Kinin B2 Receptor-Coupled Signal Transduction in Human Cultured Keratinocytes

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Kinins are key pro-inflammatory peptides that exhibit mitogenic effects in tissue-specific cellular systems. Since the life span of the keratinocyte is regulated by receptors that control proliferation and differentiation, and since both processes are affected during wound healing, we have examined the consequence of kinin B2 receptors (B2R) activation in cultured human keratinocytes. Stimulation of keratinocytes by Lys-bradykinin (LBK) induced a rapid and sustained phosphorylation of 42/44 mitogen-activated protein kinase (MAPK) that translocated to the nucleus, and decreased only after 120 min of stimulation. Kinin B1 and B2 receptor (B1R and B2R) antagonists showed that phosphorylation was mainly because of B2R activation. The GF109203X inhibitor almost completely abolished the effect of LBK, suggesting the involvement of protein kinase C in the signal cascade. MAPK phosphorylation was partially dependent on epidermal growth factor receptor transactivation as assessed by the selective inhibitor, AG1478. LBK stimulation did not result in cell proliferation, but produced a rapid c-Fos expression, nuclear translocation of nuclear factor-κB, and a moderated (pro)filaggrin synthesis, indicating that it may modulate cell differentiation. Our results support the view that kinins may affect the life span of human keratinocytes and highlight the importance that kinin peptides may have in the pathogenesis and/or progression of skin diseases.

Key words: kallikrein/keratinocyte differentiation/keratinocyte proliferation/kinins/kinin B2 receptor/MAPK/signal transduction

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Keratinocytes express several types of receptors, which once activated may induce DNA synthesis or trigger cell differentiation, events that may be relevant in the pathogenesis and/or perpetuation of skin disorders. Kinins are well-known vasoactive peptides, which exert their biological action by activating specific surface expressed B1 and B2 receptors (Bhoola *et al*, 1992). Kinin receptors belong to the superfamily of G protein-coupled receptors characterized by seven-transmembrane spanning helices although they differ in their primary structures, regulation of expression, tissue distribution, and ligand profiles. The human kinin B1 receptor (B1R) is preferentially stimulated by Lys-des[Arg^9]-bradykinin, whereas both bradykinin (BK) and Lys-bradykinin (LBK) activate the kinin B2 receptor (B2R). Most of the cellular effects produced by kinins are mediated through the B2R, that is constitutively expressed by many cell types including the keratinocyte (Schremmer-Danninger *et al*, 1995). In contrast, the B1R seems to be functionally absent under normal conditions, but is rapidly upregulated during the inflammatory response or after exposure to a noxious stimuli (Marceau, 1995). The expression of both mRNA and binding sites (Schremmer-Danninger *et al*, 1995, 1999), together with the immunoreactive substrates and the enzyme responsible for kinin generation (Poblete *et al*, 1991; Hibino *et al*, 1994; Schremmer-Danninger *et al*, 1999), have been reported in normal human skin. Functional studies have shown that BK induces phosphoinositide turnover and 1,2-diglyceride formation (Talwar *et al*, 1990) and tyrosine phosphorylation of several proteins in cultured human keratinocytes (Schremmer-Danninger *et al*, 1998).

Several studies have reported that kinins may increase DNA synthesis and cell division in several cell systems (Bhoola *et al*, 1992). some studies have, however, observed that the B2R agonist BK either does not stimulate keratinocyte proliferation (Johnson *et al*, 1992; Jung *et al*, 1999) or induces a weak response when compared with that produced by epidermal growth factor (EGF) (Coutant and Ryder, 1996). On the contrary, BK induces differentiation of PC-12 cells to a magnitude, which is similar to that produced by nerve growth factor (Kozlowski *et al*, 1989). These experiments were designed to analyze specific signaling events triggered by the B2R agonist, LBK and to evaluate the participation of this peptide in the proliferation and differentiation of human keratinocytes. Our results demonstrate that the *in vitro* stimulation of B2R induces 42/44

Abbreviations: AU, arbitrary units; B2R, kinin B2 receptor; BK, bradykinin; BrdU, 5-bromo-2′-deoxyuridine; BSA, bovine serum albumin; EGFR, epidermal growth factor receptor; HOE140, kinin B2R antagonist; IgG, immunoglobulin; LBK, Lys-bradykinin; MAPK, 42/44 mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; P-MAPK, phosphorylated MAPK; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T-MAPK, total MAPK

mitogen-activated protein kinase (MAPK) phosphorylation, which is partially dependent on EGF receptor (EGFR) transactivation. In addition, the MAPK phosphorylation pattern, the rapid c-Fos expression, the nuclear factor-κB (NF-κB) nuclear translocation, and the moderated (pro)filaggrin expression obtained after B2R stimulation provide telling evidence for the modulation of keratinocyte differentiation by B2R.

Results

Human keratinocytes in normal epidermis and in culture express immunoreactive B2R The use of previously characterized antibodies immunolabeled the B2R protein in keratinocytes, that reside in the human epidermis (Fig 1*a–c*), as well as following cell culture (Fig 1*d*, *e*). Immunoreactivity was widely distributed throughout the whole epidermis, the receptor protein being concentrated mainly on the cell surface of keratinocytes (Fig 1*b*). A similar distribution was observed for the cultured cells (Fig 1*d*). Omission and replacement of anti-B2R antibodies by non-immune rabbit immunoglobulin (IgG) and absorption with an excess of the same peptides used for immunization resulted in the absence of any staining in epidermal cells *in situ* (Fig 1*c*) or in culture (Fig 1*e*).

Stimulation of keratinocytes with LBK induces 42/44 MAPK phosphorylation Under basal conditions (untreated cells), MAPK was not significantly phosphorylated but when the cells were stimulated with different doses of LBK, a maximal response was achieved using 100 nM of the peptide (Fig 2*a*). Stimulation of cells with LBK also produced a sustained phosphorylation that lasted even after 120 min in the presence of the agonist (Fig 2*b*). In contrast, the B1 agonist des[Arg^9]-LBK induced a MAPK phosphorylation that returned to basal levels 30 min after stimulation (Fig 3*b*). Pre-treatment of keratinocytes with the kinin B2R antagonist (HOE140) prevented the phosphorylation induced by LBK, whereas the B1R antagonists des[Arg^9]-Leu^8-BK and des[Arg^9]-Leu^8-LBK (des[Arg^{10}]-Leu^8-kallidin) only had a minimal effect (Fig 3*a*). On the contrary, both antagonists prevented phosphorylation by des[Arg^9]-LBK whereas HOE140 had no effect (Fig 3*c*). These findings indicate that LBK stimulate MAPK phosphorylation in human cultured keratinocytes by activating the B2R.

