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Aging skin is functionally anaerobic: Importance of coenzyme Q₁₀ for anti aging skin care

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Abstract. The functional loss of mitochondria represents an inherent part in modern theories trying to explain the cutaneous aging process. The present study shows significant age-dependent differences in mitochondrial function of keratinocytes isolated from skin biopsies of young and old donors. Our data let us postulate that energy metabolism shifts to a predominantly non-mitochondrial pathway and is therefore functionally anaerobic with advancing age. CoQ₁₀ positively influences the age-affected cellular metabolism and enables to combat signs of aging starting at the cellular level. As a consequence topical application of CoQ₁₀ is beneficial for human skin as it rapidly improves mitochondrial function in skin *in vivo*.

Keywords: CoQ₁₀, topical application, skin aging, keratinocytes, glucose, lactate, mitochondria

Abbreviations: CoQ₁₀, coenzyme Q₁₀; ROS, reactive oxygen species, UV, ultra violet light; GLUT-1, glucose transporter type 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction; SU, subunit; ATP adenosine triphosphate; MMP, mitochondrial membrane potential; AGE, advanced glycation endproduct; AMPK, adenosine monophosphate activated protein kinase; J, Joule.

1. Introduction

Free radicals are generated in skin by two different aspects: the most exposed organ to environmental stresses is affected by UV-irradiation as a generator of ROS [7], otherwise the cell generates ROS itself, i.e. during mitochondrial respiration or inflammation reactions [14]. During the aging process, the formation of ROS is induced due to an altered mitochondrial physiology, e.g. as by-products of an impaired mitochondrial respiration [10,25]. Enhanced generation of ROS is associated with the modulation of protein structure and function, changes in gene expression, harmful changes to DNA [4, 18,27,28] and further impairment of mitochondrial protein complexes and enzymes [23], and this in turn, might further accelerate the aging process.

On the one hand the number of damaged mitochondria increases with aging, and as a consequence an impaired mitochondrial ATP synthesis can be observed [5,29]. On the other hand, a higher energy demand for repair processes successively arise at advanced age. If repair mechanisms cannot keep up

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with the ongoing ROS-induced damages detrimental changes in skin structure can occur, leading to visible signs of skin aging [2].

As coenzyme Q₁₀ (CoQ₁₀) is an integral part of the mitochondrial respiratory chain, a supplementation with CoQ₁₀ presumably leads to strengthening effects on mitochondrial function and would therefore contribute to a well-balanced cellular energy metabolism at advanced age.

In addition, the levels of CoQ₁₀ in skin decline with age and UV irradiation [12] and thereby also compromise the skin's antioxidant features, leading to an increased ROS concentration at advanced age.

Any decrease in mitochondrial energy production due to impaired mitochondrial function may presumably lead to compensatory actions in cellular metabolism which result in higher energy production via non-mitochondrial pathway, e.g. glycolysis. This goes along with hints from literature reporting a higher glucose uptake and lactate production at advanced cellular age in *in vitro* aged fibroblasts [13,32]. However, this has not yet been reported for primary skin keratinocytes isolated from aged donors. In skin, the epidermis contains a 10-fold higher level of CoQ₁₀ than the dermis [24]. The epidermis as the most outer layer of the skin is directly exposed to UV irradiation and other environmental factors and it is known that UV irradiation depletes antioxidants such as CoQ₁₀ in skin [22]. Therefore the epidermis may represent a tissue that presumably benefits most from topically applied CoQ₁₀.

The data of this study clearly indicate that primary epidermal keratinocytes isolated from old donor biopsies show signs of damage in mitochondrial function as indicated by a higher glycolytic activity unlike young donors. Additionally, the *in vivo* results demonstrate that topical application of a CoQ₁₀ containing formulation significantly improves the unstressed mitochondrial capacity and also prevents distortion of mitochondrial function followed by UVA irradiation, already after a short CoQ₁₀ application period.

However, the pathways which link the age-associated increase of glycolytic flux with malfunctioning mitochondria are still poorly understood, but there are first hints that the super-metabolic regulator AMP-activated protein kinase (AMPK) might be an overall regulator in energy metabolism [9,15].

