Biliverdin Administration Prevents the Formation of Intimal Hyperplasia Induced by Vascular Injury

Atsunori Nakao, MD; Noriko Murase, MD; Chien Ho, PhD; Hideyoshi Toyokawa, MD; Timothy R. Billiar, MD; Shinichi Kanno, MD

Background—Autologous vein grafts and balloon angioplasty are still commonly used for arterial reconstructive procedures. Their success is limited by the development of intimal hyperplasia (IH). Biliverdin (BVD), one of the by-products of heme degradation, has been shown to have potent antioxidant and antiinflammatory effects. We hypothesized that BVD administration would protect vascular tissue against vascular injury.

Methods and Results—The effects of BVD administration against IH after vascular injury were analyzed in an arterialized vein graft model and a balloon injury model in rats. BVD treatment significantly suppressed the development of IH in both models compared with those without BVD. The mechanisms by which BVD treatment inhibits IH development might include decreasing c-Jun NH2 terminal kinase activation and preventing apoptosis of endothelial cells. BVD also suppressed vascular smooth muscle cell migration in vitro.

Conclusions—BVD administration prevented IH associated with arterialized vein graft vasculopathy or balloon angioplasty–induced vessel injury. These results suggest that a treatment regimen with exogenous BVD administration could provide an effective therapeutic adjunct to facilitate transfer of experimental treatments for vascular injury to the clinic. (Circulation. 2005;112:587-591.)

Key Words: angioplasty ☐ antioxidants ☐ apoptosis ☐ reperfusion ☐ restenosis

Intimal hyperplasia (IH) resulting from vascular injury initiated by procedures such as surgical bypass grafting with vein grafts1 or percutaneous transluminal angioplasty2,3 continues to limit the success of these therapeutic interventions. Many pharmacological agents, including antithrombotics, antiplatelet agents, and angiotensin-converting enzyme inhibitors, as well as intraluminal irradiation, have been used in an attempt to attenuate this injury response.4 Infiltration/activation of circulating leukocytes, immune system–mediated injury to endothelial cells (ECs), and smooth muscle cell (SMC) migration and proliferation are thought to be central to the development of IH.5

Heme oxygenases (HOs) are ubiquitous enzymes that degrade heme to form carbon monoxide (CO), biliverdin (BVD), and free iron. HO-1 is the only inducible form of HO and has been reported to confer cytoprotection against oxidative stress.6 These by-products released from heme degradation have been reported to mediate HO-1 protective effects. CO provides cytoprotection against ischemia/reperfusion (I/R) injury in rodents7 and suppresses development of IH associated with transplant-induced arteriosclerosis and angioplasty.8 BVD administration also attenuates I/R injury after small intestinal,9 heart, and kidney transplantation10 via antioxidant and antiinflammatory effects. Injury of ECs, which are the main targets of I/R injury, leads to the upregulation of adhesion molecules and proinflammatory mediators and activates coagulation cascades, resulting in facilitation of SMC migration and proliferation in vascular tissue.11,12 Therefore, the protection from vascular EC injury due to I/R or mechanical trauma is critical for the healing response after vascular injury. We hypothesized that BVD administration would protect ECs and inhibit development of IH after vascular injury. In the present study, we sought to determine the efficacy of BVD administration against vascular injury–induced IH in 2 models of vascular injury, an arterialized vein graft model and a model of balloon angioplasty–induced vessel injury.

Methods

Animals

Inbred LEW rats, 200 to 250 g, were purchased from Harlan-Spraque Dawley, Inc (Indianapolis, Ind). Animals were fed a standard diet and water ad libitum. All animal procedures were performed in accordance with the Council on Animal Care at the University of Pittsburgh or Carnegie-Mellon University and the National Research Council’s Guide for the Humane Care and Use of Laboratory Animals.

Received September 27, 2004; revision received March 29, 2005; accepted April 12, 2005.


Correspondence to Shinichi Kanno, MD, Department of Surgery, University of Pittsburgh, School of Medicine, W1554, Biomedical Science Tower, 200 Lothrop St, Pittsburgh, PA 15213. E-mail shk34@pitt.edu

© 2005 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org DOI: 10.1161/CIRCULATIONAHA.104.509778

587
BVD Treatment
BVD HCl (Frontier Scientific) was dissolved in 0.2N NaOH, adjusted to a final pH of 7.4 with HCl, and subsequently diluted with saline (0.9% NaCl) to the final concentrations. Final pH was between 5.5 and 5.9 in each BVD solution used in this study. For the arterialized vein graft model or the in vitro wound migration assay, BVD was diluted in saline and used as stock solution. For the balloon injury model, BVD (50 mg/kg in 1 mL) or saline was injected intraperitoneally 2 hours before surgery and also immediately after angioplasty.

