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Heme Oxygenase Expression in Skin of Hairless Mouse Using Ultraviolet A (320-400 nm) Radiation as an Inducer

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This study describes RT-PCR and *in situ* hybridisation protocols, and the immunohistochemical detection method that we have developed to detect and localise cells that express HO-1 in the skin. We found that HO-1 mRNA was absent in normal mouse skin, but after UVA irradiation HO-1 mRNA was expressed in the dermal fibroblasts, and strongly in basal epidermal cells. HO-1 protein was also induced strongly in dermal fibroblasts, and also in epidermal cells. In addition, the HO substrate heme was reduced in skin microsome at 72 hrs post UVA (when HO activity is high). At the same time, the HO products bilirubin and iron levels were elevated in the cutaneous tissue. Thus in addition to a dermal response, there appears to be an epidermal HO response to UVA *in vivo* that may be relevant for immune modulation by UVA radiation.

key words: heme oxygenase, RT-PCR, *in situ* hybridisation, skin, UVA

INTRODUCTION

Heme oxygenase (HO) is a rate-limiting enzyme for degradation of heme into biliverdin, iron and carbon monoxide. Biliverdin is then reduced to its stable form bilirubin by biliverdin reductase. To date there are three isozymes of HO have been reported and named HO-1, HO-2, and recently discovered and still poorly understood HO-3. These three isozymes are the products of three separate genes. HO-1, unlike HO-2 and HO-3, is inducible both *in vitro* and *in vivo* by a variety of inducing factors such as heme, UV radiation, hydrogen peroxide, sodium arsenite, hyperthermia, endotoxin, stress, hypoxia, and nitric oxide, reviewed by Galbraith [1]. In mammals, HO-1 mRNA and/or HO-1 protein have been reported in a number of organs such as liver, brain, kidney, bone, lung, pancreas, spleen, testis, adipose tissue, eye, and including skin. However, to our knowledge the response of the HO isoforms in the skin to UVA radiation has not been characterised. Reeve *et al.* [2] have shown that UVA radiation increases the heme oxygenase activity in mouse skin and they speculated that HO-1 might be found in the dermal fibroblasts, while HO-2 (non-inducible) might be localised in the epidermal keratinocytes. In this study, we used RT-PCR, *in situ* hybridisation, and immunohistochemistry to detect and locate HO-1 mRNA and protein in the skin of the hairless mouse. In addition, we investigated the level of heme, bilirubin, and iron, which are the substrate and by-products, respectively, of HO *in vivo*. A number of studies have shown that induction of HO-1 can provide protection from oxidative stress and other damaging stimuli. The protective mechanisms due to HO are not well understood, however, HO by-products, such as carbon monoxide and bilirubin have been shown to be potential chemicals to provide protection. Therefore, HO-1 induction in skin may be important for immune modulation.

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MATERIALS AND METHODS

Mice

Male inbred albino Skh:HR-1 hairless mice, age 12-15 weeks old, were provided from the Veterinary Anatomy and Pathology breeding colony. They were maintained at 25°C under gold lighting that does not emit any UVB radiation, on a 12-h on/off cycle. They were fed stock laboratory mouse pellets and tap water *ad libitum*.

UV radiation

The UVA source has been described by Reeve *et al.* [2], consisting of a planar bank of seven 120-cm fluorescent UVA tubes (Hitachi 40W F40T10BL) held in a reflective batten at 19 cm above the radiation table, incorporating a selected sheet of 6-mm window glass as filter. This source emitted radiation >320 nm, providing 2.7×10^{-3} W/cm^2 UVA. Irradiance was measured with an International Light (Newburyport, MA) IL1500 radiometer. A group of mice were placed in a box containing food and water, and then exposed to UVA radiation for 4h (equal to 387 kJ/m^2). Temperature was controlled by electric fan.

Tissue sampling for RNA preparation and In situ hybridisation

Mice were killed at various time points (0, 1.5, 3, 6, 8, 12 and 24, h post UVA) by cervical dislocation. For RNA isolation, the dorsal skin was excised from mice and, was immediately frozen

in liquid nitrogen before storing it in -80°C freezer. For *in situ* hybridisation, the skin was embedded in O.C.T compound, quickly frozen and stored in -80°C freezer until used.

