent that consistently inhibits atherosclerosis and restenosis. It attenuates atherogenesis in animals6 and humans7 and regresses xanthomas in hypercholesterolemic patients.8 Probucol protects human coronary arteries from restenosis9-11 and promotes positive adventitial remodeling.12 In animals, probucol prevents intimal thickening after balloon injury,13 independent of both cholesterol lowering14-16 and inhibition of lipoprotein lipid oxidation.16 This beneficial effect is associated with promotion of endothelial cell growth and functional reendothelialization16 and inhibition of vascular smooth muscle cell (SMC) proliferation.16,17

The opposing effects of probucol on endothelial and SMC growth is analogous to the action of heme oxygenase-1 (HO-1), an increasing activity of which inhibits SMC proliferation18 and promotes the growth of coronary endothelial cells.19 There is also mounting evidence in vivo that HO-1 protects against vascular disease. For example, increased HO-1 decreases intimal thickening after arterial injury20 and attenuates atherosclerosis in LDL-receptor−21 and apolipoprotein E−deficient22 mice. In contrast, inhibition of HO-1 enhances atherosclerosis in Watanabe heritable hyperlipidemic rabbits.23

On the basis of the aforementioned similarities, we hypothesized that probucol’s protective effects after arterial balloon injury are mediated via induction of HO-1. We provide evidence that probucol inhibits intimal thickening via induction of HO-1 expression and heme oxygenase activity and propose that HO-1 is a therapeutic target for the inhibition of occlusive vascular disease.
Methods

Rabbit Aortic Balloon-Injury Model
Male New Zealand White rabbits (~2.5 kg; Merunga Farm, Coffs Harbor, Australia) were fed a normal diet (100 g/d) for 3 weeks before being randomized to 2 groups according to body weight and blood cholesterol concentration. Animals were then fed the limited diet without or with 0.75% (wt/wt) probucol (96% purity, Medichem), without and with aortic balloon injury (ABI) of the abdominal aorta on day 21, as described previously. Uninjured aortas were harvested on day 21 and balloon-injured aortas, on days 22 and 25. For RNA extraction, sections of the abdominal aorta were kept overnight in RNAlater (Ambion) at 4°C and then transferred to −80°C. For biochemical analyses, aortas were snap-frozen in phosphate-buffered saline containing a commercial protease inhibitor cocktail, 1 mmol/L EDTA, and 100 μmol/L butylated hydroxytoluene and then stored at −80°C.

Cell Proliferation
Rabbit aortic SMCs (RASMCs; Cell Applications; passages 6 to 11) were cultured in 12-well plates in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) and 100 U/mL streptomycin/ampicillin at 37°C in a humidified atmosphere of 5% CO2 until 60% to 70% confluent. Cells were cultured in 12-well plates in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) and 100 U/mL streptomycin/ampicillin at 37°C in a humidified atmosphere of 5% CO2 until 60% to 70% confluent. Cells were then incubated for up to 48 hours in serum-free DMEM containing 0.3% bovine serum albumin without or with the various chemicals indicated, after which they were harvested with trypsin (0.025%/EDTA (1 mmol/L) followed by cell counting with a Coulter counter (model ZF, Coulter Electronics).

HO-1–Specific siRNA
Small interfering RNA (siRNA) was designed by targeting the HO-1 sequence 5’ AACAAAAGGAAAACCCGGTCTAC 3’, according to the siRNA design guidelines (www.qiagen.com). The sequence 5’ GACTCGAGACATCGAGACA 3’ was used as the scrambled siRNA control; it does not match any mammalian sequences currently available on online databases. Double-stranded siRNAs were synthesized (Qiagen-Xeragon) and dissolved in sterile annealing buffer according to the manufacturer’s instruction.

Transfection of RASMCs
Transfection of RASMCs by Transmessenger transfection reagent (Qiagen) was optimized first by using a control, fluorescein isothiocyanate–labeled siRNA and different siRNA-to-TransMessenger reagent ratios for 4 hours in serum- and antibiotic-free DMEM. Transfection efficiency was evaluated after 24 hours by flow cytometry and counting the percentage of fluorescein isothiocyanate–labeled cells. Optimal transfection efficiency (~80%) was obtained with 3.2 μg siRNA and an siRNA-to-TransMessenger reagent ratio of 1:2.5. These conditions were used for the subsequent siRNA transfection experiments.

