Bilirubin exerts pro-angiogenic property through Akt-eNOS-dependent pathway

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Running title: Bilirubin action on angiogenesis

Abstract

Low serum bilirubin levels are associated with the risk of cardiovascular diseases including peripheral artery disease. Bilirubin is known to exert its property such as anti-oxidant effect or the enhancement of flow-mediated vasodilation, however, bilirubin action on angiogenesis remains unclear. To investigate the molecular mechanism of bilirubin on angiogenic effect, we first employed C57BL/6J mice with unilateral hindlimb ischemia surgery and divided the mice into two groups (vehicle-treated group and bilirubin treated group). The analysis of laser speckle blood flow demonstrated the enhancement of blood flow recovery in response to ischemia of mice with bilirubin treatment. The density of capillaries was significantly higher in ischemic adductor muscles of bilirubin-treated mice. The phosphorylated levels of endothelial nitric oxide synthesis (eNOS) and Akt were increased in ischemic skeletal muscles of mice with bilirubin treatment compared vehicle treatment. In *in vitro* experiments by using human aortic endothelial cells, bilirubin augmented eNOS and Akt phosphorylation, cell proliferation, cell migration and tube formation. These bilirubin actions on endothelial cell activation were inhibited by LY294002, a phosphatidylinositol 3-kinase inhibitor. In conclusion, bilirubin promotes angiogenesis through endothelial cells activation via Akt-eNOS-dependent manner.

Keywords: Bilirubin, angiogenesis, endothelial cells, eNOS

Introduction

Bilirubin is a metabolic end product following heme degradation. Hyperbilirubinemia has an aspect of cytotoxic action to cause bilirubin-induced neurological dysfunction in newborn (1). On the other hand, people with mild unconjugated nonhemolytic hyperbilirubinemia, called as Gilbert syndrome (2), have shown to be low prevalence of cardiovascular disease such as ischemic heart disease (3). Moreover, many studies have shown that serum bilirubin levels are inversely associated with the incidence of coronary heart diseases (4, 5), carotid plaque and intima-media thickness (6, 7), and stroke (8-10) even in patients without Gilbert's syndrome. Low concentration of bilirubin exerts anti-oxidant property (11), and the oxidative stress is actually lower in patients with Gilbert's syndrome than those without Gilbert's syndrome (12). Therefore, the potent protective mechanism of bilirubin on cardiovascular diseases is considered as its anti-oxidant property.

Increased serum bilirubin is an independent factor to reduce the prevalence of peripheral artery disease (PAD) (13). Angiogenesis plays an crucial role in the collatreal blood formation ischemia in response to insufficient peripheral circulation (14), and the augmentation of angiogenesis is an important therapeutic strategy for PAD. endothelial nitric oxide synthase (eNOS) has been shown to be a main regulator for angiogenesis through endothelial cells activation in both *in vivo* (14-16) and *in vitro* (17, 18). Bilirubin action on endothelial function has been also elucidated. High serum bilirubin preserves coronary flow reserve in healthy subjects (19). Endothelium dependent-flow-mediated vasodilation is higher in healthy subjects with high bilirubin (7) and Gilbert's syndrome (12). Thus, bilirubin is suggested to participate in endothelial function, however, the mechanism by which bilirubin exerts direct action

on endothelial cells has been still unclear

In the present study, we examined the molecular mechanisms of bilirubin action on endothelial function by using endothelial cells and mice ischemic hindlimb model. Bilirubin promoted vascular endothelial cells activation and angiogenesis in response to ischemia through Akt-eNOS-dependent manner.

Methods

Materials

Bilirubin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The following commercially available antibodies were used in this study: anti-phospho-Akt (Ser473), anti-total Akt, anti-phospho-eNOS (Ser1177), anti-phospho-AMPKα (Thr172), and anti-total AMPKα from Cell Signaling Technology (Beverly, MA, USA); anti-total eNOS from Santa Cruz Biotechnology (Santa Cruz, CA USA); anti-α-tubulin, as a loading control, from Calbiochem (San Diego, CA, USA); anti-CD31 (PECAM-1) from Becton, Dickinson and Company (BD; Tokyo, Japan); and anti-α smooth muscle actin (αSMA) from Sigma-Aldrich (St. Louis, MO, USA). LY294002, a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Matrigel was obtained from BD Biosciences Japan (Tokyo, Japan). The CellTiter 96 AQueous nonradioactive cell proliferation assay kit ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) reagent) was purchased from Promega KK (Tokyo, Japan). Tempol, a free radical scavenger, was purchased from Sigma-Aldrich.

Murine model of ischemic hindlimb

All experimental procedures for mice were performed in accordance with the guidelines of the Animal Research Committee, University of Tokushima Graduate School. Eight-week-old C57/BL6J mice were obtained from Nippon CLEA (Tokyo, Japan) and divided into 2 groups: Bilirubin (5 $mg \cdot kg^{-1} \cdot d^{-1}$)-treated group and a vehicle-treated group. Mice were injected intraperitoneally with either Bilirubin or vehicle 2 d before the surgery to induce ischemic hindlimb as previously described (20). In brief, mice were anesthetized with intraperitoneal pentobarbital (50 $mg \cdot kg^{-1}$) injection and subjected to unilateral hindlimb surgery. After ligation of the proximal and distal ends of the left femoral artery and vein, the entire artery and vein with side branches were excised.

Evaluation of peripheral blood flow

The blood flow in hindlimb was measured before and immediately following surgery, on postoperative days 3, 7, 14, and 28 by using a laser speckle blood flow (LSBF) analyzing system (Omega Zone, Omega Wave Co., Tokyo, Japan), as previously described (20).

Blood pressure measurement

Mice blood pressure was measured by the tail-cuff method as previously described (20).

