Cellular and Molecular Biology [TM] **55**, N°2, 89-95
DOI 10.1170/T857

ISSN 1165-158X
2009 Cell. Mol. Biol.[TM]

THE EXPRESSION OF PROTOPORPHYRINOGEN OXIDASE IN HUMAN TISSUES

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* Dedicated to the late JAH ('Chick') Campbell who passed away tragically while this study was in progress

Received, April 28th 2009; Accepted May 4th, 2009; Published July 1st, 2009

Abstract – Protoporphyrinogen oxidase is the penultimate enzyme in the haem biosynthetic pathway. In this study, the expression of protoporphyrinogen oxidase in a variety of human organs has been documented by immunohistochemical means at the light microscopy level in order to shed light on its inter- and intra-organ distribution. The expression varied amongst organs and the various cell types within an organ. The pattern of staining generally reflected presumed metabolic functionality and haem demand. Strongest staining was noted in hepatocytes, proximal convoluted tubules of the kidney, serous cells of the peribronchial gland in the lung, parietal cells of the stomach, tips of the villi in the small intestine and interstitial cells of the testis. Our results suggest that there are some significant sites of haem synthesis in addition to the liver and bone marrow, and should be borne in mind in studies related to haem or porphyrin dynamics and flux.

Key words: Immunohistological localisation, protoporphyrinogen oxidase, haem biosynthesis, porphyria.

INTRODUCTION

Protoporphyrinogen oxidase (PPOX) (EC1.3.3.4), the penultimate enzyme in the haem biosynthetic pathway, catalyses the removal of 6 electrons from protoporphyrinogen-IX to form protoporphyrin-IX. Three molecules of dioxygen are consumed and three molecules of H_2O_2 are produced (10). In humans, partial defects in this enzyme result in variegate porphyria, a condition characterised by photosensitive skin disease and a propensity to acute neurovisceral crises (for reviews see 18, 26). In plants, inhibition of PPOX by certain herbicides (including the diphenylethers) is the mode of action by which they are killed by light-dependent toxicity (5, 24). In both the above, a build up of protoporphyrinogen-IX results, which in the oxidised protoporphyrin-IX state may cause extensive photomediated cellular damage.

Abbreviations: PPOX, Protoporphyrinogen oxidase; **PBS**, Phosphate buffered saline

The human PPOX cDNA and gene have been cloned and mapped to chromosome 1q22-23 (31,33). Specifically, PPOX from a human placental cDNA library has been cloned, expressed in *Escherichia coli*, purified and characterised (11, 28). Human PPOX is a homodimer composed of subunits of Mr of ~50 000 D and contains one non-covalently bound FAD per dimer (32). Unlike yeast PPOX (6), no precursor form of human PPOX has been described . The crystal structure of human PPOX remains unsolved but that of the tobacco mitochondrial form (21) and *Myxococcus xanthus* (7) have been reported, showing 3 domains – a substrate binding, an FAD cofactor binding and a membrane binding domain.

Previous studies using subcellular fractionation of PPOX demonstrated that yeast and mammalian PPOX activities were associated with mitochondrial membrane and require detergents for solubilisation (29, 30) and that PPOX is localised to the cytosolic side of the inner mitochondrial membrane surface (16, 17). Recent crystal structure studies indicate three lobes, one of which is designated as a membrane binding domain (7, 21). It appears that human

PPOX targets the mitochondrion via a combination of N-terminal and internal targeting signals (9, 13, 27, 34).

Little is known of the tissue distribution (both intra- and inter-organ) of PPOX in humans. PPOX activity has been described in human liver (4, 8, 22), placenta (8), skin fibroblasts (1), lymphocytes (12) and Epstein-Barr virus transformed lymphoblasts (25). While all tissues are potentially able to synthesise haem, it is presumed that those primarily involved in haem production (liver and bone marrow) would demonstrate strong expression of the various haem synthetic enzymes, including PPOX. In this study we have undertaken immunohistological localisation studies of human PPOX in a variety of organs in an attempt to shed light in this regard.

