The induction of heme oxygenase-1 (HO-1) by ultraviolet A (UVA) (320–400 nm) radiation provides a protective cellular defence against oxidative stress, and has been well demonstrated in cultured human skin fibroblasts, although keratinocytes were unreactive. The UVA responsiveness of HO-1 however, has not been confirmed in intact skin. Previously, we reported that UVA-inducible HO enzyme activity in mouse skin is protective against UVB-induced immunosuppression. This study identifies the induced HO isoform and its localization in mouse skin irradiated in vivo with such an immunoprotective UVA dose. We found that HO-1 mRNA was expressed in UVA-irradiated skin, but not in normal or UVB-irradiated skin, whereas constitutive HO-2 was always present. UVA-irradiated skin had increased HO enzyme activity and bilirubin content, and decreased heme content, consistent with HO-1 induction. In situ hybridization and immunohistochemical staining localized HO-1 mRNA and protein to both epidermis and dermis, with strongest expression in basal keratinocytes and weaker expression in dermal fibroblast-like and other cells, in contrast with UVA-induced HO-1 in cultured human skin fibroblasts. This suggests that cultured skin cells may not fully represent skin functions in vivo, or that there may be inherent differences between human and hairless mouse skin HO-1 responses.

Key words: heme oxygenase/immunohistochemistry/in situ hybridization/mouse skin/UVA radiation

Heme oxygenase (HO) is a ubiquitous redox-regulated enzyme that degrades heme into biliverdin (rapidly converted to bilirubin by biliverdin reductase), carbon monoxide, and iron. To date, three isoforms of HO have been described, the constitutive forms HO-2 and HO-3 (Maines et al., 1996; McCoubrey et al., 1997), and the inducible form HO-1. In mammals, HO-1 mRNA expression has been reported in various cells and tissues, including the skin (Hanselmann et al., 2001), and its expression is considered the most sensitive and reliable indicator of cellular oxidative stress (Kutty et al., 1995; Maines, 1997). In vitro, various oxidative stressors result in the activation of the HO-1 gene in cultured skin cells, in a manner apparently specific to the cell type. For example, it has been shown that ultraviolet A (UVA) (320–400 nm) radiation and other oxidants upregulated the expression of HO-1 mRNA in cultured human skin fibroblasts (Keyse and Tyrrell, 1989; Keyse et al., 1990), whereas human keratinocytes appeared to express higher constitutive levels of both mRNA and protein of the HO-2 isoform, but did not respond to UVA radiation or oxidant stress with the induction of the HO-1 isoform (Applegate et al., 1995). In contrast, other investigators have more recently described the expression of HO-1 mRNA induced by nitric oxide in cultured human keratinocytes (Clark et al., 1997).

The induction of cutaneous HO enzyme activity in mouse skin by UVA radiation has been demonstrated to have photoimmunological relevance, and has been associated with the immunoprotective effect of the UVA waveband against immunosuppression induced by ultraviolet B (UVB) (280–320 nm) radiation or by the putative epidermal photoimmunosuppressive mediator, cis-urolonic acid (Reeve and Tyrrell, 1999). Inhibition of HO enzyme activity in the mouse abrogated the UVA immunoprotective effect, but did not affect UVB-induced suppression of contact hypersensitivity in the absence of UVA irradiation, leading to the conclusion that only the UVA-inducible form of HO provided the photoimmunoprotection, and that constitutive HO enzyme did not play this role. The cutaneous expression of the HO isoforms or their responses to UVA or UVB radiation in vivo, however, has not been characterized, nor their histological localization.

In this study, therefore, we have observed alterations in the expression of HO-1 and HO-2 mRNA expression in the skin of the hairless mouse following irradiation with either UVA or UVB, using RT-PCR. Specific probes have been developed to identify the localization of HO-1 mRNA in frozen sections of mouse skin following UVA irradiation using the in situ hybridization (ISH) technique, and further-
more the immunohistochemical localization of HO-1 protein has been correlated with the upregulation of enzyme activity in the skin. The results are consistent with the HO-1 isoform providing the reported photoimmunoprotection in vivo, and indicate that cells of both the epidermis and the dermis are induced to express HO-1 by UVA radiation.

