Rapamycin Induces Heme Oxygenase-1 in Human Pulmonary Vascular Cells
Implications in the Antiproliferative Response to Rapamycin

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Background—Rapamycin is an immunosuppressive agent with antiproliferative properties against not only lymphocytes but also vascular endothelial and smooth muscle cells, and it reduces the fibroproliferative response to vascular injury. Heme oxygenase-1 (HO-1) has also been shown to have graft protective effects and to inhibit vascular remodeling. In this study, we evaluated whether there is an interaction between rapamycin and HO-1.

Methods and Results—In human pulmonary artery endothelial or smooth muscle cells, HO-1 expression was evaluated in response to rapamycin or wortmannin, an inhibitor of the upstream modulator of mammalian target of rapamycin (mTOR) PI-3K. We also evaluated whether the inhibitory actions of rapamycin on platelet-derived growth factor–dependent proliferation was mediated by HO using the chemical inhibitor tin protoporphyrin. Rapamycin induced HO-1 expression in both pulmonary endothelial and smooth muscle cells, whereas no to little increase was seen in response to another immunosuppressive agent, cyclosporin A. HO-1 expression was also increased in response to wortmannin, suggesting that the PI-3K–mTOR pathway is required for this induction. Inhibition of HO activity resulted in a loss of the antiproliferative activity of rapamycin in growth factor–stimulated smooth muscle cells.

Conclusions—The induction of HO-1 expression by rapamycin and, more importantly, the effects of tin protoporphyrin, an inhibitor of HO activity, on the antiproliferative actions of rapamycin suggest that the effects of rapamycin may be, at least in part, modulated by its actions on HO-1. (Circulation. 2003;107:911-916.)

Key Words: heme oxygenase • sirolimus • wortmannin • cells, endothelial • cells, muscle, smooth
The protective effects of HO-1 may be related to the degradation of the pro-oxidant heme substrate\textsuperscript{18} or through the generation of its products, carbon monoxide (CO),\textsuperscript{19} biliverdin with subsequent generation of bilirubin,\textsuperscript{20} or iron with the induction of ferritin.\textsuperscript{21} CO has properties similar to those of nitric oxide\textsuperscript{22} and alters the expression of inflammatory mediators, tumor necrosis factor, interleukin-1, and interleukin-10.\textsuperscript{19} CO also has a protective role against vascular injury, because it inhibits vascular SMC proliferation.\textsuperscript{6,7} Adenovirus-mediated HO-1 gene transfer inhibits balloon injury–induced neointimal formation, potentially through the generation of CO.\textsuperscript{23,24} Rapamycin also protects against vascular injury by inhibiting vascular SMC proliferation.\textsuperscript{6,7}

In this study, we examined the possibility that the cytoprotective activities of rapamycin may include the induction of HO-1. Human pulmonary artery ECs (HPAECs) and SMCs (HPASMCs) were evaluated for HO-1 expression in response to rapamycin and whether it mediates the antiproliferative activity of rapamycin.

**Methods**

**Reagents**

Rapamycin, wortmannin, platelet-derived growth factor (PDGF), and CSA were purchased from Sigma. Tin protoporphyrin (SnPP) was from Porphyrin Products, and the Cell Proliferation Kit II (XTT) was obtained from Boehringer Mannheim. BCA protein assay reagent and Supersignal Chemiluminescent substrate were purchased from Pierce.

**Cell Culture**

HPAECs were obtained as previously described,\textsuperscript{25} and HPASMCs were obtained from Clonetics. The cells were grown in endothelial growth medium (EGM-MV) (Clonetics) plus 10% FBS (Atlanta Biologicals) at 37°C in room air/5% CO\textsubscript{2} and were passed 1:3 approximately every 4 days at confluence with 0.10% Tryptsin and 0.02 mmol/L EDTA. U937 cells were purchased from ATCC and grown in RPMI (Sigma) plus 10% FBS.