2. Material and methods

2.1. Glucose uptake and lactate production

Subconfluent primary keratinocytes were cultured in KGM-2 (Lonza, Apen, Germany) for 24 hours (37°C, 7% CO₂) and medium aliquots were collected at $t_0 = 0$ h and $t_1 = 24$ h. Glucose concentration was measured with a blood sugar detection device (Accucheck Aviva, Roche, Mannheim, Germany) and lactate concentration was determined enzymatically (Lactate Assay, Trinity Biotech, Lemgo, Germany). After fixation and staining with propidium iodide (20% ethanol, 8.3 μ M propidium iodide in phosphate buffered saline; overnight), microscope pictures (Axiovert, Zeiss, Jena, Germany) of the adherent cells were taken and cells were counted. The difference between t_0 and t_1 concentration of glucose and lactate was related to cell number.

2.2. Oligomycin incubation

For inhibiting ATP synthase, primary keratinocytes were incubated with 1 μ g/ml oligomycin (Fluka, Seelze, Germany) in the culture media for a 10 min period and subsequently washed three times prior to further analysis.

2.3. Western blot analysis

The protein samples were analysed by SDS-polyacrylamide gel electrophoresis and transferred by electroblotting onto nitrocellulose membranes. Immunodetection was performed using polyclonal antibodies against GLUT-1 (Abcam, Cambridge, UK) and GAPDH (Hytest, Turku, Finland) or monoclonal antibody cocktail recognizing subunits of the respiratory chain (Western Blotting detection Kit for OXPHOS complexes, Mitosciences, Eugene, Oregon, USA). Proteins were detected using an enhanced chemiluminescence protein detection method (Lumilight plus, Roche Diagnostics, Mannheim, Germany).

2.4. Quantitative Real-Time PCR Analysis

Total RNA was isolated from primary keratinocyte cultures using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). cDNA was prepared from 1 µg RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. The resulting cDNA was used in a PCR reaction using micro fluidic cards containing primers for target genes, i.e. GLUT-1. PCR reactions were run in duplicate and quantified with an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Cycle threshold values were normalized to 18S-rRNA by $2^{-\Delta\Delta Ct}$ method as described earlier [31].

2.5. Topical application of CoQ₁₀

Healthy volunteers topically applied a formulation containing 0.01% CoQ₁₀ twice a day for a period of 7 days on the upper arms. The study was conducted according to the Declaration of Helsinki Principles and participants gave their written informed consent. All experiments were performed and approved by the R&D, Beiersdorf AG, Hamburg, Germany.

2.6. Suction blister

Epidermal roofs were harvested from suction blisters of human skin [16]. For blistering on an area of about 5 mm in diameter, a vacuum of 300 mbar was used for about 2 h. Primary skin cells were then harvested by trypsin digestion from the freshly prepared epidermal biopsies.

2.7. UVA source

UVA irradiation (20 J per cm² for one time) was performed using a Dermalight 2020 illuminator (Dr Hönle, Planegg, Germany). The emitted dose was quantified using a UV-radiometer IL1700 (International Light, Newburyport, Massachusetts).

2.8. Evaluation of mitochondrial membrane potential (MMP)

The MMP was measured using the dye 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes, Eugene, Oregon, USA), which can be used to specifically quantify alterations in MMP in living cells [6]. Fluorescence was measured spectrometrically (EX 485 nm/EM 530 nm and EM 485 nm/EM 590 nm; Safire microplate reader, Tecan, Crailsheim, Germany). All data presented were background corrected by measuring fluorescent values without cells.

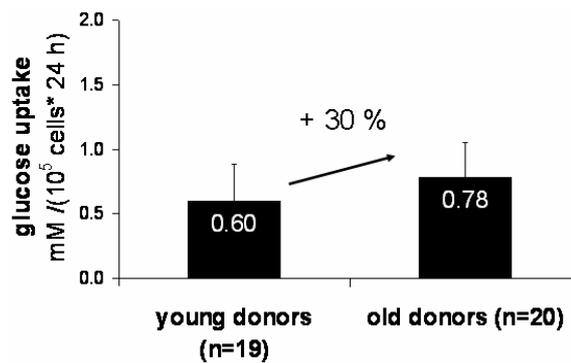


Fig. 1. glucose uptake of keratinocytes from young and old donors.