Stimulation of keratinocytes with LBK produces the translocation of phosphorylated 42/44 MAPK to the nucleus In non-stimulated controls (0 min), immunoreactivity to phosphorylated MAPK (P-MAPK) was weak, and equally distributed in the cytoplasm and nucleus (Fig 4). After 5 min of treatment with LBK, a strong reaction to P-MAPK, that lasted over 30 min, was observed inside the nucleus (Fig 4). No immunoreactivity was observed when an unrelated mouse IgG, at the same concentration, replaced the primary antibody (not shown).

Effect of inhibitors and phorbol 12-myristate 13-acetate (PMA) on 42/44 MAPK phosphorylation A significant reduction of MAPK phosphorylation was observed after preincubation of keratinocytes with 1 μM of the protein kinase C (PKC) inhibitor GF109203X, and a subsequent stimulation with LBK (Fig 5). Experiments in which cells were directly stimulated for short periods (15 min) with PMA demonstrated the availability of PKC to phosphorylate MAPK in human keratinocytes. The lack of MAPK phosphorylation after stimulation of cells with LBK prior to treatment with PMA for a long period (24 h, to downregulate PKC) also suggested the involvement of PKC (Fig 5). On the other hand, preincubation of the cells with 50 μM of the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 abolished MAPK phosphorylation completely (Fig 5). Other inhibitors

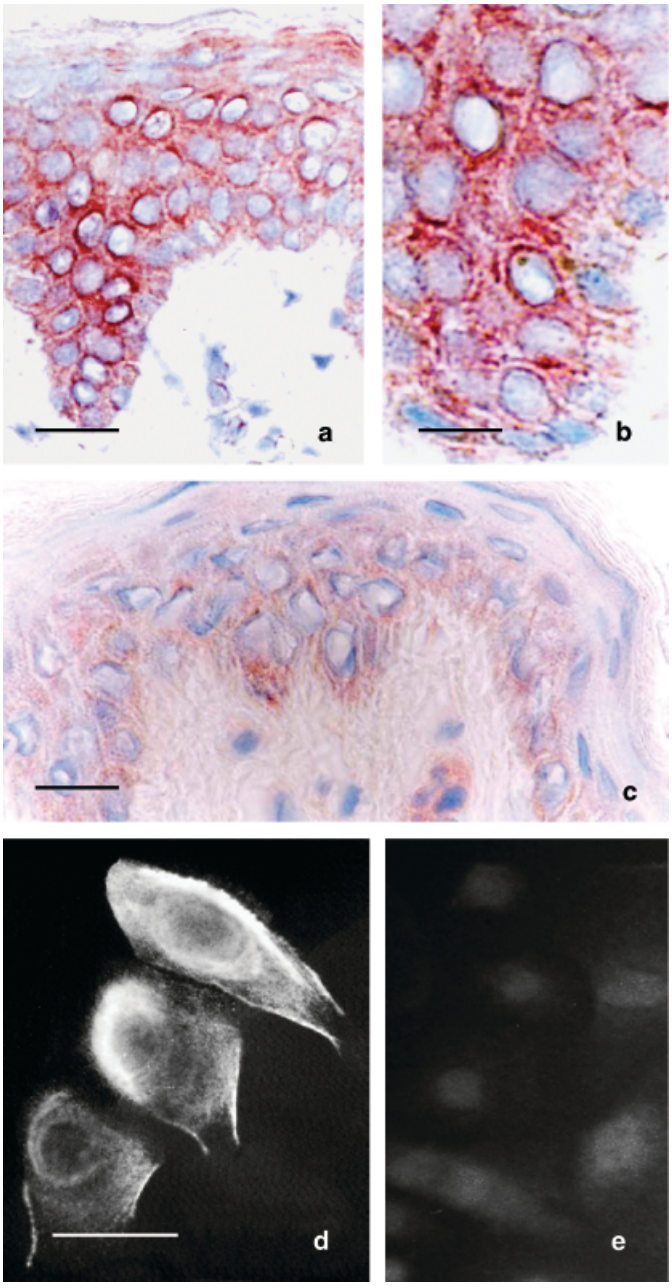

Figure 1
Human keratinocytes express immunoreactive kinin B2 receptors (B2R). (*a*, *b*) Frozen sections of normal human skin immunostained with the anti-B2R antisera mixture. Biotin–streptavidin–peroxidase ethylcarbazole system. (*d*) Visualization of kinin B2R in cultured human keratinocytes by immunofluorescence. (*c*, *e*) Controls in which the anti-B2R antibody was replaced by non-immune serum. *Scale bar* = 20 μm (*a*); 7 μm (*b*); 10 μm (*c–e*).

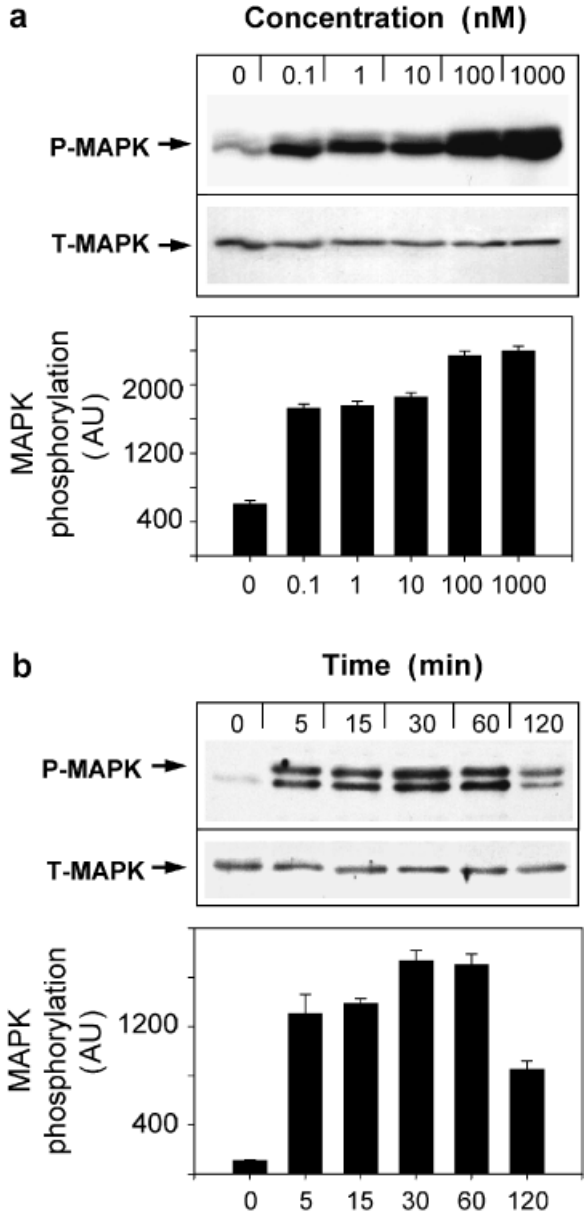

Figure 2
Lys-bradykinin (LBK) induces a sustained 42/44 mitogen-activated protein kinase (MAPK) phosphorylation. Keratinocytes were incubated with various concentrations of LBK for 5 min (*a*) or with 10^{-8} M LBK for different periods of time (*b*). Cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes that were immunoblotted with anti-phosphorylated MAPK antibodies (P-MAPK, phosphorylated MAPK). Antibodies were stripped and the membrane was incubated with anti-total MAPK (T-MAPK) antibodies (phosphorylated and non-phosphorylated, T-MAPK). AU, arbitrary units. Blots are representative of four independent experiments (n = 4).

