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MINIREVIEW

Bilirubin: An Endogenous Product of Heme Degradation with Both Cytotoxic and Cytoprotective Properties

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Bilirubin is a linear tetrapyrrole that is formed during the process of heme degradation. Heme is released from a series of hemeproteins, including hemoglobin and cytochrome P450, and metabolized by heme oxygenase to form carbon monoxide, biliverdin, and free iron. Biliverdin is subsequently transformed to bilirubin by biliverdin reductase (Fig. 1). Bilirubin is a highly lipophilic molecule despite containing hydrophilic carboxyl groups, because the latter are unavailable for interaction with water as a result of intramolecular hydrogen bonding to the pyrrole nitrogen atoms. Bilirubin is found in blood bound to plasma albumin, which transports it to the liver, where it is conjugated to hydrophilic acceptors. The major conjugates are bilirubin glucuronides formed by UDP-glucuronosyltransferase 1 (UGT*01). These polar derivatives are thereafter excreted in the bile.

The great interest in understanding the regulation of expression and enzymatic activity of heme oxygenase results from the wide array of biological effects displayed by the products of heme degradation (Maines, 1999; Shibahara et al., 2002). Carbon monoxide is a putative neural messenger and a major cardiovascular regulator but can also compete with O_2 for binding to hemoglobin. The iron released from heme has been shown to be involved in cellular toxicity as a result of its capacity to induce the formation of reactive oxygen species (ROS). Biliverdin and bilirubin are potent antioxidants and protect cells from oxidative stress on the one hand, whereas bilirubin displays neurotoxicity on the other hand. This review provides a molecular insight into the complex cytotoxic and cytoprotective effects of bilirubin under both physiological and pathological conditions.

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Cytotoxic Effects of Bilirubin

Neonatal Jaundice and Bilirubin Encephalopathy. Plasma unconjugated bilirubin (UCB) levels are usually elevated in normal infants during the first two weeks of postnatal life (< 200 μM) because of the marked and sudden breakdown of fetal erythrocytes at birth, coupled with a transient inability of the newborn to form bilirubin glucuronides in the liver and excrete them in the bile. Once the UGT*01 enzyme and the biliary excretory system reach maturity, at about 1 month of age, plasma UCB levels decrease and reach the adult levels of ~20 μM (reviewed by Gourley, 1997). The "physiologic" neonatal hyperbilirubinemia (jaundice) may worsen because of: 1) increased bilirubin production resulting from increased hemolysis (e.g., in Rh or ABO incompatibility or in G6PD deficiency); 2) delayed maturation of the hepatic conjugation system (e.g., in prematurely born neonates); 3) increased enterohepatic circulation of bilirubin; 4) genetic abnormalities (e.g., mutations in the UGT*01 gene, such as in patients with the Crigler-Najjar syndrome type 1).

When the plasma levels of UCB are excessively elevated and surpass the capacity of albumin for high-affinity binding of UCB, the unbound (free) fraction of the pigment increases. This fraction may also be elevated in the plasma of newborns with "physiologic jaundice" in association with the following conditions: 1) low blood pH (acidosis); 2) reduced capacity of plasma albumin for high-affinity binding of UCB; and 3) use of drugs that compete with UCB for binding to plasma albumin (e.g., sulfonamides). Free UCB can easily enter the cells by passive diffusion and cause toxicity. The most vulnerable site is the central nervous system. UCB binds to discrete brain areas, such as the basal ganglia (kernicterus), and produces a wide array of neurological deficits collectively known as bilirubin encephalopathy. These include irreversible abnormalities in motor, sensory (auditory and ocular), and cognitive functions (reviewed by Shapiro, 2003). New-

ABBREVIATIONS: ROS, reactive oxygen species; UCB, unconjugated bilirubin; MAP, mitogen-activated protein kinase; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl)-1*H*-imidazole; SP 600125, anthra[1,9-*cd*]pyrazol-6(2H)-one; AhR, aryl hydrocarbon receptor; LDL, low-density lipoprotein; UGT*01, UDP-glucuronosyltransferase 1.

born infants display an increased susceptibility for brain damage because of the lower UCB-binding capacity of their plasma albumin and the temporal immaturity of their blood-brain barrier. Although bilirubin encephalopathy is a subject of great clinical importance, its pathogenesis and molecular basis are still not fully understood (reviewed by Ostrow et al., 2003a).