RNA extraction

Mouse skin was cut into small pieces using a cryostat (-20°C), collected on clean aluminium foil and then placed in a 1.5 mL tube. The total RNA was extracted by a commercial modification of the phenol-chloroform-isoamyl alcohol extraction kit (Promega), and on completion of RNA extraction the sample was treated with DNAse I as instructed by the manufacturer. The total RNA concentration was determined by spectrophotometric measurement of the absorbance at 260 nm and 280 nm.

Reverse transcription for cDNA synthesis

0.4 μg of total RNA was reverse transcribed to cDNA using a commercially available kit (Promega), in total volume of 20 μL of reaction using oligo(dT) as a primer. The reaction mixture is incubated at 42°C for 1 hour, and final extension of 5 minutes at 95°C to inactivate reverse transcription enzyme.

Primers selection

Primers were selected for murine cDNA of HO-1 and HO-2 as follows: Forward 5'-GGCCCTGGAAGAGGAGATAG-3' and reverse 5'-GCTGGATGTGCTTTTGGTG-3', provided 888 bp PCR products for HO-1. HO-2 is included in this study as house keeping gene, and the primers (forward 5'- GAAGGAAGGGAC-CAAGGAAG-3' and reverse 5'-GTTTTAGGCAGAGGTG-GAGATG-3') provided 767 bp PCR products. The primers were synthesised by Life Technologies (Melbourne, Australia).

First polymerase chain reaction (PCR)

A commercial PCR kit is available from Sigma (RedTaq). The first PCR was done in a total volume of 25 μL. Amplifications were performed using Hybaid OmniGene thermal cycler which has heated lid. PCR conditions for both HO-1 and HO-2 were as follows: Initial denaturation at 95°C for 3 minutes, then 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds. After 34 cycles the temperature was held at 72°C for 10 minutes to allow final elongation. The PCR products were resolved by 1.2% agarose gel (containing ethidium bromide) electrophoresis. The gels were run in tris-borate-EDTA buffer at 80 volts for 1 hour at room temperature. They were photographed under ultraviolet light using a Kodak (Kodak DC120 Digital) camera, and stored in a file before using "QUANTITY ONE" program to analyse the image. Semi-quantitative analysis was performed on the images for HO-1 at each time point and then normalised with HO-2 using HO-1: HO-2 ratio. The highest HO-1: HO-2 ratio of the time points was selected for the *in situ* hybridisation experiment.

Probe synthesis using PCR

Klein *et al.* [8] have shown that RT-PCR can be used to make probes labelled with digoxigenin (Dig). We used a commercially available PCR DIG Probe Synthesis Kit to label our probes using "Two-Steps" PCR. First PCR was a normal PCR, the second PCR was used to incorporate Dig, and only one primer was used to make sense and anti-sense probes. The PCR condition for Dig incorporation was the same as normal PCR.

In situ hybridisation of mouse skin

Frozen skin (3 h post UVA) sections (8 μm) were cut and air dried for 10-15 minutes at room temperature (RT). They were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 10 containing 0.1% active diethyl pyrocarbonate (DEPC) for 30 minutes at RT. The sections were washed twice in PBS with 0.1% active DEPC for 15 minutes at RT, then they were equilibrated with 5x SSC (saline sodium citrate, 0.75M NaCl and 0.075M Na-citrate DEPC treated) for 15 minutes at RT. Prehybridisation was performed in a humidified chamber at 58°C for 2 h in the prehybridisation buffer containing 50% formamide, 5x SCC and 40 μg/mL of irrelevant and unlabelled pig cDNA (blocking). Then they were hybridised overnight at 58°C in 100 μL prehybridisation buffer with 400 ng/mL of Dig-labelled probe (sense or antisense alone). The next day, the sections were soaked in PBS to remove cover slips, and were washed twice in 0.1% TritonX-100 in tris-saline (0.1M tris and 0.15M NaCl pH 7.6) at 65°C for 30 minutes each. The sections were blocked for 30 minutes at RT in blocking solution containing 10% heat inactivated foetal calf serum, 3% bovine albumin in 0.1% TritonX-100 tris-saline. They were incubated with alkaline phosphatase-conjugated anti-Dig antibodies at 1: 500 dilutions in blocking solution for 2 h at 37°C. After incubation, the sections were washed twice in alkaline phosphatase substrate buffer (APSB) (0.1M tris pH 9.5, 0.1M NaCl, 50 mM $MgCl_2$) for 30 minutes each at RT using a rotating flea. Finally the slides were stained with alkaline phosphatase chromogens containing 0.33 mg/mL nitroblue tetrazolium, 0.175 mg/mL bromochloro-indolyl phosphate and 3 mM levamisole in APSB. Colour development was performed at 37°C for 30 to 60 minutes, and stopped in distilled water, air-dried, and cover slipped in DPX.

