How to Cite this article: David Stec, Claudio Tiribelli, Olufunto Badmus, and Terry Hinds, Novel Function for Bilirubin as a Metabolic Signaling Molecule: Implications for Kidney Diseases, Kidney360, Publish Ahead of Print, 10.34067/KID.0000062022

Article Type: Basic Science for Clinicians

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Key Points:

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Disclosures: D. Stec reports the following: Advisory or Leadership Role: Editorial Board for following journals: Hypertension, American Journal of Physiology Regulatory, Integrative and Comparative Physiology; and Other Interests or Relationships: American Heart Association- Chair of Peer Review Group. C. Tiribelli reports the following: Scientific Advisor or Membership. The remaining authors have nothing to disclose.


Author Contributions: David Stec: Conceptualization; Resources; Writing - original draft; Writing - review and editing Claudio Tiribelli: Conceptualization; Writing - review and editing Olufunto Badmus: Writing - review and editing Terry Hinds: Conceptualization; Resources; Writing - original draft; Writing - review and editing

Data Sharing Statement:

Clinical Trials Registration:

Registration Number:

Registration Date:

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Novel Function for Bilirubin as a Metabolic Signaling Molecule: Implications for Kidney Diseases

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Abstract
Bilirubin is the end product of the catabolism of heme via the heme oxygenase pathway. Heme oxygenase generates carbon monoxide (CO) and biliverdin from the breakdown of heme, and biliverdin is rapidly reduced to bilirubin by the enzyme biliverdin reductase (BVR). Bilirubin has long been thought of as a toxic product that is only relevant to health when blood levels are severely elevated, such as in clinical jaundice. The physiological functions of bilirubin correlate with the growing body of evidence demonstrating the protective effects of serum bilirubin against cardiovascular and metabolic diseases. While the correlative evidence suggests a protective effect of serum bilirubin against many diseases, the mechanism by which bilirubin offers protection against cardiovascular and metabolic diseases remains unanswered. We recently discovered a novel function for bilirubin as a signaling molecule capable of activating the peroxisome proliferator-activated receptor alpha (PPARα) transcription factor. This review summarizes the new finding of bilirubin as a signaling molecule and proposes several mechanisms by which this novel action of bilirubin may protect against cardiovascular and kidney diseases.
Introduction

Heme is an integral part of many enzymes throughout the body. Its incorporation into proteins such as hemoglobin, myoglobin, and cytochromes are necessary for a wide variety of biological functions, spanning oxygen transport to the synthesis of hormones and the breakdown of drugs and toxins. Heme is recycled in the body by the heme oxygenase (HO) enzymes, which, opens the porphyrin ring to release carbon monoxide (CO), biliverdin, and free iron (1). Reduction of biliverdin by the ubiquitous enzyme biliverdin reductase (BVR) forms bilirubin in the plasma and cells (2). The turnover of red blood cells in the spleen has classically been considered the primary source of plasma bilirubin (3). However, many other tissues also express the HO and BVR isozymes (4-6). The turnover of cytochrome P450 and other heme-containing proteins are also sources of bilirubin inside the cell. Studies from cells expressing the bilirubin activated fluorescence protein, UNaG, demonstrated that bilirubin transport occurs from outside the cell into different intracellular compartments, including the nucleus (7, 8). Bilirubin is transported into hepatocytes via organic anion transporting polypeptides (OATPs) and multidrug resistance proteins (MRPs) (9); however, the specific transporter responsible for the nuclear transport of bilirubin is currently unknown. Mice lacking the major BVR isozyme, biliverdin reductase A (BVRA), contain approximately 100-fold lower bilirubin levels in the plasma and exhibit higher levels of oxidative stress in the blood (10). Similarly, BVRA liver-specific KO mice on a high-fat diet have significantly increased hepatic fat content, which is attributed to the reduced signaling mechanisms of BVRA and bilirubin (11).

Low plasma bilirubin levels correlate with the development of cardiovascular disease, type II diabetes, and other metabolic complications (12-17). High plasma bilirubin levels are associated with a decreased prevalence of diabetic nephropathy, and low levels are associated with the
progression of the disease (18, 19). Higher serum bilirubin concentrations are associated with a lower risk of contrast-induced nephropathy (20). However, it is unclear whether plasma bilirubin levels are markers or mechanistically involved in cardiovascular and kidney diseases (21). Bilirubin’s antioxidant function has been known for over three decades. Bilirubin also has anti-inflammatory actions, but this mechanism remains unclear. Recent evidence demonstrates that bilirubin has a hormonal action by directly binding and activating the nuclear receptor peroxisome proliferator-activated receptor-alpha (PPARα) (22-25). Interestingly, this property of bilirubin is specific to PPARα, as it does not interact with the other PPAR isoforms, PPARγ or PPARβ/δ (22, 23). This review summarizes the evidence of bilirubin as a hormone and discusses the important finding that it activates PPARα to act as a signaling molecule that drives gene transcription changing physiological responses. We will also discuss the potential significance of this observation to the protective actions of bilirubin and the potential therapeutic implications of this pathway in several forms of kidney disease.