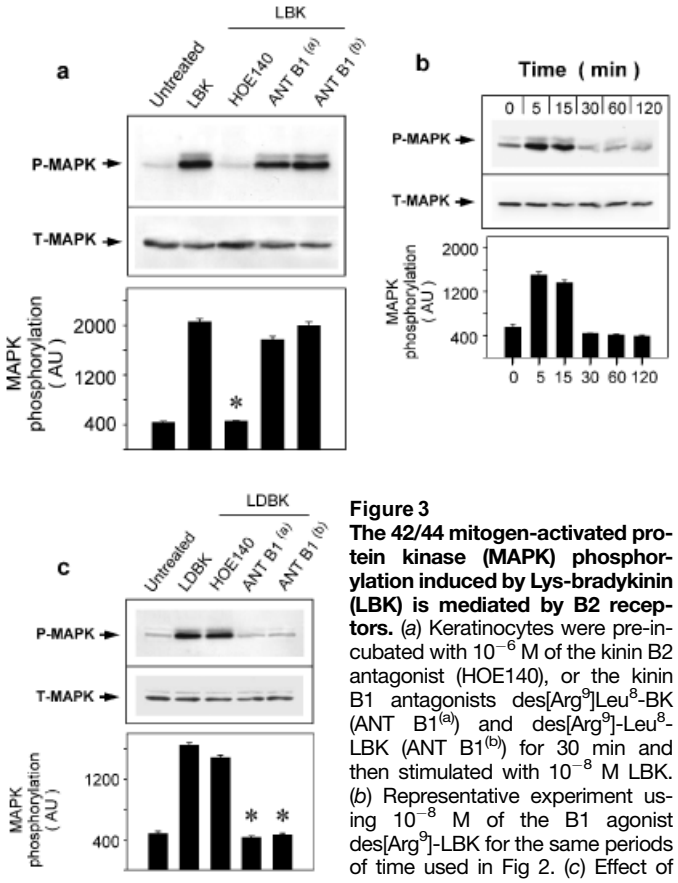

Figure 3
The 42/44 mitogen-activated protein kinase (MAPK) phosphorylation induced by Lys-bradykinin (LBK) is mediated by B2 receptors. (*a*) Keratinocytes were pre-incubated with 10^{-6} M of the kinin B2 antagonist (HOE140), or the kinin B1 antagonists des[Arg^9]Leu^8-BK (ANT B1$^{(a)}$) and des[Arg^9]-Leu^8-LBK (ANT B1$^{(b)}$) for 30 min and then stimulated with 10^{-8} M LBK. (*b*) Representative experiment using 10^{-8} M of the B1 agonist des[Arg^9]-LBK for the same periods of time used in Fig 2. (*c*) Effect of HOE140 and B1 antagonists on the phosphorylation induced by des[Arg^9]-LBK (LDBK). Values represent the mean ± SEM (n = 4). *p < 0.01.

such as 1-butanol, brefeldin A, and wortmannin gave a pattern of inhibition that was limited and only the latter produced a discrete reduction of MAPK phosphorylation (Fig 6). Keratinocytes pre-treated with 1 μM of the EGFR kinase inhibitor, AG1478, completely abolished MAPK phosphorylation when the cells were stimulated with 5 ng per mL EGF for 5 min. Concentrations lower than 1 μM (i.e. 0.1–0.5 μM) had little effect (Fig 7*a*). When keratinocytes were pre-treated with 1 μM AG1478, before stimulation with LBK, MAPK phosphorylation was reduced but not to the levels observed in untreated or EGF-stimulated cells pre-treated with the same inhibitor (Fig 7*b*).

LBK does not induce keratinocyte proliferation To investigate the proliferative action of LBK, a range of peptide concentrations (0.1–1000 nM) was tested. The 5-bromo-2′-deoxyuridine (BrdU) incorporation assay used revealed that LBK failed to induce any significant cell proliferation (Fig 8). This observation was confirmed when keratinocytes, grown under identical conditions, responded in a concentration-dependent manner to EGF stimulation (Fig 8). Keratinocyte proliferation reached a plateau at 10 nM EGF. Other positive controls, carried out when growing the cells in culture medium containing growth factors, also exhibited a high BrdU incorporation rate (not shown).

LBK activates NF-κB Immunofluorescence and western blotting of nuclear and cytoplasmic extracts showed that incubation of keratinocytes with 100 nM LBK produced a rapid and significant translocation of NF-κB (p65) to the nucleus (Fig 9). Immunofluorescence showed that LBK-induced translocation was evident between 5 and 60 min of treatment with the peptide (Fig 9*a*). Identification of p65 by western blotting pointed out that immunoreactivity was almost doubled in the nucleus of LBK-stimulated keratinocytes (Fig 9*b*).

LBK induces a rapid expression of c-Fos Expression of c-Fos was clearly visible after 1 h of stimulation and then

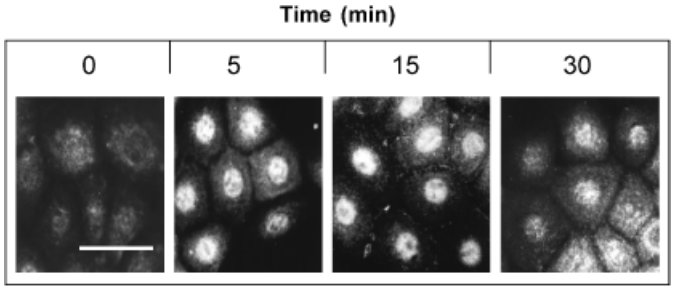

Figure 4
Lys-bradykinin (LBK) produces nuclear translocation of phosphorylated 42/44 mitogen-activated protein kinase (MAPK). Cells were grown to subconfluence and then stimulated with 100 nM LBK for 5–30 min, fixed, permeabilized, and incubated with anti-P-MAPK (phosphorylated MAPK) antibodies and with fluorescein-labeled $F(ab')_2$ anti-mouse immunoglobulin G. *Scale bar* = 25 μm.

