BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **224,** 108–114 (1996)
ARTICLE NO. 0992

Human Erythrocyte Band 7.2b Is Preferentially Labeled by a Photoreactive Phospholipid

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Received June 3, 1996

A head-group modified, photoreactive analog of phosphatidylethanolamine, N-([^{125}I]iodo-4-azidosalicylamidyl)-1,2-dilauryl-sn-glycero-3-phosphatidylethanolamine ([^{125}I]-N-ASA-DLPE), has been used in photoaffinity labeling studies of proteins of the human erythrocyte membrane. [^{125}I]-N-ASA-DLPE was shown to be preferentially incorporated into a protein with an apparent molecular weight of 31 kDa. Protein sequencing and immunoprecipitation were used to identify this protein as the erythrocyte membrane protein, band 7.2b or stomatin. A sulphydryl-reactive ligand, 4-hydroxy-3-(iodo-[^{125}I])-N-[2-(2-pyridinyldithio)ethyl]-benzenepropanamide ([^{125}I]-PDA), was also shown to preferentially label band 7.2b. We propose that band 7.2b may act as a site of transbilayer reorientation of membrane phospholipids.

Human erythrocyte band 7.2b (stomatin) is a major integral membrane protein, present at an estimated 410,000 copies per cell (1-3). This 31 kDa protein appears to be associated with the erythrocyte membrane in a monotopic fashion via the hair-pin insertion of a hydrophobic sequence into the inner leaflet of the lipid bilayer [4]. The function of band 7.2b is unknown, however it appears to play a important physiological role, as the absence of the protein is associated with the condition of hereditary stomatocytosis (5,6). These cells show an abnormal morphology, an increased fragility and a severe monovalent cation leak (Lande *et al.*, 1982). A cross-reactive polypeptide is present in a range of cell types suggesting that the role played by band 7.2b is not limited to erythroid functions (3).

A protein with an apparent molecular weight of 31 kDa has been shown to be preferentially labeled with a side chain-modified, photoreactive derivative of phosphatidylserine (7,8). The preferential labeling of this protein led these authors to postulate that this 31 kDa protein was involved in the maintenance of aminophospholipid asymmetry. Evidence was provided which suggested that the protein was an Rh blood group polypeptide (8). The 31 kDa protein was also labeled with a cysteine-reactive reagent, [^{125}I]-PDA.

In this study, we have used a head group-modified, photoreactive analog of phosphatidylethanolamine and shown that this reagent is also preferentially incorporated into a protein with an apparent molecular weight of 31 kDa. Protein sequencing and immunoprecipitation have been used to establish the identity of the labeled protein as erythrocyte band 7.2b. We also show that band 7.2b is efficiently labeled with [^{125}I]-PDA. We propose, therefore, that band 7.2b, rather than the Rh polypeptide, is the phospholipid-interacting protein in erythrocytes, and

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Abbreviations: N-[^{125}I]-ASA-DLPE, N-([^{125}I]iodo-4-azidosalicylamidyl)-1,2-dilauryl-sn-glycero-3-phosphatidylethanolamine, [^{125}I]-PDA, 4-hydroxy-3-(iodo-[^{125}I])-N-[2-(2-pyridinyldithio)ethyl]-benzenepropanamide; TLC, thin layer chromatography; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; NMR, nuclear magnetic resonance.

0006-291X/96 $18.00

discuss a possible role for band 7.2b as site for non-specific ''flip-flop'' of membrane phospholipids.

MATERIALS AND METHODS

Synthesis and iodination of N-ASA-DLPE. The synthesis of N-ASA-DLPE has been described previously (9). The synthetic product was characterized by TLC (R_f = 0.25), ^{1}H-NMR and by absorption spectroscopy, as described by Berman *et al.* (9). N-ASA-DLPE was iodinated as described previously (9). The specific activity was estimated to be approx. 10 Ci mmol^{-1}.