Capillary density and Arterioles number

Capillary density was estimated by CD31 immunohistochemistry (20, 21). Briefly, 28 d after surgery, adductor muscles were snap frozen in liquid nitrogen-cold isopentane

containing optimal cutting temperature compound and cut into 8 μm cryosections. Capillary density was expressed as the number of CD31-positive cells corrected for the number of muscle fibers. Arterioles number was also evaluated by αSMA immunofluorescence staining. Quantification of arterioles was expressed as the number of αSMA-positive cells/mm^2 of muscle area as described previously (20).

Plasma bilirubin concentration measurement

We used QuantiChrom™ Bilirubin Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions to determine plasma bilirubin concentration.

Cell culture

Human aortic endothelial cells (HAECs) were purchased from Takara Bio Inc. (Otsu, Japan) and cultured in Endothelial Cell Growth Medium MV 2 (Heidelberg, Germany), according to the manufacturer's protocol. HAECs during 5-8 times passage were used in each experiment. Cells were treated with Bilirubin dissolved in sterilized water. In some experiments, cells were pretreated with LY294002 (2 μM), tempol (100μM) or vehicle alone for 1h before bilirubin treatment.

Tube-like formation assay

Tube-like formation assay was performed by using growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA), as described previously (20, 21). Briefly, 1×10^4 HAECs were cultured per well of Matrigel-coated 96-well plate in the presence of each concentration of bilirubin or vehicle, with or without 2 μM LY294002. Cells were then incubated at 37°C for 18 h. The formation of tube-like structures were

observed using an inverted contrast microscope and captured with a CCD camera. The length of tube formation was measured using the Image J 1.42 software.

Scratch assay

Cell migration was evaluated using in vitro scratch assay (22). In brief, HAECs were seeded in 12-well plate and cultured at confluent monolayer. Cells were scratched and created in a straight line by using a p200 pipet tip. Wells were washed with culture medium to remove stripped cells, photographed at 0 h point, and then incubated in serum- and growth factor-free medium with bilirubin or vehicle for 8 h. Some experiments were pretreated with or without LY294002 1 h before. Images at 8 h were acquired at same reference point. Migrated area was measured by Image J 1.42 and calculated by subtracting the total area of the scratch at 8 h from the total area of the scratch at 0 h.

Cell proliferation assay

HAECs were seeded in 96-well plate at 1×10^4 cells pre well and incubated for 24 h. Subsequently, various concentration of Bilirubin was added for 8 h and then cell proliferation was assessed 1 h after the addition of MTS reagent by measuring absorbance at 490 nm with a plate reader.

Serum and growth factors deprivation-induced cell death assay

HAECs were seeded in 96-well plates at 1×10^4 cells per well and cultured for 24 h. Then, cells were incubated in serum- and growth factor-contained medium as control or in serum- and growth factor-free medium with bilirubin or vehicle for 48 h, with or

without LY294002 pretreatment 1 h before. Serum deprivation-induced cell death was assessed by an MTS-based assay (21).

Western blot analysis

The methods protein extraction and western blot have been described in detail (20). Immunoblotting bands were visualized using a chemiluminescence reagent, and exposed to X-ray film or scanned by C-DiGit chemiluminescent scanner (LI-COR C-DiGit Blot Scanner, Lincoln, Nebraska USA). Image J 1.42 software was used for densitometric analysis.

Statistical analysis

Data have been expressed as mean ± standard error of mean (SEM) values. An unpaired 2-tailed Student's *t*-test was used to evaluate the differences between 2 groups. For comparisons among more than 2 groups, statistical significance was assessed using a one-way analysis of variance (ANOVA), and the significance of each difference was determined by post hoc testing using Tukey-Kramer's method. *P* values of <0.05 were considered to indicate statistical significance.

Results

Bilirubin action on in vivo angiogenesis in response to ischemia

To evaluate bilirubin action on angiogenesis *in vivo*, we used a mouse model with unilateral hindlimb ischemia. There were no differences in body weight, systolic blood pressure, or pulse rate between mice with or without bilirubin treatment at 1 week after the surgery (Table 1). With respect to total plasma bilirubin concentration after an intraperitoneal injection of bilirubin, the bilirubin concentration was increased 2-fold at 1 h. This elevation was not significant and

the concentration was restored to the level in vehicle-treated mice at 6 and 24 h after injection (Table2). In LSBF analyses, mice treated with bilirubin treatment showed that the blood flow recovery after hindlimb ischemia was accelerated compared to mice treated with vehicle. The LSBF ratio of ischemic side to non-ischemic side was markedly higher at 3 d or later after ischemic hindlimb surgery in bilirubin-treated mice that in vehicle-treated mice (Figure 1A).

Increased capillary density and arterioles in ischemic adductor muscle of bilirubin-treated mice

To evaluate proangiogenic response following induction of hindlimb ischemia, CD31- and αSMA-positive cells were identified in histological sections of non-ischemic- and ischemic-adductor muscles. In accordance with the result of LSBF ratio, capillary density and arterioles number were higher in bilirubin-treated mice than in vehicle-treated mice on day 28 after surgery (Figure 1B and C).

In vivo bilirubin action on eNOS and Akt phosphorylation in ischemic hindlimb

eNOS activation plays a crucial role for angiogenesis in endothelial cells (14). PI3-kinase-Akt pathway is also shown as a key signaling for of eNOS activation (23, 24). Therefore, we examined whether bilirubin augmented the phosphorylation levels of eNOS and Akt in ischemic skeletal muscle tissue. As shown in figure 1D, eNOS phosphorylation, as well as Akt phosphorylation, were significantly increased in mice with bilirubin treatment on postoperative day 7. These results indicate that Akt-eNOS signaling pathway is involved in *in vivo* bilirubin effects on angiogenesis.