**Results**

**RT-PCR detection of HO-1 and HO-2 in UVA-irradiated mouse skin** RT-PCR amplification of skin cDNA with the HO-1 and HO-2 primer sets resulted in the production of a single DNA band in each reaction, which was of the predicted size on agarose gel electrophoresis (Fig 1). Immediately after UVA irradiation, HO-1 expression was clearly visible and persisted up to 24 h (Fig 1, Gel-3), but HO-1 mRNA was absent both in unirradiated control and UVB-irradiated skin (Fig 1, Gel-1 and Gel-2). In contrast, HO-2 mRNA was always expressed at all time points in Gel-1, Gel-2, and Gel-3.

Figure 1
RT-PCR detection of HO-1 and HO-2 mRNAs in the skin of hairless mouse at various time points. Transcripts encoding HO-1 and HO-2 were separated on 1.2% agarose gel. Gel-1, unirradiated control skin (mouse a and b); Gel-2, UVB-irradiated skin; Gel-3, UVA-irradiated skin. HO-1 and HO-2 amplifications as follows: right to left, HO-1 (0, 1.5, 3, 6, 8, 12, and 24 h post UV), DNA marker (GeneRuler 1kb DNA ladder Cat No. # SM0311, Fermentas Life Science, Brisbane, Australia), and HO-2 (0, 1.5, 3, 6, 8, 12, and 24 h post UV). HO-2 mRNA level was used as housekeeping gene. HO-1 mRNA was expressed immediately and persisted 24 h post-UVA (Gel-3), but not in control (Gel-1) or UVB-irradiated skin (Gel-2). In contrast, HO-2 mRNA was always expressed at all time points in Gel-1, Gel-2, and Gel-3.

**Synthesis of probes for ISH** The LPS stimulation of HO-1 mRNA expression in mouse liver is shown in Fig 3, right.
hand gel. Probe synthesis was followed by PCR with or without Dig labelling and resulted in bands of the expected molecular weight. Dig incorporation increases the molecular weight of the probes and they migrated more slowly in the gel during electrophoresis compared with unlabelled probe (Fig 3, left hand gel, line 4). Moreover, there was no difference in PCR amplification between sense (forward) and antisense (reverse) probes run either together or separately, consistent with the technique described by Klein et al (1995).

Localization of HO-1 mRNA and protein in UVA-irradiated mouse skin

ISH of HO-1 mRNA in frozen skin sections taken at 3 h post-UVA showed a strong signal with the antisense probe in cells of both the epidermis and dermis (Fig 4a), with staining absent with the negative control sense probe (Fig 4b). In the epidermis, HO-1 mRNA expression was seen in or near the basal region and along the epithelial lining of the hair follicle, and appeared to be intensely localized in intermittent basal keratinocytes. In the dermis, HO-1 mRNA expression was seen in fibroblast-like cells (identified by cell distribution, size and shape), and other larger cells. The localization of HO-1 mRNA was complemented by the subsequent immunohistochemical staining of HO-1 protein at 72 h post-UVA irradiation (Fig 4c), which indicated that HO-1 protein expression was also strongest in cells of the epidermis and the epithelial lining of the hair follicles, with some protein also expressed in dermal cells.

Markers of HO enzyme activity in UVA-irradiated mouse skin

In addition, at 72 h post-UVA irradiation, a marked increase in skin microsomal HO enzyme activity was detected (increased from 1.76 to 2.72 pmoles bilirubin per mg protein per min; Fig 5). The enzyme substrate, heme, was significantly reduced in the skin microsomes from 13.12 to 7.77 µg per mg protein, whereas heme concentration remained unaltered by the UVA exposure in mitochondria, organelles that are rich in heme metabolism but that do not contain the degradative HO enzyme (Fig 6). Therefore it is unlikely that photochemical destruction of heme contributed to the reduction of microsomal heme. At the same time, the enzyme product, bilirubin, was increased from 0.08 to 0.15 µmol per 100 g skin, consistent with the UVA-upregulated enzymic activity of HO in the skin (Fig 7).
Discussion

This study is the first report of the inducibility of HO-1 by UVA radiation in the intact skin. Although it is well established that UVA irradiation of cultured human skin fibroblasts results in HO-1 induction (Vile and Tyrrell, 1993; Applegate et al., 1995; Noel and Tyrrell, 1997), this response has not been observed in vivo. We report the response of HO to UVA irradiation of mouse skin in vivo, at environmentally relevant suberythemal exposures known to be immunologically modulating. The results indicate upregulation of expression of HO-1 mRNA for up to 24 h following UVA irradiation in the hairless mouse skin, whereas UVB radiation appeared to have no effect on the expression of HO in vivo. The expression of HO-1 mRNA was maximal around 3 h post-irradiation, a time point similar to that described in cultured human skin fibroblasts (4–8 h post-UVA; Applegate et al., 1995). We observed that HO-2 mRNA was constitutively expressed in the skin, but the expression of HO-2 mRNA was not altered by UV exposure.