Immunohistochemistry

Frozen skin (72 h post UVA and control) sections (8 μm) were cut and air-dried for 10-15 minutes at RT. They were fixed in 4% paraformaldehyde in PBS pH 10 for 10 minutes at RT. The sections were treated with methanol and hydrogen peroxide (ratio 141:9) for 5 minutes, then washed twice in PBS for 5 minutes at RT. They were incubated with rabbit anti-HO-1 polyclonal antibody (1/100, Stressgen) in 1% BSA and 10% sheep serum overnight at 4°C. The next day, the sections were washed twice in PBS for 5 minutes each, before incubating with goat anti-rabbit-HRP antibodies at 1:300 dilution in PBS for 2 h at 37°C. After incubation, the sections were washed twice in PBS for 5 minutes each and once with water. Finally the slides were stained with DAB, lightly counterstained with hematoxylin, dehydrated, and cover slipped in DPX.

Heme measurement

Heme determination of skin fractions was adapted from modified methods by Pandey *et al.* [4]. Microsomal and mitochondrial pellets were prepared according to Reeve *et al.* [2] and Vile *et al.* [5], and were assayed immediately for heme concentration. Twenty μL of mitochondrial or microsomal pellet suspension were placed into wells (in triplicate) of 96-well plate. In addition, 20 μL of heme standard (starting at 20 μg/mL) in 1: 2 dilutions were added to wells (in duplicate). Then 180 μL of 2.5% TritonX-100-methanol were added, and mixed for 10 seconds before measuring the absorbance at 405 nm using "Spectra Max 250" spectrophotometer. Mitochondrial and microsomal protein was measured by the method of Lowry *et al.* [6]. Heme concentration is expressed as μg/mL per mg protein.

Bilirubin measurement

Skin bilirubin determination was adapted from modified methods by Bessard *et al.* [3]. Five grams of skin was homogenised on ice in a tube containing 15 mL of 0.143M barbital-acetate (pH: 10) and 15 mL of methanol, using a Heidolph homogenizer (Kelheim, Germany) with speed of 9500 rpm for 2 minutes five times. Five mL of chloroform was added to the tube, followed by 90 minutes incubation at RT using a gentle rotating machine. Then 4 mL of 1N HCl was added to each tube and incubation was resumed for another 30 minutes, followed by centrifugation at 1000 rpm for 30 minutes at 21°C. The upper layer was removed, while orange coloured bottom layer was extracted using 5 mL syringe with a 23G needle. The extracted chloroform was place in glass tube, and centrifuged at 1000 rpm for 10 minutes before measuring the absorbance using a Perkin-Elmer spectrophotometer at 450 nm and 550 nm. Distilled chloroform was used as a blank, and bilirubin concentration is expressed in μmol per 100 g skin.

Iron measurement

Skin iron determination was adapted from modified method by Ghio *et al.* [7]. The dorsal skin was excised, weighed and then placed in a 10 mL centrifuge tube. The skin was hydrolysed in 3N HCl in 10% trichloroacetic acid in ratio of 0.2 g per 1 mL at 70°C oven for at least 16 hrs. 500 μL of clear extract was mixed with equal volume of 2% potassium ferrocyanide in 1.5 mL tube. Standards of ferric chloride starting at 20 μg/mL were serially diluted 1:2 fold in 3N HCl in 10% trichloroacetic acid, and mixed with equal volume of 2% potassium ferrocyanide in tubes. Each tube was incubated at RT for 15-20 minutes, followed by transferring 150 μL from each tube to well of 96-well plate. Each skin sample was assayed in triplicate, while standards in duplicate. The absorbance was measure at 694 nm, and iron expressed as μmol per g skin.

