
A Promising Combined Approach for Assessing Mitochondrial Turnover under Oxidative Stress

Rachel Ramchurn, Mélissa Sullerot, Séverine Escaich, Karine Laulagnier, Gaëlle Saint-Auret

GENEL, Grenoble, France

Abstract

To maintain skin turnover and renewal, Adenosine triphosphate (ATP), the energy source of skin cells, is crucial. It is required for proliferation, collagen synthesis, and DNA repair. The mitochondrion is the powerhouse of the cell and generates energy in the form of ATP. But with age and aggressions, this energy production becomes less effective and entails excess formation of deleterious Reactive Oxygen Species (ROS). Defective and dysfunctional mitochondria accumulating in cells impact the skin's functions, and ultimately its appearance. According to the pioneer work of Prof Y. Ohsumi, Nobel Prize winner for Medicine in 2016, autophagy is responsible for dumping damaged cellular material. Indeed, mitophagy, which is autophagy of mitochondria, functions as quality control scheme: the cell will eliminate defective mitochondria by fragmentation and keep the functional ones by fusion. Nevertheless, this process is a delicate balance between fusion and fragmentation. The purpose of our work was to combine several techniques to determine and investigate mitophagy hallmarks of skin primary cells under stress. In this study, we used primary keratinocytes to explore the effect of an applied oxidative stress on mitochondrial autophagy. First, the study was focused on mitochondrial visualization by a specific fluorescent dye: MitoTracker®. To label mitochondria, cells were incubated with MitoTracker® probes, which passively diffuse across the plasma membrane and accumulate in active mitochondria. Under oxidative stress, H2O2, we detected a decrease of the total labeling intensity. In parallel we also visualized increase of mitochondria fragmentation. Together, those results may indicate a fission mechanism dependent on the applied stress. To confirm this observation, we

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investigated both genetic and epigenetic impacts of oxidative stress. The expression profiles of genes implicated in mitochondrial fission, were followed by RT-qPCR. In parallel, the modulation of associated microRNAs (miRNAs), major actors of epigenetics, was analysed. Furthermore, during mitophagy the flawed mitochondria are discarded and thus the ATP concentration should be reduced. We therefore measured ATP concentration to confirm that the H202 stressed-cells contain less ATP that the unstressed ones. In conclusion, we show the effect of an oxidative stress on mitophagy using visualization techniques, RTqPCR and ATP quantification on primary keratinocytes. Optimization and combination of several techniques on primary skin cells seem to be relevant for the study of the effect of an oxidative stress on mitochondria.

Keywords: Mitophagy, Oxidative Stress, ATP, Skin, Mitochondria, Aging, Autophagy, MitoTracker, Gene expression, microRNA

The authors certify that the paper is an original.

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Introduction

Exposure to UV or environmental agents increase production of Reactive Oxygen Species (ROS) and provokes oxidative stress in cells. Unbalanced respiratory chain, located in mitochondria, is also a natural producer of ROS [1, 2]. Mitochondria are thus one of the first target of ROS and their destruction leads to cell apoptosis [1, 2]. Excess production of ROS is responsible for deleterious effects of oxidative stress, notably in skin, resulting in aging phenomenon and changes in appearance [3].

Natural anti-oxidants such as Vitamin C and E, are produced by the cell, but with aging or persistent environmental aggressions, anti-oxidants are not sufficient to overcome the effects of oxidative stress. Active ingredients are constantly under investigations to protect skin from oxidative stress. However, reliable assays are missing to test their potential.

We optimized a complete experimental set of methods to estimate the effect of an oxidative stress, notably on mitochondria, in skin cells. We induced an H2O2 stress which is commonly used to rapidly induce a strong oxidative burst with a high production of deleterious oxygen species. Then, we set-up metabolic, cell biology and genomic experimental approaches to improve measurements of oxidative stress effects on skin cells.

Materials and methods

Cell culture:

Normal Adult Human Primary Epidermal Keratinocytes (NHEK) were provided by Lonza and cultured in complete medium composed of Epilife medium (Thermofisher_MEPICF500 kit) supplemented with HKGS (Thermofisher, S0015), 60µM CaCl2, 1% Penicilline/Streptomycine (Thermofisher, 15140122) in an incubator with 5% CO2 at 37°C in a humidified atmosphere. Cells were seeded at 80,000 cells per well in a 12-well plate (RNA quantification) or per 18mm glass coverslips (mitochondria staining) 24 hours prior to experiments.