3. Results

3.1. Human skin keratinocytes derived from aged individuals show a higher glucose uptake

The effects of age dependent changes in energy metabolism were investigated in primary human skin keratinocytes. Glucose uptake in keratinocytes from young and old donors was analysed. The differences between two age groups were examined: (1) aged 19–37 years, (2) aged 61–73 years. The results are shown in Fig. 1. Within the elderly group, the level of glucose uptake was substantially increased by 30% compared to the group of young individuals (0.78 mM to 0.6 mM). Notwithstanding the expected inter-individual variability this difference is very close to statistical significance ($p = 0.086$).

3.2. Expression of glucose transporter is not altered during aging

Total mRNA level of GLUT-1 of isolated keratinocytes derived from young and old donors was analysed. Quantitative real time PCR was carried out and the results are diagrammed in Fig. 2a. The mRNA data for GLUT-1 are at identical levels in both age groups ($p = 0.704$).

We also compared GLUT-1 protein content in primary keratinocytes of young vs. old individuals by western blot analysis (Fig. 2b) followed by densitometric quantification (Fig. 2c). The GLUT-1 signal is at identical level in both age groups and therefore consistent to the collected mRNA data. These data clearly show that the amount of glucose transporter is not altered during aging of keratinocytes.

3.3. Additional glucose uptake in keratinocytes of old donors is almost entirely converted into lactate

Lactate production was measured to determine the ratio of lactate:glucose in both age groups. Figure 3 shows the results of these experiments.

Keratinocytes of the old age group show a highly significant increase (+57%) in lactate production compared to the young group ($p = 0.001$). Remarkably, this increase is not only based on the higher uptake of glucose, but on a clear shift in the glucose metabolism to lactate. Keratinocytes from young donors convert 1 mol glucose into 1.27 mol lactate whereas keratinocytes from the old age group convert 1 mol glucose into 1.53 mol lactate (Table 1).

Table 1
Glucose to lactate conversion

| Age group | Mol lactate/mol glucose ¹ |
|-----------|--------------------------------------|
| Young | 1.27 |
| Old | 1.53 |

¹Ratio of mean lactate production (mM/10⁵ cells·24 h) to mean glucose uptake (mM/10⁵ cells·24 h) per age group

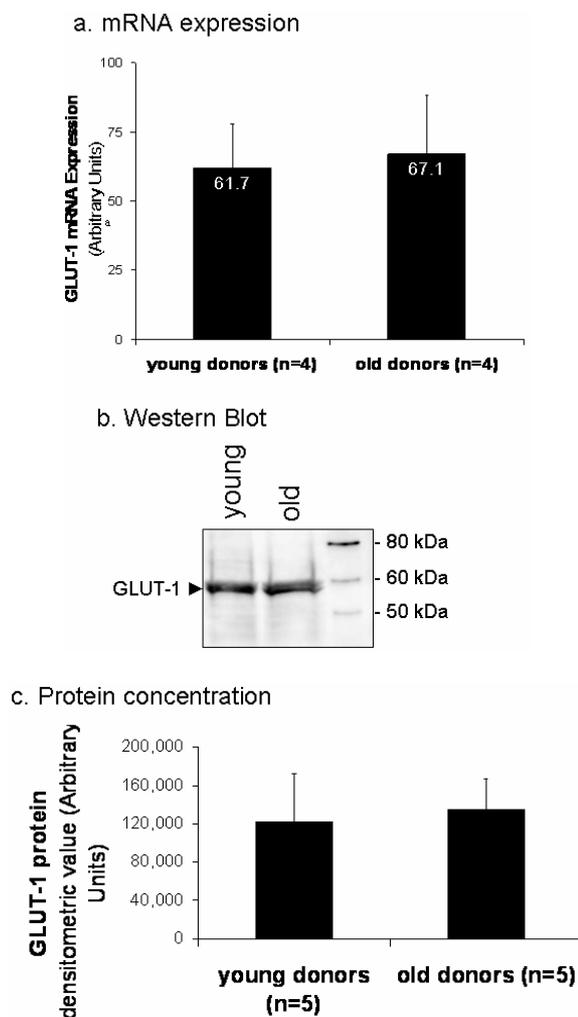


Fig. 2. (a) GLUT-1 mRNA expression in primary keratinocytes of young and old donors. (b) Western blot analysis of membrane protein isolated from keratinocytes derived from one young and old donor. A representative picture out of five experiments is displayed. (c) Densitometric quantification data from western blot analysis. The GLUT-1 protein content was measured in isolated membrane proteins from cells derived from five young and five old donors by western blot analysis using a polyclonal anti-GLUT1-antibody (1:400) and subsequent enhanced chemiluminescence detection.

