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Bilirubin redox cycling
or:
Why new evidence shows that Jim Lovell should have been a Mercury astronaut

by

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BR	Bilirubin
BV	Biliverdin
BVR	Biliverdin reductase
HO	Heme oxygenase

H_2O_2	Hydrogen peroxide
$HO^\bullet$	Hydroxyl radical
O_2	Molecular oxygen
$ROO^\bullet$	Peroxyl radical

Abstract

Bilirubin has long been recognized for its role as a main bile constituent and also as the molecule causing the yellow tint in persons with jaundice. Bilirubin is produced in the body following heme ring degradation and is often associated with serum albumin. Only recently has research suggested that this degradation product may play an important functional role in the cell as a small molecule antioxidant. Because bilirubin is very lipophilic (hydrophobic), it is situated in cell membranes at nanomolar levels. Bilirubin may prevent lipid peroxidation by removing highly oxidizing peroxyl radicals before they may attack fatty acids, such as linoleate. Bilirubin does so through a hydrogen donation reaction with a lipid peroxyl radical, resulting in biliverdin and lipid hydroperoxide. The bilirubin antioxidant may be regenerated by the rapid action of biliverdin reductase. The very high rate of catalysis accounts for the physiologically relevant role of bilirubin in the cell, in spite of the fact it is found at far lower concentrations than both α-tocopherol and glutathione. This review will highlight the pathways of formation and redox cycle participation of bilirubin as well as briefly discuss detection methods utilized in the lab.

Introduction

The bilirubin molecule has long been recognized as the leading contributor to jaundice, the yellowing of the skin. As the final product of heme catabolism, it is surprisingly toxic in the body and, because it is highly lipophilic, cannot be excreted without first undergoing an energetically-expensive modification (glucouronidation) [1]. Due to its role as the main component of waste bile, bilirubin accumulation has been viewed in the clinic as merely a marker of illness and physiologic abnormality, as was the case of astronaut Jim Lovell who was cut from Project Mercury astronaut selection in the late 1950s because of elevated bilirubin levels[1]. While elevated levels of bilirubin may have deleterious effects in neonates, including kernicterus where bilirubin deposits in brain capillaries, altering endothelial cell structure and affecting glucose transport [**2**], mild to moderate increases of bilirubin in circulation have been correlated with lower levels of cardiovascular distress in adults [**3**]. Because of these observations and the fact that large amounts of energy are consumed in synthesizing and solvating bilirubin for excretion, researchers have believed that it must contribute some other function other than as a byproduct of molecular breakdown. Only within the past few decades has its role as a small antioxidant molecule been recognized and elucidated [4, 5]. Bilirubin appears to be a more potent scavenger of peroxyl radicals than even α-tocopherol at biologically relevant O_2 tensions [4]. A role for bilirubin in redox-cycling has been revealed within the past three years showing that not only is bilirubin not an energy-wasting catabolic byproduct but, more importantly, it is one of the most relevant molecules in the body in peroxyl radical scavenging [5]. This review will highlight the

[1]From http://www.jsc.nasa.gov/history/oral_histories/LovellJA/JAL_5-25-99.pdf, page 12-5.

pathways of formation and redox cycle participation of bilirubin as well as briefly discuss detection methods utilized in the lab.

Bilirubin formation

Heme consists of a porphyrin ring that coordinates an iron atom among four nitrogen molecules. The iron ion can easily interact with and transport O_2. When hemoglobin within the blood has reached the end of its usefulness, the heme molecule is degraded to bilirubin, a tetrapyrrole consisting of four C_4H_5N aromatic rings. This degradation process occurs in a two-step reaction series involving heme oxygenase (HO) and biliverdin reductase (BVR).

