Bilirubin
A Natural Inhibitor of Vascular Smooth Muscle Cell Proliferation

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Background—Bilirubin, a natural product of heme catabolism by heme oxygenases, was considered a toxic waste product until 1987, when its antioxidant potential was recognized. On the basis of observations that oxidative stress is a potent trigger in vascular proliferative responses, that heme oxygenase-1 is antiatherogenic, and that several studies now show that individuals with high-normal or supranormal levels of plasma bilirubin have a lesser incidence of atherosclerosis-related diseases, we hypothesized that bilirubin would have salutary effects on preventing intimal hyperplasia after balloon injury.

Methods and Results—We found less balloon injury–induced neointima formation in hyperbilirubinemic Gunn rats and in wild-type rats treated with biliverdin, the precursor of bilirubin, than in controls. In vitro, bilirubin and biliverdin inhibited serum-driven smooth muscle cell cycle progression at the G1 phase via inhibition of the mitogen-activated protein kinase signal transduction pathways and inhibition of phosphorylation of the retinoblastoma tumor suppressor protein.

Conclusions—Bilirubin and biliverdin might be potential therapeutics in vascular proliferative disorders. (Circulation. 2005;112:1030-1039.)

Key Words: arteriosclerosis ■ bilirubin ■ cell cycle ■ muscle, smooth ■ signal transition

Vascular diseases such as atherosclerosis, arteriolosclerosis, and restenosis are the leading cause of mortality in the world.1 The accumulation of vascular smooth muscle cells (VSMCs) within the intima is one of the characteristic histopathological changes noted in vessels as a part of primary atheromatous plaque formation as well as restenosis after coronary (or peripheral) stenting, artery bypass grafting, or endarterectomy. These cells are thought to proliferate in the intima in response to various inflammatory and pathogenic stimuli.2 Preventing the proliferation of VSMCs is an important approach to therapy of such disorders.3,4 Heme oxygenase-1 (HO-1) has been shown effectively to inhibit VSMC growth and the development of arteriosclerotic lesions.5–8 However, the potential clinical applicability of expressing HO-1 by induction with substances such as cobalt protoporphyrin IX or by gene therapy is still fraught with problems.9,10 In contrast, the products engendered by HO-1 in the degradation of heme, including carbon monoxide11 and biliverdin/bilirubin that mediate the actions of HO-1, may well be closer to potential application in the clinic. Here we report that biliverdin and bilirubin inhibit injury-induced VSMC proliferation and thus prevent narrowing of the vascular lumen.

Bilirubin has in the past been considered a waste product of the body. When accumulated at abnormally high concentrations in early life, bilirubin is toxic and is responsible for the clinical symptoms of kernicterus.12 However, the antioxidant nature of the bile pigments suggests potential beneficial effects as well.13 Furthermore, there is accumulating evidence from epidemiological studies that individuals with high-normal or just above normal plasma bilirubin levels, including individuals with Gilbert syndrome, have a lesser incidence of coronary heart disease and carotid plaque formation.14 Nevertheless, these association studies do not necessarily implicate bilirubin as causative in the observed decreased atherosclerotic syndromes. To date, there is little evidence that bilirubin itself has a salutary effect on preventing development of atherosclerosis.15 We investigated the
effects of the bile pigments bilirubin and biliverdin on VSMC proliferation in vitro using cultured VSMCs as well as on neointimal formation after balloon injury in rats.

**Methods**

**Reagents**
Bilirubin and biliverdin dihydrochloride (ICN Biomedicals) were dissolved in 0.2M NaOH, neutralized with 1N HCl, adjusted to 10 mmol/L with PBS, sterilized by filtration, and stored at −80°C. All of the experiments in vitro as well as in vivo involving biliverdin/bilirubin were performed with minimal light. SB 203580 (Calbiochem) was dissolved with 0.1% dimethyl sulfoxide.

**Animals**
Adult male (weight, 300 to 400 g) Gunn rats (HsdBlu: GUNN10), congeneric Wistar rats (HsdBlu: GUNN10; Harlan, Indianapolis, Ind), and Lewis rats (weight, 400 to 500 g) (LEW/CrlBR; Charles River, Wilmington, Mass) were used in the balloon injury model. Primary VSMCs were obtained from Wistar rats, Lewis rats, adult male C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine), wild-type B6.129, B6.129-Ahmn1Gomp mice (gift from the National Cancer Institute, Frederick, Md), and wild-type BALB/c and BALB/c-\textsuperscript{Hmox1\textsuperscript{tm1Mlee}} (gift from Drs Yet and Perrella, Boston, Mass). Animals were housed in accordance with the guidelines from the American Association for Laboratory Animal Care and research protocols were approved by our institutional animal care and use committees.

