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ORIGINAL ARTICLE

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

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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 antioxidant 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 synthase (eNOS) and Akt were increased in ischemic skeletal muscles of mice with bilirubin treatment compared with 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.

INTRODUCTION

Bilirubin is a metabolic end product following heme degradation. Hyperbilirubinemia has an aspect of cytotoxic action to cause bilirubin-induced neurological dysfunction in newborns.[1] On the other hand, people with mild unconjugated nonhemolytic hyperbilirubinemia, called as Gilbert's syndrome,[2] have shown to have a 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 antioxidant 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 antioxidant property.

Increased serum bilirubin is an independent factor to reduce the prevalence of peripheral artery disease.[13] Angiogenesis has a 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 peripheral artery disease. 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.

MATERIALS AND METHODS

Materials

Bilirubin was purchased from Wako Pure Chemical Industries (Osaka, Japan). The following commercially available antibodies were used in this study:

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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 (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. Matrigel was obtained from BD Biosciences. 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, Tokushima University Graduate School. Eight-week-old C57/BL6J mice were obtained from Nippon CLEA (Tokyo, Japan) and divided into two groups: bilirubin-treated ($5 \, \text{mg kg}^{-1} \, \text{day}^{-1}$) group and a vehicle-treated group. Mice were injected intraperitoneally with either bilirubin or vehicle 2 days before the surgery to induce ischemic hindlimb, as previously described.[20] In brief, mice were anesthetized with i.p. pentobarbital ($50 \, \text{mg kg}^{-1}$) 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, 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 days 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, USA) according to the manufacturer's instructions to determine plasma bilirubin concentration.

Cell culture
Human aortic endothelial cells (HAECs) were purchased from Takara Bio (Otsu, Japan) and cultured in endothelial cell growth medium MV 2 (PromoCell, 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 (100 μM) or vehicle alone for 1 h before bilirubin treatment.

Tube-like formation assay
Tube-like formation assay was performed by using growth factor-reduced Matrigel (BD Biosciences), 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 (U. S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/).

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. In some experiments cells 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 per 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 for 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, LI-COR Biotechnology, Lincoln, NE, USA). Image J 1.42 software was used for densitometric analysis.

Statistical analysis
Data have been expressed as mean ± s.e.m. values. An unpaired two-tailed Student's t-test was used to evaluate the differences between the two groups. For comparisons among more than two groups, statistical significance was assessed using a two-way analysis of variance, 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

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

	Post-surgery at day 7	
	Vehicle	Bilirubin
Body weight (g)	24.3 ± 0.6	24.1 ± 0.4
Food intake (g day^{-1})	3.5 ± 0.1	3.5 ± 0.1
Systolic BP (mm Hg)	108 ± 1	106 ± 42
Diastolic BP (mm Hg)	73 ± 1	72 ± 3
Pulse rate (beats min^{-1})	664 ± 33	657 ± 20

Values are mean ± s.e.m. n = 6.

or without bilirubin treatment at 1 week after the surgery (Table 1). With respect to total plasma bilirubin concentration after an i.p. injection of bilirubin, the bilirubin concentration was increased twofold at 1 h. This elevation was significant and the concentration was restored to the level in vehicle-treated mice at 6 and 24 h after injection (Table 2). In LSBF analyses, mice treated with bilirubin treatment showed that the blood flow recovery after hindlimb ischemia was accelerated compared with that of mice treated with vehicle. The LSBF ratio of ischemic side to non-ischemic side was

markedly higher at 7 days or later, after the ischemic hindlimb surgery in bilirubin-treated mice than in vehicle-treated mice (Figure 1a).

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

To evaluate pro-angiogenic 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 (Figures 1b and c).

