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Abbreviations used: HB-EGF: heparin-binding epidermal growth factor-like growth factor, MβCD: methyl-beta-cyclodextrin, ATP: Adenosine 5'-triphosphate, EGFR: epidermal growth factor receptor.

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ABSTRACT

The Heparin-Binding EGF-like Growth Factor (HB-EGF) is an autocrine/paracrine keratinocyte growth factor which binds to the EGF (Epidermal Growth Factor) receptor family and plays a critical role during the re-epithelialization of cutaneous wound by stimulating the keratinocytes proliferation and migration. In this study, cellular stressing condition in autocrine cultures of human keratinocytes was induced by cholesterol depletion methyl-beta-cyclodextrin (MβCD). MβCD treatment induces the expression and the release of HB-EGF. By analysis of the culture media, large amounts of cellular ATP were measured particularly after 1h of MβCD treatment. To investigate whether ATP contributes to the expression of HB-EGF, the nonhydrolyzable ATP analogue, ATP-γ–S, was used to mimic the extracellular ATP released. We report that keratinocytes stimulated with ATP-γ-S induce HB-EGF expression and activate EGFR and ERK1/2. Using an antagonist of P2 purinergic receptors, we demonstrate that HB-EGF synthesis induced by lipid rafts disruption is dependent on ATP interaction with P2 purinergic receptors. Moreover, our data suggest that both MAPKs p38 and ERK1/2 are involved together or independently in the regulation of HB-EGF gene expression. These findings provide new insight into the signalling pathway by which HB-EGF is expressed after lipid rafts disruption. In summary, after lipid raft disruption, keratinocytes release large amount of extracellular ATP. ATP induces HB-EGF synthesis and release by interacting with the P2 purinergic receptor and through p38 and ERK1/2 signaling in response to a challenging environment. A release of ATP acts as an early stress response in keratinocytes.

INTRODUCTION

The skin is the most outer organ of the mammalian body and so its major function is to provide at the epidermis level, a protective barrier against dehydration and insults from the environment. The epidermis is mainly composed of keratinocytes which undergo a complex differentiation program leading to keratinization. The maintenance of the keratinized barrier is a process regulated by keratinocyte proliferation and differentiation, thus when injuries affect the epidermal barrier it is crucial to rapidly restore the original tissue. The epidermis is able to hyperproliferate in response to several stimuli, including growth factors secreted by the keratinocyte itself or by other cells (macrophages, platelets …). More particularly, keratinocytes respond to members of the epidermal growth factor (EGF) family. Here, we focus on the Heparin-Binding EGF-like Growth Factor (HB-EGF) which is an autocrine keratinocyte growth factor (Hashimoto et al., 1994; Raab and Klagsbrun, 1997). Similarly to all other members of the EGF family of growth factors, HB-EGF is synthesized as a transmembrane protein, proHB-EGF, which can be shed enzymatically by metalloproteases in order to release the soluble growth factor (Nishi and Klagsbrun, 2004). At cellular level, it has been shown that HB-EGF binds and activates the EGF receptor (EGFR), stimulating the keratinocyte proliferation and migration (Nishi and Klagsbrun, 2004). In vivo, Marikovsky et al, (1993) have first detected HB-EGF in wound fluid of pigs and HB-EGF also appeared after analysis of burn wound fluid from human, who had sustained partial thickness burns (Marikovsky et al., 1993; McCarthy et al., 1996). These findings support an important role of HB-EGF in skin wound healing and studies reveal that during the re-epithelialization of cutaneous wound, HB-EGF stimulates the migration and the proliferation of keratinocytes (Hashimoto et al., 1994; Tokumaru et al., 2000).

Cholesterol, an essential component of the plasma membrane involved in membrane structure and function, is especially present in specific microdomains called lipid rafts. Previous studies from our laboratory have shown that cellular stress with methyl-β-cyclodextrin, a molecule that extracts cholesterol from the plasma membrane and thereby disrupts of lipids rafts, strongly induces the expression of HB-EGF in human keratinocytes through the activation of p38 mitogen-activated protein kinase (Jans et al., 2004; Mathay et al., 2008). However, the mechanisms leading to the expression of HB-EGF have not been fully established.

ATP is known to be the principal intracellular energy source in cells but it has been demonstrated that a number of cell types release ATP in the extracellular environment in response to mechanical stress or biological activation (Hansen et al., 1993; Pastore et al., 2007). The first evidence that ATP has extracellular effects was noted in 1929 by Drury & Szent-Gyorgyi in their study of the effects of adenine compounds on the heart (Drury and Szent-Gyorgyi, 1929). Since this discovery of an involvement in cardiac function, many studies have revealed that extracellular ATP contributes to regulate of a variety of biological processes, including neurotransmission, smooth muscle contraction, vasodilatation, bone metabolism, cell proliferation and differentiation, platelet activation and inflammation (Agteresch et al., 1999; Birk et al., 2002; Burnstock and Knight, 2004; Hoebertz et al., 2003; Schwiebert and Zsembery, 2003). Despite this knowledge, the mechanisms and physiological roles of cellular ATP release are incompletely understood. Once released, the extracellular ATP activates P2 purinergic receptors, a family of transmembrane receptors which has been divided into two classes: the P2X (ligand-gated ion channels) receptors and the P2Y (G-protein-coupled) receptors (Boarder and Hourani, 1998). During cutaneous wound healing, skin is exposed to extracellular ATP released from platelets and damaged cells (Burrell et al., 2005; Huang et al., 1989; Weinger et al., 2005) and Dixon et al., (1999) have demonstrated that ATP released promotes keratinocyte proliferation. Moreover, during wound healing, extracellular ATP acts synergistically with growth factors such as PDGF, TGF-α or EGF to enhance DNA synthesis and to promote cell proliferation (Wang et al., 1990). Recently, Yin et al. demonstrated that ATP released from epithelial cells upon scratch wound was able to induce HB-EGF shedding (Yin et al., 2007).

In the present work, we focused on the keratinocyte stress response induced by cholesterol depletion using MβCD. We demonstrate for the first time that stress conditions like lipid rafts disruption by MβCD in human keratinocyte, induce a strong release of ATP which can be responsible for the expression and shedding of HB-EGF. These results suggest that a release of ATP, consecutive to membrane microdomains alterations in keratinocytes, induces HB-EGF expression and release which can be crucial for epidermal healing and homeostasis.