**Balloon Injury Model**
Rats were anesthetized with ketamine (87 mg/kg) and xylazine (13 mg/kg); the left common carotid artery was exposed. After we obtained proximal and distal vascular control with a 6-0 silk ligature, a 2F arterial embolectomy catheter (Baxter) was inserted via the external carotid artery 25 psi and withdrawing it slowly 3 times. The external carotid artery was then ligated, and blood flow was ensured in both of the common and internal carotid arteries.

**Bilirubin Assay**
Blood samples were collected before balloon injury. Bilirubin levels were measured with the total bilirubin assay kit (Sigma Aldrich) in duplicate.

**High-Performance Liquid Chromatography**
For cell culture experiments, bilirubin was measured with the use of high-performance liquid chromatography (HPLC). Cells were serum starved for 24 hours and then stimulated with 10% FCS in the absence or presence of biliverdin for 8 hours. The total cell lysates (200 μg protein) were subjected to size exclusion chromatography (Smart HPLC, Superdex 200 column, Amersham Biosciences). The chromatography was recorded at 280 and 439 nm to monitor the peptide and bilirubin presence in the elution profile.

**Biliverdin Treatment**
Biliverdin dissolved in PBS was injected intraperitoneally 120 and 30 minutes before and 30 minutes after balloon injury at 50 μmol/kg (n=6). For local pretreatment, biliverdin (20, 100, or 1000 μmol/L; n=6) was instilled into the common carotid artery via the external branch for 1 hour; before the balloon injury, biliverdin was washed out. PBS was used for controls.

**Histomorphometric Analysis**
Animals were euthanized 14 days after surgery, and the carotid arteries were collected and snap-frozen. Samples were sectioned (5 μm thick) every 200-μm distance and were stained with hematoxylin-eosin. Six images per vessel were acquired (resolution of 768×512 pixels) with the use of a microscope at ×10 magnification. Distances and areas were calculated by digital imaging software (AxioVision, Carl Zeiss Optics) as number of corresponding pixels. Histomorphometry was analyzed in a double-blind fashion.

**Immunohistochemistry**
Vessels were quick-frozen and stored at −80°C. Cryostat sections were fixed as previously described11 and stained (elastica van Gieson stain). Immunostaining was performed with the use of antibodies directed against SMC-α-actin (Sigma-Aldrich, St Louis, Mo) and Ki-67 (BD Pharmingen, San Diego, Calif).

**Cells**
Primary rat and mouse VSMCs were isolated and cultured as described.16 SMC phenotype was confirmed by α-actin staining. All experiments in which VSMCs were used were conducted under subconfluent conditions.

**Proliferation Assay**
This assay is described in detail in Part 1 of the online-only Data Supplement.

**Flow Cytometry**
Detailed methods for cell cycle analysis and the apoptosis assay are described in detail in Part 2 of the online-only Data Supplement.

**Western Blot Analysis**
Western blot analysis was performed as previously described.17 Details can be found in Part 3 of the online-only Data Supplement.

**Immunocytochemistry**
Detailed methods for immunocytochemistry are described in Part 4 of the online-only Data Supplement.

**Statistical Analysis**
Results were statistically analyzed with Stat View 5.0 with the use of ANOVA. Results are expressed as mean±SD or mean±SEM when indicated.