3.4. No age dependent differences in mitochondrial concentration at protein and transcriptional level

To compare the content of mitochondria in primary keratinocytes between young and old individuals, a western blot analysis was carried out to investigate the mitochondrial protein concentration. Four proteins

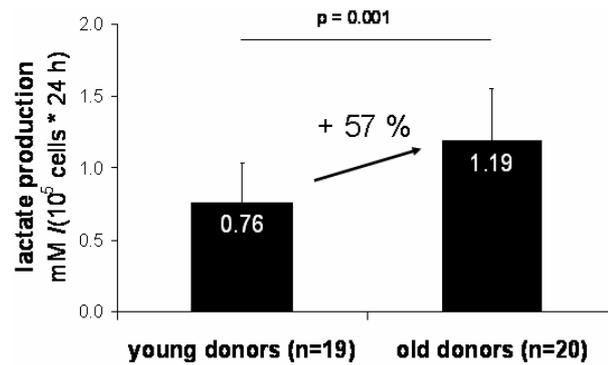


Fig. 3. Lactate production of keratinocytes from young and old donors.

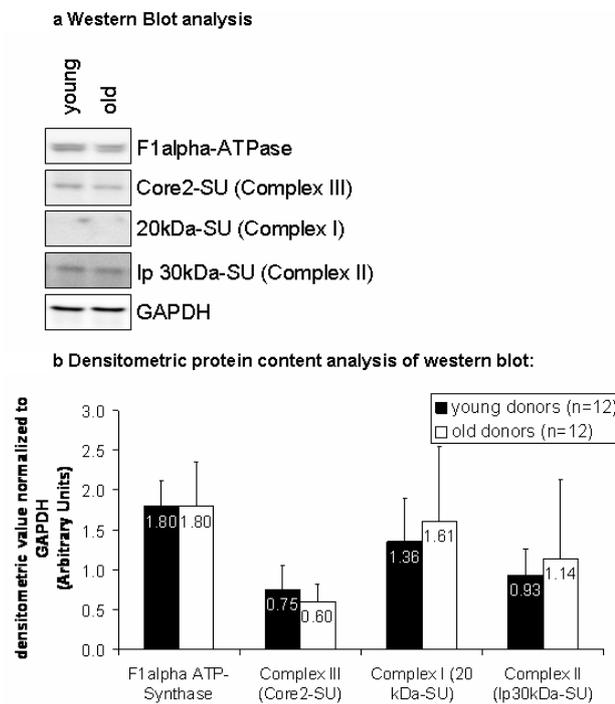


Fig. 4. (a) Western blot analysis of keratinocytes lysates for subunit (SU) content of respiratory enzymes. GAPDH was used as loading control. This is a representative result of one young and one old donor out of a total of 12 determinations per age group. (b) Densitometric protein content analysis of western blot.

of the respiratory chain, which are essential for mitochondrial function, were quantified in keratinocyte samples (Fig. 4a). Densitometric analysis of the western blot signal clearly indicates no difference between the two age groups (Fig. 4b). We have also investigated the mitochondrial ultrastructure in keratinocytes from both age groups. Transmission electron microscopy pictures revealed that there is no visible difference in mitochondrial structure or distribution (Data not shown).

In conclusion, neither the amount of mitochondria nor the structure showed visible differences in the two age groups.

