Upregulation of 8-Lipoxygenase in the Dermatitis of IκB-α-Deficient Mice

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Neonatal mice deficient in IκB-α, an inhibitor of the ubiquitous transcription factor NF-κB, develop severe and widespread dermatitis shortly after birth. In humans, inflammatory skin disorders such as psoriasis are associated with accumulation in the skin of the unusual arachidonic acid metabolite 12*R*-hydroxyeicosatetraenoic acid (12*R*-HETE), a product of the enzyme 12*R*-lipoxygenase. To examine the etiology of the murine IκB-α-deficient skin phenotype, we investigated the expression of lipoxygenases and the metabolism of exogenous arachidonic acid in the skin. In the IκB-α-deficient animals, the major lipoxygenase metabolite was 8*S*-HETE, formed together with a minor amount of 12*S*-HETE; 12*R*-HETE synthesis was undetectable. Skin from the wild-type littermates formed 12*S*-HETE as the almost exclusive lipoxygenase metabolite. Upregulation of 8*S*-lipoxygenase (8-LOX) in IκB-α-deficient mice was confirmed at the transcriptional and translational level using ribonuclease protection assay and western analysis. In immunohistochemical studies, increased expression of 8-LOX was detected in the stratum granulosum of the epidermis. In the stratum granulosum, 8-LOX may be involved in the terminal differentiation of keratinocytes. Although mouse 8*S*-lipoxygenase and human 12*R*-lipoxygenase are not ortholog genes, we speculate that in mouse and humans the two different enzymes may fulfill equivalent functions in the progression of inflammatory dermatoses.

Key words: arachidonic acid/dermatitis/lipoxygenase/NF-κB/psoriasis
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Nuclear factor κB (NF-κB) is a ubiquitous transcription factor involved in inflammatory and immune responses. In resting cells, NF-κB is retained in the cytosol by forming a complex with one of several members of the protein family inhibitor of κB (IκB). Among the seven vertebrate IκB proteins, IκB-α is recognized as the functionally most important inhibitor of NF-κB. Stimulation of resting cells through appropriate signals leads to phosphorylation and degradation of IκB, and subsequent translocation of the liberated NF-κB to the nucleus (Baldwin, 1996; Sha, 1998).

In mice deficient of IκB-α, NF-κB is constitutively active (Beg *et al*, 1995; Klement *et al*, 1996; Chen *et al*, 2000b). In the first 4–5 d after birth, the skins of IκB-α−/− and wild-type mice are phenotypically indistinguishable. On days 6–7, a dermatitis phenotype appears, and the skin becomes dry, scaly, flaky, and the development of hair is impaired. IκB-α−/− pups typically die within 9 d after birth. Neonatal lethality of the knockouts was overcome by use of the RAG2 (*recombination activating gene 2*) blastocyst complementation system to generate chimeric animals (Chen *et al*, 2000b). All mature lymphocytes of the chimeras are derived from the IκB-α−/− embryonic stem cells, and the contribution of the RAG2-deficient blastocysts is able to rescue the neonatal lethality. The RAG2−/−, IκB-α−/− chimeras are normal at birth, and the dermatitis develops at about 5 wk after birth. Histologically, the disease phenotype of the chimeras is characterized by hyperplastic epidermal keratinocytes, dermal infiltration of immunocompetent cells including lymphocytes, and formation of microabscesses. Since epidermal hyperplasia and lymphocyte infiltration are two main characteristics of psoriasis in humans, the overall phenotypic and histologic characterization of chimeric IκB-α-deficient mice suggested that these animals could serve as a highly suitable model to study molecular events of human psoriasis or severe widespread dermatitis (Chen *et al*, 2000b).

In humans, the metabolism of arachidonic acid in psoriasis and other proliferative dermatoses is characterized by the accumulation of the unusual metabolite 12*R*-hydroxyeicosatetraenoic acid (12*R*-HETE) (Hammarström *et al*, 1975; Baer *et al*, 1991). Formation of 12*R*-HETE results from the activity of 12*R*-lipoxygenase (LOX) in the psoriatic lesions (Boeglin *et al*, 1998); in normal human skin, 12*R*-LOX activity is almost undetectable. Mutations in the coding region of the 12*R*-LOX gene (*ALOX12B*) were identified recently in patients with non-bullous congenital ichthyosiform erythroderma (NCIE), a familial skin disease with a

Abbreviations: H(P)ETE, hydro(pero)xyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; IκB, inhibitor of κB; LOX, lipoxygenase; NCIE, non-bullous congenital ichthyosiform erythroderma; NF-κB, nuclear factor κB; PPAR, peroxisome proliferator activated receptor; RAG2, *recombination activating gene 2*; RP, reversed phase; SP, straight phase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

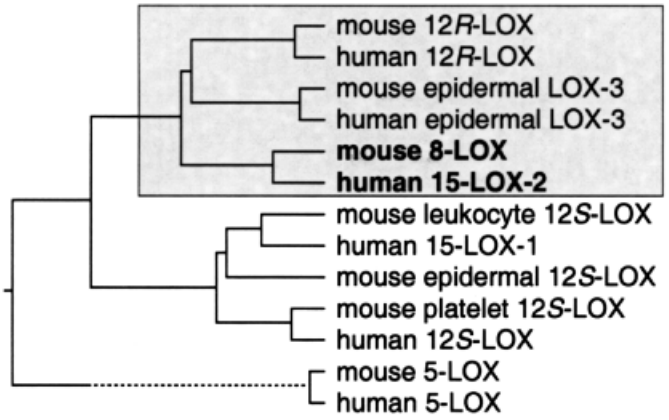

Figure 1
Phylogenetic relationship of human and mouse lipoxygenases. The subgroup of epithelial lipoxygenases is shown in a gray box. The homolog of mouse 8-LOX is the second type of 15*S*-LOX in humans (15-LOX-2) (Brash *et al*, 1997; Jisaka *et al*, 1997). The phylogenetic tree was constructed using the MegAlign program of the DNAstar software package (Lasergene, Madison, WI).

