Non-Thermal Plasma Activates Human Keratinocytes by Stimulation of Antioxidant and Phase II Pathways

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Running title: Plasma triggers hormesis-like processes in keratinocytes

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**Background:** Non-thermal plasma provides an interesting therapeutic opportunity to control redox-based processes, e.g. wound healing.

**Results:** The transcription factor Nrf2 and downstream signaling molecules were found to act as key controllers orchestrating the cellular response.

**Conclusions:** Plasma triggers hormesis-like processes in keratinocytes.

**Significance:** These findings facilitate the understanding of plasma-tissue interaction and its deduced clinical application.

**ABSTRACT**

Non-thermal atmospheric pressure plasma provides a novel therapeutic opportunity to control redox-based processes, e.g. wound healing, cancer, or inflammatory diseases. By spatial and time resolved delivery of reactive oxygen- and nitrogen species, it allows to stimulate or inhibit cellular processes in biological systems. Our data show that both, gene and protein expression, are highly affected by non-thermal plasma. The nuclear erythroid-related factor 2 (Nrf2) and phase II enzyme-pathway components were found to act as key controllers orchestrating the cellular response in keratinocytes. Additionally, the glutathione metabolism was affected which is a marker for Nrf2-related signaling events. Among the most robustly increased genes and proteins heme oxygenase 1, NADPH quinone oxidoreductase 1, and growth factors were found. The roles of Nrf2-targets, investigated by siRNA silencing, revealed that Nrf2 acts as an important switch for sensing oxidative stress events. Moreover, the influence of non-thermal plasma on the Nrf2 pathway prepares cells against exogenic noxae and increases their resilience against oxidative species. Via paracrine mechanisms distant cells benefit from cell-cell communication. The finding that non-thermal plasma triggers hormesis-like processes in keratinocytes facilitates the understanding of plasma-tissue interaction and its clinical application.

Due to the recent advances in the development of non-thermal plasma sources operating at atmospheric pressure, the treatment of living cells and tissues with a cocktail of plasma components became possible (1). Non-thermal plasma was described to have antimicrobial activity and to play an important role in killing of microorganisms (2-5). These developments resulted in a new and independent field, the plasma medicine, in which one promising approach is to promote wound closure by stimulation of cells involved. There are numerous possibilities how plasma influences cells at a molecular and genetic level (6,7). However, the knowledge about cellular signaling events subsequent plasma-treatment of eukaryotic cells or tissues is still rudimental. The molecular and cellular mechanisms of plasma-induced in vitro effects on keratinocytes have to be examined with particular emphasis on the roles of reactive oxygen (ROS) and nitrogen species (RNS), growth factors, chemokines or chemoattractants.

Keratinocytes as a cell culture model for wound healing (8) are a component of the primary skin layer and represent a major factor for tissue repair and regeneration. Under physiological conditions, ROS/RNS are being produced in the skin continuously (9). Beside other cells, keratinocytes also express ROS-detoxifying enzymes and possess an inducible defense system (10,11). It is a well-known fact that ROS/RNS are cellular modulators and signaling molecules playing integral part in immune responses, cell differentiation, or regulating angiogenesis (12,13). In addition, it was shown that keratinocytes play a crucial role in wound healing (14-17), in regulating several proteins of the coagulation cascade, platelet recruitment and activation (18,19).

Due to the fact that many healing processes are controlled by redox reactions, the application of non-thermal plasma provides an interesting therapeutic tool in redox-based wound healing. Such plasma contains variable compositions of ultraviolet light, free electrons, charged particles, as well as reactive oxygen species (ROS, e.g. HO\(_2\), O\(_2^-\), O\(_3\), H\(_2\)O\(_2\)) and nitrogen species (RNS, e.g. NO, ONOO\(^-\)) including bioactive substances (NO, H\(_2\)O\(_2\)) (20). A further favorable advantage of non-thermal plasma is the generation of reactive species at the site of interest like wounds or skin diseases where they can directly function as signaling molecules. In this context, the general applicability of cold plasmas to treat the skin or infected wounds in vivo had been investigated (21-26).

An imbalance between the production and the detoxification of reactive intermediates affects the cellular stress level. For example, the redox balance influences the maintenance of cell
proliferation rhythm like cell cycle (12). Beyond that, changes in ROS levels trigger a coordinated action of transcription factors (27). The nuclear factor erythroid 2-related factor 2 (Nrf2), a basic leucine zipper (bZIP) transcription factor, activates cellular rescue pathways against oxidative injury, inflammation or apoptosis and activates downstream signaling. Nrf2 plays a key role in regulation of genes, which encode detoxifying enzymes and antioxidant proteins, and functions in cellular defense against imbalances in redox homeostasis. Under basal conditions, Nrf2 is associated with an actin-binding protein Kelch-like ECH-associated protein 1 (Keap1) which retains Nrf2 in the cytoplasm where it is targeted for ubiquitin-mediated degradation (28). Keap1 (Kelch-like ECH-associated protein 1), a vital factor in Nrf2 signaling cascade, is a protein containing Kelch-1 like and BTB/POZ domains (29). Small amounts of constitutively nuclear localized Nrf2 maintain cellular redox homeostasis through regulation of basal expression of antioxidant genes. After release of Nrf2 from Keap1 by oxidation events at cysteine, Nrf2 translocates to the nucleus, binds to antioxidant responsive elements (AREs) in the promoters of its target genes and activates their transcription (30). Such genes encode among others ROS-detoxifying enzymes and antioxidant, and proteins such as glutathione S-transferase, cytochrome P450, NADPH quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HMOX1), γ-glutamylcysteine ligase catalytic (GCLC) and modifier subunit (GCLM), superoxide dismutases 1-3 (SOD1-3), thioredoxin (Trx), catalase (Cat), glutathione peroxidase (GPx), and non-enzymatic antioxidants like glutathione. In addition, in the absence of oxidative stress in eukaryotes, the basic region leucine zipper transcriptional regulator Bach1 binds AREs and represses transcription (31,32). Bach1 forms heterodimers with the basic leucine zipper subfamily of small Maf protein, and bind the Maf recognition element in the promoter regions of HMOX1 or NQO1 (33). Although the role of Bach1 as a regulator of gene expression after plasma treatment is uncharacterized, it was previously shown that Bach1 antagonize Nrf2-mediated induction of HMOX1 through its interaction with multiple ARE/AP-1 sites (34). Previous data suggested that plasma induces cell reactions of stress-sensing along increased expression of enzymes of the antioxidant defense system. The analysis further indicated that stimulating properties on keratinocytes make non-thermal plasma to a promising option in treatment of wounds (7). Our present study uses both a whole-genome microarray and a proteome approach to obtain insights into potential mechanisms of plasma activation in human keratinocytes. We used an atmospheric argon plasma jet kinpen (neoplas GmbH, Greifswald, Germany) to identify potential applications of non-thermal plasma to accelerate wound healing processes. Hundreds of genes and proteins were identified that were significantly changed in at least one treatment group. Independent from treatment duration, we clarify some of mechanisms of action in plasma-treated keratinocytes. In general, non-thermal plasma treatment clearly induces a strong antioxidant effect together with a plasma-related activation of Nrf2/Keap1-pathway. Further analysis revealed increased expression of Nrf2 downstream targets which play important roles for cell protection.

EXPERIMENTAL PROCEDURES

Cell culture and transfection - HaCaT (human adult low calcium high temperature keratinocytes) cells were obtained from German Center of Cancer Research (DKFZ, Heidelberg, Germany) and were cultured in Roswell Park Memorial Institute (RPMI) medium. The medium was supplemented with 10% fetal calf serum (FCS) (Sigma, Taufkirchen, Germany), 1% penicillin G/streptomycin (Lonza) and cultured under 5% CO₂ at 37°C. Primary keratinocytes NHEK were obtained from PromoCell GmbH and cultured in PromoCell medium (Heidelberg, D). Transfection of HaCaT with appropriate siRNA was carried out, when they were 50-60% confluent using X-treme gene siRNA transfection reagent (Roche, Mannheim, D) according to the manufacturer's instructions.

Measurement of hydrogen peroxide and ROS production - Hydrogen peroxide production in medium was measured fluorimetrically using 1 μM Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazin) and 10 units/ml horseradish peroxidase. Cells were re-suspended in the phosphate-buffered saline (PBS) and incubated with plasma-treated medium for 30 min at 37°C. Reagents were added and the rate of hydrogen peroxide production was quantitated at 560 nm excitation and 585 nm emission by a microplate.
Intracellular ROS production in HaCaT cells was determined utilizing the 2′,7′-dichlorofluorescein diacetate (H2DCFDA; Sigma, Taufkirchen, Germany). Briefly, 10⁶ cells in a 6 cm-culture dish were incubated with plasma-treated-medium or H₂O₂ (100 μM) for indicated times. Then, the cells were collected and incubated with 10 μM H2DCFDA for 30 min at 37°C. Cells were centrifuged, the pellets were washed twice with ice-cold PBS, and resuspended in FACS buffer. Fluorescence intensity was measured with flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Exposure of human cells to non-thermal plasma - Non-thermal plasma was produced using an atmospheric pressure argon plasma jet in air (kinpen, neoplas GmbH, Greifswald). Several treatment times (20 s, 60 s or 180 s) were chosen to clarify effects of treatment intensity on cellular changes after indirect plasma treatment. Plasma treatment was performed in an indirect treatment regimen using a motorized stage. “Indirect” means that only the liquid medium (5 mL RPMI) came into direct contact with the plasma. Immediately after treatment medium was transferred to a dish containing one million HaCaT 293, or NHEK cells allowing generated ROS/RNS to interact with the cells (7). This procedure has been found to have comparable effects on the cells as treatment of the medium when covering the cells directly (35). 2 h after plasma exposure, fresh medium was added, and cells were incubated for further 1, 4, and 22 hours before collection, centrifugation, and washing. Cells treated with 100 μM H₂O₂ served as positive controls for oxidative stress corresponding to the above described plasma treatment equivalents. To exclude effects of the carrier gas, cells treated with argon gas were used as negative controls. Subsequently, all control samples, plasma-treated and untreated cells were analyzed and compared in respect to their gene activity. For proteome approach, complete RPMI was treated in triplicates for 180 s in the same manner as described above. Medium was transferred immediately into dishes containing cells and kept in an incubator for 3 h, 6 h, or 24 h. Control cells were not plasma-treated but otherwise treated identically (Fig. 1).