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Oxidative stress:

Cells were incubated with 0.3 mM (for mRNA and miRNA quantification) or 1 mM hydrogen peroxide (for Mitochondria labeling) for 45 min at 37°C in PBS with Calcium and magnesium (Invitrogen). Cells were put back in complete medium for 2h (Mitotracker labeling) or 6h (mRNA and miRNA quantification) before further analysis.

ATP quantification:

ATP assay was carried out using Molecular Probes® ATP Determination Kit from Thermofisher (#A22066), an extremely sensitive (> 0.1 picomole of ATP) bioluminescence assay. Briefly, after oxidative treatment, cells were lysed using passive lysis buffer from Promega (E1941) and ATP quantity was determined according to manufacturer's protocol using a standard curve.

Mitochondria labeling:

Cells grown on 18 mm glass coverslips were stressed with H2O2 as described above. After 2h, cells were labeled with 150 nM MitoTracker RED CMXRos (Life technology) in cell culture medium for 30 min at 37 °C, then washed in PBS, and fixed for 10 min in 4% paraformaldehyde, washed again and stained for 10 minutes with Hoescht 33342 (Life technology) for nuclei staining. Coverslips were then mounted with Fluoromount (Sigma-Aldrich) and cells were imaged with a 40X, 63X or 100X objectives using AxioImager microscope (Zeiss). Mitotracker dye was visualized through Rhodamine filter and Hoechst with DAPI filter.

Fluorescence quantification:

Fluorescence intensity was measured on a minimum of 100 cells acquired with a 40X objective. Quantification was performed with Image J software using a dedicated Macro. The Macro allows to substract the same background from all pictures before quantification of total fluorescence intensity and normalization according to the number of cells labeled with Hoechst dye. Precise mitochondria structure was visualized with 63X and 100X objectives.

Total mRNA Extraction:

Cells grown in 12-well plates were first lysed with 350 µl Qiazol (QIAGEN) for 10 minutes at room temperature. Total RNA, including miRNA, were extracted using the RNEasy Plus Mini Kit (Qiagen)

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following the manufacturer's protocol. The absence of proteins and solvants contamination and quantification of the extracted RNA were then evaluated using NanoDrop spectrophotometer.

RT-qPCR on mRNA:

250 ng of purified RNA were reverse transcribed with the Superscript VILO cDNA Synthesis kit (Invitrogen). A control without Superscrit was carried out to ensure the absence of contamination in our samples. Amplification was performed using Platinum Quantitative PCR SuperMix-UDG Kit (Invitrogen) according to the manufacturer's instructions and performed with the CFX-connect instrument (Biorad). This analysis was performed in triplicate. The results were normalized to GAPDH expression and expression levels of RNA were calculated using the 2DD Ct method.

RT-qPCR of miRNA:

Reverse transcription was performed on 15 ng of the previously purified total RNA using miRCURY LNA Universal RT microRNA PCR kit (Applied Biosystems) following manufacturer's instructions. An enzyme-free control was carried out to ensure no contamination in our sample experiments. The quantitative PCR in real time was performed using miRCURY LNA SYBR Green PCR kit (QIAGEN) following manufacturer's instructions. This analysis was performed in triplicate. RNU1A miRNA quantification was used as an endogenous control to normalize sample experiments. The relative miRNA expression between the groups were then evaluated using the 2DD Ct method.

Statistic analysis: Data represent the means of the indicated number of experiments +/- SEM Difference between control and treated groups were analyzed using a t test Student. Each condition group was carried out in triplicate. The significance statistic was considered when $p<0,05$.

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Results

Oxidative stress inhibits ATP production by keratinocytes

ATP production by mitochondria is a general read-out of oxidative stress effects. We induced the stress with H2O2 which is commonly used to rapidly induce a strong oxidative burst. After treatment of primary human keratinocytes with 1mM H2O2, we observed a high and significant reduction of total cellular ATP produced by keratinocytes (Figure 1).

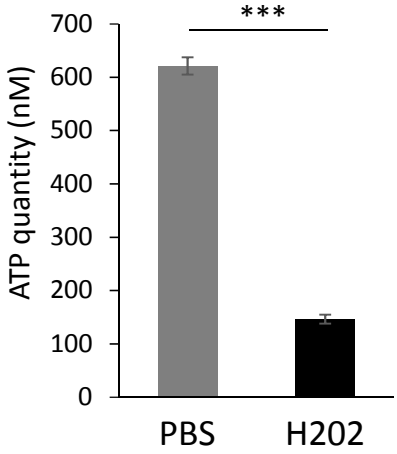

Figure 1: Oxidative stress inhibits ATP production in keratinocytes. Primary human keratinocytes were treated with 1mM H2O2 during 45min before measurement of cellular ATP in cell lysates. The mean +/- SEM of 6 experiments is shown. ***p<0.001 (unpaired, two-tailed student t test)

Determination of mitochondria defects in keratinocytes after oxidative stress.