MATERIALS AND METHODS

Antibody Production

Human PPOX was purified as previously described (23).The PPOX was blotted onto nitrocellulose paper (0.45 µM) (Schleicher and Schuell, Dassel West Germany), dissolved in DMSO and used for the innoculation of New Zealand white rabbits (20). 100µg of PPOX was used per innoculation. The strength and the specificity of the antibody were assessed by Western blotting.

Tissues

Surgically removed material was examined from liver, kidney, lung, testis, ovary, stomach, small and large intestine and placenta. Duodenum and colon specimens were used as representative tissue of small and large intestine, respectively. Only morphologically normal tissue was used. All specimens were fixed in buffered formal-saline, embedded in paraffin wax and sections cut at 5 microns. Five examples of each tissue were examined.

Immunohistochemistry

The immunoperoxidase technique was performed essentially as previously described (2) except that the swine anti-rabbit immunoglobulins utilised were biotinylated and streptavidin conjugated to the horseradish peroxidase. Briefly, the tissue sections were dewaxed and incubated in 0.3% H_2O_2 in methanol to block endogenous peroxidase. The sections were exposed to normal swine serum (1/20) in PBS for 15 min followed by incubation with a 1/100 dilution of rabbit PPOX antiserum for 60 min. The sections were exposed to biotinylated swine anti rabbit immunoglobulins (1/250) for 30 min. After exposure to streptavidin conjugated to horseradish peroxidase(1/500) the specifically bound antibody was visualised using the substrates 3-3,diaminobenzidine (0.5mg / ml of PBS) and 0.01% H_20_2. Counterstaining was with Mayers haematoxylin. The entire procedure was performed at room temperature.

Non-immune rabbit serum at the same dilution as the primary antibody was used as a negative control for each and every specimen studied. Pi glutathione S-tranferase specifically stains bile ducts in human liver (3) and a liver section thus stained was included in every run as a positive control.

The immunostaining was evaluated microscopically, using a semiquantitative scale as being strong, moderate, weak or negative.

RESULTS

All livers showed strong staining of the cytoplasm of the hepatocytes, but not the nuclei (Fig. 1A). Kupffer cells were negative and weak staining of the bile ducts was noted. No PPOX staining was apparent in the non-immune controls.

All kidneys exhibited strong staining of the cytoplasm in the proximal convoluted tubules (Fig. 1B). There was moderate to strong staining in the collecting tubules. No staining for PPOX was observed in the distal convoluted tubules or the glomeruli. All non-immune controls were negative.

The bronchial epithelium and the alveolar macrophages showed moderate staining for PPOX. In one case, peribronchial glands were present and showed negative staining in the mucous cells but strong positive staining in the serous cells (Fig. 1C). All non-immune controls were negative. All the lung specimens contained carbon pigment.

In the stomachs there was strong cytoplasmic staining of parietal cells (Fig. 1D). No positive staining was noted in any of the non-immune controls.

The staining in the intestine varied. PPOX staining in all cases was strongest in the proximal small intestine and involved cells at the tips of the villi, but not cells at the base of the crypts which were unstained. Mucosal epithelial staining became progressively weaker as more distal portions of the bowel were examined. Indeed, epithelial cell staining in the colon was weak to negative. However, mucosal macrophages also showed moderate cytoplasmic staining for PPOX. No staining of plasma cells anywhere in the gut was noted.

In the testes, cells within the seminiferous tubules stained moderately. The Leydig cells in the interstitial tissue, stained strongly. In two sections the epithelium of the epididymus was present and showed moderate staining. No staining was seen in the non-immune controls. Staining of spermatozoa was impossible to assess at our available magnification.

Of the five ovarian sections examined, primitive follicles were observed in three, and no staining for PPOX were observed in these cells.

