Atherosclerotic vascular disease is the leading cause of mortality in the Western world. Abnormal proliferation of vascular smooth muscle cells (VSMCs) plays an important role in atherogenesis and restenosis after angioplasty or stenting.1 Agents that inhibit the proliferation of VSMCs are of considerable therapeutic importance for the treatment of cardiovascular diseases. This is particularly the case for in-stent restenosis, the incidence of which has been reduced dramatically by the widespread use of stents that elute cell proliferation-inhibitory drugs. However, the incidence of in-stent thrombosis remains a significant clinical problem2 given the potentially life threatening consequences of a thrombotic event. The endothelium plays a crucial role in the prevention of in-stent thrombosis, with delayed reendothelialization likely responsible for late stent thrombosis.3 Most conventional, orally administered, drugs have failed to inhibit restenosis in humans following coronary angioplasty and stenting,4–6 one exception being probucol, a rarely used lipid-lowering drug with antiinflammatory and antioxidant properties.7 Probucol has also been reported to inhibit experimental in-stent thrombosis.8 These beneficial effects are associated with promotion of endothelial cell growth and enhancement of reendothelialization and inhibition of VSMC proliferation.9 Recent evidence suggests that the mechanisms of probucol and related compounds to inhibit VSMC proliferation, as well as experimental restenosis and atherosclerosis, are mediated via induction of the enzyme heme oxygenase (HO)-1.10,11

HO-1 is the inducible of 2 forms of heme oxygenases that degrade heme to CO, Fe2+, and biliverdin, which is then

Interplay Between Heme Oxygenase-1 and the Multifunctional Transcription Factor Yin Yang 1 in the Inhibition of Intimal Hyperplasia

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Rationale: Induction of heme oxygenase (HO)-1 protects against experimental atherosclerotic diseases, and certain pharmacological HO-1 inducers, like probucol, inhibit the proliferation of vascular smooth muscle cells and, at the same time, promote the growth of endothelial cells in vivo and in vitro.

Objective: Because such cell-specific effects are reminiscent of the action of the transcription factor Yin Yang (YY)1, we tested the hypothesis that there is a functional relationship between HO-1 and YY1.

Methods and Results: We report that probucol increases the number of YY1+ cells in rat carotid artery following balloon injury at a time coinciding with increased HO-1 expression. The drug also induces the expression of YY1 mRNA and protein in rat aortic smooth muscle cells (RASMCs) in vitro, as do other known HO-1 inducers (tert-butyldihydroquinone and hemin) and overexpression of HO-1 using a human HMOX1 cDNA plasmid. Conversely, overexpression of YY1 induces expression of HO-1 in RASMCs. Induction of YY1 expression is dependent on HO-1 enzyme activity and its reaction product CO, because pharmacological inhibition of heme oxygenase activity or CO scavenging block, whereas exposure of RASMCs to a CO-releasing molecule increases, YY1 expression. Furthermore, RNA interference knockdown of YY1 prevents probucol or adeno–HO-1 from inhibiting RASMC proliferation in vitro and neointimal formation in vivo.

Conclusions: Our findings show, for the first time, that HO-1 functionally interplays with the multifunctional transcription factor YY1 and that this interplay explains some of the protective activities of HO-1. (Circ Res. 2010;107:1490-1497.)

Key Words: Heme oxygenase-1 ■ smooth muscle cell proliferation ■ YY1 ■ carbon monoxide ■ probucol
reduced to bilirubin by biliverdin reductase.\textsuperscript{12} HO-1 is increasingly recognized as an important mediator of cellular homeostasis in conditions of stress and injury. A vast number of agents and conditions including heme, \textit{tert}-butylhydroquinone (tBHQ), hypoxia, oxidative stress, or UV irradiation stimulate HO-1 expression, primarily at the transcriptional level.\textsuperscript{13} HO-1 plays a crucial role in cell survival and in the control of cell growth in vascular diseases.\textsuperscript{14} Like its pharmacological induction, adenovirus-mediated overexpression of HO-1 in cells inhibits the proliferation of VSMCs and protects against experimental atherosclerotic diseases,\textsuperscript{15} whereas overexpression of HO-1 promotes growth of coronary artery endothelial cells.\textsuperscript{16} This differential effect of HO-1 on cell cycle progression is thought to be mediated by the HO-1 reaction product CO.\textsuperscript{17}

The HO-1–induced opposing effect on the growth of VSMCs versus endothelial cells is reminiscent of the effect of the transcription factor Yin Yang (YY1).\textsuperscript{18} YY1 (also known as \(\delta\), NF-E1, UCRBP, and CF1) is a ubiquitous and multifunctional zinc-finger transcription factor.\textsuperscript{19} It is a member of the polycom group protein family, a group of homeobox gene receptors that play critical roles in hematopoiesis and cell cycle control. YY1 regulates the expression of genes important in DNA replication, protein synthesis, and cellular responses to external stimuli during cell growth and differentiation. This regulation of gene expression is based on the ability of YY1 to initiate, activate, or repress transcription depending on the context.\textsuperscript{19} Overexpression of YY1 significantly inhibits VSMC proliferation in vitro and in vivo.\textsuperscript{20}

Based on the similar functional properties of HO-1 and YY1 related to the growth of vascular cells, we explored whether YY1 mediates the antiproliferative effect of HO-1 in VSMCs. Here we show, for the first time, that induction of HO-1 leads to the expression of YY1 and that there is a close interaction between HO-1 and YY1 function.

