

Short Thesis for the Degree of Doctor of Philosophy (Ph.D.)

Effects of external damaging factors on the calcium homeostasis of HaCaT keratinocytes

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Introduction

The epidermis

The skin is a protective barrier, it takes part in the perception, the thermoregulation, and is essential for the normal function of immune system. It is also a hormone producing organ, cholecalciferol (vitamin D3) is created in the skin by UV-B radiation. The skin has three layers, the epidermis, the dermis and the subcutis. The epidermis is developed from the ectoderma and does not contain blood vessels or nerves. Keratinisation of the epidermis starts in the 3rd month of the embrional development. The basal layer of the epidermis is built from stem cells. After the mitosis one cell remains in the basal layer, the other starts its differentiation into a keratinocyte. In the 10-12th week of the embryonary development neuroectodermal cells migrate into the epidermis. They are the ancestors of the melanocytes which produce the melanin. The keratinocytes do not produce any melanin, they get the melanin from the melanocyte, and accumulate it over their nucleus as a cap.

The HaCaT cells are commonly used models of the normal human keratinocytes. The HaCaT keratinocytes do not contain melanin, which could protect them from the UV-B radiation.

UV-B radiation

The light is a part of the electromagnetic radiation. The UV-B (280-315 nm) radiation is a part of the sunlight and it can reach the ground. Most of the UV-B light can be eliminated by window glass but not by water. UV-B is also emitted by several diagnostical and therapeutical devices. A positive effect of the UV-B radiation is to create vitamin D3 in the skin. On the other hand, the most common biological effect of the UV-B radiation is sunburn (erythema), and it also irritates the conjunctiva and the cornea. Taken together, UV-B radiation has mostly negative effects: it causes acute and chronic skin damages.

These negative effects consist of damages of the DNA, RNA, protein and membrane, which can lead to carcinogenesis.

Function of the keratinocytes in wound healing

During the first day of wound healing, macrophags appear in the wound bed. These macrophags produce cytokines to control the wound healing. In the wound edge, the integrine mediated connections between the keratinocytes break up before the cells are mobilised. The keratinocytes flatten to cover an as large area as they can, and start to move into the woud bed. The speed of their movement is approximately 0.5 mm/day. Mitosis of the keratinocytes is enhanced by epidermal growth factor and keratinocyte growth factor (KGF). Diabetes and the excess of gucocorticoids depress the production of KGF, and delay the wound healing.

Purinergic signalisation in keratinocytes

The purinergic compounds are essential componenents of the DNA, RNA, and energy storing molecules. The first publication about their extracellular effect was appeared in 1929. ATP could be secreted into the extracellular space as a cotransmitter or it can be also released from dying cells. Because of the latter, the ATP is a danger sign for most primitive life forms. The purinergic receptors are so common in living organisms, that it is hard to find cells not expressing any purinoreceptors. ATP appearing in the extracellular space can bind to ionotropic P2X and metabotropic P2Y purinergic receptors, which are localised in the surface membrane.

Healthy human epidermal keratinocytes express P2Y₁, P2Y₂, P2X₅ and P2X₇ receptors. P2Y₁ and P2Y₂ receptors could be detected in the cells of stratum basale, their funcion can be to control proliferation. P2X₅ receptors were detected in the cells of stratum spinosum, while P2X₇ receptors were detected in

the stratum corneum. Their appearance can be connected with controlling differentiation of the keratinocytes, or the induction of apoptotic cell death.

Protein kinases and phosphoprotein phosphatases

The reversible phosphorylation caused by protein kinases has essential role in the response of cells to extracellular signals. Usually the terminal phosphate group of the ATP molecule is used for the phosphorylation. The phosphoprotein phosphatase (PP) enzymes can remove phosphate groups from different proteins by hydrolytic cleavage. 2-3% of the genetic information in the eucaryotic cells codes protein kinases and phosphoprotein phosphatases. 30% of the intracellular proteins can be phosphorylated by protein kinases. The phosphorylation of the serine/threonine groups is more common than that of the tyrosine groups.