disorder of impaired keratinization (Jobard *et al*, 2002). Prompted by the knowledge that alterations in the lipoxygenase metabolism of arachidonic acid are involved in several human hyperproliferative epidermal disorders including psoriasis, we investigated lipoxygenase expression and activity in the affected skin of IκB-α knockout mice.

12*R*-LOX was a likely candidate as an altered lipoxygenase in the affected skin of IκB-α-deficient mice due to available information with respect to human psoriasiform disease and NCIE. We did not limit our investigation to this isoform however, as a multitude of lipoxygenases are expressed in mouse skin, and there is high homology within the family of lipoxygenases across species (Fig 1). Here we report the characterization of the lipoxygenase activity in this cutaneous model using animals deficient in IκB-α.

Results

Arachidonic acid metabolism in the skin of wild-type and IκB-α-deficient mice The metabolism of exogenous radiolabeled arachidonic acid was analyzed in epidermal homogenates of IκB-α-deficient mice and control littermates. In the IκB-α−/− animals, the entire dorsal epidermis was affected by the dermatitis phenotype, and the affected skin was used for the experiments. The control mice showed higher conversion of substrate to lipoxygenase-derived products than the IκB-α-null mice (12%, 25%, and 27% in controls compared to 3%, 6%, and 10% in the IκB-α-deficient littermates). Product analysis showed that this was associated with a dramatic switch in enzymatic activities.

Initial analysis of radiolabeled arachidonic acid metabolites using reversed-phase HPLC (RP-HPLC) showed the formation of lipoxygenase-derived HETEs as the most prominent radiolabeled products, together with less amounts of early eluting polar products that corresponded in retention time to prostaglandins (Fig 2, *A* and *B*). The

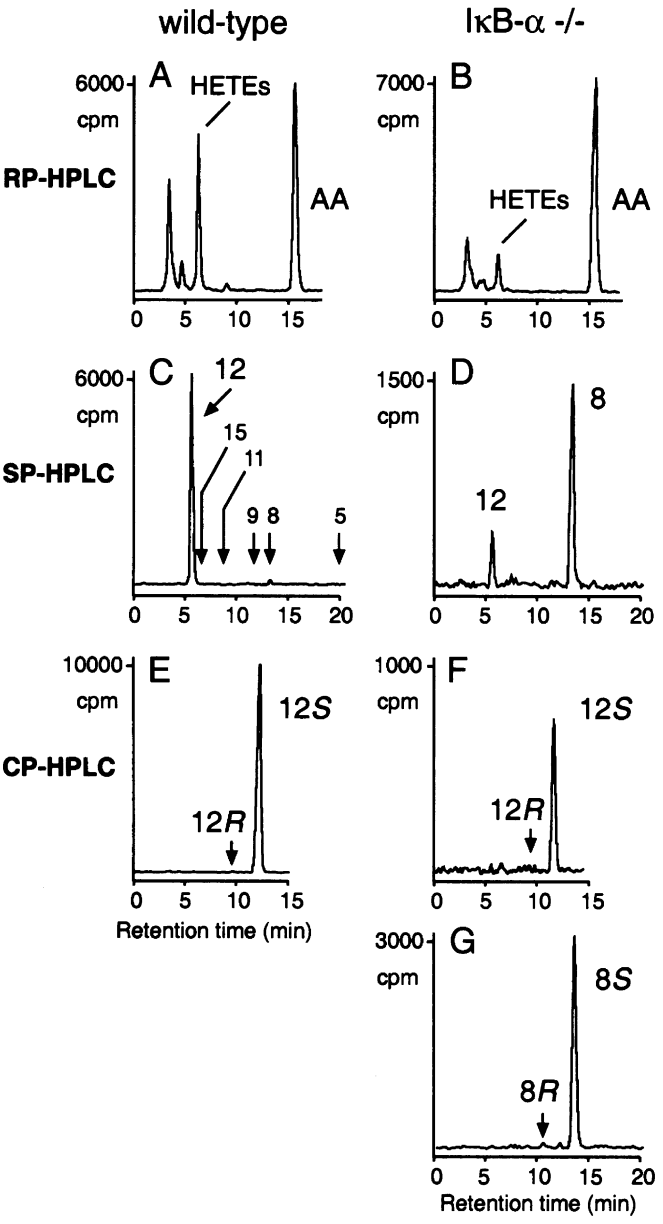

Figure 2
HPLC analysis of the metabolism of [1-^{14}C]arachidonic acid in skin homogenates of wild-type and IκB-α-deficient mice. The panels on the left show results from wild-type, and the panels on the right show results from IκB-α−/− mice. (*A*) and (*B*) show chromatograms from the initial RP-HPLC analysis where the individual HETE isomers co-elute. In (*C*) and (*D*), the collected HETE fractions from RP-HPLC have been re-chromatographed as the methyl esters using SP-HPLC. In (*C*) the retention times of HETE isomers are indicated by arrows. (*E–G*) Chiral phase HPLC analysis of 12-HETE (wild-type, panel *E*), 12-HETE (IκB-α−/−, panel *F*), and 8-HETE (IκB-α−/−, panel *G*) collected from SP-HPLC. Incubations with [1-^{14}C]arachidonic acid, extraction, and HPLC conditions are described in *Materials and Methods*. The chromatograms shown are representative of several independent experiments.