\section*{Methods}

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and includes information regarding the following: materials used in the study, rat aortic smooth muscle cells (RASMCs), RNA isolation and real-time quantitative PCR, isolation of total cell extracts, Western blotting, and heme oxygenase activity.

\subsection*{Vascular Injury, Intimal Hyperplasia, and Immunohistochemistry}

Rabbit aortic balloon injury was performed as described previously,\textsuperscript{9} with approval from the University of New South Wales Animal Ethics Committee. Rat carotid balloon injury was performed and the extent of intimal hyperplasia was assessed as described previously,\textsuperscript{11} with approval from the University of Sydney Animal Ethics Committee (see Online Data Supplement for more details). For immunohistochemistry, see the Online Data Supplement.

\subsection*{HO-1 and YY1 Overexpression and Knockdown}

The preparation of the HO-1 overexpression construct is described in the Online Data Supplement. Where indicated, YY1 was transiently overexpressed using plasmid pCB6\textsuperscript{+}.YY1, with pCB6\textsuperscript{+} serving as a control vector.\textsuperscript{21} Plasmids were purified by CsCl gradient and used for transient transfection of RASMCs using FuGENE 6 (Roche Diagnostics). To knockdown HO-1 and YY1, stealth RNAi duplexes (Invitrogen) were used to transiently transfect RASMCs using \textit{X-treme GENE} transfection reagent (Roche Diagnostics) (see Online Data Supplement).

\subsection*{Statistical Analysis}

Data represent averaged results from 3 to 6 separate experiments as indicated, with error bars indicating SEM unless stated otherwise. Statistical significance was assessed using the Wilcoxon Mann Whitney Rank Sum test, with \(P<0.05\) considered significant.

\section*{Results}

\subsection*{Probucol Decreases Neointimal Formation in Response to Injury in Part via Increasing Medial Expression of YY1}

We reported previously that oral administration of probucol effectively attenuates balloon injury–induced intimal hyperplasia in the abdominal aorta of rabbits and in the common carotid artery of Zucker rats by increasing HO-1 expression and heme oxygenase activity.\textsuperscript{11} Similarly, probucol treatment increased medial expression of HO-1 in the carotid artery of Sprague–Dawley rats 4 days after injury (Figure 1A), a time point shown previously to coincide with maximal HO-1 expression.\textsuperscript{10} At the same time, medial expression of the zinc-finger transcription factor YY1 was also observed, and this too was increased by probucol in both the abdominal aorta of rabbits (Online Figure I) and the carotid artery of rats (Figure 1B). These results suggest that in these models, probucol induces the expression of both HO-1 and YY1 in medial cells following vascular injury.

To assess the potential functional relevance of this coexpression, we examined the impact of YY1 knockdown on the ability of probucol to inhibit intimal thickening. Initial pilot studies carried out in Sprague–Dawley rats confirmed that topical application of YY1 small interfering (si)RNA in pluronic gel to the artery at the time of injury was effective in reducing subsequent YY1 expression in medial cells even at 2 weeks after injury (Online Figure II, A). Such blockade of YY1 expression increased intimal hyperplasia, whereas balloon injury in control Sprague–Dawley rats treated with YY1 scrambled (sc)RNA caused only modest intimal hyperplasia (Online Figure II, B). Having established efficient in vivo knockdown of YY1, we assessed the impact of this on the ability of probucol to inhibit intimal hyperplasia in carotid arteries of Zucker rats where, compared with Sprague–Dawley rats, balloon injury causes a more substantial hyperproliferative response, amenable to intervention studies. Con-
consistent with our previous results, probucol prevented intimal hyperplasia in control Zucker rats treated with YY1 scRNA (Figure 1C). Application of YY1 siRNA only slightly increased intimal hyperplasia in control rats that did not receive probucol, whereas YY1 siRNA very substantially, but not completely, reversed the inhibitory effect of probucol on intimal hyperplasia (Figure 1C). Control experiments performed in Sprague–Dawley rats also showed that treatment with YY1 siRNA increased the number of PCNA cells in the neointima (Online Figure II, C). Together, these results indicate that the ability of probucol to inhibit injury-induced intimal hyperplasia can be explained largely by upregulation of YY1 in medial cells.