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

eNOS activation has a crucial role for angiogenesis in endothelial cells.[14] PI3-kinase-Akt pathway is also shown as a key signaling for

Table 2 Changes of plasma bilirubin concentration

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

Values are mean ± s.e.m. $n = 4–6$.
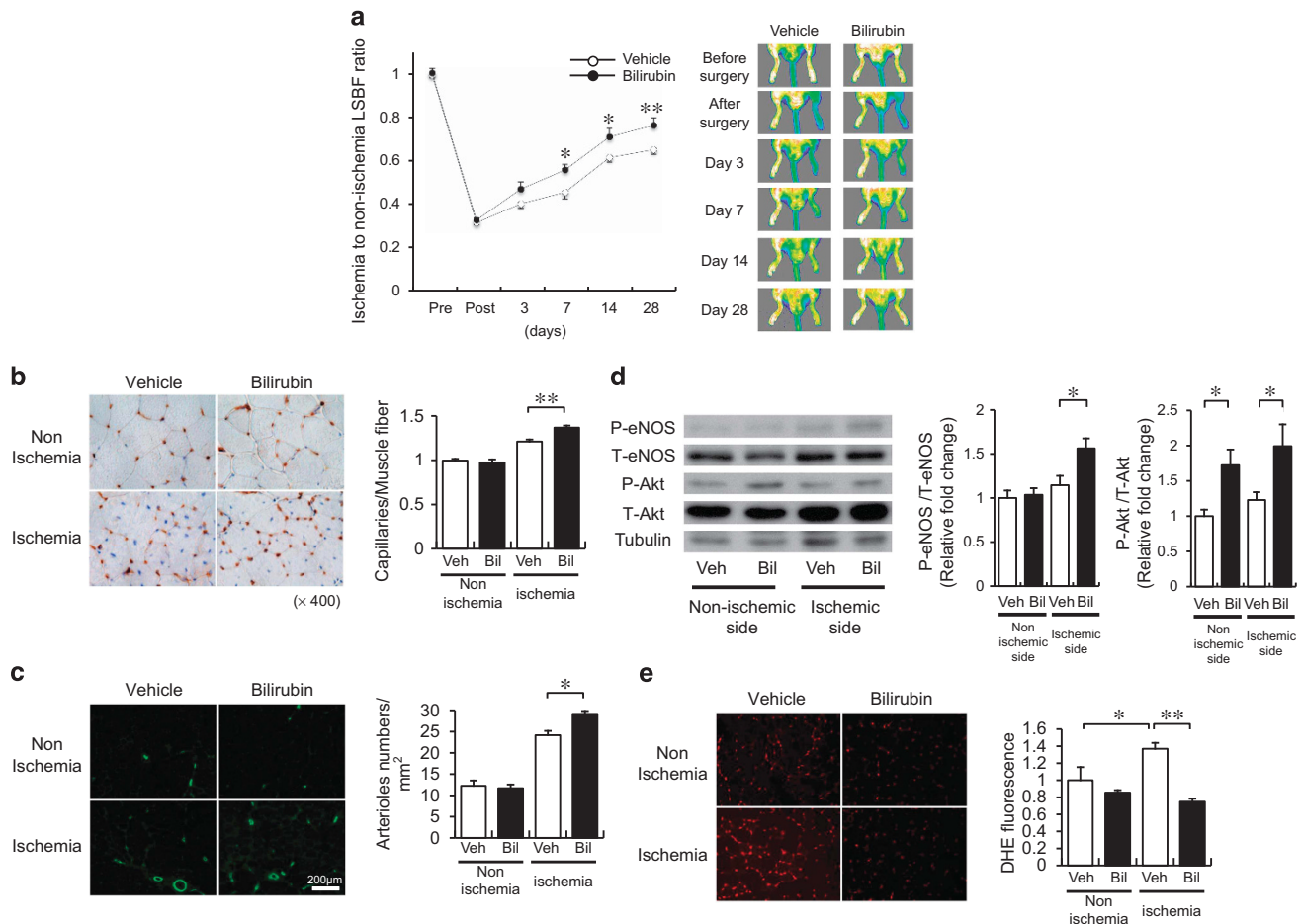$^a P < 0.01$ vs the vehicle-treated group.