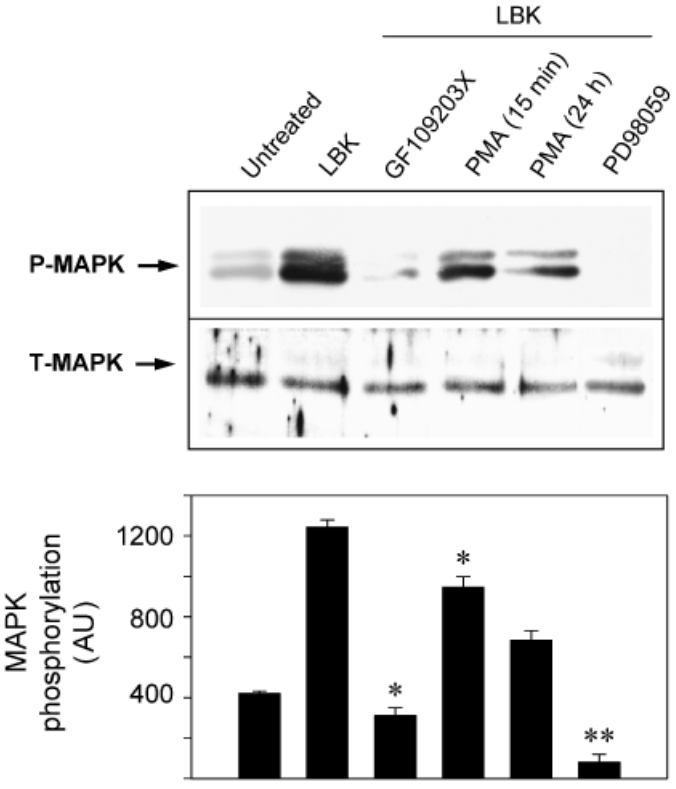

Figure 5
42/44 mitogen-activated protein kinase (MAPK) phosphorylation is mediated by protein kinase C (PKC) and mitogen-activated protein kinase kinase (MEK). Cells were stimulated with 10^{-8} M Lys-bradykinin (LBK) after pre-incubation with the PKC inhibitor GF109203X (1 μM, for 30 min), the phorbolester phorbol 12-myristate 13-acetate (PMA) (500 nM for 24 h), and the MEK inhibitor PD98059 (50 μM for 30 min). Additionally, keratinocytes were incubated directly with 50 nM PMA for 15 min. Values represent the mean ± SEM (n = 3). $^{*}p < 0.05$ and $^{**}p < 0.01$, with respect to LBK-stimulated cells.

declined gradually after 3–6 h (Fig 10*a*). Immunocytochemistry using the same antibody revealed intense c-Fos immunoreactivity in the nuclei of keratinocytes after 1 h of stimulation with the peptide (Fig 10*b*). c-Fos expression under basal conditions and after 6 h of LBK stimulation was almost undetectable.

B2R stimulation induces a moderated (pro)filaggrin synthesis A clear expression of the terminal differentiation

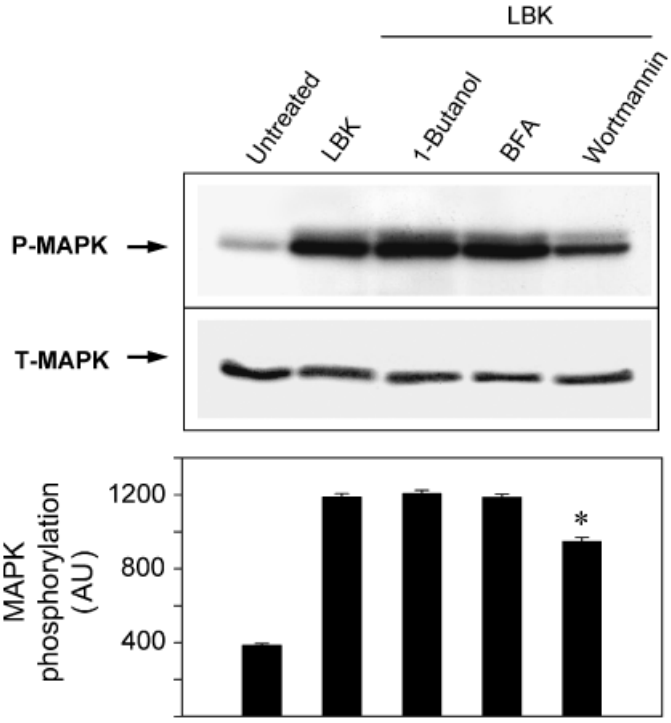

Figure 6
Effect of 1-butanol, brefeldin A, and wortmannin on 42/44 mitogen-activated protein kinase (MAPK) phosphorylation. Keratinocytes were stimulated with 10^{-8} M Lys-bradykinin (LBK) after 30 min of pre-incubation with 2% v/v 1-butanol, 10 μg per mL brefeldin A (BFA), and 50 nM wortmannin. Values represent the mean ± SEM (n = 3). $^{*}p < 0.05$, with respect to LBK-stimulated cells.

marker, (pro)filaggrin, was observed in control experiments carried out with the CD40 agonist M89 (Fig 11*b*). In contrast, a moderated increase in (pro)filaggrin expression was observed after stimulation with 100 nM LBK (Fig 11*a*). This increase was reduced when stimulation of cells was carried out in the presence of an excess of the HOE140, but only partially when the B1R antagonist des[Arg^9]-Leu^8-BK was used (Fig 11*a*). The effect produced by LBK was not observed when keratinocytes were stimulated after reaching confluence (not shown). By comparison, stimulation of keratinocytes with an unrelated mouse IgG_1 (control for M89) at the same concentration did not induce (pro)filaggrin expression in the same magnitude as that produced by the M89 ligand.