individual HETE isomers were not resolved under these RP-HPLC conditions, and therefore the HETE products were collected as a pooled fraction from RP-HPLC and analyzed further using SP-HPLC. In the epidermis of control mice, 12-HETE was the almost exclusive component in the HETE fraction (Fig 2*C*), and chiral analysis revealed the configuration as >98% 12*S*-HETE (Fig 2*E*). This is the product expected of any of the three 12*S*-LOX enzymes expressed

in mouse skin (Heidt *et al*, 2000; Siebert *et al*, 2001). In the IκB-α−/− mice, 8-HETE was the major metabolite together with about 20%–25% in abundance of 12-HETE (Fig 2*D*). Stereochemical analysis of the minor 12-HETE product in the IκB-α−/− skin homogenates revealed, as in the controls, a purely 12*S*-configuration (Fig 2*F*). The 12*S* chirality ruled out the participation of the 12*R*-LOX enzyme in the biosynthesis of 12-HETE. Stereochemical analysis of the 8-HETE formed in the IκB-α−/− skin homogenates showed that it was exclusively of the *S*-configuration (Fig 2*G*), as expected for its biosynthesis by the mouse skin 8*S*-LOX (Jisaka *et al*, 1997; Krieg *et al*, 1998). Formation of other HETE isomers such as 5-HETE or 15-HETE was not observed in the control or in the IκB-α−/− mice.

Based on previous reports on increased 12*R*-HETE formation in human psoriasis and in other abnormalities of epidermal differentiation, we carefully analyzed for the expression of 12*R*-LOX activity in the IκB-α−/− and wild-type mice. The recombinant mouse 12*R*-LOX does not oxygenate free arachidonic acid, and to date only the methyl ester of arachidonic acid has been reported to be a substrate (Krieg *et al*, 1999). Therefore, skin homogenates were incubated with [1-^{14}C]arachidonic acid methyl ester and analyzed using HPLC conditions as described above. The formation of 12*R*-HETE methyl ester was not detected in these analyses. Northern and RPA assays, as well as western blot analyses using a polyclonal antibody raised against recombinant human 12*R*-LOX failed to detect expression of 12*R*-LOX in skin homogenates of either IκB-α-deficient or control animals (not shown).

Analysis for 12-lipoxygenase isozymes Due to limitations of sensitivity, we were unable to identify the enzyme(s) responsible for the 12*S*-HETE synthesis in the wild-type and IB-α-deficient animals. The most likely candidate is the platelet-type 12*S*-LOX, an enzyme with an activity profile what most closely matches what we observe. The platelet-type 12*S*-LOX forms purely 12*S*-HETE from arachidonic acid whereas both the leukocyte-type and epidermal-type 12*S*-LOX enzymes form 15*S*-HETE as a significant second metabolite (Chen *et al*, 1994; McDonnell *et al*, 2001). 15-HETE was not evident on our HPLC chromatograms (Fig 2). Although the epidermal-type 12-LOX has been cloned from mouse skin, it is actually the least likely candidate because the recombinant protein is known to possess exceptionally low catalytic activity (Funk *et al*, 1996; Burger *et al*, 2000). Using specific commercial antibodies, we could not detect expression of platelet-type 12*S*-LOX. We used three different antibodies raised against the leukocyte-type 12*S*-LOX or its human homolog and could not detect expression of this LOX. Given that we were unable to identify 12-LOX in the skin samples, we are limited in what we can say about the downregulation of the enzyme in the knockout animals. Although it is not clear why constitutive activation of NF-κB is associated with the reduced 12-LOX activity that we observe in the IκB-α-deficient mouse skin, a role for 12-lipoxygenases is implied in normal skin differentiation (e.g. for the platelet-type 12*S*-LOX in the normal barrier functioning (Johnson *et al*, 1999)), and the appropriate stage for its expression may be bypassed in this dermatitis model.

Western and ribonuclease protection analyses Transcriptional regulation of 8-LOX was analyzed using RNA isolated from the skins of chimeric RAG2−/−, IκB-α−/− mice (Chen *et al*, 2000a, 2000b). The chimeras were used to generate adult mice and thus circumvent the fact that the IκB-α−/− animals die around neonatal day 9. The chimeric mice were generated using the RAG2 (*recombination activating gene 2*) blastocyst complementation system. Using this technique, IκB-α−/− lymphocytes were transferred into RAG2−/− recipient mice, and the resulting chimeras live into adulthood. Two of three chimeric mice generated using this method developed a psoriasiform skin disease (Chen *et al*, 2000b). Expression analysis by RNase protection assay using RNA from the two affected animals and of two control littermates proved upregulation of 8-LOX mRNA in the diseased animals (Fig 3*A*). Subsequent western blot analyses confirmed the induction of 8-LOX protein in skin homogenates of IκB-α−/− mice as compared to wild-type animals (Fig 3*B*).