Bilirubin treatment suppresses superoxide production after ischemic stress

We analyzed the effect of bilirubin on oxidative stress by using DHE staining for superoxide detection, because bilirubin exerts anti-oxidant property (11). Superoxide production was elevated in ischemic muscle on day 7 after surgery, and bilirubin reduced the ischemia-induced superoxide production (Fig. 1E).

Bilirubin effects on eNOS and Akt phosphorylation and cell activation in HAECs

To investigate the involvement of bilirubin effect on Akt-eNOS pathway in detail, we performed *in vitro* experiments by using HAECs. Correspondently to the results i*n vivo*, bilirubin augmented Akt and eNOS phosphorylation in a time- and dose-dependent manner (Figure 2A and B). MTS-based cell proliferation assay showed that bilirubin treatment significantly increased cell proliferation (Figure 3A). To test the preventive action of bilirubin on cell death, HAECs were treated with bilirubin or vehicle and incubated for 48 h in serum- and growth factor- free medium. As shown in Figure 2B, bilirubin diminished serum and growth factor starvation-induced cell death assessed by an MTS-based assay (Figure 2B). Tube-like formations were also pronounced by bilirubin treatment compared to vehicle treatment alone (Figure 3C). Moreover, scratch assay demonstrated that bilirubin promoted cell migration in a dose-dependent manner (Figure 3D). AMPK as well as Akt are important upstream signaling activators of eNOS phosphorylation (25); however, our results showed that bilirubin was not involved in AMPK activation (Figure 2A and B).

Bilirubin exerts eNOS phosphorylation and cell activation through PI3-kinase-Akt-dependent pathway

Next, we analyzed whether Akt pathway was involved in bilirubin induced eNOS phosphorylation and ECs activation by using LY294002, a PI3-kinase inhibitor. Pretreatment with LY294002 significantly suppressed bilirubin-induced eNOS

phosphorylation (Figure 4A). Bilirubin effect on serum- and growth factor-starved cell death was canceled by LY294002 pretreatment (Figure 4C). Moreover, bilirubin induced ECs activation such as cell proliferation, migration, and tube like formation were all abolished by LY294002 pretreatment (Figure 4B, D, and E). These results suggested that bilirubin action on eNOS phosphorylation and endothelial cells activation is involved in PI3-kinase-Akt mediated pathway.

Discussion

In the present study, bilirubin also increased Akt and eNOS phosphorylation, and augmented capillary density and arterioles number in ischemic muscles, leading to the enhancement of blood flow recovery in *in vivo* mouse ischemic hindlimb model. In accordance with *in vivo* findings, bilirubin also increased eNOS phosphorylation, and activated cell proliferation, migration and tube formation in HAECs, contributing to the precipitation of angiogenic response. The mechanism of bilirubin on endothelial cells activation is involved in Akt-eNOS-dependent manner.

In clinical studies, high serum levels of bilirubin are negatively regulates the prevalence of various disorders including cardiovascular diseases such (4-10), chronic kidney disease (26), type 2 diabetes (27), and its complications such as neuropathy (28) and nephropathy (29). Low bilirubin is a potent endogenous anti-oxidant (11), and many studies have reported the relationship between bilirubin effects and anti-oxidant property in various diseases. In experimental studies, the anti-oxidant effect of bilirubin and biliverdin (the precursor of bilirubin) protects diabetic nephropathy and impaired glucose tolerance through suppressing NADPH oxidase (30, 31). Hyperbilirubinemia suppresses angiotensin II-induced hypertension through ameliorating endothelium-dependent vasodilation via the reduction of vascular

oxidative stress in Gunn rats (32) or mice treated with a drug that competes with bilirubin (33). In clinically, oxidative stress is lower in patients with Gilbert syndrome (12). Moreover, Dekker et al. have clarified that a bilirubin-increasing drug ameliorated plasma anti-oxidant capacity in patients with type 2 diabetes mellitus (34). Therefore, the protective mechanism of bilirubin has been generally considered as exerting through the suppression of oxidative stress. In accordance with the above-mentioned findings, bilirubin treatment reduced the increased oxidative stress in ischemic skeletal muscle. Oxidative stress causes endothelial dysfunction and impaired revascularization in response to ischemic hindlimb (35, 36). Our previous study also showed that deferoxamine, an iron chelator, diminished ischemia-induced oxidative stress in adductor muscles through inhibiting Fenton reaction (20). In concordance with our findings, vitamin C, nifedipine, and dipyridamole ameliorate ischemia-induced angiogenesis by inhibiting oxidative stress (37-39). Conversely, anti-oxidants inhibit eNOS activity (40). We tested the effect of an anti-oxidant, tempol, on bilirubin-induced activation of the Akt-eNOS pathway in HAECs, and found that tempol did not affect bilirubin-induced Akt and eNOS phosphorylation (data not shown). Although further examination is necessary to clarify this issue, the anti-oxidant effect of bilirubin could contribute to promote angiogenesis in part.

There is a close relationship between endothelial function and oxidative stress (41). Indeed, patients with Bartter's/Gitelman's syndromes, characterized by normal to hypotension, have reduced oxidative stress and higher NO-dependent vasodilatation (42). Serum bilirubin is also shown to augment endothelial cells activation indicated as coronary flow reserve and flow mediated vasodilatation (7, 12, 19). Bilirubin-increasing drug also improved endothelium-dependent vasodilation in Type2 diabetic patients (34). The bilirubin effect on endothelium was suggested to exert through the

reduction of oxidative stress. Although the protective effect of bilirubin on various organs is also due to anti-oxidant property as mentioned above, bilirubin might have additional effects independently to anti-oxidant action. Bilirubin suppresses vascular smooth muscle cells hyperplasia through the inhibitory effect of cell cycle at G1 phase via the inhibition of p38 mitogen activated protein kinase signaling and retinoblastoma tumor suppressor protein in rat (43). Bilirubin also ameliorates insulin sensitivity through the inhibitory properties of endoplasmic reticulum stress and inflammation (44). Thus, bilirubin might exert its protective action through multiple mechanisms in addition to anti-oxidant property.

