Identification of Enzymes Responsible for the Metabolism of Heme in Human Platelets*

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The major enzymes involved in the degradation of heme were identified in human platelets. It was determined that heme oxygenase activity levels in umbilical cord blood platelets were higher, whereas biliverdin reductase activity levels were comparable with that found in platelets from adults. In membranes prepared from adenosine diphosphate-activated platelets, UDP-glucuronosyltransferases (UGTs) form the major erythrocyte cytosolic heme catabolic pathway in humans and most mammalian species (1, 2). When senescent red blood cells migrate to the spleen, the globin portion of the hemoglobin molecule is removed and recycled to component amino acids. The heme moiety is metabolized by the sequential action of HO to form the linear tetrapyrrolic biliverdin and BVR to form bilirubin. Bilirubin is transported to the liver, where specific UGTs catalyze conjugation with glucuronic acid. Bilirubin glucuronides are then secreted into the bile and ultimately excreted via the intestines (3).

The inducible form of heme oxygenase, HO-1, is expressed in many tissues including the liver, where it is reportedly higher in human neonates than in adults (4). In contrast to the brain and testes, total HO activity in the liver declines with maturation (5). During the first week of life when fetal hemoglobin is degraded, there is an increase in HO activity attributed to increased expression of HO-1. Subsequently, there is a rapid decline in HO-1 expression while the expression of HO-2 gradually increases with maturity until, under normal conditions, HO-2 is the predominant form expressed in the liver (6).

Although heme degradation occurs primarily in the spleen and liver, heme-metabolizing enzymes are expressed in many tissues, suggesting role(s) that are distinct from red blood cell turnover. Heme metabolism is particularly important in response to cell injury, where it may play a role in cell-cell communication and antioxidant defense (7–15). Carbon monoxide, which is produced as a consequence of heme oxidation, has been proposed to function as an intracellular messenger partially regulating cGMP levels (11). Three isoforms of heme oxygenase are known to exist. HO-1 was first identified as a 32-kDa heat shock protein and is inducible by hyperthermic, oxidative, and other stresses and is widely distributed throughout the body, including the liver and spleen (12, 13, 16–19). HO-2 is the major glucocorticoid-regulated constitutive form present predominantly in adult liver connective and nervous tissue and has a molecular mass of approximately 39 kDa (6, 20, 21). The recently identified HO-3 has a molecular mass of approximately 33 kDa and is closely related to HO-2 but is the product of a different gene. HO-3 is found in many tissues, including the liver, is reportedly immunochimically distinct from the other isoforms, and is not as efficient a heme metabolizer (22).

The cytosolic enzyme BVR catalyzes the final step in heme metabolism in mammals and some fish species by the conversion of biliverdin IXα to bilirubin IXα. The human enzyme has been purified and found to resolve into four isoelectric zones and two molecular mass forms (40.7 and 39.6 kDa) in the liver, whereas variants were detected in the kidney. Although the primary sequence of the human enzyme differs extensively from the rat enzyme, they share the unique property of having two distinct pH optima and requiring different cofactors for each pH value: NADH at low pH and NADPH at alkaline pH (23).

The capacity for heme metabolism is developmentally regulated. HO-1 is preferentially expressed in the liver rather than the spleen during late gestation but during the early neonatal period hepatic expression is diminished concurrently with the development of activity in the spleen (6, 24). Conversely, UGT activity toward bilirubin (and UGT1A1 expression) is absent from fetal liver but develops in the early neonatal period (25, 26). These developmental changes have physiological and path-
ological consequences. For example, the developmental deficiency in hepatic bilirubin-UGT is a contributing factor in the high incidence of jaundice in human newborns (27). UDP-glucuronosyltransferases also exist as multiple isoforms. UGT1A1 is the primary isoform responsible for the conjugation of bilirubin, although UGTs exhibit broad overlapping substrate specificity, and other isoforms, such as UGT1A4, are capable of catalyzing this reaction (28).