Analysis of cell viability and programmed cell death - Cells were seeded in 6-well plates, and after 24 h of cell attachment, the cells were exposed to plasma-treated medium as indicated. The viability of HaCaTs was assessed by dye-based method: calcein (green)/propidium iodide (PI, red) staining for living and dead cells, respectively. Apoptotic cells were detected by terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) stain. The cells were fixed and permeabilized using a mixture containing 0.1% Triton X-100 in phosphate buffered saline (PBS), followed by treatment with the Click-iT® TUNEL Alexa Fluor® 594 Imaging Assay (Invitrogen, Germany) at 37°C for 60 min. The apoptotic cells (pink) were visualized using fluorescence microscopy (Zeiss AG, Oberkochen, Germany).

Sample preparation for gene expression profiling - Total RNAs from each group were purified, isolated using RNA mini kit (BioSell, Germany) and RNA integrity was confirmed using the bioanalyzer2010 (Agilent) as described (7). Briefly, cDNA was synthesized from 10 μg of total RNA using cDNA Synthesis Kit (Life Technology, Darmstadt, Germany) in the presence of oligo dT primer (200 ng/mL). Double strand cDNA were labeled with fluorescent Cy3 dye. A global gene expression study was carried out using multiplex arrays containing a 4-plex format (4x72K) with 24,000 different human specific probes per array (n≥3). Hybridization and washing of gene chips were done according to the supplier's instruction and slides were analyzed by a Microarray Laser Scanner (MS 200). If not other stated, all kits, microarrays and software programs were provided from Roche NimbleGen (Mannheim, Germany).

Gene expression data acquisition and processing - Signal intensity values were translated into gene ID lists including expression values using DEVA1.1 software. Background-corrected signal intensities were determined and processed using a Robust Multichip Averaging (RMA) analysis (36). Quantile normalization of microarray data, statistical tests, and further filtering methods were accomplished by analysis software. Data handling and all calculations including cluster analysis were performed using Partek Genomic Suite (PGS). To find the differentially expressed genes, expression data were grouped according to treatment conditions and statistically analyzed using multiple testing corrections (37). The Gene ontology (GO) terms were determined by uploading the gene list on the Panther classification system. Gene family classification from Panther represents various gene
families that are over or under represented for all scenarios. Ingenuity System Pathway Analysis (IPA) was applied to summarize the effect of gene expression changes and to obtain top biological functions, pathways and networks associated with plasma treatment.

Protein separation and digestion - Total cellular protein was harvested by lysis using RIPA buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 2 mM EDTA, Roche complete protease inhibitor cocktail, 2 mM phenylmethanesulfonylfluoride). After clearance by centrifugation (10,000 G, 4°C), supernatant was used for further analysis). Proteins were fractionated by SDS gel electrophoresis (ProGel Tris Glycerin 10%) for 120 min and 125 V in electrophoresis buffer (20 mM TRIS; 200 mM glycine, 3.5 mM SDS, pH 8.3). After washing (2x 2 min, millipore water) and fixation for 20 min in methanol:water:acetic acid (50:40:10), gel was cut into lanes and 12 fractions each. In-gel protein digestion was performed using sequencing grade trypsin 1:50 (Promega, Mannheim, Germany) overnight at 37°C. After peptide extraction from the gel pieces using a step gradient of formic acid/acetonitrile and concentration/ acetonitrile removal (aqueous settings, SpeedVac Concentrator, Eppendorf, Hamburg, Germany), peptide mixture was subjected to liquid chromatography/mass spectrometry (LC/MS).

Liquid chromatography and high resolution mass spectrometry (LC/MS) - Peptide mixture was analyzed by nano LC/MS. Briefly, peptides were separated on a 30 cm / 75 μm inner diameter fused silica column (Aeris, 3 μm, RP18, Phenomenex, Aschaffenburg, Germany) using a Proxeon nanoLC II (Thermo Fisher Scientific) and a water 0.1% acetic acid/acetonitrile 0.1% acetic acid gradient (0.3 μl/min/ 120 min). Eluent was ionized by electrospray ionization technique (NanoSpray III source) and ions analyzed using an ABsciex TripleTOF 5600 mass spectrometer. All samples were injected twice. Raw data were then analyzed for peptide and protein identification using ProteinPilot 4.5 software (Paragon algorithm). Identified proteins were relatively quantified using PeakView, protein quantitation tool. After normalization in MarkerView, further data processing to obtain information on protein expression pattern and statistical analysis was done by Partek, Panther database and Ingenuity Pathway Analysis (IPA).

Gene validation using quantitative real time PCR - To confirm changes in the expression of selected genes, RNA samples from each treatment group, including those analyzed by microarray, were assayed by quantitative real-time reverse transcriptase PCR (qRT PCR). qRT PCR was performed on 96-well LightCycler 480 qPCR system (Roche Diagnostics Ltd, Germany) according to the manufacturer’s protocols. For quantification of several mRNAs by qPCR, one μg of RNA was transcribed into cDNA as described previously (7). Using SYBR Green I Master (Roche Diagnostics Ltd, Germany) qRT PCR was conducted by 20 ng of cDNA in triplicate with internal and no template controls. Primer sequences of target genes Nrf2, Keap1, NQO1, HMOX1, SOD, Cat, VEGFA, HBEGF, GCLC, GCLM, and GSR were purchased from Roche (Roche Diagnostics Ltd, Mannheim, Germany). The protocol included pre-incubation at 95 °C for 3 min, 45 cycles of 95 °C for 10 s, annealing for 20 s at 55 °C, and amplification for 1 s at 72 °C. A SYBR Green detection reporter system was used and the reactions generated a melting temperature dissociation curve enabling quantitation of the PCR products. The housekeeping gene RPL13A, whose expression was unaffected by plasma, was used as an internal control for normalization in parallel with each gene of interest. To verify reproducibility, each sample was analyzed in triplicate in three independent experiments for each gene. The expression of the single genes was analyzed using ΔΔCT method. The final value for gene expression in each plasma-treated sample was determined as a ratio of the gene expression in the respective sample related to the control.

Western blot analysis - HaCaT cells were incubated with cell culture medium treated under the same condition as described above. Following incubation for distinct time at 37°C, cells were rinsed once with ice-cold PBS, spun down and lysed in ice-cold lysis buffer RIPA containing protease and phosphatase inhibitors (cOmplete Mini, phosSTOP; Roche, Mannheim, Germany) and freshly added 2 mM phenylmethanesulfonylfluoride (PMSF; Carl Roth, Karlsruhe, Germany). Subsequently, cells were ultrasonicated (Labsonic M Ultrasonicator, Sartorius) applying 2 pulses at 50% of nominal power with a duty cycle of 50 and kept on ice for 30 min. Lysates were centrifuged at 10,000 g. Supernatants were isolated and protein
concentration was adjusted in all samples prior to heating to 95°C for 5 min in 5 x sample buffer (1.25 M Tris, 10% SDS, 50% glycerol, 10% β-mercaptoethanol, 0.02% bromphenol blue) and subjected to SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) on precast 10% PAGE Gels (Abcam, Cambridge, UK). Proteins were blotted onto Roti®PVDF membranes (Carl Roth, Karlsruhe, Germany). Subsequently, unspecific binding was blocked with 3% bovine serum albumin (BSA, Roth, Karlsruhe, Germany) in Tris-buffered saline (20 mM Tris, 13.7 mM NaCl) containing 0.1% Tween (TBS T) for 30 min. After this, the membrane was incubated with the corresponding primary antibody 1:200 at 4°C over night. Antibodies against Nrf2 (sc-13032), HMOX1 (sc-1796), NQO1 (sc-6464), and Keap1 (sc-15246) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Incubation was followed by three washing steps with TBST and incubation with horseradish peroxidase-coupled secondary antibodies 1:10,000 for 1 h at room temperature. After three washing steps in TBST, membranes were incubated with Serva Light Polaris (Serva Electrophoresis GmbH, Heidelberg, Germany) and imaged using the ImageQuantLAS4000 (GE Healthcare, Chalfont St. Giles, UK). The same membranes were stripped and reprobed with antibodies directed against β-actin (sc-9104) as a loading control. Band intensities were quantified using ImageQuantTL Software (GE Healthcare, Chalfont St. Giles, UK), normalized to actin, and expressed as the percentage of fold change compared to the corresponding control.