Using the techniques of ISH and immunohistochemistry, the cellular localization of HO-1 mRNA and protein was examined. Both HO-1 mRNA and protein appeared to have a similar distribution in the skin. The strongest expression of HO-1 was in discrete cells in the basal layer of the epidermis and the hair follicle lining, but there were also positively staining fibroblast-like cells throughout the dermal layer. The epidermal location of HO-1 may be relevant for its immunoprotection, since the photoimmunosuppressive initiating molecule, cis-urocanic acid, is also produced in the epidermis and the stratum corneum. Nevertheless, the detection of strong epidermal staining was unexpected, since a comparative study of matched pairs of cultured human skin fibroblasts and keratinocytes, as examples of the major cell types in the dermis and epidermis, respectively, showed clearly that the keratinocytes, while expressing a low basal level of HO-1 mRNA, were inert to oxidative stress induced by either UVA irradiation or hydrogen peroxide in vitro; only the fibroblast cell lines responded with HO-1 mRNA upregulation (Applegate et al., 1995). The UVA dose of 250 kJ per m² was somewhat less than we have used in vivo (387 kJ per m²), but we speculate that the UVA radiation would have been attenuated significantly by the stratum corneum in the mouse, whereas irradiation in vitro would not encounter such a barrier, and therefore the dose received by the skin cells in either model would have been similar. On the other hand, in vivo experiments in wound healing in the skin of the mouse demonstrate mild to strong upregulation of HO-1 expression in the keratinocytes (Hanselmann et al., 2001; Kampfer et al., 2001) at the wound site, and furthermore, HO-1 mRNA overexpression was described in the keratinocytes of the hyperproliferative epidermis of the human psoriatic skin (Hanselmann et al., 2001). Thus in the in vivo situation, both murine and human keratinocytes appear to have activatable HO-1. The epithelium of other tissues such as the rat lung alveoli can also be induced to express HO-1 mRNA by chronic hypoxia (Carraway et al., 2000). Possibly the response of this gene is determined by the stressor, because cultured human SV40-transformed keratinocytes have been induced to express HO-1 mRNA by the NO donor, sodium nitroprusside (Clark et al., 1997). We speculate that in vivo, there is signalling between stress-activated cells of the different cutaneous strata, and that the primary UVA target might be the dermal fibroblast, which releases mediators to activate the epidermal keratinocytes. Such a mediator might be fibroblast-derived NO (Clark et al., 1997) or a cytokine like IFN-γ, which we have found to be necessary for HO enzyme activity upregulation by UVA radiation in the mouse (Reeve and Domanski, 2003), or a variety of other cytokines such as

Figure 5
Heme oxygenase activity in skin microsomes at 72 h post-UVA was significantly (p < 0.05) induced. Error bars = SEM.

Figure 6
Microsomal heme was significantly (p < 0.05) reduced at 72 h post-UVA compared with unirradiated microsomes, but not in the mitochondria. Error bars = SEM.

Figure 7
Skin bilirubin was significantly (p < 0.05) elevated at 72 h post-UVA in skin compared with unirradiated skin. Error bars = SEM.
interleukin (IL)-1, IL-6, tumor necrosis factor-\(\alpha\) reportedly involved in HO-1 induction by other stressors (Miltani et al., 1992; Rizzardini et al., 1998; Terry et al., 1999).

Although we have not determined the exact identity of the HO-1 expressing cells, their location and size in the epidermis indicate that these are basal keratinocytes. The intermittent pattern of positive cells aligned along the basement membrane suggests that the HO-1 response may be regulated by the proliferative status of the basal cell (Smith and Rees, 1994), and this is likely to be under much less complex control in cultured cells. In the dermis, the positive cells have the distribution and shape of fibroblasts, and the larger cells also expressing HO-1 may be inflammatory cells such as macrophages, which would be consistent with the healing skin wound, in which the cells expressing HO-1 at least as strongly as the keratinocytes were the infiltrating macrophages or other inflammatory cells (Hanselmann et al., 2001; Kampfer et al., 2001). On the other hand, the overt inflammatory reaction resulting from 3MED of UVB irradiation did not induce HO-1, suggesting that the two wavebands have different targets and cellular responses for inflammation, the induction of UVB inflammation being predominantly a non-oxidative process. Further studies should confirm the identity of these critical cell populations.