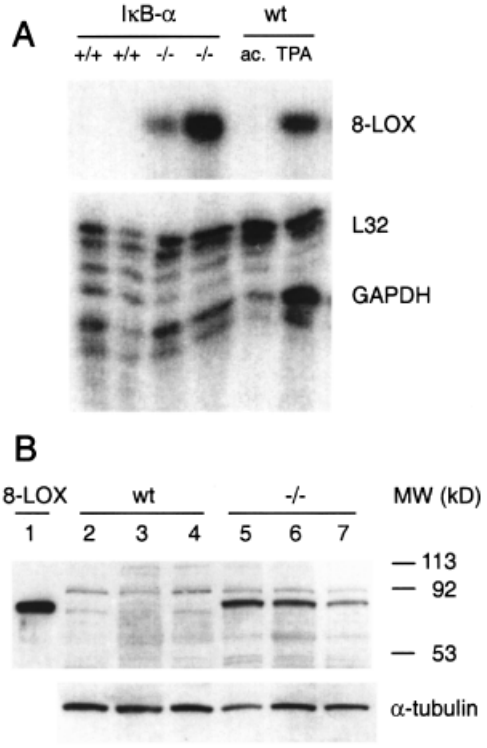

Figure 3
Induction of 8-LOX expression in the dorsal skin of IκB-α−/− mice. In (*A*) total RNA isolated from RAG2−/−, IκB-α−/− mice was used. RNase protection assay indicates upregulation of 8-LOX mRNA in the skins from diseased animals (−/−) as compared with those from control animals (+/+). The different levels of 8-LOX mRNA in the two chimeric animals are most likely due to differences in the relative contribution of RAG2−/−, IκB-α+/+ *versus* RAG2+/+, IκB-α−/− cells in the skin of the two animals. The two lanes on the right show normal mouse skin treated with the vehicle acetone (ac.) or with TPA to induce 8-LOX expression. RNA was isolated using TRI reagent, and 5 μg each was used in the assay using a probe specific for mouse 8-LOX (≈ 400 bp). L32 and GAPDH (both ≈ 150 bp) were used as loading controls. (*B*) Western blot detection of 8-LOX expression in the dorsal skin of wild-type (*lanes 2–4*) and IκB-α−/− mice (*lanes 5–7*) using a polyclonal antibody raised against human 15-LOX-2. For the skin samples, 30 μg of protein was loaded onto a 10% SDS-PAGE gel and blotted on a 0.2 μm nitrocellulose membrane. In *lane 1*, a standard of mouse 8-LOX expressed in HEK 293 cells was loaded. A mouse monoclonal antibody raised against α-tubulin was used as loading control.