**Immunofluorescence microscopy** - HaCaT, NHEK and 293 cells were grown on glass coverslips in 6 well plates. Cells were transfected with siRNA for Nrf2, Keap1, or Bach1. 72 hours after transfection, cells were incubated with plasma-treated medium for 20 min and washed twice with PBS, fixed with 4% (w/v) paraformaldehyde for 20 min at room temperature. Cells were permeabilized with PBS/0.2% Triton X-100 for another 20 min, and blocked with PBS/1% bovine serum albumin (BSA).Slides were washed twice with PBS/1% BSA and incubated at 4°C for 24 h with primary antibody Nrf2 at a 1:200 dilution in PBS. Actin cytoskeleton was stained with phalloidin-FITC. Afterwards, cells were washed twice in PBS and incubated with secondary antibody Alexa Fluor 546-conjugated goat anti-rabbit (1:700, Life Technology, Darmstadt, Germany) for 1 h. Coverslips were washed again in PBS, and mounted onto glass microscope slides using Vectashield® HardSet™ Mounting Medium. Images were obtained using an Axio Observer Z1 (Zeiss AG, Oberkochem, Germany). **Enzyme activity assay.** Cellular NADPH quinone oxidoreductase 1 (NQO1, P15559) activity was obtained by measuring the menadione reduction in presence or absence of the NQO1 inhibitor dicoumarol using a commercially available kit (Abcam, Cambridge, UK). Protocol was followed as suggested by the supplier except that extraction buffer was supplemented by protease inhibitors (1 mM PMSF). Cells were treated like described in standard methods and lysed after 6 h or 24 h after treatment. 50 µL of lysate were assayed. Human glutathione peroxidase (GPx) activity was determined indirectly by the quantification of the enzymatic creation of GSSG under cumene hydroperoxide impact and its reduction by glutathione reductase using a commercially available kit (Cayman, Ann Arbor, USA). Protocol was followed as suggested by the supplier and samples were used undiluted (20 µl). Cells were prepared like described before, and cell lysate was obtained 3 h, 6 h, and 24 h after treatment by scraping on ice using a 50 mM Tris buffer pH 7.5, supplemented by 5 mM EDTA and 1 mM dithiothreitol. Protein contents were determined using Biorad RCDC assay and an appropriate standard curve prepared in extraction buffer.

**Statistical analysis** - Statistical significance was estimated by two-way analysis of variance (ANOVA) followed by a calculation of means and standard deviations. Student’s t-test was used to determine the degree of statistical significance between values from different experimental groups. Each experiment was performed at least three times. Treshold was set to 3fold, and the difference was considered statistically significant when \( p \leq 0.05 \) (>3fold change). All statistical analyses were performed using Graphpad Prism 6, Partek, and IPA. Statistical analysis to evaluate the effects of siRNA on gene expression was carried out by using on-way ANOVA.

**RESULTS**

**ROS generation, cell viability and apoptosis assays after plasma treatment** - In addition to various molecular sources including NADPH oxidase and mitochondrial electron leakage that
may contribute to ROS formation in response to oxidative stress (38), the role of plasma as a trigger of oxidative stress level alterations has not been extensively investigated. Plasma-generated reactive species were assigned to act a major part of plasma effects and add to the cellular production level of endogenous ROS/RNS (39,40) (Fig. 1). Consequently, the study investigated the plasma-induced ROS formation in the plasma-treated medium and in HaCaT cells. As illustrated in figure 2, plasma treatment has a significant impact on ROS levels, in particular on H$_2$O$_2$ formation: We obtained a treatment-time dependent generation of H$_2$O$_2$ from 15 μM (20 s) to 32 μM (60 s), and, 100 μM (180 s) in the plasma-treated medium, respectively (Fig. 2A). To assess changes in the intracellular ROS levels, H2DCFDA conversion was quantified by flow cytometry. 30 min after plasma exposure to medium, the ROS level was approximately two times higher in short-term plasma-treated cells (20 s) and four times higher in long-term plasma-treated cells (180 s) compared to the untreated controls. Similar results were obtained when ROS production was measured after 60 min incubation. 180 s of plasma treatment led to a comparable ROS generation as H$_2$O$_2$ treatment (100 μM), whereas shorter treatment times show a significant lower ROS formation level (Fig. 2B).

In addition, cell viability measurements were used to monitor the vitality of cells after plasma treatment (Fig. 2C). Even after longer exposure to plasma we detected a surviving rate of more than 90% of cells. Terminal deoxyribonucleotidetransferase-mediated nick-end labeling (TUNEL) assay confirmed low cytotoxic and apoptotic effects in HaCaT cells. The treatment regime chosen excludes a significant role of apoptosis after plasma exposure. At the highest plasma treatment time we only found a marginal increase of the number of TUNEL-positive cells up to 10% (Fig. 2D).

**Gene expression profiling in HaCaT cells and functional enrichment analysis** - Further, all plasma-samples were analyzed and compared in respect to their gene activity. The data of gene expression profiling have been uploaded in “NCBI’s Gene Expression Omnibus” (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE58395). Evaluation of the data by Partek Genomic Suite (PGS) allowed the identification of regulated genes, according to non-thermal plasma features. Gene expression data of both increased and decreased gene groups were determined to identify regulated genes represented by several experimental groups. Our findings demonstrate that a large number of genes are differentially expressed after incubation with plasma-treated medium. All transcripts were clustered into two distinct expression patterns, following a hierarchical clustering of similarity (data not shown).

Aiming at evaluating the genes, pathways and biological processes involved in the observed response against plasma, several treatment times (20 s, 60 s or 180 s) were chosen to clarify effects of treatment intensity on cellular changes after indirect plasma treatment. Moreover, a comparison between 3 h, 6 h, 24 h, and control groups was carried out. If not indicated otherwise, all experimental groups were named according to their incubation time (e.g. “3 h-group”) or to their treatment time (e.g. “20 s-group”). Venn diagrams were constructed to identify common and exclusively up- or down-regulated genes correspondingly to sampling time. After 3 h, we obtained total data for approximately 1.200/560/400 differentially expressed genes for each treatment time (3-fold change, p ≤ 0.05, Fig. 3A). The number of modulated genes increased to 2.100/2.350/5.000 genes after 6 h identified as statistically significant in the study (Fig. 3AII). After 24 h we found a total number of 300/120/700 regulated genes, respectively (Fig. 3AIII). Venn diagrams also visualize the overlapping results between the differentially regulated genes found at 3 h (150 genes for all experimental groups), 6 h (900 genes), and 24 h (10 genes) vs. control comparison (red numbers). In addition, we constructed Venn diagrams for each treatment time group (e.g. 20 s, 60 s, 180 s) to show the distribution of genes within the “treatment time-groups” (Fig. 3B-BII). Highest numbers of differentially expressed genes were detected for 6 h group and after long-term plasma treatment. Yellow circle depicts the total number of all differentially expressed genes within the indicated times.

After identifying the profiles of differentially expressed genes, we carried out functional enrichment analysis to reveal transcripts putatively involved in signaling pathways (41). Following statistical testing procedures, removal of the transcripts with no Entrez gene ID and fold change
cutoff (genes with fold change ≥3), we found between 27 and about 2,500 of regulated genes due to plasma treatment. Biology-focused pathway Protein Analysis Through Evolutionary Relationships (Panther) classification system was used to better understand significance of the differentially expressed genes in relation to each other, and to identify signaling cascades involved. Over and underrepresented families were detected by comparison with untreated control. From all differentially expressed transcripts, about 80% were found to have annotation in data base. Based on GO classification, the altered genes were functionally enriched (Fig. 4). Placement of genes into “molecular functions” finds genes -amongst others- which were strongly associated with transcriptional functions (e.g. transcription factors as most over-represented gene family, data not shown), nucleic acid binding and signaling molecules (Fig. 4A). One relevant category represented in “molecular function class” was that of enzymes for regulation of antioxidant activity, e.g. oxidoreductases (data not shown). To identify biological processes that could discriminate plasma-treated from non-treated clusters, we classified genes into 11 categories according to their functional role in “biological processes” (Fig. 4B). E.g., modulated genes from each group were mainly related to metabolic (42-48%), and cellular (33-34%) processes, whereas genes belonging to “biological adhesion” (6%), “cellular organization” (5-7%), and “apoptosis” (3%) were less regulated following plasma treatment.

Pathway analysis reveals the main biological functions modulated by non-thermal plasma - Ingenuity Pathway Analysis (IPA) was performed in order to compare expression measurement as well as an interpretation of differential gene and protein expression on molecular level. Our study evaluates that oxidative stress induced by plasma treatment. Biology-focused pathway Protein Analysis Through Evolutionary Relationships (Panther) classification system was used to better understand significance of the differentially expressed genes in relation to each other, and to identify signaling cascades involved. Over and underrepresented families were detected by comparison with untreated control. From all differentially expressed transcripts, about 80% were found to have annotation in data base. Based on GO classification, the altered genes were functionally enriched (Fig. 4). Placement of genes into “molecular functions” finds genes -amongst others- which were strongly associated with transcriptional functions (e.g. transcription factors as most over-represented gene family, data not shown), nucleic acid binding and signaling molecules (Fig. 4A). One relevant category represented in “molecular function class” was that of enzymes for regulation of antioxidant activity, e.g. oxidoreductases (data not shown). To identify biological processes that could discriminate plasma-treated from non-treated clusters, we classified genes into 11 categories according to their functional role in “biological processes” (Fig. 4B). E.g., modulated genes from each group were mainly related to metabolic (42-48%), and cellular (33-34%) processes, whereas genes belonging to “biological adhesion” (6%), “cellular organization” (5-7%), and “apoptosis” (3%) were less regulated following plasma treatment.