Discussion

The existence of a kinin system in human skin is a well-documented fact. The kinin-forming enzyme, tissue kallikrein, has been localized in the secretory fundus of sweat glands (Poblete *et al*, 1991), and several authors have described its biochemical properties (Mann *et al*, 1980; Mayfield *et al*, 1989; Hibino *et al*, 1994). Recently, the mRNA for *true* kallikrein (KLK1, tissue kallikrein, kininogenase) and those for the B1R and B2R have been reported for the normal human skin, and in biopsies of patients with some skin diseases (Schremmer-Danninger *et al*, 1999). Furthermore, high-molecular-weight kininogen has been visualized in the interstitial tissue space of guinea-pig epidermis (Yamamoto *et al*, 1987), a fact also observed in the human epidermis

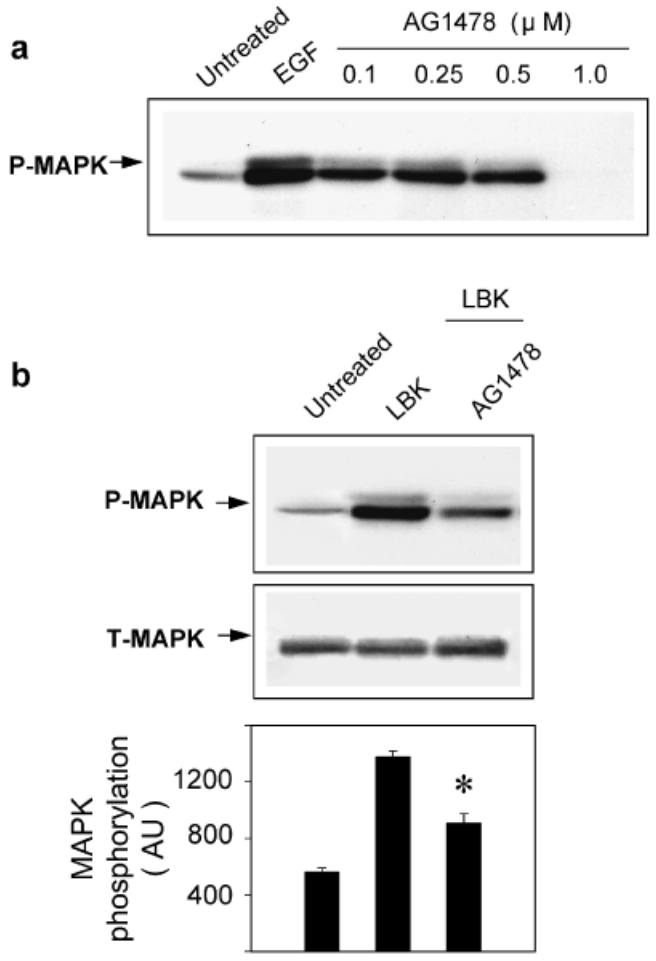

Figure 7
Lys-bradykinin (LBK) induces a partial epidermal growth factor receptor (EGFR) transactivation. (*a*) Keratinocytes were first pre-treated with various concentrations of AG1478 for 30 min and then stimulated with 5 ng per mL of EGF for 5 min. (*b*) In a separate set of experiments, keratinocytes were pre-treated with 1 μM AG1478 for 30 min and then stimulated with 10^{-8} M LBK. Values represent the mean ± SEM (n = 3). *p < 0.01.

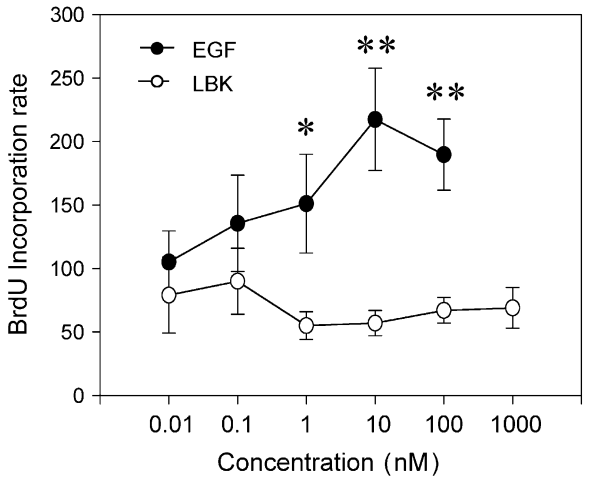

Figure 8
Lys-bradykinin (LBK) does not induce keratinocyte proliferation. Cells were grown on 96-well plates and stimulated with various doses of LBK (open circles) or epidermal growth factor (EGF) (closed circles). The 5-bromo-2′-deoxyuridine (BrdU) incorporation rate was determined by a colorimetric cell proliferation immunoassay and measuring absorbance at 450 nm. Values represent the mean ± SEM (n = 4). *p < 0.001; **p < 0.0001.

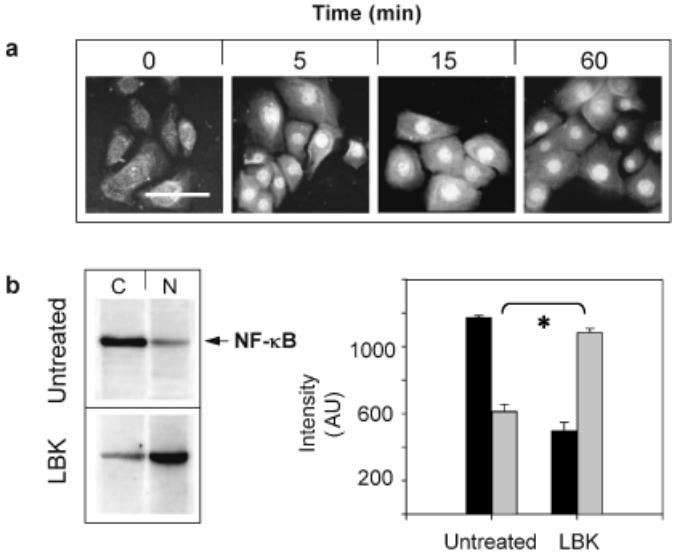

Figure 9
Lys-bradykinin (LBK) activates nuclear factor-κ B (NF-κB) inducing its translocation to the nucleus. (*a*) Cells were grown to subconfluence and then stimulated with 100 nM LBK, fixed, permeabilized, and incubated with an anti-p65 NF-κB subunit antibody and a fluorescein-labeled $F(ab')_2$ anti-rabbit immunoglobulin G. *Scale bar* = 25 μm. (*b*) Nuclear (N) and cytoplasmic (C) fractions, isolated from untreated and LBK-stimulated cells, were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose, and immunoprinted with anti-p65 antibody. Values represent the mean ± SEM (n = 3). *p < 0.01.

when antibodies to both kininogens are used (C. D. Figueroa, unpublished data). These data indicate that both kallikreins and the kinin-containing substrates are normally present in human skin, making viable the concept that kinins are formed in this organ.

Our results indicate that the human epidermis and cultured keratinocytes express immunoreactive and functional B2R, which respond to agonist stimulation *in vitro*, with 42/44 MAPK phosphorylation, NF-κB nuclear translocation, a rapid c-Fos expression, and a moderated (pro)filaggrin synthesis. In contrast, B2R stimulation does not result in keratinocyte proliferation, although high doses of the peptide were used, an observation that is in agreement with other studies in which this possibility had been investigated (Johnson *et al*, 1992; Jung *et al*, 1999).