MATERIALS AND METHODS

Antibodies and chemicals

MBCD, Suramin, CRM197 and Apyrase were obtained from Sigma-Aldrich (Munich, Germany). PD 98059 was from Calbiochem VWR (Leuven, Belgium). Goat anti-human HB-EGF antibody was obtained from R&D Systems (Abingdon, UK). Mouse anti-phospho-ERK1/2, rabbit anti-ERK1/2, rabbit anti-p38, anti-phospho-p38 and rabbit anti-human EGFR antibody were purchased from Cell Signaling (Leiden, The Netherlands). Rabbit anti-human phospho-EGFR Tyr1173 antibody was from Biosource (Nivelles, Belgium). Serum-free keratinocyte growth medium was from Lonza (Verviers, Belgium). Serum-free keratinocyte complete culture medium (Epilife and HKGS) and keratinocyte autocrine culture medium (Epilife without HKGS) were from Cascade Biologics (Mansfield, UK).

Culture of human keratinocytes

Normal human adult abdominal skin samples were obtained from plastic surgery (Dr Bienfait, Clinique St Luc, Namur-Bouge, Belgium). Superficial skin samples were cut with a dermatome and keratinocytes were isolated following the trypsin float technique (Wille et al., 1984). Primary cultures were initiated in KGM2. Proliferating primary cultures were trypsinized and keratinocytes were plated into secondary cultures at 6 X 10^3 cells/cm^2 in complete culture medium (EpiLife containing HKGS). When the cells covered approximately 50% of the culture substratum, keratinocytes were switched to an autocrine medium (EpiLife alone) which does not contain any growth factor (Minner et al., 2010). In such conditions, keratinocytes proliferate autonomously until the confluence of the culture is reached concomitantly with cell growth arrest (Poumay and Pittelkow, 1995). All experiments were performed at confluence in autocrine culture conditions.

Keratinocytes treatments

For cholesterol depletion, confluent keratinocyte cultures were incubated in the presence of 7.5mM MβCD for 1 hour followed by incubation in autocrine culture medium for different periods (recovery times). This current working MβCD concentration has been previously used for treatment of keratinocytes (Jans et al., 2004; Lambert et al., 2008) as Jans *et al.* (2004) showed that 1h of MβCD is efficient to decrease significantly the concentration of cholesterol

in keratinocytes. MβCD loaded with cholesterol was used as control to avoid any effect not resulting from cholesterol extraction (Mathay et al., 2008; Mathay and Poumay, 2010).
For scratch wound assay, multiple linear scratch wounds were made with a 200 µl pipette tip in confluent keratinocytes cultures. Then keratinocytes were washed with PBS to remove cellular debris and were incubated in fresh autocrine culture medium for different indicated periods (recovery times).

Protein extraction and Western blotting

Before lysis, cells were washed with phosphate-buffered saline (PBS) and then scraped into twice concentrated Laemmli sample buffer (62.5mM Tris–HCl, 2% SDS, 8.7% glycerol, 0.05% bromophenol blue, 0.2% dithiothreitol). The proteins of the cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (GE Healthcare Bio-Sciences, Uppsala, Sweden). Blocking of the membrane in PBS/1% Tween 20/5% skimmed milk (blocking buffer) was followed by incubation of the membrane with primary antibody diluted in blocking buffer. After three washing steps, the membrane was incubated with a HRP-conjugated secondary antibody in blocking buffer. Finally, a POD Chemoluminescence Substrate (Roche Diagnostics, Mannheim, Germany) was used in order to visualize the recognized protein bands. For the visualisation, an ImageQuant 350 (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used.

Measurement of the release of HB-EGF

Release of HB-EGF in the culture medium was quantified by quantitative sandwich enzyme-linked immunosorbent assay (ELISA), using the DuoSet ELISA human HB-EGF kit from R&D systems (Abingdon, U.K.). Using a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.), the optical density was determined at 540 nm (wavelength correction at 450 nm). The amount of HB-EGF present in the culture supernatants was calculated on the basis of a standard curve with the Softmax Pro 5.2 program.

Lactate dehydrogenase (LDH) release

Lactate dehydrogenase (LDH) release was measured with the CyTox-ONE^{TM} Homogeneous Membrane Integrity Assay from Promega (Leiden, Netherlands) according to the manufacturer's protocol. Fluorescence was measured using a fluorescence plate reader (Thermo Scientific, Zellick, Belgium) and LDH release was determined using background control and positive control cell lysis in Triton X-100 (maximum LDH release).

Measurement of extracellular ATP

Release of extracellular ATP was quantified using the Molecular Probes' ATP determination kit based on the requirement of luciferase for ATP to produce light. After treatment (scratch wound or cholesterol depletion), the media were harvested and luciferase activity in the presence of these media was measured using a luminometer. The amount of extracellular ATP was calculated in reference to a standard curve.

Measurement of cell survival: MTT assay

MTT solution (Sigma-Aldrich, Bornem, Belgium) dissolved in culture medium was used at a concentration of 0.5 mg/ml during 1h. After this incubation, the MTT solution was discarded and isopropanol was added to each well for 1h at room temperature. The optical density of MTT extraction solution was determined using a microplate reader (550 nm) (Molecular Devices, Sunnyvale, CA, U.S.A.).

Cholesterol extraction and quantification

Cells were scraped into demineralised water before being sonicated (1 minute). Lipids and proteins were then separated in chloroform/methanol (2:1). NaCl (0.05 M) was added to the organic phase, which was then washed twice with 0.36 M $CaCl_2$/methanol (1:1). 1% Triton X-100/acetone was added and samples were evaporated in air flow with SpeedVac SC100 (Thermo Electron Corporation). The extracts were then solubilised with demineralised water before measurement of cholesterol.

Cholesterol was quantified using the Amplex Red Cholesterol Assay Kit (Invitrogen) according to the instructions of the manufacturer.

Statistical analysis

Data were analysed by analysis of variance (ANOVA 1) after testing the homogeneity of variance (Bartlett). Post hoc comparisons were performed by pairwise Scheffe's test (*$p<0.05$, **$p<0.01$, ***$p<0.001$). All data represent a mean of three independent experiments.