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 ± s.e.m., *$P < 0.05$, **$P < 0.01$ vs mice treated with the vehicle, $n = 9$ in each group. (b) Immunohistochemical analysis of capillaries in adductor muscles. Left panel: representative anti-CD31 immunohistochemical staining. Right panel: quantification of capillary density at 28 days after surgery. Values are expressed as means ± s.e.m., **$P < 0.01$ 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 ± s.e.m., **$P < 0.01$ 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 days after surgery. Right panel: densitometric analysis of eNOS and Akt phosphorylation. Values are expressed as mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, $n = 12$ 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.
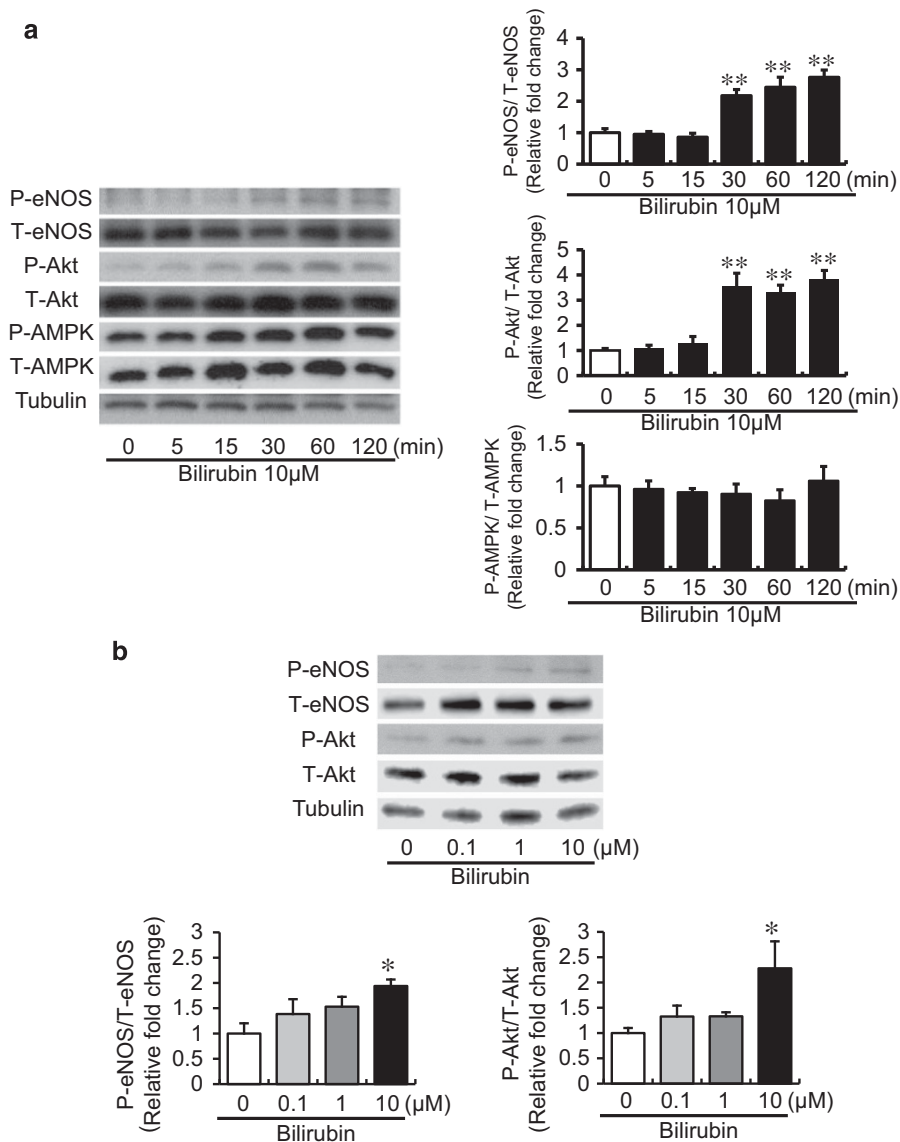
a

b

Figure 2 The effects of bilirubin on Akt-eNOS pathway in HAECs. (a) Time-dependent changes in eNOS and phosphorylation in HAECs following bilirubin 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 ± s.e.m. **$P < 0.01$, $n = 8$ 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 ± s.e.m. *$P < 0.05$, $n = 8$ in each group.