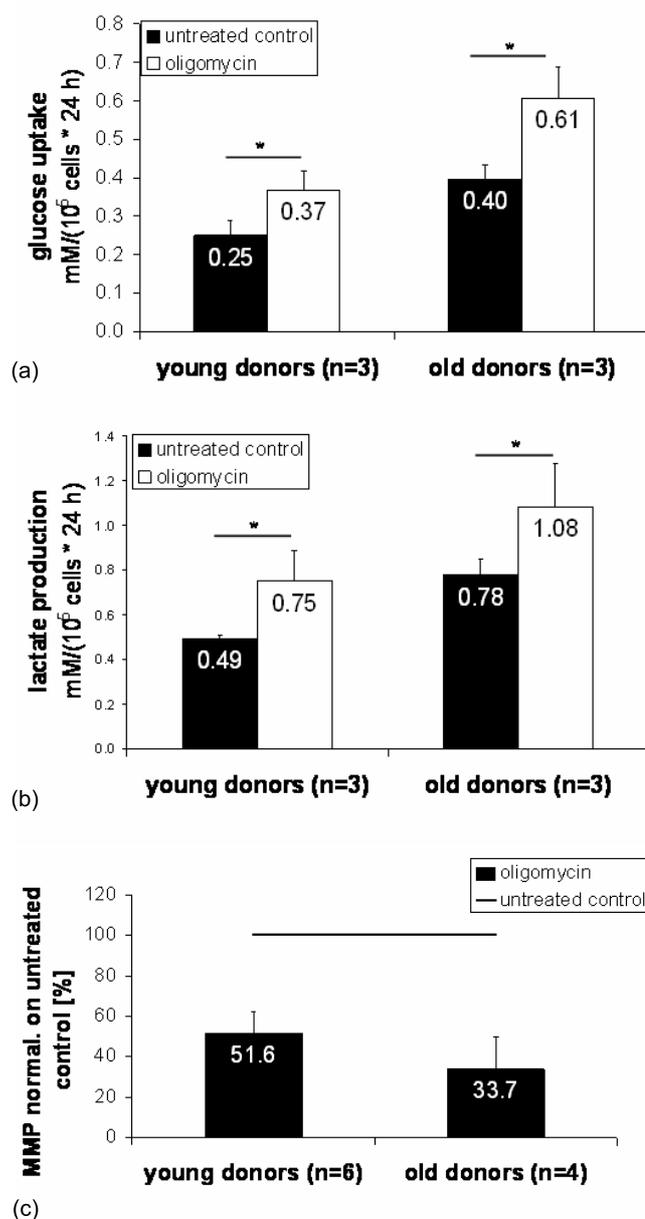


Fig. 5. (a) Oligomycin significantly increases glucose uptake of keratinocytes from young and old donors. (b) Oligomycin significantly increases lactate production of keratinocytes from young and old donors. (c) Oligomycin significantly decreases MMP in keratinocytes from both age groups. (*, significant difference; $p < 0.05$).

3.5. Effects of oligomycin on glucose uptake, lactate production and mitochondrial membrane potential

An essential protein of mitochondrial ATP production, ATP synthase, was inhibited by oligomycin to mimic age-associated changes. The glucose uptake and lactate production were analysed to determine the effect of oligomycin on glycolytic flux. Additionally, the mitochondrial membrane potential of

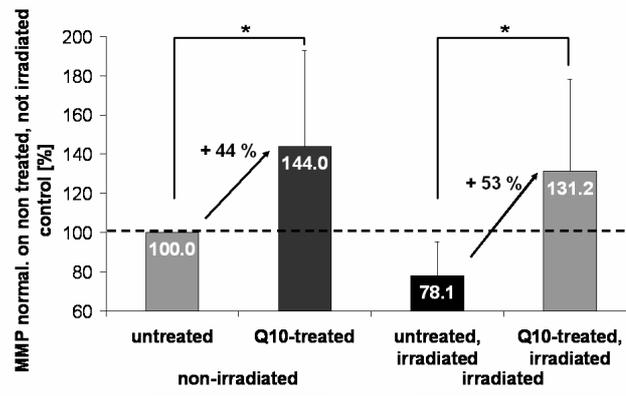


Fig. 6. Mitochondrial membrane potential (MMP) protection following topical application of CoQ₁₀. All data represent mean \pm SD and were related to untreated, non-irradiated control (=100%). (*, significant difference; $p < 0.05$).

untreated and oligomycin-treated keratinocytes was investigated.