Preliminary studies suggested to us that human platelets also have the capacity to metabolize heme and its degradation products (29). This report indicates that heme metabolizing enzymes are expressed in human platelets so that the potential exists for heme to be metabolized completely to bilirubin-glucuronide in situ. The platelet enzymes also appear to reflect the developmental regulation of expression reported for the hepatic forms.

EXPERIMENTAL PROCEDURES

Materials—Histopaque 1077 and 1119, platelet aggregation kits, uridine 5′-diphosphoglucuronic acid, hemin, biliverdin, direct bilirubin assay kits, bilirubin Lin-TROL, and bilirubin standards (total and direct), were from Sigma. Bilirubin was from Acros Organics (Pittsburgh, PA). [14C]UDP-glucuronic acid was purchased from NEN Life Science Products. Polyclonal antibodies to rat heme oxygenase-1, heme oxygenase-2, and biliverdin reductase were purchased from StressGen (Victoria, BC, Canada). Anti-UGT C-terminal peptide antibody was provided by Chris Patton (Gentest Corporation, Woburn, MA). Recombinant UGTs and adult human liver microsomes were also purchased from Gentest Corporation. Additional human liver microsomal and cytosol fractions that were used as positive controls were frozen samples stored at −80 °C. The neonatal human liver sample was a 2-day-old term infant who died of sudden infant death syndrome and was originally obtained with appropriate Institutional Review Board approval from Dr. Linda Perrot (Department of Pathology, Arkansas Children’s Hospital, Little Rock, AR). The rat liver S9 preparation was a 9,000 × g supernatant from an adult male Sprague-Dawley rat. Blood samples were obtained according to an Institutional Review Board-approved protocol (NCI-2888). Electrophoresis and Western blot reagents were from Novex (San Diego, CA). Detection of immunoreactive bands was performed using the ECL chemiluminescence method from Amersham Pharmacia Biotech. In some instances, alkaline phosphatase visualization was used. Quantitation of immunoreactive bands was performed on a model 300A Computing Densitometer from Molecular Devices (Sunnyvale, CA).

Platelet Isolation and Activation—Blood samples from healthy adult volunteers (32 ml) and human neonates (8–16 ml, from umbilical cord) were collected in "yellow-top" ACD tubes (Becton-Dickinson, Franklin Lakes, NJ) and stored at room temperature until processed (<12 h). Individually collected blood components were then isolated by centrifugation on discontinuous gradients of Histopaque-1077 and Histopaque 1119, using a modification of the manufacturer’s protocol (Sigma procedure number 1119). Briefly, whole blood was layered on top of gradients and centrifuged at 700 × g for 30 min (room temperature); lymphocytes and platelets comigrated as a distinct band in the top third of the tube of each gradient. These fractions were collected and diluted 3-fold with phosphate-buffered saline containing 15% ACD solution and centrifuged (500 × g, 10 min) to achieve an initial separation. Lymphocytes appeared in the pellet along with contaminating platelets, whereas the contaminant contained mostly platelets. Cell pellets were resuspended in 15% ACD/phosphate-buffered saline buffer and centrifuged at 150 × g (10 min) twice, and both “washes” were added to the original platelet pool. The diluted cell suspension was then centrifuged (500 × g, 20 min) to obtain a platelet pellet that was resuspended in a small volume of 0.25 M sucrose, 10 mM triethanolamine, and 5 mM 2-mercaptoethanol. The diluted cell suspension was then centrifuged (500 × g) for 10 min at 4 °C. The resulting supernatant was re-centrifuged at 100,000 × g for 1 h at 4 °C. For activation of the platelets for UGT assays, washed platelets (approximately 109 cells/ml) were incubated with 20 μM ADP and 50 μM CaCl2 for 10 min at 37 °C. After incubation, the platelets were placed on ice until they could be assayed for bilirubin glucuronidation activity.