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 antioxidant property.[11] Superoxide production was elevated in ischemic muscle on day 7 after surgery, and bilirubin reduced the ischemia-induced superoxide production (Figure 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. Correspondingly to the results in *vivo*, bilirubin augmented Akt and eNOS phosphorylation in a time- and dose-dependent manner (Figures 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 with the vehicle treatment alone (Figure 3c). Moreover, scratch assay
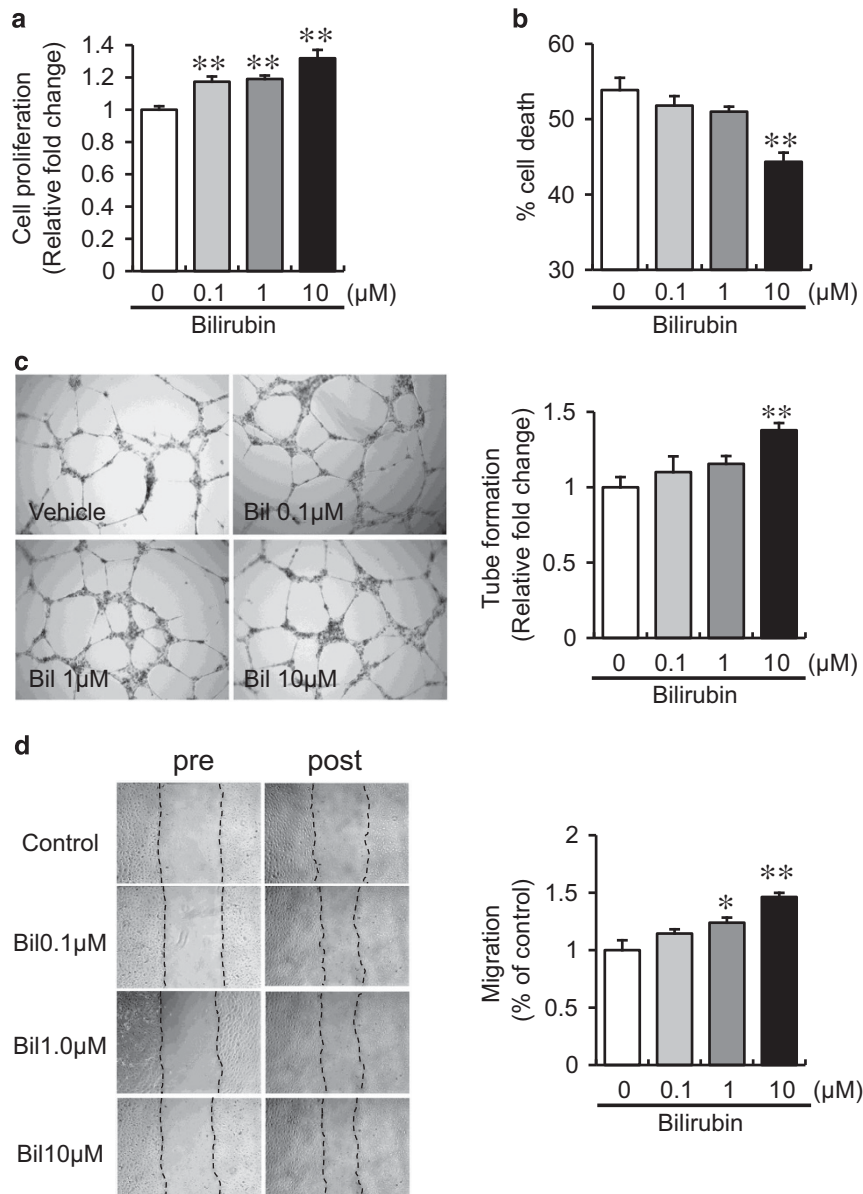
Figure 3 Dose effect of 0.1–10 μM bilirubin on endothelial cells activaiton. (**a**) Bilirubin action on endothelial cell proliferation. Cell proliferation was increased by bilirubin treatment in a dose-dependent manner. Values are expressed as mean ± s.e.m. *P<0.05, **P<0.01, n=16 in each group. (**b**) 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 ± s.e.m. **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 percentage of migrated area. Values are expressed in terms of mean ± s.e.m. *P<0.05, n=4 in each group.