Evidence for bilirubin as a selective PPARα modulator (SPPARM)

Bilirubin is an open-chain tetrapyrrole formed by oxidative cleavage of porphyrin in heme (Figure 1). The half structure of the bilirubin tetrapyrrole has similarities to the molecular structure of known PPARα agonists such as WY 14,643 and fenofibrate (Figure 1) (25). Subsequent molecular modeling analysis also demonstrated that bilirubin fits into the binding domain of PPARα similar to that of another agonist GW735 (25). Evidence for the activation of PPARα by bilirubin is derived from several sources, including the autofluorescence of bilirubin when bound to proteins, and the activation of a PPARα- Gal4 transcription factor reporter system (22, 26). Both of these approaches estimate the EC50/Kd of bilirubin binding and activation of the LBD of PPARα.
to be between 5-9 μM, which is equivalent to the range of normal plasma bilirubin concentrations (0.3-0.5 mg/dL). Thus, these data suggest the bilirubin binds PPARα at normal plasma levels. In contrast, bilirubin binds the Mas-related G-protein Coupled Receptor (MRGPR) at much higher levels as with an EC$_{50}$ of 145 μM, corresponding to plasma bilirubin levels of 8-9 mg/dL typically observed in severe jaundice (27).

**PPARα mediates the transcriptional response to bilirubin**

If bilirubin binds to PPARα *in vitro* and *in vivo*, it should alter the target cells' transcriptional response in a PPARα-dependent fashion. To determine the role of PPARα in the transcriptional response to bilirubin, we performed a series of experiments using lentiviral expression of shRNA targeting PPARα in HepG2 liver cells. HepG2 cells treated with a scrambled shRNA served as controls (*Figure 2*). After treatment with biliverdin which is converted to bilirubin inside the cells, RNA sequencing analysis revealed 398 genes exhibited a 2-fold or greater change in expression compared to only 23 genes in the shRNA treated cells (*Figure 2*) (24). The change in expression observed in the shRNA-treated cells may be due to bilirubin's antioxidant or anti-inflammatory actions. PPARα also mediates the *in vivo* effects of bilirubin treatment. Intraperitoneal (i.p.) bilirubin treatment in wild-type mice results in decreases in body weight and fasting blood glucose, increases in hepatic *Fgf21* and *Cyp4a* mRNA levels, and serum FGF21 levels (25). Mice containing the human Gilbert’s mutation are moderately hyperbilirubinemic and also exhibit increases in hepatic Fgf21 and Cyp4a mRNA levels (28). These changes were lost in PPARα knockout mice (*Figure 3*) (25). These data demonstrate that the direct effect of bilirubin on transcriptional responses is mediated via PPARα. It will be essential to distinguish the role of PPARα in the *in vivo* response to bilirubin treatment using tissue-specific PPARα knockout (KO).
mice. We have recently reported that hepatocyte-specific PPARα KO mice exhibit exacerbated high fat diet-induced hepatic steatosis and increased hepatic inflammation and serum hyperlipidemia (29). We also showed that adipose-specific PPARα KO mice had significantly higher adiposity (30) and that pegylated bilirubin suppressed white adipose tissue (WAT) size resucing adiposity (26). It will be interesting to determine whether bilirubin treatment can reverse these parameters in mice deficient in hepatic or adipocyte PPARα, as has recently been described (30, 31).

