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Original article

Absence of the biliverdin reductase-a gene is associated with increased endogenous oxidative stress

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ABSTRACT

Bilirubin, a byproduct of heme catabolism, has been shown to be an effective lipid-soluble antioxidant *in vitro*. Bilirubin is able to inhibit free radical chain reactions and protects against oxidant-induced damage *in vitro* and *ex vivo*. However, direct evidence for bilirubin's antioxidant effects *in vivo* remains limited. As bilirubin is formed from biliverdin by biliverdin reductase, we generated global *biliverdin reductase-a* gene knockout ($Bvra^{-/-}$) mice to assess the contribution of bilirubin as an endogenous antioxidant. $Bvra^{-/-}$ mice appear normal and are born at the expected Mendelian ratio from $Bvra^{+/-}$ x $Bvra^{+/-}$ matings. Compared with corresponding littermate $Bvra^{+/+}$ and $Bvra^{+/-}$ animals, $Bvra^{-/-}$ mice have green gall bladders and their plasma concentrations of biliverdin and bilirubin are approximately 25-fold higher and 100-fold lower, respectively. Naïve $Bvra^{-/-}$ and $Bvra^{+/+}$ mice have comparable plasma lipid profiles and low-molecular weight antioxidants, *i.e.*, ascorbic acid, α-tocopherol and ubiquinol-9. Compared with wild-type littermates, however, plasma from $Bvra^{-/-}$ mice contains higher concentrations of cholesteryl ester hydroperoxides (CE-OOH), and their peroxiredoxin 2 (Prx2) in erythrocytes is more oxidized as assessed by the extent of Prx2 dimerization. These data show that $Bvra^{-/-}$ mice experience higher oxidative stress in blood, implying that plasma bilirubin attenuates endogenous oxidative stress.

1. Introduction

Bilirubin is the end product of heme catabolism in mammals. Heme is degraded by heme oxygenase to carbon monoxide, ferrous iron and biliverdin, and the latter is then reduced to bilirubin by biliverdin reductase [1]. Adult humans produce ~300 mg bilirubin each day [2]. The bilirubin formed is essentially insoluble in water and hence tightly bound to albumin for transport in the circulation [3]. When the binding capacity of albumin for bilirubin is exceeded, the yellow pigment can accumulate in the brain and cause toxicity and neurologic dysfunction. Bilirubin is removed from the circulation by hepatic glucuronyl transferase-mediated conjugation with glucuronic acid. The resulting conjugated bilirubin is secreted into bile, and responsible for the typical yellow appearance of the gall bladder.

Considered originally to be a metabolic waste product, bilirubin in its free, albumin-bound and conjugated forms, has since been recognized to possess potent antioxidant activities *in vitro* [4–6]. These include the efficient inhibition of linoleic acid oxidation by peroxyl radicals [4,5] and protection of circulating lipids from oxidation *via* interaction with α-tocopherol (α-TOH) [6]. In addition to scavenging lipid peroxyl radicals and α-tocopheroxyl radical (α-TO•), bilirubin attenuates oxidative damage to proteins [7,8]; scavenges hypochlorous acid [9], nitric oxide and reactive nitrogen species [10]; and inhibits chloramine formation and myeloperoxidase-mediated protein oxidation [11].

Suggestions that bilirubin may be an important antioxidant *in vivo* were initially based on experiments showing protective effects of the pigment in cultured cells. For example, bilirubin formation induced by hemin treatment, protects vascular smooth muscle cells against oxidative stress [12]. Bilirubin also attenuates vascular endothelial dysfunction in response to oxidized low-density lipoprotein [13]. In human cardiomyocytes, albumin-bound bilirubin is a powerful protector against oxidant toxicity [14]. Antioxidant properties of bilirubin have also been reported in several animal models of oxidative stress. For example, bilirubin protected against oxidative stress in hyperbilirubinemic Gunn rats exposed to hyperoxia, as assessed by a decrease in thiobarbituric acid-reactive substances [15]. Hemin treatment or exogenously added bilirubin preserved myocardial function in an animal model of cardiac ischemia/reperfusion injury [16]. Moreover, *ex vivo* treatment of perfused rat hearts with conjugated bilirubin improved

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post-ischemic functional outcomes and decreased myocardial oxidative damage [17].