Photoaffinity labeling of proteins in human erythrocyte membranes using radioiodinated N-ASA-DLPE. Samples of fresh human erythrocytes (Red Cross Blood Bank, Melbourne) were washed twice and resuspended in 5mM sodium phosphate, pH 7.5, 150 mM NaCl (PBS). Samples (1 ml) containing approximately 5×10^7 cells were maintained at 37°C during the addition of N-[^{125}I]-ASA-DLPE (10 μL, 0.5 μCi, 50 pmol) from a stock in ethanol. The exogenously added phospholipid represented approximately 1% of the endogenous phospholipid in the erythrocyte samples. The samples were incubated for 30 min at 37°C, under reduced light, then pelleted at 2,200 g for 5 min, and resuspended in 30 μl of the supernatant fluid. Photoactivation was induced by irradiating the samples with ultraviolet light (365 nm, 37°C, 10 min) using a hand-held source (Mineralight UVGL-58) positioned 10 cm from the surface of the samples. Following photoactivation, the samples were resuspended in 1.5 ml of 5 mM phosphate, pH 8, to lyse the erythrocytes. The cell membranes were pelleted at 3,700 g for 7 min and the pellet was immediately mixed with an equal volume of electrophoresis sample buffer (10). The samples were analysed by SDS-PAGE and stained with Coomassie blue. Radiolabeled proteins were visualized by phosphorimage analysis (Molecular Dynamics). For competition experiments, p-azidosalicylic acid (10 μl, 10 nmol, synthesized in our laboratories) and salicylamide (10 μl, 10 nmol, Aldrich) were added from stocks in PBS, together with the N-[^{125}I]-ASA-DLPE. In some experiments, the labeled membranes were incubated with 5 mM sodium phosphate, pH 8, for 30 min at 37°C to extract the peripheral membrane proteins, prior to analysis by SDS-PAGE.

Labeling of erythrocyte membranes with [^{125}I]-PDA. 2-(2-pyridinyldithio)-ethanamine) (PDA) was prepared according to the method of Johnson and Chenoweth (11). [^{125}I]-PDA was prepared using a modification of the procedure of Connor and Schroit (12). A solution of PDA (200 nmol) in 40 μl of chloroform/methanol/triethylamine (1:2:0.01) was added to [^{125}I]-Bolton-Hunter reagent (Amersham, 2 mCi, 1 nmol), which had been dried under nitrogen, and the solution allowed to stand for 4 days at 4°C. The reaction mixture was applied to a TLC plate (silica gel 60, Merck) which was developed using ethyl acetate/toluene (1:1). [^{125}I]-PDA migrated with an R_f = 0.2. The product was visualized by autoradiography and extracted twice with 10 ml of chloroform/methanol (8:2 v/v). The extracts were dried under nitrogen in siliconized glass tubes and redissolved in chloroform. The specific activity of the preparation of [^{125}I]-PDA was 2 Ci μmol^{-1}. For labeling experiments, [^{125}I]-PDA (13 μCi) was dried, under nitrogen, onto the surface of an Eppendorf tube. An erythrocyte sample (1 ml, approx. 2×10^8 cells) was added and the mixture was incubated for 30 min at 4°C with agitation. The cells were washed once with PBS, and lysed and washed four times with 5 mM sodium phosphate, pH 7.5. The membrane pellet was analysed by SDS-PAGE (12% acrylamide) under non-reducing conditions and stained with Coomassie blue. Radiolabeled proteins were visualized by phosphorimage analysis (Molecular Dynamics).

Protein sequence analysis. The Coomassie blue-stained band corresponding to the 31 kDa labeled protein (approximately 10 μg protein) was excized from a preparative SDS-polyacrylamide gel, washed three times for 10 min in 10 ml water, cut into small pieces and vacuum dried. The protein was digested with trypsin *in situ* using a modification of the procedure of Tetaz *et al.* (13). Briefly, the gel pieces were rehydrated in 1 ml of 0.1 M NH_4HCO_3, pH 8, containing 0.5 μg of modified trypsin (Promega) and the mixture was transferred to an empty HPLC precolumn and incubated at 37°C for 20 hours. The tryptic peptides were eluted from the precolumn onto a reverse phase column (VYDAC, 250 × 1 mm, 300A, C8) by pumping Buffer A (0.1% trifluoroacetic acid/water) through the column at high (>100 bar) pressure. The peptides were eluted using a gradient of 0 - 6% B for 0 - 2 min, 6 - 50% B for 2 - 110 min, 50 - 70% B for 110 - 120 min, 70 - 100% B for 120 - 130 min, at 50 μl/min, where solution B is 0.09% trifluoroacetic acid /70% acetonitrile in water. Best peak fractions were loaded directly onto glass fibre filters and the peptides were sequenced using a gas-phase sequencer (Applied Biosystems Inc.).