In a global proteome approach we confirmed many of the differentially expressed genes. A total of 3819 proteins were detected in all HaCaT lysates. About 400 of it were up regulated more than 2fold by the plasma treatment whereas 350 proteins were down regulated (>2fold) (42). As expected, IPA predicted an increased oxidative stress on the basis of the detection and regulation of several proteins which act as sensors and/or effectors for this condition. Among these, the peroxiredoxins 1 to 6 and catalase were noticeable. In correlation to protein expression results, several ROS-scavenging enzymes like glutathione peroxidases (GPx1, 3), glutathione reductase, and peroxiredoxins displayed enhanced mRNA expression (7). The strongly increased abundance of heat shock proteins (HSP90 and HSP40 derivatives) also indicates thermal or chemical stress. Proteins involved in thiol group reduction or coupling (GSTK1, GSTO1, GSTP1) showed an increased abundance within the investigated time line. Thus, the glutathione metabolism was affected which is a marker for the Nrf2-related signaling events (NF2L2, 69 molecules, IPA p-value 3.5*10⁻⁸). Among the most robustly increased proteins NQO1 and carbonyl reductase 1 (CRB1) were found (Fig. 5). However, some major downstream proteins could not be detected by LC/MS, e.g. HMOX1 could not be detected while HMOX2 was found. Moreover, response to stress by activation of those factors culminates in enhanced protein synthesis and transcriptional activity. Briefly, the transcription factors FOS, JUNB/D were detected in the microarray as differentially expressed (Table 1): both are components of the activator protein 1 (AP-1) complex. Furthermore, we obtained a plasma-induced altered expression of genes and proteins that were associated with the mitogen-activated protein kinase (MAPK) signaling. Many of the downstream factors of MAPK signaling were identified (e.g. IL7/10/27, IL3RA, IL4R, IL1RAAPI1/2, FOS, TGFB1, TNFRSF13B/18, HIF, MAPK3, MAP2K3/4, JUNB/D, FOS etc., see Table 2, 3). Some of the significantly changed genes submitted to IPA, as well as the genes mapped to the cluster 1 or 2 (data not shown) were used for subsequent analyses. Overrepresented
signaling pathways following plasma treatment were not only genes of MAPK and Nrf2 and antioxidant response signaling but also genes of p53, TGFβ, PPARα, RAR activation, and cell cycle G1 checkpoint regulation. Further pathways dealing with the regulation of downstream targets were found in plasma-modulated groups, e.g. protein kinase C (PKC), phosphatidylinositol 3-kinase (PI-3K), and the Janus Kinase/Signal transducers and activators of transcription (JAK/Stat) pathways. For longer treatment times we found a regulation in nNOS and MSP-RON signaling pathways, which play important roles in inflammation and in late stages of wound healing (see details in Table 2).

With the assistance of IPA, the differentially expressed transcripts were organized and classified into networks. Although many of the networks are commonly involved in gene expression, cellular function, growth and proliferation, one of the top networks also include those that contribute mainly to “cellular and connective tissue development and molecular transport” for the “3 h, and 180 s-group”, as well as “small molecule biochemistry” for the “6 h-group”. In this context, networks discover an induction of targets of the JUN pathway within the transactivation of HMOX1 on mRNA (data not shown). We than asked what underlying molecular and cellular functions are associated with the mode of action in plasma-induced activation of cellular signal transduction. IPA annotation revealed that plasma was significantly involved in nucleic acid metabolism (3/6 h), small molecule biochemistry (3/6 h), cell signaling (3/6 h), cellular growth and proliferation (3/24 h), cellular development (3/24 h), cell-to-cell signaling and interaction (6/24 h), amino acid metabolism (6 h), and energy production (24 h) (see details in Table 3).

Non-thermal plasma activates phase II enzymes through Nrf2 pathway to scavenge ROS - To confirm Nrf2 signaling on single cell level, we analyzed subcellular localization of Nrf2 after plasma exposure. HaCaT cells were incubated with the plasma-treated medium for the indicated period of time. Immunofluorescence staining with anti-Nrf2 antibody has shown that protein translation is actively switched on after plasma treatment and that this is partly due to the activation of Nrf2. This intra-cytosolic transcription factor is in its reduced form bound to Keap1 (data not shown). Thus, untreated cells showed predominantly localization of Nrf2 in the cytosol (Fig. 6A-E). As expected, we confirmed the cytoplasmic-nuclear trafficking of endogenous Nrf2 after plasma (Fig. 6AI-EI) and H2O2 treatment (100 µM, Fig. 6AI-DII) by immunofluorescence microscopy. These findings were also verified and confirmed in primary NHEK keratinocytes by indirect immunofluorescence staining of Nrf2. Upon 60 s of non-thermal plasma treatment of NHEK cells, Nrf2 is released and translocate into the nucleus where it binds to specific DNA elements. In the untreated sample, there was no increase in Nrf2 expression or nuclear accumulation visible (Fig. 6F).

To further study the role of Nrf2-mediated antioxidant response and plasma-induced changes in redox balance, we performed quantitative PCR (qPCR) and western blot analysis of Nrf2 and Keap1. All time point was chosen for further analysis of plasma treatment time dependent gene expression. In concordance with the results obtained from microarray and LC/MS, transcript and protein levels of Nrf2, and Keap1 were not significantly altered after longer incubation times (3 to 24 hour). Nrf2 and Keap1 expression levels were mainly enhanced after very short incubation times (about 20 min, Fig. 7). However, applying immunofluorescence microscopy, no significant changes were seen in the expression and localization of Keap1 in untreated cells (upper panel, Fig. 7C) and cells after plasma treatment (Fig 7CI).

The results obtained by microarrays, and LC/MS showed an activation of the downstream targets of Nrf2 in plasma-treated keratinocytes. Corresponding to Nrf2 activation, enhanced levels of HMOX1, and NQO1 mRNA were exclusively associated with the presence of plasma. Furthermore, a robust increase in protein expression of NQO1 was found in the proteome approach (Fig. 4, left). To verify potential downstream activation of Nrf2 target genes, a time course of the gene expression was assessed using qPCR. The expression of HMOX1 and NQO1 mRNA was induced in a treatment time dependent manner. HMOX1 gene expression showed a maximal, 12fold up regulation after 180 s of plasma treatment (Fig. 8A). NQO1 was up regulated up to 2fold after 60 s of plasma treatment (Fig. 8AI). Gene expression did no longer increase with treatment times longer than 60 s of plasma treatment. Western blot analysis confirmed these
findings: NQO1 protein was found to be increased after plasma treatment in the analyzed data set whereas HMOX1 protein was at most 2-fold higher in comparison to control (Fig. 8B, BI).

A treatment time dependent increase in enzyme activity of NQO1 was detected. High sensitivity, even sensitive treatments (10 µM H₂O₂ or 20 s plasma), lead to a measureable enzyme activity increase. NQO1 enzyme is synthesized de novo – this effect can be observed after 24 h only whereas after 6 h, no effect was seen (Fig. 9A-A’). After 24 h, inverse effect to expectations was measured for GPx enzyme activity: for intense treatments, enzyme activity is decreased. After 3 h, there was a slight increase in enzyme activity for moderate treatment intensities while longer treatment time did not result in any measureable changes. After 6 h, no effect could be detected (Fig. 9B-B’).

Inhibitory effect of siRNA on ARE-activity in keratinocytes - With non-thermal plasma as a regulatory agent causing gene response, we found a potent modulator of Nrf2 activity and its downstream signaling. To better understand how plasma impacts Nrf2-ARE signaling, we used siRNA transfection assay to knockdown target genes of Nrf2/Keap1-pathway in HaCaT cells. As shown by immunofluorescence, at the 72 h time point after transfection with siRNA directed against Nrf2, Nrf2 was decreased in treated samples compared with the non-transfected control group (Fig. 10B). Quantification of qPCR was done, and representative pictures of knockdown were shown: siRNA silencing directed against Nrf2 led to a significant decrease in Nrf2, Keap1, HMOX1, and NQO1 expression in all samples (Fig. 10C).

Interestingly, a number of other transcripts were down-regulated following knockdown, suggesting that Nrf2 may control certain repressor activities. The mRNAs whose expression decreased most included the following: PRDX1, which reduced to 48%; SOD1 and SOD2 which reduced to 34% and 69%, respectively; GCLC and GCLM which reduced to 24% and 27%, respectively. Comparable silencing effects of Keap1 knockdown were obtained for genes of antioxidant defense system (e.g. PRDX1, SOD1/2, GCLC, GCLM), whereas expression of catalase (Cat), and growth factors: HBEGF, VEGFA were enhanced (Fig. 10D).

Because Bach1 is a repressor of the oxidative stress response, we examined the function of Bach1 in HaCaT cells subjected to plasma exposure. It is not clear which ARE-driven genes are regulated by Bach1 after plasma exposure in HaCaT cells. Immunofluorescence of Bach1 following plasma treatment has shown a cytoplasmic as well as nuclear staining (data not shown). qPCR revealed an strong effect after knockdown of Bach1 on ARE-driven gene expression by studying HMOX1 because Bach1 is known to repress this oxygenase gene (34). HMOX1 was up-regulated 12-fold following Bach1 silencing, and is therefore negatively controlled by this protein to a much greater extent than had been observed for any of the genes by Keap1. Relative to HMOX1, only very modest increases of 2 - 2.5-fold in the mRNA were observed for Cat and VEGFA mRNA (Fig. 10E), and no increase was detected in NQO1 mRNA (data not shown).

Next, we performed knockdown in the presence and absence of plasma treatment using siRNA directed against Nrf2 genes. As demonstrate, non-thermal plasma induces nuclear translocation of Nrf2 (Fig. 11A). Nrf2 gene silencing (72 h before plasma exposure) using siRNA abrogates the ability of plasma to increase mRNA for both enzymes after 20 min of cell incubation (Fig. 11B). In contrast, the exposure of cells to prolonged plasma treatment time (180 s) showed a clearly increase of silenced Nrf2 and accumulation in the cytoplasm as well in the nucleus of HaCaT cells (Fig. 11C).

DISCUSSION

Given its potential to kill microorganism and to interact with cells or tissues, non-thermal atmospheric pressure plasma appears as a promising biomedical tool for the treatment of various skin diseases including chronic wounds (24-26,43). In this study, we investigated the effects on mRNA and protein level in order to identify possible applications of non-thermal plasma in keratinocytes, e.g. in acceleration of wound healing and re-epithelialization. Thus, detailed knowledge about molecular signaling and the identification of responsible genes or proteins may represent valuable clinical targets for a wound therapy.