In Situ Detection of Apoptotic Cells
Paraffin-embedded cross sections of rabbit aortas were stained for apoptotic nuclei with an in situ cell death detection kit (Roche Molecular Biochemicals) according to the manufacturer’s recommendations. In brief, sections were treated with proteinase K and exposed to diaminobenzidine black nickel chromogen. Sections were incubated with a terminal dUTP nick end-labeling (TUNEL) reaction mixture, treated with converter-peroxidase solution, and exposed to diaminobenzidine black–nickel chromogen. Slides were counterstained with hematoxylin and analyzed by light microscopy for total and TUNEL-positive medial wall cells.

RNA Extraction and Real-Time RT-PCR
Frozen tissue was homogenized in Trizol reagent (Life Technologies) using matrix D in a FastPrep machine (BIO 101). For cultured cells, Trizol reagent was added directly to cells. RNA was extracted according to the manufacturer’s instruction, followed by treatment with RQ1 DNase I (Promega). Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was performed with MMLV (Life Technologies) and the SYBR Green PCR kit (Applied Biosystems) in an ABI 7700 sequence detector (Applied Biosystems). Expression of HO-1 gene was determined as the amount of HO-1 mRNA relative to mRNA for hypoxanthine phosphoribosyltransferase (HPRT) by using the comparative Ct method described in the ABI 7700 sequence detector user bulletin 2. Sense and antisense primers for HO-1 were 5’-GGAGTTTACGTCAGACAAAGGA-3’ and 5’-ACCGGGAAGTACGTGCTTGA-3′ respectively. Primers for HPRT were as described.

HO Activity
The activity of HO was determined in microsomes prepared from aortas and RASMCs. In brief, frozen tissue was pulverized in LN2, resuspended in phosphate-buffered saline with protease inhibitors (Roche), and then homogenized. Cells were scraped with a rubber policeman in the same buffer and lyzed by 3 freeze-thaw cycles. The tissue homogenate or cell lysate was then centrifuged at 15 000g at 4°C for 20 minutes, and the supernatant was then collected and subjected to ultracentrifugation at 100 000g at 4°C for 1 hour. The resulting microsomal pellet was suspended in buffer A (250 mmol/L sucrose, 20 mmol/L Tris, pH 7.4). For the HO activity assay, 400 to 600 μg microsomal protein was mixed with 200 μg rat liver microsomes, 1 mmol/L NADPH, 2 mmol/L D-glucose-6-phosphate, 1 U glucose-6-phosphate dehydrogenase, and 1 μL of 2.5 mmol/L hem (Sigma) in 25% dimethyl sulfoxide (DMSO) in 100 μL of buffer A on ice. The mixture was incubated at 37°C in the dark for 1 hour, the reaction stopped by adding 100 μL of ethanol/DMSO (95:5, vol/vol), the sample was centrifuged at 13 000g for 5 minutes, and the supernatant was used for further analysis. HO enzyme activity was determined by formation of bilirubin as detected by high-performance liquid chromatography, as described, with slight modifications. In brief, the supramant resulting from the ethanol-DMSO extraction was subjected to an LC18 column eluted with a linear gradient generated by changing from 100% solvent A (methanol/100 mmol/L ammonium acetate, pH 5.1: 3.2; vol/vol) to 100% solvent B (100% methanol) over 18 minutes, with bilirubin detected at 455 nm.

Statistical Analysis
All data are expressed as mean±SEM. Unpaired Student’s t tests were used for between-group comparisons, and significance was accepted at the 95% confidence interval.

Results

Aortic HO-1
In response to ABI, the aortic content of mRNA for HO-1 initially (day 21) decreased (0.5±0.05) and then increased (2.2±0.3) on day 25 relative to the content of HPRT mRNA (Figure 1A). Such a biphasic response in HO-1 induction after mechanical injury is consistent with previous reports. Treatment with probucol increased HO-1 mRNA level (Figure 1A) independent of whether rabbits underwent ABI (relative increase, 2.0±0.15 and 1.9±0.7 on days 22 and 25, respectively; P<0.01 vs control for both time points) or not (2.8±0.4-fold on day 21; P<0.01 vs control). In parallel, probucol increased HO activity by 2.1-, 1.3-, and 1.9-fold compared with control on days 21, 22, and 25 respectively (Figure 1B, P<0.01 for all 3 time points), as assessed by bilirubin formation (Figure 1C).