The phosphoprotein phosphatases are categorized by the aminoacid side-chains they use as a substrate of dephosphorylation. We can distinguish serine/threonine (PP1, PP2A, PP2B, PP2C and the recently discovered PP4, PP5, PP6 and PP7) and tyrosine phosphoprotein phosphatases.

Serine/threonine phosphatase inhibitors

We used two different serine/threonine phosphatase inhibitors in our experiments. Okadaic acid (OA) was isolated from the *Halichondria okadai* marine sponge, but actually it is produced by marine algae. It inhibits the protein serine/threonine protein phosphatase 1, 2A and 2B subgroups. Its highest inhibitory effect is on PP2A, then on PP1 and it is less effective on PP2B.

Calyculin A (CLA) was isolated from the marine sponge *Discodermia calyx*. It is a more effective serine/threonine phosphatase inhibitor than the okadaic acid. Calyculin A can potently inhibit both PP2A and PP1. Neither calyculin A nor okadaic acid has any effect on acidic/alkaline phosphatases or phosphotyrosine protein phosphatases.

Aims

The skin is a protecting barrier composed of mostly keratinocytes. We examined the effects of UV-B irradiation and scratch on the calcium homeostasis of HaCaT keratinocytes. Our aims were to

- examine the effect of UV-B irradiation on the viability of HaCaT keratinocytes
- examine the effect of UV-B irradiation on the purinoreceptor expression and calcium homeostasis of the keratinocytes
- measure the ATP-induced apoptosis connected to P2X₇ receptors.

Further experiments revealed spontaneous intracellular calcium transients in HaCaT keratinocytes. So our next aims were to

- characterize the spontaneous intracellular calcium transients
- examine the effects of scratch on these calcium transients
- examine the effects of phosphatase inhibitors on these calcium transients and on the migration of keratinocytes.

Materials and methods

Culturing HaCaT keratinocytes

HaCaT keratinocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mmol/L L-glutamine, 10% fetal calf serum and antibiotics (50 NE/ml penicillin, 50 µg/ml streptomycin and 1.25 µg/ml fungizone) at 37 °C temperature in a 5% CO₂ atmosphere.

RT-PCR

For RT-PCR analysis total RNA was isolated from the cultures with Qiagen RNeasy® Micro Kit. Reverse transcription was performed by Omniscript reverse transcription kit and oligo(dT) primers. Amplification of specific cDNA sequences was performed with specific primers that were designed based on nucleotide sequences published on the internet. PCR samples were examined on 1.5% agarose gel, stained with ethidium-bromide. GAPDH was used as an internal control. The quantitative analysis of the results was performed with the ImageJ program.

Western-blot analysis

For the detection of purinoreceptors total cell lysates were prepared from the cultures. Samples were sonicated, then 1/5 volume of 5-fold concentrated electrophoresis sample buffer was added, and were boiled for 10 min at 100 °C. Samples were transferred electrophoretically to nitrocellulose membranes. After blocking with 5% non-fat dry milk in PBS, membranes were incubated with the primary antibodies overnight at 4 °C. After washing with PBST (PBS supplemented with 0.1% Tween 20), membranes were incubated with a peroxidase-conjugated secondary antibody. Signals were detected by enhanced chemiluminescence reaction.

Irradiation of the keratinocytes with UV-B

During the irradiation keratinocytes were cultured in 10 cm diameter Petri dishes and kept in 0.5 ml of PBS to avoid desiccation of the monolayer culture. A broadband UV-B irradiation was applied in a 40 mJ/cm² dose for 1 minute.

Determination of viable cell numbers

The number of viable cells was determined by measuring the conversion of the tetrazolium salt MTT to formazan. Cells were plated in 96-well multititer plates (5000 cells/well density) and were incubated with 0.5 mg/ml MTT for 2 h, and the concentration of formazan crystals (as the indicator of number of viable cells) was determined using a colorimetric assay according to the manufacturer's protocol. Data were expressed as mean $\pm$ SEM.