Expression of HO-1 and YY1 in RASMCs In Vitro
Addition of probucol to cultured RASMCs caused the time-dependent transient increase in the expression of HO-1 (Figure 2A) and YY1 mRNA (Figure 2B). Probucol treatment also increased the cellular content of HO-1 and YY1 protein for up to 24 hours (Figure 2C). Augmented expression of YY1 in RASMCs appeared to be a general feature of pharmacological induction of HO-1, because BHQ (Figure 3) and heme (Online Figure III) also increased the expression of both HO-1 and YY1. In addition, transient transfection of RASMCs with human HMOX1 cDNA increased HO-1 and YY1 mRNA and proteins (Figure 4). In all cases examined, expression of HO-1 mRNA preceded that of YY1 mRNA (Figure 2A and 2B; Figure 3A and 3B; Figure 4A and 4B; Online Figure III, A and B), suggesting that HO-1 induction lies upstream of YY1 expression.

We next investigated whether mitogen-activated protein kinase (MAPK) signaling was involved in the HO-1–mediated expression of YY1 in RASMCs, using BHQ as a model agonist. Blocking extracellular signal-regulated kinase (ERK) had no effect on HO-1 or YY1 expression (Online Figure IV). In contrast, inhibition of c-Jun N-terminal kinase (JNK) phosphorylation attenuated, whereas inhibition of p38 kinase, augmented HO-1 and YY1 induction (Online Figure IV). Similar results were obtained in RASMCs transfected with human HMOX1 cDNA (data not shown). These data suggest that JNK positively and p38 negatively regulates HO-1 induction in RASMCs by BHQ and that both signaling paths are upstream of HO-1. The observation that expression of YY1 mirrored that of HO-1 in these experiments lends further credit to the notion that HO-1 induction is linked closely to YY1 expression.
Figure 3. tBHQ increases the expression of HO-1 and YY1 in RASMCs. RASMCs were treated with 5 μmol/L tBHQ for up to 24 hours before HO-1 and YY1 mRNA (A and B) and protein (C) were determined as described in Methods. Changes in mRNA levels of HO-1 (A) and YY1 (B) determined in 3 separate experiments, each performed in triplicate. C, Blots representative of 3 separate experiments are shown. The extent of HO-1 (gray bars) and YY1 (black bars) protein expression in the 3 separate experiments was quantified by expressing the respective Western blot band intensity relative to that of actin. The 0 hour value was set as 1. *Significantly different to 0 hour value.

We next examined the effect of YY1 expression on HO-1 levels. Transient transfection of RASMCs with YY1 using plasmid pCB6−YY1 resulted in increased HO-1 protein expression in the 3 separate experiments was quantified by expressing the respective Western blot band intensity relative to that of actin. The 0 hour value was set as 1. *Significantly different to 0 hour value.

**HO-1–Mediated Expression of YY1 is Dependent on Heme Oxygenase Enzyme Activity and CO**

HO-1 protein has recently been reported to be localized to the nucleus of cells exposed to hypoxia, and to mediate activation of transcription factors independent of its enzymatic activity. In the case of RASMCs treated with probucol, however, HO-1 activity was required for the increased YY1 expression. Thus, tin-protoporphyrin-IX (SnPP) (a pharmacological inhibitor of heme oxygenase) completely abrogated the ability of probucol to increase YY1 expression (Figure 5A), whereas the inhibitor did not block HO-1 protein expression. Control experiments confirmed that SnPP completely blocked the increase in heme oxygenase activity seen in RASMCs treated with probucol (Online Figure VI).

HO-1–mediated induction of YY1 is likely dependent on CO generated as a product of HO-1 enzyme activity. This is because the inclusion of hemoglobin, which avidly binds CO, completely blocked the ability of tBHQ to induce YY1 in RASMCs, without materially affecting the expression of HO-1 (Figure 5B). Conversely, addition of the CO-releasing molecule, tricarbonyldichlororuthenium (II) dimer (CORM2), increased YY1 protein (Figure 5C).

**Inhibition of RASMC Proliferation by HO-1 Is Dependent on YY1**

A phenotypic manifestation of VSMCs treated with probucol or related compounds is HO-1–dependent inhibition of cell growth. We confirmed this property of probucol for RASMCs, using serum inducible bromodeoxyuridine incorporation as the read-out (Figure 6A). Similar to the situation with HO-1, knockdown of YY1 abrogated the ability of probucol to inhibit RASMC growth, irrespective of whether cell proliferation was assessed by bromodeoxyuridine incorporation (Figure 6B) or by cell counting (data not shown). Effective knockdown of HO-1 and YY1 by the respective siRNA was confirmed by Western blot (data not shown). Similar results were obtained with RASMCs that expressed human HO-1 as a result of adenovirus-mediated transfection. Compared with the corresponding LacZ-transfected control cells, RASMCs expressing HO-1 grew slower (Figure 6C), and this growth inhibition was abrogated significantly by YY1 siRNA but not YY1 scRNA (Figure 6D). In contrast to RASMCs, adenoviral transfection with *HMOX1* had no material effect on the growth of bovine aortic endothelial cells (Online Figure VII) despite efficient HO-1 protein expression (Online Figure VII, inset). These data demonstrate that HO-1–mediated induction of YY1 expression is functionally related to the control of RASMCs but not bovine aortic endothelial cell growth. Furthermore, the results suggest that there may be species and/or vascular bed–specific differences with regard to the growth effect of HO-1, given the earlier finding by others that overexpression of human HO-1 promoted the growth of rabbit coronary endothelial cells.