HO-1 in RASMCs
Previous studies have shown that inhibition of ABI-induced intimal thickening by probucol is associated with...
enhanced reendothelialization and inhibition of vascular SMC proliferation. 16 Because HO activity has been linked to the control of cell growth, 20 we assessed the effect of probucol on HO-1 in endothelial cells and SMCs. Treatment of primary porcine aortic endothelial cells with probucol (100 μmol/L) appeared to decrease HO-1 mRNA (50±12% and 60±15% of control at 6 and 8 hours, respectively), although this did not reach statistical significance (P>0.05). By contrast, treatment of RASMCs with probucol increased HO-1 mRNA in a time-dependent manner (Figure 2A), and this trend was mirrored by an increase in HO activity (Figure 2B). After 24 hours, cells with and without probucol treatment produced 3.3±0.3 and 1.7±0.4 nmol bilirubin per milligram protein per hour, respectively (P<0.01). Basal expression of HO-1 and its induction by probucol required active transcription, because pretreatment of cells for 30 minutes with 0.5 mg/mL actinomycin D (ActD; Sigma) to inhibit transcription completely abolished the increase in HO-1 mRNA (Figure 2C).

**Figure 1.** Probucol induces HO-1 mRNA expression and HO activity. A, HO-1 mRNA expression and B, HO activity in aortas of 4 control (open bars) and 4 probucol-treated (closed bars) rabbits on days 21, 22, and 25. C, Representative high-performance liquid chromatograms showing formation of bilirubin in aortas of control (lower line) and probucol-treated (upper line) rabbits on day 25. AU indicates arbitrary units; OD, optical density; and RT, retention time. All other abbreviations are as defined in text. *P<0.01 vs control.

**Figure 2.** Probucol increases HO-1 transcription and activity in RASMCs. A, HO-1 mRNA expression in control (open bars) and 100 μmol/L probucol-treated (closed bars) RASMCs cultured in serum-free DMEM for times indicated (n=3). B, HO activity in control (open bars) and 100 μmol/L probucol–treated (closed bars) RASMCs cultured in serum-free DMEM for times indicated (n=3). C, HO-1 mRNA expression in control (open bars) and 100 μmol/L probucol-treated (closed bars) RASMCs cultured in serum-free DMEM for 24 hours in absence or presence of 0.5 mg/mL ActD added 30 minutes before probucol (n=3). *P<0.01 vs control; **P<0.001 vs cells without ActD. n.s. indicates not significant. All other abbreviations are as defined in text.

**HO and RASMC Proliferation**

Addition of probucol inhibited the proliferation of cultured RASMCs (Figure 3), as reported previously. 16 Addition of the competitive HO inhibitor tin protoporphyrin (20 μmol/L, Porphyrin Products) abolished this antiproliferative effect of probucol (108±3.3% vs 67±5.5%, P<0.01). In contrast, addition of the inactive copper protoporphyrin (CuPP; 20 μmol/L, Porphyrin Products) tended to decrease cell proliferation, an effect that was due to cytotoxicity as assessed by trypan blue dye exclusion (not shown). More important, CuPP (20 μmol/L) failed to abrogate growth inhibition by probucol (65±5.2% and 67±5.5% for probucol and CuPP, respectively; Figure 3).

We next transfected RASMCs with anti–HO-1 siRNA to knock down HO-1 induction and examined the effect of this on probucol’s ability to inhibit cell proliferation. Transfection of RASMCs with anti–HO-1 siRNA but not scrambled
Probucol Induces Apoptosis of SMCs In Vivo

Previous studies have established that increasing HO-1 causes inhibition of proliferation of cultured SMCs via stimulation of apoptosis.26 It is also known that probucol decreases arterial injury–induced SMC proliferation in vivo and that 3 weeks after ABI, the numbers of medial cells are decreased in vessels of probucol-treated compared with normal rabbits.16 Consistent with in vivo inhibition of SMC proliferation, injured aortas from probucol-treated rabbits 4 days after ABI already contained fewer medial cells than sinus corresponding control aortas, although this difference did not reach statistical significance (Table). Further, probucol significantly increased the proportion of apoptotic cells in the media (16.5±0.9% vs 9.6±1.0% of cells for probucol vs control, \( P<0.01 \)), as assessed by the TUNEL assay (Table).

Discussion

Probucol consistently inhibits intimal thickening in animals and restenosis in humans, and these protective effects are commonly attributed to its cholesterol-lowering and antioxidant activities. However, probucol reduces postangioplasty restenosis in humans by improving vascular remodeling,12 and our previous studies in the rabbit ABI model of intimal thickening showed that in vivo protection by probucol is related to the promotion of reendothelialization and inhibition of SMC proliferation, rather than inhibition of aortic lipoprotein lipid oxidation and cholesterol lowering.16 Here, we show that probucol induces HO-1 mRNA and HO activity in SMCs in vivo and in vitro and that this is responsible for the drug’s ability to inhibit proliferation of SMCs, likely via promotion of apoptosis.