**Results**

**Hyperbilirubinemian Prevents Neointimal Hyperplasia After Balloon Injury In Vivo**
We first tested the hypothesis that bilirubin itself has a salutary effect on suppressing vascular neointimal formation. The carotid artery in wild-type Wistar rats (HsdBlu: GUNN10) developed maximum neointimal hyperplasia at 2 weeks after balloon injury. The neointima consisted mainly of VSMCs as confirmed by the Ki-67 antigen, which indicates that these cells were actively proliferating (Figure 1A). A significant number of neointimal VSMCs were positive for the Ki-67 antigen, which indicates that these cells were actively proliferating (Figure 1A). In contrast, the artery of hyperbilirubinemiac Gunn rats (HsdBlu: GUNN10), congeneric with the Wistar rats, showed very few proliferating cells (data not shown) and minimal neointimal hyperplasia 2 weeks after balloon injury compared with the control (Figure 1B). Neointimal hyperplasia was quantified by the intima/media ratio (0.50±0.13 versus 1.41±0.28 in controls; P<0.001) and luminal cross-sectional area narrowing, which represents the degree of vascular stenosis within these vessels (18.0±4.4% versus 44.2±7.8% in controls; P<0.001; Figure 1C). We confirmed that Gunn rats, lacking the enzyme UDP-glucuronosyltransferase that is responsible for glucuronidation and thus excretion of bilirubin,18 had significantly higher...
serum bilirubin levels than control wild-type rats (12.0±2.5 versus 0.9±0.5 mg/dL in controls; *P<0.001; Figure 1D).

Biliverdin Ameliorates Neointimal Hyperplasia Associated With Balloon Injury

We also tested whether administration of biliverdin, the precursor of bilirubin, to wild-type rats has an effect on neointima formation similar to that of endogenous bilirubin. The decision to use biliverdin was based on the fact that biliverdin is water soluble, thus facilitating the use of the molecule.19 We assessed whether systemic treatment of wild-type Lewis rats by administering biliverdin intraperitoneally 3 times during the perioperative course at 50 μmol/kg would suppress the development of neointimal hyperplasia after balloon injury. This dose induces tolerance to cardiac allografts in mice.17 Biliverdin significantly suppressed neointima formation as quantified by intima/media ratio (1.07±0.13 versus 1.82±0.17 in controls; *P<0.05) and luminal cross-sectional area narrowing (30.2±2.9% versus 43.2±1.8% in controls; *P<0.05; Figure 1E). We also asked whether even a short local exposure of the carotid artery lumen to biliverdin would ameliorate neointimal hyperplasia after balloon injury. Biliverdin (at a concentration of 100 μmol/L) was administered by intra-arterial local instillation for 1 hour into the carotid artery before balloon injury. Local exposure to biliverdin for 1 hour was sufficient to significantly decrease formation of neointimal hyperplasia compared with PBS-treated controls when examined 2 weeks after injury. This protective effect was associated with lower numbers of Ki-67–expressing cells in the neointimal lesion (data not shown) and in a significantly reduced intima/media ratio (0.71±0.50 versus 1.66±0.38 in controls; *P<0.05) and luminal cross-sectional area narrowing (18.0±8.1% versus 51.3±6.7% in controls; *P<0.001; Figure 1F). Biliverdin at 1000 μmol/L applied locally likewise significantly inhibited intimal proliferation, whereas we did not observe a significant inhibition at 20 μmol/L (data not shown).

Bilirubin/Biliverdin Inhibits VSMC Proliferation Through Cell Cycle Arrest

To understand the underlying mechanism by which bilirubin/biliverdin inhibits neointimal hyperplasia, we examined the effect of bilirubin on VSMC proliferation in primary cell cultures. After 48 hours of serum starvation, stimulation with 10% fetal calf serum (FCS) led to extensive proliferation of both rat primary (data not shown) and mouse primary VSMCs as assessed by [3H]thymidine incorporation 48 hours after stimulation (Figure 2A). Addition of bilirubin or biliverdin into the culture wells at the time of serum stimulation inhibited proliferation of both rat (data not shown) and mouse primary VSMCs. This effect was dose dependent in that higher concentrations of bilirubin/Biliverdin resulted in lower levels of VSMC proliferation (Figure 2A). Because we were interested in dissecting the molecular mechanisms of these effects, we continued our studies in mice rather than rats. The antiproliferative effect of bilirubin/biliverdin was not due to the induction of VSMC apoptosis; significant proportions of neither apoptotic nor necrotic cells were detected in the