RESULTS

Lipid rafts disruption in keratinocytes induces the expression and the shedding of HB-EGF which subsequently binds to the EGFR.

Previous studies of human keratinocytes have shown that cholesterol depletion by MβCD induces the activation of p38 and ERK1/2 MAPKs, followed by the expression of proHB-EGF which has been shown to depend on p38 activity (Mathay et al., 2008). Although keratinocytes can produce several growth factors of the EGF family including amphiregulin, betacellulin, epiregulin, transforming growth factor-α and HB-EGF, it has been shown that after treatment for 1h with MβCD or 1h of MβCD treatment followed by 1h of recovery period, HB-EGF is the only member of the family that is rapidly and strongly induced in these conditions (Mathay et al., 2010).

In this work, time-course experiments have been performed after lipid rafts disruption (1h MβCD) in order to study the recovery period varying from 1 hour to 18 hours in normal autocrine culture medium. Figure 1a confirms that protein expression of proHB-EGF is indeed expressed following stress conditions in keratinocytes where cholesterol has been extracted from the plasma membrane. Immediately after lipid rafts disruption, EGFR and the MAPK ERK1/2 and p38 were phosphorylated. However, whereas the phosphorylations of EGFR and p38 MAPK were rapidly down-regulated, the ERK1/2 MAPK remained activated during the full period of recovery.

HB-EGF is synthesized as a precursor transmembrane protein that has to be cleaved enzymatically to release a soluble growth factor (mature HB-EGF) which then can bind and activate the EGFR. Thus, to investigate whether the mature form of HB-EGF was shedded and secreted in the cellular environment following cholesterol depletion by MβCD, we analysed HB-EGF concentration in the culture medium using the ELISA technique. Figure 1b illustrates that HB-EGF is mainly secreted after long recovery periods (4h, 8h and 18h) in accordance with the maximum of expression of its precursor (1h, 2h, 4h, 8h) as seen in figure 1a. When the GM6001 metalloprotease inhibitor was added, we observed a decrease in HB-EGF concentration in the culture medium particularly after 4h, 8h and 18h of recovery periods, confirming the implication of metalloprotease in the shedding of HB-EGF (data not shown).

To explore if the mature form of HB-EGF secreted was able to activate the EGFR, cells were incubated after cholesterol depletion in the presence of CRM197, a non-toxic mutant of

diphtheria toxin. CRM197 binds to the proHB-EGF as well as to the mature form of HB-EGF, impairing the binding between the EGFR and HB-EGF and subsequently inhibiting the mitogenic action of HB-EGF. CRM197 does not bind to other growth factors of the EGF family (Mitamura et al., 1995). We focused on the timing where HB-EGF is principally secreted and found that after 4h and 8h of recovery times, the phosphorylation of EGFR decreases in the presence of CRM197 (Figure 1c), demonstrating that part of the EGFR phosphorylation following cholesterol depletion is partly due to receptor binding by HB-EGF ligand. Comparing data in Figure 1a, there was a surprising slight increase in the phosphorylation of EGFR after 4h of recovery. However generally, the phosphorylation of EGFR decreases during the recovery periods.

These results suggest that perturbation of lipid rafts in keratinocytes plasma membrane of induces a stress response which results in the synthesis and shedding of HB-EGF by epidermal cells as found during skin wound repair at the margin of the healing epidermis (Mathay et al., 2008). A similar pattern of precursor expression and mature form release is observed when keratinocytes are wounded in a scratch wound assay (Figure 2a and b), indicating that HB-EGF, due to its mitogenic and chemotactic properties, is likely released by this cell type in order to participate to healing of the epidermal tissue. To investigate the importance of HB-EGF synthesis and release in scratch wound assays, cells were incubated in the presence or absence of CRM197. We report that keratinocytes migration was reduced in the presence of CRM197, suggesting involvement of HB-EGF in cell migration and proliferation (Figure 2c).

These data suggest that HB-EGF must be considered as a key factor in epidermal stress response, as it was already suggested by studies revealing that HB-EGF neutralization with CRM197 or with specific neutralizing antibody produces an impaired wound closure (Block et al., 2004; Xu et al., 2004).

ATP-γ-S treatment induces HB-EGF synthesis.

Membrane cholesterol depletion, like other cellular stress, induces an HB-EGF synthesis (Mathay et al., 2008). However, the mechanisms leading to the expression of HB-EGF have not been fully established although a role for p38 MAPK was identified. It is known that ATP signalling in epithelial cells can play a critical role in tissue wound repair since ATP is a known extracellular signalling molecule inducing cell proliferation and migration and thus promoting wound closure (Klepeis et al., 2004; Weinger et al., 2005). Because the shedding

of HB-EGF has already been linked to extracellular ATP in other epithelial cells (Yin et al., 2007), we wondered whether ATP could play a role in the early stages of keratinocyte stress response. To investigate whether ATP can contribute directly to cellular protein expression of HB-EGF, ATP-γ–S, the nonhydrolyzable ATP analogue, was added to culture medium in order to mimic an extracellular ATP release. Figure 3 illustrates that EGFR and ERK1/2 were activated 30 minutes after ATP-γ–S stimulation in keratinocytes, followed by expression of HB-EGF as detected after 1h and up to 4h. The HB-EGF concentration released in the medium were also measured simultaneously, revealing a weak shedding of HB-EGF after 4h and 8h only (data not shown), probably due to very low concentrations of released HB-EGF. In addition, increasing concentrations of ATP-γ–S stimulate EGFR phosphorylation and HB-EGF protein expression (Figure 3b).

This result demonstrates that some extracellular ATP is able to induce the synthesis of HB-EGF in keratinocytes. Moreover, in good accordance with response to the cholesterol depletion, we also observe phosphorylation of EGFR and ERK1/2 after ATP-γ–S treatment, suggesting that early cell response after cholesterol depletion could be due to some release of ATP by keratinocytes.

Cholesterol depletion induces ATP release from keratinocytes.