UCB Toxicity in Neural Cells. Early studies established that mitochondria might be a major target for UCB neurotoxicity, as demonstrated by impairment in mitochondrial function leading to the uncoupling of oxidative phosphorylation (Zetterstrom and Ernster, 1956; Cowger et al., 1965; Diamond and Schmid, 1967; Menken and Weinbach, 1967; Mustafa et al., 1969). Additional effects of UCB in neural tissues and neuronal cell lines include inhibition of DNA and protein synthesis, changes in carbohydrate metabolism, and modulation of neurotransmitter synthesis and release. Most of the early neural toxicity data were obtained in cell cultures using excessively high UCB concentrations, exceeding its very low aqueous solubility (Hahm et al., 1992) and the high-affinity binding capacity of plasma albumin (Kaufmann et al., 1969; Brodersen, 1979). Moreover, the source of the albumin or plasma that is often used in binding experiments is adult blood, whereas that obtained from newborns has a diminished binding capacity for UCB (Kapitulnik et al., 1972; Alayoff et al., 1980). These facts suggest that many of the published in vitro toxicity findings may be irrelevant to the in vivo conditions prevalent in most cases of neonatal jaundice (Ostrow and Tiribelli, 2001; Ostrow et al., 2003b). Additional confounding factors in the early in vitro studies are: the type of cell used (astrocytes versus neurons), species differences in the cellular systems employed, dependence of the effect on the chosen endpoint of toxicity, and length of exposure to UCB.

Recent studies employed UCB concentrations that are below saturation of albumin and may be comparable with the UCB levels found in the central nervous system of most jaundiced infants, as well as better defined cell culture systems and appropriate endpoints. Amit and Brenner (1993), using primary cultures of fetal rat glial cells (which consist mainly of astrocytes), showed that UCB affects cell morphology, cell viability, and mitochondrial function. UCB toxicity was dependent on its concentration and on the UCB/albumin molar ratio. It is interesting that the toxic effects of UCB were directly related to the cells' age in culture. Both cell viability and mitochondrial function were considerably impaired upon exposure to UCB of cells cultured for 2 days, whereas extension of the culture time abolished their sensitivity to the toxic effects of UCB. These data correlate with the increased in vivo neurotoxicity of UCB in newborns compared with adults.