Figure 1. Bilirubin/biliverdin suppresses neointimal hyperplasia associated with balloon injury. A, Immunohistochemistry for α-actin and Ki-67 antigen of wild-type (WT) Wistar carotid arteries, 2 weeks after balloon injury; I indicates intima; M, media, and A, adventitia. Arrows indicate cells positive for Ki-67 antigen. B, Immunohistochemistry of control wild-type Wistar and Gunn rat carotid arteries, 2 weeks after balloon injury. C, Intima/media ratio and luminal cross-sectional area narrowing (LCAN) of wild-type Wistar and Gunn rat carotid arteries at 2 weeks after balloon injury. D, Plasma bilirubin levels measured in wild-type Wistar and Gunn rats before balloon injury (n=6; values are mean±SD). E, Histomorphometry of the carotid arteries at 2 weeks after balloon injury with or without systemic biliverdin treatment. F, Histomorphometry of the carotid arteries at 2 weeks after balloon injury with or without local biliverdin instillation. Values are mean±SEM (n=6) in each group. *P<0.001, #P<0.05 vs control.
serum-stimulated VSMCs in the presence of bilirubin or biliverdin. This result was constantly seen even when bilirubin/biliverdin were added at a concentration of 200 μmol/L (Figure 2B). Rather, we observed cell cycle arrest as the sole mechanism by which bilirubin/biliverdin inhibits VSMC proliferation. Twenty-four hours after FCS stimulation, 48.2% ± 0.4% of control VSMCs were at the G0/G1 phase, whereas the remaining cells entered the S or the G2/M phase. In contrast, there were significantly more VSMCs held at the boundary of the G0/G1 phase when these cells were treated with bilirubin/biliverdin (Figure 2A).
with bilirubin (64.3±1.7%; P<0.01; n=3) or biliverdin (76.4±2.7%; P<0.01; n=3; Figure 2C).

### Biliverdin Is Converted to Bilirubin in VSMCs In Vitro

Because biliverdin mimicked the effects of bilirubin on VSMC proliferation, we assessed whether the biliverdin that was added into the culture wells was converted to bilirubin. HPLC analysis of total cell lysates of VSMCs treated with biliverdin for 8 hours showed that the peaks at 439 nm (bilirubin) were 3.6 times (±0.4; P<0.05; n=3) higher than those of control cells to which no biliverdin has been added (Figure 2D), suggesting that biliverdin, at least in part, is converted to bilirubin in the VSMCs.

### Bilirubin Arrests Cell Cycle Progression in VSMCs by Inhibition of Rb Phosphorylation

Because bilirubin arrested cell cycle progression in late G1 in 10% FCS–stimulated VSMCs, we investigated the effect of bilirubin on the key downstream cell cycle regulators of the G1 to S phase transition. Stimulation of the VSMCs with 10% FCS resulted in increased expression of cyclin-dependent kinases (cdk) cdk2 and cdk4 and the cyclins A, D1, and E (Figure 3A and 3B). Bilirubin treatment suppressed expression of cdk2 and cyclin A, D1, and E, whereas expression levels of cdk4 were not affected after bilirubin treatment compared with the control (Figure 3A and 3B). These findings were consistent with inhibition of hyperphosphorylation of the retinoblastoma tumor suppressor protein (Rb) under bilirubin treatment, as demonstrated in nuclear extracts of serum-stimulated VSMCs analyzed 18 hours after 10% FCS stimulation (Figure 4A and 4B). Eighteen hours after 10% FCS stimulation, most of the Rb was found in the nuclei (Figure 4C), consistent with the findings that total protein levels of Rb were higher in the nuclear extracts of 10% FCS–stimulated cells (Figure 4B). After bilirubin treatment, there was less Rb found in the nuclei (Figure 4B), and a significant portion of Rb was kept in the cytosol, similar to what was seen in growth-arrested cells (Figure 4C).

Yin Yang-1 (YY1) is a zinc finger DNA-binding transcription factor known to regulate the expression of genes with important functions in DNA replication, protein synthesis, and cellular response to external stimuli during cell growth and differentiation.20,21 The functions of YY1 are interrelated to Rb.20 We found significantly less YY1 expression at several time points with bilirubin treatment (Figure 4D), consistent with reduced DNA synthesis.

### Bilirubin Mediates its Antiproliferative Actions Through Inhibition of p38 Mitogen-Activated Protein Kinase Activation

p38 mitogen-activated protein kinase (MAPK) is an important mediator in cellular activation and mitogenesis.22,23 Thus, we examined the effect of bilirubin on phosphorylation of p38 MAPK. Stimulation of VSMCs with 10% FCS resulted in a rapid increase of phosphorylated forms of p38 MAPK (8.0±0.7-fold increase) compared with the nonstimulated quiescent state (Figure 5A and 5B). Bilirubin significantly inhibited phosphorylation of p38 MAPK at 5, 15, and 30 minutes after stimulation compared with the control (Figure 5A and 5B).

