Gene Expression to Genetical Genomics

ORIGINAL RESEARCH

Correlation Between Peroxisome Proliferation and Up-Regulation of Cytochrome P450 CYP4A and Peroxisomal Beta-Oxidation Fatty Acyl CoA Oxidases (AOX) in the Koala (*Phascolarctos Cinereus*)

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Abstract: Peroxisomes are membrane bound cytoplasmic organelles that are involved in lipid metabolism and other biological functions. In rat and mouse, profound xenobiotic-induced peroxisome proliferation has been reported, with a marked increase in number and size of peroxisomes in liver parenchymal cells and induction of lipid metabolising enzymes, in particular cytochrome P450 CYP4A and peroxisomal β-oxidation palmitoyl CoA oxidases (AOX1). The present study investigates whether the previously observed higher hepatic CYP4A and AOX1 expression in the koala (*Phascolarctos cinereus*), a unique Australian marsupial, compared with rat and human is associated with peroxisome proliferation. Visualisation and quantification of peroxisomes were performed on liver samples from three koalas utilising transmission electron microscopy, with rat and bandicoot livers being used for comparative purposes. Numerous catalase positive peroxisomes, which clearly stand out by their black single membrane globular structures, were detected in all test ultra-thin sections from koala livers. A higher average number of peroxisomes per hepatocyte was observed for the koala, an obligate eucalyptus feeder, compared with non-eucalyptus feeders rat and bandicoot. No species differences in the average size of peroxisomes were detected. This is the first morphological study examining hepatic peroxisomes in an Australian marsupial. The results suggested that dietary eucalyptus constituents might possess peroxisome proliferating activities.

Keywords: marsupials, eucalyptus terpenes, cytochrome P450 CYP4A, peroxisomal beta-oxidation, fatty palmitoyl CoA oxidases AOX1

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Introduction

Peroxisomes are membrane bound cytoplasmic organelles that are involved in lipid metabolism and many other biological functions including respiration, gluconeogenesis, thermogenesis, and purine catabolism. In most animal cells, peroxisomes are widely distributed and their number, volume, and density remain relatively constant under various physiological conditions.[1,2] Peroxisomes were first identified in 1954 as single-membrane limited cytoplasmic organelle that contains a finely granular matrix and were named "microbody".[3] In 1956, microbodies were found in the parenchymal cells of rat liver.[4] Subsequent studies in the 1960s revealed the presence of microbodies with some variation in size and sharp in various vertebrate liver and kidney cells. These microbodies were also found to have different kinds of nucleoid or crystalline core.[5–7] The enzymes urate oxidase, D-amino acid oxidase, and catalase were then characterised in rat liver microbodies, with the term "peroxisomes" being introduced in 1965 to emphasise the biochemical property of the microbody enzymes to generate hydrogen peroxide.[2,8,9] In 1968, a histological assay was developed to identify catalase, the marker enzyme of peroxisomes, and initiated the identification of certain peroxisomes in various cell types.[10] Important enzymes found in peroxisomes include enzymes of the fatty acid β-oxidation system, acyl transferase, catalase, urate oxidase, and D-amino acid oxidase.[2,8–10]

