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Original research article

Can the effect of cold physical plasma-derived oxidants be transported via thiol group oxidation?

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ABSTRACT

Purpose: Intra- and intercellular redox-signaling processes where found responsible in various physiological and pathological processes with cellular thiol groups as important signal transducers. Using cold atmospheric plasma (CAP), a similar oxidation pattern of thiol groups can be achieved. Hence, it must be clarified which role extracellular thiol groups play in mediating CAP effects and whether or not the effects of short-lived reactive species can be preserved in a molecule like cysteine.
Methods: Physiological buffer solutions containing the amino acid cysteine were treated by an MHz argon plasma jet with molecular gas admixtures (kINPen) and transferred to cultured human keratinocytes. Cell proliferation, migratory activity, and metabolism were investigated. High-resolution mass spectrometry was used to estimate the impact of plasma generated species on thiol groups.
Results: While treated physiologic cysteine concentrations showed no impact on cell behavior, artificially high concentrations decreased proliferation, migration and lactate secretion. GSH levels inside cells were stabilized.
Conclusion: Extracellular thiol groups scavenge plasma-generated species and form a multitude of covalent modifications. Unexpectedly, human keratinocytes show only small functional consequences for treated physiologic cysteine concentrations. Results for high concentrated cysteine solutions indicate an improved cytostatic/cytotoxic impact by plasma treatment suggesting a potential application as a "preserving agent" of the chemical energy of plasma-derived species.

1. Introduction

Cold atmospheric pressure plasma (CAP) shows promising results in different medical applications, e.g. the promotion of acute and chronic wound healing, or in the treatment of malignant diseases [1,2]. Atmospheric cold plasma used in clinical approaches, e.g. kINPen MED, comprise of a mix of potentially bioactive entities: electrical fields, ultraviolet radiation, and reactive oxygen and nitrogen species (ROS/RNS). Besides the direct application of plasmas, numerous reports have been published describing the significant impact of plasma treated liquids, indicating that radiation and electrical fields are not (solely) responsible for some plasma effects [3,4]. Recently, the injection of plasma treated medium showed promising effects in a peritoneal carcinomatosis model in mice inducing apoptosis in cancer cells, reducing tumor size and leading to prolonged survival [5]. These in vitro and in vivo results strongly indicate that plasma generated ROS and RNS deposited in the liquid environment play a major role, presumably via interfering with the cellular and intercellular redox signaling processes [6–8]. Among ROS, the comparably stable hydrogen peroxide is one of the dominant compounds observed, along with the medium lived superoxide anion radical and the short-lived hydroxyl radical. In respect to RNS, peroxynitrite and nitric oxide are under debate [9–14]. An influence of nitrate or nitrite which are frequently detected in plasma treated liquids within a wide concentration range of a few $\mu mol\,L^{-1}$ to a few $mmol\,L^{-1}$ on cell viability of mammalian cell lines remains unsettled. Published data indicate that the chemical environment strongly modulates their role [15–17]. It has been suggested, that aquaporins facilitate the entrance of plasma-derived ROS/RNS into the cytosol, especially for hydrogen peroxide [18–20]. In contrast, many plasma-derived species are highly reactive and very short lived with free migration radiuses in the low µm-range [21], precluding an unaltered entrance via pore proteins in many treatment setups. Such, the

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ultimately responsible ROS and/or RNS are not fully identified yet.

Biological systems are complex, with a number of potential reaction partners such as sugars, lipids, or proteins and their respective monomers present [22]. Accordingly, it may be hypothesized that cold plasma exerts its activity in part via the chemical interaction with organic molecules, leading to the generation of (stable) mediators of the plasma-derived chemical potential. Proteins and numerous amino acids are assumed to be susceptible to plasma-derived reactive oxygen or nitrogen species [23–25]. Especially sulfur groups are excellent targets and may undergo a number of oxidative and nitrosative modifications with potential bioactivity (reactive sulfur species, RSS) [26]. Many RSS are able to modify redox-sensitive proteins to trigger a biological response [27]. They can derive from cysteine, or are plant metabolites, e.g. sulforaphane and allicin [28].

