Liver X receptor β : maintenance of epidermal expression in intrinsic and extrinsic skin aging

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Abstract Aging in human skin is the composite of time-dependent intrinsic aging plus photoaging induced by chronic exposure to ultraviolet radiation. Nuclear hormone receptors coordinate diverse processes including metabolic homeostasis. Liver X receptor β (LXR β) is a close human homologue of *daf-12*, a regulator of nematode longevity. LXR β is positively regulated by sirtuin-1 and resveratrol, while LXR β -null mice show transcriptional profiles similar to those seen in aged human skin. In these studies, we examined LXR β expression in aged and photoaged human skin. Volunteers were recruited to assess intrinsic aging and photoaging. Epidermal LXR β mRNA was examined by in situ hybridization while protein was identified by immunofluorescence. No significant changes were observed in either LXR β mRNA or protein expression between young and aged volunteers (mRNA $p=0.90$; protein $p=0.26$). Similarly, LXR β protein expression was unaltered in photoaged skin ($p=0.75$). Our data therefore suggest that, while not playing a major role in skin aging,

robust cutaneous expression implies a fundamental role for LXR β in epidermal biology.

Keywords Nuclear hormone receptors · Skin · Aging · Liver X receptor

Introduction

Unlike aging in other human organs, skin aging follows dual primary pathways. Chronological or intrinsic aging is a subtle process that significantly impacts on skin function only at advanced age, while photoaging, induced by chronic exposure to solar ultraviolet radiation, produces a more severe phenotype with an earlier onset. Despite their disparate pathogeneses, intrinsic skin aging and photoaging share fundamental molecular characteristics (Giacomoni and Rein 2001; Griffiths 2001). Liver X receptor β (LXR β) is a member of the nuclear hormone receptor (NHR) family, transcription factors that orchestrate cellular responses to hormonal and metabolic ligands. Oxysterol ligands (Janowski et al. 1999; Fu et al. 2001) activate LXR α (LXR α) and LXR β transcription of a large number of genetic loci (Stulnig et al. 2002) involved in diverse functions but primarily related to the control of lipid and glucose homeostasis (Laffitte et al. 2003). LXR transcription is activated by binding to glucose in vitro (Mitro et al. 2007). However, based on current evidence glucose does not appear to be a physiologically important LXR β ligand in vivo

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(Denechaud 2008). A number of recent studies implicate LXR β in the aging process. Based on DNA sequence similarity, LXR β , jointly with LXR α , is the closest human homologue to the nematode NHR *daf-12* (Mooijjaart et al. 2005) which regulates the dauer diapause response to stress and nutrient levels in *Caenorhabditis elegans*. Genetic manipulation of *daf-12* can substantially extend the mean and maximum lifespan of nematodes carrying specific mutant alleles for *daf-2*, an insulin/IGF-1 receptor homologue (Larsen et al. 1995; Gems et al. 1998). Additionally, lifespan extension of genetically normal nematodes by laser germline ablation requires functional *daf-12* (Hsin and Kenyon 1999; Gerisch et al. 2001). The broad spectrum NAD-dependent deacetylase sirtuin-1 promotes the deacetylation of LXR β and increases its transcriptional activity (Li et al. 2007). This is interesting as sirtuin-1 is thought to be a major target of the polyphenol resveratrol, a putative mimetic of calorie restriction that has been shown to extend lifespan in yeast, nematodes, fruit flies, and fish (Howitz et al. 2003; Wood et al. 2004; Valenzano et al. 2006). The closely related receptor LXR α is transcriptionally up-regulated by resveratrol in macrophages in vitro (Sevov et al. 2006). LXR α has been associated with human lifespan in a longitudinal genetic study of a cohort aged 85 years at baseline and monitored for 6–8 years (Mooijjaart et al. 2007). In this cohort, one LXR α haplotype was moderately protective against all-cause mortality and highly protective against mortality caused by infectious disease. In mice, LXR α is dominant throughout most of the body, but LXR β is uniquely influential in the skin: oxysterol induction of epidermal differentiation markers is abolished in LXR $\beta^{-/-}$ but not in LXR $\alpha^{-/-}$ mice. LXR $\beta^{-/-}$ mice also exhibit a thinner epidermis than wild type mice, whereas LXR $\alpha^{-/-}$ mice do not demonstrate any specific skin defect (Komuves et al. 2002). We therefore elected to focus on LXR β in our studies. LXR signaling is down-regulated in human cell models of photoaging; while a hairless, albino UV-irradiated mouse responds to pan-LXR agonists with dose-dependent decreases in skin thickness, which also occurs in photoaging (Chang et al. 2008). Microarray has revealed that the altered expression patterns between normal and LXR $\beta^{-/-}$ mouse skin bear a notable resemblance to changes between young and aged human skin (Ly et al. 2000). This finding gives rise to the hypothesis that LXR β signaling is reduced

in aging. During aging, the expression profiles of a number of NHRs are known to alter in various tissues (Tohgi et al. 1995; Enderlin et al. 1997; Pallet et al. 1997). Our laboratory has previously shown that expression of the related NHR retinoic acid receptor alpha is increased approximately twofold in both intrinsically aged and photoaged human skin (Watson et al. 2004; Tsourelis-Nikita et al. 2004). We therefore aimed to study the expression of LXR β in human skin by comparing LXR β expression in intrinsically aged (young versus aged photoprotected skin) and extrinsically aged (photoprotected versus photoexposed) human skin.