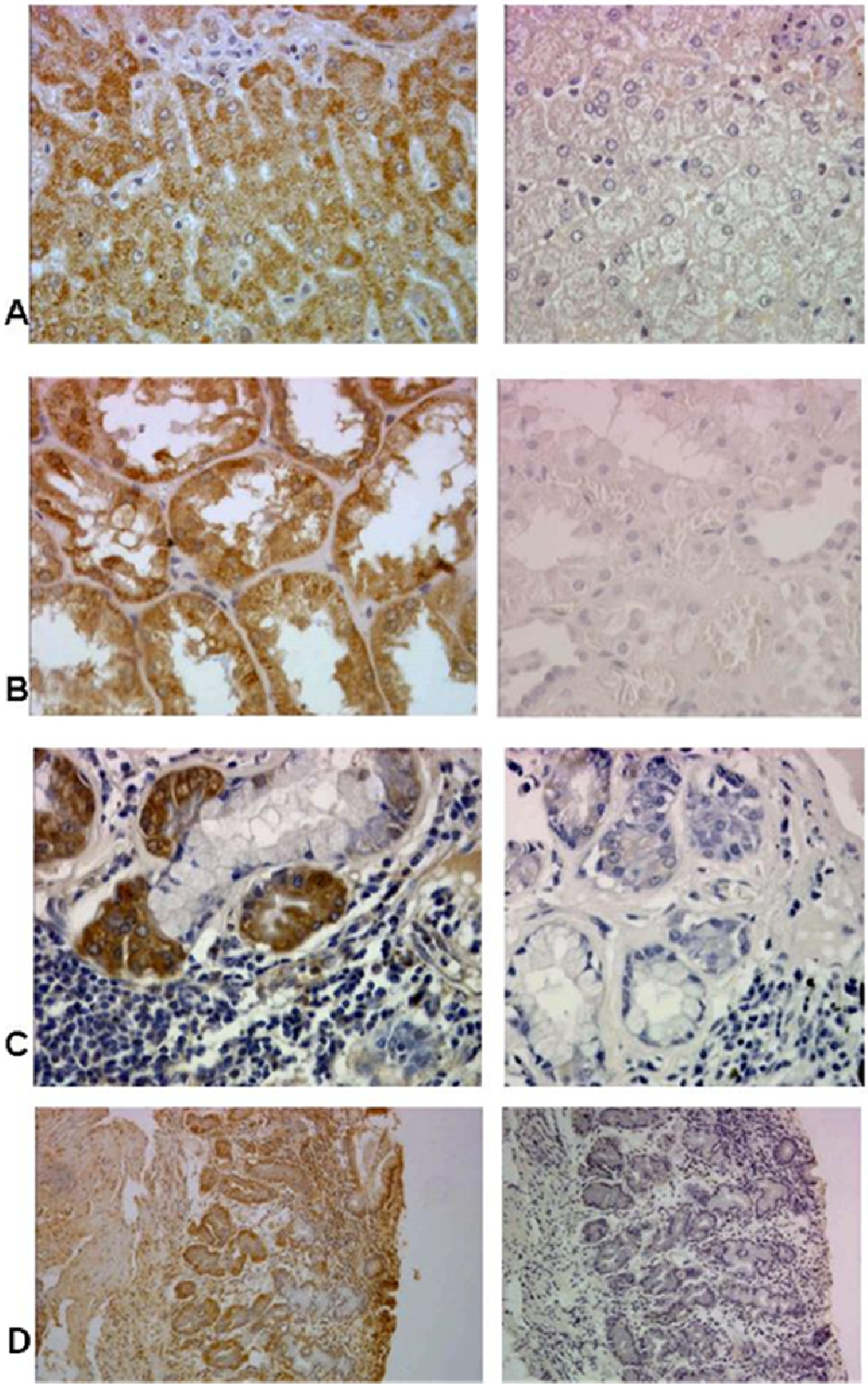

Figure 1. Examples of immunohistological localisation of PPOX in human tissues. PPOX staining shown in the left panel and corresponding non-immune control, on right. Positive PPOX staining is seen as brown colour.
A. Liver, showing strong staining of hepatocytes (final magnification X400); B. Kidney, showing strong staining of the proximal convoluted tubules (final magnification X400); C. Lung, showing strong staining of the serous cells of the peribronchial glands (final magnification X400); D. Stomach, showing strong staining of the parietal cells (final magnification X100).

In four of five sections, the mature follicular cells showed negative staining. However, in one case the epithelium of one mature follicle showed strong staining. All non-immune controls were negative.

In all placental tissue sections examined, the syncitiotrophoblasts showed a moderate staining of the cytoplasm for PPOX. All non-immune controls were negative.