To investigate the role of RSS and other thiol derivatives as potential transducers of plasma-derived liquid chemistry, the ubiquitously in mammalian tissue present amino acid cysteine was used as a model compound. The impact of argon-oxygen plasma jet derived cysteine derivatives was tested on human epithelial cells, especially with regard to cytotoxicity, cell proliferation, migration capability, and processes of cell metabolism.

2. Materials and methods

2.1. Cell culture

HaCaT keratinocytes were cultivated in Roswell Park Memorial Institute (RPMI1640) cell culture medium (PanBiotech, Germany) supplemented with 1% glutamine, penicillin/streptomycin (final concentration 100 IU/ml penicillin; 100 µg/ml streptomycin), and 8% heat-inactivated fetal bovine serum (FBS; all Sigma, Germany) at 37 °C and 5% CO_2 in a humidified atmosphere. To sub-culture cells twice a week they were incubated with 5 mM Ethylenediamine tetraacetic acid (EDTA; Sigma, Germany) in phosphate buffered saline (PBS) buffer for 10 min and trypsinized with 0.025% trypsin/EDTA. To determine the number of cells, a sample was lysed and the nuclei counted using a Buerker counting chamber. 1.5 million cells were seeded in a 75 cm^2 cell culture flask (Sarstedt, Germany). For assays, the cells were collected with the same procedure.

2.2. Cold physical plasma treatment

The plasma treatment (CAP) was performed using an atmospheric pressure argon plasma jet (kINPen 09; neoplas tools, Germany) with a working gas flow of three standard liters per minute (slm) dry argon (argon N50, Air Liquide, France). 1% of molecular gas admixture oxygen N48 (Air Liquide, Paris, France) was added to achieve oxidative conditions. This feed gas composition is known for a limited generation of nitric oxides while emphasizing oxidative species [29]. A computer controlled xyz-table (CNC, Germany) was used to control the jets movements. A volume of 1 mL was treated in 24 well-plates (Sarstedt, Germany) for up to 10 min, with 8 mm distance from the jet's nozzle to the liquid surface. Water loss caused by prolonged exposure to the gas flux during treatment was compensated to ensure correct osmolality. On the day of the experiment, crystalline cysteine (BioUltra, Sigma-Aldrich, Germany) was dissolved in diluted PBS to assure correct osmolality in the desired concentration, e.g. of 2 mM, 10 mM, or 100 mM. For control, buffer without cysteine was treated. If desired, 100 Units catalase were added directly after treatment to eliminate hydrogen peroxide.

2.3. Cysteine derivative profiling

The cysteine derivatives produced by the plasma treatment were characterized by high-resolution mass spectrometry, using a TripleTOF5600 mass spectrometer (Sciex Ltd., Germany). For the mass spectrometric analysis, control or treated solutions were diluted 1:1 with water/methanol/ammonia 50/49.85%/0.15%. Via direct infusion (10 µL min^{-1}) and negative electrospray ionization using a TurboV source (150 °C, 35 L/min curtain gas, 20 L/min ion source gas 1, 25 L/min ion source gas 2, −4 kV ion spray voltage) samples were analyzed. Spectra were acquired in a mass range from 50 to 400 m/z. Peaks found in the MS spectra were fragmented using a collision energy of −24 eV and a declustering potential of −10 kV to identify the compounds on the base of his fragmentation pattern.

2.4. Cell proliferation assay

The proliferation of keratinocytes was explored using the resazurin transformation assay. 3 500 cells per well were plated in the inner 60 wells of a 96 well-plate (tissue culture treated polystyrene, flat bottom; Sarstedt, Germany) to assure similar conditions 24 h prior experiment. Medium was removed, cells were washed with PBS and 100 µL treated liquid was transferred onto the cells. A serial dilution was performed to simulate different treatment times. PBS served as an untreated control, 20 µM of hydrogen peroxide (Sigma, Germany) as the positive control. This concentration was predetermined to reduce HaCaT cell proliferation to 50% of untreated control [30]. After 30 min incubation time, the liquid was replaced by fresh medium and the cells were incubated for 71 h. After washing the blue dye resazurin (Alfa Aesar, Germany) was added to the cells in a concentration of 100 µM in fresh medium. Viable cells reduce it to the pink colored high fluorescent resorufin. The fluorescence intensity was read after an hour incubation at 535 nm excitation and 590 nm emission in a plate reader. No-cell controls were used to determine assay background. Cell proliferation was calculated as percent of control.