Materials and methods

All materials were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise indicated.

Subjects and skin biopsies

Intrinsic aging study Two cohorts of healthy male volunteers were recruited; 18–30 years old ($n=6$) and 70–75 years old ($n=7$). Punch biopsies (4 mm) were taken from the photoprotected hip.

Extrinsic aging (photoaging) study Healthy adult volunteers were recruited ($n=9$; four males, five females, 52–76 years). Punch biopsies (6 mm) were taken from photoprotected hip, photoprotected upper inner arm (anatomical site control), and photoaged forearm of each individual.

Biopsies were taken under local anesthesia (1% lignocaine), embedded in optimal cutting temperature compound (OCT; Tissue-Tek; IN, USA) and stored at -70°C . Frozen sections (7 μm) were prepared for analysis by in situ hybridization and immunohistochemistry. The studies were approved by The Salford and Trafford Research Ethics Committee and all volunteers gave written, informed consent.

In situ hybridization

Oligonucleotide probes were designed against exon 8 of LXR β mRNA (Table 1). The probes were synthesized by Sigma-Genosys (Pampisford, UK), and labeled using a Photoprobe[®] biotin kit (Vector

Table 1 Details of oligonucleotide probes

Probe name	Probe length (bases)	Probe sequence
LXR β antisense	30	5' CTT CTT GAA GGA CTT CAC CTA CAG CAA GGA 3'
LXR β sense	30	5' TCC TTG CTG TAG GTG AAG TCC TTC AAG AAG 3'

Laboratories; CA, USA). Biopsy sections were pre-treated to preserve tissue morphology and total RNA. Briefly, tissue was fixed (4% paraformaldehyde), acetylated (100 mM triethanolamine, 150 mM NaCl, 26 mM acetic anhydride), sequentially dehydrated in ethanol, and then delipidated using chloroform.

Hybridizations were performed using standard protocols (Harrison and Pearson 1990; Watson et al. 2001). After blocking with hybridization buffer (5 $\times$ SSC, 50% deionized formamide, 1 $\times$ Denhardt's solution, 0.02% denatured salmon sperm ssDNA, 0.01% lyophilized yeast tRNA, 0.01% poly(A) potassium salt) to reduce non-specific binding, sections were incubated overnight at 26°C with sense or antisense probe in hybridization buffer at 20 μ g/ml. Unbound probe was removed by 3 $\times$ 20 min washes in 1 $\times$ SSC at 48°C. Hybridization and stringency wash temperatures were calculated as previously described (Sambrook et al. 1989; Farrell 1998). Bound probe was visualized using horseradish peroxidase-conjugated avidin followed by 3,3'-diaminobenzidine tetrahydrochloride chromogen plus nickel at room temperature for 10 min (Vector Laboratories; CA, USA).

LXR β mRNA expression was quantified by counting the number of positive epidermal cells in high power ($\times$ 400) microscope fields. Twelve sections were analyzed per site, per volunteer. This raw count was then normalized for the epidermal thickness of each patient, measured by image analysis (QWin, Leica; Germany). In addition to the sense strand control, RNase-treated, excess unlabelled probe and zero probe treatments were performed.

Immunofluorescence

To identify LXR β protein in whole skin sections, tissue was fixed (50% methanol, 50% acetone), solubilized (0.5% Triton X-100 in TBS) and blocked (1% normal goat serum, 1% bovine serum albumin in TBS). Primary LXR β antibody (mouse anti-human, Perseus Proteomics; Japan) was applied overnight in block at 0.2 mg/ml. Sections were then incubated

with fluorescently tagged anti-IgG antibody (goat anti-mouse fluorescein isothiocyanate conjugate, Dako, Denmark). LXR β expression was quantified in the epidermis by image analysis (QWin, Leica, Germany). Twelve sections were analyzed per site, per volunteer. Negative controls were performed without primary antibody. For nuclear colocalization, sections were briefly incubated with 10 μ g/ml 4', 6-diamidino-2-phenylindole (DAPI) solution before mounting.