**RNA Isolation and Northern Analysis**

Ten micrograms of total RNA were isolated by the guanidine–acid phenol method and purified with RNeasy mini kits (Qiagen). The RNA was fractionated by size on a 1% agarose/6% formaldehyde gel and transferred to a nylon membrane (GeneScreen, NEN), and crosslinked with ultraviolet light. The RNA was hybridized to a \textsuperscript{32}P-labeled human HO-1 cDNA and rehybridized with a radiolabeled human GAPDH cDNA. The membranes were subjected to autoradiography with an intensifying screen at −85°C, and densitometric analysis was performed using NIH image software.

**Protein Isolation and Western Analysis**

Cells were washed twice with ice-cold PBS, and 10\textsuperscript{5} cells/mL were lysed with Triton lysis buffer. Protein concentrations were determined, and 40 μg of total protein was separated by use of a 10% SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membranes were blocked overnight with 5% nonfat dry milk in 10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, and 0.05% Tween 20 and incubated for 1.5 hours with a 1:1000 dilution of the anti–HO-1 antibody (StressGen). The membrane was incubated with the secondary antibody, peroxidase-conjugated goat anti-rabbit IgG antibody, for 1 hour, and the labeled protein bands were examined by use of a chemiluminescence method according to the manufacturer’s recommendations (Pierce).

**HO Activity Assay**

HO activity was measured by bilirubin generation as previously described.\textsuperscript{26} HPAECs or HPASMCs were grown to confluence in 10-cm tissue culture dishes. After treatment, HPA cells were washed, scraped with a rubber policeman, and pelleted at 3000g for 10 minutes. The pellet was resuspended in 0.1 mol/L KPO\textsubscript{4} and 2 mmol/L MgCl\textsubscript{2}, and the cells were frozen (−80°C) and thawed 3 times to break up the cell membrane. The samples were sonicated on ice, and 10 μL was taken to determine protein concentration. The remaining sonicate was centrifuged at 12 000g at 4°C for 20 minutes, and the supernatant was added to the reaction mixture (400 μL) containing 3 mg rat liver cytosol, 20 μmol/L hemin, 2 mmol/L glucose 6-phosphate, 0.2 units glucose 6-phosphate dehydrogenase, and 0.8 mmol/L β–NADPH and incubated at 37°C for 1 hour in the dark. One milliliter of chlorofrom was added to extract the bilirubin, and the change in optical density at 464–530 nm was measured. The concentration of bilirubin produced in 60 minutes was calculated using the extinction coefficient, 40 mmol/L \textsuperscript{-1} cm\textsuperscript{-1} for bilirubin per mg protein.

**Cell Proliferation**

Proliferation/metabolic activity was evaluated with the tetrazolium-based assay XTT (Boehringer Mannheim) according to manufacturer’s instructions. HPASMCs (1×10\textsuperscript{4}) were incubated in 96-well plates in cell culture media with or without PDGF (10 ng/mL), rapamycin (10.0 μmol/L), or SnPP (5 μmol/L). XTT at a concentration of 0.3 mg/mL was added to each well for 8 hours at 37°C and with 6.5% CO\textsubscript{2}, and the absorbance of the samples was measured between 450 and 500 nm with a reference wavelength >650 nm.

**Statistical Analysis**

Results are expressed as mean±SEM and are representative of at least 3 independent experiments. HO-1 mRNA, protein, and activity levels and XTT cell proliferation assays for the various conditions were evaluated on the basis of 1-way ANOVA using GraphPad InStat software.

**Results**

To determine whether the cytoprotective effects of rapamycin are mediated by HO-1, we first evaluated HO-1 expression in response to rapamycin in HPAECs and HPASMCs. We observed an increase in HO-1 mRNA levels in both HPAECs and HPASMCs. Figure 1A illustrates a Northern analysis showing HO-1 mRNA levels in HPAECs and HPASMCs after 2 to 24 hours of exposure to 10 μmol/L rapamycin. The peak induction was observed after 10.0 μmol/L for 4 hours. The peak induction was observed after 10.0 μmol/L rapamycin in HPAECs and in HPASMCs. Higher concentrations were not tested because 100.0 μmol/L of rapamycin resulted in cell injury as seen by phase-contrast microscopy (data not shown).