Determination of apoptosis

To detect the rate of apoptosis, the Dead Cell Apoptosis Kit with Annexin V FITC and PI was used. Cells were plated in 96 multititer plates with black walls and clear bottoms (5000 cells/well density). To detect the total cell number CyQUANT® Cell Proliferation Assay was used, according to the manufacturer's protocol. To evoke apoptosis 180 μ M ATP was administered to the cells. Solution was changed after every 12 hours. The apoptotic cell number was normalized to the total cell number.

Whole cell calcium measurements

Changes in intracellular [Ca²⁺]_i of the cells were measured with Fura-2-AM fluorescent dye. The cells were loaded with the dye for 60 min in a 37 °C incubator. Excitation wavelength was alternated between 340 and 380 nm by a Photon Technology International (PTI) Deltascan dual wavelength monochromator, while the emission was monitored at 510 nm using a photomultiplier. [Ca²⁺]_i was calculated from the ratio of fluorescence intensities

($R = F_{340}/F_{380}$) using an in vivo calibration ($R_{\min} = 0.2045$, $R_{\max} = 8.315$, $K_d \beta = 1183$):

$$[Ca^{2+}]_i = K_D \cdot \beta \cdot (R - R_{\min}) / (R_{\max} - R).$$

Cells were continuously washed with normal Tyrode solution using a background perfusion system. ATP was directly applied onto the cells through a perfusion capillary tube.

Confocal microscopy

Calcium transients were monitored with the Zeiss 5 LIVE confocal laser scanning microscope. HaCaT keratinocytes were incubated with 10 μ M Fluo-4-AM for 30 min at 37 °C. Calcium imaging was performed in normal Tyrode solution. Fluo-4 was excited with an argon ion laser. All measurements were performed at room temperature. Images were analyzed by using an automatic event detection program, developed in our department. Fluorescence of the images (F) was normalized to baseline fluorescence (F_0) and amplitudes of the transients were determined as $\Delta F/F_0$.

Time-lapse photography

An inverse microscope was equipped with a high sensitivity video camera, and was connected to a custom-built image acquisition computer system. Custom-designed illumination was developed to minimize heat- and photo-toxicity. Photographs were taken in every minute. The measurements were performed at 37 °C in a 5% CO₂ atmosphere.

Cell migration assay

Cell migration was measured in Boyden-chambers by using fibronectin-coated (FN) Quantitative Cell Migration Assay. HaCaT cells were incubated in the absence or in the presence of 50 nM OA and 10 nM CLA for 18 hours.

Migration of the cells to the other side of the membrane was assessed by measuring the optical density of dye in filtered cells at 540 nm in a plate reader.

Results

Effects of UV-B irradiation on the viability of HaCaT keratinocytes

In our experiments cells were treated with a 1 minute long, 40 mJ/cm² dose of UV-B irradiation. As measured at several time points after irradiation, the number of viable cells– expressed as the percentage of control – decreased substantially as assessed using the MTT assay. Not only did the number of cells decrease but their morphology was also altered. The irradiated cells shrank and lost their extensions.

ATP-evoked Ca²⁺ transients in HaCaT keratinocytes

Both control and irradiated cells responded to challenges of extracellular ATP by elevating their intracellular Ca²⁺ level ([Ca²⁺]_i). In the irradiated cells the amplitude of the ATP-evoked Ca²⁺ transients was lower, and after the transient the [Ca²⁺]_i returned to its resting level more slowly than in control cells. The resting calcium level and the maximal rate of rise of the calcium transients were also lower in the irradiated cells.