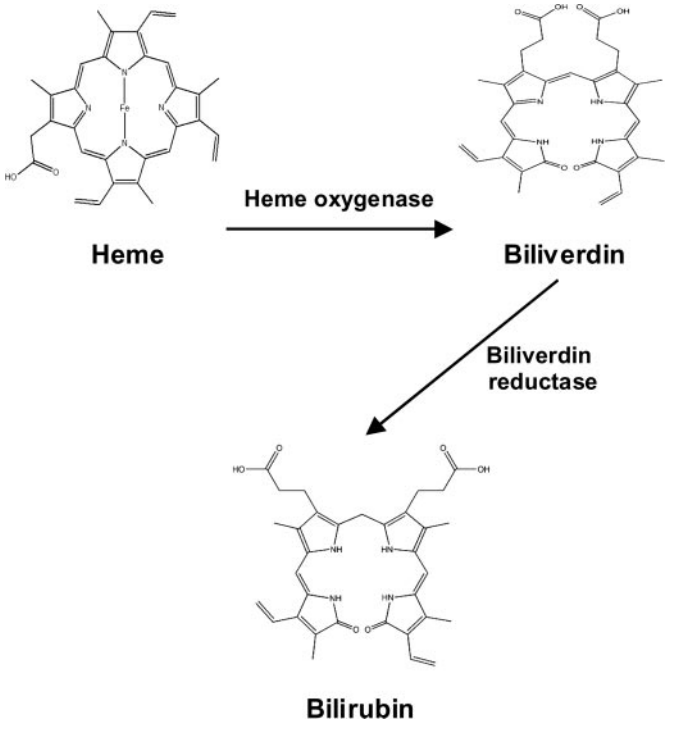

Fig. 1

Grojean et al. (2000) reported that low levels of UCB (0.5 μM) induce programmed cell death (apoptosis) in primary cultured neurons from the embryonic rat forebrain. The apoptotic process involves caspase activation and requires the participation of glutamatergic *N*-methyl-D-aspartate receptors. Moreover, UCB enhanced the effects of hypoxia in these immature neurons by facilitating glutamate-mediated apoptosis (Grojean et al., 2001). On the other hand, UCB inhibited glutamate uptake in cultured rat cortical astrocytes, which play a major role in the transport of synaptically released glutamate (Silva et al., 1999). This inhibition was directly correlated with the UCB/albumin molar ratio and was observed at a molar ratio as low as 0.8. This effect of UCB was pH-dependent and occurred at pH 7.4 and 8.0, but not at 7.0, suggesting that the monoanionic species of UCB is responsible for the inhibition of glutamate uptake in astrocytes. In accordance with these findings in cell cultures, intrastriatal injections of *N*-methyl-D-aspartate, an excitatory glutamate analog, caused an increased atrophy of the striatum and hippocampus in jaundiced (jj) compared with nonjaundiced (Jj) Gunn rats (McDonald et al., 1998). It is interesting that astrocytes are more susceptible than neurons to the UCB-mediated inhibition of glutamate uptake and MTT reduction (an indicator of mitochondrial function). In contrast, neurons are more sensitive than astrocytes to the UCB-induced loss of cell viability (as measured by the release of LDH), disruption of the cytoskeleton, and apoptotic cell death (Silva et al., 2002). These findings stress the importance of the cell type and endpoint used for analysis of UCB-mediated neurotoxicity.

The molecular events leading to apoptotic cell death were characterized in cultures of developing rat brain neurons exposed to purified UCB (Rodrigues et al., 2002a). UCB stimulated neuronal apoptosis even at nonsaturating UCB/albumin molar ratios of 1.0 or less. Cytochrome *c* was extensively released from the mitochondria and accumulated in the cytosol. UCB induced mitochondrial depolarization by diminishing the mitochondrial transmembrane potential, and increased the translocation of the pro-apoptotic Bax protein to mitochondria, leading ultimately to activation of caspase 3. The apoptotic effect of UCB may be mediated by its physical interaction with the mitochondrial membrane. UCB increased lipid polarity and fluidity, as well as protein mobility, resulting in an increased permeability of this membrane and release of cytochrome *c* (Rodrigues et al., 2002b,c). Ursodeoxycholic acid, a mitochondrial membrane-stabilizing agent that prevents the changes in mitochondrial transmembrane

potential, almost completely abolished the UCB-induced membrane perturbation in isolated mitochondria (Rodrigues et al., 2002b) and inhibited the UCB-mediated apoptosis of neurons and astrocytes (Silva et al., 2001).

Rodrigues et al. (2002d) reported that the apoptotic effect of UCB in primary cultures of rat neurons and astrocytes decreases as a function of age-in-culture of these cells, thus confirming the earlier findings of Amit and Brenner (1993) in primary cultures of fetal rat glial cells. In contrast, the UCB-induced mitochondrial membrane permeabilization and cytochrome *c* release were 2-fold greater for mitochondria derived from older rats compared with younger rats (Rodrigues et al., 2002d). These authors concluded that the young rats are relatively resistant to UCB toxicity. This conclusion is in disagreement with the greater sensitivity of human newborn infants to the neurotoxic effects of UCB. It is unfortunate that these authors did not compare mitochondria from young and old, jaundiced and nonjaundiced Gunn rats, because the in vivo exposure of neural cells to UCB might be a more relevant experimental system than the in vitro exposure in cultures of isolated cells. Further studies, using neural cells derived from jaundiced animals, are therefore needed to determine whether mitochondrial injury is the sole mechanism of UCB-induced neurotoxicity.