Heme Oxidase Assays—Heme oxygenase activity was determined by the method of Tenhunen et al. (30) with the modifications of the assay detailed by Lincoln et al. (31). Bilirubin production was measured by a differential spectrum between the reaction mixture and a blank that did not contain an NADPH-regenerating system. Each incubation contained 5 mM deferoxamine, 25 μM heme, 20 μg/ml bovine serum albumin, 0.1 mM potassium phosphate, pH 7.4, and either 0.05 mg of rat liver S9 or 0.1 mg of platelet homogenate in a volume of 0.1 ml. The samples were pre-incubated at 37 °C for 5 min prior to the addition of an NADPH-regenerating system and then incubated for an additional 10 min. The reaction was stopped by the addition of 0.1 ml of ethanol-Me2SO (95:5, v/v), and the samples were chilled on ice for 5 min and then centrifuged at 18,000 × g for 10 min at 4 °C. Supernatants were pipetted into a 96-well microtiter plate, and their absorbance at 450 nm was recorded. Activities were calculated using an extinction coefficient of 40 μM−1 cm−1 for bilirubin formation. To confirm that the increased absorbance was in fact due to the formation of bilirubin, samples were analyzed colorimetrically using a Sigma Diagnostic kit. Biliverdin reductase activity was assayed by the method of Tenhunen et al. (32). The assay mixture consisted of 100 mM Tris-HCl, pH 8.5, 10% glycerol, 5 μM [14C]bilirubin, enzyme, and NADPH and was preincubated with the addition of an NADPH-regenerating system, and bilirubin formation was calculated as described previously (23). UDP-GlcUA-dependent UGT activity toward bilirubin was determined colorimetrically by a modification of Walters and Gerarde (33) as developed by Sigma Diagnostics (procedure number 550/551). Briefly, bilirubin solution was prepared according to Van Roy et al. (34). Activated platelets (100 μl; approximately 0.1 mg of protein) were incubated with 100 nmol of bilirubin solution in the presence or absence of 2 μM UDP-GlcUA at 37 °C for 1 h. The formation of bilirubin glucuronides was detected colorimetrically and quantified by comparison with bilirubin glucuronide standards. Confirmation of the product as a bilirubin glucuronide was performed by HPLC according to the method of Coumbarie et al. (35). Briefly, platelet microsomes (approximately 100 μg of protein) incubated for 1 h in 15 μl of incubation mixture containing 30 nmol of 14C-labeled UDPGlcUA, 6 nmol of bilirubin, 6 nmol of saccharo-1,4-lactone, and 1 μg of alamethicin in Tris-HCl buffer, pH 7.4. The incubation was terminated by the addition of 60 μl of acetonitrile followed by 30 μl of water. The resulting mixture was sonicated for 10 min and then centrifuged at 20,000 × g for 5 min. 80 μl of the supernatant was placed in an autoinjector vial for HPLC analysis. HPLC was performed on a Waters HPLC system equipped with a Whatman PAC column. Solvent A consisted of 100% acetonitrile, whereas solvent B was 10% tetrabutyl ammonium hydrogen sulfate in water. Products were eluted using a flow rate of 1.5 ml/min, and a gradient of 100% solvent A to 100% solvent B from 0 to 20 min. Detection of products was performed using a Packard Radiomatic 525 inline radioactivity detector (Packard Instrument Co., Meridian, CT) and a Waters 490 detector.

Western Blot Analysis—Membrane fractions and cytosol were prepared by subjecting the platelets to three 10-s bursts on a Sonifier Cell Disruptor (Ultrasonics Inc., PlainView, NY; setting 1) on ice. Platelet cytosol for the BVR assays was prepared by sonication followed by centrifugation at 18,000 × g for 10 min at 4 °C. The resulting supernatant was re-centrifuged at 100,000 × g for 1 h at 4 °C. For activation of the platelets for UGT assays, washed platelets (approximately 109 cells/ml) were incubated with 20 μM ADP and 50 μM CaCl2 for 10 min at 37 °C. After incubation, the platelets were placed on ice until they could be assayed for bilirubin glucuronidation activity.