In susceptible species such as rat and mouse, xenobiotic-induced peroxisome proliferation has been reported, which is characterised by a profound increase in number and size of peroxisomes in liver parenchymal cells and an increase in liver weight.[11] Xenobiotic-induced peroxisome proliferation has also been found to be related to fatty acid metabolism due to the presence of fatty acid β-oxidation enzymes in peroxisomes. A class of structurally diverse compounds, which are collectively classified as peroxisome proliferators (PPs), have been identified to cause peroxisome proliferation. This occurs via activation of peroxisome proliferator activated receptor-alpha (PPAR-α).[12,13] Exposure to PPs has been found to induce expression of genes encoding peroxisomal fatty acid β-oxidation enzymes such as palmitoyl-CoA oxidase (AOX1) and cytochrome P450 CYP4As.[12–14] Rat liver peroxisomes contain three acyl-CoA oxidases, namely palmitoyl-CoA oxidase, pristanoyl-CoA oxidase, and trihydroxycoprostanoyl-CoA oxidase. Among the three, only palmitoyl-CoA oxidase (AOX1) is inducible by peroxisome proliferators.[15] While rodents are highly responsive to PPs, human and marmosets are weakly or non-responsive. This might be due to having less abundance or lack of hepatic PPAR-α. PPAR-α was first isolated from mouse liver,[16] subsequently cloned from rat,[17] guinea-pig,[14] human,[18,19] also Amphibia,[20,21] Reptilian,[22] different teleostei species,[23–29] and S. purruatus.[30] *Xenopus* PPAR-α has been suggested to be activated by naturally occurring fatty acids.[20,21] In fish, the transcription of PPAR-α is also activated in response to a wide spectrum of mammalian PPAR ligands.[31]

Unlike most other species, the koala (*Phascolarctos cinereus*) diet is restricted to eucalyptus leaves, which contain a high proportion of monoterpenes. Previous studies conducted in our laboratories have suggested a possible dietary induction of hepatic CYP4A enzymes in the koala due to eucalyptus terpenes which may act as potent activators of the nuclear receptor PPAR-α.[32–34] Our subsequent work identified the presence of functional koala liver *AOX1* and *AOX2* isoforms and suggested higher expression of AOX mRNA in the koala liver as compared to rat and human.[34,35] In humans, rat and other species studied, both *CYP4A* and *AOX* are PPAR-α target genes.[36–38] In a more current study, PPAR-α has been isolated from the koala liver and its expression was characterised across species.[39] The aim of the present study was to examine whether our previously observed higher hepatic CYP4A and AOX expression in the koala, a unique specialist eucalyptus feeder, compared with rat and human, non-eucalyptus feeders, is associated with peroxisome proliferation. This is the first morphological study examining hepatic peroxisomes in an Australian marsupial.

Materials and Methods

Materials

Glutaraldehyde, hydrogen peroxide, and sodium cacodylate trihydrate were obtained from Sigma (St. Louis, MO, USA). Durcupan ACM mixtures 1 and 2 were purchased from Fluka AG, Chemische Fabrik (Bucks, England, UK). DAB (3',3'-diaminobenzidine) was obtained from Biorad (Sydney, NSW, Australia). All solvents and other chemicals were of analytical grade.

Hepatic tissues

Livers were scavenged from road injured male koalas and a bandicoot, ultimately euthanised using phenobarbital. Male Hooded Wistar rats (*Rattus norvegicus*) were used as the source of rat livers. All animals were sexually mature and the Ethic Committees of the appropriate institutions have approved the use of these tissues.

Methods

Visualisation of kola peroxisomes was performed on liver specimens from three koalas. Two rats and a bandicoot were used for comparative purposes. For each animal tissue, several tissue blocks were dissected, randomly from different areas of the liver, and subjected to the EM procedure as described shortly. Blocks without staining with DAB from each animal were used as negative controls.

Specimen preparation

Morphological examination of liver peroxisomes by electron microscopy (EM) in the koala, a eucalyptus feeder, was accomplished using livers of rat and bandicoot, non-eucalyptus feeders, as control samples. All livers sections for light and electron microscopy were dissected within five minutes after sacrifice of the animals, and then immersed immediately in glutaraldehyde solution.

Fixation and DAB staining

Pre-fixation: Fresh tissues were cut out into small blocks with a razor blade and fixed by immersion in at least 10 × volumes of cold 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 hours at 4 °C. The fixed tissues were rinsed twice in cacodylate buffer, each of 10 minutes, with constant rotation, and then stored in the same buffer overnight until required at 4 °C.