Hemoglobin is degraded in the liver and, if the heme molecules enter the bloodstream, they may be deposited in tissues that can break down the ring and safely sequester the iron atom. Such tissues include the brain, which contains heme oxygenase 2 (HO2) to deal with the heme molecule. Heme oxygenase 1 (HO1) is located in the liver to immediately degrade the heme that did not travel through the bloodstream. In order for HO1 or HO2 to catalyze the reaction from heme to its product,

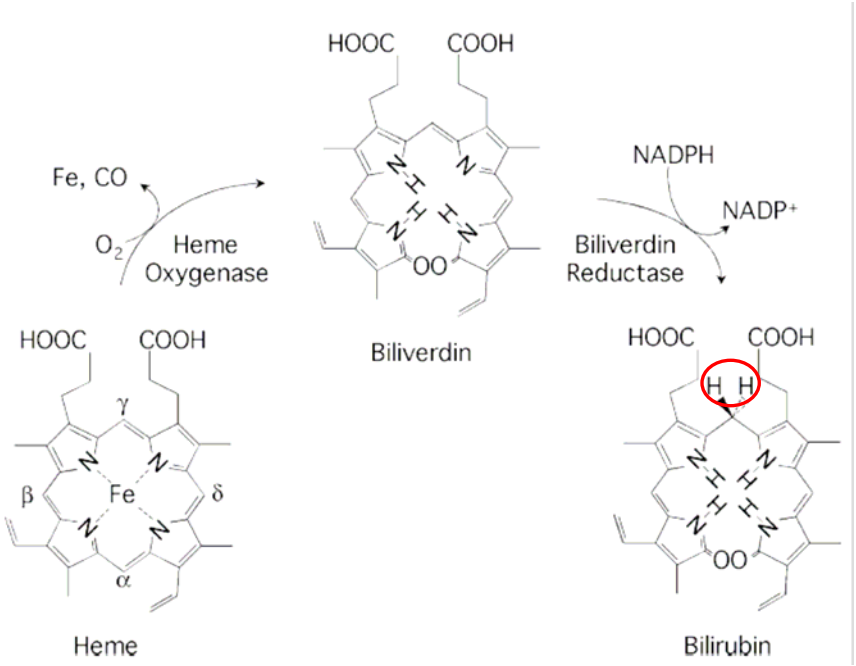

Figure 1. Oxidative heme catabolism to biliverdin and bilirubin. Molecular oxygen and NADPH are requisite in the ring cleaving reaction. Bilirubin is thought to have a pair of reactive hydrogens located on the outside of the ring, which are emphasized in the structure at right. From [7].

biliverdin, oxygen and NADPH must be present to accept and donate electrons, respectively, to allow the porphyrin ring to be cleaved (**Figure 1**). Carbon monoxide, water, and the oxidized $NADP^+$ cofactor are byproducts of this reaction. Biliverdin is a water-soluble molecule and may be readily flushed out of biological systems.

Biliverdin reductase (BVR) catalyzes the second step of heme degradation. The biliverdin substrate synthesized during the HO reaction enters the active site of BVR where it may accept two electrons, one of which is in the form of a hydrogen atom, to make bilirubin (**Figure 1**). This reaction product is a highly lipophilic, insoluble molecule, rendering it potentially physiologically toxic. The BVR enzyme catalyzes a kinetically favorable and therefore rapid reaction, having a first-order rate constant (k) of 20 s^{-1} [6].

Bilirubin redox chemistry

Because premature neonates present symptoms of jaundice more frequently than full term neonates, the role of bilirubin and phototherapy have been extensively investigated [2, 4, 9, 12, 14]. While bilirubin is considered to be a small antioxidant molecule, a redox cycle between biliverdin and bilirubin using BVR is the full pathway required to scavenge radicals and is discussed in further detail below [5].