In summary, this study has demonstrated the UVA induction of HO-1 mRNA, protein, and enzyme activity in vivo in the skin of the hairless mouse. In contrast to the in vitro data available, HO-1 appears to be upregulated strongly in the basal keratinocytes of the epidermis, and is also expressed in dermal fibroblast-like cells and other dermal cells. Irradiation of mouse skin with UVB did not induce HO-1, whereas the constitutive HO-2 was detectable in the skin and remained unaltered by UV exposure. This HO-1 response to a dose of UVA radiation known to protect mice from UVB-induced immunosuppression confirms our earlier suggestion (Reeve and Tyrrell, 1999) that the photomimicry protection associated with UVA-induced HO enzyme activity in the skin was attributable to inducible HO-1, and not the constitutive HO-2 isoform.

**Materials and Methods**

**Mice** Male inbred albino Skh:hr-1 hairless mice, aged 12–15 wk, were provided from the Veterinary Science breeding colony. They were housed in wire-topped plastic cages on compressed paper bedding (Fibrecycle Pty Ltd, Mudgeeraba, Australia) and tap water ad libitum. All procedures were approved by the University of Sydney Animal Ethics Committee and complied with the state Animal Research Act 1985.

**UV radiation** The UVA source has been previously described (Reeve and Domanski, 2002), consisting of a planar bank of seven 120-cm fluorescent UVA tubes (Hitachi 40W F40T10BL, Tokyo, Japan) held in a reflective batten at 19 cm above the irradiation table, and incorporating a selected sheet of 6 mm window glass as a filter. This source emitted radiation of wavelength above 320 nm, providing 2.7 \(\times\) \(10^{-2}\) W per cm\(^2\) UVA and an effectively negligible 2.3 \(\times\) \(10^{-8}\) W per cm\(^2\) UVB. The UVB source consisted of a single UVB tube (Phillips TL-40W/12 RS, Eindhoven, The Netherlands) emitting 2.5 \(\times\) \(10^{-4}\) W per cm\(^2\) UVA and 4.1 \(\times\) \(10^{-4}\) W per cm\(^2\) UVB. Irradiance was measured with an International Light (Newburyport, Massachusetts) IL1500 radiometer, with UVA and UVB detectors (SEE 015/UVA and SEE 240/UVB, Newburyport, MA) calibrated to the spectral irradiances of the sources. Groups of three mice or less were exposed unrestrained in their cages with the wire tops removed, to 387 kJ per m\(^2\) UVA radiation (approximately 4 h), which is a suberythemal dose equivalent to the UVA content of approximately 6 \(\times\) minimal erythematic dose (MED) of solar UV radiation (Reeve and Tyrrell, 1999), or to 5.5 kJ per m\(^2\) unfiltered UVB radiation, equal to 3 \(\times\) MED. Temperature was controlled by an electric fan.

**RNA extraction from skin** Mice (three per group) were euthanized by cervical dislocation before and at various time points (0, 1.5, 3, 6, 8, 12, and 24 h) post-irradiation. Using sterile scissors and forceps, approximately 1 \(\times\) 2 cm of mid-dorsal skin was excised from mice, immediately frozen in liquid nitrogen, and stored at \(-80^\circ\)C until RNA extraction. The frozen skin was cut into 16 \(\mu\)m slices using a cryostat at \(-20^\circ\)C, to facilitate the extraction of total RNA by the phenol–chloroform–isoamyl alcohol method. After treatment with DNase I (SV Total RNA Isolation System, Promega, Madison, Wisconsin), RNA was quantitated spectrophotometrically by absorbance at 260 and 280 nm (one absorbance unit = 40 \(\mu\)g per mL RNA), and stored at \(-80^\circ\)C until assay.

**RT-PCR analysis of HO-1 and HO-2 expressions** Murine HO-1 cDNA sequence (accession no. X13356) and murine HO-2 cDNA (accession no. AF029874) can be located on the Genbank website (http://www.ncbi.nlm.nih.gov/). HO-1 primers were designed [forward 5'-GGGCCCTGGAAGGAAGATAG-3' and reverse 5'-GCTTG-ATGTCCTTTTGGTGTG-3'] to provide an 888 bp PCR product. Primers for HO-2, also used as a control housekeeping gene (Kutty et al., 1995), were designed [forward 5'-GAAAGGAAGGCACAAGGAGG-3' and reverse 5'-GTTTGGCAAGGCTGAGATG-3'] to generate 767 bp PCR products. The primers were synthesized by Life Technologies (Melbourne, Australia).