Detection of purinergic receptors in connection with UV-B irradiation

Our lab previously identified the P2X₁, P2X₃, P2X₇ and P2Y₂ receptors to be the most common purinergic receptors on normal HaCaT cells. With Western blot both the ionotropic and metabotropic purinoreceptors were detected after 8, 12, 24, 36h of UV-B irradiation. The protein level of P2X₁ was less in the irradiated cells as compared to control (mock-treated) cells in almost every time points. In the case of P2X₃ receptor, after the irradiation, an extra band appeared below the P2X₃ protein band. This might indicate that UV-B irradiation damaged some of these receptors. Examining the P2X₇ protein levels we could detect the characteristic (80 kDa) band of the intact receptor only in the non-irradiated cells. In the irradiated cells we could detect only an approximately 20

kDa band, which could represent the monomeric form of this receptor. The expression of the P2Y₂ receptor was higher after 8 hours from the irradiation, although, this change was not detectable after 12 hours. After 24 and 36 hours from the UV-B treatment, the expression of P2Y₂ receptor decreased in the irradiated cells.

The mRNA expression of P2X₇ receptor was lower in almost every case after irradiation, compared to control. After irradiation the mRNA level of the P2Y₂ receptor decreased, and after 36 hours from the irradiation, the mRNA of this receptor disappeared from the UV-B treated cells.

Determination of the ATP induced apoptosis

Although the basal rate of apoptosis (in the absence of ATP) was already slightly smaller in UV-B irradiated cells (78±19% of non-irradiated control, n=8), this difference did not prove to be statistically significant (p>0.2). If apoptosis was induced by the addition of ATP into the bathing medium, the rate of apoptosis was significantly smaller in UV-B irradiated cells (61±22% of control, n=8; p<0.02), in line with the absence of functional P2X₇ receptors on these cells.

Detection of spontaneous calcium oscillations of HaCaT keratinocytes

Confluent HaCaT keratinocyte cultures displayed slow and repetitive spontaneous [Ca²⁺]_i transients as observed by confocal laser scanning microscopy.

To assess the underlying mechanisms responsible for the Ca²⁺ oscillations we tested a number of chemical agents. Tetracaine in 200 µM – assumed to affect only RyR – neither modified the percentage of oscillating cells nor the frequency of oscillations. On the other hand, the IP₃R inhibitor Xestospongine C, and tetracaine in 1 mM (which concentration is known to inhibit IP₃R as well), significantly reduced both parameters. Similar observation was made with 10

μM thapsigargin which depletes the ER Ca^{2+} stores by inhibiting the SERCA pumps.

The percentage of cells showing Ca^{2+} oscillations in scratched cultures

In scratched cultures untreated by phosphatase inhibitors the percentage of oscillating cells decreased significantly near the scratch as compared to unscratched cultures. In case of scratched CLA- or OA-treated cultures this significant decrease was also observable compared to their unscratched counterparts. On the other hand, the ratio of oscillating cells was significantly higher in cells treated with phosphatase inhibitors both before and after the scratch. This was true for all cases except for the unscratched untreated vs unscratched CLA-treated cultures. These observations clearly suggest that while neither CLA nor OA influenced the effects of scratching, they both interfered with the basic mechanism responsible for the generation of the spontaneous increases in $[\text{Ca}^{2+}]_i$.

Not only the percentage of the oscillating cells but also the frequency of calcium oscillations was significantly lower in the scratched control (untreated) cultures than in the unscratched control. The presence of the phosphatase inhibitors did not alter the frequency of oscillations significantly, as measured before and after the scratch was made. Nevertheless the same tendency was present in untreated and in CLA and OA treated cells, namely, the frequency of calcium transients was less in cells next to the scratch as compared to cells from unscratched cultures. Furthermore, the presence of phosphatase inhibitors also raised the frequency of oscillations in cells from both unscratched and scratched cultures as compared to control cells, although these differences were not significant. The ratio of cells showing Ca^{2+} -oscillations next to the scratch was higher in the presence of phosphatase inhibitors than under control conditions.