The oligomycin-induced disruption of mitochondrial function results in a significant increase in glucose uptake in keratinocyte derived from young (+48%) and old donors (+38%) (Fig. 5a). A similar effect induced by oligomycin on lactate production was discovered for the young (+53%) and the old age group (+38%) (Fig. 5b). Oligomycin incubation lead to a rise in glucose uptake (0.37 mM / 10⁵ cells per 24 h) and lactate production (0.75 mM / 10⁵ cells per 24 h) in keratinocytes from young donors, which were at the same level to values detected in the basic, unstressed state of keratinocytes from the old age group (for glucose uptake i.e. 0.40 mM / 10⁵ cells per 24 h; for lactate production i.e. 0.78 mM / 10⁵ cells per 24 h). Furthermore, the mitochondrial membrane potential is significantly diminished following oligomycin incubation *in vitro* (Fig. 5c). Keratinocytes from old donors respond more sensitive to oligomycin in regard to MMP, however not significantly different from keratinocytes derived from young donors ($p = 0.152$).

3.6. Detrimental effects after age-inducing irradiation in mitochondrial function can be reversed by topically applied CoQ₁₀

Mitochondrial membrane potential as a parameter of cutaneous energetic capacity was measured *in vivo* after CoQ₁₀ application. In addition, the stabilizing effects of CoQ₁₀ in regard to UV irradiation were investigated.

Healthy volunteers topically applied a CoQ₁₀-containing creme formulation twice a day in a seven days period. Afterwards, epidermal primary keratinocytes were isolated via suction blister, irradiated and examined for MMP (Fig. 6). CoQ₁₀ application clearly resulted in a significant amelioration (+44%) of MMP compared to the untreated control.

Irradiation with 20 J per cm² UVA light lowered MMP to 78.1% of the untreated control. CoQ₁₀ pretreatment lead to a protection of MMP against UV irradiation and resulted in significant stabilization (+53%) of MMP compared to the untreated, irradiated value.

4. Discussion

The skin is permanently exposed to environmental stressors, which influence the physiological behaviour of cutaneous cells and thus the skin as an entire organ. During the aging process of skin cells, these stressors, UV irradiation and mainly extrinsic and intrinsic ROS in particular, procure an enhanced intracellular ROS concentration which lead to a damage of cellular components and mitochondrial function [23]. The impairment of mitochondrial function can lead to a compensatory response at cellular level in terms of a rise in anaerobic glycolysis [19,30].

This study shows an age dependent change in energy metabolism in skin keratinocytes and the beneficial effects of CoQ₁₀ topical application on cutaneous energetic capacity *in vivo*.

Primary keratinocytes derived from old donors show a higher glucose uptake compared to the cells obtained from a young donor panel (Fig. 1). As it is well-known, that the uptake of glucose in keratinocytes is dependent on the amount of glucose transporter proteins, mainly on glucose transporter type 1 (GLUT-1) [26], we investigated whether the observed increase in glucose uptake correlates with a higher expression of GLUT-1. Since GLUT-1 mRNA and protein levels are not altered between the two age groups (Fig. 2), we can conclude that the amount of GLUT-1 is not the rate limiting step for glucose uptake in keratinocytes under these conditions. Furthermore, we tried to elucidate whether the increased uptake causes accumulation of glucose in the cell or is further utilized for energy production. The increased lactate production in keratinocytes from the old age group (Fig. 3) clearly indicates a suboptimal utilization of glucose and a shift in metabolism towards an increased anaerobic glycolysis. These data correlate with results from other experiments showing a higher glucose uptake and lactate production caused by a loss of mitochondrial DNA [19] or cells suffering from a severe respiratory chain deficiency [30]. Taking this into consideration our data show no differences in mitochondrial content and structure during aging in skin keratinocytes (Fig. 4). This clearly states that the number of mitochondria does not change, but rather their function. This decline of mitochondrial function may explain the observed age-associated glycolytic activity as some kind of compensatory counterregulation. Stimulation of mitochondrial dysfunction by inhibition of ATP synthase (Fig. 5) supports this assumption, as it leads to a comparable rise in glucose uptake and lactate production in keratinocytes from young donors, which are almost comparable to values detected in the basic, unstressed state of keratinocytes from the old age group (Fig. 5a, b). This also indicates that oligomycin incubation might be a suitable and applicable way to mimic age-associated changes in mitochondrial energy metabolism of keratinocytes *in vitro*. Moreover, keratinocytes from old donors evidently respond more sensitive to oligomycin concerning the parameter of MMP, which might be another hint for the existence of some functionally predamaged and fragile mitochondria in old donor cells (Fig. 5c). In this context one can speculate that the chosen primary cell culture system, on basis of primary skin cells derived from young and old donors model of aging, might be a suitable and reliable model to investigate the effects caused by aging in closer detail, despite a high donor variability [29]. *In vitro* aged cell culture models may bare the risk to be affected by a selection of cells favoured to the cell culture conditions, which occurs during long term cell culture and thereby might distort age related effects.