**Bilirubin alters the binding of coregulators to the PPARα transcription factor**

PPARα and other nuclear receptors are regulated at multiple levels by ligand binding. Ligand binding can result in conformational changes in the protein, and such changes can modify a specific set of coregulators that can impact gene-specific transcriptional activity. The coregulator interactome that modifies PPARα transcriptional activity is largely unknown, as are the effects of different PPARα ligands on the coregulator profile and their subsequent impact on transcriptional activity. In order to examine the effect of bilirubin on the coregulatory profile of PPARα, we utilized PamGene nuclear hormone receptor (NHR) chip that employed MARCoNI (microarray assay for real-time coregulator-nuclear receptor interaction) technology on white adipose tissue from bilirubin treated obese mice as well mice that exhibit a humanized form of Gilbert’s polymorphism (Figure 4) (26). WAT from these different hyperbilirubinemia mouse models gave remarkably similar results in that PPARα transcriptional activity was significantly higher. Bilirubin treatment and mice with moderate hyperbilirubinemia due to the human Gilbert’s polymorphism both exhibited enhanced binding of coactivators of the nuclear receptor coactivator (NCOA) family, MED1 (Mediator Complex Subunit 1), and peroxisome proliferator-
activated receptor-gamma coactivator 1-alpha (PGC-1α) (Figure 4). Bilirubin reduced the transcriptional binding of corepressors such as CCR4-NOT transcription complex subunit 1 (CNOT1), nuclear receptor-interacting protein 1 (NRIP1, also known as RIP140), and nuclear corepressor (NCOR) (26). Therefore, bilirubin remolds transcriptional activators and repressors of PPARα to increase its transcriptional activity. Activation of PPARα in white adipose tissue activates β3 adrenergic receptor (Adrb3) and uncoupling protein 1 (UCP1), inducing proton leak across the inner mitochondrial membrane, increasing oxygen consumption, promoting mitochondrial biogenesis, and improving mitochondrial function (Figure 4) (26). β3 adrenergic receptor stimulation also increases lipolysis and thermogenesis, decreasing adiposity and improving insulin sensitivity. Loss of adipocyte BVRA results in a decrease in bilirubin generation, decreased Adrb3 expression, reduced PPARα, and lower mitochondrial content (32). Similarly, we found that CRISPR knockout of BVRA in mouse proximal tubule cells caused lipotoxicity and reduced mitochondrial function (33). These data demonstrate the profound effect that bilirubin treatment has on mitochondrial activity.

**Bilirubin, PPARα, and the kidney**

Heme oxygenase-1 (HO-1) is an important regulator of blood pressure and kidney function (34-37). HO-1 exerts its effects through the breakdown of free heme, which is a potent oxidant and can join in the Fenton reaction to produce toxic free hydroxyl radicals. HO-1 also exerts its effect by generating carbon monoxide (CO) and bilirubin.

CO has many functions in the kidney. CO protects the kidney against excessive vasoconstriction, especially in conditions of decreased nitric oxide (NO) production (38-40). CO stimulates soluble guanylate cyclase (sGC) to increase the levels of Guanosine 3′,5′-cyclic
monophosphate (cyclic GMP or cGMP), stimulating a second messenger pathway that ultimately leads to activation of large-conductance calcium-activated potassium (KCa) channels and vasodilation (41, 42). CO also regulates renal tubule function through its effects on ion channels, generation of cGMP, and its impact on the generation of reactive oxygen species (ROS) (43-46).

Bilirubin has vascular and tubular effects on the kidney. Bilirubin lowers blood pressure and improves renal vascular resistance and glomerular filtration rate in angiotensin II-dependent hypertension (47, 48). Bilirubin improves renal hemodynamics by increasing the bioavailability of nitric oxide (47). Hyperbilirubinemic Gunn rats are also resistant to the pressor actions of angiotensin II (49, 50). Bilirubin treatment increases the bioavailability of NO presumable through decreases in ROS production that prevents the formation of peroxynitrite (51). Bilirubin is a potent antioxidant in the body that can directly scavenge ROS and prevent their formation through its effects on ROS generating enzymes (52, 53). It was thought that the antioxidant activity of bilirubin in the kidney improves renal vascular resistance and glomerular filtration rate. However, prior antioxidant treatment did not attenuate the blood pressure-lowering actions of bilirubin in angiotensin II-hypertensive mice. In this study, treatment with the antioxidant apocynin did not lower blood pressure to the same level as bilirubin treatment alone despite similar antioxidant activity (54), suggesting that bilirubin lowers blood pressure by pathways independent of its antioxidant actions.