RESULTS

Table I shows a comparison of HO activities derived from human platelet homogenates from both neonates and adults. Platelet HO activity in neonates was significantly greater than adult value by Student’s t test (p < 0.02), although both activities were lower than the rat liver S9 fraction used as a positive control. As shown in Fig. 1, a very similar developmental pattern was found in immunoreactive protein from platelets. Fig.
TABLE I

Enzymatic activities of heme oxygenase, biliverdin reductase, and bilirubin UDP-glucuronosyltransferase in liver and in platelets from human adults and umbilical cord blood

| Enzyme source                      | Heme oxygenase activity pmol/min/mg | Biliverdin reductase activity nmol/min/mg | Bilirubin UGT activity pmol/min/mg |
|------------------------------------|-----------------------------------|------------------------------------------|-----------------------------------|
| Rat liver S9 fraction              | 1340.4 ± 102.1                    | 98 ± 0.45 (n = 4)                        | ND                                |
| Adult platelets                    | 207.4 ± 32.4 (n = 8)              | 585†                                     | 34.5 ± 5.9 (n = 8)†                |
| Neonatal platelets                 | 539.9 ± 115.5 (n = 8)             | 4%                                       | ND                                |
| Adult human liver microsomes       | 585†                              | 176                                      | ND                                |
| Neonatal human liver microsomes    |                                   |                                          | ND                                |
| Lymphoblastoid-expressed UGT1A1    |                                   |                                          | ND                                |
| ADP-activated adult platelets      |                                   |                                          | ND                                |
| ADP-activated neonatal platelets    |                                   |                                          | ND                                |
| Serum                              |                                   |                                          | ND                                |
| Human liver cytosol                | 20.0 ± 0.68                       | 28.6 ± 3.5 (n = 8)                       | ND                                |
| Adult platelet cytosol             |                                   |                                          | ND                                |
| Neonatal platelet cytosol          |                                   |                                          | ND                                |

a Significantly greater than adult value by Student’s t test (p < 0.02).

b ND, activity not detected.
c Values are for the individual samples used for the Western blot.
d Significantly greater than unactivated value by Student’s t test (p < 0.01).

**Fig. 2. Western blot analysis of liver and platelet cytosol with anti-biliverdin reductase antibody.** Cytosolic proteins from adult human liver and platelet membrane fractions from adults and neonates (10 µg of protein each) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and exposed to antibodies directed against rat biliverdin reductase. Lane 1, adult human liver cytosol; lane 2, adult human platelet cytosol; lane 3, neonatal platelet cytosol.

Comparative analysis of heme oxygenase, biliverdin reductase, and bilirubin UDP-glucuronosyltransferase activities in liver and in platelets from human adults and umbilical cord blood.

**Fig. 1. Western blot analysis of liver and platelet membranes with anti-heme oxygenase antibodies.** Microsomal proteins from adult human liver and platelet membrane fractions from adults and neonates (10 µg of protein each) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and exposed to antibodies to rat heme oxygenase-1 (A) or rat heme oxygenase-2 (B). Lanes 1, adult human liver microsomes; lanes 2, adult human platelet membranes; lanes 3, human neonate platelet membranes.

1A compares the expression of proteins reacting with a polyclonal antibody to rat HO-1 in human liver (lane 1), to platelet membranes from an adult human (lane 2), and to platelet membranes from newborn umbilical cord blood (lane 3). The level of expression of platelet-derived immunoreactive protein was comparable with or greater than the liver-derived form, although the molecular mass was slightly higher. Platelets from a human neonate expressed levels of immunoreactive protein that were four times higher than those of adults, as determined by densitometry. By contrast, the constitutive form, HO-2, was expressed at slightly higher levels in platelets from adults compared with newborns (Fig. 1B, lanes 2 and 3). Although expression was highest in the liver (Fig. 1B, lane 1), the developmental pattern of HO-2 expression in platelets was similar to that previously reported for the hepatic form.