**Discussion**

The present study shows that an increase in cellular HO-1 is associated with the enhanced expression of the zinc-finger transcription factor YY1 and that this is both dependent on enzymatic activity of HO-1 and functionally important with regard to the impact of HO-1 on the proliferation of VSMCs. These observations may help explain the antirestenotic and antiatherosclerotic activities of HO-1 and pharmacological inducers of this enzyme.
Several lines of evidence support the concept that the multifunctional transcription factor YY1 is a down-stream effector of HO-1. Thus, independent of whether expression of HO-1 was achieved through pharmacological or molecular means, its transcript always increased before that of YY1. Also, blockade of HO-1 enzymatic activity, as well as addition of hemoglobin to scavenge the reaction product CO, completely abrogated the increase in YY1 expression, without measurably lowering the extent of HO-1 protein induction. In addition, chemically generated CO alone was able to increase YY1 protein expression. Interestingly, however, overexpression of YY1 also increased HO-1 protein, suggesting that the interaction between the 2 proteins is complex and may involve a feed forward amplification. In any case, the fact that knockdown of YY1 effectively abrogated the ability of probucol to inhibit intimal hyperplasia provides direct evidence that the interaction between HO-1 and YY1 is functionally important in vivo.

Our findings extend existing knowledge of how induction of HO-1 may translate into cellular effects via the transcription factor YY1. By modulating cellular heme levels, heme oxygenase activity can regulate the transcription factor Bach1, a heme-binding protein that negatively regulates the expression of genes involved in heme metabolism, oxidative stress and globin synthesis,23,24 including HO-1. Such HO-1/heme-dependent regulation of the transcription factor Bach1 is conceptually different from the HO-1–mediated expression of YY1 reported here, because YY1 is not known to avidly bind heme. A previous study reported hypoxia- or heme-induced nuclear entry of a C-terminally cleaved form of HO-1 to be associated with activation of oxidant-responsive transcription factors including activator protein-1.22 In that study, however, activation of transcription factors was independent of enzymatic activity of HO-1,22 whereas the HO-1–induced expression of YY1 described here required enzymatic activity. Other previous studies reporting altered expression of transcription factors used the heme oxygenase reaction product CO as a pharmacological agent rather than studying the effects of cellular HO-1 activity. Examples of such factors include hypoxia-inducible factor-1,25–27 peroxisome proliferation-activated receptor-γ,28 early growth response-1,29 and E2F1.30 Downregulation of E2F1 or E2F3 inhibits the proliferation of SMCs.31 YY1 is a partner protein for several members of the E2F transcription factor family.32 E2F and YY1 binding sites in the promoter of Cdc6 are important for Cdc6 activation and regulation of G1/S phase transition by E2F2 or E2F3, although E2F1 does not appear to interact with this promotor,33 and we are unaware of any previous reports on E2F1/YY1 interactions.

To date, there has been only circumstantial evidence pointing toward a possible regulatory link between HO-1 and YY1. Serum deprivation decreases HO-1 in cultured astrocytes34 and YY1 in NIH3T3 cells.35 Conversely, serum repletion of RASMCs increases HO-126 and augments HO-1 and YY1 expression (our unpublished data). Also, experimental ER stress induces HO-1 in VSMCs37 and YY1 in human umbilical vein endothelial cells,38 and both proteins have been reported to be involved in the protection of cells against ER stress.37,39

The present observation that HO-1 inhibits VSMC proliferation via YY1 also extends the known functional connection between the 2 proteins. HO-1 is a key enzyme involved in the reutilization of body iron, making it available for erythropoiesis.40 HO-1 is primarily responsible for the degradation of hemoglobin heme of aged red cells that are...
removed from the circulation by macrophages of the reticuloendothelial system. The iron generated by the action of HO-1 is released to transferrin, transported to the bone marrow, and reused in de novo synthesis of heme. The resulting heme controls the expression of globin genes in erythrocye progenitor cells of the bone marrow, and hence erythropoiesis.40 Interestingly, globin expression is also regulated by YY1, and this plays an important role in globin switching during development. YY1 regulates the expression of the α41,42 and β43 globin clusters, while repressing the expression of embryonic globin.44 In addition, YY1 controls body iron homeostasis by modulating the expression of the transferrin receptor on endothelial cells,45 a process intimately linked to cellular uptake of iron.