Because extracellular ATP was demonstrated as being able to induce HB-EGF synthesis, the question was then to know whether cholesterol depletion could induce some ATP release. In order to investigate whether keratinocytes do release ATP after cholesterol depletion, the concentrations of ATP were measured in the culture media. Briefly, confluent keratinocyte cultures were treated for 1h with MβCD followed by incubation in autocrine culture medium for different periods of recovery. The culture media were harvested and analysed using the luciferase-luciferin ATP bioluminescent assay. Figure 4 illustrates that during incubation 1h with MβCD, keratinocytes release a high concentration of ATP (355 nM) which corresponds to an approximately 21-fold increase over the control condition (16 nM). During the first hour of recovery time, we observe a reduced release of extracellular ATP secreted in the culture medium compared with the sole incubation with MβCD (1h). This release is also seen up to 4 hours of recovery, and then returns to normal values after 8h of recovery. These results strongly suggest that the extracellular ATP released by keratinocytes during cholesterol depletion could explain the induced HB-EGF expression after this treatment.

The question was then to determine whether some cell lysis could be responsible for ATP release. To quantify eventual cell lysis, we measured the cytosolic lactate dehydrogenase (LDH) activity in the culture media after MβCD treatment and during the recovery period. The cell membrane is impermeable to LDH, but when the integrity of the plasma membrane is altered or disrupted, LDH is rapidly released into the culture medium and therefore can be considered as a marker for cell damage. Keratinocyte cultures were grown to confluence and treated for 1h with MβCD, followed by recovery periods in autocrine culture medium. The culture medium was collected and then analysed to measure the amount of LDH activity released during the recovery period. Figure 5a shows that MβCD did not cause any immediate damage to the cell membrane. Some LDH release progressively happened during recovery, but its kinetics could not explain the cellular release of ATP, discarding damages to plasma membranes as a potential explanation. MTT assays were also performed and showed that cholesterol depletion does not affect cellular viability (data not shown). Finally, measurements of cholesterol have shown that during recovery periods, cholesterol is progressively re-synthesized, reaching a cholesterol concentration in keratinocytes after 17h of recovery period that is similar to the initial concentration measured in control conditions (Figure 5b).

HB-EGF synthesis induced by lipid rafts disruption is dependent on ATP interaction with purinergic receptors.

Released ATP stimulates cells via the activation of the P2 purinergic receptors in plasma membranes (Klepeis et al., 2004). To elucidate whether the expression of HB-EGF observed in MβCD treatment could be due to the interaction between the extracellular ATP with the P2 purinergic receptors, we added suramin, a known antagonist of the P2 purinergic receptor. Suramin is a non-selective antagonist P2 receptor inhibitor (Dunn and Blakeley, 1988). Keratinocyte cultures were incubated 1h with suramin, followed by 1h of MβCD treatment before washing and incubation for cell recovery in autocrine culture medium containing 200 µM of suramin (Figure 6a). Results illustrate downregulations of the phosphorylations of EGFR and ERK1/2, suggesting that the lipid rafts-induced activation of EGFR and ERK1/2 is potentially mediated by the P2 purinergic receptor signalling pathway. On the other hand, the p38 MAPK was still phosphorylated in the presence of suramin whilst HB-EGF synthesis was completely impaired. This was surprising because previous data have shown that HB-EGF synthesis, particularly during short recovery periods after disruption of lipid rafts, is

dependent on p38 MAPK activity (Mathay et al., 2008). Thus, to control the efficiency of p38 activity after suramin treatment, the phosphorylation of Hsp27, a down-stream target of p38 activity in keratinocytes (Garmyn et al., 2001), was checked by Western blot analysis (Figure 6a). The state of phosphorylation of Hsp27 is in concordance with the activity of p38, indicating that the MAPK p38 activity is not affected by suramin. To confirm the implication of extracellular ATP in the synthesis of HB-EGF after cholesterol depletion, keratinocytes were treated with apyrase. Figure 6b shows that in the presence of apyrase, HB-EGF protein expression is also downregulated.

Because these data indicate that suramin is responsible for some downregulation of ERK1/2 phosphorylation, followed by a strong decrease in HB-EGF protein expression (Figure 6a), we decided to assess whether the MAPK ERK1/2 could participate in the synthesis of HB-EGF. For this purpose, the MEK inhibitor PD98069 was employed. Interestingly, this experiment revealed that inhibition of ERK1/2 phosphorylation by PD98069 decreases detection of HB-EGF protein after 1h, 2h and 4h of recovery (Figure 7a). However, since the expression of HB-EGF is not completely abolished by this treatment, one can deduce that the induction of the synthesis of HB-EGF is probably dependent on an interaction between p38 and ERK1/2. Indeed, Sharma et al. (2003) observed that a two-way cross-talk between p38 and ERK1/2 MAPKs in corneal epithelium exists and that, when ERK1/2 pathway is interrupted by specific inhibitor, the MAPK p38 is concomitantly up-regulated (Sharma et al., 2003). Moreover, our previous results showed that the inhibition of p38 did not completely abolish the protein expression of HB-EGF after long time (8h and 18h) recovery periods (Mathay et al. 2008). For this reason, we analysed the effect of PD98069 combined with the p38 MAPK inhibitor PD169316 during the cell response to lipid rafts disruption. Figure 7b shows a complete impairing of HB-EGF protein expression in the presence of both inhibitors, supporting a two-way cross-talk between p38 and ERK1/2 in controlling the induction of HB-EGF.

Altogether our results demonstrate that extracellular ATP acts as an early keratinocytes stress response able to induce ERK1/2 signalling through suramin-antagonized receptor (likely the P2-purinergic receptor), leading to strong cooperation with p38 in order to induce the transient expression of HB-EGF by this cell type.

DISCUSSION

Previous data from our laboratory have shown that cellular stresses, such as cholesterol depletion or oxidative stress, strongly induced the expression of HB-EGF in human keratinocytes (Jans et al., 2004; Mathay et al., 2008). However the mechanisms leading to the synthesis of HB-EGF have not been fully understood.