Western blot

HaCaT and COS-1 cells were cultured at 37°C under 5% CO₂ until 80% confluent. Proteins were extracted in RIPA buffer by standard methodologies (Berton et al. 1994). Samples (24.4 μ g total protein) were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) resolving gels. Proteins were then transferred to PVDF membranes (Amersham; UK) by electroblotting and blocked in 5% non-fat milk powder at 4°C overnight. Proteins were identified using LXR β (mouse anti-human; Perseus Proteomics, Japan; 1:700 dilution) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; mouse anti-rabbit, Santa Cruz Biotechnology; CA, USA; 1:100 dilution) antibodies. Secondary peroxidase-conjugated antibodies were applied (goat anti-mouse IgG-HRP conjugates; BioRad; Herts, UK; 1:1,250 dilution). After washing, bound antibody was visualized employing enhanced chemiluminescence plus (ECL⁺, G.E. Healthcare; UK) and X-ray film (Amersham; UK).

Statistical analysis

Groups were compared statistically by Student's unpaired two-tailed *t* test (intrinsic aging study) or repeated measures ANOVA (extrinsic aging study) using SPSS 14.0 (SPSS, IL, USA), taking significance at the 95% confidence interval.

Results

LXR β is expressed in human epidermis

LXR β was detected in human skin at both the mRNA and protein levels. We found the expression of LXR β mRNA (Fig. 1) and protein (Fig. 2) to be largely confined to the epidermis, with minimal staining in the dermis. Double staining using DAPI to label nuclear DNA showed no colocalization with LXR β reactivity, which displays a pericytoplasmic distribution (Fig. 3).

In situ hybridization analysis of LXR β mRNA

LXR β mRNA expression was identified by in situ hybridization in human skin sections, with epidermal

expression quantified and normalized for individual epidermal thickness prior to statistical analysis. Comparison of young and intrinsically aged epidermis revealed no significant difference in expression (Student's two-tailed unpaired t test; $p=0.90$; mean $\pm$ SE; young 0.69 ± 0.08 ; aged 0.70 ± 0.06 ; Fig. 1).

Immunofluorescent analysis of LXR β protein

LXR β protein was identified by immunofluorescence in samples from both intrinsically and extrinsically aged skin (Figs. 2 and 4). Epidermal fluorescence was quantified by image analysis prior to statistical analyses. We observed no significant differences between young and intrinsically aged skin (Student's unpaired two-tailed t test; $p=0.26$; mean $\pm$ SE; young 62.4 ± 9.8 ; aged 77.5 ± 8.4 ; Fig. 2). Similarly, we could

Fig. 1 In situ hybridization staining for LXR β mRNA in human skin. Nuclear LXR β mRNA staining is localized predominantly in the epidermis. *Dashed lines* chart the approximate course of the dermal-epidermal junction. **a** Sense strand control, very little difference is observed in LXR β

mRNA expression between **b** young and **c** intrinsically aged skin. **d** *Box and whisker plot* of LXR β mRNA expression in young and aged epidermis. Mean $\pm$ SE; young 0.69 ± 0.08 ; aged 0.70 ± 0.06 . *Scale bar* 50 μ m. Photographs were taken under identical standardized microscopy conditions

Fig. 2 LXR β protein expression does not alter with increasing age in photoprotected human skin. LXR β antibody reactivity is distributed in the cell periphery in photoprotected skin. Neither the distribution nor the amount of fluorescence alters with increasing age. Representative photographs of photoprotected skin from **a** young and **b** intrinsically aged individuals. **c**

Negative control. **d** Box and whisker plot of LXR β protein expression in young and intrinsically aged epidermis. Mean $\pm$ SE; young 62.4 ± 9.8 ; aged 77.5 ± 8.4 . Scale bar 50 μ m. Photographs were taken under identical standardized microscopy conditions

not detect a significant difference between photo-protected and photoexposed anatomical sites (repeated measures ANOVA; $p=0.75$; mean $\pm$ SE; hip 42.4 ± 8.9 ; upper inner arm 45.0 ± 8.8 ; forearm 44.9 ± 10.0 ; Fig. 4).

Western analysis of antibody specificity

To validate the specificity of our monoclonal LXR β antibody, we conducted a western blot in keratinocytic (HaCaT) and fibroblastic (COS-1) cell line protein extracts. A single reactive band was present at 50 kDa, the predicted molecular weight of LXR β (Fig. 5). GAPDH was also blotted to ensure even protein loading of all gels. Expression of LXR β was observed in keratinocytic HaCaT cells and in the fibroblast cell line, COS-1.

Discussion

In these studies we analyzed the expression of LXR β , a nuclear hormone receptor that mediates lipid and glucose homeostasis, in aged and photoaged human skin. In agreement with a previous study (Russell et al. 2007) we found that LXR β mRNA and protein were uniformly and abundantly expressed by the keratinocytes of the intrafollicular epidermis. In contrast, signal in the dermis was consistently low for both mRNA and protein and could not be robustly distinguished from non-specific background staining. These studies therefore concentrated on LXR β expression in the epidermis.