UCB has been recently shown to decrease oligodendrocyte viability in a concentration- and time-dependent fashion (Genc et al., 2003). UCB caused apoptotic cell death, induced nitric-oxide synthase mRNA expression, and increased nitrite production in cultured rat oligodendrocytes. Nitric-oxide synthase inhibition partly blocked the UCB-induced cytotoxicity, thus providing a role for nitric oxide in this process. These findings suggest that oligodendrocytes, which are the myelin-forming cells in the central nervous system, may be another important target of UCB in addition to neurons and astrocytes. This suggestion is supported by the relatively high concentrations of UCB that have been found in the myelin fraction of rat brain after intravenous administration of UCB (Hansen et al., 2001) and by the myelin alterations that have been described in jaundiced Gunn rats (O'Callaghan and Miller, 1985).

Lin et al. (2003) investigated the effect of UCB on the activation of p38 MAP kinase, a member of the MAP kinase superfamiliy of proteins that function as critical mediators of signal transduction from the cell surface to the nucleus. p38 MAP kinase, which is activated by phosphorylation on Thr-180 and Tyr-182 by a variety of cellular stress events, has been implicated in neuronal apoptosis induced by nitric oxide (Ghatan et al., 2000). UCB induced death of cultured rat cerebellar granule neurons at low concentrations (1–17 μM) in a concentration-dependent fashion. The phosphorylation of p38 MAP kinase was significantly increased after 1 h of exposure to 5 μM UCB. This effect was blocked by pretreating neurons with SB 203580, an inhibitor of p38 MAP kinase, but not by SP 600125, a specific inhibitor of c-Jun NH_2-terminal kinase 2/3. In addition, inhibition of the latter MAP kinases did not protect the neurons from UCB-mediated toxicity, indicating that the effect of UCB on MAP kinase signaling may be specific. It is surprising that the p38 MAP kinase inhibitor did not prevent neuronal death at higher UCB concentrations (10 and 20 μM), suggesting that additional factors occurring upstream or downstream of the p38 MAP kinase pathway may contribute to UCB-mediated neuronal toxicity.

UCB Toxicity in Non-Neural Cells. In contrast to the great interest in elucidating the molecular mechanisms of UCB-induced neurotoxicity, fewer studies addressed the possibility that UCB is also toxic for non-neural cells.

Blood erythrocytes are the immediate target for binding of free (unbound) UCB. This binding is greatly increased at UCB/albumin molar ratios higher than 1.0 (Kaufmann et al., 1967), leading to morphologic changes, cell lysis, and loss of membrane lipids (Brito et al., 2000). Alterations of membrane dynamic properties of erythrocytes were accompanied by the release of phospholipids and cholesterol (Brito et al., 2001). The loss of inner-located phospholipids induced the externalization of phosphatidylserine (Brito et al., 2002). This membrane perturbation was increased by acidosis, indicating that the UCB species interacting with the membrane is the uncharged diacid (Brito et al., 2001).

Both UCB and biliverdin stimulated apoptosis of cultured rat aortic smooth muscle cells (Liu et al., 2002). Relatively low levels of UCB (5 μM) induced a significant change in DNA laddering. UCB induced apoptosis in cultured bovine brain endothelial cells (Akin et al., 2002), but this effect was not concentration-dependent in the range of UCB concentrations studied (10–100 μM).

UCB induced apoptosis also in murine hepatoma Hepa 1c1c7 cells (Seubert et al., 2002). However, higher UCB concentrations ($>$ 25 μM) were required to cause apoptosis in these cells compared with neural cells (0.5–1 μM), suggesting that the latter are more sensitive to UCB-induced apoptosis. The effect of UCB in Hepa 1c1c7 cells was partially dependent on the presence of the Aryl hydrocarbon receptor (AhR), because AhR-deficient mutant cells were less responsive to UCB than the wild-type cells. Moreover, pretreatment with the AhR antagonist α-naphthoflavone reduced the extent of UCB-induced apoptosis in wild-type but not in mutant cells. The AhR is an intracellular protein that is activated to a transcriptionally active form by environmental pollutants such as dioxins and polycyclic aromatic hydrocarbons. A direct role for the involvement of the AhR in premature ovarian failure, via induction of the pro-apoptotic Bax protein, was demonstrated in mouse oocytes exposed to polycyclic aromatic hydrocarbons (Matikainen et al., 2001).