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**Figure 3.** Bilirubin arrests VSMC cell cycle progression by downregulating cyclins A, D1, and E and cdk2. A, Western blot analysis of cell cycle regulators involved in G1 to S phase transition in growth-stimulated primary VSMCs at various time points in the presence or absence of bilirubin (200 μmol/L). Blots are representative of 3 independent experiments. B, Optical densities (OD) measured in the Western blots shown in A, normalized to β-actin. Values are mean±SEM of 3 independent experiments. &P=NS, *P<0.05 vs 10% FCS–stimulated control.
We obtained additional evidence that inhibition of p38 MAPK correlated with suppression of VSMC proliferation. Blockade in stimulated VSMCs of p38 MAPK with SB203580, a specific inhibitor of p38α and p38β activation, significantly inhibited [3H]thymidine uptake in a dose-dependent manner, with increasing concentrations of SB203580 resulting in decreasing levels of VSMC proliferation (Figure 5C). This was, as with bilirubin, mainly due to the inhibition of cell cycle progression from G1 to the S phase (Figure 5D) and was not due to apoptosis or necrosis when examined at 20 μmol/L SB203580, the highest dose tested (data not shown). Addition of SB203580 resulted in reduced expression levels of cdk2 and YY1, to an extent similar to that after bilirubin treatment (Figure 5E). However, cyclin D1 expression, which was suppressed by bilirubin, was not affected by SB203580 treatment (Figure 5E).

Discussion

Until recently, no physiological functions have been attributed to bilirubin, a potentially toxic tetapyrrole. Three lines of evidence now suggest that bilirubin may have salutary functions. First, bilirubin is a very potent antioxidant that can efficiently scavenge chemically generated peroxyl radicals. Second, several studies have shown that individuals with high-normal or slightly above normal levels of bilirubin have a lesser incidence of atherosclerosis-related diseases than individuals with low-normal levels. These findings do not, however, necessarily implicate bilirubin as the protective...
agent given that they are based solely on association studies. Finally, the administration of bilirubin or its precursor biliverdin to rodents can suppress unwanted reactions such as ischemia/reperfusion injury\textsuperscript{24,25} and allograft rejection.\textsuperscript{17} However, before this study, there were no experimental findings that address the issue of the role of biliverdin/bilirubin in VSMC proliferation.

In the present study, using a clinically relevant rat model of vascular injury, we show that higher blood concentrations of bilirubin in Gunn rats clearly suppress the development of vascular neointimal hyperplasia after balloon injury. In this setting the smooth muscle cells from the injured vascular wall undergo substantial growth inhibition in response to injury. The bilirubin levels present in Gunn rats are several times higher than those seen in humans with high-normal to slightly above normal plasma bilirubin levels. That there are beneficial effects of the elevated bilirubin levels in both cases could reflect either of 2 situations: (1) that the levels of bilirubin in rats and humans needed to provide a salutary effect to prevent intimal hyperplasia are different; or (2) that the levels in Gunn rats are far above those needed to suppress neointimal hyperplasia. We posit that the latter explanation is more likely.

In an approach that might find use clinically for the treatment of vascular proliferative diseases, we instilled biliverdin locally for 1 hour into the carotid arteries before balloon injury. This relatively short pretreatment resulted in significantly diminished neointima formation 2 weeks after balloon injury. This rapid action of biliverdin in vivo is consistent with our findings that biliverdin, even more potently than bilirubin, suppressed p38 MAPK activation in stimulated VSMCs in vitro after 15 minutes of incubation.