Effect of phosphatase inhibitors and scratching on the resting $[\text{Ca}^{2+}]_i$

After scratching, the resting $[Ca^{2+}]_i$ of the cells near the scratch was significantly higher in both control, both in CLA or OA treated cells as compared to unscratched control or phosphatase inhibitor-treated cultures. The Phosphatase inhibitors did not alter the resting $[Ca^{2+}]_i$ of HaCaT keratinocytes.

Characterisation of the Ca^{2+} transients.

In unscratched cultures in the presence of phosphatase inhibitors the amplitudes of the spontaneous Ca^{2+} transients were significantly higher than without the inhibitors. No such increase was observable analyzing the data of cells next to the scratch in any of the drug treatments. Furthermore, except for the CLA treated cells, the difference in the amplitude values measured in unscratched cultures and in cells near the scratch was not significant.

In control and in OA treated cultures the full time at half maximum (FTHM) values were significantly higher in the vicinity of the scratch as compared to untouched areas. This difference was not statistically significant in case of CLA treated cells. In unscratched cultures CLA treatment decreased, while OA treatment increased the FTHM values. In cells near the scratch, the CLA treatment decreased significantly, while OA treatment did not change the FTHM values as compared to the scratched control cells.

The influence of the scratching on both parameters was parallel in control cultures, i.e. the amplitude as well as the FTHM increased. After the treatment by phosphatase inhibitors the measured values of these parameters (except for CLA treatment on FTHM) were also augmented.

Time-lapse and Boyden-chamber experiments to examine the migration of HaCaT keratinocytes

In the time-lapse experiments the control cells started to cover the scratched area, and they totally filled it in by the 17th hour of the experiment. In contrast, CLA and OA treated cells were not able to cover the scratched area

during the same time interval, furthermore, in case of OA treatment cells lost their extensions and their connections with their neighbors by the end of the experiment.

In Boyden-chamber experiments OA in 50 nM concentration decreased the chemoattractant-induced cell migration by about 80%. Similarly, 10 nM CLA also had a massive inhibitory effect on cell migration decreasing the normalized cell migration by 90%.

Discussion

In our experiments, we examined how keratinocytes respond to UV-B irradiation in respect to their purinergic calcium signaling. In the events of programmed cell death one of the first steps is the increase in the intracellular calcium concentration of the cell. Several studies have linked the activation of purinoreceptors especially that of the P2X₇ receptor to the initiation of apoptotic cell death. *In vivo* these purinoreceptors may be activated by ATP coming from other dead cells, or physiologically released from nerves, uroepithel and endothel. In the human squamous cell carcinoma line A431 micromolar concentrations of ATP were mitogenic and increased intracellular calcium levels. ATP has also been shown to activate cell proliferation in MCF-7 breast cancer cells and in human ovarian tumor cells. Importantly though high concentrations of ATP and BzATP (specific agonist of the P2X₇ receptor) caused a significant decrease in A431 cell number. In our experiments, after irradiation, the P2X₇ receptors were diminished in the cells, and we detected less P2X₇ receptor mRNA. This might indicate that cells which were damaged to some extent but survived the irradiation were not as capable of initiating apoptotic cell death. This in turn could give rise to a population of cells with damaged DNA but low apoptotic capabilities thus enabling malignantly transformed cells to persist. It should be noted, however, that while DNA damage in the skin is commonly described in association with UV-B irradiation the direct degradation of proteins following exposure to UV-B light is far less frequently observed.