Western blot analysis. Proteins were electrophoretically transferred from SDS-polyacrylamide gels to PVDF-plus membranes (Micron Separation Inc.) and probed with a murine monoclonal anti-band 7.2b antibody (3). The anti-band 7.2b antibody was kindly donated by Dr. R. Prohaska, University of Vienna, Vienna. Immuno-reactive bands were detected using the enhanced chemiluminescence system (Amersham).

Immunoprecipitation. Membranes prepared from approximately 2×10^8 erythrocytes, which had been labeled with [^{125}I]-N-ASA-DLPE or approximately 10^9 erythrocytes, which had been labeled with [^{125}I]-PDA, were solubilized in a buffer containing 0.2% SDS and 1% Triton X-100 as described by Hiebl-Dirschmied *et al.* (3). The solubilized sample was clarified by centrifugation (10,000 g, 10 min) and mixed with 500 μl of culture supernatant from a hybridoma producing anti-band 7.2b antibodies plus 50 μl of a 50% slurry of Protein A-Sepharose beads. The mixture was agitated for 2 h at room temperature and washed four times with 0.5% (v/v) Triton X-100, 150 mM NaCl, 20

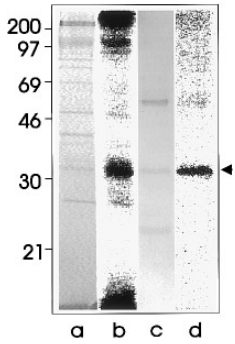

FIG. 1. Photoaffinity labeling of phospholipid-interacting proteins in human erythrocytes and immunoprecipitation of band 7.2b. Approximately 5×10^7 washed human erythrocytes were incubated with [^{125}I]-N-ASA-DLPE (0.5 μCi, 50 nM) and exposed to UV illumination. Membrane samples were prepared and subjected to SDS-PAGE (12 % acrylamide) and visualized by phosphorimage analysis (Lanes a,b). Alternatively, labeled membrane sample was solubilized in 0.2% SDS/ 1% Triton X-100 (as described Materials and Methods) and immunoprecipitated using an anti-band 7.2b monoclonal antibody and protein A-Sepharose beads. The precipitated proteins were analysed by SDS-PAGE (12% acrylamide) and visualized by phosphorimage analysis (Lanes b,d). Lanes a and c are Coomassie blue-stained profiles of Lanes b and d. The 31 kDa protein is indicated with an arrowhead.

mM Tris (pH 7.5) and once with phosphate-buffered saline. The bound proteins were analysed by SDS-PAGE (12% acrylamide) and radiolabeled bands were visualized by phosphorimage analysis (Molecular Dynamics). Alternatively, the [^{125}I]-PDA-labeled membrane sample was mixed with 1 ml of 1% (v/v) Triton X-100 in 20 mM Tris, pH 7.5, 5 mM EDTA, 1 mM PMSF, and incubated under rotation for 10 min at room temperature. The samples were clarified by centrifugation (10,000 g, 10 min) and mixed with 500 μl of rabbit anti-human haemoglobin antiserum (Sigma) plus 50 μl of a 50% slurry of Protein A-Sepharose beads. The mixture was rotated for 3 h at room temperature and washed four times with 0.5% (v/v) Triton X-100, 150 mM NaCl, 20 mM Tris (pH 7.5) and once with 20 mM Tris, pH 7.5. The bound proteins were analysed by SDS-PAGE (12% acrylamide) under non-denaturing conditions and radiolabeled bands were visualized by phosphorimage analysis (Molecular Dynamics).