**Figure 5.** Bilirubin exerts its antiproliferative effect on VSMCs through inhibition of p38 MAPK phosphorylation. A, Western blot analysis of phosphorylated and total p38 MAPK in growth-stimulated VSMCs in the presence or absence of bilirubin (200 μmol/L). Various time points after stimulation are shown. Blots are representative of 3 independent experiments. B, Relative ratio of phosphorylated vs total protein expression levels of p38 MAPK of the experiment shown in A. Values are mean±SD of 3 independent experiments. C, [\textsuperscript{3}H]Thymidine incorporation of cultured VSMCs at 48 hours after stimulation in the presence or absence of the specific p38 MAPK inhibitor SB203580 at various concentrations. Values are mean±SEM of 3 independent experiments conducted in triplicate. D, Cell cycle progression in VSMCs at 24 hours after 10% FCS stimulation in the presence or absence of SB203580 (20 μmol/L). Analysis shown is representative of 3 independent experiments. E, Western blot analysis of growth-stimulated VSMCs in the presence or absence of SB203580 (20 μmol/L). Cell lysates were probed with antibodies against cdk2, cyclin D1, and YY1. Blots are representative of 3 independent experiments. &P=NS, #P<0.05, *P<0.001 vs 10% FCS-stimulated control.
Bilirubin/biliverdin arrested cell cycle progression in the late G1 phase in growth-stimulated cultures of primary VSMCs by affecting the cell cycle machinery, a target that has been implicated for use in the treatment of vascular proliferative diseases.3,4

Previous studies have suggested that bilirubin at supranormal concentrations binds to the aryl hydrocarbon receptor (Ahr), which is a well-known ligand for dioxin.26,27 Binding of Ahr results in multiple cellular events including G1 cell cycle arrest mediated via hypophosphorylation of Rb.28 We thus asked whether the growth-inhibiting effect of bilirubin on VSMCs is initiated via Ahr. We found that Ahr is not required for the antiproliferative effect of bilirubin as tested in VSMCs of Ahr knockout mice (data not shown).

Others have recently shown that administration of bilirubin in vitro induces apoptosis in VSMCs.8 We have not seen this in our system. With the use of 10% FCS, bilirubin at concentrations up to 200 μmol/L was not cytotoxic, nor did it induce necrosis or apoptosis. The lack of apoptosis in our model is likely important given recent reports that apoptosis of VSMCs can trigger the development of neointima formation.29

Bilirubin peaks analyzed by HPLC in whole cell lysates increased significantly (AU 3.6±0.4 times versus control) measured 8 hours after biliverdin treatment. Considering the fact that the bilirubin and biliverdin effects on VSMC proliferation were independent of HO-1 expression, as tested in VSMCs from HO-1 knockout cells (data not shown), we assume the bilirubin we measured was converted from biliverdin rather than being generated by HO-1 induction. However, we do not know whether it is the bilirubin generated from biliverdin that mediates the biliverdin effects, whether the biliverdin exerts effects on VSMC proliferation itself, and whether, as suggested by others, the redox cycle sustained by the enzyme biliverdin reductase19 is involved in the observed effects. Biliverdin reductase was strongly expressed in the VSMCs used, but no increase was observed after 8 hours of biliverdin treatment, as analyzed by Western blot (data not shown).

The cyclin-dependent kinases cdk2 and cdk4 are key mediators of cyclin D1 expression during G1 to S phase progression of the cell cycle by forming complexes with cyclin A, E, and D1. These complexes phosphorylate a large number of proteins, resulting in hyperphosphorylation of Rb, which then releases transcription factors that promote DNA synthesis.20,30 Bilirubin potently inhibited phosphorylation of Rb and affected Rb localization in a fashion similar to that seen in growth-arrested VSMCs.

Bilirubin exerts its growth suppressor functions in VSMCs at least in part by modulating the p38 MAPK signaling pathway. Bilirubin significantly inhibited phosphorylation of p38 MAPK in VSMC cultures. Several groups have shown that on growth stimulation, MAPKs are phosphorylated and thus activated.23,31 Our data suggest that p38 MAPK is likely to be crucial for the bilirubin effect on the VSMCs, a conclusion strengthened by the observation that the p38α/β-specific inhibitor SB203580 mimicked the effects of bilirubin on VSMC growth inhibition as well as on cdk2 and YY1 expression. Furthermore, it has been reported that transfection of rat carotid arteries before balloon injury with dominant-negative mutants of p38 MAPK inhibits neointima formation.32 However, pharmacological inhibition of p38 MAPK did not downregulate cyclin D1 expression, in contrast to observations after bilirubin treatment. It may be that inhibition of JNK 1/2, which was seen under bilirubin treatment (data not shown), has functional consequences in addition to those related to the inhibition of p38 MAPK. Cyclin D1 expression can be dependent on JNK 1/2.33 Furthermore, JNK 1/2 is implicated in balloon injury: it is overexpressed after injury,34 and transfection of rat carotid arteries with dominant-negative mutants of JNK prevents neointima formation after injury.35 Others have recently demonstrated that bilirubin inhibits phosphorylation of ERK 1/2 in human airway pulmonary smooth muscle cells36; however, in the VSMCs used, we did not observe inhibition of ERK 1/2 after bilirubin treatment, which might be explained by the different cell type studies.