We show here that HO-1 activity is required, and the heme oxygenase reaction product CO responsible for, the upregulation of YY1 expression. This suggests an involvement of CO in the cellular signaling that links HO-1 with YY1. Because there is precedence for CO regulating the expression of a transcription factor via ERK,29 we asked whether MAPK signaling was involved in the increase of YY1 by HO-1. Indeed, our results suggest that p38 negatively and JNK positively regulates HO-1 induction by tBHQ in RASMCs. These results agree with previous findings reporting p38 to negatively regulate tBHQ-mediated induction of other genes containing antioxidant response elements in their promoter regions, irrespective of the cell type involved.46-47 In contrast to our results obtained in RASMCs, JNK activation does not appear to be involved in tBHQ-induced gene induction in other cell types.47,48 The role of ERK in tBHQ-induced transcriptional activation seems to be cell-specific, because ERK is a negative regulator in H4IE cells48 and a positive regulator in Hepa1c1c7 cells.49 Irrespective of this, our studies with the MAPK inhibitors provide a separate line of evidence in support of an interdependence of HO-1 and YY1.

Pharmacological induction of HO-1 or overexpression of HO-1 inhibits VSMC proliferation in vivo1,15 and in vitro.10,50 This is most commonly attributed to CO production,30,36 although bilirubin/biliverdin have also been reported to inhibit VSMC growth.51 In VSMCs, CO can activate soluble guanylate cyclase, leading to increased cGMP,52 activation of p38 MAPK,53 and induction of the cell cycle inhibitor p21Waf1/Cip1.54 CO also selectively inhibits the expression of cyclin A and markedly decreases the phosphorylation of retinoblastoma,36 ie, processes that contribute to the inhibition of cell cycle progression. In apparent contrast, inhibition of VSMC growth by YY1 overexpression has been reported to repress p21Waf1/Cip1,55 preventing the assembly of the p21Waf1/Cip1–cdk4–cyclin D1 complex and thereby blocking phosphorylation of retinoblastoma protein. In light of our finding that HO-1 induction increases YY1 and that both proteins are involved in mediating the antiproliferative effect on RASMCs, further studies are warranted to clarify the involvement of p21Waf1/Cip1 along the HO-1/YY1 axis.

It remains unknown how HO-1 overexpression or pharmacological HO-1 induction exerts cell type–specific inhibition...
or enhancement of cellular proliferation. However, our present study suggests that YY1 is intimately linked to the actions of HO-1 and, as such, may affect functions in addition to the control of cell growth.

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Disclosures

None.

References

What Is Known?

- The uncontrolled proliferation of vascular smooth muscle cells (VSMCs), ie, neointimal hyperplasia, remains a key problem after cardiovascular intervention.
- Enhancing the expression of heme oxygenase (HO)-1 (an enzyme that metabolizes heme to carbon monoxide, iron, and biliverdin) attenuates neointimal hyperplasia via both the inhibition of VSMC proliferation and the promotion of formation of an intact endothelium.
- Enhancing the expression of the transcription factor Yin Yang (YY)1 also inhibits VSMC proliferation but without promoting endothelial regrowth.

What New Information Does This Article Contribute?

- The transcription factor YY1 is a downstream effector of carbon monoxide produced by HO-1.
- Inhibition of neointimal hyperplasia in vivo by HO-1 is mediated in part via increases in the transcription factor YY1.

H.O-1 plays a key role in the homeostasis of body iron by degrading heme to carbon monoxide, iron, and biliverdin. More recently, HO-1 has also been shown to have a number of additional functions, the underlying mechanisms of which remain largely unknown. Its expression in blood vessels is associated with inhibition of diseases, including atherosclerosis, transplant rejection, and neointimal hyperplasia following injury. HO-1 also inhibits the proliferation of VSMCs in vitro and in vivo. We now show that in VSMCs, an increase in the expression of HO-1 increases levels of the transcription factor YY1 via HO-1–dependent carbon monoxide production. The increase in YY1 was essential for the ability of HO-1 to inhibit VSMC proliferation in vitro and neointimal formation in vivo. These findings uncover a previously unrecognized link between intermediary metabolism and transcriptional regulation and suggest that increasing HO-1 may be a novel therapeutic strategy for preventing neointimal hyperplasia and restenosis.
Interplay Between Heme Oxygenase-1 and the Multifunctional Transcription Factor Yin Yang 1 in the Inhibition of Intimal Hyperplasia

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Data Supplement (unedited) at:

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Online Data Supplement

Materials

Probucol (4',4'-(isopropylidene dithio)bis(2,6-di-tert-butyl)phenol) was from Medicam, Spain. Sn(IV) Protoporphyrin-IX dichloride (SnPP) and hemin were from Frontier Scientific, and the signalling inhibitors PD98059, SP600125 and SB202190 were obtained from Calbiochem. All other chemicals were obtained from Sigma-Aldrich. Antibodies against HO-1, YY1 and β-actin were purchased from Stressgen (HO-1) and Santa Cruz Biotechnologies (YY1 and β-actin).