In the present study, we demonstrated that bilirubin augmented eNOS and Akt phosphorylation, as well as ischemia-induced revascularization, in mice with ischemic hindlimb. In *in vitro* experiments, bilirubin activated endothelial cells function through eNOS-dependent pathway, and these bilirubin-induced effect were cancelled by PI3-kinase inhibitor, suggesting that direct effects of bilirubin on eNOS activation in Akt-dependent signaling. eNOS is well recognized as a crucial regulator of proangiogenic action. Indeed, it has been shown that eNOS activation leads the enhancement of capillaries and arterioles formation in mice with hindlimb ischemia (14, 15, 20). Moreover, Akt is an important signaling pathway of eNOS phosphorylation (23, 24). Several studies have shown bilirubin effect on Akt activation. Bilirubin treatment enhanced Akt phosphorylation in skeletal muscle of high fat diet-induced diabetic mice (44). Anti-inflammatory action of bilirubin exerts IL-10 regulation via Akt-dependent pathway (45). According to our findings, only recent article have reported that bilirubin restored endothelial dysfunction through ameliorating the reduced Akt-eNOS-NO signaling in aorta of type 2 murine diabetic model (46). Our findings strongly suggest that bilirubin promotes angiogenesis

through activating endothelial function via Akt-eNOS-dependent axis. Bilirubin treatment augmented Akt and eNOS phosphorylation in the ischemic side, as opposed to only Akt phosphorylation in the non-ischemic side. Bilirubin augments Akt phosphorylation in various tissues and cell types including skeletal muscles (Dong et al. Endocrinology 2013). Therefore, bilirubin could increase Akt phosphorylation not only in vascular endothelial cells but also in all other tissues including skeletal muscles. On the other hand, eNOS is generally expressed only in vascular endothelial cells. In the present study, capillaries and arterioles increased in number in the ischemic muscles, but not in the non-ischemic muscles with or without bilirubin treatment. Therefore, bilirubin-induced an increase in Akt phosphorylation in not only vascular endothelial cells but also in all other tissues including muscles, although the increase in eNOS phosphorylation in ischemic muscles might be influenced by the increase in the number of capillaries and arterioles. Moreover, the detailed mechanism of bilirubin action on PI3-kinase-Akt pathway has remained unclear. Further study is necessary for elucidating the upstream mechanism of bilirubin action on this signaling pathway.

In conclusion, bilirubin promotes endothelial cells activation through Akt-eNOS-dependent pathway, contributing to the enhancement of revascularization in murine model with unilateral ischemic hindlimb. This finding indicates a new protective effect of bilirubin against insufficient peripheral artery circulation.

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Disclosures

The authors have nothing to disclose.

References

1. Dennery PA, Seidman DS, Stevenson DK. Neonatal hyperbilirubinemia. *N Engl J Med.* 2001;344(8):581-590.
2. Owens D, Evans J. Population studies on Gilbert's syndrome. *J Med Genet.* 1975;12(2):152-156.
3. Vitek L, Jirsa M, Brodanova M, et al. Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels. *Atherosclerosis.* 2002;160(2):449-456.
4. Schwertner HA, Jackson WG, Tolan G. Association of low serum concentration of bilirubin with increased risk of coronary artery disease. *Clin Chem.* 1994;40(1):18-23.
5. Hopkins PN, Wu LL, Hunt SC, James BC, Vincent GM, Williams RR. Higher serum bilirubin is associated with decreased risk for early familial coronary artery disease. *Arterioscler Thromb Vasc Biol.* 1996;16(2):250-255.
6. Ishizaka N, Ishizaka Y, Takahashi E, Yamakado M, Hashimoto H. High serum bilirubin level is inversely associated with the presence of carotid plaque. *Stroke.* 2001;32(2):580-583.
7. Erdogan D, Gullu H, Yildirim E, et al. Low serum bilirubin levels are independently and inversely related to impaired flow-mediated vasodilation and increased carotid intima-media thickness in both men and women. *Atherosclerosis.* 2006;184(2):431-437.
8. Perlstein TS, Pande RL, Creager MA, Weuve J, Beckman JA. Serum total bilirubin level, prevalent stroke, and stroke outcomes: NHANES 1999-2004. *Am J Med.* 2008;121(9):781-788 e781.
9. Kimm H, Yun JE, Jo J, Jee SH. Low serum bilirubin level as an independent predictor of stroke incidence: a prospective study in Korean men and women. *Stroke.* 2009;40(11):3422-3427.
10. Li RY, Cao ZG, Zhang JR, Li Y, Wang RT. Decreased serum bilirubin is associated with silent cerebral infarction. *Arterioscler Thromb Vasc Biol.* 2014;34(4):946-951.
11. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science.* 1987;235(4792):1043-1046.
12. Maruhashi T, Soga J, Fujimura N, et al. Nitroglycerine-induced vasodilation for assessment of vascular function: a comparison with flow-mediated vasodilation. *Arterioscler Thromb Vasc Biol.* 2013;33(6):1401-1408.
13. Perlstein TS, Pande RL, Beckman JA, Creager MA. Serum total bilirubin level and prevalent lower-extremity peripheral arterial disease: National Health and Nutrition Examination Survey (NHANES) 1999 to 2004. *Arterioscler Thromb Vasc Biol.* 2008;28(1):166-172.