Mitochondria can be stained with fluorescent dyes such as Mitotracker probes that label functional mitochondria in living cells. After induction of damage by ROS production, mitochondrial activity undergoes an overall decrease and mitochondria are fragmented. These two features can be measured with Mitotracker dye [4].

We thus labeled keratinocytes in culture with Mitotraker dye after H2O2 exposure. In figure 2A, fields of cells treated or not with H2O2, are shown. Total fluorescence quantification in figure 2B demonstrates a significant 20% decrease of Mitotracker labeling in H2O2 conditions. Hoechst staining does not show any nuclear condensation suggesting that there is no apoptosis immediately after

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treatment. We therefore concluded that the labeling of mitochondria was significantly affected by oxidative stress.

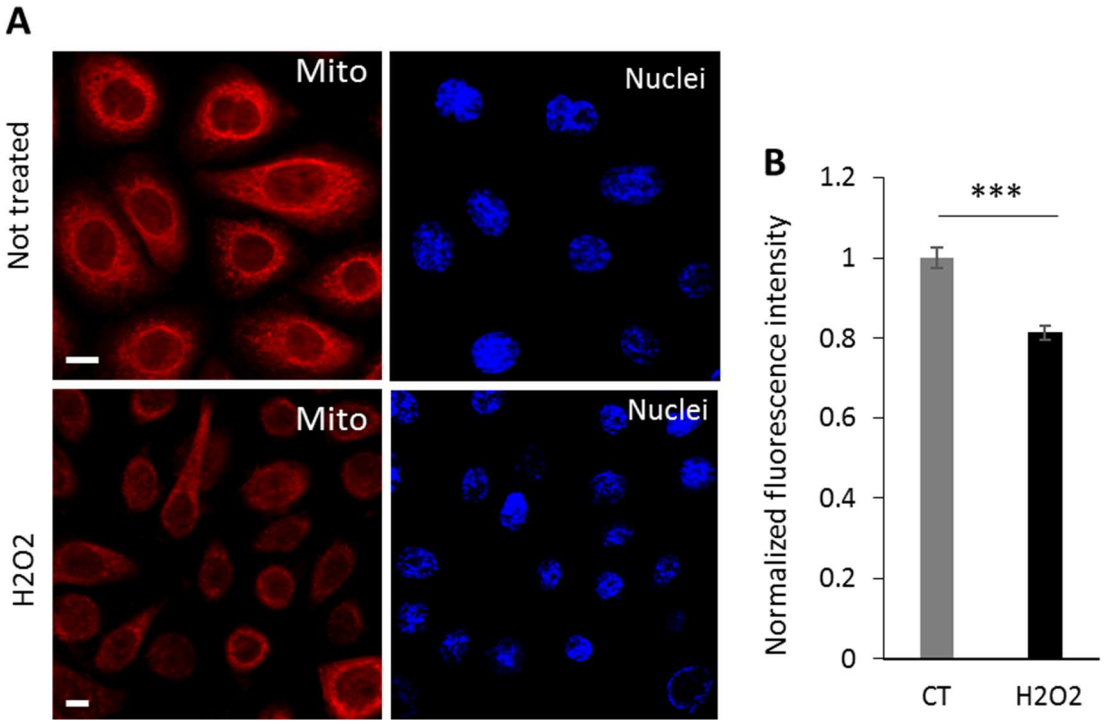

Figure 2: Effect of oxidative stress on mitochondria labelling in keratinocytes. Primary human keratinocytes were treated with 1mM H2O2 and living mitochondria were labeled with Mitotracker Red CMXRos (red) and cell nuclei with Hoescht dye (blue). **A.** Fluorescence from control (upper) or H2O2-treated cells (lower) was imaged with a 40X objective. Bar= 10µm **B.** Quantification of total fluorescence intensity with Image J software is shown. ***p<0.001 (unpaired, two-tailed student t test).

Using the same probe and a higher magnification, we visualized the mitochondrial structure more precisely. In figure 3, we can observe a radical change of Mitotracker labeling between control conditions where a filamentous network is visible and after H2O2 treatment where isolated fragmented structures clearly appear. This fragmentation is a well-documented mechanism resulting from an imbalance of the fusion-fission dynamics of mitochondria towards fission [1, 2]. These experiments show that H2O2 treatment of keratinocytes induces fragmentation mitochondria.