2.5. Cell migration assay

To evaluate the cell migration and velocity of HaCaT cells in response to cysteine derivatives treatment the scratch assay was used. 50 000 cells per well were seed in a 24 well-plate (tissue culture treated polystyrene, flat bottom; Sarstedt, Germany) in complete medium and kept in an incubator for 24 h. The medium was replaced by deficiency medium with only 1% FBS to decrease the proliferation for another 24 h incubation. Confluent cells were scratched with a 1000 µL pipette tip to create a scratch with a width of around 1000 µm. After washing with PBS cells were stimulated with the test liquids for one hour. Different dilutions of treated and untreated cysteine were under observation. Subsequently, test liquids were replaced by deficiency medium. As negative control, 2 µM cytochalasin B (Sigma, Germany) in medium was used. The width of the scratch was monitored over 24 h using time-lapse microscopy (Zeiss, Germany) and data collected every 10 min using AxioVision software (Zeiss, Germany). The migration velocity was calculated from the distance reduction for different time periods.

2.6. Metabolic profiling

To explore viability and cell metabolism directly after stimulation with plasma treated liquids a modified version of the proliferation assay was used. 10 000 cells per well were seeded in 96 well-plates and received the same treatment as described in 2.4. After 30 min incubation, the treated liquid was removed and 50 µM resazurin in 100 µl full medium was added. Resazurin conversion was measured at different time points from 20 min to 6 h after adding the dye. To explore how different metabolic pathways are influenced diverse luminescence assays were used. Directly after stimulation intracellular total glutathione (GSH) and oxidized glutathione (GSSG) were measured using GSH/GSSG-Glo Assay and glucose uptake using Glucose Uptake-Glo Assay. Lactate secretion after 8 h was evaluated using Lactate-Glo Assay (all Promega, Germany). Assays were performed according to the manufacturer's protocol with recommended controls. For all experiments 10 000 cells per