Several human studies indirectly suggest bilirubin to provide benefit *in vivo*. For example, plasma bilirubin concentrations inversely associate with risk of cardiovascular disease, including coronary artery disease [18], atherosclerosis [19] and stroke [20]. Individuals with Gilbert syndrome, who have moderately increased plasma bilirubin as a result of decreased activity of bilirubin uridine diphosphate glucuronosyltransferase have lower risk of cardiovascular disease [21]. In these subjects markers of oxidative stress, such as serum malonyldialdehyde and urinary 8-hydroxy-2′-deoxyguanosine, are also decreased compared with the normal population [22].

Despite this association between bilirubin and cellular protection, evidence that bilirubin at physiological concentrations directly protects against oxidative stress *in vivo* remains limited. Therefore, we generated a *biliverdin reductase-a* gene-deficient (*Bvra*$^{-/-}$) mouse to more directly assess the contribution of bilirubin as an endogenous antioxidant *in vivo*. Herein, we report that *Bvra*$^{-/-}$ mice which lack circulating bilirubin, have higher oxidative stress. Our findings support the notion that normal concentrations of bilirubin contribute to the endogenous antioxidant defense *in vivo*.

2. Materials and methods

2.1. Animals

Biliverdin reductase-a *(Bvra)* gene deficient mice were generated by homologous recombination in embryonic stem (ES) cells (Ozgene, Perth, WA, Australia) (Fig. 1A). In this procedure, exon 3 of the *Bvra* gene was replaced *via* a targeting vector that was created by PCR from C57BL/6J genomic DNA using the plasmid pBR322 as the vector backbone. The targeting vector construct contained a cDNA sequence of exons 2, 4 and 5 of the *Bvra* gene, with a phosphoglycerine kinase promoter driving a neomycin resistance gene cassette (PKG_*neo*) that was flanked by flippase recognition target (FRT) sites. The targeting vector was electroporated into Bruce4 ES cells derived from a C57BL/6 congenic strain. The antibiotic-resistant heterozygous knockout ES cells were expanded and injected into developing mouse embryos, which were then implanted in pseudopregnant females to generate chimeras. The resultant chimeras were mated to Albino BL/6 mice to obtain germ line-transmitting heterozygotes, with ES cell coat color offspring genotyped by Southern blot. To remove the PKG_*neo* cassette, homozygous knockout offspring were mated to a whole body FlpE deleter strain. After deletion of the PKG_*neo* selection cassette, *Bvra*$^{+/+}$, *Bvra*$^{+/-}$ and *Bvra*$^{-/-}$ littermates were obtained from *Bvra*$^{+/-}$ x *Bvra*$^{+/-}$ breeding pairs. Male and female mice, 8–12 weeks old, were used for experiments. All procedures were carried out according to the Australian NHMRC Guidelines for Animal Research and were approved by the Animal Care and Ethics Committees of the Garvan Institute of Medical Research/St Vincent's Hospital. Genotyping was performed on genomic DNA isolated from ear punch biopsies *via* the ISOLATE II Genomic DNA Kit (Bioline, Sydney, NSW, Australia). PCR amplification was performed using the DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) with primers purchased from Integrated DNA Technologies: *Brva* universal forward primer, 5′- CTGGGGTTGC CAGCTTCCCT -3′; *Bvra* wildtype reverse primer, 5′- CTGGACACATAT CCAATCAGGTTTA -3′; *Bvra* knockout reverse primer, 5′- ATAGGAAC TTCGGCGCGCCTGATAT -3′. The combination of these primers resulted in PCR products of 233 bp in *Bvra*$^{+/+}$, 233 and 139 bp in *Bvra*$^{+/-}$, and 139 bp in *Bvra*$^{-/-}$.

2.2. Collection of blood, plasma and tissue samples

Mice were anesthetized by isoflurane inhalation. Blood was collected by cardiac puncture using a 25-gauge needle and transferred into heparin-coated micro tubes (Sarstedt, Nümbrecht, Germany). To obtain plasma, heparinized blood was centrifuged at 2000 × *g* and 4 °C for 15 min. Immediately following cardiac puncture, mice were gravity-perfused (90 mm Hg) with phosphate buffered saline (PBS). Brain, heart, liver and spleen were collected for Western blot and mRNA analyses. Tail tips were collected to confirm genotype of mice.