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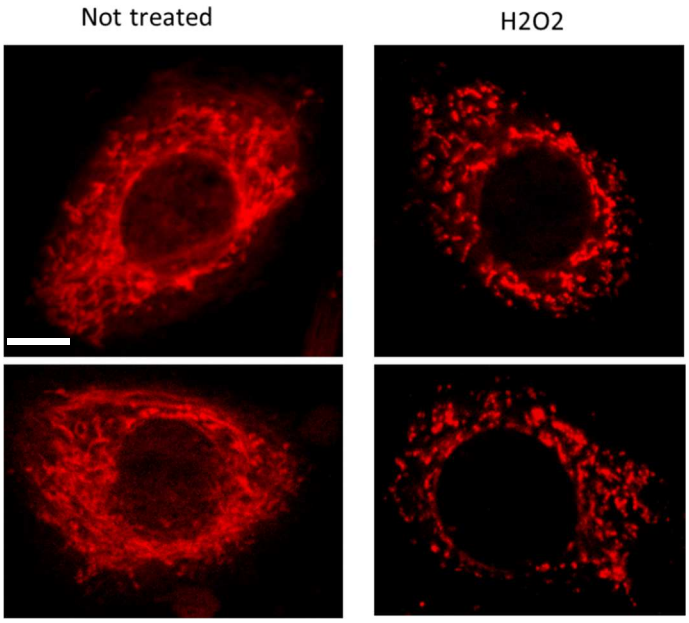

Figure 3: Mitochondria morphology after oxidative stress. Cells were treated and labeled as in figure 2. Mitochondria morphology was observed in control cells (left) and in H2O2-treated cells (right). Two pictures for each treatment are shown. Bar = 5 µm

Gene and miRNA expression profiles after oxidative stress in keratinocytes

We then carried on our analysis of oxidative stress effect on keratinocytes at the genomic level. We measured the expression of genes implicated in DNA damages (MLH1), apoptosis (cytochrome c, CYC1), anti-oxidative response (NF2E2L), autophagy (Beclin1) and finally genes involved in mitochondrial function, i.e. fission (Fis1), respiratory functions (GCAT, SHMT2) and inhibition of ATP production (ATP5IF1) [1, 5, 6, 7]. Among these mechanisms, autophagy that specifically clears the cell of damaged fragmented mitochondria is called "mitophagy" and is of crucial for cell survival [2]. As shown in figure 4A, we found that these genes were all upregulated after oxidative stress in keratinocytes. These gene expression changes occur 6h after treatment indicating a rapid modification of cell homeostasis.

MicroRNAs are also known to regulate these mechanisms [8, 9]. As shown in figure 5B, three microRNAs, miR23a-3p, miR34a-5p, miR21-5p, were significantly reduced under oxidative stress, thereby demonstrating that oxidative stress can rapidly affect miRNA expression in our experimental model.