If its antioxidant properties fail to explain bilirubin's blood pressure lowering effect, what other mechanisms does bilirubin lower blood pressure? This question was difficult to answer until the discovery of bilirubin as a selective activator of PPARα (25). PPARα plays a vital role in regulating blood pressure and kidney function. PPARα induction with fibrates lowers blood pressure in several models of hypertension in rodents (55-58). Mice with global deficiency of
PPARα exhibit alterations in pressure-natriuresis and salt-sensitive hypertension (59). Fenofibrate treatment also lowers blood pressure in salt-sensitive but not salt-resistant hypertensive patients (60). PPARα agonists protect against diabetic nephropathy and acute kidney injury (61-63). PPARα is a master regulator of fatty acid β-oxidation as well as numerous signaling pathways that impact renal function. Stimulation of fatty acid β-oxidation decreases the accumulation of free fatty acids in renal cells, reducing cellular toxicity and improving cell damage in several models of renal disease. One of the pathways in the kidney that is affected by treatment with fibrates is the forkhead box proteins (FoxOs) (64, 65). Alterations in FoxO phosphorylation led to dysregulation of FoxO activity which contributes to insulin resistance, type 2 diabetes, and hyperlipidemia (66, 67). Fox01 is highly expressed in the proximal tubule and expressed at low levels in the glomerulus; whereas, FoxO3a is highly expressed in collecting tubules and podocytes (64). FoxOs can reduce intracellular ROS production by increasing the expression of antioxidants like manganese superoxide dismutase (MnSOD) (65). Fenofibrate treatment protects against ischemia-induced acute kidney injury through alterations in apoptotic pathways and enhancement of nitric oxide synthase activity (68, 69). In isolated podocytes, PPARα agonists upregulate the expression of nephrin and protect against injury (70-72). PPARα agonists attenuate mesangial cell proliferation, extracellular matrix synthesis and production of inflammatory cytokines (73-75). Thus, increased renal PPARα activity offers protection via several mechanisms (Figure 5).

In rodent models of ischemia-induced acute renal injury, bilirubin administration is protective (76). Kidneys undergoing transplant in renal transplant recipients undergo a period of ischemia-reperfusion, which is associated with increased levels of ROS production, Plasma bilirubin levels negatively correlate with late transplant rejection (7 yrs post transplant) in renal transplant recipients (77). Immunoglobulin A (IgA) nephropathy is a kidney disease due to excess
build up of IgA in the kidney resulting in severe inflammation and glomerular injury. Low plasma bilirubin levels are associated with an increased incidence of end stage renal disease and worse outcomes in patients with IgA nephropathy (78, 79). In IgA vasculitis with nephritis (IgAV-N) low plasma bilirubin is associated with greater progression of the disease (80). The Gilbert’s polymorphism (UGT1A1*28) and plasma bilirubin levels have been demonstrated to protect against cardiovascular events in hemodialysis and peritoneal patients (81-83).

Serum bilirubin levels negatively correlate with the development of diabetic nephropathy and end-stage renal disease (19, 84-87). Bilirubin treatment is also protective in several preclinical models of acute kidney injury (76, 88). There are several mechanisms by which bilirubin offers protection against acute and chronic kidney injury, including increases in the bioavailability of NO preserving renal blood flow (48, 51), attenuation of the inflammatory response (89, 90), and its antioxidant activity. The specific role of PPARα activation in the protective effects of bilirubin in models of kidney injury has not yet been examined. These types of studies will require animal models in which PPARα is selectively knocked out in the whole kidney, defined nephron segments, vascular or immune cells.

Low serum bilirubin levels are associated with several chronic cardiovascular, metabolic, and renal diseases (21). Novel strategies to increase serum bilirubin and formulations of bilirubin are needed to translate the therapeutic potential of bilirubin (91). Antagonism of hepatic UDP glucuronosyltransferase family 1 member A1 (UGT1A1) increases serum bilirubin levels due to reduced bilirubin conjugation in the liver reducing blood levels. Treatment with protease inhibitors such as indinavir and atazanavir acts as UGT1A1 antagonists, raising serum bilirubin levels, improving vascular function, and lowering blood pressure (31, 62). However, these drugs have side effects that preclude their widespread use clinically. Natural products such as silymarin
derived from milk seed thistle also target hepatic UGT1A1 and increase serum bilirubin levels (92, 93). Lastly, molecular tools like antisense morpholinos or RNA interference (RNAi) targeting hepatic UGT1A1 can be developed to increase serum bilirubin levels.

Exogenous bilirubin treatment has been used therapeutically to treat acute kidney injury in preclinical models. The low solubility of bilirubin in aqueous solutions prevents its use in clinical settings. However, recent formulations of bilirubin have been designed to increase its water solubility. For instance, nanoparticles consisting of bilirubin coupled to polyethylene glycol (PEG-BR) alleviate inflammation and the development of nonalcoholic fatty liver disease (NAFLD) in specific mouse models of the diseases (31, 94-96). Recently, hyaluronic acid-coated polylysine-bilirubin nanoparticle (nHA/PLBR) that selectively accumulate in the kidney attenuated inflammation, preserved mitochondrial function, and inhibited tubule cell apoptosis in a model of ischemia-induced acute kidney injury (97). These studies highlight the potential use of bilirubin, chemically modified to increase solubility, as a therapeutic in cardiovascular, metabolic, and kidney diseases.