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 (Figures 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 endothelial cells 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 endothelial cells activation such as cell proliferation, migration and tube-like formation were all abolished by LY294002 pretreatment (Figures 4b, d and e). These results suggest that bilirubin action on eNOS phosphorylation and endothelial cells activation is involved in PI3-kinase-Akt mediated pathway.

DISCUSSION

In the present study, bilirubin increased Akt and eNOS phosphorylation, and augmented capillary density and arterioles number in ischemic muscles, leading to the enhancement of blood flow recovery
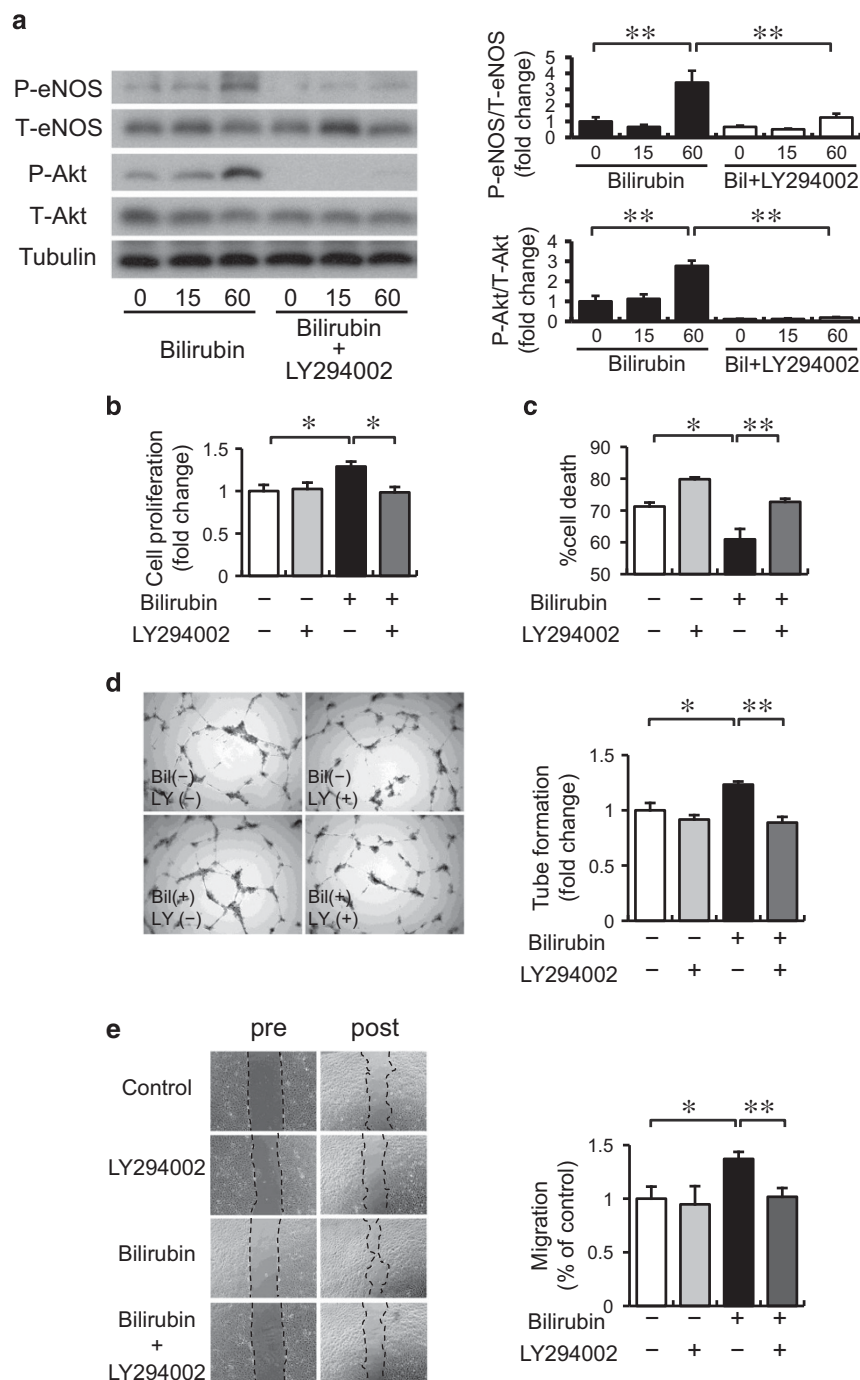
Figure 4 The involvement of PI3-kinase-Akt pathway on bilirubin-induced endothelial cell activation. (**a**) The induction of eNOS phosphorylation by bilirubin was abolished by LY294002. Left panel: representative blots of phospho-eNOS and Akt, and total eNOS, Akt and tubulin. Right panel: densitometric analysis of eNOS and Akt phosphorylation. The values have been expressed in terms of mean ± s.e.m. **$P<0.01$, $n=8$ in each group. (**b**) Bilirubin-induced cell proliferation was abolished by LY294002. Values are expressed as mean ± s.e.m. *$P<0.05$, $n=16$ 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 ± s.e.m. *$P<0.05$,**$P<0.01$, $n=16$ in each group. The augmentatio of (**d**) tube formation and (**e**) cell migration by bilirubin were inhibited by LY294002. The values have been expressed in terms of mean ± s.e.m. *$P<0.05$, **$P<0.01$, $n=6$–8 in each group.