Although hydrogen peroxide is known for its cytotoxic effects, in recent years it has been shown to play a crucial role in eukaryotic signal transduction (44). H₂O₂ is an important second messenger for growth factors released during the
wound healing process, and has been shown to increase keratinocyte viability and migration in a wound model (45). Plasma-generated reactive intermediates add to the cellular production level of endogenous ROS (7). Regarding this, a notable fact is that reactive species are produced in the gas phase of the plasma jet itself; then they are solved in the liquid or secondary intermediates formed in the plasma-treated solution (46). Importantly, $\text{H}_2\text{O}_2$ is a principal component of “plasma cocktail” produced by the kinpen (47). The highest plasma treatment (180 s) led to the production of about 100 $\mu$M $\text{H}_2\text{O}_2$ in the treated medium volume. Intracellular ROS level was up to four times higher in comparison to control, and leads to an increased sensitivity of treated cells to plasma. However, human cells have to be treated with caution due as very high amounts of ROS/RNS generated by longer plasma treatment may inhibit cell growth or induce apoptotic pathways (48). Nevertheless, no differences in survival of plasma-treated HaCaT cells were detected under the treatment regimen chosen for this study. Moreover, apoptotic effects were nearly negligible at all treatment times. In a previous study, it was demonstrated that incubation of cells with plasma-treated medium showed an acute death rate under 10% in HaCaT cells (49), which could be confirmed with the present data on viability assays. However, the magnitude of apoptosis is strongly dependent on the investigated cell type. Monocytes are less sensitive than CD4+ T helper cells, whereas Jurkat and THP-1 immune cells displayed a higher survival rate compared with their human blood counterparts (50).

The cellular effects of non-thermal plasma have been demonstrated in vitro in a variety of cell types (e.g. keratinocytes (39,51), fibroblast (52), immune cells (1,35,50,53). However, the knowledge about cellular signaling events in plasma-treated human cells is still rudimental. In this study, a global gene expression microarray analysis was performed to understand the whole transcriptional response of human keratinocyte cell line HaCaT to non-thermal plasma and to analyze the total cellular proteome under the same conditions. An indirect treatment with an argon driven plasma jet was done to identify correlation of intracellular signaling with duration of plasma treatment and subsequent incubation time as well as correlation between mRNA and protein expression. Due to the generation of ROS/RNS by non-thermal plasma, the question was how plasma triggers the transcription and translation of oxidative stress responding proteins to contribute as a promoting agent in wound healing.

Primarily, we assessed the levels of 24.000 transcripts in plasma treated HaCaTs and obtained marked alterations in the expression of hundreds of genes in all experimental groups (data set: GSE58395). Upon plasma treatment, HaCaT cells regulated much more genes in the “6 h-group” compared to 3 h or 24 h or to immune cells (50) indicating the highest changes in gene expression activity after middle-term incubation time. A possible explanation for the higher number of regulated genes in HaCaTs in contrast to plasma treated immune cells could be that the latter have a totally different behavior regarding oxidative stress events, e.g. monocytes produce reactive species themselves (54). Moreover, the distribution of genes depicted in Venn diagrams clearly shows that the incubation time of plasma is much more important than the treatment time between 20 s and 180 s. This finding supports the assumption that plasma-generated stable species in liquids induce the cellular response (55). Additionally, plasma-activated reactions were obtained up to several hours after plasma treatment in post-treatment kinetic measurements (56).

Summarizing identified changes, the altered transcripts were classified into several categories according to their functional roles. Amongst others (e.g. metabolism, transport, redox homeostasis), the regulation of transcription, signal transduction, and antioxidant activities belong to classes with most alterations. The vast bulk of induced or repressed genes after plasma treatment include transcription factors, receptors, signaling molecules, and genes involved in antioxidant activity like oxidoreductases (7). Secondly, between 350 (down-regulated) and 400 (up-regulated) proteins were identified by liquid chromatography (LC) and mass spectrometry (MS). Analysis of the proteome data set showed for the plasma treated cells a comparable normal cell physiology as was indicated by eIF2 signaling, eIF 4/p70S6K signaling, or t-RNA charging (protein translation etc.). The protein ubiquitination pathway was found to be emphasized. This is in agreement with increased demand of protein degradation, provoked by oxidative (or other plasma chemistry derived) modification of proteins. Under this perspective,
the active protein de-novo synthesis machine might result from increased protein degradation and shows the necessity to replace proteins. By LC/MS, overall, 10% of proteins were found to be regulated by the plasma treatment: among the most regulated proteins antioxidant acting factors and enzymes were identified (42). According to this finding, the regulatory effects of plasma on genes and proteins of the antioxidant defense system or on molecules involved in redox-homeostasis were in the focus of our investigations.

An increased abundance of intracellular reactive species needs to be controlled by the cell. An important switch to control ROS and/or RNS levels is the intra-cytosolic transcription factor Nrf2 which is in its reduced form bound to Keap1 (57). By IPA prediction, Nrf2 signaling was ranked among the most active regulatory network and canonical pathway (Fig. 11D). For the data sets of gene activation, 16 Nrf2 targets were found in the “20 s-group”; 16 molecules in “60 s-group”; 26 molecules in “180 s-group”, and predominantly in the “3 h incubation group”. For the protein expression data set, the most Nrf2-regulated proteins were found after 24 h.

Transcript and protein levels of Nrf2 were mainly enhanced after short incubation times (20 min or 3 h, respectively; Fig. 7A, B). The fluctuating expression may result from a negative feedback loop fed by the increased expression of anti-oxidant proteins and the accordingly increased ubiquitination of the protein. However, similar to an oscillating p53 expression (58), fluctuating Nrf2 protein expression may also reflect a modulation of the anti-oxidative response intensity or may be necessary for the specific function of individual Nrf2 targets. Data from Pekovic-Vaughan et al. revealed a rhythmic nuclear abundance of endogenous Nrf2 protein in tissues and cells that accompanies the rhythmic levels in the total pool of Nrf2 protein (59). We suggest that the function of Nrf2 is to act as a key molecule that promotes and maintains transcriptional noise to interfere with stress signals evoked by plasma treatment. These results suggest that alterations in the status of ROS intermediates in dependence of chemical reactions in liquids (55) cause fluctuations in expression levels of such signaling molecules.

Consistent with the expression pattern in the literature (29,60-61), Nrf2 immediately translocates to the nucleus as response to plasma, and binds to ARE sequences in the promoters of its target genes. Afterwards, a significant mRNA expression of antioxidant and phase II detoxification enzymes and proteins like GPxs, catalase, SOD, HMOX1, NQO1, MAP kinases, and molecules of JUN pathway were triggered. Therefore, Nrf2 acts as an important switch for sensing oxidative stress events triggered by the plasma treatment. Further, the result showed that protein translation is actively switched on, and that this is partly due to the activation of Nrf2. For example, NQO1, a member of the NADPH dehydrogenase family encoding a cytoplasmic 2-electron reductase gene, and CBR1, a carbonyl reductase, were shown to be significantly increased after plasma treatment. In perfect correlation with global measurements we also found a treatment time dependent increase in enzyme activity of NQO1. However, there was a slight divergence between transcriptomic data (global and qPCR) and proteomic data (western blot, LC/MS) observed which might be explained as follows: NQO1 as a major cellular antioxidant is permanently transcribed from the corresponding in metabolically active cells. As protein accumulates, it may act as a repressor of transcription and signaling. Accordingly, an increase in transcription might not be necessary for shorter plasma treatment times. In addition, a fast ubiquitination of NQO1 is described for a one amino acid polymorphism (NQO1*2) (62). In this case, the half life time of the enzyme drops from 18 h (wild type) to 1.2 h. One may speculate that a similar mechanism – however not yet identified – may be in charge of controlling the NQO1 levels during normal conditions. In this case, excess NQO1 would be degraded by a proteasomal mechanism. With rising oxidative stress ubiquitination decreases and protein stability increases. This would lead to the situation observed: while mRNA levels keep at control rate, protein degradation stops under stimulus (plasma) and apparent protein expression increases. If stimulus keeps rising, transcription soars as do protein level and activity, with now both global gene and proteome date being in accordance.

The strongly up-regulated CRB1 is involved in nitroso glutathione recycling, hinting on a possible role of RNS in this scenario (46). Nevertheless, the expression of enzymes involved in glutathione synthesis like GCLC/GCLM were not found to be up regulated after 3 to 24 hours. Other proteins like heat shock proteins (HSP90s,
HSP40s) or cytosolic and membrane-bound forms of glutathione S-transferase (GSTO1) show an increased abundance regardless of sampling time (up to 24 h). HSPs are observed after cellular stress and serve mainly by preventing misfolding or aggregation of cellular proteins (42). GSTs function in the detoxification of electrophilic compounds, including products of oxidative stress, by conjugation with glutathione (63).

GPx has shown a more complex behavior than NQO1. The protein activity seems to be in poor correlation with global gene or protein data which both indicate an increase in expression. One possible explanation could be that GPx needs to be processed before being active: the active center selenocysteine is included in a posttranslational mechanism. If selenium is in limitation in the assay medium, the production of functional protein might be reduced. Additionally, the potential oxidation of selenium to selenium oxide leads to a GPx inactivation as selenium is equally likely to be oxidized as sulfur (64). Therefore, we detected a slight reduction of GPx enzyme activity, especially in case of longer treatment.

Plasma treatment quickly changed mRNA status of Keap1 but did not change the protein expression level after longer treatment times. Hydrogen peroxide marginal decreased the levels of Keap1 protein after 3 h indicating its ubiquitin-dependent proteasomal degradation. We concluded that only concentration dependent differences were detected. Nevertheless, slight difference in reactions between plasma and H2O2 on Keap1 expression resulted in the fact that many other compounds (secondary radicals) in plasma interact with cells and provoke differences in their gene and protein expression (46,47, 55).