Table 1. Summary of PPOX inter- and intra-tissue staining

TISSUE	PPOX STAINING
LIVER	
Hepatocytes	Strong
Bile ducts	Weak
Kupffer cells	Nil
LUNG	
Bronchial epithelium	Moderate
Alveolar macrophages	Moderate
Peribronchial glands	
- Mucous cells	Nil
- Serous cells	Strong
KIDNEY	
Proximal convoluted tubules	Strong
Collecting tubules	Moderate
Distal convoluted tubules	Nil
Glomerulis	Nil
STOMACH	
Parietal cells	Strong
SMALL INTESTINE	
Mucosal epithelium	Moderate
Tips of villi	Strong
Base of crypts	Nil
COLON	
Mucosal epithelium	Weak
Mucosal macrophages	Moderate
OVARY	
Primitive follicles	Nil
One mature follicle	Strong
PLACENTA	
Cytotrophoblasts	Moderate
TESTES	
Seminiferous cells	Moderate
Interstitial cells	Strong

DISCUSSION

In this study antibody to human PPOX was successfully employed to localise PPOX at the light microscopic level using the indirect peroxidase anti-peroxidase staining technique. The presence of PPOX in a variety of tissues was demonstrated.

The pattern of staining generally represents the presumed haem requirements of the particular tissues examined. Haem containing proteins include many of the important electron carriers in the respiratory chain in mitochondria (e.g. NADH reductase, cytochrome reductase and cytochrome oxidase), and many ubiquitous biological oxidative and peroxidative reactions are catalysed by haem containing enzymes (e.g. cytochrome P450s, peroxidases and catalases). Thus, in tissues with large numbers of mitochondria, with high metabolic requirement for ATP, and/or where there is a high redox demand, one would expect a higher degree of staining for PPOX than in metabolically or oxidatively inactive tissue.

The liver is a multifunctional organ involved in several metabolic activities. Hepatocytes are metabolically highly active cells, with abundant mitochondria, and given their multiple oxidative reactions and peroxidations would create a high haem demand. The strong PPOX staining in hepatocytes is in keeping with this. In contrast, the less metabolically active bile duct epithelium showed only weak staining. The bile ducts transport bile to the intestine – drainage by the bile ducts should not require huge amounts of energy and this result is therefore not unexpected. Similarly, the lack of staining in the Kupffer cells probably correlates with their lesser energy demand compared to hepatocytes under basal conditions. Interestingly, a previous study in a single rat liver (19) showed strong expression of porphobilinogen deaminase (an earlier, non-mitochondrial, haem synthetic enzyme) in the Kuppfer cells. This may be a similar situation to mature eythrocytes, which have no mitochondria, and therefore cannot synthesise haem. Yet porphobilinogen deaminase is present in their cytosol, representing a 'leftover' from their immature 'blast' stages, when haem synthesis is highly active. Alternatively, as the rat liver Kupffer cells in that study were present in the sinusoids (19) they may represent activated cells, in response to some form of biochemical/physiological stress.

In the kidney, the proximal convoluted tubular cells are metabolically active cells, with abundant mitochondria and highly specialized apical and basolateral surfaces. They are involved in the reabsorption of approximately 75% of ions and water from the renal tubules into blood capillaries, via an active sodium-potassium ATPase pump. PPOX was clearly present. The distal convoluted tubules are relatively less active and possess fewer mitochondria. Hence the lack of staining for PPOX. Similarly the glomeruli are less active, involved in the filtration of water and dissolved substances down a pressure gradient, and this process requires less energy production by the cells. It follows therefore that these cells will stain less, if at all, for PPOX. Interestingly, the collecting tubules did stain for PPOX. Jorgensen et al (19) reported strong staining for porphobilinogen deaminase in both the distal and collecting tubules in their rat study. The presence of porphobilinogen deaminase in the distal tubules in the rat may reflect its cytosolic intracellular location as compared to the mitochondrial location of PPOX.

In the lung the bronchial lining epithelium is composed predominantly of ciliated columnar cells involved in the co-ordinated movement of mucus along the respiratory tract with scattered, intermingled mucus-producing goblet cells. The function of the former require energy. Positive staining for PPOX in the ciliated bronchial epithelium is consistent with such energy-requiring processes. Similarly, the serous cells in bronchial glands which contain secretory granules, for the secretion of peptide compounds, stain strongly. In contrast, the mucus cells which produce and store mucus contain fewer mitochondria hence, lacked staining for PPOX.
The parietal cells of the stomach have large numbers of mitochondria, with a high oxidative capacity, which produce the ATP needed for the hydrogen/potassium ATPase needed for the production of HCl and other factors. This functionality is reflected by strong staining for PPOX.