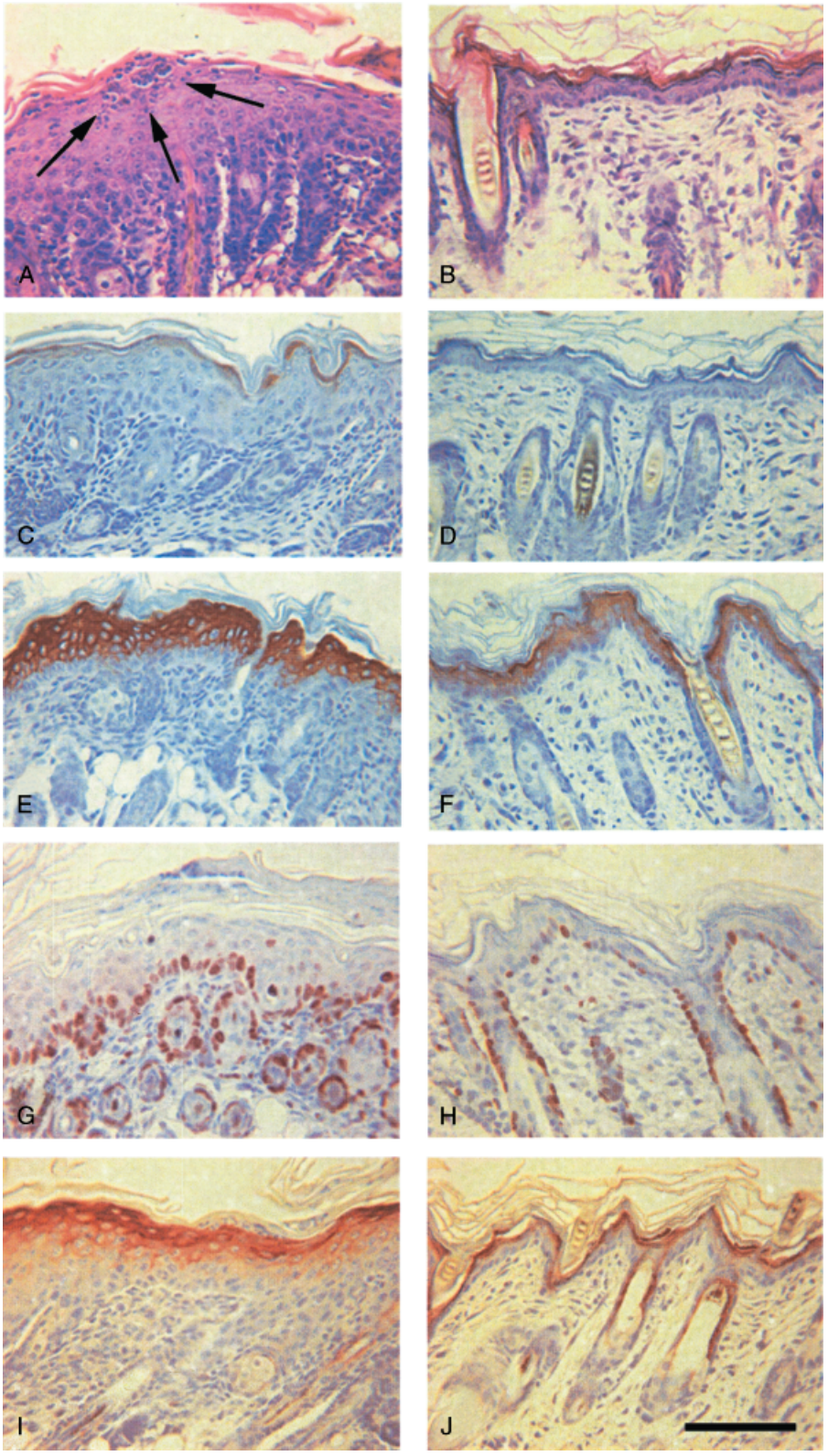

Figure 4
Immunohistochemical analysis of 8-LOX expression and of morphological differences between the dorsal skins of IκB-α-deficient (left panels) and wild-type mice (right panels). (*A*) The epidermis is greatly thickened in the outer epidermis of the IκB-α−/− mice; a small microabscess is visible (indicated by arrows). (*B*) The epidermis is unremarkable in the wild-type mice. (*A*, *B*) Hematoxylin and eosin staining. (*C*) Positive immunoreactivity for 8-LOX expression was detected in the stratum granulosum of IκB-α-deficient mice. (*D*) Immunoreactivity for 8-LOX was not detected in the epidermis of wild-type mice. (*E*) The distribution of keratin-1 in IκB-α−/− mice indicates an expanded suprabasal compartment leading to a thickening in the stratum corneum. (*F*) Keratin-1 immunoreactivity in the suprabasal epidermal compartment in wild-type mice shows the normal suprabasal distribution. (*G*) The majority of cells along the basal layer are stained with the proliferative marker Ki67 in the IκB-α−/− mice. (*H*) Only a few proliferative cells are present in the epidermis of this wild-type mouse. (*I*) The involucrin marker shows immunoreactivity in the expanded differentiated compartment of the outer epidermis of an IκB-α-deficient mouse. (*J*) The involucrin marker shows minimal thickness in the wild-type mouse. *Size bar*, 50 μm.

Immunohistochemical analyses The results of the arachidonic acid incubations and RPA and western analyses indicated increased expression and activity of 8-LOX in the knockout animals. In order to localize this aberrant expression, immunohistochemical analysis was performed on dorsal skin sections.

The antibody used for immunohistochemistry has been shown to detect 8-LOX expression in mouse epidermis (Jisaka *et al*, 1997; Muga *et al*, 2000). Sections of dorsal skins from 6-to7-d-old wild-type and IκB-α-deficient mice were fixed in 10% formaldehyde, embedded, sectioned, stained with standard hematoxylin and eosin, and immunostained to determine the distribution of 8-LOX (Fig 4). Additional immunostaining with markers for immune cells (T lymphocytes and macrophages) and for the various epidermal compartments was performed (Fig 4). Gram

staining and periodic acid staining were unremarkable, indicating that the cause for the disease was genetic and not due to infection.

The knockout mice in this study showed a rapid onset of dermatitis that required their euthanization at 8–9 d of age. Several hallmarks of epidermal hyperproliferation and severe dermatitis are obvious in the skin samples. Figure 4*A* illustrates a small microabscess in the outer epidermis. This was a prominent feature, and many of the abscesses were considerably larger. The number of epidermal layers was greatly increased in the knockout mice as compared to the wild-type. In addition to the morphologically obvious thickening, quantitative morphometric measurements were performed. The average epidermal thickness in the IκB-α-deficient mice was 50.5 ± 5.1 μm and in the wild-type was 16.7 ± 4.8 μm (Fig 4*A* and *B*). In the IκB-α-deficient mice, a prominent signal for 8-LOX expression was localized to the stratum granulosum (Fig 4*C*). Staining for 8-LOX was absent in the epidermis of wild-type mice as expected from the results of the activity and expression analyses (Fig 4*D*). Keratin-1 was used as a marker for the suprabasal region of the epidermis. Staining with an antibody against keratin-1 revealed an apparent expansion of the number of layers of keratinocytes within the differentiated region of the epidermis in the IκB-α-deficient mice as compared to the control mice (Fig 4*E* and *F*). An overall higher cell density was observed in the dermis of the IκB-α-deficient mice as compared to the wild-type littermates.

Since a thickened epidermis can be the result of an increase in cell proliferation and/or an increase in the outer, more differentiated compartment, we conducted additional immunohistochemical assays with specific markers. The extent of the proliferative compartment was revealed with Ki67 immunostaining (Fig 4*G* and *H*). The number of proliferating cells was greatly increased along the basal layer in the IκB-α-deficient mice as compared to the wild-type. It is noteworthy that the labeled nuclei are largely restricted to the basal layer and are not expanded above the most basal layer as one would expect to see in psoriatic disease. The numbers of proliferating cells are greatly increased in the keratinocytes associated with hair follicles. This was an expected finding since this is the time of active growth of the first coat of fur. Additional staining with an immunomarker that is restricted to the outer, most highly differentiated skin layers (involucrin) confirmed that the most differentiated epidermal compartment was expanded in IκB-α-deficient mice (Fig 4*I* and *J*). Although neutrophilic infiltrates were readily observed in the hematoxylin- and eosin-stained sections and were a prominent feature in the outer epidermis and sloughing flaky layers, CD4 + cells were not increased in the IκB-α-deficient mice (data not shown). The numbers of macrophages as highlighted by the macrophage marker F4/80 were likewise not significantly changed between the IκB-α-deficient and the wild-type animals.