14. Murohara T, Asahara T, Silver M, et al. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest.* 1998;101(11):2567-2578.

15. Kureishi Y, Luo Z, Shiojima I, et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med.* 2000;6(9):1004-1010.

16. Chen LL, Yin H, Huang J. Inhibition of TGF-beta1 signaling by eNOS gene transfer improves ventricular remodeling after myocardial infarction through angiogenesis and reduction of apoptosis. *Cardiovasc Pathol.* 2007;16(4):221-230.

17. Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest.* 1997;100(12):3131-3139.

18. Murohara T, Witzenbichler B, Spyridopoulos I, et al. Role of endothelial nitric oxide synthase in endothelial cell migration. *Arterioscler Thromb Vasc Biol.* 1999;19(5):1156-1161.

19. Gullu H, Erdogan D, Tok D, et al. High serum bilirubin concentrations preserve coronary flow reserve and coronary microvascular functions. *Arterioscler Thromb Vasc Biol.* 2005;25(11):2289-2294.

20. Ikeda Y, Tajima S, Yoshida S, et al. Deferoxamine promotes angiogenesis via the activation of vascular endothelial cell function. *Atherosclerosis.* 2011;215(2):339-347.

21. Ikeda Y, Tajima S, Izawa-Ishizawa Y, et al. Bovine milk-derived lactoferrin exerts proangiogenic effects in an Src-Akt-eNOS-dependent manner in response to ischemia. *J Cardiovasc Pharmacol.* 2013;61(5):423-429.

22. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc.* 2007;2(2):329-333.

23. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature.* 1999;399(6736):601-605.

24. Fulton D, Gratton JP, McCabe TJ, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature.* 1999;399(6736):597-601.

25. Levine YC, Li GK, Michel T. Agonist-modulated regulation of AMP-activated protein kinase (AMPK) in endothelial cells. Evidence for an AMPK -> Rac1 -> Akt -> endothelial nitric-oxide synthase pathway. *J Biol Chem.* 2007;282(28):20351-20364.

26. Tanaka M, Fukui M, Okada H, et al. Low serum bilirubin concentration is a predictor of chronic kidney disease. *Atherosclerosis.* 2014;234(2):421-425.

27. Abbasi A, Deetman PE, Corpeleijn E, et al. Bilirubin as a potential causal factor in type 2 diabetes risk: a Mendelian randomization study. *Diabetes.* 2014.

28. Fukui M, Tanaka M, Shiraishi E, et al. Relationship between serum bilirubin and albuminuria in patients with type 2 diabetes. *Kidney Int.* 2008;74(9):1197-1201.

29. Riphagen IJ, Deetman PE, Bakker SJ, et al. Bilirubin and progression of nephropathy in type 2 diabetes: a post hoc analysis of RENAAL with independent replication in IDNT. *Diabetes.* 2014;63(8):2845-2853.

30. Fujii M, Inoguchi T, Sasaki S, et al. Bilirubin and biliverdin protect rodents against diabetic nephropathy by downregulating NAD(P)H oxidase. *Kidney Int.* 2010;78(9):905-919.

31. Ikeda N, Inoguchi T, Sonoda N, et al. Biliverdin protects against the deterioration of glucose tolerance in db/db mice. *Diabetologia.* 2011;54(8):2183-2191.

32. Pflueger A, Croatt AJ, Peterson TE, et al. The hyperbilirubinemic Gunn rat is resistant to the pressor effects of angiotensin II. *Am J Physiol Renal Physiol.* 2005;288(3):F552-558.

33. Vera T, Granger JP, Stec DE. Inhibition of bilirubin metabolism induces moderate hyperbilirubinemia and attenuates ANG II-dependent hypertension in mice. *Am J Physiol Regul Integr Comp Physiol.* 2009;297(3):R738-743.

34. Dekker D, Dorresteijn MJ, Pijnenburg M, et al. The bilirubin-increasing drug atazanavir improves endothelial function in patients with type 2 diabetes mellitus. *Arterioscler Thromb Vasc Biol.* 2011;31(2):458-463.

35. Galasso G, Schiekofer S, Sato K, et al. Impaired angiogenesis in glutathione peroxidase-1-deficient mice is associated with endothelial progenitor cell dysfunction. *Circ Res.* 2006;98(2):254-261.

36. Haddad P, Dussault S, Groleau J, et al. Nox2-containing NADPH oxidase deficiency confers protection from hindlimb ischemia in conditions of increased oxidative stress. *Arterioscler Thromb Vasc Biol.* 2009;29(10):1522-1528.

37. El-Azab MF, Hazem RM, Moustafa YM. Role of simvastatin and/or antioxidant vitamins in therapeutic angiogenesis in experimental diabetic hindlimb ischemia: effects on capillary density, angiogenesis markers, and oxidative stress. *Eur J Pharmacol.* 2012;690(1-3):31-41.

38. Kito T, Shibata R, Kondo M, et al. Nifedipine ameliorates ischemia-induced revascularization in diet-induced obese mice. *Am J Hypertens.* 2012;25(4):401-406.

39. Pattillo CB, Bir SC, Branch BG, et al. Dipyridamole reverses peripheral ischemia and induces angiogenesis in the Db/Db diabetic mouse hind-limb model by decreasing oxidative stress. *Free Radic Biol Med.* 2011;50(2):262-269.

40. Polytarchou C, Papadimitriou E. Antioxidants inhibit human endothelial cell functions through down-regulation of endothelial nitric oxide synthase activity. *Eur J Pharmacol.* 2005;510(1-2):31-38.

41. Higashi Y, Kihara Y, Noma K. Endothelial dysfunction and hypertension in aging. *Hypertens Res.* 2012;35(11):1039-1047.