Similarly, in the small intestine, the cells at the tips of the villi, which are involved in the absorption of nutrients and the synthesis of digestive enzymes, stained strongly. These cells contain numerous mitochondria to provide the energy requirement for these activities. On the other hand, the cells at the base of the crypts of Lieberkühn, did not stain for PPOX, consistent with their lower metabolic requirement.

In the testes, the stratified columnar cells lining the seminiferous tubules are a proliferating population involved in spermatogenesis. This cellular activity creates an energy demand which was reflected by moderate PPOX staining. Between these cells are the secretory Leydig cells which produce and secrete the male hormone, testosterone. These are very active populations of cells, hence the strong staining for PPOX.

The primitive follicles in the ovary contain a population of dividing cells which are relatively inactive, hence they stain negatively for PPOX. In mature follicles most of the cells are still dividing, but at a certain stage of development some of the cells will start producing and secreting female sex hormones, oestrogen and progesterone. These reactions involve haem-containing cytochrome P450 enzymes as well as the haem-containing microsomal epoxide hydrolase and are thus likely to create a demand for haem. There is thus a variable energy requirement (and hence number of mitochondria) in these cells depending on their stage of development. Consequently, these follicles may stain positively for PPOX and may explain the positive staining in one of the five sections. Similarly, the cytotrophoblasts in the placenta, are active cells, producing oestrogen and progesterone, human chorionic gonadotrophin, somatomammotrophin and endothelial growth factor. As all placentas examined were full-term, no cytotrophoblasts were observed but the trophoblast consisted of a layer of syncitiotrophoblasts, which stained strongly, in keeping with their metabolic activity.

The presence of PPOX was demonstrated in a wide variety of organs and generally the presence/absence in a particular tissue appears to represent it's haem requirement. PPOX, as one of the haem synthetic enzymes, appears to be a good marker for this. Yet, a previous study investigating the localisation of porphobilinogen deaminase in rats, demonstrated certain differences in distribution (19). This may simply reflect the cytosolic-mitochondrial location differences between the two enzymes. Nevertheless, we suggest that caution must be exercised in extrapolating the level of expression of one haem synthesising enzyme to another, or possibly to haem demand. Furthermore, there may well be variables in immunoaccessibility of one antibody, compared to another.

However, our findings, using a polyclonal PPOX antibody raised in rabbit, support a correlation between PPOX expression and

presumed haem demand, suggesting that it may be useful as an indicator of haem production at a light microscopic level as, to our knowledge, there is no direct stain for haem.

Finally, our results, and those of Jorgensen et al (19), imply that there are some significant sites of haem synthesis other than the liver and bone marrow, which should not be ignored in studies concerned with haem or porphyrin dynamics and flux in the human body. Although immunohistological localisation is non-quantitative, based on our observations, it is quite conceivable that organs such as the kidney, may contribute a significant portion to the porphyrin excretory pool. Our findings therefore lend weight to earlier studies which strongly suggest that the kidneys play an important, even predominant role in the etiology of excess urinary porphyrins, particularly in individuals with renal impairmant and/or disordered porphyrin metabolism (14, 15). Indeed, it would be interesting to perform similar localisation studies in tissue obtained from variegate porphyric individuals to ascertain differences and/or induction of PPOX under porphyric conditions, should such tissue become available for study. Further studies, including analysis of skin tissue may provide insight into the potential contribution of locally synthesised, as opposed to, transported-deposited porphyrins leading to the skin disease associated with variegate porphyria.

ACKNOWLEDGEMENTS

We wish to thank Dr Henry Hairwadzi, the late Prof Pauline Hall, Prof Dhiren Govender, Dr Ahmed Motala, Dr Richard Kirsch and Dr Peter Berman for helpful discussion. This work was funded in part by the Medical Research Council of South Africa, and the University of Cape Town Research Committee.

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