Discussion

Psoriasis is a chronic hyperproliferative and hyperinflammatory skin disease characterized by hyperplasia and impaired differentiation of epidermal keratinocytes. The inflammatory reaction is caused by infiltration into the epidermis of immunocytes, e.g., T lymphocytes and macrophages. In humans, psoriatic lesions have abnormal arachidonic acid metabolism, leading to the accumulation of the unusual product 12*R*-HETE as the major metabolite (Hammarström *et al*, 1975; Baer *et al*, 1991). The formation of 12*R*-HETE in psoriasis is the result of the activity of 12*R*-LOX, which has very low levels of activity in normal human skin (Boeglin *et al*, 1998). The physiological consequences of 12*R*-HETE synthesis by 12*R*-LOX are not understood, and our original hypothesis was that the IκB-α-deficient mice would represent an appropriate model to investigate these consequences. Analysis of the LOX metabolism of arachidonic acid in the skins of IκB-α-deficient mice revealed the unexpected result of prominent formation of 8*S*-HETE in the IκB-α−/− animals. The wild-type animals, by contrast, formed mainly 12*S*-HETE. Formation of 12*R*-HETE was not observed in the IκB-α−/− or the wild-type animals. We can conclude that either this mouse model of psoriasis differs from the human condition with respect to the role of its arachidonic acid metabolites or, another tenable possibility, that the functions of human 12*R*-LOX in psoriasis and related dermatoses are taken over by the 8-LOX enzyme in the mouse.

Seven functional LOX genes are expressed in the mouse, all of which can be detected in mouse skin. These are the platelet, leukocyte, and epidermal types of 12*S*-LOX, 5-LOX, 8-LOX, 12*R*-LOX, and eLOX3 (Fig 1) (Brash, 1999; Heidt *et al*, 2000). The latter three enzymes form a subgroup of epithelial-specific lipoxygenases located on a gene cluster on mouse chromosome 11, and the proteins share about 50% amino acid identity (Heidt *et al*, 2000). In the epidermis of most mouse strains, 8-LOX protein and activity are almost undetectable or absent (Fürstenberger *et al*, 1991; Hughes and Brash, 1991). Expression of the enzyme is highly inducible upon topical application of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), although the extent of this induction is strain dependent (Gschwendt *et al*, 1986; Hughes and Brash, 1991). TPA treatment of the skin leads to a phenotype of inflammation with epidermal hyperplasia within 24 h of treatment. Mouse 12*R*-LOX has a peculiar substrate specificity, and so far arachidonic acid methyl ester is the only substrate reported to be oxygenated by the recombinant enzyme *in vitro* (Krieg *et al*, 1999). A naturally occurring substrate for mouse 12*R*-LOX has yet to be identified. Likewise, 12*R*-HETE has not been detected as an endogenous arachidonic acid metabolite in mouse skin. Based on the temporal expression pattern of 12*R*-LOX in the skin during mouse embryonic development, it was suggested that the enzyme might have a functional role in development of the epidermis (Sun *et al*, 1998). The identification and cloning of mouse eLOX3 was reported several years ago, however, for the recombinant and expressed enzyme, no oxygenation of arachidonic acid or any other polyunsaturated fatty acid substrate has yet been described (Kinzig *et al*, 1999). Recent studies identified a catalytic activity for human eLOX3 in the conversion of HPETE substrates to specific epoxyalcohol products (Yu *et al*, 2003).

Upon immunohistochemical analyses, we detected expression of 8-LOX in the stratum granulosum of the affected

epidermis of the knockouts while there was no staining in the wild-type animals. In the stratum granulosum, 8-LOX co-localized with keratin-1, indicating that this LOX is expressed in differentiating keratinocytes. This was further confirmed using staining for involucrin as an additional marker of epidermal differentiation (Fig 4). A similar localization also occurred in transgenic mice overexpressing 8-LOX under the influence of a loricrin promoter; 8-LOX was expressed in the suprabasal epidermis and the phenotype was characterized by epidermal thinning and a high degree of differentiation and keratinization (Muga *et al*, 2000). In the same study, it was found that the 8-LOX metabolite 8*S*-HETE could act as a ligand for the nuclear receptor PPARα, and thus mediate calcium-induced keratinocyte differentiation through a pathway involving PPARα (Muga *et al*, 2000; Thuillier *et al*, 2002). 8*S*-HETE is generally recognized as a potent PPARα ligand (Yu *et al*, 1995), although 8*R*-HETE and 12*R*-HETE were equipotent with 8*S*-HETE in inducing mouse keratinocyte differentiation in a low-calcium medium (Thuillier *et al*, 2002). Given these considerations, it seems plausible that 8-LOX expression in IκB-α−/− mice is part of a program initiated in an attempt to terminally differentiate the large number of proliferating keratinocytes.