DAB staining and post-fixation: The glutaraldehyde pre-fixed blocks were stained for catalase by incubating in at least 10 × volumes of a freshly prepared solution, which contains 0.05% (w/v) DAB in 0.1 M Tris-HCl buffer (pH 8.5) and 0.02% (v/v) hydrogen peroxide, for 60 minutes at 37 °C with gentle agitation. Blocks were then rinsed in cacodylate buffer several times, each of 10 minutes, with constant rotation. DAB-stained blocks were post-fixed for 1 hour at room temperature in 1% (w/v) osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.4).

Dehydration

Prior to dehydration, osmium-fixed blocks were rinsed twice in cacodylate buffer, each of 10 minutes, and then washed several times with distilled water, each of 10 minutes. Dehydration of the sections were performed in a graded series of ethanol concentrations (v/v) in distilled water on a rotor as follows: 50% ethanol for 10 minutes, 70% ethanol for 10 minutes then stored overnight, two changes of 70% ethanol each of 10 minutes, two changes of 90% ethanol each of 10 minutes, absolute ethanol for 10 minutes, and then absolute ethanol for 1 hour.

Infiltration and embedding using epoxy resin Durcupan ACM mixtures

Durcupan mixtures number 1 and 2 were prepared as described by the manufacturer (Fluka AG). Infiltration was accomplished on a rotor by gradual and continuous replacement of the dehydrating agent with an embedding medium as follows: 50% (v/v) Durcupan mixture 1 in absolute ethanol for 1 hour at room temperature, 75% (v/v) Durcupan mixture 1 in absolute ethanol for 1 hour at room temperature, 100% (v/v) Durcupan mixture 1 at 50 °C for 1 hour overnight, and then 50% (v/v) Durcupan mixture 2 in Durcupan mixture 1 at 50 °C for 1 hour. Tissue blocks were then placed in small embedding capsules in Durcupan mixture 2 and polymerised at 60 °C for 48 hours, allowed to cool for 4 hours, and the plastic capsule was then removed.

Sectioning and visualisation

Prior to sectioning, semi-thin sections of 0.5 to 1 μm were cut, stained with 10% (w/v) toluidine blue in 5% (w/v) aqueous borax solution, and examined by light microscopy. Morphology studies were then performed using a transmission electron microscope. Ultra-thin sections of 70 to 100 nm, with pale gold interference colour, were obtained to enable adequate penetration of the electron beam for reasonable resolution and contrast.

Quantification and determination of size

Many ultra thin sections were cut from several tissue blocks for quantification and measurement of peroxisomes. Tissue blocks were chosen randomly from different areas of the liver and the dissecting was repeated using the same procedure for each liver sample, to ensure consistency in counting and size measurement. For each individual animal, counting was performed on at least 50 hepatocytes.

The measurement of the size of peroxisomes was accomplished on at least 40 peroxisomes from many different hepatocytes. Due to the small number of animals in the control group, it was not feasible to perform statistical analysis.

Results and Discussion

Visualisation of koala peroxisomes by EM

This is the first EM morphological study examining hepatic peroxisomes in an Australian marsupial. Representative electron micrographs of koala peroxisomes are shown in Figure 1 and 2A. Representative electron micrograph of rat peroxisomes is shown in Figure 2B.