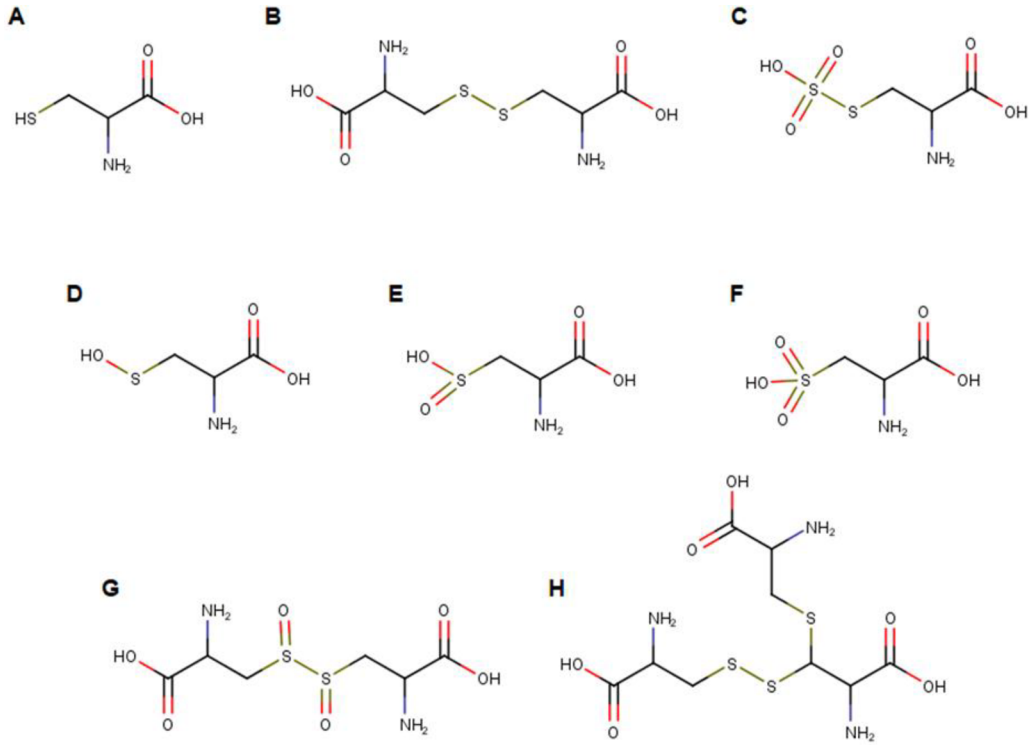

Fig. 1. Cysteine derivatives observed by high-resolution mass spectrometric analysis after argon plasma jet treatment of cysteine (A) (100 mM, 10 min, 3 slm argon/1% oxygen, kinpen09). Dominant products are cystine (B), cysteine sulfonic acid (F), and oxidized cystine derivatives (G, H). For further details see the supplementary information, Bruno et al. [33], and Lackmann & Wende et al. [34].

well were seed in the inner 60 wells of 96 well-plates and kept in an incubator for 24 h. Medium was removed and the cells were washed with PBS. Then 100 µl treated liquid was transferred onto the cells. As a negative control the untreated liquid was added to the cells. Plates where incubated for 30 min. After a washing step the procedure between the assays differed. Total GSH and GSSG were measured immediately. A no-cell control was used to reflect assay background. Glucose uptake over 10 min was determined using buffer without glucose as negative control. For determination of reductive potential and lactate secretion 150 µL fresh medium were added per well. 8 h after stimulation supernatant samples were collected and lactate was measured using medium as background control. To explore metabolic activity over this time period medium with 50 µM resazurin was added to cells instead of just medium and fluorescence was determined after 8 h. Metabolic activity was also measured 24 h after stimulation as described in 2.4.

2.7. Quantification of intracellular ROS

The fluorogenic probe CellROX Deep Red Reagent (Thermo Fisher, Germany) was used to determine the amount of ROS inside the cells. 20 000 cells per well were seed in 8 well µ-slides (ibidi, Germany) 24 h prior to experiment. The cells were washed with PBS and incubated for 30 min with test liquids. Incubation with 10 µM CellROX DR in medium for another 30 min followed. As positive control 50 µM menadione was added to the CellROX reagent during incubation. After washing three times with PBS cells were counterstained with 1 µg/mL Hoechst 33,342 (ImmunoChemistry Technologies, USA). After another 15 min imaging was done using a Leica SP5 confocal microscope (Leica, Germany). Mean fluorescence intensity values (MFI) of five 250 µm x 250 µm images with at least 40 cells each were calculated using las x software (Leica, Germany). For better comparability, the MFI ratios compared to a PBS control were calculated and displayed for each condition.

2.8. Cytometric bead array

Cells were prepared and stimulated as described in 2.6. After washing 150 µL fresh medium were added per well. 24 h later cell supernatant samples were collected and pooled. Samples were frozen at −20 °C. On the day of the assay samples were defrosted and centrifuged. A cytometric bead array for IL-6, IL-8, IL-12p70, and TNF-α was performed following the manufacturer´s protocol (RayBiotech, USA) with a CytoFLEX 24 (Beckman, Germany).

2.9. Quantification of stable reactive species and statistical analysis

Hydrogen peroxide was determined for selected conditions using the xylenol orange assay (Thermo Scientific, Germany) according to the manufacturer´s protocol. OD was measured at 560 nm and a calibration curves determined using authentic hydrogen peroxide was used for quantification. Nitrite and Nitrate were determined for selected conditions using a Dionex ICS 5000 ion chromatography system using the conditions suggested by the manufacturer for the 7-Anion standard solution. Photometric detection of both ions was favored over conductivity measurements for sensitivity reasons. Standard curves were used for quantification purposes. To minimize interference with cysteine the concentration of 2 mM was used for both assays. Values are expressed in mean + SD for three independent experiments carried out at least in duplicates, except for viability assay with two independent experiments in sextuplicates. Statistical analysis was done with two-way-ANOVA using GraphPad Prism 7. P values under 0.05 were considered significant.