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 an Akt-eNOS-dependent manner.

In clinical studies, high serum levels of bilirubin are negatively regulates the prevalence of various disorders including cardiovascular diseases such as[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 antioxidant,[11] and many studies have reported the relationship between bilirubin effects and

antioxidant property in various diseases. In experimental studies, the antioxidant 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] Clinically, the oxidative stress is lower in patients with Gilbert's syndrome.[12] Moreover, Dekker et al.[34] have clarified that a bilirubin-increasing drug ameliorated plasma antioxidant capacity in patients with type 2 diabetes mellitus. 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, antioxidants inhibit eNOS activity.[40] We tested the effect of an antioxidant, 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 antioxidant 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 the endothelial cells activation indicated as coronary flow reserve and flow-mediated vasodilatation.[7,12,19] Bilirubin-increasing drug also improved endothelium-dependent vasodilation in type 2 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 the antioxidant property as mentioned above, bilirubin might have additional effects independent of antioxidant 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 the antioxidant 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 effects were cancelled by the 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 pro-angiogenic 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.[44] 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.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

1 Dennery PA, Seidman DS, Stevenson DK. Neonatal hyperbilirubinemia. N Engl J Med 2001; 344: 581–590.
2 Owens D, Evans J. Population studies on Gilbert's syndrome. J Med Genet 1975; 12: 152–156.
3 Vitek L, Jirsa M, Brodanova M, Kalab M, Marecek Z, Danzig V, Novotný L, Kotal P. Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels. Atherosclerosis 2002; 160: 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: 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: 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: 580–583.
7 Erdogan D, Gullu H, Yildirim E, Tok D, Kirbas I, Ciftci O, Baycan ST, Muderrisoglu H. 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: 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: 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: 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**: 946–951.