Probucol solutions

Probucol stock (100 mmol/L) was prepared by dissolving a known amount of the drug in the appropriate volume of ethanol (99.5% v/v). This stock solution was then diluted 1/100 (v/v) in phosphate buffered saline (50 mmol/L) containing 3% (w/v) essentially fatty acid free bovine serum albumin, and incubated at 37 °C for several hours until the solution clarified. This working solution (1 mmol/L probucol) was then diluted further 1/10 (v/v) in culture media, yielding a final probucol concentration of 100 µmol/L probucol. Control samples were treated the same, except that 100% ethanol was used instead of the probucol stock solution.

Rat Aortic Smooth Muscle Cells (RASMC)

RASMC were obtained from Cell Applications, cultured in DMEM containing 1 g/L glucose (Invitrogen) and 10% fetal calf serum, and used within passage eight at 4 – 8 x 10^5 cells.

Animals and balloon injury

Two groups of male New Zealand White rabbits (1.8-2.2 kb, Merunga Farm, Coffs Harbour, Australia) were fed 100 g per day of normal chow ± 1% (wt/wt) probucol, for 32 days with
water provided ad libitum. Aortic balloon-injury was carried out on day 28 and resulted in complete endothelial denudation.1 Sprague Dawley rats (male, 14-16 weeks old) were obtained from Animal Resource Centre, Perth, Australia. Obese Zucker rats (male, 11–13 weeks old) were obtained from Monash Animal Services (Melbourne, Australia). Rats were fed chow without or with added probucol 1% (wt/wt) for 2 weeks before balloon injury of the left common carotid artery, as described previously.2 Complete endothelial denudation was confirmed by Evan's Blue staining immediately after balloon injury.2

For in vivo inhibition of YY1, 50 µg of siRNA YY1 (rat, 5'r(GAG GUG AUU CUG GUG CAG A)dTdT)3') or scRNA YY1 (control) (5'r(UAG GCU UGA AGA GGU CGA U)dTdT)3') was mixed at 4 °C with pluronic gel F127 (BASF, 0.25%) and 10 µL FuGENE 6 for a final volume of 200 µL, and applied topically around the left common carotid artery immediately after injury and following ligation of the external carotid artery.3 After surgery, animals were fed their respective diet for up to another 2 weeks.

Immunohistochemistry and lesion assessment

Four days or two weeks after balloon injury, the animals were sacrificed, carotid arteries were perfused and fixed with 4 % (v/v) paraformaldehyde in 50 mmol/L phosphate-buffered saline (PBS), harvested and embedded in paraffin. Cross-sections (5 µm) were stained with hematoxylin and eosin for lesion assessment. For immunohistochemistry, cross-sections were stained using mouse monoclonal anti-YY1 antibody (Santa Cruz; dilution 1:150), rabbit polyclonal anti-HO-1 antibody (Stressgen; dilution 1:100), or mouse monoclonal anti-PCNA antibody (Santa Cruz; 1:200) and using avidin-biotin-horseradish peroxidase for detection (Vectorstain Elite ABC kit; Vector Laboratories). An upright light microscope (Zeiss, Jena, Germany) was used for image capture, and the total intima/media cross-sectional area and HO-1 positive stained area were quantified with ImageJ software (http://rsb.info.nih.gov/ij/).
Total and YY1+ cells were counted in a blinded manner either manually at high magnification (40x objective) or using Magellan software (University of New South Wales).

**RNA isolation and real time quantitative RT-PCR**

Cells were washed twice with saline and total RNA was isolated using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. RNA was re-suspended in 20 µL H₂O-DEPC and RNA concentration and purity determined spectrophotometrically. 500 ng RNA were used for preparation of cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using Oligo(dT) as primer and including an RNase H digestion step. The equivalent of 20 ng RNA was then used in real time quantitative PCR reaction using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Primers HO-1-F (5’ GAACCTTTCAGAAGGGTCAGG 3’) and HO-1-R (5’ AAGTAGAGCGGGGCCTAGAC 3’) were used to amplify HO-1 cDNA, whereas YY1-F (5’ AGAAGCAGGTGCAGATCAAG 3’) and YY1-R (5’ ATCAGGAGGTGAGTTCTCTC 3’) were used for YY1 amplification. As internal standard, porphobilinogen deaminase (PBGD) cDNA was determined using PBGD-F (5’ AGATTCTTGATGACTGCCTTC 3’) and PBGD-R (5’ TGAAAGACAACACGACACGACAAGATCAAG 3’). The PCR protocol on a Rotor-Gene 3000 (Corbett Research) was as follows: 2 min at 50 ºC, 10 min at 95 ºC, 40 cycles of 10 sec at 95 ºC and 1 min at 56 ºC. Melting curve analyses and sequencing of the amplification products were performed to verify the specificity of the amplification. HO-1 and YY1 mRNA levels were determined as HO-1 or YY1 mRNA relative to PBGD mRNA, using the comparative CT method.