RESULTS

A photoreactive derivative of dilauryl phosphatidylethanolamine which bears an azidosalicylate moiety on the phospholipid head group was used in photoaffinity labeling studies of human erythrocyte membranes. N-[^{125}I]-ASA-DLPE was incorporated into the erythrocyte membrane from an ethanol solution, and allowed to equilibrate for 30 min at 37°C, prior to exposure to ultraviolet illumination. Following photoactivation, the erythrocytes were lysed hypotonically and the membrane pellets subjected to SDS-PAGE. Phosphorimage analysis revealed preferential incorporation of N-[^{125}I]-ASA-DLPE into a protein with an apparent molecular weight of 31 kDa (Figure 1, Lanes a,b). Insertion of the photoaffinity ligand into high molecular weight complexes and into a polypeptide corresponding to the major erythrocyte membrane protein, band 3, was also observed (Figure 1, Lanes a,b). There was no incorporation of N-[^{125}I]-ASA-DLPE into proteins in the absence of ultraviolet illumination (data not shown), indicating that photolabeling occurs exclusively through the activated nitrene intermediate, or its rearrangement products. Similarly, photoactivation of the phospholipid analog prior to addition to the erythrocyte sample prevented insertion of the radiolabel (data not shown) again indicating that no light-independent reactions were occurring. Two water-soluble compounds, p-azidosalicylic acid and salicylamide, which bear the structure of the photoreactive moiety of N-[^{125}I]-ASA-DLPE did not inhibit the photolabeling of the erythrocyte proteins when added in a 250-fold molar excess (data not shown). This indicates that the labeled proteins are associated with the phospholipid moiety rather than with the aryl-azide moiety prior to photoinsertion of the probe.

The 31 kDa protein was not solubilized during a low ionic strength treatment of erythrocyte

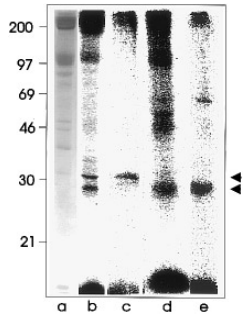

FIG. 2. Photoaffinity labeling of [^{125}I]-PDA-reactive proteins in human erythrocytes. Approximately 2×10^8 washed human erythrocytes were incubated with [^{125}I]-PDA (13 μCi, 6.5 nM). Membrane samples were prepared and subjected to SDS-PAGE (12 % acrylamide) and visualized by phosphorimage analysis (Lanes a,b). Lane a is a Coomassie blue-stained profile of Lane b. [^{125}I]-PDA-labeled membrane samples were solubilized in 0.2% SDS/ 1% Triton X-100 and immunoprecipitated with an anti-band 7.2b monoclonal antibody and protein A-Sepharose beads (pellet, Lane c; supernatant, Lane d), or solubilized in 1% Triton X-100 and immunoprecipitated with an anti-haemoglobin antiserum (pellet, Lane d). The precipitated proteins were analysed by SDS-PAGE (12 % acrylamide) and visualized by phosphorimage analysis. The 31 kDa and 28 kDa proteins are indicated with arrowheads.

membranes (data not shown) suggesting that it is membrane-embedded. The radiolabeled protein co-migrated with a relatively major Coomassie blue-staining polypeptide. This band was excized from a preparative gel and subjected to tryptic digestion and sequence analysis. A search of the sequenced peptide (LLAQTTLR) revealed an exact match with erythrocyte integral membrane protein band 7.2b or stomatin (14). Western analysis, using a monoclonal antibody which recognizes band 7.2b, also showed that band 7.2b migrated with an apparent molecular weight equivalent to that of the labeled protein (data not shown). This identification was confirmed by immunoprecipitation. Labeled membranes were solubilized in 0.2% SDS/ 1% Triton X-100. The [^{125}I]-N-ASA-DLPE-labeled 31 kDa protein was immunoprecipitated from the detergent-solubilized membranes using a monoclonal anti-band 7.2b antibody (Figure 1, Lanes c,d). No labeled proteins were precipitated in the absence of antibody (not shown).

A protein with an apparent molecular weight of 31 kDa has previously been shown to be preferentially labeled with a cysteine-reactive reagent, [^{125}I]-PDA (7,8). To determine whether this sulphydryl-reactive protein was distinct from band 7.2b, we used [^{125}I]-PDA to label human erythrocytes. Under the conditions of our experiments, we found that [^{125}I]-PDA was incorporated into proteins with apparent molecular weights of 31 kDa and 28 kDa (Figure 2, Lanes a,b). Samples of the labeled membranes were solubilized in 0.2% SDS/ 1% Triton X-100 and the 31 kDa protein was confirmed as band 7.2b by immunoprecipitation using the monoclonal anti-band 7.2b antibody (Figure 2, Lane c). The 28 kDa protein was immunoprecipitated, from Triton extracts, using an anti-haemoglobin antiserum (Figure 2, Lane d) suggesting that some covalent dimers of haemoglobin are formed under the non-reducing conditions used in this experiment.