It has been shown that cholesterol depletion using MβCD disorganizes the structures of lipid rafts (Kabouridis et al., 2000) and initiates keratinocytes signalling pathways including the activation of EGFR (Jans et al., 2004; Lambert et al., 2008). The present work precisely focuses on the keratinocyte stress response consecutive to cholesterol depletion and which has been shown previously to include a strong induction of HB-EGF expression (Mathay et al., 2008). Here, cells and culture media following cholesterol depletion, during a recovery period (Lambert et al., 2008; Mathay et al., 2008) were analysed by Western blotting and ELISA and revealed that HB-EGF is mainly released 4h, 8h and 18h following lipid rafts disruption. Using the non-toxic mutant of diphtheria toxin, CRM197, we provide evidence that HB-EGF secreted in the culture media is likely binding the EGFR, partly inducing its phosphorylation after 4h and 8h of recovery. Since HB-EGF expression has been also demonstrated as dependent on p38 phosphorylation in stress conditions (Mathay et al., 2008), further characterization of the early stress response in keratinocyte was initiated.

An important role for ATP in keratinocyte proliferation, differentiation and cell-to-cell communication has previously been underlined (Burrell et al., 2005; Dixon et al., 1999). In vivo, during skin wound healing, keratinocytes are exposed to high levels of ATP released from platelets, damaged cells including keratinocytes, and such exposure promotes epidermal healing. In addition, because a recent study has shown that extracellular ATP is responsible for HB-EGF shedding from epithelial cells after scratch wound (Yin et al., 2007), we investigated whether the nonhydrolyzable ATP analogue, ATP-γ-S, was able to induce HB-EGF expression in autocrine keratinocyte culture model. Interestingly, HB-EGF was indeed detected after ATP-γ-S treatment, together with phosphorylation of EGFR and ERK1/2. Because related signaling pathways were also activated after MβCD treatment, we investigated whether keratinocytes do release ATP in their environment after cholesterol depletion. Using an ATP bioluminescent assay, we showed that keratinocytes release large amount of extracellular ATP upon cholesterol depletion. However, other stresses, like

oxidative stress or treatment with phorbol ester, which are known to also increase HB-EGF expression in keratinocytes, do not induce release of ATP in the culture media (data not shown).

Mechanisms responsible for the release of ATP are multiple and probably differ from cell type to another. There are several potential mechanisms, including cytolysis, ATP-binding cassette (ABC) protein, neighboring ion channels or exocytosis of vesicles (Bodin and Burnstock, 2001; Schwiebert and Zsembery, 2003). Because MβCD extracts cholesterol from plasma membrane and thereby disrupts lipid rafts, we evaluated the damage to cell membrane by measurement of the release of lactate dehydrogenase (LDH) after MβCD treatment of keratinocytes. No significant release of LDH was observed, suggesting good integrity of the cell membrane. Many mitochondrial proteins are found in plasma membrane such as ATP synthase complex which are able to generate extracellular ATP in cell environment (Kim et al., 2004; Zhao et al., 2004). Kim et al. (2004) have also demonstrated that plasma membrane lipid rafts do contain ATP synthase complex. One possible explanation could be that the disruption of lipid rafts by MβCD causes the activation of ATP synthase. On the other hand, Feranchak et al. (2010) have reported the vesicular localisation of ATP in a model of liver cell line, suggesting that exocytosis could be involved in ATP release.

Once released, ATP can be rapidly degraded or interacts and activates in an autocrine or paracrine manner the P2 purinergic receptors (Schwiebert and Zsembery, 2003). Among P2 purinergic receptors, $P2Y_1$ and $P2Y_2$ receptors were identified in the basal layer of the epidermis and implicated in keratinocytes proliferation (Dixon et al., 1999; Greig et al., 2003). After cholesterol depletion, the released ATP acts as an extracellular mediator by interacting with P2 purinergic receptors, leading to HB-EGF synthesis. Indeed, when keratinocytes are incubated with suramin, an antagonist of P2 purinergic receptors, followed by MβCD treatment, the HB-EGF synthesis was abolished as well as the phosphorylation of EGFR and ERK1/2.

The release of extracellular ATP is therefore an early signal after cholesterol depletion, identified to be at least partially upstream of the EGFR activation. Recently, Liu et al. have suggested a mechanism for the transactivation of the EGFR by the P2Y2 receptor. They have identified two SH3-binding domains in the P2Y2 receptor that are necessary for the G-protein-coupled receptor (GPCR) to bind directly and activate the Src nonreceptor tyrosine kinase, leading to Src-dependent transactivation of several receptor tyrosine kinases, including the EGFR (Liu et al., 2004). Similarly, Buzzi et al. have suggested that in human

colon cancer Caco-2 cells stimulated by ATP, GPCR may transactivate the EGFR via activation of tyrosine kinases such as Src, or via activation of metalloproteinases to generate EGFR ligands (Buzzi et al., 2009).

ERK1/2 is known to be implicated in cell survival and proliferation (Seger and Krebs, 1995). Recently, Satoh *et al.* demonstrated that ERK2 can contribute to wound healing after a partial-thickness burn (Satoh et al., 2009). Thus we investigated whether the activation of ERK1/2 could be involved in HB-EGF expression. Although previous data had indicated that HB-EGF synthesis was dependent on p38 activation, ERK inhibition appears to partially impair the early HB-EGF expression in keratinocytes. These findings provide new insight into the signalling pathway by which HB-EGF is expressed after lipid rafts disruption. Our data suggest that after p38 and ERK1/2 activation, both MAPKs are involved together or independently in the regulation of HB-EGF gene expression. This concurs with studies showing that inhibition of one of these two MAPKs impairs wound healing (Satoh et al., 2009; Sharma et al., 2003).

In conclusion, after lipid raft disruption by cholesterol extraction, keratinocytes respond to this cellular stress by release large amounts of extracellular ATP. This release, by interacting with the P2 purinergic receptor, induces HB-EGF synthesis and shedding (Figure 8). This growth factor is considered as a good marker of the keratinocyte's response to a challenging environment and could even be involved in atopic dermatitis (Mathay et al., 2008). Thus our data suggest that the release of extracellular ATP acts as an early stress response in cholesterol-depleted keratinocytes.