BVR activity was measured in human platelet cytosol. As shown in Table I, the activity from adult human platelets was comparable with the activity found in human liver cytosol. BVR activity in umbilical cord blood platelets was slightly higher than either of the adult preparations. Fig. 2 compares the expression of BVR in human liver cytosol (lane 1), in adult platelet cytosol (lane 2), and in platelet cytosol from human neonates (lane 3). Immunoreactive forms were present at approximately 40 kDa in both liver and platelet cytosols.

Platelets were also assayed for UGT activity toward bilirubin. In preliminary studies, washed platelets were shown to have negligible glucuronidation capacity, regardless of whether the platelets were intact or sonicated. Incubation with detergents (CHAPS, Brij 58, and Triton-100) or with the channel-forming peptide alamethicin also failed to activate platelet glucuronidation capacity. However, when a series of platelet activators/aggregators (i.e., collagen, thrombin, epinephrine, and ADP) were examined for their ability to stimulate the glucuronidation of bilirubin, ADP was found to be an effective activator. When platelets from adult volunteers were exposed to 20 µM ADP, UDP-GlcUA-dependent conjugation of bilirubin was significantly higher by Student’s t test (p < 0.01) than in unactivated platelets (Table I). By contrast, activity was not detected in either unactivated or ADP-activated platelets from neonates or in adult or neonatal serum. Additional analysis of bilirubin glucuronide formation using [14C]UDP-glucuronic acid and HPLC separation of products confirmed these findings (Fig. 3). Fig. 3A is a representative radioactive trace of an incubation with activated platelets, and Fig. 3B is the corresponding UV profile. Fig. 3Bb shows the radioactive trace of an incubation using unactivated platelets, whereas Fig. 3C is the corresponding UV profile. The glucuronide peaks were dependent on the presence of both UDPGlcUA and bilirubin in the incubation mixture and could be converted to free bilirubin and glucuronic acid by β-glucuronidase, and their retention times...
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discussed under “Experimental Procedures.” Panel A shows the radioactive trace of an incubation using activated platelets, whereas panel C shows the corresponding UV trace. Panel B shows the radioactive trace of an incubation using unactivated platelets, and panel D is the UV trace of panel B. b, m, d, and u denote free bilirubin, bilirubin mono-glucuronide(s), bilirubin di-glucuronide, and UDPGlcUA peaks, respectively.

Corresponded to those produced by incubations of rat or human liver microsomes. As shown in Table I, bilirubin glucuronide formation is considerably higher in adult human liver microsomes than in either neonatal liver microsomes or in activated adult platelets. Glucuronide formation by neonatal platelets was not detected, either colorimetrically or by HPLC.

Developmental regulation of UGT expression in platelets was demonstrated by Western blot using an anti-peptide antibody directed against an exon common to all members of the UGT1A family. A representative Western blot is shown in Fig. 4. UGT-specific immunoreactivity (approximately 53–55 kDa) was demonstrated in adult and neonatal human liver, recombinant UGT1A1, UGT1A4, platelet membrane fractions from adult humans, and platelet membrane fractions from human neonatal umbilical cord blood. With this antibody preparation, the amount of immunoreactive UGT in platelets from neonates (Fig. 4, lane 6) was almost undetectable compared with that of platelets from adults (Fig. 4, lane 5). This pattern of development is similar to many liver-derived isoforms of UGT, including the bilirubin-specific form. The molecular mass of the UGT1A immunoreactive protein corresponds to that of the major UGT1A band in adult human liver but was of a lower apparent molecular mass than either recombinant UGT1A1 or UGT1A4.

DISCUSSION

Heme-metabolizing enzymes are found primarily in the liver and spleen, but many cell-types express them. To our knowl-