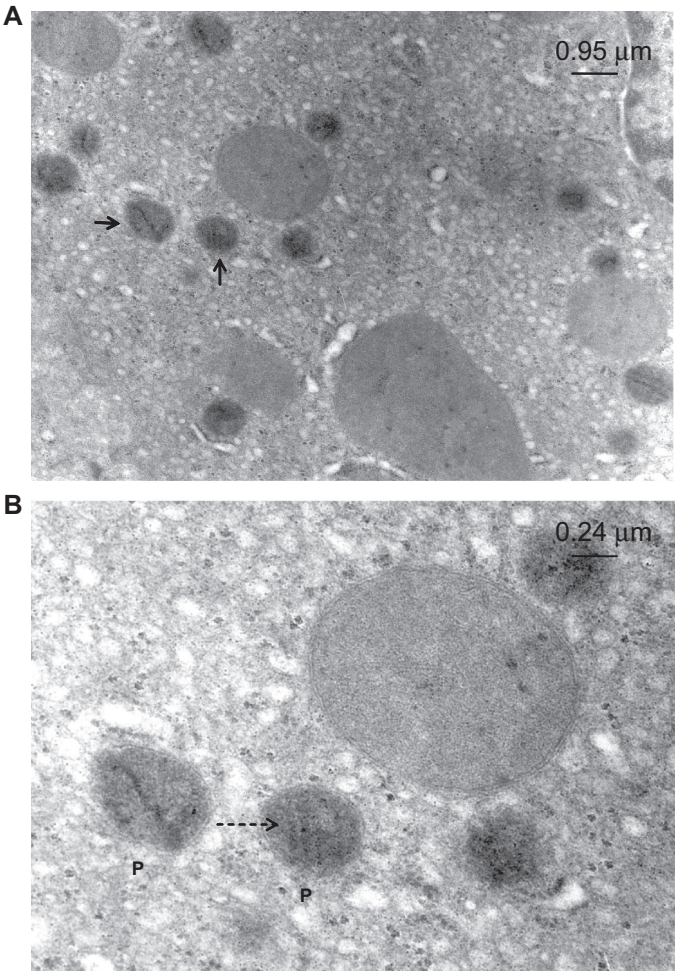

Figure 1. Upper panel (**A**) DAB-cytochemical visualisation of peroxisomes in koala liver by electron microscopy (original magnification × 10500). Test ultra-thin section of koala liver stained with diaminobenzidine (DAB) showing peroxisomes, indicated by an arrow, that clearly stand out by their single-membrane bound globular structures and a regular striation within peroxisomes. Lower panel (**B**) DAB-cytochemical visualisation of peroxisomes in koala liver by electron microscopy (original magnification × 4 × 10500). Test ultra-thin section of koala liver stained with diaminobenzidine (DAB) that shows peroxisomes, indicated by a symbol P, in Figure 1A being enlarged 4 folds featuring striation, indicated by an arrow, within peroxisomes.