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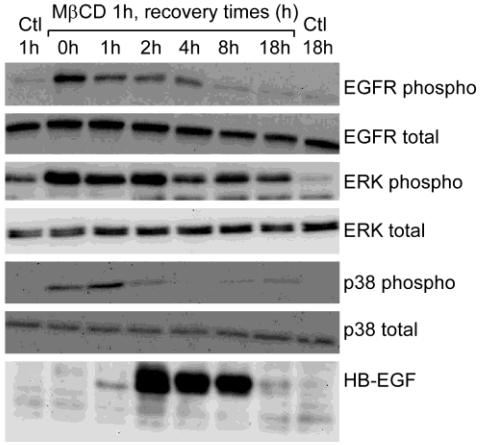
FIGURE LEGENDS

Figure 1: HB-EGF is synthesized and released after cholesterol depletion in human keratinocytes cultures.

a. Confluent keratinocytes were left untreated (Ctl 1h and Ctl 18h) or were treated 1h with MβCD (7.5 mM) then followed by several recovery periods in culture medium (0, 1h, 2h, 4h, 8h and 18h). After each treatment, proteins were extracted and analysed by Western blotting using specific antibodies (EGFR, p38, ERK, their phosphorylated forms and HB-EGF). b. The culture media were harvested and analysed by enzyme-linked immunosorbent assay (ELISA) to measure the concentration of HB-EGF released after cholesterol depletion. The results shown are representative data obtained

in three experiments and were analysed by ANOVA 1 after testing the homogeneity of variance (Bartlett). Post hoc comparisons were performed by pairwise Scheffe's test (**p<0.01). c. Confluent keratinocytes were left untreated (Ctl 1h and Ctl 18h) or treated 1h with MβCD (7.5 mM) following by several recovering periods in culture medium (2h, 4h, 8h and 18h) in the presence of CRM197 (10 μg/ml). After each treatment, proteins were extracted and analysed by Western blotting using specific antibodies against EGFR and its phosphorylated form.

a.



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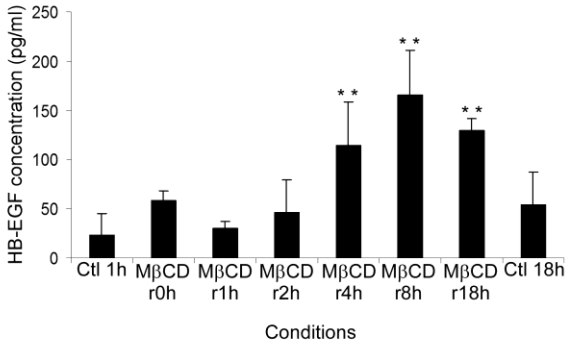

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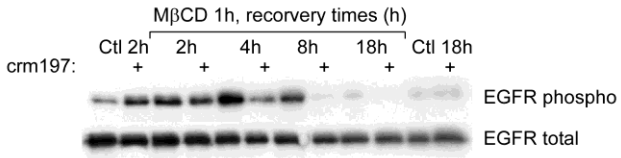

Figure 2: HB-EGF is synthesized and released after scratch wound in human keratinocytes cultures.

a. Confluent keratinocytes were left untreated (Ctl 1h and Ctl 18h) or cultures were wounded followed by several recovery periods in culture medium (0, 1h, 2h, 4h, 8h, 18h and 24h). Proteins were then extracted and analysed by Western blotting using specific antibodies (EGFR, p38, ERK, their phosphorylated forms and HB-EGF). b. The culture media were harvested and analysed by enzyme-linked immunosorbent assay (ELISA) to measure the concentration of HB-EGF released after cholesterol depletion. The results shown are representative data obtained in three experiments and were analysed by ANOVA 1 after testing the homogeneity of variance (Bartlett) (*p<0.05, **p<0.01, ***p<0.001). Post hoc comparisons were performed by pairwise Scheffe's test. c. Scratch-wounded keratinocytes were incubated in the presence or in absence (Ctl) of CRM197 (10ng/ml). Cells were photographed by phase contrast microscopy immediately after the scratch-wound (0h) or after 18h of recovery period.

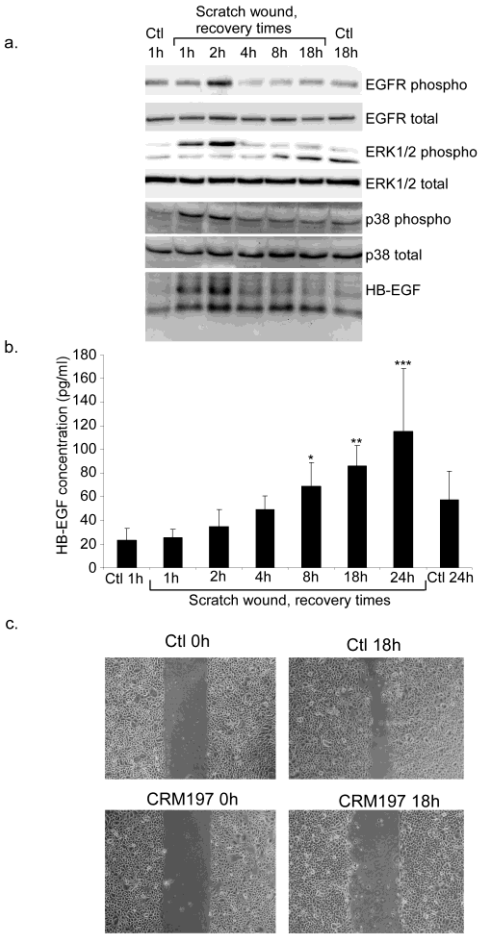

Figure 3: ATP-γ-S treatment induces HB-EGF expression.

a. Confluent keratinocytes were left untreated (Ctl 30 min and Ctl 8h) or were treated with ATP-γ-S (50 µM) during 30 min, 1h, 2h, 4h and 8h and cells lysates were analysed by Western blotting using specific antibodies against EGFR, ERK, their phosphorylated forms and HB-EGF. b. Confluent keratinocytes were left untreated (Ctl 1h) or treated with several concentrations of ATP-γ-S (12.5, 25, 50 or 100 µM) during 1h. Cells lysates were then analysed by Western blotting using specific antibodies against EGFR, pEGFR and HB-EGF.