42. Davis PA, Pagnin E, Dal Maso L, et al. SIRT1, heme oxygenase-1 and NO-mediated vasodilation in a human model of endogenous angiotensin II type 1 receptor antagonism: implications for hypertension. *Hypertens Res.* 2013;36(10):873-878.

43. Ollinger R, Bilban M, Erat A, et al. Bilirubin: a natural inhibitor of vascular smooth muscle cell proliferation. *Circulation.* 2005;112(7):1030-1039.

44. Dong H, Huang H, Yun X, et al. Bilirubin increases insulin sensitivity in leptin-receptor deficient and diet-induced obese mice through suppression of ER stress and chronic inflammation. *Endocrinology.* 2014;155(3):818-828.

45. Wegiel B, Baty CJ, Gallo D, et al. Cell surface biliverdin reductase mediates biliverdin-induced anti-inflammatory effects via phosphatidylinositol 3-kinase and Akt. *J Biol Chem.* 2009;284(32):21369-21378.

46. Liu J, Wang L, Tian X, et al. Unconjugated bilirubin mediates heme oxygenase-1-induced vascular benefits in diabetic mice. *Diabetes*. 2014.

Figure legends

Figure 1. Evaluation of revascularization in ischemic hindlimbs of mice with bilirubin or vehicle treatment. (A) Left panel: Quantitative analysis of blood flow recovery by laser speckle blood flow (LSBF) before surgery and on postoperative days 0, 3, 7, 14, and 28. Right panel: Representative LSBF images for hindlimb ischemia in vehicle or bilirubin-treated mice. Values are expressed as means ± SEM, **P* < 0.05, ***P* < 0.01 vs. mice treated with vehicle, *n* = 9 in each group. (B) Immunohistochemical analysis of capillaries in adductor muscles. Left panel: Representative nti-CD31 immunohistochemical staining. Right panel: Quantification of capillary density at 28 d after surgery. Values are expressed as means ± SEM, ***P* < 0.01 vs. mice treated with the vehicle, *n* = 6 in each group. (C) Left panel: Representative αSMA immunofluorescence staining. Right panel: Quantification of arteriolar numbers in adductor muscles. Values are expressed as means ± SEM, ***P* < 0.01 vs. mice treated with the vehicle, *n* = 6 in each group. (D) The phosphorylated levels of eNOS and Akt in adductor muscles of mice treated with vehicle or bilirubin. Left panel: Representative blots of phospho- and total eNOS, and Akt at 7 d after surgery. Right panel: Densitometric analysis of eNOS and Akt phosphorylation. Values were expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01, *n* = 12 in each group. (E) Left panel: Representative superoxide detection by dihydroethidium (DHE) staining for adductor skeletal muscles from vehicle- or bilirubin-treated mice. Right panel: Quantification of DHE fluorescence intensity in skeletal muscles from vehicle- or bilirubin-treated mice 7 days after surgery. *P < 0.05, **P < 0.01, n = 5 in each group.

Figure 2. The effects of Bilirubin on Akt-eNOS pathway in HAECs. (A) Time-dependent changes in eNOS and phosphorylation in HAECs following bLF administration (10 μM). Upper panel: Representative blots of phospho-eNOS, Akt and AMPKα, and total eNOS, Akt and AMPKα, and tubulin. Lower panel: Densitometric analysis of eNOS, and Akt phosphorylation. The values have been expressed in terms of mean ± SEM. $^{**}P < 0.01$, $n = 8$ in each group. (B) Dose-dependent Bilirubin actions on eNOS and Akt phosphorylation at 1 h after bilirubin treatment. Left panel: Representative blots of phospho-eNOS and Akt, total eNOS and Akt, and tubulin. Right panel: Densitometric analysis of Akt and eNOS phosphorylation. Values are expressed in terms of mean ± SEM. $^{*}P < 0.05$, $n = 8$ in each group.

Figure 3. Dose effect of 1−100 μM bilirubin on endothelial cells activaiton (A) Bilirubin action on endothelial cell proliferation. Cell proliferation was increased by bilirubin treatment in a dose-dependent manner. Values are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 16$ in each group. (B) The Effect of bilirubin on serum stravation-induced cell death. Cell death was prevented by 10 μM bilirubin. Values are expressed in terms of mean ± SEM. $^{**}P < 0.01$, $n = 16$ in each group. (C) Left panels; Representative figures of tube formation at 18 h after bilirubin stimulation. The Effect of bilirubin on tube-like formation. Right panel; Quantitative analysis of tube formation. Endothelial cell tube formation was provoked by bilirubin in 10 μM concentration. $^{**}P < 0.01$, $n = 6$ in each group. (D) Biliribin effect on cell migration. Left panels; Representative pahse contrast images at 0 h and 8 h after scratch. Right panel; Quantitative analysis of % migrated area. Values are expressed in terms of mean ± SEM. $^{*}P < 0.05$, $n = 4$ in each group.

Figure 4. The involvement of PI3-kinase-Akt pathway on bilirubin-induced

endothelial cell activation. (A) The induction of eNOS phosphorylation by bilirubin was abolished by LY294002. Left panel: Representative blots of phospho-eNOS and Akt, and total eNOS, Akt, and tubulin. Right panel: Densitometric analysis of eNOS and Akt phosphorylation. The values have been expressed in terms of mean ± SEM. $^{**}P < 0.01$, $n = 8$ in each group. (B) Bilirubin-induced cell proliferation was abolished by LY294002. Values are expressed as mean ± SEM. $^{*}P < 0.05$, $n = 16$ in each group. (C) The prevntive effct of bilirubin on serum stravation-induced cell death was abolished by LY294002. Values are expressed in terms of mean ± SEM. $^{*}P < 0.05$,$^{**}P < 0.01$, $n = 16$ in each group. The augementatio of (D) tube formation and (E) cell migration by bilirubin were inhibited by LY294002. The values have been expressed in terms of mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 6$–8 in each group.