Cytoprotective Effects of Bilirubin

The pioneering studies of Stocker et al. (1987a,b) introduced the concept that UCB, which was until then regarded as a toxic waste product of heme catabolism, has a beneficial role at low, "physiological" plasma concentrations by acting as a potent antioxidant that scavenges peroxyl radicals as efficiently as α-tocopherol. Moreover, when bound to human albumin and at concentrations encountered in normal human plasma, UCB prevents in vitro the oxidation of albumin-bound fatty acids as well as of the protein itself. One mole of albumin-bound UCB can scavenge two moles of peroxyl radicals while being itself oxidized to its nontoxic metabolic precursor biliverdin. Additional studies have shown that UCB, when bound to bovine serum albumin, is oxidized by hydroxyl ($^{\bullet}OH$), hydroperoxyl ($HO_2^{\bullet}$), and superoxide anion ($O_2^{\bullet-}$) radicals (in decreasing order of oxidation rates). UCB protects albumin from $^{\bullet}OH$-induced oxidative damage and strongly inhibits the $^{\bullet}OH$-mediated formation of protein car-

bonyls (Neuzil and Stocker, 1993). UCB is also an antioxidant in vivo, as shown by the reduced oxidative injury to serum proteins and lipids in the first days of life in hyperbilirubinemic neonatal Gunn rats exposed to hyperoxia (Dennery et al., 1995).

Cardiovascular Effects. UCB inhibits oxidation of low-density lipoprotein (LDL) lipids by interacting with LDL's α-tocopherol (Neuzil and Stocker, 1994). This effect, which is exhibited by both unconjugated and conjugated bilirubin (Wu et al., 1996), may reduce the risk of atherogenesis. It is interesting that oxidized LDL induces heme oxygenase-1 expression and activity in vascular endothelial and smooth muscle cells, leading to the formation of the antioxidant UCB and thus preventing further oxidative damage and atherogenesis (reviewed by Siow et al., 1999). In this context, an inverse relationship was found between serum UCB levels and severity of atherosclerosis in men, in a *meta*-analysis of 11 published studies (Novotny and Vitek, 2003). Moreover, a low prevalence of ischemic heart disease was found in patients with Gilbert syndrome (Vitek et al., 2002). These patients exhibit a sustained and mild unconjugated hyperbilirubinemia (with plasma UCB levels between 20 and 100 μM) because of a partial deficiency of hepatic UGT*01.

UCB, at concentrations as low as 100 nM, has also been shown to restore myocardial function and minimize both infarct size and mitochondrial damage in a rat model of cardiac ischemia-reperfusion injury (Clark et al., 2000a). Exposure of rat cardiomyocytes to 0.5 μM UCB during a hypoxic challenge considerably diminished the cell injury caused by subsequent reoxygenation and reduced the generation of ROS during hypoxia (Foresti et al., 2001).

Additional targets for the protective effects of BR in the cardiovascular system include vascular smooth muscle (Clark et al., 2000b) and endothelial cells (Oberle et al., 2003), which were exposed to oxidative stress. Pretreatment of aortic endothelial cells with nitric oxide-releasing compounds, which increase heme oxygenase activity and UCB production, protected these cells against H_2O_2-mediated cytotoxicity (Motterlini et al., 1996). It is interesting that heme oxygenase-1 up-regulation in diabetic rats reduced the production of superoxide anion and the endothelial cell sloughing associated with hyperglycemia (Quan et al., 2004). In addition, UCB inhibited the oxidative stress-induced chemotactic activity of monocytes (Morita et al., 2003) and caused a delay in vascular thrombus formation (Lindenblatt et al., 2004).