A commercially available kit (Promega, Reverse Transcription System, Madison, Wisconsin) was employed to make first-strand cDNA. Total RNA (0.4 \(\mu\)g) was incubated in 20 \(\mu\)L of reverse transcription reaction using olio(dT) as a primer according to the manufacturer’s protocol and the reaction mixture was incubated at 42 \(\circ\)C for 60 min, with a final incubation of 5 min at 95 \(\circ\)C inactivate reverse transcriptase. Once the reaction was completed, the tubes were placed on ice and immediately PCR was performed using a commercial PCR kit (Sigma-Aldrich, RedTaq DNA, St Louis, Missouri), according to the manufacturer’s instruction. Amplifications were performed using a Hybaid OmniGene thermalycler (State Agricultural Biotechnology Centre, Murdoch, West Australia). PCR conditions for both HO-1 and HO-2 were as follows: initial denaturation at 95 \(\circ\)C for 3 min, then 94 \(\circ\)C for 30 s, annealing at 56 \(\circ\)C for 30 s, and extension at 72 \(\circ\)C for 30 s. After 34 cycles, the temperature was held at 72 \(\circ\)C for 10 min to allow final extension.

The PCR products (5 \(\mu\)L) and 5 \(\mu\)L of 0.5 \(\mu\)g of DNA molecular weight marker (GeneRuler 1kb DNA ladder, Gronigen, The Netherlands) were resolved electrophoretically on a 1.2% agarose gel (containing 0.125 \(\mu\)g per mL of ethidium bromide) in tris–borate EDTA buffer pH 8 at 80 V for 1 h at room temperature (RT). Gels were photographed under ultraviolet light using a digital camera (Kodak DC120 Digital, Kodak Australia Pty, Ltd, Cobura, Australia) and images were analyzed using the “Quantity One” (Biorad version 4.1.1) program. Semi-quantitative analysis was performed on the images for HO-1 for each time point and then normalized with respect to HO-2. The analysis for one of three individual mice, each having responded similarly, is presented as the HO-1:HO-2 ratio in arbitrary image analysis units.

**ISH for HO-1 analysis in mouse skin** Approximately 1 cm\(^2\) of the mid-dorsal skin was excised from groups of three unirradiated control mice, and mice at 3 h post-UVA when HO-1 mRNA...
expression reached the optimum level indicated by RT-PCR. The skin samples were frozen at −20°C in a triple layer interspersed with OCT embedding compound (Tissue-Tek, Elkhart, Indiana), from which vertical sections could be cut.

Digoxigenin (Dig)-labelled sense and antisense probes for ISH were prepared from murine hepatic RNA extracted 90 min following intraperitoneal injection of lipopolysaccharide (LPS; Sigma-Aldrich, St Louis, Missouri), as described by Rizzarini et al (1998) for the activation of the HO-1 gene. The RNA was reverse transcribed and the cDNA (HO-1, 888 bp) was used as the template for probe synthesis. A commercial PCR Dig probe synthesis kit (Roche, Indianapolis, Indiana) was used to label the probe for HO-1 mRNA. The Dig-labelled probes were purified according to Klein et al (1995), precipitated with ethanol at −80°C, washed, air dried, resuspended in water, and stored at −20°C. Dig-labelled probe concentration was determined by the absorbance at 260 nm.

HO enzyme activity HO activity in the skin microsomal fraction (72 h post-UVA and unirradiated control) was determined by a slight modification of the method previously described by Reeve and Tyrrell (1999). Briefly, the reaction mixture of 200 μL contained NADPH (100 μM), glucose-6-phosphate (1 mM), glucose-6-phosphate dehydrogenase (0.2 U), hemin (42 μM), desferrioxamine (5 mM), mouse liver cytosol as a source of biliverdin reductase, and 2–3 mg microsomal protein (boiled for 5 min for negative controls). The reaction was conducted at 37°C on a shaker for 60 min, terminated by cooling on ice, and centrifuged at 4°C for 10 min at 7000 × g. Aliquots of 150 μL of the supernatants were placed in a glass 96-well microplate, and the difference in absorbance at 470 and 540 nm was measured using a Spectra Max 250 spectrophotometer (Molecular Devices Corp, Sunnyvale, CA). The total protein content was determined using the method of Lowry et al (1951) and HO activity expressed as picomoles of bilirubin per mg protein per min, using the bilirubin extinction coefficient = 60,700 per M cm.