The induction of cytoprotective enzymes in response to stress was regulated primarily at the transcriptional level. The argon plasma treatment produced a significant increase in heme oxygenase 1 (HMOX1) mRNA levels up to 12-fold. The outcome of this is that HMOX1 activity is not only induced by its substrate heme but also by non-heme substances such as plasma. In oxidative environments like skin injury or wounds, H2O2 releases the potent oxidant agent heme which is degraded by HMOX1 to iron ion, carbon monoxide (a putative neurotransmitter), biliverdin and bilirubin (66). As known, the HMOX1 promoter contains multiple ARE binding motifs suggesting transcriptional regulation not only over AP-1 but also over Nrf2 (67). The heme-HMOX system including transporter, degrading or binding proteins of heme as well as antioxidant enzymes protect the skin from heme contribution to several diseases (68). Additionally, it was shown that HMOX1 influences wound healing especially in vascularization (69), and plays an important role in the regulation of vascular endothelial growth factor expression (70,71). Therefore, evidence is accumulating that the heme-HMOX system forms a novel and important target in plasma applications. However, HMOX1 could not be detected by LC/MS while HMOX2 was found. Analysis of the identified peptides indicated that, although both enzymes share a high homology, no disambiguation occurred. Either HMOX1 is translated later than 24 h past treatment, in a small amount only, or it might result in no distinct peptides which hamper LC/MS detection.

To show functional relevance of plasma induced Nrf2 pathway in HaCaT cells, the effect of Nrf2 and Keap1 silencing on expression of antioxidant enzymes was investigated. The finding that Nrf2 inhibition by siRNA silencing show down-regulation of phase II gene induction program in HaCaTs (Fig. 10) supports the importance of Nrf2 in cellular defense. An Nrf2 lack is associated with heightened sensitivity to oxidative stress (72,73). In contrast, the activation of Nrf2 by plasma treatment after silencing (Fig. 11C) would lead to an activation of many ARE-dependent target genes (e.g. HMOX1, NQO1, SOD, GSR) suggesting a possible protection mechanism of non-thermal plasma against cytotoxic alterations on redox levels. Such mechanisms could support processes like wound healing in which signal molecules stimulate ongoing processes. Interestingly, Keap1 knockdown did not activate the ARE gene battery. After silencing of Keap1, we found a strong down-regulation of Nrf2, HMOX1, and NQO1 showing that inhibition of Keap1 is critical for regulating of intracellular redox conditions in keratinocytes. In contradiction to the literature, the cytosolic interaction between Nrf2 and Keap1 entails that if one of the interaction partners is knocked down, either Nrf2 or Keap1, also the other one is down-regulated. As one result of the knockdowns, the ubiquitination of proteins can occur faster. Experiments by Itoh et al. suggest a delicate balance of Nrf2 protein stability due to its high turnover rate. Presence of Keap1 was important to reduce proteasomal degradation (74). In contrast,
we observed an increase in VEGF expression after Keap1 knockdown indicating other roles of Nrf2 than the anti-oxidative protection of cells (Fig. 10D). Interestingly, VEGF expression was indeed shown to be increased by plasma treatment in the cellular environment (49). The growth factor has multiple effector cells, among which are keratinocytes and endothelial cells. In chronic wounds, an increased oxygenation of the tissue is desirable. Our data indicate that this might be achieved by the plasma treatment. Taken together, the interference of non-thermal plasma with the Nrf2/Keap1 axis prepares eukaryotic cells against exogenic noxae and increases their resilience against oxidative species. Via paracrine mechanisms also distant cells benefit from cell-cell communication triggered by the treatment.

In contrast, Bach1 heterodimers function as repressor of gene expression by binding on AREs. Usually, Bach1 is inactivated in the presence of oxidative stress which allows transcriptional activation of genes by Nrf2, and transcriptional repression in the absence of oxidative stress (75,76). We found a plasma-induced repression of the function of Bach1 which evokes a preferential binding of Nrf2 on AREs and an activation of target genes such as HMOX1 (data not shown). Using knockdown directed against Bach1, we investigated the role of Bach1 as a potential target for oxidative gene expression regulation after plasma treatment. Data indicated that Bach1 silencing by small interfering RNA is associated with induction of genes like HMOX1, VEGFA and catalase indicating that Bach1 is a critical repressor of these genes in HaCaT cells. As expected, we demonstrated a strong, up to 12-fold increase in HMOX1 expression, showing that Bach1 - together with its competitor Nrf2 - regulates cellular responses to oxidative stress after plasma treatment through specific induction of HMOX1. Taken together, we propose that a plasma stimulus resulting in ROS generation leads to an activation of the transcription factor Nrf2, and to an antioxidant defense response by altered expression of down-stream targets like HMOX1, NQO1, Bach1 (Fig. 11D). Additionally, some proteins show distinctive incubation time dependence, e.g. different mitogen-activated protein kinases (MAPKs). MAPK signaling was one of the most overrepresented pathways after plasma treatment on gene as well as on protein level. Moreover, several cell types showed a plasma-mediated activation of different MAP kinases indicating that they are important intracellular plasma responses (50). MAPKs play an important role in wound healing (77), and they mainly regulated the phosphorylation of transcription factors (78). p38-MAPK pathway is activated by a diverse array of stress stimuli that include inflammatory cytokines, death ligands (e.g. TNF), transforming growth factor (TGF)-β-related polypeptides and environmental factors like oxidative stress (79). Response to stress by activation of those factors culminates in enhanced transcriptional activity and protein synthesis as we found by microarray and gelfree LC/MS proteomics (eIF2 signaling, eIF4/p70S6K signaling or t-RNA charging). Since an induction of JUNB/D and FOS could be identified in this study, the activation of the activator protein AP-1 in the JUN pathway will be one possible activation mechanism following plasma treatment. A discrete increase of H2O2 triggers most of the proliferation signaling pathways by activation of transcription factors like NF-κB, p53, RAF, HIF-α, glucocorticoid receptor, and AP-1 (80). The latter, AP-1, belonging to the JUN family, is an inducible transcription factor, and regulates gene expression in response to a variety of stimuli like ROS (81). The members of AP-1 are involved in various molecular mechanisms inducing proliferation, differentiation, and cell fate determination (82). JUND was found to be essential for cytokine secretion which is important for a proper wound healing (83). Furthermore, plasma accelerates the expression of the keratinocytes-derived granulocyte-macrophage colony stimulating factor (CSF2). Both, an up regulated gene expression of CSF2 and an increased secretion of its synthesized protein GM-CSF measured by ELISA (data not shown) were observed. An overexpression of CSF2 propose beneficial effects in wound healing (e.g. re-epithelialization) via the induction of secondary cytokines, and improves wound healing by recruitment of leukocytes, enhances keratinocyte proliferation and increases angiogenesis by VEGF up regulation (49, 84).

Further understanding of the protection offered by Nrf2 expression may provide clues to new modalities for in vivo skin protection and prevention of skin disorder. Stress tolerance and promotion of cell survival is increased by Nrf2 activation (85). A decline in Nrf2 function also sensitzes cells to oxidative stress during aging; increased Nrf2 levels are noted in the cells of long-
lived mice, and high Nrf2 levels protect these cells against oxidant-induced damage. Several studies have suggested that beneficial effects of chemopreventive drugs on suppression of carcinogenesis are mediated through activation of Nrf2 (86). In contrast, some studies suggest an oncogenic characteristic of Nrf2 causing constitutive activation and resistance to chemotherapy (87). Just a transient (and not a permanent) activation of Nrf2 by non-thermal plasma should not be associated with cancer formation. This assumption is in accordance with results of colony transforming assay, showing that a plasma exposure to HaCaT cells has no cancerogenic properties (data not shown). Moreover, several plasma treatment times were used to analyze the mutagenicity induced in V79 Chinese hamster cells. Using the hypoxanthine-guanine phosphoribosyl transferase mutation assay, results further show that a plasma treatment did not induce mutagenicity at the Hprt locus (88).

Applying non-thermal plasma it is feasible to manipulate cellular reactions simultaneously at diverse levels. Our data show that both gene and protein expression is highly affected by non-thermal plasma, and highlights the pivotal role played by Nrf2 in regulating cellular defenses against oxidative stress. Plasma-induced regulation of Nrf2/Keap1/Bach1 is a mechanism to induce multiple ARE-genes and proteins in keratinocytes. These findings suggest that non-thermal plasma provides an interesting therapeutic tool to control redox-based processes, e.g. wound healing. Results are useful for the identification of molecular targets for novel therapeutic strategies in wound management. By plasma-based delivery of reactive oxygen- and nitrogen species, plasma stimulate or inhibit cellular processes and triggers hormesis-like processes in keratinocytes.
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Acknowledgements - The authors thank L. Kantz for her distinguished technical support. For critical reading of the manuscript the authors thank J.C. Schmidt.

FOOTNOTES

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3The abbreviations used are: ARE, antioxidative response element; Nrf2, nuclear factor erythroid-related factor 2; Keap1, Kelch-like ECH-associated protein 1; HMOX1, heme oxygenase 1; NQO1, nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidoreductase 1; qPCR, quantitative real-time polymerase chain reaction; siRNA, short inhibitory RNA

4This work was realized within the framework of the multi-disciplinary research center ZIK plasmatis which is funded by German Federal Ministry of Education and Research (grant number 03Z2DN11).

FIGURE LEGENDS

FIGURE 1. Schematic setup of the plasma treatment using kinpen and sample preparation for gene and protein expression profiling. A-B) The kinpen source (neoplas tools, Greifswald, Germany) produces a jet-type plasma, with an effluent containing a variable mixture of active components: reactive molecules, radicals, electrons, ions, different types of radiation (electric fields, thermal and ultraviolet radiation). C) For assessment of transcriptome and proteome changes cell culture medium was plasma treated for the lengths of time indicated and applied to the HaCaT keratinocyte cell culture. Central element of the whole experimental procedure is the transcriptome analysis using microarrays containing 4x72K gene data sets. Data analysis using Partek Genomic Suite, Panther database, and Ingenuity Pathway analysis, summarizes gene expression levels of each transcript and allows conclusions on cell physiology under the following conditions: 0 s, 20 s, 60 s, and 180 s treatment of cell culture medium including “two hour incubation” of HaCaT cells. Afterward, cells were incubated with fresh medium for 1 h, 4 h, and 22 h. Liquid chromatography and mass spectrometry were done for the “180 s treatment group”.