Hepatic Effects. Pretreatment of rats with hemin, an inducer of heme oxygenase-1, or with biliverdin, a product of heme oxidation by this enzyme, prevented acetaminophen-induced hepatotoxicity (Chiu et al., 2002). Hepatotoxicity results from the formation of a highly reactive electrophilic metabolite of acetaminophen that binds covalently to cellular macromolecules, and from reactive oxygen and nitrogen intermediates produced by hepatic parenchymal and nonparenchymal cells. Biliverdin, and most probably its metabolite UCB, protect the liver via their free radical chain-breaking antioxidant effects. In addition, UCB administration has been shown to prevent acetaminophen-induced glutathione depletion (Noriega et al., 2000).

Hyperbilirubinemia may also have a protective role in hepatobiliary disease. Accumulation of bile acids in the livers of patients with cholestasis generates hepatocellular damage, manifested as either hepatocyte necrosis or apoptosis. Both unconjugated and conjugated bilirubin inhibited bile acid-induced apoptosis in rat hepatocytes and suppressed the generation of ROS by these cells (Granato et al., 2003). In contrast, UCB may have a detrimental effect when liver failure after major hepatic resection is accompanied by hyperbilirubinemia. Moreover, UCB impairs the bactericidal activity of neutrophils through scavenging of ROS (Arai et al., 2001), and may be therefore responsible for the septic complications associated with this condition. The latter findings suggest that the clinical scenario, as well as the particular cellular target of ROS, may determine whether UCB will exhibit protective or toxic effects.

UCB induced cytochrome P4501A1 in jaundiced Gunn rats (Kapitulnik et al., 1987; Kapitulnik and Gonzalez, 1993), thus enabling the elimination of UCB in these glucuronidation-deficient animals (Kapitulnik and Ostrow, 1978). Relatively low concentrations of UCB (10 μM) activated the AhR in cultured hepatoma cells (Sinal and Bend, 1997; Phelan et al., 1998). The activated receptor translocates from the cytoplasm to the nucleus and induces the transcription of genes such as that encoding for cytochrome P4501A1 (Gu et al., 2000). Recent findings from our laboratory suggest that the activation of AhR nuclear translocation and function by UCB does not require its binding to the receptor, as is the case with dioxins and polycyclic aromatic hydrocarbons. The above effects of UCB are probably mediated by its antioxidant properties (Maklakov et al., 2004). The UCB-mediated activation of AhR and induction of cytochrome P4501A1 can be of obvious benefit, because it may prevent UCB neurotoxicity by stimulating the oxidative degradation and elimination of UCB when glucuronidation is impaired.

Effects on the Immune System. Both UCB and biliverdin display immunoprotective effects on murine liver and cardiac grafts. A short-term (5 min) rinse with micromolar concentrations of UCB, during ex vivo organ reperfusion or preceding in vivo transplantation, attenuated biliary dysfunction and cell injury of rat livers harvested as grafts at 4°C (Kato et al., 2003). UCB mimicked the effect of heme oxygenase-1 preconditioning of the ischemic grafts. Likewise, a brief treatment with biliverdin induced tolerance to H-2 incompatible mouse heart allografts (Yamashita et al., 2004). Biliverdin markedly prolonged graft viability, decreased intragraft leukocyte infiltration in vivo, and inhibited T cell proliferation in vitro. These effects of biliverdin are mediated by suppression of IL-2 production, via inhibition of activation of nuclear factor of activated T cells and nuclear factor κB.

UCB, by virtue of its antioxidant properties, prevented both short- and long-term experimental autoimmune encephalomyelitis, the rat model for human multiple sclerosis, and suppressed ongoing clinical manifestations of the disease when given after its onset (Liu et al., 2003).