**Over-expression of HO-1**

To obtain a vector for over-expression of human HO-1, total RNA was isolated from human coronary artery smooth muscle cells (Cell Applications) grown for 24 h in the presence of 25
μmol/L hemin and cDNA prepared using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using Oligo(dT) as primer. Primers hFLHO-1 F1 (5’ GACGAATTCGGATGGAGCGTCCGCAACCC3’) and hFLHO-1 R (5’ CTTCAGTGCCCACGGTAAGGA 3’) were used to amplify the entire HO-1 cDNA. The PCR product was gel purified (Qiaquick gel extraction kit, Qiagen) and ligated into pGEMT-easy (Promega). The insert was subsequently excised with EcoRI and sub-cloned into pcDNA3 (Invitrogen). The pcDNA3-HO-1 construct was digested with XbaI to verify correct orientation, while the correct sequence of the insert was verified by DNA sequencing.

For adenoviral expression, the HO-1 ORF was excised from pcDNA3-HO-1 by BamHI/NotI digestion and sub-cloned into the entry vector pENTR-1A (Invitrogen). The HO-1 ORF was subsequently cloned into pAd/CMV/V5-DEST vector with LR recombinase (Invitrogen). The adenoviral vector carrying HO-1 was digested with PacI (New England Biolabs), and the digestion product used to produce crude and amplified adenovirus stock according to the manufacturer’s instructions.

Stealth RNAi for HO-1 and YY1 knockdown

To knockdown HO-1 and YY1, the following stealth RNAi duplexes (Invitrogen) were used:
sense Hmox-1 RNA (5’ GCAGAGAAUUCUGAGUCAUGAGGA 3’), anti-sense Hmox-1 RNA (5’ UCCUCAUGAACUCAGAAUUCUCUGC 3’), sense YY1 RNA (5’ CCAAGAACAAAUAGCUUGCCCUCUAUA 3’), anti-sense YY1 RNA (5’ UAUGAGGGCAAGCUAUUGUUCUUGG 3’), control sense RNA (5’ CCAAAACAAUAGCUUGCCCUCAGAUA 3’), control anti-sense RNA (5’ UAUCUGAGGGCAAGCUAUUGUUAUGG 3’).

Preparation of total cell extracts
Tissue culture plates were transferred onto ice and cells washed twice with cold PBS. Cells were lysed on ice in RIPA buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EGTA, 0.1 % SDS, 1 % Triton-X100, 0.5 % Na-deoxycholate), containing complete protease inhibitor (Roche Diagnostics) for 5 min followed by 5 min centrifugation at 16,000 g and 4 °C. The protein concentration in the supernate was determined using the BCA protein assay A reagent (Pierce).

**Heme oxygenase activity assay**

The activity of heme oxygenase was determined in microsomes prepared from RASMC. In brief, cells were scraped with a rubber policeman in phosphate-buffered saline with protease inhibitors (Roche) and lysed by 3 freeze-thaw cycles. The cell lysate was centrifuged at 15,000 g at 4 °C for 20 min and the supernate then collected and subjected to ultracentrifugation at 100,000 g at 4 °C for 1 h. The resulting microsomal pellet was suspended in buffer A (250 mmol/L sucrose, 20 mmol/L Tris, pH 7.4). For the heme oxygenase 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 heme (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 h, the reaction stopped by adding 100 µL of ethanol/DMSO (95:5, v/v) and the sample centrifuged at 13,000 g for 5 min. The resulting supernate was used for further analysis. Heme oxygenase enzyme activity was determined by formation of bilirubin as detected by high-performance liquid chromatography, as described previously, with slight modifications. In brief, the supernate 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, v/v) to 100% solvent B (100% methanol) over 18 min, with bilirubin detected at 455 nm.
**SDS PAGE and Western Blot analysis**

Cell extracts containing 5-10 µg protein were denatured, size separated using the 10% NuPAGE gelsystem (Invitrogen) and transferred onto nitrocellulose membranes (GE Healthcare Bio-Sciences). After blocking in 5 % skim milk in TTBS (20 mmol/L Tris, pH 7.5, 0.9 % NaCl, 0.1 % Tween), the membranes were exposed for 60 min to polyclonal rabbit anti-rat HO-1 antibody (Stressgen-895; diluted 1:1000 in blocking solution), monoclonal mouse anti-human YY1 (H-10; Santa Cruz Biotechnology; diluted 1:1000 in blocking solution), or polyclonal goat anti-actin antibody (C-11; Santa Cruz Biotechnology; diluted 1:1000 in blocking solution). Secondary antibodies goat anti-rabbit IgG-HRP (Dako; diluted 1:1500 in blocking solution), rabbit anti-mouse IgG-HRP (Dako; diluted 1:1500 in blocking solution), or rabbit anti-goat IgG (Sigma; diluted 1:2500 in blocking solution) were added for 1 h. After each incubation step the membranes were washed 4 times for 5 min in TTBS. Finally, bands were visualized by chemiluminescence (ECL detection reagents and Hyperfilm ECL, GE Healthcare Bio-Sciences) and analyzed by densitometry using the Geldoc system (Bio-Rad) and QuantityOne software (Bio-Rad).