3. Results and discussion

3.1. Cysteine oxidation product profile shows active oxidized compounds

Cysteine and glutathione have been used as reporter substance to compare and standardize plasma discharges before [31,32]. By the impact of plasma-generated species various covalent cysteine derivatives were generated (Fig. 1 and supplementary Fig. S1) as was expected. Due to the working gas containing 1% O_2, predominantly ROS and subsequently oxidative modifications were observed. Both treatment time and initial cysteine concentration determined the observed product profiles. Some products were found as intermediates mainly,

e.g. cystine (A), sulfenic (D), and sulfinic acid (E). Cysteine sulfonic acid (F) accumulates and might be a stable end product. Of special interest are the oxidized cystins (G, H), and cysteine-S-sulfonate (C) which are regarded as reactive sulfur species (RSS) [26]. The amount of cysteine modified by the treatment varies between 100% (2 mM initial concentration), 60% (10 mM), and up to 10% (100 mM). Further details on plasma treatment derived cysteine chemistry can be found in Bruno et al. [33].

Oxidized disulfides, such as formed by the plasma treatment are able to modify thiol groups in proteins forming mixed disulfides, sulfenic acid, or sulfinic acid [35]. Effects are even resistant to different antioxidants suggesting an influence on cells can occur in spite of active defense mechanisms. Such posttranslational modifications are part of redox signaling and could contribute to the mediation of cold plasma-derived signals. Besides, oxidized disulfides can influence metabolic enzymes like glyceraldehyde-3-phosphate dehydrogenase with consequences for the reduction of GSSG [35]. Another compound of interest is cysteine-S-sulfonate. It was recently found to be an agonist of the NMDA receptor, which activation leads to calcium influx influencing further cell signaling [36].

3.2. Deposition of long-lived ROS and RNS in CAP treated buffers

By the plasma treatment of liquids the long-lived species hydrogen peroxide, nitrite, and nitrate are deposited and can be used as marker compounds. The overall deposition of nitrate and nitrite was found to be low, caused by an oxygen dominated chemistry Fig 2. In the absence of cysteine mostly no nitrite is detectable, while small µM amounts were found in its presence. Nitrate deposition was increased for long treated cysteine solution, yet still at biologically negligible concentrations (no impact on cell proliferation observed for maximal tested 250 µM, data not shown). No differences in hydrogen peroxide deposition were measured immediately after treatment. These findings are supported by the slow direct reaction of hydrogen peroxide with cysteine at physiological pH conditions with reactions rates around $3\,M^{-1}\,s^{-1}$ [34,37].

3.3. Impact of CAP derived cysteine derivatives on cell proliferation

The reaction products obtained from short to moderate plasma treatment of 2 mM cysteine in PBS revealed no significant impact on cell proliferation (Fig. 3). This may be due to a low concentration of active cysteine derivatives and/or the absence of any long-term effects of those compounds in respect to cell cycle control. The undesired effect of hydrogen peroxide, which is known to reduce mammalian cell proliferation at higher concentrations considerably, while small concentration exerts stimulating effects [13,30] was inhibited successfully by the added catalase. Such, treated control (plasma treated PBS) showed identical proliferation as untreated cell control, indicating no other long-lived species influencing cell proliferation was deposited.