The peroxide problem

H_2O_2, hydroxyl radicals ($HO^\bullet$), and peroxyl radicals ($ROO^\bullet$) have some of the greatest potential to oxidize other cellular molecules. The same iron that is coordinated in the heme molecule and removed by heme oxygenase may undergo Fenton chemistry with H_2O_2 to produce $HO^\bullet$ (**Equation 1**). The radicals may then initiate lipid

peroxidation in the cell when the unpaired electron interacts with an electron on a linoleic acid molecule (LH) creating a linoleic radical ($L^{\bullet}$). This new radical may initiate a chain reaction when O_2 is present, even at low tensions (**Equation 2**) to create an ever increasing amount of linoleic acid peroxyl radicals ($LOO^{\bullet}$).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-} + O_2 \quad (1)$$

$$HO^{\bullet} \xrightarrow{LH} L^{\bullet} \xrightarrow{O_2} LOO^{\bullet} \quad (2)$$

(LH: $LOO^{\bullet}$ → $L^{\bullet}$)

Bilirubin as an antioxidant

Evidence for the role of bilirubin in oxidative processes was first recognized in the 1950s following observations that small quantities of bilirubin contribute to increased stability of vitamin A and β-carotene [reviewed in 7]. Like other antioxidants, bilirubin has a conjugated double bond system with a pair of reactive bisallylic hydrogen atoms (**Figure 1**) [4]. Bilirubin is a versatile antioxidant in that it may scavenge both 1 and 2 e^{-} oxidants [9]. *In vitro* studies have shown that, upon adding bilirubin to a system undergoing chain propagation, bilirubin disappeared when no heme oxygenase was present to replenish it and lipid peroxidation decreased (200 μM compared to only 6 μM of bilirubin consumed) [4]. One hypothesis for the mechanism of this action is that bilirubin may donate a hydrogen atom to an incident $ROO^{\bullet}$ (**Equation 3**) [10]. The

$$LOO^{\bullet} + BR \longrightarrow LOOH + BR^{\bullet} \quad (3)$$

$$BR^{\bullet} \longleftrightarrow BV \quad (4)$$

$$BV \xrightarrow{BVR} BR \quad (5)$$

resulting bilirubin radical may isomerize to the biliverdin species (**Equation 4**), which may be rapidly reduced back to bilirubin by BVR (**Equation 5**) at which point it is ready to scavenge once again.

Comparisons of lipid peroxidation incidence in the presence of bilirubin with α-tocopherol and β-carotene at atmospheric and physiologic oxygen tension (~20% and 2%, respectively) are shown in **figure 2**. While bilirubin does not chain-break as effectively as α-tocopherol at either oxygen tension in linoleic acid, it is more effective at physiologic O_2 levels. At this tension bilirubin is even more effective at reducing lipid peroxidation in phosphotidylcholine than α-tocopherol (**Figure 2**). Ascorbate studies have been conducted on plasma derived from premature and full-term babies to compare levels with bilirubin [11]. Though directly after birth, cord blood contained higher levels of ascorbate and lower levels of bilirubin when compared to that seen in term blood samples, five days post partum saw a dramatic increase in the bilirubin antioxidant activity in premature samples. This activation of bilirubin correlated directly with the total plasma antioxidant activity, whereas no other molecule showed any such