While the localization of LXR β mRNA and tissue distribution of LXR β protein were as expected, intracellular reactivity was observed in the cell periphery, not the

Fig. 3 Colocalization of **a** a nuclear stain (DAPI) with **b** LXR β antibody reactivity. **c** is a composite overlay of **a** and **b**. No appreciable colocalization is visible, which indicates a peripheral

cytoplasmic or membranous pattern of LXR β antibody reactivity. Additionally, no specific cellular staining is visible in the dermis. Scale bar 50 μ m

Fig. 4 Photoaging does not perturb the expression pattern of LXR β . Representative photomicrographs from **a** photoprotected hip, **b** photoprotected upper inner arm, and **c** photoaged forearm of the same individual. **d** Box and whisker plot of

LXR β expression in photoprotected and photoaged anatomical sites. Mean $\pm$ SE; hip 42.4 $\pm$ 8.9; upper inner arm 45.0 $\pm$ 8.8; forearm 44.9 $\pm$ 10.0. Scale bar 50 μ m. Photographs were taken under identical standardized microscopy conditions

Fig. 5 Western blotting produces a single reactive band in both keratinocyte and fibroblast cell line extracts. Protein was extracted from HaCaT and COS-1 cell lines, separated by

SDS-PAGE prior to western blotting for LXRβ and GAPDH as a loading control. A single reactive band for LXRβ was found at its predicted molecular weight (50 kDa)

nucleus. To verify the specificity of our antibody we conducted a western blot using samples from keratinocyte and fibroblast cell lines. A single reactive band co-migrated with weight markers at the predicted molecular weight of LXRβ (50 kDa), evidence for specificity of the antibody. Reactivity was clearly detected in the keratinocyte and fibroblast cell line extracts at equal intensity, which agrees with the previous work by Russell and co-workers (2007). However, the dermis contains a much lower cell density than the epidermis and is prone to autofluorescence and non-specific staining, which may explain why dermal signal could not be reliably detected by in situ hybridization and immunofluorescence.

Cytoplasmic expression of LXRβ has previously been recorded in a study using fluorescent LXR fusion proteins, which showed that, in contrast to LXRα, LXRβ is partially exported from the nucleus to the cytoplasm under low ligand conditions (Prüfer and Boudreaux 2007). As additional support for the cytoplasmic expression of LXRβ in keratinocytes, a pan-LXR polyclonal antibody used by Russell et al. (2007) in whole skin sections showed both nuclear and cytoplasmic reactivity.

In Western blots using the same monoclonal LXRβ antibody as our studies, Russell and co-workers (2007) found that while a single reactive band was present in other differentiated skin cells and cell lines, primary human keratinocytes showed two bands at approximately 50 and 55 kDa. This suggests that two distinct isoforms of LXRβ may be produced in epidermal keratinocytes by alternative exon splicing or post-translational modification. Speculatively, these isoforms might localize differentially, with the antibody epitope present or accessible only in the non-nuclear population.

Our results show that the epidermal mRNA and protein expression of LXRβ do not differ signifi-

cantly between photoprotected skin sections from our young and intrinsically aged volunteer cohorts. One factor affecting NHR expression is ligand availability (Enderlin et al. 1997; Pallet et al. 1997). While total serum oxysterol levels do not appear to correlate with age, fasting glucose and 24-hydroxycholesterol, an endogenous LXR ligand, are altered in aged sera (Janowski et al. 1996, Lutjohann et al. 1996, Lindeman et al. 2003). However, it is not known whether the increased serum concentrations of these ligands equate to increased intracellular concentrations.

In skin, chronic ultraviolet light exposure produces the accelerated aging phenotype. We have studied the effects of photoaging on LXRβ protein expression by taking skin biopsies from two photoprotected sites (hip and upper inner arm) and from photoaged forearm of otherwise healthy adults. Comparing these anatomical sites in nine volunteers revealed no significant differences in epidermal LXRβ protein expression. Inter-individual differences were significant, showing baseline variability or perhaps the effect of an unidentified factor in this cohort.

Our data suggest that human skin aging has no major effect on LXRβ expression. Although a larger sample size may allow confident identification of subtle changes in expression as significant, the largest difference between any two group means in our data is less than 20%. This is considerably lower than the 200% up-regulation of the related NHR retinoic acid receptor α observed in aged and photoaged epidermis (Watson et al. 2004; Tsourelis-Nikita et al. 2004). Like other NHRs, LXRβ is nested in complex signaling pathways with a number of possible points of regulation to modify LXRβ activity without affecting LXRβ expression. The specifics of LXRβ's role in epidermal biology, the biological response to resveratrol via sirtuin activation

of LXR β and skin aging are open to elucidation by future studies.

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