Effects on Neural Cells. Doré et al. (1999) have demonstrated a protective role for very low nanomolar concentrations of UCB in cultured rat primary hippocampal neurons exposed to 75 μM H_2O_2. The oxidative stress-induced neuronal injury was almost completely prevented by pretreating the cells with a 10 nM UCB/human serum albumin solution (molar ratio = 1). These findings highlighted, in a cellular system, the physiological relevance of the antioxidant properties of UCB demonstrated earlier by Stocker et al. (1987a,b) in in vitro systems. UCB was also neuroprotective when added without

albumin but was less potent than in the albumin-bound form. The neuroprotective effect of UCB decreased at higher concentrations (250 nM), at which UCB might probably act in this system as a toxic, pro-oxidant compound. This toxic effect of UCB was particularly evident in neuronal cultures not exposed to H_2O_2, suggesting that UCB is degraded in the presence of H_2O_2. Very low UCB concentrations (10 nM) also reduced the number of apoptotic cells and increased survival of cultured mouse olfactory receptor neurons treated with a heme oxygenase inhibitor (Chen et al., 2003).

The findings of Doré et al. (1999) raised a very puzzling question: how can nanomolar concentrations of UCB antagonize the effect of an almost 10,000-fold higher H_2O_2 concentration? The answer was provided by the elegant study of Barañano et al. (2002), who demonstrated that the potent physiologic antioxidant properties of UCB result from the following redox cycle: 1) in the presence of ROS, UCB (in its albumin-bound form) is oxidized to biliverdin and 2) biliverdin is converted back to UCB by the enzyme biliverdin reductase. When HeLa cells or primary cerebral cortical neurons were depleted of biliverdin reductase, and consequently of UCB, by RNA interference of its transcript, a marked increase was observed in cellular ROS levels, thus leading to increased apoptosis.

It has recently been suggested that UCB may protect mouse astrocytes from its own toxicity (Gennuso et al., 2004). In these cells, which play an important role in scavenging ROS and preventing brain cytotoxicity, a low concentration of unbound UCB (40 nM) rapidly up-regulated expression of the multidrug resistance-associated protein 1 (Mrp1), an ATP-dependent transporter that mediates the export of organic anions (probably including UCB). In addition, UCB promoted the translocation of this transporter from the Golgi to the plasma membrane, thus reducing the intracellular concentration of UCB and its toxicity to astrocytes.

Effects on Airway Smooth Muscle Cells. Further proof for the antioxidant and cytoprotective role of low concentrations of UCB was obtained in a different cellular model of ROS-mediated injury. UCB (1 μM), as well as the reducing antioxidant *N*-acetyl-cysteine, inhibited mitogen-induced cell proliferation, ROS production, and phosphorylation of the MAP kinases extracellular signal-regulated kinase 1/2 in human airway smooth muscle cell cultures (Taillé et al., 2003). These results are of clinical significance because an increase in airway smooth muscle cell mass is characteristic of airway remodeling in asthmatic patients. Bronchial smooth muscle cells from these patients show abnormal proliferation in vitro. UCB can also modulate ROS production and ROS-mediated phosphorylation of myosin light chain in cultured guinea pig tracheal smooth muscle cells under physiological conditions (Samb et al., 2002). These two reports, together with that of Lin et al. (2003), highlight the important role of MAP kinase signaling in mediating the cellular effects of UCB. Thus, activation (increased phosphorylation) of the p38 MAP kinase by UCB causes cytotoxicity, whereas inhibition of extracellular signal-regulated kinase 1/2 phosphorylation is cytoprotective. However, it should be kept in mind that these opposed effects of UCB were observed in different cell types—neurons and smooth muscle cells, respectively, suggesting a cell-specific pattern for the UCB-mediated modulation of cellular events.

Protection from Nitrosative Stress. In addition to the pivotal role played by UCB in the defense against ROS in a variety of cell types, recent findings provide support for the involvement of UCB in physiological scavenging of nitric oxide-derived reactive substances and its potential ability to counteract intracellular nitrosative stress reactions (Mancuso et al., 2003).

