Unconjugated Bilirubin Causes Changes In Intracellular Redox Status, Changes To The Disulphide Proteome, And Upregulation Of Several Genes Involved In ER Stress

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Introduction

- Unconjugated bilirubin (UCB) is an endogenous metabolite generated by the oxidation of heme, a ubiquitous, iron-containing molecule present in hemoproteins, including hemoglobin and cytochrome P450.
- Heme oxygenase (HO) catalyzes the rate-limiting step in heme degradation, resulting in the production of equimolar amounts of ferrous iron, carbon monoxide and biliverdin, which is rapidly reduced to UCB by biliverdin reductase.
- This metabolic pathway is important from a toxicological perspective because induction of HO-1, the inducible isoform of HO, occurs as an adaptive response to many injurious stimuli including ultraviolet A radiation, hydrogen peroxide (H_2O_2), heavy metal exposure and nitric oxide, resulting in increased formation of UCB.
- UCB was shown to be a potent antioxidant at nM concentrations[1]. However, if plasma UCB concentrations rise above homeostatic levels (> 20 μM), UCB can enter cells by passive diffusion and cause toxicity. The central nervous system of the very young, prior to maturation of the blood-brain barrier, is the most vulnerable site of UCB toxicity, however, toxicity has also been reported in non-neuronal tissues such as erythrocytes and aortic smooth muscle cells[2].
- Previous work in our laboratory has shown that UCB causes apoptosis in Hepa 1c1c7 cells, a murine hepatoma cell line. The apoptotic response is characterized by increased oxidative stress as shown by increased generation of ROS and increased lipid peroxidation[3,4]. We have also demonstrated that HepG2 cells, a human hepatoma cell line, are relatively resistant to UCB mediated apoptosis.

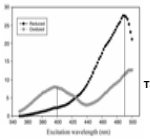
Objective

The objective of the present study was to employ novel, redox sensitive, green fluorescent proteins as well as redox two dimensional gel eletrophoresis to detect changes in intracellular redox status following treatment of cells with nM or μM concentrations of UCB.

Methods

Measuring Intracellular Redox Status

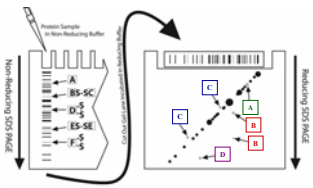
Intracellular redox status was measured using novel redox sensitive green fluorescent proteins (roGFPs)[6,7]. This technique uses mutant GFPs which have two introduced cysteine residues present near the chromophore (S147C & Q204C). Under oxidizing conditions a reversible intramolecular disulfide bond is formed between these two cysteine thiols. Disulfide bond formation results in increased excitability at 405 nm and decreased excitability at 490 nm (Emission 530 nm)



Taken from Dooley, C. T. *et al*. J. Biol. Chem. 2004; 279: 22284-93

Redox-Two Dimensional Gel Electrophoresis

Redox-two dimensional gel electrophoresis (R2D PAGE) was used to determine the oxidation of intracellular proteins. Cell lysates, cytosolic or membrane fractions, were separated according to size in the first dimension under non-reducing conditions. Gel slabs from the first dimension were reduced with dithiothreitol and then separated according to size in a second dimension under reducing conditions. After silver staining a diagonal line of proteins is observed with off diagonal proteins corresponding to redox regulated proteins.



A – No Disulphide Bonds
B – Heterodimer
C – Intramolecular Disulphide Bond
D - Homodimer

Results

Pro- and antioxidant concentrations of UCB cause changes in the intracellular redox status as detected by roGFP2s

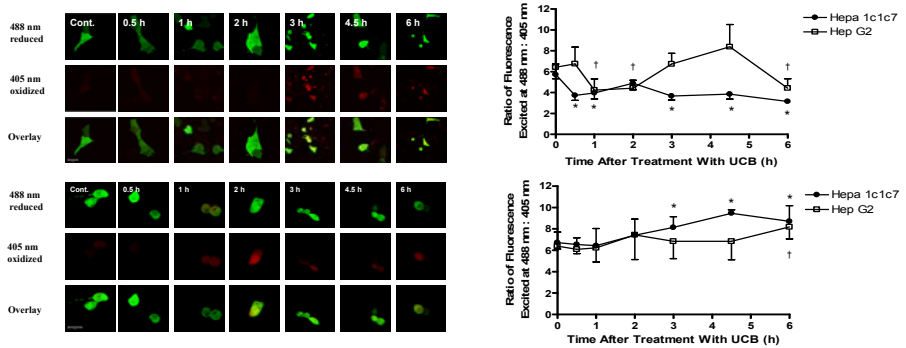


Figure 1: Hepa 1c1c7 or HepG2 cells transfected with roGFP2 were treated with either 50 μM (prooxoidant) or 70 nM (antioxidant) UCB and visualized with a Zeiss LSM-510 META laser scanning microscope using dual excitation (405 and 488 nm) and an emission filter of 505 nm. Left panel; Images of roGFP2 expressing Hepa 1c1c7 cells (A) and Hep G2 cells (B) after treatment with 50 μM UCB. Right panel; Quantified fluorescence data for both Hepa 1c1c7 and Hep G2 cells after treatment with either 50 μM (C) or 70 nM (D) UCB. n = 4; *, P < 0.05 according to an ANOVA with a Dunnett's post test for Hepa 1c1c7 cells; †, P<0.05 accoriding to an ANOVA with a Dunnett's post test for Hep G2 cells.