RESULTS

RT-PCR

HO-1 mRNA was expressed immediately and persisted 24 h post UVA, but not in control or UVB irradiated skin (pictures not shown). In contrast, HO-2 mRNA was always expressed at all time points using both UVA or UVB. Using "QUANTITY ONE" program to analyse the image, HO-1: HO-2 ratio peaked at 3 h post UVA (Table 1). This time point was selected for *in situ* hybridisation.

Table 1. Ratio HO-1/HO-2 in Units

Time (h) post UVA	0	1.5	3	6	8	12	24
Ratio HO-1/HO-2	0.49	0.45	0.73	0.29	0.52	0.46	0.17

Probe synthesis

Using one primer alone produced similar intensity as combining both forward and reverse primer indicative of equal efficiency during PCR amplification (data not shown). However, Dig incorporation increases the molecular weight of the probe, which migrated slower in the gel during electrophoresis compared with unlabelled (using dNTP) probe.

In situ hybridisation

HO-1 mRNA expression at 3 h post UVA showed a strong signal with the antisense probe in cells of both epidermis and dermis, but was absent with the negative control sense probe. In the epidermis HO-1 mRNA expression was seen in or near the basal region, and along the hair follicle lining. In the dermis, HO-1 mRNA expression was seen in fibroblast cells (identified by distribution and cell shape).

Immunohistochemistry

HO-1 protein expression at 72 h post UVA was seen strongly in the epidermis, including along the cells lining the hair follicle. In the dermis, HO-1 protein was seen in fibroblast cells (identified by cell size and shape). HO-1 protein expression was absent in the negative control. This result is consistent with the *in situ* hybridisation such that both epidermis and dermis can express HO-1.

Heme measurement

Heme is reduced at 72 h post UVA (compared to non-UVA) in skin microsomes, but not in mitochondria (act as controls) (Table 2)

Bilirubin measurement

Bilirubin is elevated at 72 h post UVA in skin, compared to non-UVA (Table 3).

Iron measurement

Iron is slightly elevated at 72 h post UVA in skin, compared

Table 2. Heme expressed as μg / mL per mg protein

Treatment	Microsomes	Mitochondria
Non-UVA	7.77 ± 0.81	7.30 ± 0.57
UVA	13.12 ± 0.57	7.34 ± 0.28

Table 3. Bilirubin is expressed as μmol per 100 g skin

Treatment	Skin Bilirubin
Non-UVA	0.08± 0.01
UVA	0.15± 0.02

Table 4. Iron is expressed as μmol per g skin

Treatment	Skin Iron
Non-UVA	1.30± 0.16
UVA	1.40± 0.14

to non-UVA.

DISCUSSION

RT-PCR is a very sensitive method to detect expression of mRNA in tissues or cells. Unlike *in situ* hybridisation, it can not be used to locate the cell that produces it. By combining RT-PCR and *in situ*, we were able to show that HO-1 mRNA is expressed both in epidermis and dermis after UVA irradiation but not in non-UVA skin. Strong signals of HO-1 mRNA were detected in cells in epidermis, including cells lining hair follicle, while in dermis HO-1 mRNA was expressed in other cells (large shaped cells) in addition to fibroblasts. The finding of HO-1 expression in the epidermis is consistent with the results shown by Hanselman *et al.* [9] and Clark *et al.* [10]. Both groups have found that keratinocytes (present in epidermis) can express HO-1 mRNA, contrasting with the evidence shown by Applegate *et al.* [11]. HO-1 can be expressed essentially in every tissue upon appropriate stimulation, and may be Applegate *et al.* did not use sufficient UVA stimulus resulting in no induction of HO-1 in the human cultured keratinocytes. Expression of HO-1 mRNA in epidermis is supported by our immunohistochemistry results which showed HO-1 protein is expressed in the epidermis, not just in dermis. Other supporting evidence includes reduction of heme levels in skin 72 h post UVA, while bilirubin and iron levels were elevated, confirming HO-1 induction must take place in skin, since constitutive form (HO-2) alone will not contribute to reduction and/or elevation of heme oxygenase substrate or by-products.

CONCLUSION

In conclusion, we have shown for the first time that UVA radiation induces HO-1 mRNA and protein *in vivo* in cells of both epidermis and dermis of the mouse. This extends the evidence for induction of HO-1 in fibroblasts and keratinocytes when these cells were stimulated *in vitro*.

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