Moderate increases in plasma bilirubin are protective to the kidney; however, higher levels can be detrimental in some conditions. For example, severely jaundiced patients with liver failure can exhibit cholemic nephropathy (98). Hyperbilirubinemia is also associated with postoperative acute kidney injury in patients undergoing cardiac surgeries (99). Hyperbilirubinemia is also associated with greater risk of contrast-induced acute kidney injury (100). High bilirubin levels on admission are associated with the development of acute respiratory distress syndrome and increased mortality in septic patients (101, 102). The shift in the protection of bilirubin in cases of liver dysfunction is not fully understood and warrants further investigation.
Conclusions

A wealth of population and clinical data demonstrate the protective effects of plasma bilirubin levels against cardiovascular and kidney disease. Despite this evidence, the mechanism(s) by which bilirubin offers protection against cardiovascular and kidney disease remains unknown. Bilirubin, long thought of as a metabolic waste product of heme metabolism, has several essential functions that are often overlooked by practicing physicians. The antioxidant and anti-inflammatory actions of bilirubin have been known for some time. However, emerging evidence demonstrates that bilirubin can activate nuclear hormone receptors such as PPARα. This data suggests that bilirubin has hormonal functions in specific tissues. The extent to which bilirubin's hormonal actions protect the kidney in various forms of kidney injury needs to be studied further so that clinical nephrologists can fully translate and practice the potential therapeutic benefit.

Disclosures: D. Stec reports the following: Advisory or Leadership Role: Editorial Board for following journals: Hypertension, American Journal of Physiology Regulatory, Integrative and Comparative Physiology; and Other Interests or Relationships: American Heart Association- Chair of Peer Review Group. C. Tiribelli reports the following: Scientific Advisor or Membership. The remaining authors have nothing to disclose.

Funding: This work was supported by the National Institutes of Health 1R01DK121797-01A1 (T.D.H.) and 1R01DK126884-01A1 (D.E.S.), the National Heart, Lung, and Blood Institute K01HL-125445 (T.D.H.) and P01 HL05197-11 (D.E.S.), and the National Institute of General
Medical Sciences P20GM104357-02 (D.E.S.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Acknowledgments: All figures were made using BioRender.com

Author Contributions: David E. Stec: Conceptualization; Writing - original draft; Writing - review and editing. Claudio Tiribelli: Conceptualization; Writing - review and editing. Olufunto O. Badmus: review and editing. Terry D. Hinds, Jr.: Conceptualization; Writing - original draft; Writing - review and editing.
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**Figure Legends**

**Figure 1**- Structural similarities between bilirubin and known PPARα agonists, WY-14,643 and fenofibrate.

**Figure 2**- Transcription profiling of wild-type and PPARα deficient HepG2 cells treated with bilirubin. Knockdown of PPARα decreases the transcriptional response to bilirubin treatment by greater than 15 fold.

**Figure 3**- Summary of the effect of bilirubin treatment in wild-type (WT) and PPARα knockout (KO) mice.

**Figure 4**- Bilirubin regulates the transcriptional coregulators that bind to PPARα, which affects the expression of target genes such as β3 adrenergic receptor (ADRB3) and uncoupling protein 1 (UCP1) to affect adiposity, glucose tolerance, and mitochondrial function.

**Figure 5**- Summary of the effects of PPARα activation in the kidney. PPARα agonists improve pressure-natriuresis and lower blood pressure in hypertension. PPARα agonists also affect β-oxidation, the FoxO pathway, and nitric oxide (NO) generation to improve tubule cell survival as well as renal vasodilation.
Figures

WY-14,643

Fenofibrate

Bilirubin

Figure 1
Figure 2

HepG2 Cells

PPARα

WT

RNASEq

398 genes > 2 fold change

PPARα KD

Lentiviral PPARα shRNA

23 genes > 2 fold change
Figure 3

WT

↓ Body Weight
↓ Fasting blood glucose
↑ Hepatic FGF21
↑ Plasma FGF21

PPARα KO

↔ Body Weight
↔ Fasting blood glucose
↔ Hepatic FGF21
↔ Plasma FGF21