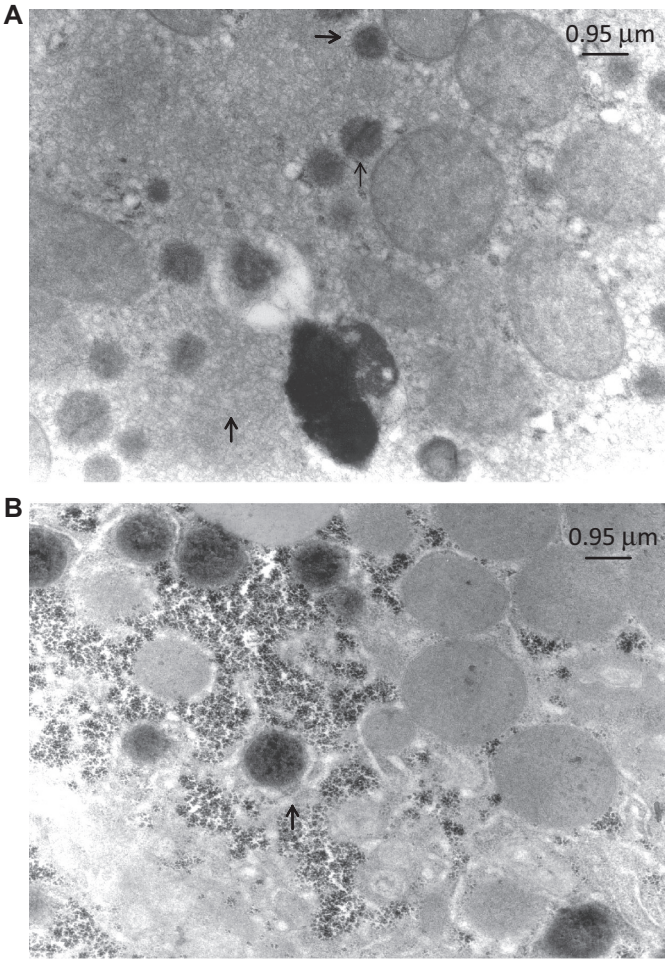

Figure 2. DAB-cytochemical visualisation of peroxisomes in koala and rat livers by electron microscopy (original magnification × 10500). Test ultra-thin section of liver stained with diaminobenzidine (DAB) showing peroxisomes, indicated by an arrow, featured by their single-membrane bound globular structures and regular dark striations within peroxisomes. Upper panel (**A**) Koala liver. Lower panel (**B**) Rat liver, being used as control animal.

Numerous catalase positive peroxisomes were detected in all test ultra-thin sections from koala livers. As shown in Figure 1, the koala peroxisomes were observed to clearly stand out as dark globular structures that are surrounded by a single membrane and characterised by a very regular striation within peroxisomes. This was consistent with that described in other species.[6,7] The koala peroxisomes were distinguished from stained lipid droplets and other globular structures of similar sizes by their single-membrane bound structures and their core-striation characteristics. This dense core or nucleoid has been described as crystalline, semi-crystalline, crystalloid, or multilamellated and is the most striking structural component of liver peroxisomes.[2] A representative image of such structures is shown in Figure 1B, where four

peroxisomes in Figure 1A have been enlarged 4 folds, with the striation being clearly visible in the peroxisome located in the lower centre, the middle one in the group of three peroxisomes below the mitochondrion.

It was observed that peroxisomes did not stand out in the EM micrographs of negative ultra-thin sections that were not incubated with DAB. This is because there was formation of precipitated DAB polymer, catalysed by peroxisome marker enzyme catalase, resulting in increased electron density and improved visibility of peroxisomes in ultra-thin sections stained with DAB.[40]

Quantification and measurement of peroxisomes

The average number of peroxisomes per hepatocyte and the average size of peroxisome from each species are shown in Table 1.

As shown in Table 1, a higher average number of peroxisomes per hepatocyte was observed for the specialist eucalyptus feeder koala compared to non-eucalyptus feeders rat and bandicoot. Although the density of peroxisome often varies with the zone distribution of the hepatocytes, it is unlikely that this zone variation has a significant implication in the interpretation of the results of this study. This was because several tissue blocks from each species were cut randomly from different parts of the liver and many ultra-thin sections from each block were selected. The observed higher number of peroxisomes per hepatocyte in the koala might be one of the indications of peroxisome proliferation, suggesting that dietary eucalyptus terpenes may act as peroxisome proliferators. In rat livers, eucalyptus terpenes such as citral have been found to induce CYP4A and peroxisomal β-oxidation enzymes and caused a marked increase in liver weight.[41] No species differences in the average size of peroxisomes were detected. The average size values of peroxisomes for koala, rat, and bandicoot livers are within the range of values reported in most species studies (0.3 to 1 μm).[42]

In summary, the current study described the first morphological study that examines hepatic peroxisomes in an Australian marsupial. The results shown the presence of more peroxisomes in hepatocytes of koala, a specialist eucalyptus feeder, compared to those of rat and bandicoot, non-eucalyptus feeding species, suggesting that dietary eucalyptus terpenes may possess peroxisome proliferating activities. While rat and human have been reported to be highly responsive and non-responsive to peroxisome proliferators, respectively, this study suggested that the marsupial koala may be a responsive species, given that the numbers of peroxisomes are the sole important criterion.

Table 1. Quantification and measurement of peroxisomes in koala, rat, and bandicoot livers.

Samples	Number of peroxisomes per hepatocyte	Size (μm)
Koala (n = 3)	20 ± 2	0.403 ± 0.032
Rat (n = 2)	11	0.504
Bandicoot (n = 1)	9	0.375

For koala, values were expressed as mean SD of three animals. Results for rat were expressed as a mean of two animals, and those for bandicoot were from a single animal.

Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors report no conflicts of interest.

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