Table.1 Body weight, Blood pressure and pulse rate at day 7 after ischemic hindlimb surgery

	Post-surgery at day 7	
	Vehicle	Bilirubin
BW (g)	24.3 ± 0.6	24.1 ± 0.4
Food intake (g/day)	3.5 ± 0.1	3.5 ± 0.1
Sys. BP (mmHg)	108 ± 1	106 ± 42
Dia. BP (mmHg)	73 ± 1	72 ± 3
Pulse rate (beats/min)	664 ± 33	657 ± 20

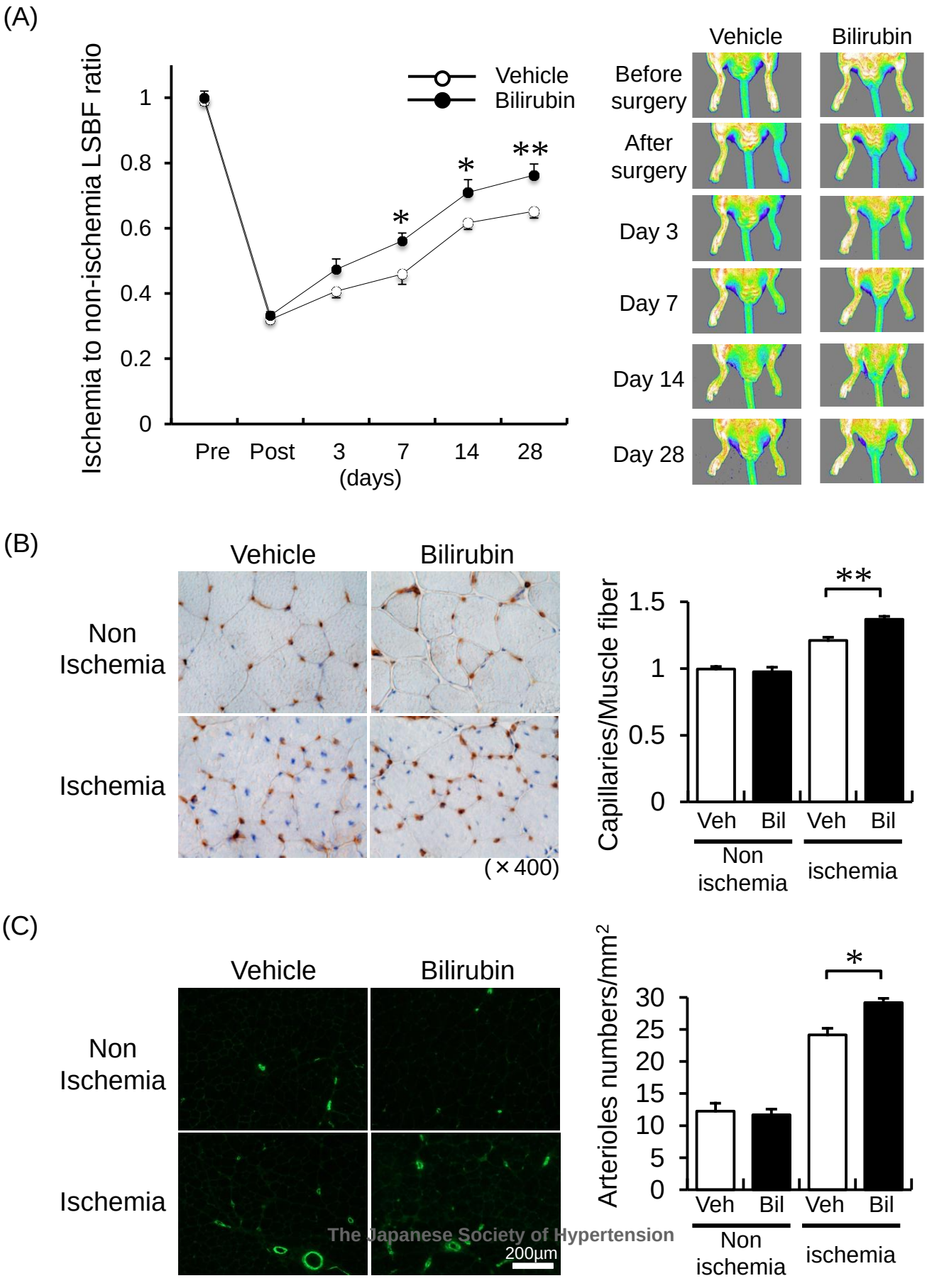
Values are mean ± SEM. n=6, respectively.