edge, this is the first report of this enzyme system in platelets, although macrophages have been shown to mediate the breakdown (and detoxification) of heme to bile acids at sites of injury (37). Mammalian systems are also known to possess pathways of heme degradation that are carried out by enzymes other than the heme oxygenase system. Heme can be degraded by xanthine oxidase, hydrogen peroxides, and NADH-cytochrome P-450 reductase, but in these cases the product is a mixture of pyrrolic compounds instead of biliverdin or CO (38). We initially found immunoreactive protein and enzymatic activities for BVR and for bilirubin UGT, which led to attempts to identify HO isoform(s) that might be present in platelets. The three isoforms of HO identified thus far are all different gene products and are thought to be immunochemically distinct, although they share the same substrate specificity, cofactor requirements, and mechanistic characteristics (22, 39). In this study, the protein detected in platelets using an anti-rat polyclonal antibody to HO-1 exhibited a molecular mass comparable with that reported for the newly identified HO-3 rather than that of human HO-1. HO-3 was first isolated in rat brain but later was detected in a variety of tissues and organs, including the liver (22). Although rat HO-3 was reported to be immunochemically distinct from rat HO-1 in the original studies, it is possible that the polyclonal HO-1 antibody that we used in the present study cross-reacted with human HO-3 or with a novel HO isoform. Alternatively, the differences in molecular mass between the immunoreactive proteins found in human liver and platelets could be a result of differential post-translational modifications. However, when platelet membranes were probed with a polyclonal antibody to rat HO-2, the immunoreactive protein was the same apparent size as that reported in the literature and that observed in our human liver sample. The liver sample used here expressed high levels of HO-2 immunoreactive protein. The enzymatic activity toward hemin found in the platelets, therefore, cannot be attributed to a specific isoform at this point. Regardless of the identity of the isoforms present in platelets, it is noteworthy that they exhibit the same pattern of developmental regulation observed for hepatic isoforms.

Biliverdin is a biologically active compound that has been shown to inhibit viral replication in vitro (40). BVR is the enzyme responsible for the biotransformation of biliverdin to bilirubin and, along with heme oxygenase, is also found in a variety of tissues and organs. The presence of this enzyme in platelets provides the next step in the pathway of heme degradation. The presence of UGT activity toward bilirubin in activated platelets provides the final stage in heme degradation. The UGT1A immunoreactive protein observed in adult human platelets showed an apparent molecular mass that corresponded to a major UGT1A band in human liver but was smaller than either recombinant UGT1A1 or UGT1A4. This
suggests that either platelet bilirubin UGT activity is catalyzed by a UGT1A isoform other than 1A1 or 1A4 or that the apparent molecular mass of recombinant UGT1A1 differs from that of the constitutively expressed isoform. However, platelet bilirubin UGT activity was dependent on activation of the platelets with ADP. A defined sequence of events occurs when platelets are exposed to ADP. Discoid-shaped resting platelets are changed rapidly to spherical shape. Change in the physical form of the platelet is followed by platelet aggregation and release of dense granule contents (41). Whether ADP itself or a substance released from platelet dense granules is responsible for activation of bilirubin UGT activity has not been established. This finding could indicate that platelet-specific heme metabolism, along with the subsequent detoxification of bilirubin via glucuronidation, plays a role in restoring homeostasis at sites of injury (thrombus formation). In normally circulating platelets the bilirubin would be expected to diffuse out of the platelets and be conjugated by hepatic UGTs. But within a blood clot or at the site of injury this route of bilirubin detoxification would not be available. Hence, it is advantageous for the activated platelets to conjugate bilirubin in situ.

Recently, the presence of UGTs, along with other protein components of the glucose-6-phosphatase system, has been demonstrated in human embryonic and fetal red blood cells but not in adult red blood cells (42). The developmental regulation of this system differs from the hepatic UGTs, where neonatal expression is low (resulting in an initial deficiency in the ability to conjugate bilirubin), but increases with maturation. In addition to normal developmental changes, hereditary deficiencies in bilirubin UGT activity are known to exist. These deficiencies are responsible for Crigler-Najjar Type I and Type II syndromes as well as Gilbert's syndrome, a more common deficiency that affects more than 5% of the population (43). Interindividual variability in some cases, have been shown to arise from the same genes as those found in other tissues (44). In this study, it was demonstrated that the developmental profiles and enzymatic activities of platelet heme oxygenase, biliverdin reductase, and UGT(s) seem to correlate with the activity found in the liver.

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