Microsurgical Procedure for Vein Grafting
A 20-mm-long segment of inferior vena cava (IVC) was excised and preserved in 2 mL heparinized saline solution or BVD solution (10 μmol/L) at 4°C as shown in Figure 1. A 15-mm segment of the abdominal aorta of the recipient animal was resected, and the divided ends of the artery were irrigated with heparinized saline solution. The IVC graft was interposed into the aortic defect according to the cuff technique. Arterial occlusion time ranged from 5 to 10 minutes. When the arterial occlusion clamps were removed, immediate restoration of blood flow was confirmed. Animals were humanely killed 14 days after grafting. The vein graft samples were snap-frozen in LN2 and kept at −80°C until analysis.

Balloon Injury Model
The left carotid artery was exposed and subjected to balloon catheter injury as previously described.13 In brief, an arteriotomy was made in the external carotid artery, and a 2F Fogarty catheter (Edwards Lifesciences) was inserted into the common carotid artery. The balloon was inflated to 5 atm of pressure for 5 minutes. After the balloon was deflated and the external carotid artery was ligated, the common carotid artery was flushed to ensure return of blood flow through the common and external carotid artery.

Histomorphometric Analysis
Vein grafts and carotid arteries were harvested at 14 days after injury. Vessels were fixed in 10% paraformaldehyde and embedded in paraffin at room temperature or OCT compound at −80°C. The slides were stained with hematoxylin/eosin, Verhoeff–van Gieson’s, or Masson’s trichrome stain. Images of the cross section of the vessels were photographed with a Zeiss microscope (Axioskop) and placed on a color scanner (EPSON Smart Panel). After the digitized images were obtained, areas were measured by computer planimetry with NIH Image (v1.62) software and expressed as percent area of intimal/intima + media) in each cross section.

Immunoblot Analysis
Protein taken from the snap-frozen samples of vein grafts was separated on sodium dodecyl sulfate–polyacrylamide gels and electroblotted to a polyvinylidene difluoride membrane. Phosphorylated c-Jun NH2-terminal kinase (JNK) 1/2 and JNK2 were detected with a polyclonal antibody (New England Biolabs, Inc) and polyclonal antibodies (Santa Cruz Biotechnology, Inc), respectively. Phosphorylated p38 mitogen-activated protein kinase (MAPK), p38MAPK, phosphorylated extracellular signal-regulated kinase (ERK) 1/2, ERK1/2, and phosphorylated c-Jun were detected with polyclonal antibodies (Cell Signaling Technology Inc).

Endothelial Apoptosis
In situ detection of endothelial apoptosis in paraffin-embedded graft tissues was performed with a VasoTACS KIT (Trevigen, Inc) according to the manufacturer’s protocol.

Reductive Capacity
Freshly frozen samples of vein graft tissues taken 1 hour after reperfusion and control normal samples were placed in saline or BVD solution for 5 minutes, homogenized, and analyzed for total antioxidant power (Oxford Biomedical Research) with the supernatant as previously described.3,10 This assay measures reductive capacity by detecting the reduction of Cu2+ to Cu+.

Wound Migration Assay
Confluent monolayers of vascular smooth muscle cells on 35-mm type-I collagen–coated dishes were wounded with a razor blade14 and incubated with or without pretreatment with BVD 12 hours before wounding. The cells that had migrated across the edge of the wound were counted after 24 hours.

Statistical Analysis
Results in this study were expressed as mean±SD. Statistical analysis was performed with Student t test or ANOVA where appropriate. A value of P<0.05 was considered statistically significant.

Results
The capacity of BVD administration to inhibit IH was first assessed in a model of vascular remodeling involving the arterization of vein grafts. Minimal IH was seen in vein segments exposed to only 5 minutes of cold ischemia with or without BVD exposure (Figure 2A) When the vein grafts were exposed to an extended period of cold ischemia (2 hours), a marked increase of IH was seen (Figure 2B). Incubation in BVD ex vivo completely blocked the IH seen with cold ischemic storage (Figure 2C and 2D).

To explore the mechanisms mediated in the BVD effect, MAPK activation was assessed at 7 days after grafting. Phosphorylated JNK1/2 was easily detected in the vein graft tissues was performed with a VasoTACS KIT (Trevigen, Inc) according to the manufacturer’s protocol.
EC death may be a factor in the level of IH; therefore, the presence of apoptosis was assessed in the grafts at 14 days. A 4-fold increase in apoptotic ECs was noted in grafts from group 3. Apoptotic rates were reduced by 57% in the BVD-treated group (group IV) (Figure 3B).

To assess the role of redox stress as a factor in BVD protection, the reductive capacity in the vein grafts was measured by an antioxidant power assay kit. The incubation in BVD solution or minimal cold ischemia did not change the reductive capacity. BVD administration for the normal animals did not affect the reductive capacity. The reductive capacity of the 2 hour–preserved vein grafts was significantly impaired 1 hour after reperfusion. However, BVD treatment maintained that capacity (Figure 3C).

