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Mitochondrial targeting of bilirubin regulatory enzymes: An adaptive response to oxidative stress

Siti Nur Fadzilah Muhsain [a,b], Matti A. Lang [a], A'edah Abu-Bakar [a,*]

[a] The University of Queensland, National Research Centre for Environmental Toxicology (Entox), 4072 Brisbane, Queensland, Australia
[b] Faculty of Pharmacy, University Teknologi Mara, Malaysia

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ABSTRACT

The intracellular level of bilirubin (BR), an endogenous antioxidant that is cytotoxic at high concentrations, is tightly controlled within the optimal therapeutic range. We have recently described a concerted intracellular BR regulation by two microsomal enzymes: heme oxygenase 1 (HMOX1), essential for BR production and cytochrome P450 2A5 (CYP2A5), a BR oxidase. Herein, we describe targeting of these enzymes to hepatic mitochondria during oxidative stress. The kinetics of microsomal and mitochondrial BR oxidation were compared. Treatment of DBA/2J mice with 200 mg pyrazole/kg/day for 3 days increased hepatic intracellular protein carbonyl content and induced nucleo-translocation of Nrf2. HMOX1 and CYP2A5 proteins and activities were elevated in microsomes and mitoplasts but not the UGT1A1, a catalyst of BR glucuronidation. A CYP2A5 antibody inhibited 75% of microsomal BR oxidation. The inhibition was absent in control mitoplasts but elevated to 50% after treatment. An adrenodoxin reductase antibody did not inhibit microsomal BR oxidation but inhibited 50% of mitochondrial BR oxidation. Ascorbic acid inhibited 5% and 22% of the reaction in control and treated microsomes, respectively. In control mitoplasts the inhibition was 100%, which was reduced to 50% after treatment. Bilirubin affinity to mitochondrial and microsomal CYP2A5 enzyme is equally high. Lastly, the treatment neither released cytochrome *c* into cytoplasm nor dissipated membrane potential, indicating the absence of mitochondrial membrane damage. Collectively, the observations suggest that BR regulatory enzymes are recruited to mitochondria during oxidative stress and BR oxidation by mitochondrial CYP2A5 is supported by mitochondrial mono-oxygenase system. The induced recruitment potentially confers membrane protection.

Introduction

In mitochondria, bilirubin (BR) is constitutively generated from heme in two successive reactions: heme oxygenase-catalysed degradation to biliverdin (BV), followed by biliverdin reductase-catalysed reduction to BR (Converso et al., 2006). At low constitutive level the free-radical quencher BR prevents accumulation of radical oxygen species (ROS) in mitochondria (Jansen et al., 2010), but at above 10 μM it disrupts mitochondrial membrane permeability, which in turn causes apoptosis (Rodrigues et al., 2002). Therefore, mitochondrial BR needs to be well regulated especially under oxidative stress conditions when BR production is markedly elevated following the induction of heme oxygenase-1 (HMOX1). However, little is known about the regulatory mechanism of mitochondrial BR.

The normal route of BR excretion in mammals is via the bile and involves the conversion of BR into its glucuronides, a reaction solely catalysed by the microsomal uridine-diphosphate-glucuronosyltransferase 1A1 (UGT1A1) (Tukey and Strassburg, 2000). This enzyme however is not present in mitochondria (Radominska-Pandya et al., 2005). In 1969, Brodersen & Bartels described a BR oxidase in guinea-pig brain mitochondria. The reaction products include BV (Brodersen and Bartels, 1969). Subsequently, similar BR oxidase activity was described in rat liver mitochondria (Cardenas-Vazquez et al., 1986). Bilirubin oxidation was thus proposed as the main metabolic pathway for mitochondrial BR. However, the brain and liver enzymes were not identified.

Importantly, we have recently discovered that the liver microsomal cytochrome P450 2A5 (CYP2A5) can function as an inducible BR oxidase that catalyses BR oxidation to BV (Abu-Bakar et al., 2011). In response to oxidative stress CYP2A5 induction paralleled but followed the induction of microsomal HMOX1 by several hours (Abu-Bakar et al., 2005). The CYP2A5 induction was delayed until cellular BR concentrations were elevated above the K_m of 1–2 μM. At these concentrations BR was shown to stabilise the labile CYP2A5 protein by delaying its degradation (Abu-Bakar et al., 2011, 2012). These observations would be consistent with BR inducing its own metabolism and CYP2A5 serving to clear BR

Abbreviations: BR, bilirubin; ROS, reactive oxygen species; CYP, cytochrome P450; CYP2A5, cytochrome P450 2A5; HMOX1, heme oxygenase-1; UGT1A1, UDP-glucuronosyltransferase 1A1; BVR, biliverdin reductase.