Treatment with UCB oxidizes proteins in the cytosol while reducing proteins in the membranes

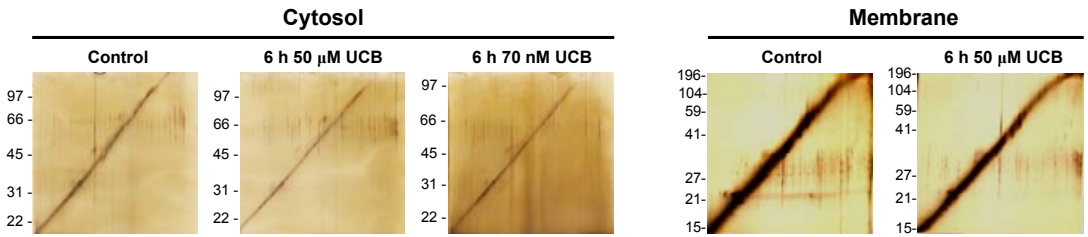


Figure 2: Hepa 1c1c7 cells were treated with either a prooxidant (50 μM) or an antioxidant (70nM) concentration of UCB for 6 h. Crude membrane fractions and cytosol fractions were obtained by spinning cell lysates at 14 000 g for 10 min. Proteins were resolved by redox 2D page and detected by silver staining. For cytosol 20 μg of protein was resolved and for membranes 50 μg of protein was resolved.

Microarray analysis demonstrates a number of genes involved in ER stress are upregulated following treatment with prooxidant concentrations of UCB

Genes Involved in ER stress Upregulated Following Treatment With 50 μM UCB for 6 h

Gene	Fold Change
ERdjb1	7.6
Creb homologous protein (CHOP)	6.3
BiP/GRP78	3.9
Homocysteine-induced ER proteins (herp)	3.3

Figure 3: Microarray anlysis of genes involved in ER stress found to be redox regulated following treatment with antioxidant (70 nM) and prooxidant (50 μ) concentrations of UCB. Genes are expressed as fold change of Hepa 1c1c7 cells treated with 50 μM UCB as compared to Hepa 1c1c7 cells treated with 70 nM for 6 h. Microarray analysis was performed at the London Regional Genomics Facility using an Affeymetrix Mouse Genome 2.0 gene chip.

Conclusions

- Initially, treatment of Hepa 1c1c7 cells with 50 μM UCB resulted in reversible oxidation that was reversed by the cells' metabolism by 2 h, followed by significant re-oxidation at later time points.
- Hep G2 cells were less susceptible to UCB mediated oxidation than were Hepa 1c1c7 cells.
- Treatment of Hepa 1c1c7 cells with an anti-oxidant concentration of UCB (70 nM) resulted in pronounced reduction of the intracellular environment by 3 h post-treatment. This effect was not observed in the Hep G2 cell line until 6 h posttreatment.
- Protein disulphide bond formation readily occurs within the cytoplasm of control as well as oxidant-stressed cells and a number of proteins were found to be differentially redox regulated following treatment with UCB as compared to the control.
- UCB reduces PSSP, PSSG and intramolecular disulfide bonding within the ER/MIT membrane fractions of Hepa 1c1c7 cells. As the fraction is rich with ER (which has a more oxidized redox status than mitochondria), UCB acts as an antioxidant, reducing ER proteins containing ionized thiol residues (PS) that are redox regulated.
- Identification (by mass spectrometry) and analysis of the time-dependent oxidation of proteins that compose the disulfide proteome allows time-dependent consideration of the correlation between structural alteration and function of proteins containing reactive cysteine thiol residues that contribute significantly to cell death via apoptosis or necrosis.
- A number of genes involved in the response to ER stress were found to be upregulated suggesting that ER stress may play a role in UCB toxicity, in addition to the apoptosis that results from mitochondrial signaling that we previously described.

References

1. Stocker R et al. Bilirubin is an antioxidant of possible physiological importance. Science 1987; 235: 1043-6.
2. Shapiro SM. Bilirubin toxicity in the developing nervous system. Pediatr Neurol 2003; 29: 410-21.
3. Seubert JM et al, Bend JR: Apoptosis in murine hepatoma Hepa 1c1c7 wild-type, C12 and C4 cells mediated by bilirubin. Mol Pharmacol 2002; 62: 257-64.
4. Oakes GH and Bend JR. Early steps in bilirubin-mediated apoptosis in murine hepatoma (Hepa 1c1c7) cells are characterized by aryl hydrocarbon receptor-independent oxidative stress and activation of the mitochondrial pathway. J Biochem Mol Toxicol 2005; 19: 244-55.
5. Dooley CT et al. Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. J Biol Chem 2004; 279: 22284-93.
6. Hanson GT et al. Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. J Biol Chem 2004; 279: 13044-53.
7. Leichert LI and Jakob U. Global methods to monitor the thiol-disulphide state of proteins in vivo. Antioxid Redox Signal. 2006; May-June; 8(5-6): 763-72.

Acknowledgments

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