SMC migration and proliferation are features of IH. To determine whether BVD had a direct effect on SMCs, a wound migration assay was performed to measure the effect of BVD on SMC migration. In control SMC cultures, active cell migration was easily detected 24 hours after wounding. Coincubation with BVD suppressed SMC migration in both in 1 and 10 \( \mu \)mol/L solution without loss of cellular viability (Figure 4).

To determine whether BVD administration in vivo also influenced the development of IH, BVD was administered in rats subjected to carotid artery balloon injury. As expected,
balloon injury resulted in the development of IH 14 days after injury. This was significantly decreased by 61% in animals receiving BVD (50 mg/kg) 2 hours and just after balloon injury (Figure 5).

Discussion

Atherosclerosis contributes to ≈50% of deaths in the United States and Europe, being the predominant process underlying myocardial ischemia, cerebral ischemia, and peripheral arterial insufficiency. Current therapies, such as angioplasty or surgical bypass with vein grafts, involve exuberant SMC proliferation that leads to IH and restenosis. The vascular healing response comprises several phases. First, disruption of ECs initiates platelet and leukocyte adhesion with the release of numerous chemotactic and mitogenic factors. After this initial phase, medial SMCs begin to proliferate and migrate to form a neointima. This proliferative phase lasts 2 to 4 weeks. Subsequent increases in neointimal lesion size occur through continued upregulation of extracellular matrix synthesis and deposition, which can persist for months.

In the present study, we demonstrated that BVD administration had vasculoprotective effects similar to those seen with HO-1 overexpression. This suggests that potent protective effects of BVD and bilirubin might account for some of the protective effects of HO-1. Because bilirubin is oxidized to BVD and then recycled by biliverdin reductase back to bilirubin, a cycle for the regeneration of bilirubin could maintain the antioxidant activity. Bilirubin is most likely involved in antioxidant activity via H donation to an incipient radical in this recycling; however, BVD also has antioxidant activity owing to formation of a resonance-stabilized, carbon-centered radical resulting from the addition of radicals. Additional experiments will be required to determine whether the protection afforded by BVD administration is due to BVD itself, bilirubin, or both.

Oxidant injury can activate cell signaling pathway such as JNK. JNK phosphorylation has been associated with cell death in I/R. In our model of ex vivo BVD exposure, we saw a reduction of JNK phosphorylation, c-Jun phosphorylation, and EC apoptosis. Although these results only associate the events with the beneficial effects of BVD administration, it is likely that the antioxidant actions of BVD persist in the grafts for periods adequate to interfere with the activation of this pathway that is essential to the injury response leading to IH.

Because BVD is rapidly converted to bilirubin, BVD is normally not detectable in the blood. After a single intraperitoneal injection of BVD (50 mg/kg), the serum bilirubin level increased and peaked at 1.07 ± 0.5 mg/kg at 30 minutes after injection, from <0.04 mg/kg at the normal level, and thereafter returned quickly toward normal levels by 2 hours. Although the potential actions of bile pigments in humans range from itching to severe neuronal damage, primarily of basal ganglia as observed in kernicterus, a more general toxic action is assumed to result from damage of lipid bilayers of biological membranes. We have not found any adverse effects of BVD treatment up to 14 days of follow-up in our protocol. Further study would be needed for to determine the long-term outcome of BVD treatments.

CO, another product of the HO system, also has been shown to prevent IH and reduce organ injury in models of I/R. Thus, CO was thought to account for mediating the protective effects of HO-1 overexpression in some models. Our data are the first to show the potent effects of BVD in models of IH, indicating a possible cooperative benefit between CO and BVD during upregulation.

In summary, this study showed that BVD treatment suppresses 2 key steps in IH development in vascular injury. BVD administration may protect ECs from free radical–mediated insult and inhibit SMC migration by decreasing JNK activation. These results suggest that a treatment regimen with exogenous BVD administration, before vascular grafting as well as balloon angioplasty, could be an effective treatment strategy to limit IH.
Acknowledgment
This work was supported by National Institutes of Health grants HL 066949 and GM044100, and a Thomas E. Starzl Transplantation Institute Young Investigator Grant. We thank Mike Tabacek, Lisa Chedwick, and Donna B Stolz, PhD (Center for Biologic Imaging, University of Pittsburgh, Pittsburgh, Pa) for excellent technical support.

References
Biliverdin Administration Prevents the Formation of Intimal Hyperplasia Induced by Vascular Injury
Atsunori Nakao, Noriko Murase, Chien Ho, Hideyoshi Toyokawa, Timothy R. Billiar and Shinichi Kanno

_Circulation_. 2005;112:587-591; originally published online July 18, 2005; doi: 10.1161/CIRCULATIONAHA.104.509778

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/112/4/587

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/