The MAPK phosphorylation induced by LBK was mainly related to B2R stimulation, as demonstrated by the use of two B1 and one B2 receptor antagonists. Moreover, the phosphorylation pattern observed after stimulation of keratinocytes with the B1 agonist des[Arg^9]-LBK was completely different from that elicited by LBK. Time-course experiments suggested that LBK might be associated to keratinocyte differentiation, since sustained MAPK phosphorylation (up to 120 min) has been aligned to cell differentiation rather than proliferation by other authors (Harada *et al*, 2001). Moreover, one previous study shows that BK causes the differentiation of PC-12 cells in a magnitude similar to that produced by nerve growth factor and potentiates the neurite-extending effect when added in combination with the growth factor (Kozlowski *et al*, 1989). Several studies have implicated transcription factors (which

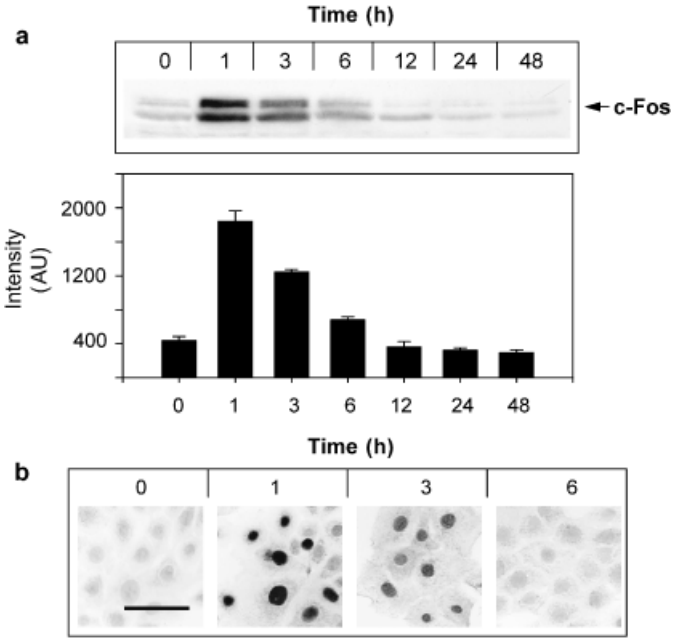

Figure 10
Lys-bradykinin (LBK) induces an early expression of c-Fos. (*a*) Keratinocytes were incubated for various periods of time with 10^{-8} M LBK. Cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose and immunoprinted with anti-c-Fos antibody. Values represent the mean ± SEM (n = 3). (*b*) In parallel experiments, cells were fixed, permeabilized, and immunostained with the anti-c-Fos antibody and the biotin/streptavidin–peroxidase ethylcarbazole system. *Scale bar* = 25 μm.

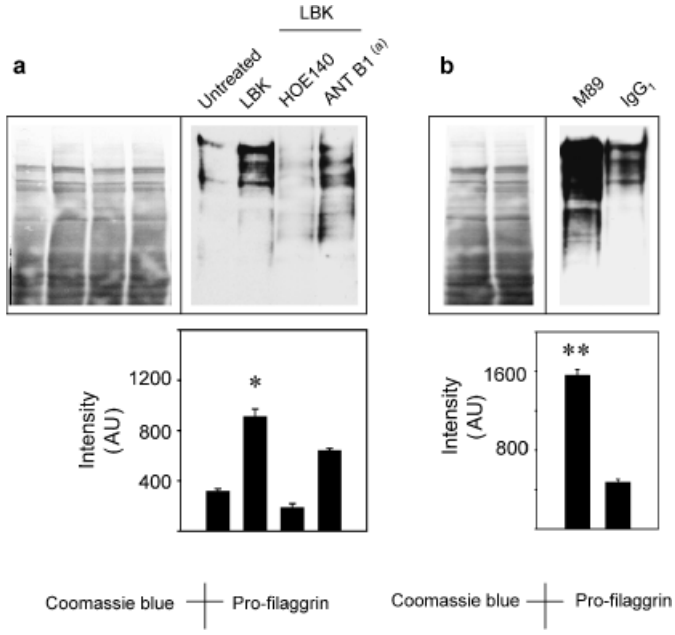

Figure 11
Lys-bradykinin (LBK) stimulates a moderated (pro)filaggrin synthesis. (*a*) LBK (10^{-7} M) was used as a single dose and in the presence of ANT B1$^{(a)}$ (the B1 receptor antagonist des[Arg^9]Leu^8-BK, 10^{-6} M) or HOE140 (the B2R antagonist, 10^{-6} M). (*b*) As positive controls, keratinocytes were stimulated with anti-CD40 monoclonal antibody, M89; immunoglobulin (Ig) G_1 is an isotype-matched mouse IgG used as a control for M89. To ensure that an equal amount of protein is present in each lane, the nitrocellulose membranes were stained with Coomassie blue. Blots are representative of three independent experiments and values represent the mean ± SEM (n = 3). $^*p < 0.05$, with respect to untreated cells and HOE140; $^{**}p < 0.001$, with respect to IgG_1.

bind to AP-1 regulatory sites), and NF-κB as key regulators of the keratinocyte differentiation program. Our experiments indicate that stimulation of human keratinocytes with LBK induces a rapid c-Fos expression. Previous studies on HaCat epidermal cells have shown that BK causes a rapid (10 min) and transient accumulation of c-Fos and c-Jun mRNA (Coutant and Ryder, 1996). Further, a somewhat broader pattern of distribution for c-Fos has been observed in the spinous and granular layers, but almost completely absent in the basal stratum of the normal human epidermis (Eckert and Welter, 1996; Angel *et al*, 2001). This expression pattern in proliferation-incompetent cells has led to the postulation that in the human epidermis this AP-1-binding protein may be linked to a differentiation program rather than cell proliferation. Similarly, nuclear translocation of NF-κB has been detected only in non-proliferative cells of the suprabasal layers of human epidermis, suggesting a role for this molecule in the switch from proliferation to growth arrest and differentiation. In fact, functional and pharmacological blockade of NF-κB produces a hyperplastic epithelium *in vivo*, whereas overexpression of active p50 and p65 NF-κB subunits generates hypoplasia and growth inhibition (Seitz *et al*, 1998). Our study shows that LBK activates NF-κB inducing its nuclear translocation, thereby supporting a role for B2R in the differentiation of human keratinocytes. Interestingly, tumor necrosis factor-α and interferon-γ, which stimulate nuclear translocation of NF-κB, also inhibit keratinocyte proliferation (Takao *et al*, 2003), whereas lipopolysaccharide and dexamethasone do not activate NF-κB and at the same time have a small effect on keratinocyte proliferation. Our results add new evidence to early articles on MAPK phosphorylation (Clerk *et al*, 1996; Velarde *et al*, 1999), NF-κB activation (Pan *et al*, 1996), and c-Fos expression (El-Dahr *et al*, 1998) in different cell systems after B2R stimulation. An extra support for a role of B2R agonists in keratinocyte differentiation is our observation of moderated (pro)filaggrin expression following stimulation of subconfluent cells with LBK, a novel effect that has not been described before in human keratinocytes. Comparative control experiments carried out to activate CD40, a molecule associated to keratinocyte differentiation (Concha *et al*, 2003), confirmed that the response evoked by LBK was indeed moderated.