**BrdU incorporation assay**

RASMC were seeded at 500 cells/well in a 96-well plate and cultured at 37 ºC for 3 days. The cells were then washed twice with sterile saline and further cultured for 30 h in serum free medium containing 0.3 % bovine serum albumin ± 5, 10 or 100 µmol/L probucol. After addition of BrdU labelling reagent and 5 % fetal bovine serum the cells were incubated a further 18 h at 37 ºC.

To assess whether knockdown of HO-1 or YY1 affected the ability of probucol to inhibit RASMC proliferation, 500 cells were seeded per well in a 96-well plate and cultured for 72 h prior to transient transfection with HO-1, YY1 or control stealth RNAi for 24 h. The cells were then serum-starved ± 100 µmol/L probucol, and fetal bovine serum and BrdU
subsequently added as above. BrdU incorporation was determined using a horseradish peroxidase-coupled anti-BrdU antibody, followed by peroxidase reaction with tetramethylbenzidine. The reaction was stopped by addition of $\text{H}_2\text{SO}_4$. Absorption was measured at 450 nm.

References


Online Figure I. Probucol increases YY1 positive cells in neointima in rabbit aortic balloon injury model. Representative immunohistochemistry images of aortic segments obtained 4 days after balloon injury of NZW rabbits fed normal chow without (A) or with 1% (w/w) probucol (B) for a total of 18 days, including 14 days prior to injury. Sections were stained with anti-YY1 antibodies. Black and empty block arrows indicated YY1+ and YY1− nucleus. Bar represents 50 µm. (C) Percentage of medial cell nuclei positive for YY1 in aortic sections from control and probucol-treated rabbits. Four areas (1,600 µm²) from two sections per animal (n = 3 per treatment) were counted, with ~100 nuclei per area, and percentages averaged.
Online Figure II. Knock down of YY1 increases intimal hyperplasia and cell proliferation. (A) Medial YY1 expression, (B) intima-to-media ratio and (C) neointimal PCNA⁺-cells in the carotid artery 14 days post-balloon injury of Sprague Dawley rats fed normal chow and treated with YY1 scRNA or YY1 siRNA at the time of injury. Left, representative images. Right, results from three animals per treatment group, with data obtained from three serial sections per segment. *P<0.05 significantly different to YY1 scRNA control.
Online Figure III. Heme induces expression of YY1 in RASMC. RASMC were treated with 10 µmol/L hemin for up to 6 (A, B) and 24 h (C) before HO-1 and YY1 mRNA (A, B) and protein (C) were determined as described in Materials and Methods. Changes in mRNA levels of HO-1 (A) and YY1 (B) determined in three separate experiments, each performed in triplicate. Blots representative of three separate experiments are shown. The extent of HO-1 (grey bars) and YY1 (black bars) protein expression in the three separate experiments was quantified by expressing the respective Western blot band intensity relative to that of actin. The 0 h-value was set as 1. *Significantly different to 0 h-value.
Online Figure IV. JNK and p38 are positive and negative regulators of interdependent induction of HO-1 and YY1 by tBHQ. RASMC were treated with 5 mmol/L tBHQ and 50 μmol/L PD98059 (PD), 20 μmol/L SP600125 (SP) or 10 μmol/L SB202190 (SB) for 16 h. Total cell extracts were then subjected to Western Blots against HO-1, YY1 and actin as described in Materials and Methods. The results shown are representative of three independent experiments.
Online Figure V. Over-expression of YY1 increases HO-1 in RASMC. Left, Western blots of total cell extracts from RASMC transfected with pCB6+ (control) or pCB6+-YY1 (pYY1) for 72 h. Right, the extent of YY1 (black bars) and HO-1 (grey bars) protein expression was quantified by expressing the respective Western blot band intensity relative to that of actin. The control backbone (pCB6+) value was set as 1. Results shown are derived from three separate experiments. *Significantly different to pCB6+. 
Online Figure VI. SnPP attenuates probucol-induced increase in heme oxygenase activity. Heme oxygenase activity was determined in RASMC cultured for 16 h in the presence of 100 µmol/L probucol, 10 µmol/L SnPP or 100 µmol/L probucol plus 10 µmol/L SnPP. Results shown are from three separate experiments. *P<0.05 compared with other groups.
Online Figure VII. Over-expression of HO-1 does not affect endothelial cell proliferation. Upper panel, bovine aortic endothelial cells were treated with adeno-HO-1 in a dose-dependant manner (MOI 1-100). Total proteins were harvested 72 h after transduction, separated on SDS-PAGE and transferred into a membrane. The membrane was blotted with HO-1 and YY1 primary antibodies and the relevant secondary antibody bodies. Lower panel, BAEC at 2,000 cells per well were seeded onto 96-well plates before transduction with adeno-LacZ (MOI 100) (empty columns) or adeno-HO-1 (MOI 100) (grey columns) for the indicated time. Cells were then trypsinized and counted using an automatic cell counter (Beckman Coulter). The results shown are mean + SEM from an experiment performed in triplicate. A second, separate experiment gave similar results.