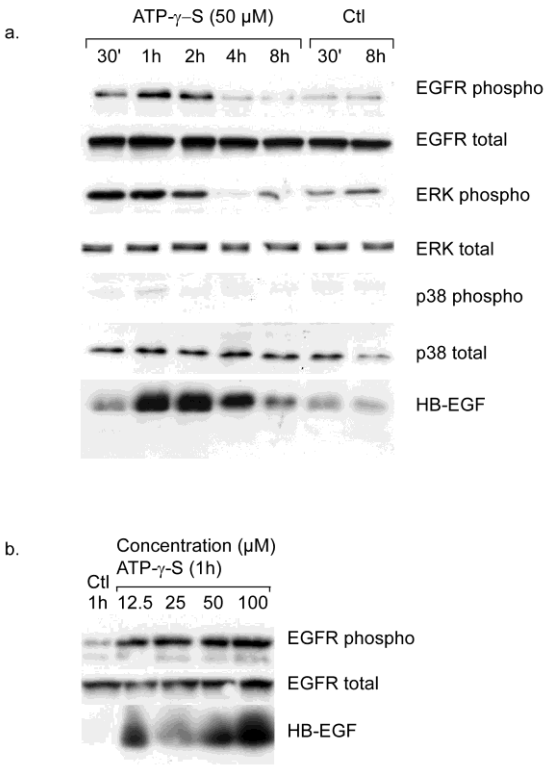

Figure 4: Cholesterol depletion induces the released of ATP in the culture media.

Confluent keratinocyte were treated 1 h with MβCD (7.5 mM) followed by recovery time (0h, 1h, 2h, 4h, 8h or 18h). The culture media were harvested for each condition and the concentrations of extracellular ATP were analysed using an ATP Bioluminescent Assay Kit. The results shown are representative data obtained in four experiments and were analysed by ANOVA I after testing the homogeneity of variance (Bartlett). Post Hoc comparisons were performed by pairwise Scheffe's test (*p<0.05, **p<0.01, ***p<0.001).

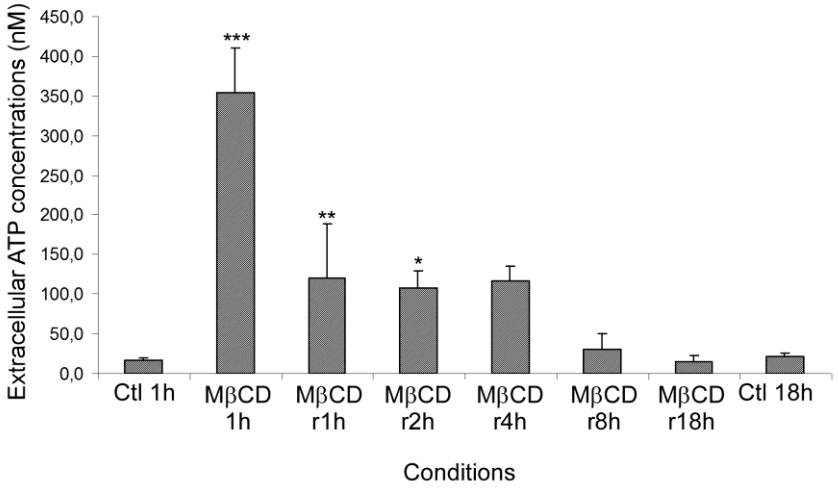

Figure 5: The integrity of plasma membrane is not affected after 1h of MβCD treatment.

Confluent keratinocytes were left untreated (Ctl 1h or Ctl 18h) or were treated 1h with MβCD (7.5 mM) followed by recovery periods varying from 1 hour to 18 hours in normal autocrine culture. a. Culture media were analysed and the results are expressed as percentages of the maximum LDH release and represent the mean absorbance ± SD (n = 3). b. Cholesterol content was extracted and quantified with Amplex Red Cholesterol Assay kit. After testing the homogeneity of variance (Bartlett), data were analysed by ANOVA 1 and Post Hoc comparisons were performed by pairwise Scheffe's test (*p<0.05, ***p<0.001).

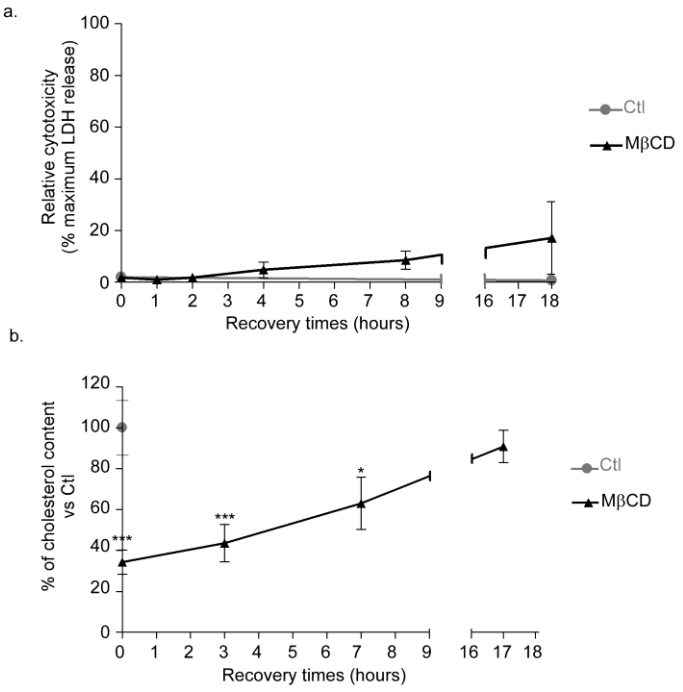

Figure 6: HB-EGF synthesis after lipid raft disruption is dependent on extracellular ATP released in keratinocytes.

a. Confluent keratinocyte cultures were pretreated 1h with suramin (200 µM) followed by 1h of MβCD treatment (7.5mM). Then cells were incubated in autocrine culture medium during several recovering periods (0, 1h, 2h, 4h, 8h and 18h). After each treatment, proteins were extracted and analysed by Western blotting using specific antibodies against EGFR, p38, ERK, HSP27 and their phosphorylated forms and HB-EGF.

b. Confluent keratinocyte cultures were treated 1h with 1h MβCD (7.5mM) in the presence of apyrase (10U/ml) or the vehicle. Then cells were incubated in autocrine culture medium during several recovering periods (0, 1h, 2h, 4h, 8h and 18h) in the presence of apyrase (10U/ml) or the vehicle. After each treatment, proteins were extracted and analysed by Western blotting using specific antibodies against EGFR, p38, ERK, and their phosphorylated forms and HB-EGF.