Other P2X and P2Y receptors also have important functions in regulating the proliferation and differentiation of cells. The metabotropic P2Y₁ and P2Y₂ receptors were shown to control the proliferation of keratinocytes. When a skin cancer evolves from healthy keratinocytes, the pattern of these purinoreceptors may change. We found that UV-B irradiation decreased the amount of both

P2X₁ and P2Y₂ receptors, while essentially destroyed the P2X₇ receptors. In particular, P2Y₂ receptors have been associated with promoting proliferation in both normal human keratinocytes and in HaCaT cells. In our experiments, we demonstrated that the viable cell number was decreased after UV-B irradiation, in which the decreased amount of P2Y₂ receptors might also have played a role. Indeed, the experiments revealed that the mRNA of the P2Y₂ receptor was less in the UV-B treated than in control cells, and 36 hours after the irradiation the mRNA of this receptor disappeared from the irradiated cells. Although 8 hours after the irradiation the protein level of the P2Y₂ receptor was higher in the UV-B treated than in the control cells, the amount of this receptor was decreased in the UV-B treated cells at every other point in time. By losing much of these receptors, cells could lose the control over the proliferation, differentiation or both, and thus be shifted towards a malignant transformation.

In line with the altered expression of purinoreceptors, differences in the calcium transients obtained in response to challenges with ATP were also observed. The amplitude and the rate of rise of these calcium transients were lower in the irradiated cells, which may reflect the lower amount of purinergic receptors on these cells. The latter might also be caused by the shift from mostly ionotropic (P2X) to partly metabotropic (P2Y) response in the irradiated cells. For $[Ca^{2+}]_i$ to return to its resting level took longer times in the irradiated cells. This could indicate that other proteins, for example the calcium pump was also damaged by UV-B irradiation. This notion should, however, be taken with caution since resting $[Ca^{2+}]_i$ was lower in irradiated than in control cells.

Scratching a confluent layer of keratinocytes and subsequent filling of the wound bed with new cells represent a wound healing model often applied to study the mechanism of wound closure and restoration of barrier function of this cell type. Protein kinase and phosphatase enzymes together with the changes in $[Ca^{2+}]_i$ have been shown to possess a significant role in the regulation of cell migration and wound healing. The latter is especially important in case of the

skin which is the first defense line of the body. Despite of the numerous studies there still is no clear consensus whether changes in $[Ca^{2+}]_i$ and phosphatase activities have parallel or antagonistic roles.

Our present results show that in cells from scratched regions the frequency of Ca^{2+} -oscillations are significantly decreased compared to the cells from the untouched areas and the ratio of oscillating cells is also reduced. The characteristic parameters of these oscillations (amplitude, FTHM), however, were significantly higher in the scratched area. These observations suggest that the Ca^{2+} release processes in cells next to the scratch are less frequent but last longer and result in a greater change in $[Ca^{2+}]_i$ as compared with untouched cells. Other cell types like Cajal and extraocular muscle cells also show spontaneous calcium elevations, which are similar to those observed here on HaCaT cells considering both their amplitude and their time course.

On the other hand, enhancing the phosphorylation level of proteins by inhibition of Ser/Thr specific protein phosphatases with cell-permeable CLA and OA increased resting $[Ca^{2+}]_i$ and the frequency of Ca^{2+} -oscillations in cells of both unscratched and scratched areas, however, cells close to the scratch still exhibited fewer number of oscillations than the unscratched ones. This $[Ca^{2+}]_i$ increasing effect of phosphatase inhibitors is in accordance with previous results suggesting that phosphatase inhibition may raise $[Ca^{2+}]_i$ and Ca^{2+} entry via enhancing the phosphorylation level of proteins involved in Ca^{2+} -transport such as phospholamban, ryanodine receptor and plasma membrane Ca^{2+} channel. In non-scratched cultures the characteristic parameters of the Ca^{2+} -transients (amplitude, FTHM) were calculated to be higher after the treatment by CLA and OA (except for FTHM in case of CLA treatment). These parameters showed remarkable alteration after scratching on phosphatase inhibitors treated cell cultures. Comparing the values measured on non-scratched cultures to the parameters obtained in cells next to the scratch, a synergistic relationship between phosphatases and the scratch-induced changes in $[Ca^{2+}]_i$ could be

excluded. The magnitude of the scratch-induced $[Ca^{2+}]_i$ elevation was not significantly potentiated either by CLA or by OA treatment. The changes in the examined parameters observed in control cultures after scratching were not amplified by the application of phosphatase inhibitors rather they were diminished or vanished, however the application of phosphatase inhibitors on non-scratched cells had similar effect on the measured parameters as the scratch did.