In order to find a way to efficiently and rapidly target this loss of mitochondrial function *in vivo*, the effects of CoQ₁₀ were investigated in an *ex vivo* study (Fig. 6). Epidermal keratinocytes significantly benefit from topically applied CoQ₁₀ *in vivo* already after a seven days application period, thus stating that CoQ₁₀ can overcome mitochondrial dysfunction by enhancing MMP. Moreover, CoQ₁₀ treatment protects cutaneous energetic capacity against UV irradiation in an overcompensatory manner.

These data clearly correlate with findings from earlier experiments, which exhibit that CoQ₁₀ stabilizes mitochondrial function, attenuates oxidative effects in human skin cells and additionally exerts positive

effects on cell vitality [2,3]. Another reason for the beneficial effects of CoQ₁₀ application might be explained by the replenishment of its age- and UV-induced loss [12,22].

The pathways, which link the age-associated increase of glycolytic flux with malfunctioning mitochondria are still not fully understood. It can only be speculated about the causes of an increase in anaerobic glycolysis at advanced age. It might be a cellular strategy to minimize ROS production by dysfunctional mitochondria or to compensate lower mitochondrial function by a higher glycolytic activity. To elucidate this further, a new target is needed that encompasses the whole energetic pathway network. AMPK can be speculated to be such a key player, as AMPK plays a central role in the control of cellular energy balance in many tissues with high energy expenditure [21] and is regarded as the “fuel gauge” in energy extensive tissues [8].

Although the role of AMPK in skin remains obscure, we anticipate a central energy regulating function in cellular metabolism. We performed several experiments showing that the phosphorylation of AMPK, thus its activation, in skin keratinocytes can be induced by substances that mimic high AMP levels (data not shown), clearly indicating its functional expression in human skin. We assume that AMPK has a similar balancing effect on cellular energy metabolism in skin keratinocytes as described in other tissues. AMPK might be an indicator for examining energetic failure and the resulting events at cellular level. In future prospects we focus on elucidating whether AMPK could act as a further biomarker of energetic capacity in skin.

It is known from the literature that high glycolytic fluxes and glucose accumulation are sources of endogenous damage, because most glycolytic intermediates favour the formation of advanced glycation endproducts (AGEs) [11] via reactive carbonyl groups, that are able to modify protein amino groups in the cytosol. Moreover, it is reported that the intracellular concentration of these glyating agents - such as highly reactive methylglyoxal - is determined by the rate of glycolytic activity [1,20]. Methylglyoxal, glyoxal and other autooxidated derivatives of sugars induce AGE's that negatively affect essential features of skin cells and extracellular matrix proteins. Kueper and colleagues have reported earlier, that AGE formation results in a loss of contractile capacity and cytoskeleton integrity in human skin fibroblasts [17], which possibly affects tissue cohesion and leads to visible effects of skin aging. Whether comparable effects of AGE formation can be reversed by optimal glucose utilization facilitated by CoQ₁₀ application, remains to be elucidated for keratinocytes.

In summary, our work has proven the age-dependent loss of aerobic, mitochondrial activity in epidermal keratinocytes, leading to a shift from aerobic to anaerobic, glycolytic pathway, which is inevitably linked to a suboptimal utilization of glucose. The stabilization of mitochondrial function as shown to be facilitated by topical CoQ₁₀ already after a short application period, emphasizes the importance of CoQ₁₀ for an application in age associated deficiencies and again emphasizes CoQ₁₀'s fast efficacy in cosmetic formulations and its beneficial effects for anti aging skin care.

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