To determine whether HO-1 induction was a general response to immunosuppressive agents, we also examined the effect of CSA on HO-1 expression. Exposure up to 10.0 μmol/L CSA resulted in little if any increase in HO-1 mRNA levels in either HPAECs or HPASMCs (Figure 3, A and B); however, like rapamycin, CSA increased the expression of HO-1 in a monocytic cell line, U937 (Figure 3C). This
demonstrates a differential response between rapamycin and CSA depending on the cell type.

To verify that the increase in HO-1 expression in response to rapamycin also reflects an increase in HO-1 protein levels and, more importantly, activity level, we evaluated HO-1 protein and activity in response to rapamycin in both HPAECs and HPASMCs (Figure 4). HO-1 protein and activity were increased in both cell types after 24 hours of rapamycin exposure. HO-1 protein increased 6- to 11-fold in human pulmonary vascular cells, and activity was increased 4-fold, which was similar to hemin, a known stimulus for HO-1.

Rapamycin binding to FKBP12 results in the inhibition of mTOR protein kinase activity. TOR proteins are similar to the lipid kinases, especially phosphoinositide 3-kinase (PI3K), belonging to a larger protein kinase family called the phosphoinositide kinase–related kinases. PI3K activity is critical in growth factor–dependent cell cycle progression and mediates the activation of mTOR by extracellular stimuli. To determine whether this pathway is involved in HO-1 induction, cells were treated with wortmannin, a relatively specific PI3K inhibitor. Exposure of HPASMCs to wortmannin resulted in an increase in HO-1 mRNA levels similar to rapamycin exposure. Figure 5 illustrates HO-1 mRNA

![Figure 1](image1)

A. RNA was isolated from untreated HPASMCs (C) or exposed to vehicle methanol (M) for 4 hours or to rapamycin 10 μmol/L (R) for 2, 4, or 24 hours. An HO-1 and GAPDH mRNA was identified by Northern analysis. B. Graph showing relative fold induction of HO-1 mRNA levels in HPAECs or HPASMCs exposed to 10 μmol/L rapamycin compared with untreated cells, control. There were 2 to 8 independent experiments for various time points.

![Figure 2](image2)

A. Northern analysis of HPAECs exposed to increasing concentrations of rapamycin (0 to 10.0 μmol/L) for 4 hours illustrating HO-1 and GAPDH mRNA levels. B. Graph of relative fold induction of HO-1 mRNA levels in HPAECs and HPASMCs exposed to increasing concentrations of rapamycin for 4 hours (n=2 to 8 independent experiments).
levels in HPASMCs after 2 to 24 hours of exposure to wortmannin and to increasing concentrations of wortmannin. HO-1 mRNA levels were increased 2 and 4 hours after exposure and responded to both low and higher concentrations (0.01 to 10.0 \( \mu \text{mol/L} \)). Similar results were also observed in HPAECs (data not shown).

Rapamycin inhibits growth factor–dependent proliferation of ECs, fibroblasts, and SMCs.\(^3,4\) In addition, activity of HO-1 or one of its products, CO, has also been shown to suppress vascular SMC proliferation in response to an injury.\(^15,23,24\) Like previous studies, we found that rapamycin inhibited PDGF-dependent human SMC proliferation (Figure 6). To determine whether the inhibitory actions of rapamycin on PDGF-dependent proliferation may be mediated by its induction of HO-1, we exposed cells to rapamycin, PDGF, and SnPP, an inhibitor of HO activity. The addition of SnPP resulted in a loss of the suppressive effects of rapamycin on PDGF-stimulated cell growth (Figure 6), suggesting that rapamycin-mediated increases in HO activity contribute to the antiproliferative effects of rapamycin.

**Discussion**

Rapamycin is a new immunosuppressive agent with a unique mechanism of action compared with CSA or FK506.\(^1\)\(^-\)\(^4\) In addition to its antiproliferative effects on lymphocytes, rapamycin has potent antiproliferative activity on a variety of cell types, including SMCs, ECs, and fibroblasts, compared with CSA or FK506.\(^3,4\) Rapamycin inhibits the kinase mTOR, whereas CSA and FK506 are calcineurin inhibitors. Previous studies have compared the antiproliferative effects of rapa-
mycin, CSA, and FK506 in vascular SMCs in vitro and in models of neointimal proliferation. These studies demonstrated that the antiproliferative effects of CSA on vascular cells are mediated indirectly through its immune effects on lymphocytes without any local effects attributed to CSA directly. Interestingly, FK506 antagonizes the antiproliferative properties of rapamycin in vascular SMCs, because both agents bind to the same cytosolic receptor, FKBP12.