2.3. RNA extraction and analysis

Animal tissues were homogenized with 1 mL TRIzol (Thermo Fisher Scientific) and RNA isolated following the manufacturer's instructions. RNA pellets were re-suspended in nuclease-free water and RNA concentrations determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Complementary DNA was prepared using the Superscript III First Strand Synthesis kit (Thermo Fisher Scientific) following the manufacturer's instruction. Real-time quantitative PCR was performed on a LightCycler 480 System (Roche, Basel, Switzerland) using the SensiFAST Probe No-ROX Kit (Bioline). The amount of mRNA was determined relative to *β-actin* mRNA using the comparative CT method [23]. PCR products were verified by melt curve analysis. The PCR primers (Integrated DNA Technologies, Coralville, IA, USA) used were: *β-actin* forward, 5′-GGATGCAGAAGGAGATCACTG-3′; *β-actin* reverse, 5′-CGATCCACACGGAGTACTTG-3′; *Bvra* forward, 5′-AGCCGCT GGTAAGCTCC-3′; *Bvra* reverse, 5′-ACCAACCACTACCACACCAAA-3′; *Bvrb* forward, 5′-TTCTCAGCTTTTCCGGCCCT-3′; *Bvrb* reverse, 5′-CCT CATAACCTGCTTGCACCG-3′; *Hmox1* forward, 5′-AGGTACACATCCAA GCCGAGA-3′; *Hmox1* reverse, 5′-CATCACCAGCTTAAAGCCTTCT-3′; *Hmox2* forward, 5′-AGCACATGACCGAGCAGAAAA-3′; *Hmox2* reverse, 5′-GCTCCGTGGGGAAATATAAGGG-3′; *Ttpa* forward, 5′-TCTACAGAGA ACACTAATGAGCAATGTG-3′; *Ttpa* reverse, 5′- TGGTGAAGCCATGTG GAAAGT-3′.

2.4. SDS-PAGE and immunoblotting

Tissues were homogenized in SDS-urea lysis buffer (6.7 M urea, 10% glycerol, 10 mM Tris pH 6.8, 1% SDS, 1 mM DTT, 1 mM PMSF, 1 mM protease inhibitor cocktail) (Roche). Homogenates (30 μg protein) were loaded onto 10% SDS-polyacrylamide gels (NuPage, Thermo Fisher Scientific). Electrophoresis was performed at 100 V for 90 min, and separated proteins transferred onto nitrocellulose membranes by the iBlot2 Dry Blotting System (Thermo Fisher Scientific). Membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) at room temperature for 1 h and incubated with primary antibodies (BVRA antibody, ADI-OSA-450, 1:1000, Enzo Life, Farmingdale, NY, USA; β-actin antibody, 691001, 1:5000, MP Biomedicals, Santa Ana, CA, USA) at 4 °C overnight. Membranes were washed and proteins detected using IRDye 800CW goat-anti-rabbit IgG or IRDye 680RD goat anti-mouse IgG (LI-COR Biosciences, Lincoln, NE, USA). Proteins were visualized using the Odyssey Model 9120 Gel Documentation System and analyzed by densitometry using the Image Studio Lite software (LI-COR Biosciences).

2.5. Hematology and clinical biochemistry

Hematological and clinical biochemical analyses were performed at the Veterinary Pathology Diagnostic Services, The University of Sydney. Red blood cells, hemoglobin, hematocrit, mean corpuscular volume, platelets, white blood cells, neutrophil, lymphocyte, monocyte, eosinophil and basophil were determined in EDTA blood with the XT-2000i Hematology Analyzer (Sysmex, Kobe, Japan) by fluorescence flow cytometry technology. Standard clinical biochemistry analyses were performed on heparinized-plasma samples using the Konelab 20i Chemistry Analyzer (Thermo Fisher Scientific) to determine the concentrations of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, urea, creatinine, albumin and iron.