11 Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987; **235**: 1043–1046.

12 Maruhashi T, Soga J, Fujimura N, Idei N, Mikami S, Iwamoto Y, Kajikawa M, Matsumoto T, Hidaka T, Kihara Y, Chayama K, Noma K, Nakashima A, Goto C, Higashi Y. Nitroglycerine-induced vasodilation for assessment of vascular function: a comparison with flow-mediated vasodilation. *Arterioscler Thromb Vasc Biol* 2013; **33**: 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**: 166–172.

14 Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, Isner JM. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 1998; **101**: 2567–2578.

15 Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med* 2000; **6**: 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**: 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**: 3131–3139.

18 Murohara T, Witzenbichler B, Spyridopoulos I, Asahara T, Ding B, Sullivan A, Losordo DW, Isner JM. Role of endothelial nitric oxide synthase in endothelial cell migration. *Arterioscler Thromb Vasc Biol* 1999; **19**: 1156–1161.

19 Gullu H, Erdogan D, Tok D, Topcu S, Caliskan M, Ulus T, Muderrisoglu H. High serum bilirubin concentrations preserve coronary flow reserve and coronary microvascular functions. *Arterioscler Thromb Vasc Biol* 2005; **25**: 2289–2294.

20 Ikeda Y, Tajima S, Yoshida S, Yamano N, Kihira Y, Ishizawa K, Aihara K, Tomita S, Tsuchiya K, Tamaki T. Deferoxamine promotes angiogenesis via the activation of vascular endothelial cell function. *Atherosclerosis* 2011; **215**: 339–347.

21 Ikeda Y, Tajima S, Izawa-Ishizawa Y, Kihira Y, Ishizawa K, Yoshida S, Aihara K, Tsuchiya K, Tamaki T. Bovine milk-derived lactoferrin exerts proangiogenic effects in an Src-Akt-eNOS-dependent manner in response to ischemia. *J Cardiovasc Pharmacol* 2013; **61**: 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**: 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**: 601–605.

24 Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999; **399**: 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**: 20351–20364.

26 Tanaka M, Fukui M, Okada H, Senmaru T, Asano M, Akabame S, Yamazaki M, Tomiyasu K, Oda Y, Hasegawa G, Toda H, Nakamura N. Low serum bilirubin concentration is a predictor of chronic kidney disease. *Atherosclerosis* 2014; **234**: 421–425.

27 Abbasi A, Deetman PE, Corpeleijn E, Gansevoort RT, Gans RO, Hillege HL, van der Harst P, Stolk RP, Navis G, Alizadeh BZ, Bakker SJ. Bilirubin as a potential causal factor in type 2 diabetes risk: a Mendelian randomization study. *Diabetes* 2014; **64**: 1459–1469.

28 Fukui M, Tanaka M, Shiraishi E, Harusato I, Hosoda H, Asano M, Hasegawa G, Nakamura N. Relationship between serum bilirubin and albuminuria in patients with type 2 diabetes. *Kidney Int* 2008; **74**: 1197–1201.

29 Riphagen IJ, Deetman PE, Bakker SJ, Navis G, Cooper ME, Lewis JB, de Zeeuw D, Lambers Heerspink HJ. Bilirubin and progression of nephropathy in type 2 diabetes: a

30 post hoc analysis of RENAAL with independent replication in IDNT. *Diabetes* 2014; **63**: 2845–2853.

30 Fujii M, Inoguchi T, Sasaki S, Maeda Y, Zheng J, Kobayashi K, Takayanagi R. Bilirubin and biliverdin protect rodents against diabetic nephropathy by downregulating NAD(P)H oxidase. *Kidney Int* 2010; **78**: 905–919.