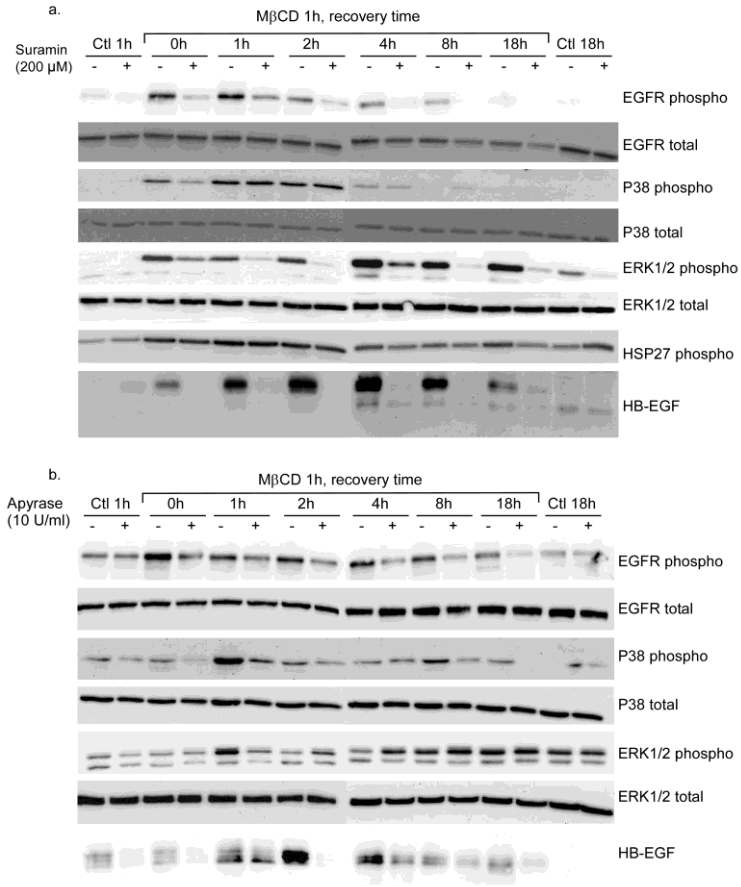

Figure 7: Early HB-EGF protein expression induced by disruption of lipid rafts is dependent on ERK and p38 MAPK activity.

a. Confluent keratinocyte cultures were left untreated or were pretreated 1h with the ERK inhibitor, the PD98069 (2 µM) then left untreated or treated with MβCD (7.5mM) for 1h in the presence of PD98069 (2 µM) or the vehicle. Then cells were incubated in autocrine culture medium during several recovering periods (0, 1h, 2h, 4h, 8h and 18h) in the presence of PD98069 (2 µM) or the vehicle. Cells lysates were then analysed by Western blotting using specific antibodies against EGFR, ERK, their phosphorylated forms and HB-EGF.

b. Confluent keratinocyte cultures were pretreated 1h with PD98069 (2 µM) and PD169316 (15 µM). Then cultures were treated 1h with MβCD (7.5mM) in the presence of both inhibitors which were also part of the recovery medium (0, 1h, 2h, 4h, 8h and 18h). Cells lysates were then analysed by Western blotting.

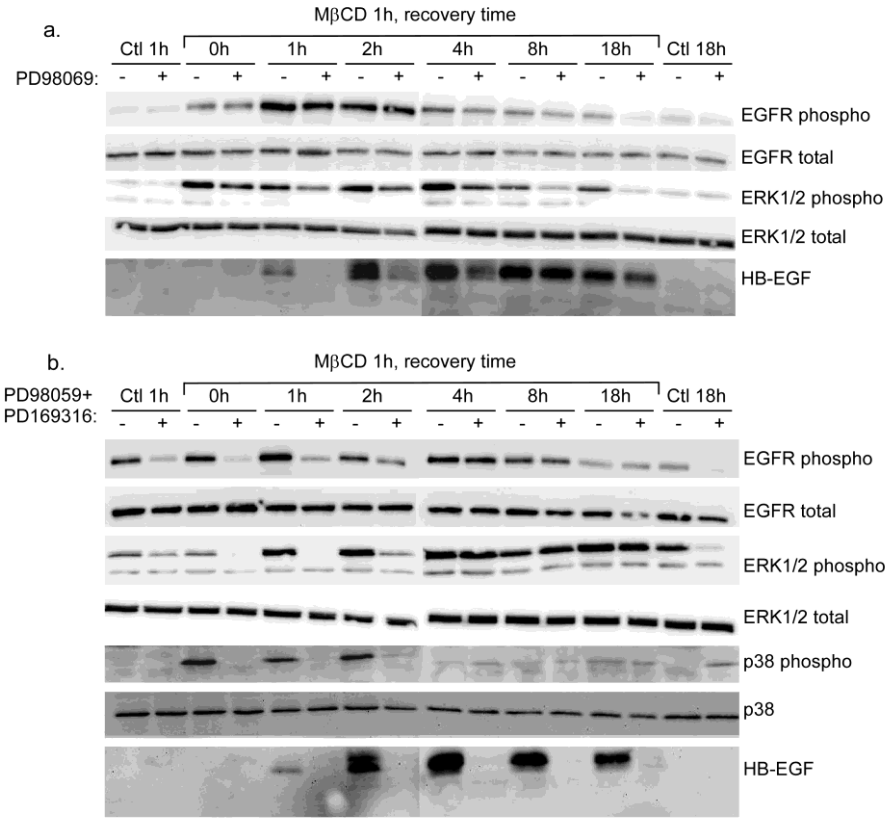

Figure 8: HB-EGF synthesis and release induced by MβCD treatment is controlled by extracellular ATP and involves both p38 and ERK1/2 signaling pathways in keratinocytes.
After cholesterol depletion, keratinocytes release large amounts of extracellular ATP. By interaction with P2 purinergic receptors, ATP induces EGFR phosphorylation, HB-EGF synthesis and release through signaling pathways involving p38 and ERK1/2. Once released, HB-EGF further activates EGFR.