Still standing key question is how changes in $[Ca^{2+}]_i$ and the phosphorylation state of certain proteins may contribute to the proliferation and migration of the cells during the wound healing process. According to the current literature this issue is controversial. On the one hand a burst increase in $[Ca^{2+}]_i$ was reported to stop directional movement of keratinocytes and an increased phosphorylation level of myosin II together with an increased stress fiber formation resulted in a decreased hepatic cell migration. On the other hand, protein-serine/threonine kinase inhibitors enhance the formation and extension of lamellipodia, a process believed to mark the start of events that lead to the migration of keratinocytes and wound healing. In addition, inhibition of PP2A by 10 nM okadaic acid resulted in an increased extent of migration.

Movement of cells requires remodeling of the actin-cytoskeleton that includes formation of actin-myosin stress fibers via the Ca^{2+} -dependent phosphorylation of the 20 kDa light chain of myosin II (MLC20) and RhoA/Rho-kinase induced phosphorylation and inactivation of myosin phosphatase. It was shown that following wounding a rapid phosphorylation of MLC20 occurred as a prelude of cell polarization and migration which required a cytosolic Ca^{2+} flux and upstream activation of the p38/MAPK. This early and rapid phosphorylation of MLC20 resulted in translocation of myosin IIA to the cell cortex primarily and this actin-myosin interaction was implicated in the membrane repair of wounded cells. In addition, RhoA/Rho-kinase was found to be essential for contraction and directed migration of keratinocytes.

Accordingly, inhibition of the protein phosphatases by CLA or OA would be expected to favor cell migration via increasing $[Ca^{2+}]_i$ as well as promoting the phosphorylation of both MLC20 and the inhibitory phosphorylation of the myosin phosphatase regulatory subunit as shown earlier. Nevertheless, CLA and OA were shown here to apparently halt migration of keratinocytes in both the wound healing assay and in the transwell migration assay. Myosin phosphorylation plays important roles in the cell attachment-detachment process especially during retraction of the tail which requires the force exerted by the actin-myosin stress fibers. As cell migration involves repeated attachment-detachment processes it should be coupled with cyclic phosphorylation-dephosphorylation of MLC20. Our findings support the idea that phosphatase inhibitors cause a maintained phosphorylation of MLC20 with long-lasting stress fibers which eventually suppresses migration.

With respect to these findings the decrease of Ca^{2+} -oscillations in cells close to the scratch may help keratinocytes to move into the gap between the cells caused by scratching. On the other hand lowering $[Ca^{2+}]_i$ may also be advantageous for the proliferation of keratinocytes since this process could be inhibited by increasing influx of Ca^{2+} into the cells.

Summary

In this thesis the effect of UV-B radiation on purinergic receptor expression of HaCaT keratinocytes was examined. We revealed that HaCaT keratinocytes show spontaneous repetitive calcium transients. The effects of two phosphatase inhibitors (CLA and OA) on these spontaneous calcium oscillations, and the keratinocyte migration were also examined.

UV-B irradiation decreased the number of viable cells and changed the morphology of the cells. UV-B irradiation decreased the amount of both P2X₁ and P2Y₂ receptors and essentially destroyed the P2X₇ receptors in surviving cells. Morphology of ATP-induced Ca²⁺ transients were altered in irradiated cells compared to control. The amplitude and the rate of rise of the transients were decreased and the return to resting [Ca²⁺]_i prolonged. This observation is consistent with the finding that in control cells mostly ionotropic, while in irradiated cells mostly metabotropic receptors were underlying the response to ATP. These alterations in the expression pattern of purinergic receptors and in the Ca²⁺ transients could explain the observed decreased tendency for ATP-induced apoptosis and thus enable the malignant transformation of keratinocytes.