The thickened outer compartment in the skins of the IκB-α−/− mice may be the end-stage response to excessive proliferation in the underlying basal epidermis. Immunostaining with a proliferative marker provided an indirect indicator that signals in the basal compartment of the epidermis are likewise affected by the deficiency in IκB-α. Although the numbers of proliferating cells were greatly increased in the IκB-α-deficient mice, surprisingly the labeled nuclei remained restricted to the basal compartment. This observation does not resemble abnormalities in psoriasiform lesions where the proliferative cell layer is typically expanded to several layers deep. Thus at least in this early stage of development (6–7 d after birth), the phenotypic signs in these mice have not had sufficient time to develop into a full-blown psoriasiform skin disease. An alternative possibility is that the phenotype developed by the IκB-α-deficient animals is a unique type of dermatosis that is not akin to psoriasis. The role of 8-LOX in both phenotypes, however, may be identical based on the fact that expression was detected in the chimeras and in the IκB-α−/− animals.

The idea that the mouse 8-LOX might assume a function in promoting keratinocyte differentiation is compatible with current concepts of the role of its human homolog. The phylogenetic counterpart of the mouse 8-LOX in humans is the second type of mammalian 15-lipoxygenase, 15-LOX-2 (Fig 1). In humans, this lipoxygenase is mainly expressed in secretory epithelial tissue, e.g., sebaceous glands of hair follicles, and the secretory epithelium in the prostate (Shappell *et al*, 2001b). In the prostate, 15-LOX-2 appears to modulate the differentiation of prostate epithelial cells (Shappell *et al*, 2001a). Its expression is lost in prostate neoplasia and adenocarcinoma (Shappell *et al*, 1999), and it acts as a negative regulator of the cell cycle in normal prostate epithelial cells (Tang *et al*, 2002). Overall, the physiological function of 15-LOX-2 is related to a differentiation phenotype, and it has been shown that 15-LOX-2-derived arachidonic acid metabolites can serve as ligands for nuclear receptors of the PPAR family (Shappell *et al*, 2001a).

Multiple studies have shown that elevated NF-κB activity can alter cellular proliferation (Seitz *et al*, 1998; Bell *et al*, 2003; Hinata *et al*, 2003). In this model, the hyperactivation of NF-κB caused by the absence of the major inhibitor IκB-α results in hyperplasia of epidermal keratinocytes and a psoriasis-like skin disease. Our data show that under these circumstances the level of 8-LOX expression increases in the stratum granulosum of the epidermis. This may be suggestive that elevated 8-LOX is not a causal component of the pathophysiology of the disease, but may be induced in an attempt to differentiate the excess cell population. This conclusion has important implications for the potential role and significance of arachidonic acid-metabolizing enzymes in therapeutics. Further insights into the participation of 8-LOX in the progression of cutaneous disease in IκB-α−/− mice could be derived from the use of genetically altered animals, 8-LOX knockouts, and 8-LOX overexpressing transgenic mice. Conceptually, a potential role of the 8-LOX as an inducing factor in keratinocyte differentiation is in clear distinction to a role in promoting the phenotype of psoriasis in the chimeric IκB-α−/− animals. To induce differentiation is to counteract the lack of differentiation, which is one of the hallmarks of psoriasis. Thus, a prospect for future studies is to examine the effects of manipulating 8-LOX expression. A central issue still to be resolved is whether the same function within the pathophysiology of psoriasis is fulfilled by phylogenetically different enzymes, 12*R*-LOX in human psoriasis and 8-LOX in the mouse model. The results of this study suggest such a possibility.

Materials and Methods

Generation of IκB-α-deficient mice The IκB-α knockout animals were generated as described before (Chen *et al*, 2000a). The animal studies have been approved by the local IRB.

Analysis of [1-^{14}C]arachidonic acid metabolites in skin homogenates Neonatal mice were selected as wild-type/heterozygote or −/− based on the appearance of the skin, and the genotype was confirmed by Southern analysis. As previously reported, the epidermal thickening and red, flaky phenotype develops only in homozygote null animals (Klement *et al*, 1996; Chen *et al*, 2000b; Huber *et al*, 2002). Six- to seven-day-old wild-type and IκB-α−/− mice were anesthetized using carbon dioxide and euthanized by decapitation. A section of dorsal skin was collected and placed on a piece of filter paper on a block of dry ice. The epidermis of the frozen skin was scraped off with a scalpel as described (Hughes and Brash, 1991) and transferred to a tube containing 300 μL 50 mM Tris-HCl and 150 mM NaCl (pH 7.5). After brief sonication (5–10 s), the samples were centrifuged briefly at 1500 × *g* in a benchtop centrifuge. To 100 μL of the supernatant, 100 μM [1-^{14}C]arachidonic acid (NEN Life Science Products, Boston, Massachussets) was added in 1 μL ethanol, and the samples were incubated with shaking at 37°C for 45 min. Products were extracted using the procedure of Bligh and Dyer (1959) including 1 μg triphenylphosphine to ensure that any arachidonate hydroperoxides were reduced to HETE. After the addition of 250 ng each of unlabeled racemic HETE as internal standards for HPLC analysis (to establish the precise retention times using an online UV detector), the formation of radiolabeled products was analyzed

by injecting a 20% aliquot of the samples on a Waters Symmetry (Waters, Milford, MA) C18 column (5 μm particle size, 25 × 0.46 cm) eluted with methanol/water/acetic acid (90/10/0.01, by volume) at a flow rate of 1 mL per min. The column effluent was monitored using an HP 1040A diode array detector coupled to a Packard Radiomatic Flo-one radioactive flow detector (Packard, Downers Grove, IL). The RP-HPLC solvent system only partly resolves 8- and 12-HETE. Therefore, the rest of the sample was run on RP-HPLC, and the HETE region of the chromatogram was collected and further analyzed by SP-HPLC using a Beckman Ultrasphere Silica 5-μm column (25 × 0.46 cm) eluted with hexane/isopropyl alcohol/acetic acid (100/2/0.1, by volume) (Beckman, Fullerton, CA).