Nitric oxide donors induced expression of heme oxygenase-1 in bovine aortic endothelial cells (Foresti et al., 1997). The specific nitric oxide scavenger hydroxocobalamin decreased endothelial heme oxygenase activity. Moreover, the nitric oxide-mediated induction of heme oxygenase-1 was also significantly reduced by *N*-acetyl-cysteine, a precursor of glutathione synthesis, via stabilization of nitric oxide through the formation of *S*-nitrosothiols. These results indicate that a reactive derivative of nitric oxide is associated with the nitric oxide-mediated induction of heme oxygenase-1. Accordingly, peroxynitrite ($ONOO^-$), a strong oxidant formed when nitric oxide reacts with the superoxide anion, was a potent inducer of heme oxygenase-1 expression (Foresti et al., 1999). Peroxynitrite also increased apoptosis and cytotoxicity, and peroxynitrite scavengers decreased these effects. It is interesting that pretreatment of the endothelial cells with hemin, an inducer of heme oxygenase-1, increased UCB production and decreased the peroxynitrite-mediated apoptosis. Moreover, agents that release nitric oxide or nitroxyl, as well as peroxynitrite, caused UCB and biliverdin decomposition in vitro (Kaur et al., 2003). These findings suggest that UCB and biliverdin protect cells from the damage caused by uncontrolled nitric oxide production. Accordingly, UCB, in the micromolar concentration range, provided significant protection against peroxynitrite-mediated protein oxidation in human blood plasma (Minetti et al., 1998).

An interesting synergistic interaction between UCB and nitric oxide in the modulation of the endothelial heme oxygenase-1 pathway has been described recently (Foresti et al., 2003). Exposure of aortic endothelial cells to a medium containing increased UCB levels (derived from hemin-treated cells) markedly potentiated heme oxygenase-1 expression and enzymatic activity induced by nitric oxide donors. A heme oxygenase inhibitor abolished this effect. Both the iron liberated during heme breakdown and the nitroxyl anion formed from nitric oxide partially contributed to this potentiation phenomenon. In addition, the UCB-containing medium augmented the release of nitric oxide from the nitric oxide donors. These results suggest that the signaling actions of nitric oxide that are related to the induction of heme oxygenase-1 are affected by heme metabolites such as UCB, thus increasing the protection of vascular endothelium against nitrosative stress.

The above findings suggest the existence of an important regulatory loop that contributes to the cellular defense against oxidative and nitrosative reactive substances: increased production and/or release of nitric oxide, in conjunction with increased formation of superoxide anion, stimulate the formation of peroxynitrite. Peroxynitrite induces expression of heme oxygenase-1, which increases in turn the formation of biliverdin and UCB. The latter protect cells from peroxynitrite-mediated protein oxidation and cytotoxicity by virtue of their potent free radical scavenging properties. UCB further potentiates the nitric oxide-mediated induction of heme oxygenase-1, thus amplifying its protective effects.

Conclusions and Perspectives

UCB, a major product of heme degradation, is much more than a simple endogenous waste product. Its neurotoxic properties, clinically manifested under conditions of excessive UCB production and/or impaired elimination, have been known for many decades. However, the molecular mechanisms responsible for UCB neurotoxicity have only recently begun to be unraveled.

The landmark findings of the group of Bruce Ames in Berkeley laid the ground stone for understanding the important physiological role of low concentrations of UCB in counteracting oxidative and nitrosative stress-mediated injury in many cellular systems. UCB has been shown to confer cardiovascular, hepatic, neural, and immunoprotection, by virtue of its potent free radical scavenging capacity. Moreover, it is becoming clear that low plasma concentrations of UCB are associated with a low incidence of cardiovascular disorders in humans (e.g., atherosclerosis and ischemic heart disease). Nevertheless, scavenging of ROS may not always be beneficial, as exemplified by the UCB-induced impairment of the bactericidal activity of neutrophils. Thus, UCB should be considered as a metabolic "double-edged sword". The final outcome of its cellular effects—toxicity or protection—will depend on: 1) UCB plasma/tissue concentration and extent of binding to plasma albumin; 2) target cell/tissue involved; 3) type of insult; 4) cellular redox state; and 5) developmental stage.

Because the up-regulation of heme oxygenase-1, and the resulting increased production of UCB, seem to constitute physiologic responses to both oxidative and nitrosative stress, it may be justified to speculate that low plasma/tissue concentrations of UCB are "good for us", because they could protect sensitive tissues against oxidative/nitrosative insults during critical periods of development and prevent the occurrence of diseases associated with cellular stress.

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