Taken together, our data suggest that bilirubin treatment inhibits VSMC growth by impaired activation of MAPKs, resulting in 2 events. First, Rb phosphorylation is inhibited via inhibition of expression of cyclins D1, A, and E as well as cdk2. These events impede the release of transcription factors such as YY1 that are important for VSMC growth. Second, there is reduced expression of the transcription factor YY1 itself, resulting in a suppressed capacity to induce DNA synthesis and transcriptional activity.

We suggest that the potent antioxidant effects of bilirubin, at least in part, may explain our findings. It is known that redox alterations promote neointimal growth in induced VSMC proliferation and that the superoxide-generating enzyme NADPH oxidase plays a pivotal role in this scenario.37 Bilirubin is a highly efficient radical scavenger and has the potential to oppose the cellular redox alterations, such as scavenging of peroxyl radicals,13 that occur in response to vascular injury. Recent data showing that antioxidants suppress p38 MAPK and JNK, but not ERK activation, support this suggestion.38 Whether or not it is the antioxidant effect of biliverdin/bilirubin that accounts in part or in full for the present findings, we present here new information with regard to signaling and gene expression related to biliverdin/bilirubin.

We tested bilirubin/biliverdin in part to assess whether we could engender experimental evidence supporting bilirubin as the molecule that explains the association of high-normal levels of bilirubin in humans with fewer atherosclerotic-related disorders. We find that bilirubin has effects experimentally, in cells and in animal models tested here, that would help to explain the population associations, thus providing support for the suggestion that bilirubin may participate in the maintenance of vascular homeostasis.