For chiral analysis of 8-HETE and 12-HETE, the products were methylated using ethereal diazomethane and re-purified by SP-HPLC using a Beckman Ultrasphere Si 5-μm column (25 × 0.46 cm) eluted with hexane/isopropyl alcohol/acetic acid (100/1/0.1, by volume) at a flow rate of 1 mL per min. Chiral separation was performed on a Chiralpak AD column (Chiral Technologies, Exton, Pennsylvania) (25 × 0.46 cm) using a solvent of hexane/methanol (100/2, by volume) at a flow rate of 1 mL per min (Schneider *et al*, 2000). Elution from chiral phase HPLC was monitored using a Hewlett-Packard 1040A diode array detector coupled to the radioactive flow detector.

Ribonuclease protection assay RNA expression levels were analyzed in the skins of control and chimeric RAG2−/−, IκB-α−/− mice. These mice display a thickened epidermal phenotype when compared to the IκB-α−/− mice, but live into adulthood (Chen *et al*, 2000b). For RNA preparation, the complete back skins of RAG2−/−, IκB-α−/− chimeras and control mice were removed and homogenized in 2 mL of Tri Reagent (Molecular Research Center, Cincinnati, OH) using a polytron homogenizer. RNA extraction was completed following the manufacturer's instructions. 8-LOX mRNA levels in the skin were analyzed using the RiboQuant Multi-Probe RNAse Protection Assay System according to the manufacturer's instructions (PharMingen, San Diego, California). Five micrograms of total skin RNA was analyzed per reaction using ^{32}P-labeled anti-sense RNA templates specific for 8-LOX and the internal loading controls L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RNA templates specific for L32 and GAPDH were transcribed using an mCK-3b Custom Template Set (PharMingen) according to the manufacturer's instructions. An RNA template specific for 8-LOX was transcribed in a separate reaction using a cDNA template and T7 RNA polymerase to produce undigested and protected RNA templates of 478 and 402 nucleotides, respectively. To produce the 8-LOX DNA template, the *Hind*III/*Xho*I fragment of 8-LOX in pCR 2.1 (Jisaka *et al*, 1997) was subcloned into the corresponding sites of pBS SK(−) (Stratagene, La Jolla, California) and linearized with *Nco*I.

Western analysis The protein extracts were quantified using the Bradford assay (Bio-Rad, Hercules, CA), and aliquots of 30 μg of protein of the skin homogenates were separated by SDS-polyacrylamide gel electrophoresis and then transferred electrophoretically to a 0.2 μm nitrocellulose membrane (Bio-Rad). The membranes were probed using rabbit polyclonal antibodies specific for mouse 8-LOX (Jisaka *et al*, 1997) and human/mouse 12*R*-LOX. The 12*R*-LOX antibody is suitable to detect about 10 ng of recombinant 12*R*-LOX in western analyses (1:500 dilution). The following antibodies were used for the detection of additional LOX isozymes: human 5-LOX (rabbit polyclonal, Cayman Chemical, Ann Arbor, MI), human platelet 12*S*-LOX (rabbit polyclonal, Oxford Biomedical, Oxford, MI), mouse leukocyte 12*S*-LOX (rabbit polyclonal, Cayman Chemical), human 15-LOX-1 (sheep polyclonal from Dr Joseph Cornicelli and rabbit polyclonal from Dr Douglas Conrad). Recombinant human 5-LOX, human 15-LOX-1, and human platelet lysate were loaded as positive controls. For loading controls, a mouse monoclonal antibody raised against α-tubulin was used in a 1:1000 dilution (Santa Cruz, CA). Donkey anti-rabbit IgG or sheep anti-mouse IgG linked with horseradish peroxidase (Amersham Life Science, Piscataway, NJ) was used as the secondary antibody. Specifically bound protein was detected using the ECL method and detection of chemiluminescence by exposure to Hyperfilm (Amersham Life Science).

Immunohistochemical analysis Dorsal skins were harvested and fixed in 10% formaldehyde solution and processed for paraffin embedding. Tissues were sectioned, stained with hematoxylin and eosin, indicators of possible pathogenic organisms (gram staining, periodic acid Schiff stain), and a series of immunostaining assays was performed using standard markers to determine the status of epidermal proliferation and differentiation. Immunostaining for 8-LOX was carried out as described previously (Jisaka *et al*, 1997). The proliferating population of cells was demonstrated with immunostaining for Ki67. This was performed at a dilution of 1:1500 (Novacastra Labs, Newcastle, UK). The suprabasal compartment of the epidermis was selectively highlighted using keratin-1 immunostaining with a polyclonal antibody in a 1:500 dilution (Covance, Richmond, California). The most differentiated epidermal compartment was highlighted with involucrin immunostaining at a dilution of 1:500 (Covance). Tissues were further reacted with an immunomarker for T-lymphocytes (CD4) at a dilution of 1:50 (Santa Cruz) and an immunomarker for macrophages (F4/80) at a dilution of 1:100 (Serotec). All reactions were completed with an Envision+ kit from Daco (Carpintera, California), and the DAB chromagen was used. Epidermal thickness was measured in a blinded fashion from three knockout and two wild-type animals using 20 random data points along the dorsal surface of each animal.

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