Excessive proliferation of SMCs is a key contributor to intimal thickening after ABI and restenosis after angioplasty. We now provide pharmacologic and molecular evidence for HO-1 induction’s being responsible for the observed probucol-mediated inhibition of SMC proliferation. Both induction of HO-1 and inhibition of RASMC proliferation by probucol were lost completely by the HO-1 inhibitor tin protoporphyrin and HO-1–specific siRNA. In contrast, inactive CuPP and transfection with scrambled siRNA affected neither HO-1 nor cell proliferation. Increased aortic HO activity was observed as early as 4 days after ABI, at which time the injured vessel remained essentially devoid of endothelium. Also, probucol upregulated HO-1 in cultured RASMCs but not in aortic endothelial cells, suggesting that the drug acted directly on SMCs.

It is established that HO-1 confers protection against cellular proliferation and lesion formation after arterial injury. For example, compared with control cells, vascular SMCs from HO-1–deficient mice display enhanced DNA synthesis and growth, and wire injury of femoral arteries results in greater intimal thickening in HO-1–deficient than in control mice.20 Conversely, transfer of the HO-1 gene to porcine arteries attenuates injury-induced proliferation of SMCs and intimal thickening.20 In serum-starved porcine vascular SMCs, HO-1 inhibits DNA synthesis in part via enhanced G1/G0 growth arrest,20 reminiscent of the effect of probucol on cultured RASMCs.16 In rat aortic SMCs, overexpression of HO-1 decreases proliferation by stimulating programmed cell death.19 Consistent with this concept, we observed the proportion of apoptotic cells to increase significantly in the media of probucol-treated compared with control rabbits (Table). Together, these studies suggest that probucol inhibits intimal thickening after balloon injury by directly inhibiting SMC proliferation via induction of HO-1 and apoptosis.

It is presently unclear how probucol induces HO-1 and how this leads to inhibition of SMC proliferation. HO-1 is a stress protein induced under many conditions and by several agents.27 The latter include oxidants such as hydrogen peroxide28 but also reductants including phenols, such as curcumin,29 suggesting that induction of HO-1 is under redox control and may provide antioxidant protection.30 Because probucol can engage in redox reactions,31 it is conceivable that the drug induces HO-1 via changes in cellular redox status. However, preliminary experiments indicate that probucol does not alter the redox status or concentration of glutathione in RASMCs (R. Mashima and
R. Stocker, 2002, unpublished data). Therefore, additional studies are required to clarify how probucol induces HO-1 in SMCs and whether this involves redox reactions.

HO-1 is the inducible form of HO that degrades heme to iron, carbon monoxide (CO), and biliverdin that is then converted to bilirubin.27 HO-1 could principally regulate cell growth by a decrease in cellular heme, an increase in 1 or several of its products, or a combination thereof. A presently unresolved key question is the extent to which altered HO-1 activity translates into changes in the cellular concentrations and localization of heme, iron, CO, and biliverdin/bilirubin. Notwithstanding these limitations, there is convincing evidence that CO at 250 ppm suppresses intimal thickening after balloon injury in vivo and inhibits SMC proliferation in vitro, likely involving cGMP and p38 mitogen-activated protein kinase.32 Similarly, micromolar concentrations of biliverdin and bilirubin can inhibit vascular SMC proliferation by stimulating apoptosis.18

Like probucol,16 HO-1 regulates cell growth in a cell-specific manner, as exemplified by the opposing effect on SMC and endothelial cell proliferation (see Introduction). Similarly contrasting effects are seen with programmed cell death, as overexpression of HO-1 promotes and blocks apoptosis in SMCs and endothelial cells, respectively.18,33 In endothelial cells, gaseous CO mimics and hemoglobin suppresses inhibition of apoptosis induced by HO-1,33 suggesting that the latter is mediated by HO-1–derived CO. If so, this raises the possibility that CO produced by SMCs as a result of probucol-mediated HO-1 induction diffuses to and acts on adjacent endothelial cells to suppress apoptosis. This could help explain why probucol effectively promotes reendothelialization in vivo,16 a process that itself inhibits intimal thickening.34

In conclusion, the present study shows that induction of HO-1 is responsible for the ability of probucol to inhibit SMC proliferation and intimal thickening. The results add not only to the growing list of protective properties of probucol but also to the list of therapeutic agents that induce HO-1.35 These agents include nitric oxide,36 aspirin,37 and rapamycin.38 These findings lend further support for the notion that HO-1 protects against cardiovascular diseases such as intimal thickening,20 atherosclerosis,21–23 and transplant-associated arteriosclerosis.33 They highlight the potential of HO-1 as a therapeutic target for the alleviation of occlusive vascular disease.

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