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References

5. Yet SF, Layne MD, Liu X, Chen YH, Lith B, Sbinga NE, Perrell MA. Absence of heme oxygenase-1 exacerbates atherosclerotic lesion for-
6. Durante W. Heme oxygenase-1 in growth control and its clinical appli-
oxygenase-1 gene expression stimulates apoptosis in vascular smooth
10. Pislaru S, Janssens SP, Gersh BJ, Simari RD. Defining gene transfer
potential interventions and routes of delivery. Circulation. 2001;103:
11. Otterbein LE, Zuckerbraun BS, Haga M, Liu F, Song R, Usheva A,
Kawamura K, Ishikawa K, Wada Y, Kimura S, Matsumoto H, Kohro T,
Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Biliverdin is a
natural product of heme catabolism, induces tolerance to cardiac allografts.
12. Barnes-Ellerbe S, Knudsen KE. Puga A. TCDD blocks androgen-
dependent cell proliferation of LNCaP cells through modulation of RB
muscle cell proliferation requires both p38 and BMK1 MAP kinases. Arch
14. Yeary RA, Grothaus RH. The Gunn rat as an animal model in com-
Heme oxygenase-1-derived bilirubin ameliorates postischemic myo-
vascular response to injury: the redox hypothesis of restenosis. Car-
major physiologic cytoprotectant. Proc Natl Acad Sci U S A. 2002;99:
Kreuzer J. JNK and c-Jun but not ERK and c-Fos are associated with
2003;33:11–17.
bilirubin-dependent modulation of ERK1/2 phosphorylation. J Biol
Heme oxygenase-1 expression in experimental pulmonary hypertension in
21. Palko L, Bass HW, Beyrouthy MJ, Hurt MM. The Yin Yang-1 (YY1) pro-
gene undergoes a DNA-replication-associated switch in localization
22. Waskiewicz AJ, Cooper JA. Mitogen and stress response pathways: MAP
kinase cascades and phosphatase regulation in mammals and yeast. Curr
23. Shennan S, Hamilton J, Cooper JA. Potential role of MAPKs in vascular 
24. Bartek J, Bartkova J, Lukas J. The retinoblastoma protein pathway and
discussion of a locally delivered caspase inhibitor in a balloon injury model.
25. Shenk T, Usheva A. Interaction between YY1 and the retinoblastoma
protein undergoes a DNA-replication-associated switch in localization
before expecting gene therapy: putting the horse before the cart. Circu-
26. Otterbein LE, Zuckerbraun BS, Haga M, Liu F, Song R, Usheva A,
Stachulak C, Bodyak N, Smith RN, Csizmadia E, Tyagi S, Akamatsu Y,
Shenk T, Usheva A. Interaction between YY1 and the retinoblastoma
protein undergoes a DNA-replication-associated switch in localization
before expecting gene therapy: putting the horse before the cart. Circu-
27. Hoffman EC, Reyes H, Chu FF, Sander F, Conley LH, Brooks BA,
Hankinson O. Cloning of a factor required for activity of the Ah (dioxin)
28. Barnes-Ellerbe S, Knudsen KE. Puga A. TCDD blocks androgen-
dependent cell proliferation of LNCaP cells through modulation of RB
of JNK, p38, and ERK in platelet-derived growth factor–induced vascular
proliferation, migration, and gene expression. Arterioscler Thromb Vasc
30. Schwabe RF, Bradham CA, Uehara T, Hatano E, Bennett BL,
Schoonhoven R, Brenner DA. c-Jun-N-terminal kinase drives cyclin D1
expression and proliferation during liver regeneration. Hepatology. 2003;
37:824–832.
Kreuzer J. JNK and c-Jun but not ERK and c-Fos are associated with
32. Izumi Y, Kim S, Namba M, Yasumoto H, Miyazaki H, Hoshiga M,
Kaneda Y, Morishita R, Zhan Y, Iwao H. Gene transfer of dominant-
negative mutants of extracellular signal-regulated kinase and c-Jun NH2-
terminal kinase prevents neointimal formation in balloon-injured rat
G, Fadel E, Yamaguchi T, Marthan R, Auhier M, Boczkowski J. Heme
oxygenase inhibits human airway smooth muscle proliferation via a
bilirubin-dependent modulation of ERK1/2 phosphorylation. J Biol
34. Zhan Y, Kim S, Namba M, Yasumoto H, Miyazaki H, Hoshiga M,
Kaneda Y, Morishita R, Zhan Y, Iwao H. Gene transfer of dominant-
negative mutants of extracellular signal-regulated kinase and c-Jun
NH2-terminal kinase prevents neointimal formation in balloon-injured rat
G, Fadel E, Yamaguchi T, Marthan R, Auhier M, Boczkowski J. Heme
oxygenase inhibits human airway smooth muscle proliferation via a
bilirubin-dependent modulation of ERK1/2 phosphorylation. J Biol
Our study demonstrates that biliverdin and bilirubin, natural products of the degradation of heme by heme oxygenase-1, potently block vascular smooth muscle cell (VSMC) proliferation after balloon angioplasty in rats. VSMC proliferation is a major component of the early phase of atherosclerosis and restenosis after angioplasty; the proliferation of the VSMC leads to vascular narrowing. Population studies in humans have also related bilirubin to atherosclerotic-type disease. It has now been extensively documented that there is a strong association between high-normal (or just above normal) levels of bilirubin and a lesser frequency of atherosclerosis-like diseases. Individuals with Gilbert syndrome, who have several times the normal levels of bilirubin, are also protected. To test whether bilirubin would inhibit VSMC proliferation in an experimental system, we studied rats that have congenitally high levels of bilirubin. Those rats responded with significantly less VSMC proliferation in response to injury than did their congenic wild-type controls. In addition, biliverdin and bilirubin inhibited VSMC growth in vitro by cell cycle arrest. The combination of our findings that bilirubin per se inhibits VSMC proliferation in vitro and intimal proliferation in vivo as well as studies in humans provides strong support for our conclusion that bilirubin, when present at high enough levels, is a powerful protective agent against neointimal hyperplasia that is seen in atherosclerosis.
Bilirubin: A Natural Inhibitor of Vascular Smooth Muscle Cell Proliferation
Robert Öllinger, Martin Bilban, Anna Erat, Alberto Froio, James McDaid, Shivraj Tyagi, Eva Csizmadia, Aurelio V. Graça-Souza, Angela Liloia, Miguel P. Soares, Leo E. Otterbein, Anny Usheva, Kenichiro Yamashita and Fritz H. Bach

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