Finally, our results indicate that PKC is clearly implicated in MAPK phosphorylation since the GF109203X inhibitor completely abolished the stimulatory effect of LBK. Furthermore, downregulation of PKC after prolonged phorbolester treatment resulted in a reduction of MAPK phosphorylation after stimulation with LBK. On the other hand, the MAPK phosphorylation induced by LBK was not reduced by brefeldin A and 1-butanol, but it was partially diminished by wortmannin, a phosphoinositide 3 kinase inhibitor. These experiments suggest that phospholipase D and the small guanosine 5′-triphosphate (GTP)-binding protein adenosine 5′-diphosphate ribosylation factor (ARF) may not participate in the differentiation program activated by LBK. Previous studies have shown that BK activates phospholipase D transiently, an effect that does not seem to be of sufficient magnitude to trigger differentiation in mouse keratinocytes.

Experiments orientated to elucidate whether LBK-stimulated-MAPK phosphorylation was dependent on EGFR transactivation showed that phosphorylation was only partially mediated by EGFR activation, since the selective inhibitor of EGFR kinase, AG1478 (tyrphostin), did not reduce MAPK phosphorylation to the levels observed in non-stimulated or EGF-stimulated cells pre-treated with the inhibitor. Transactivation of EGFR, mediated by stimulation of G protein-coupled receptors, occurs in diverse cell types (Daub *et al*, 1997) and in several of these receptors, including the B2R (Adomeit *et al*, 1999). Our results indicate that MAPK phosphorylation induced by LBK is only partially regulated by EGFR phosphorylation, confirming previous studies showing that in addition to the ligand itself, EGFR transactivation also depends on the cell type involved. A similar observation has been reported for A431 cells where BK independently activates MAPK via a pathway that is sensitive towards inhibitors of phosphoinositide 3 kinase and PKC, but not to AG1478, corroborating that EGFR transactivation is not necessarily a prerequisite for G protein-coupled receptors-induced activation of MAPK (Graness *et al*, 2000).

In summary, B2R agonists do not induce cell proliferation, but trigger a cellular pathway that may initiate a mild keratinocyte differentiation, a response highly relevant for the life span of keratinocytes during skin wound healing and inflammation.

Materials and Methods

Tissue samples Patients who underwent reductive surgery and had given written consent to the attending surgeon donated the skin samples, which were used in this study. Written consent from the patients and sample collection followed the guidelines stipulated by the Medical Ethical Committee of Universidad Austral de Chile and the Declaration of Helsinki principles.

Visualization of B2R Skin tissue samples were rapidly frozen in liquid nitrogen and stored until use. Frozen sections (8 μm thick) were air-dried and washed with PBS, pH 7.4. Next, they were treated with 0.5% IgG-free bovine serum albumin (BSA) for 20 min and then incubated with a mixture of well-characterized anti-peptide antibodies (1:6000) directed to the intra- and extra-cellular domains of the B2R (Figueroa *et al*, 1995), kindly provided by Prof. Werner Müller-Esterl of the Institute for Biochemistry II, Johann Wolfgang Goethe-University, Frankfurt, Germany. Bound IgG were detected with the LSAB + biotin/streptavidin–peroxidase kit (Dako, Carpinteria, California) and peroxidase was visualized by incubation with 3-amino-9-ethylcarbazol (Dako). Sections were finally contrasted with hematoxylin and mounted with Mowiol (Polysciences Inc., Warrington, Pennsylvania). Bound IgG were also detected by a fluorescein-labeled $F(ab')_2$ anti-rabbit IgG. Controls included omission of primary antibody, incubation of the anti-peptide antibody with an excess of the same peptides used for immunization, or its replacement by non-immune serum of the same origin (Figueroa *et al*, 1995).

Human keratinocyte culture Cultures were prepared as previously described (Concha *et al*, 2003). Briefly, skin pieces split cut with a keratome device were incubated for 1 h at 37°C with 0.05% trypsin (Life Technologies, Rockville, Maryland) to obtain epidermal sheets that were then pooled in Hanks' balanced salt solution (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah). A single-cell suspension was prepared using a fine scissors, repeated pipetting, and filtration through gauze. Keratinocyte culture was established on lethally irradiated 3T3 fibroblast (10×10^3 cells per cm^2) in a 3:1 mixture of Dulbecco's-modified Eagle's and Ham's F-12 media (Life Technologies) containing 10% fetal bovine serum, 0.4 μg per mL hydrocortisone, 5 μg per mL insulin, 10^{-10} M cholera toxin, 10 μg per mL EGF, and antibiotics. Cells were cultured until subconfluence and then passaged by dissociation with 0.25% trypsin-EDTA (Life Technologies) and reseeded on irradiated fibroblasts. Cells were used after two or three passages and in all experiments they were grown in a defined keratinocyte serum-free and low calcium medium (DK-SFM; Life Technologies).

MAPK phosphorylation Quiescent subconfluent keratinocytes (grown for 24 h in the absence of growth factors) were stimulated with 0.1–1000 nM of LBK (Bachem Inc., Torrance, California) for various periods of time. Experiments using 100 nM of the kinin B1 agonist des[Arg^9]-LBK were carried out as a comparison. Stimulation was stopped by addition of 100 μL of ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.25% deoxycholic acid, 1% Nonidet P40, 1 mM EDTA, 1 mM Na_3VO_4, 250 μg per mL *p*-nitrophenyl phosphate, 1 mM phenylmethane–sulfonyl fluoride, 1 mM NaF, 1 μg per mL leupeptin, 1 μg per mL pepstatin, and 1 μg per mL aprotinin). Cells were scraped, sonicated, and the amount of protein for each time point was measured. Equal amount of protein (25 μg), dissolved in a sample buffer containing 2.5% mercaptoethanol, was subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred onto nitrocellulose filters. After blocking of non-specific-binding sites with 5% skimmed milk, blots were incubated overnight with a monoclonal anti-phospho 42/44-MAPK antibody (Cell Signaling Technology Inc., Beverly, Massachusetts) in 0.05 M Tris-HCl containing 0.1% Tween 20 (Sigma, St. Louis, Missouri) and 5% BSA. Bound antibodies were detected using a chemiluminescence kit (Pierce, Rockford, Illinois). The total amount of MAPK present in each sample was assessed on the same membrane using a polyclonal anti-MAPK antibody (1:1000). In some cell types, this antibody reacts mainly with the 42-kDa band (Cell Signaling Technology). For this purpose, the antibodies used for the first immunodetection were previously stripped for 30 min at 50°C with 62.5 mM Tris-HCl, pH7.4, containing 100 mM mercaptoethanol and 2% SDS.