Skin heme measurement Skin microsomes (mitochondria as controls) were analyzed for heme level (72 h post-UVA and unirradiated control skin), using a method adapted from Pandey et al (1999). Microsomal and mitochondrial pellets were prepared according to Reeve and Tyrrell (1999) and Vile and Tyrrell (1993), respectively. Briefly, 20 μL triplicate samples of mitochondrial or microsomal pellet suspension (equivalent to approximately 2–3 mg protein) or duplicate samples of a standard solution of heme (concentration range 0.15–20 μg per mL) were placed into wells of a 96-well microplate. Then 180 μL of 2.5% Triton X-100 in methanol was added and mixed for 10 s before measuring the absorbance at 405 nm using the Spectra Max 250 spectrophotometer. Mitochondrial and microsomal proteins were measured by the method of Lowry et al (1951). Heme concentration is expressed as μg per mg protein.

Skin bilirubin measurement Skin bilirubin determination was adapted from Bessard et al (1982). Cut skin pieces (72 h post-UVA) were homogenized on ice using a Heidolph stainless steel cutting homogenizer (Kelheim, Germany) with a speed of 9500 rpm for 2 min five times (avoid over-heating) in 15 mL of 0.143 M barbital-hydrogen peroxide, and 3 mM levamisole, and 5 mM levamisole in APSB. The reaction was conducted at 37°C for 2 h at 37°C. After incubation, the sections were washed twice in PBS for 5 min each, then centrifuged at 4°C for 2 h at 37°C. After incubation, the sections were washed twice in PBS for 5 min each and once with water. Finally, the slides were stained with DAB reagent set (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland), lightly counterstained with hematoxylin, dehydrated, mounted, and cover slipped in DePeX mounting medium (BDH, Australia). They were photographed and the images were captured as above.

A number of controls were performed to demonstrate the specificity of immunohistochemical staining as follows: incubation (a) omitting the primary rabbit anti-HO-1 antibody (as a traditional negative control); (b) omitting the secondary goat antirabbit antibody; and (c) omitting both primary and secondary antibodies.

Immunohistochemical detection of HO-1 in mouse skin Frozen sections were cut (8 μm) from mid-dorsal skin taken at 3 h post-UVA and uniradiated control skin, mounted on silane-coated slides, and air-dried for 10–15 min at RT. They were fixed in cold 4% paraformaldehyde in PBS pH 10 for 10 min at RT. The sections were treated with methanol:hydrogen peroxide 14:1:9 for 5 min, and washed twice in PBS for 5 min at RT. They were incubated with rabbit anti-HO-1 polyclonal antibody (1/100, Stressgen, Victoria, Canada) in 1% bovine serum albumin and 10% sheep serum overnight at 4°C. The next day, the sections were washed twice in PBS for 5 min each, before incubating with goat antirabbit-HRP antibody (Stressgen, Victoria, Canada) at 1:300 dilution in PBS for 2 h at 37°C. After incubation, the sections were washed twice in PBS for 5 min each and once with water. Finally, the slides were stained with DAB reagent set (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland), lightly counterstained with hematoxylin, dehydrated, mounted, and cover slipped in DePeX mounting medium (BDH, Australia). They were photographed and the images were captured as above.
absorbanes, multiplied by the extinction coefficient of bilirubin (60,700 per M per cm), and is expressed in mmol per 100 g skin.

**Statistical analysis** Significance of the differences between groups was determined using Student’s t test.

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**References**


Pandey AV, Joshi SK, Tekwani BL: A colorimetric assay for heme in biological samples using 96-well plates. Anal Biochem 268:159–161, 1999

Reeve VE, Domanski D: Refractoriness of UVA-induced protection from photoinmunosuppression correlates with heme oxygenase response to repeated UVA exposure. Photochem Photobiol 76:401–405, 2002


Terry CM, Clikeman JA, Hoidal JR, Callahan KS: TNF-α and IL-1α induce heme oxygenase-1 via protein kinase C, Ca2+ and phospholipase A2 in endothelial cells. Am J Physiol 278:H1493–H1501, 1999


Vile GF, Tyrrell RM: Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. J Biol Chem 268:14678–14681, 1993