* Corresponding author at: Entox, The University of Queensland, 39 Kessels Road, Coopers Plains, 4108 Queensland, Australia. Fax: +61 7 3274 9003.
E-mail addresses: sitinurfadzilah077@ppinang.uitm.edu.my (S.N.F. Muhsain), m.lang@uq.edu.au (M.A. Lang), a.abubakar@uq.edu.au (A. Abu-Bakar).

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generated by HMOX1 to ensure the levels of this essential but toxic compound do not exceed a safe threshold.

We have proposed that the significance of this regulatory mechanism is to ensure a rapid increase in intracellular antioxidant capacity during oxidative stress, by increasing the levels of BR, followed by an efficient elimination of any excess of BR as soon as cellular ROS reached physiological level (Abu-Bakar et al., 2013). The kinetics of CYP2A5 catalysing BR oxidation seem ideal for this purpose as the K_m for BR is roughly one tenth of the cytotoxic concentrations (Abu-Bakar et al., 2005, 2012).

Earlier observations that a fraction of microsomal HMOX1 is targeted to mitochondria in response to oxidative stress (Converso et al., 2006; Srivastava and Pandey, 1996) indicate an increased mitochondrial BR production. The fact that the CYP2A5 is also targeted to mitochondria in response to oxidative stress (Genter et al., 2006; Honkakoski et al., 1988) raises an interesting question if mitochondria are equipped with a similar system to that of microsomes in regulating local BR homeostasis.

In addressing this question, we explored the induction profile of key BR regulatory enzymes in the microsomes and mitochondria of the mouse liver in response to oxidative stress. We also investigated the contribution of mitochondrial CYP2A5 to BR oxidation. We observed that oxidative stress — indicated by elevated intracellular protein carbonylation and nucelo-Nrf2 levels — markedly increased HMOX1 and CYP2A5 proteins and activities in the microsomes and mitochondria. The induction of CYP2A5 in mitochondria was stronger than in the microsomes. We also observed that mitochondrial CYP2A5 oxidised BR with an affinity similar to that of the microsomal enzyme but required the mitochondrial electron transfer chain for its activity. During oxidative stress the mitochondrial CYP2A5-catalysed BR oxidation was highly significant, whilst under normal condition oxidation by ROS was the dominant pathway. By contrast, the microsomal BR oxidation was mainly driven by the CYP2A5 both under normal and oxidative stress conditions. Additionally, protein carbonyl content in microsomes was increased by 200% but in mitochondria it was reduced by about 25% of control. This suggests marked oxidative damage to microsomal proteins, whilst the mitochondrial proteins seemed to be well protected, which is further supported by observed lack of mitochondrial cytochrome *c* leakage into the cytosol and intact membrane potential in treated mice. Collectively, our observations indicate that induced recruitment of key bilirubin regulatory enzymes to mitochondria potentially confers mitochondrial protection against severe oxidative damage.

Materials and methods

Chemicals and antibodies. Coumarin, mesoporphyrin, umbelliferone (7-hydroxycoumarin), pyrazole, β-NADPH, glycerol, mannitol, leupeptin, Hepes, phenylmethylsulfonyl-fluoride (PMSF), digitonin, Percoll, bovine serum albumin (BSA), ethylene glycol tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), dimethyl sulfoxide (DMSO) and Tween 20 were purchased from Sigma-Aldrich (Sydney, Australia). Bilirubin, mesobilirubin, biliverdin and sucrose were from Frontier Scientific, Inc. (Utah, USA) and Ajax Chemical (Sydney, Australia), respectively. Dithiothreitol (DTT) was purchased from Applichem (Germany). Goat anti-rabbit, goat anti-mouse, mouse anti-goat and rabbit anti-chicken antibodies conjugated with horseradish-peroxidase were from Thermo Fisher Scientific Inc. (Victoria, Australia). Rabbit polyclonal anti-COX IV and anti-VDAC antibodies were from Cell Signalling Technology (NY, USA) whereas anti-cytochrome P450 reductase antibody was from Assay Designs (NY, USA). Rabbit polyclonal anti-Nrf2 (ab92946), anti-biliverdin reductase (ab19260), anti-β-actin (ab8227), and mouse monoclonal anti-HMOX1 (ab13248) antibodies were sourced from Abcam (Cambridge, UK). Rabbit polyclonal anti-Lamin B1 (sc20682) and goat polyclonal anti-UGT1A1 (sc27419) antibodies were purchased from Santa Cruz Biotechnology Inc. (Texas, USA). Rabbit polyclonal anti-CYP2A5 antibody and anti-adrenodoxin reductase antibody was sourced from Dr Risto Juvonen, University of Kuopio, Finland and Dr Sergej Usanov, National Academic of Sciences, Republic of Belarus, respectively. The specificity of these antibodies in inhibiting CYP2A5 activity (coumarin 7-hydroxylase) and adrenodoxin reductase activity, respectively, have been well documented in various models (Abu-Bakar et al., 2005; Asikainen et al., 2003; Chernogolov and Usanov, 1997; Guzov et al., 1993; Honkakoski et al., 1988, 1993; Honkakoski and Lang, 1989; Juvonen et al., 1988; Kaipainen et al., 1984; Usanov et al., 1984, 1989, 1990).