Previous studies have also shown that although CSA attenuates neointimal proliferation in the setting of transplantation, it does not reduce neointimal proliferation after balloon injury. However, rapamycin has unique direct antiproliferative effects on vascular SMCs, popularizing its use, rather than that of CSA or FK506, as an agent in stents to prevent vascular restenosis.

HO-1 was recently identified as a graft survival gene with protective actions against early complications such as reperfusion injury of liver isografts, chronic injury, and arteriosclerosis in a cardiac allograft model. In addition, HO-1 expression has been linked to the graft survival actions of the immunosuppressive peptide derived from the HLA class I heavy chain, with the protective effect of this peptide being abolished by inhibition of HO activity.

In this study, we examined the possibility that the cytotoxic and/or antiproliferative properties of rapamycin may be, at least in part, mediated through the induction of HO-1. We found that rapamycin differentially induces the expression of HO-1 in HPAECs, HPASMCs, and U937 cells compared with CSA, which induces the gene in U937 cells but not in HPAECs or HPASMCs. Rapamycin exposure resulted in an increase in both HO-1 mRNA and protein levels, and the increase in HO activity in the HPA vascular cells was similar to that of the potent inducer hemin. This leads to the possibility that some of the actions of rapamycin may be mediated through the induction of HO-1. One such possibility is its antiproliferative activities on nonlymphocytic cells. Rapamycin differentially inhibits growth in both ECs and SMCs, and rapamycin increases HO-1 expression in these cell types compared with CSA.

Chronic rejection results from an abnormal fibroproliferative response with an increase in mesenchymal cells leading to obstruction. Neointimal proliferation after vascular injury is another example of a pathological response to wound healing. The potent antiproliferative actions of rapamycin on vascular SMCs have led to studies evaluating the potential use of rapamycin in vascular injury. Oral rapamycin has been shown to reduce the neointimal formation after balloon-induced injury in an animal model, and implanted stents coated with rapamycin inhibited neointimal formation in animals and in human subjects undergoing coronary angioplasty.

Interestingly, HO-1 is induced in vascular injury, and this induction is believed to be a cytoprotective response against vascular injury, whether it is transplant arteriosclerosis or restenosis in a localized vascular injury. We found that the antiproliferative effects of rapamycin against PDGF-stimulated cell growth was blocked with the chemical inhibitor of HO activity, SnPP. This raises the possibility that the antiproliferative effects of rapamycin against vascular injury and SMC proliferation may be mediated by HO-1 and is consistent with findings by Volti et al that HO-1 induction inhibits vascular SMC proliferation.

The actions of rapamycin are mediated by the formation of a complex with FKBP12, and this complex inhibits the activity of mTOR. The antiproliferative affects of rapamycin on SMCs is also dependent on its binding to FKBP12 and therefore most likely through its inhibition of mTOR activity. Growth factor activation of mTOR is believed to be downstream to a wortmannin-sensitive PI-3K activity, which is an important mediator in growth factor–dependent cell cycle progression.

We found that wortmannin also induced HO-1 expression, suggesting that the PI-3K–mTOR signaling pathway is involved in HO-1 induction. This is a critical pathway in growth factor–dependent proliferation, and HO-1 actions with respect to proliferation may also involve this pathway.
Rapamycin, an immunosuppressive agent, has unique properties distinct from those of the classic calcineurin inhibitors, such as a more potent antiproliferative activity on nonlymphocytic cells. We found that HO-1 is differentially expressed in human pulmonary vascular cells by rapamycin and that the antiproliferative effects may be mediated by this induction. These studies identify a potentially unique graft survival activity of rapamycin, the induction of HO-1, which has not been previously recognized.

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References

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