31 Ikeda N, Inoguchi T, Sonoda N, Fujii M, Takei R, Hirata E, Yokomizo H, Zheng J, Maeda Y, Kobayashi K, Takayanagi R. Biliverdin protects against the deterioration of glucose tolerance in db/db mice. *Diabetologia* 2011; **54**: 2183–2191.

32 Pflueger A, Croatt AJ, Peterson TE, Smith LA, d'Uscio LV, Katusic ZS, Nath KA. The hyperbilirubinemic Gunn rat is resistant to the pressor effects of angiotensin II. *Am J Physiol Renal Physiol* 2005; **288**: F552–F558.

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**: R738–R743.

34 Dekker D, Dorresteijn MJ, Pijnenburg M, Heemskerk S, Rasing-Hoogveld A, Burger DM, Wagener FA, Smits P. The bilirubin-increasing drug atazanavir improves endothelial function in patients with type 2 diabetes mellitus. *Arterioscler Thromb Vasc Biol* 2011; **31**: 458–463.

35 Galasso G, Schiekofer S, Sato K, Shibata R, Handy DE, Ouchi N, Leopold JA, Loscalzo J, Walsh K. Impaired angiogenesis in glutathione peroxidase-1-deficient mice is associated with endothelial progenitor cell dysfunction. *Circ Res* 2006; **98**: 254–261.

36 Haddad P, Dussault S, Groleau J, Turgeon J, Michaud SE, Ménard C, Perez G, Maingrette F, Rivard A. Nox2-containing NADPH oxidase deficiency confers protection from hindlimb ischemia in conditions of increased oxidative stress. *Arterioscler Thromb Vasc Biol* 2009; **29**: 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**: 31–41.

38 Kito T, Shibata R, Kondo M, Yamamoto T, Suzuki H, Ishii M, Murohara T. Nifedipine ameliorates ischemia-induced revascularization in diet-induced obese mice. *Am J Hypertens* 2012; **25**: 401–406.

39 Pattillo CB, Bir SC, Branch BG, Greber E, Shen X, Pardue S, Patel RP, Kevil CG. 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**: 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**: 31–38.

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

42 Davis PA, Pagnin E, Dal Maso L, Caielli P, Maiolino G, Fusaro M, Paolo Rossi G, Calò LA. 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**: 873–878.

43 Ollinger R, Bilban M, Erat A, Froio A, McDaid J, Tyagi S, Csizmadia E, Graça-Souza AV, Liloia A, Soares MP, Otterbein LE, Usheva A, Yamashita K, Bach FH. Bilirubin: a natural inhibitor of vascular smooth muscle cell proliferation. *Circulation* 2005; **112**: 1030–1039.

44 Dong H, Huang H, Yun X, Kim DS, Yue Y, Wu H, Sutter A, Chavin KD, Otterbein LE, Adams DB, Kim YB, Wang H. Bilirubin increases insulin sensitivity in leptin-receptor deficient and diet-induced obese mice through suppression of ER stress and chronic inflammation. *Endocrinology* 2014; **155**: 818–828.

45 Wegiel B, Baty CJ, Gallo D, Csizmadia E, Scott JR, Akhavan A, Chin BY, Kaczmarek E, Alam J, Bach FH, Zuckerbraun BS, Otterbein LE. Cell surface biliverdin reductase mediates biliverdin-induced anti-inflammatory effects via phosphatidylinositol 3-kinase and Akt. *J Biol Chem* 2009; **284**: 21369–21378.

46 Liu J, Wang L, Tian X, Liu L, Wong WT, Zhang Y, Han QB, Ho HM, Wang N, Wong SL, Chen ZY, Yu J, Ng CF, Yao X, Huang Y. Unconjugated bilirubin mediates heme oxygenase-1-induced vascular benefits in diabetic mice. *Diabetes* 2014; **64**: 1564–1575.