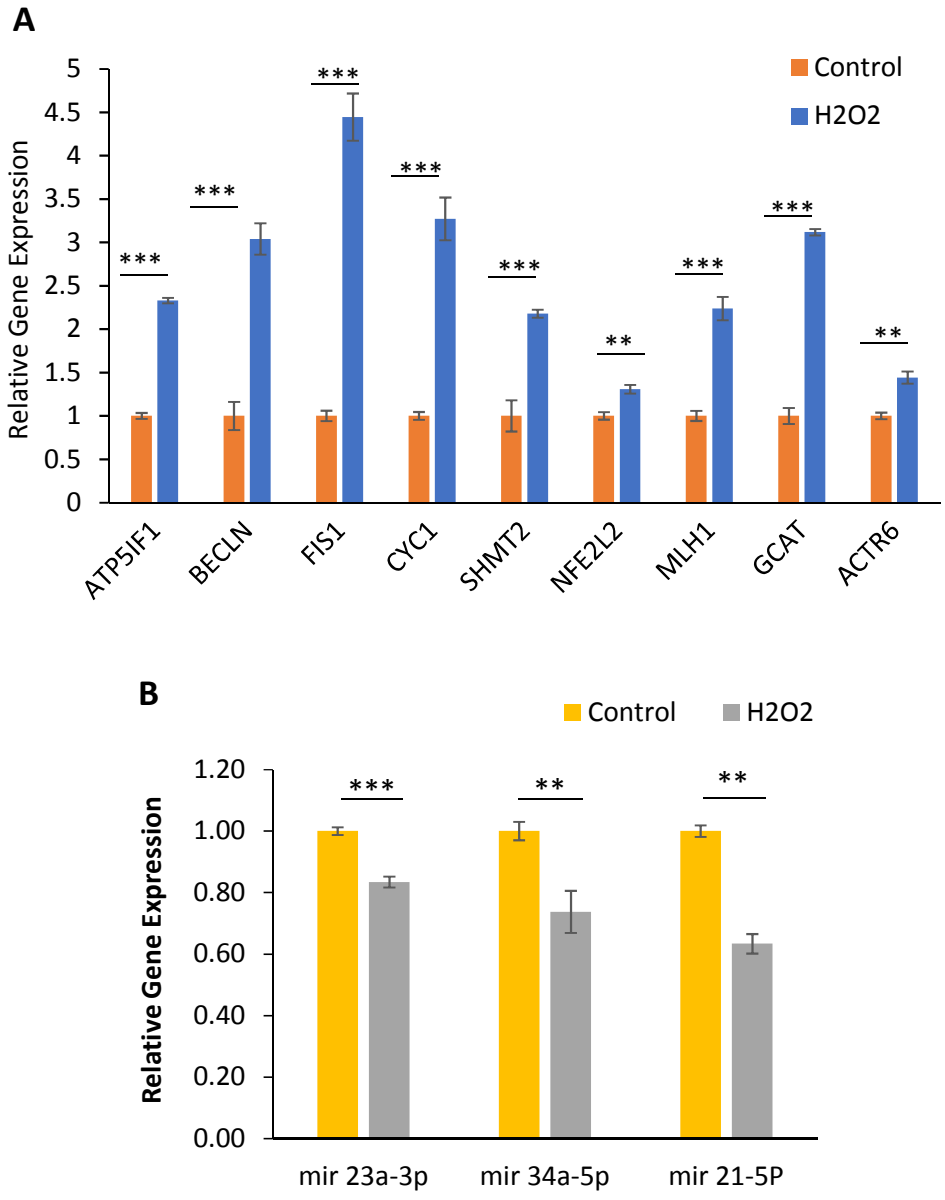

Figure 4: Effect of oxidative stress on expression of genes and microRNA. 6h after H2O2 exposure, keratinocytes were lysed and expression of specific genes (A) and microRNA (B) were analysed by RT-qPCR. Expression of genes were normalized to GAPDH (mRNA) or RNU1A (miRNA). Means of triplicates +/- SEM are presented. Statistics were made with unpaired, two-tailed student t test. ***p<0.001; **p<0.01.

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Discussion

We have used human primary keratinocytes as a cellular model to estimate effects of oxidative stress since these cells are the first protection barrier against environmental stress. Four cell functions have been chosen as read-outs of oxidative stress effects: cellular ATP level, mitochondrial activity and quantification of specific mRNA and microRNA involved in oxidative stress response.

3h after incubation of keratinocytes with H2O2, ATP level dropped. Also, 20% less mitochondrial labeling was observed indicating that mitochondria global activity, characterized by membrane potential and/or number of mitochondria, is affected. In normal conditions, mitochondria form a highly dynamics filamentous network regulated by constant fusion and fission processes. In stress or pathologic conditions, this dynamic is dysregulated, and fission prevails. Accordingly, we observed in figure 3 that mitochondria were strongly fragmented by the H2O2 stress.

6h after oxidative stress, nine genes implicated in response to oxidative stress were upregulated. Precisely, Fis1, that regulates mitochondrial fission [5], was increased by 4.5-fold which is in agreement with our mitochondria labeling. Beclin 1, which is a major regulator of autophagy [6], was increased by 3-fold suggesting the induction of autophagy. This process is known to be consecutive to mitochondrial fission and is required for their clearance, notably under oxidative stress in keratinocyte cell lines [6, 8]. ATPIF1, an activator of mitophagy pathway which works by blocking the ATPase activity [7], was increased by 2.5 times, which is in agreement with the decrease of ATP that we observed. Cytochrome C (CYC1) was increased by 3 times indicating the induction of apoptosis under oxidative stress.

We also found a reduction of miR34a and miR21 that were both implicated in oxidative stress response [9]. miR34a reduction is important for induction of autophagy while miR21 decrease impedes mitochondria functions [10, 11). These studies are thus in agreement with our results. Finally, we detected a significant decrease of miR23a, which downregulation has been correlated with an increased autophagy, in agreement with Beclin 1 upregulation and mitochondria fragmentation that we observed [12].

Altogether, the protocols that we optimized offer a combined and broaden approach to further analyze pro- or anti-oxidants effects of future active ingredients on skin cells, notably during aging process.

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