FIGURE 2. Assessment of hydrogen peroxide and ROS production as well as cell viability and DNA fragmentation in HaCaT cells. A) Cells were incubated with plasma-treated medium for the length of time indicated, and generation of H$_2$O$_2$ was measured using Amplex Red assay (n=3). After 20 s, 60 s, and 180 s ca. 15 µM, 32 µM and 100 µM H$_2$O$_2$ was generated in plasma-treated medium. B) ROS levels were quantified after short (20 s) and long-term (180 s) plasma treatment following 30 min or 60 min incubation of cells with plasma-treated medium. H$_2$O$_2$ (100 µM) exposure to HaCaT cells shows the same ROS level as long-term treatment. C) Cell viability measured by calcein/PI assay in HaCaT cells exposed to increasing plasma treatment times. Representative images of randomly selected microscopic pictures of HaCaT cells stained with calcein/PI after plasma exposure showed no dose-dependent cytotoxic effect in plasma-treated groups: there was not a strong decrease of viability at higher concentrations ranging from 0-180 s (low or no green fluorescence, red fluorescence indicating cell death). D) Apoptosis was assessed using the TUNEL assay. Representative microscopic pictures of TUNEL-positive apoptotic HaCaT cells (red) and counter staining with Hoechst (blue). Cells exhibiting pink fluorescence are positive for both death cells, and nuclei. TUNEL analysis revealed that the number of apoptotic cells in plasma-treated cells was marginally higher than in un- or low-treated cells. Scale bar 50 µm.

FIGURE 3. Venn diagrams for regulated genes for each experimental group. A) About 1.500 genes (3 h incubation), 6.000 genes (6 h), and 1.000 genes (24 h) were detected (great circle, yellow). Indicated number of genes was found under either plasma condition and incubation time tested. Expression of
red colored genes was exclusively affected in all experimental groups by plasma. B) Venn diagrams for 20 s, 60 s, and 180 s treatment groups. Diameters of circles are to scale.

**FIGURE 4.** Global gene expression in functional categories and protein classes using Panther software. A-B) Diagrams of microarray results (representing the number of differentially expressed genes) revealed similarities and differences in all transcriptome profiles found for 20 s, 60 s, and 180 s of plasma treatment. Open access Panther program was used to analyze the gene lists for each experimental group (analysis of 1.500, 6.000 or 1.000 sequences after 3 h, 6 h, and 24 h incubation) to find all categories with the “molecular function” (left) and “biological process” (right) functions domain of GO (p≤0.05). Bars represent the number of genes with both, enhanced or decreased expression. C) Nrf2-associated pathway genes were identified in a gene expression microarray analysis: left, down-regulation; right, up-regulation.

**FIGURE 5.** Nrf2-related protein expression by human keratinocytes after plasma treatment according to ingenuity pathway analysis (IPA) data reduction. Cellular proteins identified/quantified by liquid chromatography and high resolution mass spectrometry (180 s treatment, 8 h incubation, see experimental procedures). A) IPA analysis revealed 40 proteins related to NRF signaling to be up-regulated (thereof 23 proteins >2fold), indicating the presence of oxidative stress. Among the up-regulated proteins enzymes like NQO1 or CBR1 stand out (see discussion). B) 29 proteins related to Nrf2 signaling were down-regulated (29 proteins, 16 thereof >-2fold). Glutathion-S-transferases show complex pattern with up- or down regulation of different types, e.g. GSTO1 and GSTM3. Bar represents mean of 5 data points ± SD.

**FIGURE 6.** The cytoplasmic-nuclear trafficking of Nrf2 after non-thermal plasma treatment in HaCaT and NHEK cells. Untreated cells depicted in the left panel (A-E). Cells were incubated with plasma-treated medium for 60 s (15 min of incubation, middle panel, AI-EI). Cells were treated with 50 µM H$_2$O$_2$ (AII-DII). Subcellular localization of Nrf2 was detected by indirect immunofluorescence staining with anti-Nrf2 (red, A) and visualized with fluorescence microscopy. The nuclei were counterstained with DAPI (blue, B), and the actin cytoskeleton was stained with phalloidin-FITC antibody (green, C); merge (D). Histogram of subcellular distribution of Nrf2, actin and nuclei (E, EI). F) The Nrf2 staining was identical performed in NHEK cells (green, actin cytoskeleton; red, Nrf2; blue, DAPI). Scale bar 20 µm.

**FIGURE 7.** Validation of the expression of target genes by quantitative real-time PCR (qPCR). HaCaT cells were plasma- or H$_2$O$_2$-treated for indicated times. qPCR was performed for Nrf2 (A), and Keap1 (AI). B) Representative western blot analysis of Nrf2 (B) and Keap1 (BI) protein expression. Data in bar graph are the mean ± SD of three independent measurements. C) Immunofluorescence of cytoplasmic Keap1 expression after plasma treatment (CI) in HaCaT cells. Scale bar 50 µm.

**FIGURE 8.** Plasma-induced regulation of HMOX1 and NQO1 antioxidant enzymes. A) qPCR analysis of the plasma effect on HMOX1 and NQO1 mRNA expression in HaCaT cells. Results from qPCR confirmed mRNA gene expression of HMOX1 an NQO1 measured in microarrays (MA, bottom lane in tables). B) Representative western blot analysis of the plasma effect on HMOX1 (B) and NQO1 (BI) protein expression for all time points indicated. Data in the lower diagram are the mean ± SD of three independent measurements.

**FIGURE 9.** Enzyme activity of NQO1 and GPx. A) NQO1 activity after 6 h (A) and 24 h (A’) after plasma treatment. Treatment time dependent increase in enzyme activity was detected. B) Glutathione peroxidase activity after 3 h (B), 6 h (B’) and 24 h (B”). After 3 h a treatment time dependent increase
was detected, while after 6 h no effect on GPx activity was measured. After 24 h a decrease for long treatment times was observed.

**FIGURE 10.** Representative pictures of immunofluorescence microscopy of the effect of Nrf2 siRNA on Nrf2 expression. A) HaCaT cells were transfected with a control scrambled siRNA (A) or Nrf2 siRNA (B) and incubated for 72 h. Scale bar 50 µm. C) Quantification of qPCR of Nrf2 and Keap1 siRNA knockdown. Representative semi-quantitative PCR pictures for Nrf2 and Keap1 siRNA knockdown for Nrf2, Keap1, HMOX1, NQO1. D) qPCR of different enzymes of antioxidant defense enzymes after knockdown of Nrf2 and Keap1. E) Bach1 siRNA knockdown led to a significant up-regulation of HMOX1, catalase (Cat), and VEGFA. Data represent the means ± SD of three independent measurements.

**FIGURE 11.** Plasma treatment abolish siRNA-induced knockdown of Nrf2. A) Immunofluorescence microscopy showing nuclear trafficking of Nrf2 after plasma treatment. B) HaCaT cells were transfected with a control scrambled siRNA or Nrf2 siRNA, and incubated for 72 h. After 60 s of plasma treatment (20 min of incubation), plasma effects on Nrf2 localization were obtained. C) Increased plasma treatment time up to 180 s and prolonged incubation time (1 h) abolish siRNA-induced knockdown of Nrf2 and enhanced expression of Nrf2 in the cytosol and the nuclear translocation. D) Functional annotation, pathway, network analysis, and graphical summary of Nrf2 activation in keratinocytes after plasma treatment: The top gene interaction network associated with plasma-treated HaCaTs were predicted by IPA and presented for all groups. About 30 genes are involved in the network. A solid line represents a direct and a dashed line an indirect interaction between target genes; red: up-, and green: down-regulation. Plasma activates Nrf2 signaling and induces an antioxidant response. Amongst others, HMOX1, FOS, JUN, JUNB/D, and Bach1 were regulated by plasma. Regulation of several components is simplified depicted in the drawing.
**TABLES**

**Table 1** Gene expression changes (= fold change vs. control) of components of activator protein AP-1 (JUN pathway) for the action of plasma in HaCaT cells (n.d. = not detected).

| Genes | Treatment time | Incubation time |
|-------|----------------|-----------------|
|       |                | 3 h | 6 h | 24 h |
| **FOS** | 20 s | 1.23 | 2.13 | n.d. |
|        | 60 s | 1.39 | 1.69 | n.d. |
|        | 180 s | 3.94 | 2.64 | n.d. |
| **JUNB** | 20 s | 3.63 | 4.03 | 1.73 |
|         | 60 s | 1.8 | 1.56 | 3.72 |
|         | 180 s | 4.15 | 4.29 | 1.85 |
| **JUND** | 20 s | 4 | 4.12 | n.d. |
|         | 60 s | 1.9 | 2.12 | n.d. |
|         | 180 s | 5.54 | 4.03 | n.d. |
Table 2 IPA annotation of the top pathways altered by plasma for each incubation time (3 h, 6 h, 24 h groups) in HaCaT cells.