Effect of kinin receptor antagonists and inhibitors To establish the kinin receptor type activated, quiescent keratinocytes were pre-incubated for 30 min with an excess (10^{-6} M) of the HOE140 (Aventis Pharma Deutschland GmbH, Frankfurt, Germany) or the B1R antagonists des[Arg^9]-Leu^8-BK (Sigma) and des[Arg^9]-Leu^8-LBK (des[Arg^{10}]-Leu^8-kallidin; Bachem Inc.). To investigate the pathway involved in MAPK phosphorylation, cells were pre-incubated for 30 min with 1 μM GF109203X (Calbiochem), a PKC inhibitor; 50 μM PD98059 (Calbiochem, San Diego, California), an MAPKK (MEK) inhibitor; 50 nM PMA for 15 min or 500 nM for 24 h; 10 μg per mL brefeldin A, an inhibitor of small GTP-binding protein ARF; 2% v/v 1-butanol, a phospholipase D inhibitor, and 50 nM wortmannin, a phosphoinositide 3 kinase inhibitor. The potent and selective inhibitor of EGFR kinase, AG1478 (tyrphostin), was used to determine whether MAPK phosphorylation was influenced by EGFR transactivation. For this purpose, cells were pre-incubated with 1 μM of AG1478 for 30 min and then stimulated with LBK (10^{-8} M) for 5 min. The concentration of AG1478 to be used was determined by pre-incubating keratinocytes with various concentrations of the inhibitor before stimulation with EGF (5 ng per mL) for 5 min.

Cell proliferation assay Keratinocytes were seeded on 96-well plates and cultured at subconfluence. After 48 h in the absence of growth factors, cells were stimulated with various doses of LBK. The culture medium was changed after 24 h, the cells stimulated again with the same dose of LBK, and pulsed with BrdU for another 24 h. The incorporation rate of BrdU was determined by a colorimetric immunoassay according to the manufacturer's protocol (Roche Diagnostics GmbH, Penzberg, Germany). Control experiments were carried out stimulating keratinocytes with EGF. Four

experiments were carried out for each concentration point and each point was performed in duplicate.

Activation of the transcription factors c-Fos and NF-κB Activation of c-Fos and NF-κB was investigated by western blotting and immunocytochemistry using commercial antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, California). The antibody to c-Fos, directed to the amino terminus of the human molecule, does not cross-react with Fos B, Fra-1, or Fra-2. NF-κB was identified using an antibody that recognizes the 65 kDa subunit. For western blotting, both antibodies were used at a 1:1000 dilution. In addition, activation of NF-κB was assessed on cytoplasmic and nuclear extracts using the method of Gerber *et al* (1992). Briefly, quiescent keratinocytes were stimulated with 100 nM LBK for 60 min as described before and then lysed with 400 μL of extraction buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 1 μg per mL leupeptin, 1 μg per mL aprotinin, and 1 mM phenylmethane–sulfonyl fluoride). After 20 min on ice, 25 μL of 10% Nonidet P-40 was added and then centrifuged 10,000 *g*. for 2 min at 4°C. The supernatant (cytoplasmic extract) was collected and the pellet treated with nuclear extraction buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM EGTA, 0.4 M NaCl, 1 mM dithiothreitol, 1 μg per mL leupeptin, 1 μg per mL aprotinin, and 1 mM phenylmethane–sulfonyl fluoride), vortexed for 1 min and left to stand on ice for 10 min. After centrifugation 10,000 *g*. for 10 min at 4°C, the supernatant (nuclear extract) was collected. Proteins of both extracts were separated by SDS-PAGE and p65 subunit detected by chemiluminescence as described above.

Immunocytochemistry The intracellular distribution of P-MAPK, NF-κB, and c-Fos was investigated by stimulating keratinocytes, grown on permanox tissue culture chambers (Nunc Brand Products, Naperville, Illinois), with 100 nM LBK. Cells fixed for 10 min with 4% paraformaldehyde dissolved in PBS, pH 7.4, were permeabilized with methanol at −20°C and incubated with anti-phospho 42/44-MAPK (1:100), anti-p65 (1:20), or anti-c-Fos (1:800). Bound IgG were detected with the aid of fluorescein-labeled $F(ab')_2$ antibodies (Roche Diagnostics GmbH) or with the LSAB + kit to increase sensitivity (Dako).

Evaluation of (pro)filaggrin expression Expression of (pro)filaggrin has been widely used to evaluate keratinocyte terminal differentiation and under SDS-PAGE is normally observed as a 400 kDa product accompanied by several proteolytic breakdown products (Fleckman *et al*, 1985). Quiescent subconfluent keratinocytes were stimulated once with 100 nM LBK and cultured for 2 extra days. The (pro)filaggrin production was estimated by SDS-PAGE on a 6% polyacrylamide gel and immunoprinting with the AKH-1 monoclonal antibody (1:1000; Biomedical Technologies, Stoughton, Massachusetts). Controls were carried out simultaneously by culturing cells in the absence of ligand and using B1R and B2R antagonists. Positive controls were performed with the M89 monoclonal antibody that activates CD40 to elicit keratinocyte differentiation; its specificity was assessed by replacing M89 by an unrelated mouse IgG_1 (Concha *et al*, 2003). Homogenates were prepared in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM β-mercaptoethanol, 9 M urea, 1 mM phenylmethane–sulfonyl fluoride, 1 mM NaF, 1mM Na_3VO_4, 1 μg per mL aprotinin, 1 μg per mL pepstatin, and 1 μg per mL leupeptin).

Quantitative image analysis The intensity of immunoreactive protein bands, visualized after immunoblotting, was quantified using an automated image digitizing system (Un-Scan-It, Silk Scientific Inc., Orem, Utah) as previously described (Figueroa *et al*, 2001).

Statistical analysis Statistical evaluation of intensity in immunoblots and cell proliferation assays was carried out with the Student *t* test. Values are expressed as mean ± SEM, and significance was considered acceptable at the 5% level ($p < 0.05$).

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