DISCUSSION

The human erythrocyte membrane contains a number of integral membrane proteins of which band 3 (anion exchange protein) (1.2×10^6 copies/ cell), glycophorin A (1×10^6 copies/ cell), and band 7.2b (stomatin) (0.4×10^6 copies/ cell), are the most abundant (1,3,15). The function of stomatin is unknown, however, stomatin is absent in a number of cases of hereditary stomatocytosis, a condition which is characterized by a dramatic cation leak (5). Stomatin has, thus, been proposed to play some role in cation transport (5), possibly by interacting with a cation channel (3). Stomatin, itself, is unlikely to act as a cation channel as

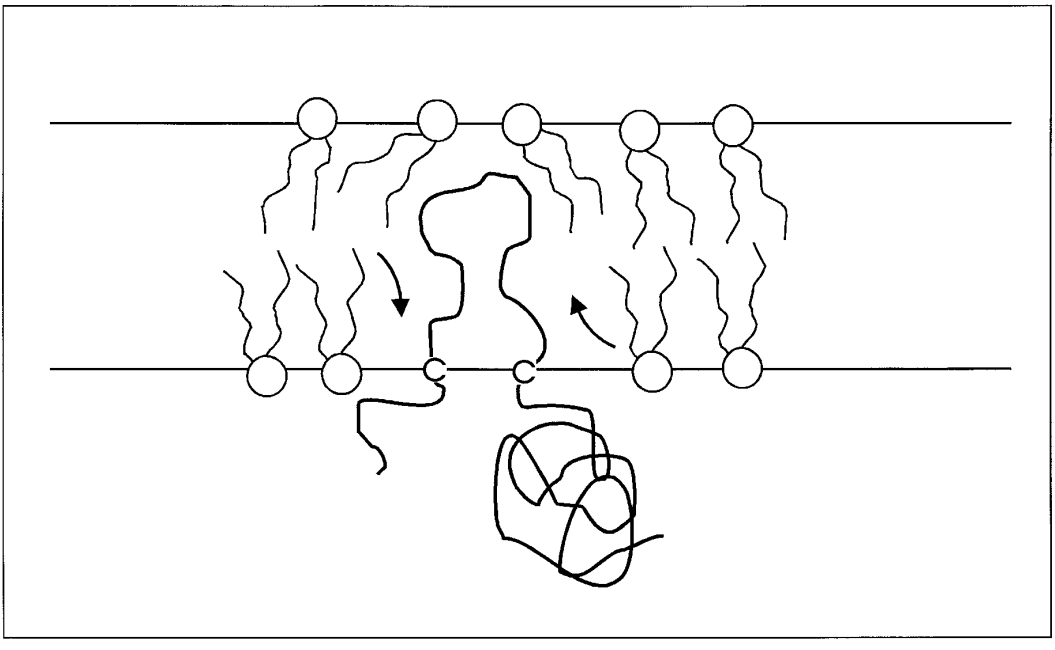

FIG. 3. Diagram illustrating the postulated role of band 7.2b (stomatin) in the non-specific ''flipping'' of phospholipid molecules between the bilayer leaflets. Band 7.2b is thought to be inserted into the membrane in a monotypic fashion (4). The protein is also palmitylated although the site of fatty acid attachment has not been established (2). The monotopic topology of band 7.2b may create imperfections in the protein-lipid interface, thus facilitating the transbilayer movement of phospholipids.

it appears to be associated with the membrane in a monotopic fashion, apparently via insertion of a hydrophobic hair-pin loop (4).

In this study, stomatin was shown to be preferentially labeled with a derivative of phosphatidylethanolamine bearing a photoreactive moiety on its polar head group. This preferential labeling may represent a specific interaction of the exogenously-added phospholipid analog with band 7.2b. The photoreactive phospholipid molecules will be initially incorporated into the outer leaflet of the erythrocyte membrane, however the resulting expansion of the outer leaflet will drive the relocation of a proportion of the labeled lipid to the inner leaflet. Assuming that the proposed monotopic topology of stomatin is correct, two of the three cysteine residues within the band 7.2b sequence will be located near the cytoplasmic surface of the membrane, close to the phospholipid head-groups (see Ref. 4 and Figure 3). The cysteine residue may offer well-located nucleophillic sites for the highly reactive intermediates which are formed upon activation of the azido salicylic moiety (see Haley (16), for a detailed discussion of the reactions involved in photoaffinity labeling).