| Group | Pathway                        | p value     | Ratio       | Molecule                                                                 |
|-------|--------------------------------|-------------|-------------|---------------------------------------------------------------------------|
| 3 h   | Nrf2-mediated oxidative stress | 7.31E-02    | 12/144      | AKR7L, Bach1, CYP2C19, FOS, HMOX1, JUNB/D, MAF, MAFK, MAP3K7, MAPK1, PIK3C2G |
|       | p38 MAPK signaling              | 8.02E-02    | 8/76        | DUSP1, HIST2H3C, IL1RAPL1/2, MAP3K7, MEF2C, PLA2G2E, TIFA                |
|       | PKC signaling                   | 6.95E-03    | 10/92       | CAMK2B/D, CFOS, FYN, HLA-DRB5, MAP3K7, JUNB/D, MAF, MAFK, MAPK1, PIK3C2G |
|       | IL10 signaling                  | 1.42E-02    | 7/54        | FOS, MAPK1, PIK3C2G, SOCS1/3/5                                          |
|       | JAK/Stat signaling              | 9.18E-02    | 6/56        | FOS, MAPK1, PIK3C2G, SOCS1/3/5                                          |
|       | GM-CSF signaling                | 9.18E-02    | 6/57        | CAMK2B/D, CSF2, CSF2RA, MAPK1, PIK3C2G                                   |
| 6 h   | MSP-RON signaling pathway       | 7.8E-03     | 14/32       | ACTA1/2, ATM, CCI2, CSF1, CSF2RB, IL3RA, JAK2, KLK3, PIK3C2G, PIK3R1/3, TLR2/4 |
|       | 3-phosphoinositide biosynthesis | 7.65E-03    | 21/67       | ASP5, ATM, CDC25C, DUSP1/8, PIK3C2G, PIK3R1/3, PIPIK5K1B, PAPDC2, PPP1R16/1B, PSEN1, PTPN20A/B, PTPR1, PTPR2, SOCS3, TPTE2 |
|       | TGF-β signaling                 | 6.25E-02    | 25/75       | ACVR1B, ACVR2A, AMH, BCL2, BMP2, CDC42, GSC, HNF4A, INHA, JUN, MAPK1, MAPK3, MAPK4, MAPK5, NOS1, NODAL, RNF110, RUNX2, SMAD1, SMAD2, SMAD3, SMAD6, SOS2, TGFB1, ZFYVE9, ZNF423 |
| 24 h  | nNOS signaling                  | 1.07E-04    | 4/12        | CAMK2, CHRNA1, NOS1, RYR2                                                 |
|       | γ-glutamyl cycle                | 1.44E-03    | 3/6 (0.5)   | GGT1, GGTL1/2                                                              |
|       | MSP-RON pathway                 | 8.48E-03    | 5/32        | CSF1, CSF2RB, PIK3C2G, TLR2/4                                            |
Table 3  IPA annotation of the top molecular and cellular functions for the action of plasma in HaCaT cells.

| Group                          | Molecular/cellular functions         | Ratio                  | #    | Molecules                                                                 |
|------|----------------------------------|------------------------|------|---------------------------------------------------------------------------|
| 3 h  | nucleic acid metabolism          | 8.93E-04 - 3.27E-02    | 13   | AKAP5, CALCA, CRH, GHRH, GHRHR, GLP1R, GLP2R, HMOX1, HTR1A, HTR1B, MRAP, NPPC, RAMP3, TSHR |
|      | small molecule biochemistry      | 8.93E-04 - 4.79E-02    | 17   | AKAP5, CALCA, CRH, GHRH, GHRHR, GLP1R, GLP2R, GRM1/5, HMOX1, HTR1A, HTR1B, MRAP, NPPC, RAMP3, TSHR, TSPO, UROC1 |
|      | cell signaling                   | 3.01E-03 - 3.27E-02    | 25   | AKAP5, CALCA, CRH, GHRH, GHRHR, GLP1R, HTR1A, HTR1B, KCNH2, MRAP, RAMP3, TSHR |
|      | cellular growth and proliferation | 7.86E-03 - 1.07E-02    | 16   | IGFBP5, MAPK1, Nr4A1/3, PPARα, RUNX2, TCF4                                 |
|      | cellular development              | 8.4E-03 - 1.07E-02     | 15   | IGFBP5, MAPK1, NR4A1/3, PPARα, TCF4                                        |

| 6 h  | nucleic acid metabolism          | 1.24E-04 - 4.13E-02    | 53   | ABCA1, AKAP5, AQP1, CALCA, CRH, CRHR1, CRMP1, CYP3A4, DRD1-4, EIF2AK3, GCG, GLPR1, GPBAR1, HMOX1, HTR1A/B, MTNR1A, NME2, NPY, POMC, PTGER2/3, PTH1R, RAMP1-3, TBXA2R, TSHR,.... |
|      | small molecule biochemistry      | 1.24E-04 - 3.71E-02    | 64   | ABCA1, AKAP5, CALCA, CRH, CRHR1, CRMP1, CYP3A4, DRD1-4, EIF2AK3, GCG, GLPR1, GPBAR1, HMOX1, HTR1A/B, MTNR1A, NME2, NPY, OXER1, POMC, PTGER2, PTGER3, PTH1R, RAMP1, RAMP2, RAMP3, TAAR1, TBXA2R, TSHR,.... |
|      | cell signaling                   | 1.18E-03 - 2.68E-02    | 58   | CNR1, CRH, CRHR1, PSIP1, TGM2, TLR2-5                                     |
|      | cell-to-cell signaling and interaction | 4.13E-03 - 2.68E-02  | 10   | DAO, DDC, GAD1, HDC, HNF4A, SDS, TAT, UROC1                              |
|      | amino acid metabolism            | 7.37E-03 - 3.71E-02    | 9    | DAO, DDC, GAD1, HDC, HNF4A, SDS, TAT, UROC1                              |
| 24 h               | cellular development                  | 1.66E-03-4.31E-02 | 19 | BAX, CACNA1G, CSF2RB, CTGF, FUT4, FST, IL6, ITGB1, PCYT1B, PRKCA, PTGS2, RYR2, S1PR1/3, SLURP1, TCF4, TLR2/4 |
|-------------------|--------------------------------------|-------------------|----|-----------------------------------------------------------------------------------|
|                   | cellular growth and proliferation     | 1.66E-03-4.31E-02 | 16 | BAX, CACNA1G, CSF2RB, CTGF, FUT4, FST, IL6, ITGB1, PCYT1B, PRKCA, PTGS2, RYR2, S1PR1/3, SLURP1, TCF4, TLR2/4 |
|                   | cell cycle                            | 1.86E-03-4.31E-02 | 6  | BAX, CDKN2C, IL6, PRKCA/B, PPARα                                                  |
|                   | cell-to-cell signaling and interaction| 1.86E-03-4.37E-02 | 9  | CSF1, NOS1, KCNH2, SNCA, TLR2/4                                                   |
|                   | energy production                     | 1.86E-03-4.31E-02 | 6  | ADH4, ALDH1A1, HSD17B2, PPARα, SLC16A7, RDH16                                      |
Figures

Figure 1

A

2 h incubation replacement with fresh medium

0-180 s

B

charged species (electrons/ions)

radicals/reactive species

(v)UV radiation

thermal radiation

electromagnetic fields

plasma

C

Human HaCaT keratinocytes

Isolation of RNA
Cy3 labeling of cDNA

Microarray hybridization on NimbleGen 4x44K Array

Nano liquid chromatography
Mass spectrometry

Gene expression data analysis

Protein expression data analysis

20 s: 3 h 6 h 24 h
60 s: 3 h 6 h 24 h
180 s: 3 h 6 h 24 h

Genes regulated by Nrf2

Oxidative stress responsive genes

Pathway and network analysis

Validation of selected genes in HaCaTs/NHEKs
Figure 2

A

| Plasma H₂O₂ (µM) | 20 s | 60 s | 180 s |
|------------------|------|------|-------|
| 20 s             | ~15  |      |       |
| 60 s             | ~32  |      |       |
| 180 s            | ~100 |      |       |

B

ROS levels (ratio to control) vs. incubation time

- 20 s
- 180 s
- H₂O₂ (100 µM)

Incubation time:
- 30 min
- 60 min
Figure 3

A

3 h incubation
~1,500 genes

20 s: 773
60 s: 172
6 h: 151
24 h: 115

20 s: 137
60 s: 93
6 h: 587
24 h: 63

6 h incubation
~6,000 genes

20 s: 471
60 s: 69
6 h: 894
24 h: 93

24 h incubation
~1,000 genes

20 s: 143
60 s: 91
6 h: 132
24 h: 10

B

20 s treatment
~2,800 genes

3 h: 587
6 h: 3,515
24 h: 63

60 s treatment
~2,600 genes

409
137
58
24 h: 63

24 h: 6

180 s treatment
~5,300 genes

3 h: 149
6 h: 1,926
24 h: 28

187
82
12
24 h: 100
Figure 4

A

GO: molecular function

B

GO: biological process

C

Nr1f2-associated pathway genes

gene short name

fold change

Figure 4

A

GO: molecular function

B

GO: biological process

C

Nr1f2-associated pathway genes

gene short name

fold change
Figure 8
Figure 9

A. NQO1 activity (6 h)

A'. NQO1 activity (24 h)

B. GPx activity (3 h)

B'. GPx activity (6 h)

B''. GPx activity (24 h)
Figure 10
Figure 11

A) 60 s plasma treatment, 20 min incubation

B) Nrf2 knockdown + 60 s plasma, 20 min incubation

C) Nrf2 knockdown + 180 s plasma, 1 h incubation

D) IPA annotation

- **Activation of Nrf2**
- **Regulation**
- **AREs binding sites**
  - Anti-oxidant defense by altered expression of down-stream targets
  - Influence on cellular activity (e.g. CSF2)
Non-Thermal Plasma Activates Human Keratinocytes by Stimulation of Antioxidant and Phase II Pathways
Anke Schmidt, Stephan Dietrich, Anna Steuer, Klaus-Dieter Weltmann, Thomas von Woedtke, Kai Masur and Kristian Wende

J. Biol. Chem. published online January 14, 2015

Access the most updated version of this article at doi: 10.1074/jbc.M114.603555

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