Table.2 Changes of plasma bilirubin concentration

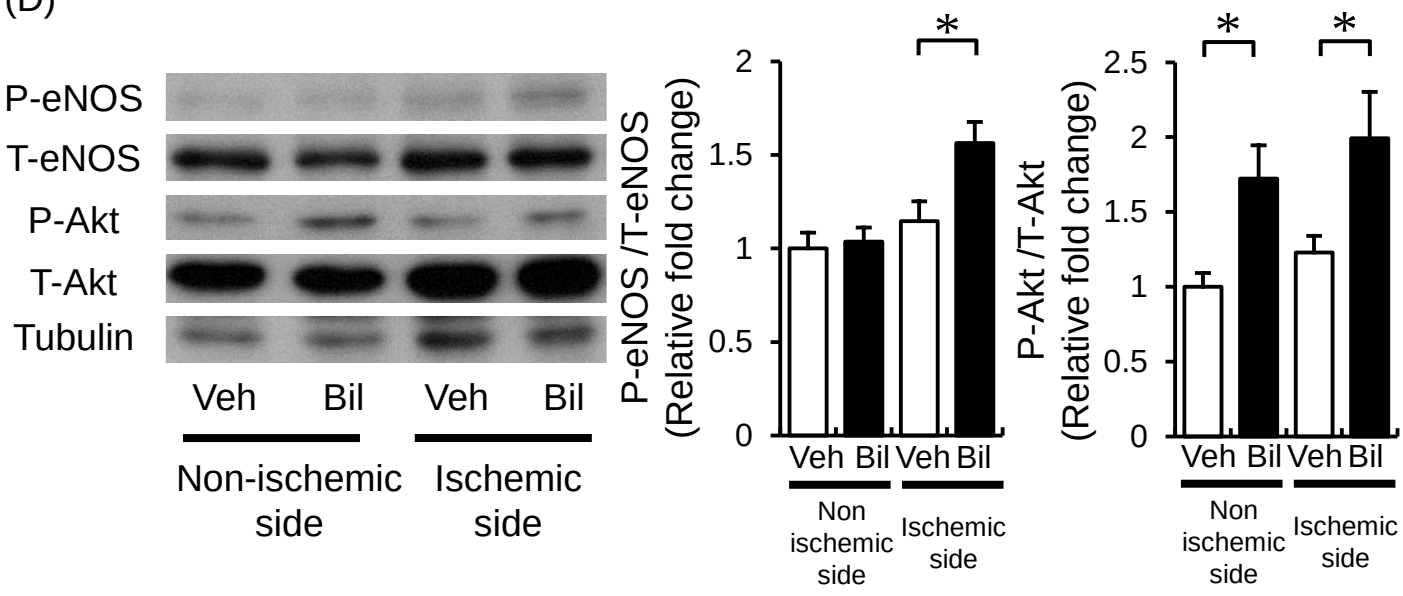
Time after treatment (h)	1	6	24
Vehicle-treated mice (mg/dl)	0.28 ± 0.03	0.36 ± 0.08	0.36 ± 0.14
Bilirubin-treated mice (mg/dl)	0.55 ± 0.04**	0.35 ± 0.04	0.39 ± 0.06

**p<0.01 vs vehicle treatment group

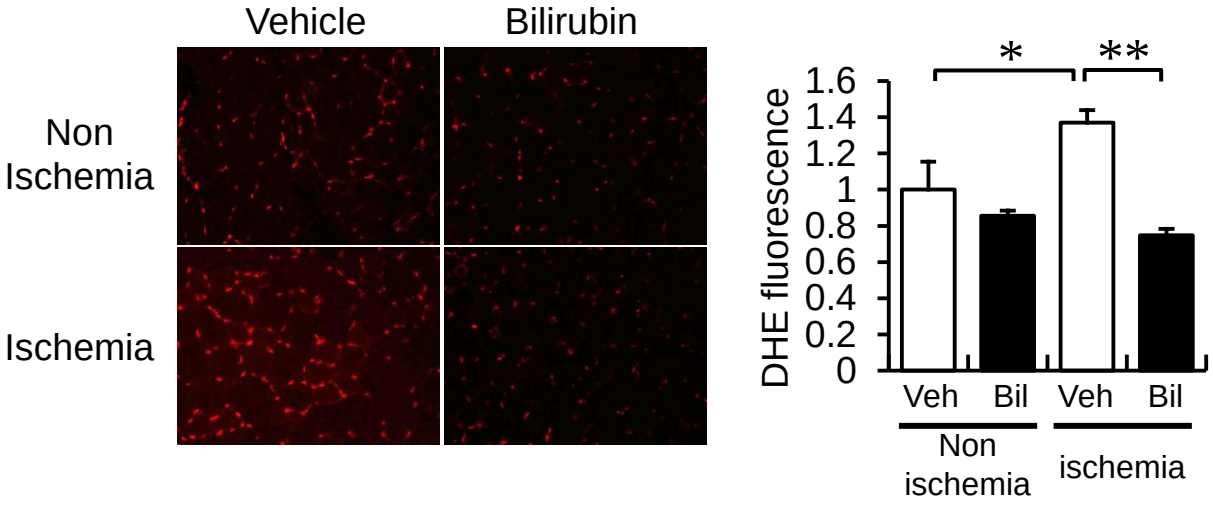
Values are mean ± SEM. n=4-6, respectively.


(A)
Ischemia to non-ischemia LSBF ratio
1
0.8
0.6
0.4
0.2
0
Vehicle
Bilirubin
*
*
**
Pre
Post
3
7
14
28
(days)
Vehicle
Bilirubin
Before surgery
After surgery
Day 3
Day 7
Day 14
Day 28
(B)
Vehicle
Bilirubin
Non Ischemia
Ischemia
(×400)
Capillaries/Muscle fiber
1.5
1
0.5
0
**
Veh
Bil
Veh
Bil
Non ischemia
ischemia
(C)
Vehicle
Bilirubin
Non Ischemia
Ischemia
The Japanese Society of Hypertension
200μm
Arterioles numbers/mm2
30
25
20
15
10
5
0
*
Veh
Bil
Veh
Bil
Non ischemia
ischemia

(D)


P-eNOS
T-eNOS
P-Akt
T-Akt
Tubulin
Veh
Bil
Veh
Bil
Non-ischemic side
Ischemic side
P-eNOS /T-eNOS (Relative fold change)
2
1.5
1
0.5
0
*
Veh
Bil
Veh
Bil
Non ischemic side
Ischemic side
P-Akt /T-Akt (Relative fold change)
2.5
2
1.5
1
0.5
0
*
*
Veh
Bil
Veh
Bil
Non ischemic side
Ischemic side

(E)


Vehicle
Bilirubin
Non Ischemia
Ischemia
DHE fluorescence
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
Veh
Bil
Veh
Bil
Non ischemia
ischemia