Treatment of animals. All experimental procedures involving handling of laboratory animals were conducted in accordance with the Australian National Health and Medical Research Council (NHMRC) Code of Practice for care and use of experimental animals, and approved by the Queensland Forensic and Scientific Services Animal Ethics Committee (FSS-AEC Approval No. 10P01). Male DBA/2J mice were purchased from the Animal Resource Centre, Western Australia. They were group-housed, maintained at a 12 h light/dark cycle, and had access to standard rodent chow ad libitum. The mice were randomly allocated into treated and control groups (6 mice per group). Induction of oxidative stress was achieved by intraperitoneal administration of 200 mg pyrazole/kg bwt/day for three days (Bae et al., 2012; Gilmore and Kirby, 2004). The animals in control group were given normal saline only. Twenty-four hours after the final dose the animals were euthanised with CO_2/O_2 overdose and their livers excised.

Preparations of subcellular fractions. Microsomes and mitochondria were prepared from fresh liver at 0–4 °C as described by Genter et al. (2006) with some modifications. Briefly, the fresh livers were homogenised in isolation buffer (2 mM Hepes, 70 mM sucrose, 220 mM mannitol, 2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.01 μg/μl leupeptin) using a motor-driven Potter-Elvehjem homogeniser (Sartorius, Göttingen, Germany). The homogenate was centrifuged (2000 ×*g*) for 10 min at 4 °C. The supernatant was aspirated into a clean tube and further centrifuged (14,000 ×*g*) for 15 min at 4 °C. The supernatant (post-mitochondrial fraction) was used to prepare microsomes. The mitochondrial pellet was washed by resuspending in isolation buffer and centrifuged again at 14,000 ×*g* for 15 min (4 °C). Resulting pellet was resuspended in isolation buffer, underlay with 27% sucrose using a 20-gauge blunt-end needle and centrifuged for 15 min at 14,000 ×*g* (4 °C). The pellet was washed and resuspended in isolation buffer. To increase the purity of mitochondrial fractions the pellet was treated with an appropriate volume of 0.13 mg digitonin/mg protein to strip off the outer membrane that is associated with microsomes. This mixture was incubated on ice with occasional agitation for 15 min at 4 °C. The reaction was stopped by resuspending the mixture with isolation buffer and centrifuged (12,000 ×*g*) for 15 min at 4 °C. The resulting pellet was suspended in the buffer and centrifuged again for 15 min at 12,000 ×*g*. The mitoplasts were resuspended in dilution buffer (2 mM Tris–HCl, pH 7.4, 1 mM EDTA, 20% glycerol), aliquoted and stored at −80 °C until required. Enzyme activity assays and Western immunoblot were performed with mitoplasts as the enzymes studied (HMOX1, CYP2A5, UGT1A1), and cytochrome *c* is integrated in the inner mitochondrial membrane (Cortese et al., 1998; Gallet et al., 1997; Genter et al., 2006; Honkakoski et al., 1988; Rytomaa and Kinnunen, 1995), which is intact in the mitoplasts. Prior to enzyme activity assays the purified mitoplasts were brought down by centrifugation, suspended in dilution buffer and sonicated (4 × 30 s with 30 s intervening cooling time) to disrupt the inner membrane to allow NADPH and substrates to reach the enzymes of interest.

Nuclei fractions were prepared in the cold (2–4 °C) as previously described (Wang, 1967) with modifications. Briefly, the fresh livers were rinsed in ice-cold PBS. The livers were cut into small pieces in 6 ml of