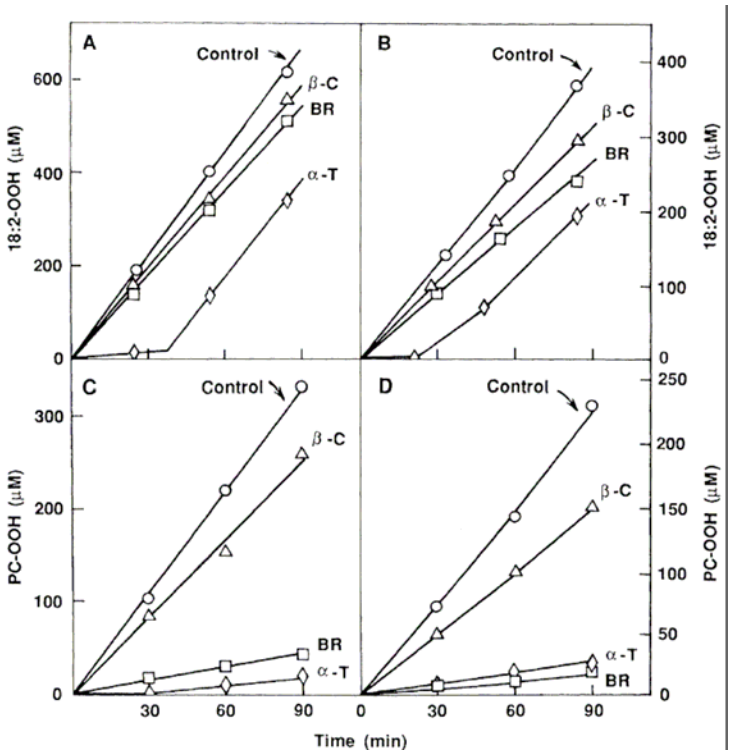

Figure 2. Inhibition of lipid peroxidation by various antioxidant treatments (β-C, β-carotene; BR, bilirubin; α-T, α-tocopherol) at atmospheric (A, C) and physiologic (B, D) oxygen tensions in homogeneous solutions of linoleic acid (A, B) or phosphatidylcholine (C, D). At biologically relevant O_2 levels, bilirubin (BR) improves its antioxidant capacity by 16% [4].

pattern [11]. Together, these data suggest that slightly increased bilirubin is required for antioxidant defenses, particularly in populations that have increased oxidative stress.

Recent studies have shown that, though physiologic bilirubin concentration is only 0.1% that of glutathione, it is a more potent antioxidant and must therefore have more than stoichiometric activity [5, 12]. In order to scavenge reactive oxygen species, bilirubin is oxidized to biliverdin by H_2O_2, and BVR must replenish bilirubin levels by reducing biliverdin. Fortunately, the reaction is very rapid and the cell is able to deactivate ROS that are at 10,000-fold greater concentration than that of bilirubin [5]. In this manner, bilirubin plays an important role in decreasing oxidative stress without achieving toxic levels of accumulation.

Detecting bilirubin

One of the most common methods used to detect bilirubin is spectrophotometry. Bilirubin has an absorbance in the visible range of the light spectrum (approximately 450 nm, depending on solvent) and an extinction coefficient of 55,000 $M^{-1}cm^{-1}$ at 450.75 nm in chloroform [13]. This characteristic accounts for the visible yellowish hue and the photoreactivity of bilirubin. Absorbance difference spectrophotometry employs two lamps, one shining at the absorbance of bilirubin and a second, dimmer lamp used to determine its molecular reactive properties. Because the incidence of light (*i.e.* during phototherapy and spectroscopic methods) can isomerize bilirubin, it is important to be certain that the species being studied is actually bilirubin and not one of its photoproducts [14]. Absorbance difference spectroscopy is able to differentiate between these species for study.

Bilirubin may be detected and differentiated from photoisomers and other similar molecules (*i.e.* biliverdin) using reverse-phase high-performance liquid chromatography (HPLC). This process can separate molecules on the basis of the solute partitioning that utilizes a stationary liquid phase and a mobile liquid phase. One solvated species will be retained more greatly by silicon beads of 10 microns in diameter packed within the chromatograph's column one of the phases so bilirubin separation from other blood serum components is based on solubility [15]. This technique is especially useful in quantitatively determining the amount of bilirubin within total serum inputs.

Summary

Though bilirubin may be toxic at high levels due to its hydrophobicity, mild to moderate increases are beneficial because of the antioxidative potential against peroxyl radicals in the cell and its potential to decrease lipid peroxidation within the cell. This recent evidence suggests that Jim Lovell today could actually be considered an even stronger astronaut candidate because his higher levels of bilirubin in circulation are correlated with a decreased risk of cardiovascular disease.

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