A protein with an apparent molecular weight of 31 kDa has previously been photolabeled using an acyl chain-modified phosphatidylserine analog (7,8). Schroit *et al.* (8) used immunoprecipitation of Triton X-100-solubilized membranes to identify the labeled protein as the Rhesus antigen-associated polypeptide. As this protein was also labeled with the sulphydryl-reactive reagent, [^{125}I]-PDA, it appears to be the same polypeptide as that under examination in the present study. The reasons for the discrepancy between the two studies, with regard to the identity of this protein, is not entirely clear. It is possible that both band 7.2b and the Rh protein are labeled by the reagents used in the two studies, however, given that we achieved close to quantitative immunoprecipitation of the labeled 31 kDa protein using an anti-band

7.2b antibody, we believe that the major site of labeling is band 7.2b. The conditions used for immunoprecipitation of the Rh protein in the studies of Schroit *et al.* (8) did not involve an SDS-containing solubilisation buffer. The Rh protein has been shown to be present as a component of a large complex of proteins within the erythrocyte membrane, and these complexes are thought to survive Triton X-100 solubilisation (17). It is possible that band 7.2b is associated with the Rh protein in Triton X-100 extracts and can be co-precipitated with this protein.

The preferential interaction of the photoreactive phosphatidylserine with the 31 kDa protein led Schroit *et al.* (7,8) to suggest that this protein may function as a component of the amino phospholipid translocase. The photoreactive phospholipid analog, used in this study, does not have a primary amine in its head group and, therefore, would not be expected to be recognized by the amino phospholipid translocase. Our data, therefore, argue against a role for band 7.2b as the ATP-dependent amino phospholipid translocase. None the less, the preferential interaction of the phospholipid analogs with band 7.2b may indicate an important role for this protein in controlling lipid organisation in the erythrocyte membrane.

The human erythrocyte membrane exhibits an asymmetric distribution of phospholipids; the choline-containing phospholipids are restricted mainly to the outer leaflet, while the amino phospholipids are located mainly at the inner leaflet. The asymmetric distribution is maintained by the action of an amino phospholipid translocase, that actively transports phosphatidylserine and phosphatidylethanolamine to the inner leaflet (18,19). The identity of this translocase has not yet been established, although a protein from chromaffin granules, which appears to perform a similar function, has recently been cloned and characterized (20). The uni-directional ATP-dependent translocation of aminophospholipids would create an imbalance in the proportion of phospholipid in the two leaflets, unless compensated by the simultaneous movement of other phospholipid molecules to the outer leaflet (21). It has been suggested, therefore, that a separate protein may be involved in the non-specific reorientation, or ''scrambling'' of phospholipids in erythrocytes and other cells types (see 22,23, for reviews). We propose that band 7.2b may fulfil this role. The hair-pin insertion of band 7.2b in the membrane bilayer may create a region of imperfection in the protein-lipid interface which could act as a site of phospholipid redistribution (see Figure 3). Interestingly, one would predict that the absence of this protein in hereditary stomatocytosis would be associated with an imbalance in the phospholipid leaflets, such that the rate of inward translocation of amino phospholipids might exceed the outward movement of lipid molecules. The bilayer couple hypothesis (24,25) predicts that this imbalance would result in a stomatocytic morphology similar to that observed in hereditary stomatocytosis. Further work is required to test this hypothesis regarding the physiological role of band 7.2b.

ACKNOWLEDGMENTS

Expert technical assistance was provided by Ms. Gabriel Thorn and Ms. Rosemary Condron. We thank Dr. R. Prohaska, Institute of Biochemistry, University of Vienna, Austria, for helpful advice and for donating the anti-band 7.2b antibody, and Dr. D. Daleke, Indianna University, Indianna, USA, and Dr. A. Schroit, the University of Texas, Texas, USA, for useful discussions. This work was supported by the National Health and Medical Research Council, Australia.

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