Scratching confluent HaCaT monolayers decreased the number of cells displaying repetitive Ca²⁺ oscillations as well as the frequency of their Ca²⁺-transients in cells close to the wounded area and initiated migration of the cells into the wound bed. In contrast, calyculin-A (CLA) and okadaic acid (OA), known cell permeable inhibitors of protein phosphatase-1 and 2A, increased the level of resting [Ca²⁺]_i and suppressed cell migration and wound healing of HaCaT cells. Furthermore, neither CLA nor OA influenced how scratching affected Ca²⁺ oscillations. It is assumed that changes in and alterations of the phosphorylation level of Ca²⁺-transport and contractile proteins upon phosphatase inhibition mediates cell migration and wound healing.

Publications

Register Number: DEENKÉTK/201/2013.

Item Number:

Subject: Ph.D. List of Publications

Candidate: Olga Ruzsnavszky

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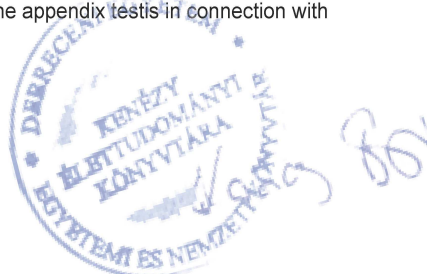
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Ruzsnavszky, O.**, Dienes, B., Oláh, T., Vincze, J., Gáll, T., Balogh, E., Nagy, G., Bátor, R., Lontay, B., Erdődi, F., Csernoch, L.: Differential Effects of Phosphatase Inhibitors on the Calcium Homeostasis and Migration of HaCaT Keratinocytes.
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3. Kistamás, K., **Ruzsnavszky, O.**, Telek, A., Kosztka, L., Kovács, I., Dienes, B., Csernoch, L., Józsa, T.: Expression of anti-Müllerian hormone receptor on the appendix testis in connection with urological disorders.
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DOI: <http://dx.doi.org/10.1016/j.ceca.2011.03.012>
IF:3.766

Total IF: 21.933

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The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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List of conference presentations connecting to the topic of the thesis:

Olga Ruzsnavszky: HaCaT keratinocytes exhibit spontaneous intracellular calcium transients, Annual Symposium of the Doctoral School of Molecular Medicine, University of Debrecen, Debrecen 2011. June 6-7

Olga Ruzsnavszky: Effect of UV-B irradiation on the expression of purine receptors of HaCaT keratinocytes, Gene-Environment Multidisciplinary Meeting, Szeged, 2010. July 5

List of posters connecting to the topic of the thesis:

M. Gönczi, A. Telek, **O. Ruzsnavszky**, L. Csernoch: „Effect of UVB irradiation on calcium handling and purinoreceptor expression of HaCaT keratinocytes” (Kyoto, Japan, 2008)

A. Telek, M. Gönczi, Á. Balogh, **O. Ruzsnavszky**, É. Remenyik, L. Csernoch: „Effects of UVB irradiation on the purinergic expression pattern of the HaCaT keratinocytes” (Purine Club Meeting, Leipzig, Germany, 2007)

Ruzsnavszky Olga, Oláh Tamás, Dienes Beatrix, Vincze János, Gáll Tamás, Balogh Enikő, Nagy Gábor, Bátori Róbert, Lontay Beáta, Erdődi Ferenc, Csernoch László: HaCaT keratinocitákon megfigyelhető spontán intracelluláris kalciumtranziensek és azok módosítása sebzéssel és foszfataz inhibitorokkal (LXXVI. Annual Meeting of the Hungarian Physiological Society, Debrecen, 2012)

Ruzsnavszky O., Balogh Á., Telek A., Gönczi M., Remenyik É., Csernoch L.: „UV-B besugárzás hatása HaCaT keratinocyták purinoreceptor expressziójára” (LXXI. Annual Meeting of the Hungarian Physiological Society, Pécs, 2007)