To increase the formation of potentially active compounds cysteine concentration was increased to 100 mM and the treatment time was extended to 10 min. No effects of exposure to high untreated cysteine concentrations on cell proliferation were observed (see Fig. S2). A treatment with Ar/O_2 plasma (CAP) derived cysteine derivatives significantly decreased cell proliferation rate, even when H_2O_2 was removed by catalase. Undiluted, the treated cysteine solution lead even to complete cell death (Fig. 4A), while dilutions to about 50% did not change the cell proliferation rate. Also, CAP treated PBS alone showed no impact. When catalase addition was waived, the treated PBS reduced cell proliferation most likely due to the formation of long-living ROS (Fig. 4B). The effect was similar to a comparable H_2O_2 treatment without plasma (see supplementary information, Fig. S2), verifying a dominant effect of this compound in plasma – cell interaction [12,38]. Hydrogen peroxide also enhanced the impact of plasma generated cysteine derivatives: even when diluted to 1/3 of the nominal concentration resazurin transformation was lower than buffer control. This behavior of the cells was not expected, as cysteine is known to react with H_2O_2 to form cystine [37] and a significant portion of cysteine is still unaffected after the treatment ($\approx$ 90%), it was assumed to act as a scavenger. Instead, cysteine derivatives seem to emphasize the influence of H_2O_2. The reason for this is not clear so far, but the observation might be explained by increased energy demand for cysteine and cystine metabolism directed at GSH production and subsequent lack of energy equivalents to control oxidative challenges [39]. Considerable cytotoxicity is known for high concentrations of this amino acid in neurons [40]. The catabolism is depending on the regulation of cysteine dioxygenase (CDO). Although this enzyme is highly regulated, the artificial high concentrations could lead to an additional burden of the cells with possible enhanced vulnerability. For shorter treatment times (5 min) weaker effects of the generated cysteine derivatives were observed, and with catalase addition impact on cell proliferation was negligible (see supporting information). This suggests, that higher oxidized cysteine derivatives might be responsible for the observed cell death after 10 min and/or that the resulting concentration of active compounds is higher in this condition. Other than expected, osmotic pressure remained at 290 – 295 mOSmol kg^{-1} regardless of the treatment (see Fig. S3).

Additionally, a 10 mM cysteine solution was examined (Fig. 5A). In contrast to the 100 mM solution, even a treated, undiluted 10 mM cysteine solution did not lead to complete cell death. However, a significant decrease in cellular proliferation rate, especially for 5 min and 10 min treatment, was observed. After a short treatment (2 min), a slight stimulation of proliferation was observed which might be attributed to the scavenging of H_2O_2 by the thiol groups and subsequent lower effective H_2O_2 concentration after CAP. When catalase is added, all effects on metabolic activity were abolished (Fig. 5B). The biochemical background for this observation is assumed to be the same as for the 100 mM cysteine and related to disturbances in energy and redox equivalent metabolism (e.g. glutathione). Additional redox signals cannot be received and translated properly. This suggests, that in cancer cells, which often present a non-normal energy metabolism resting on catabolic processes plus having a limited oxidative stress resilience, the influence of plasma treated concentrated cysteine solutions on proliferation rate could be significant.

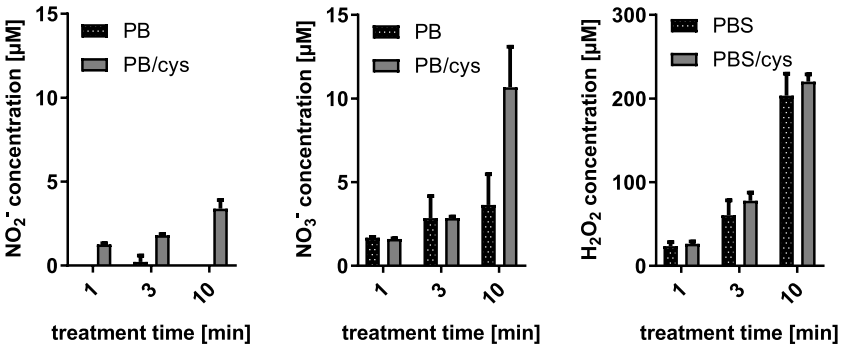

Fig. 2. Deposition of long-lived species in phosphate buffer (nitrite/nitrate) in the absence or presence of cysteine. If cysteine is present, nitrite and nitrate deposition was increased. Hydrogen peroxide concentrations immediately after treatment were indifferent of cysteine, indicating a slow direct reaction. mean + SD of 3 experiments in duplicates.

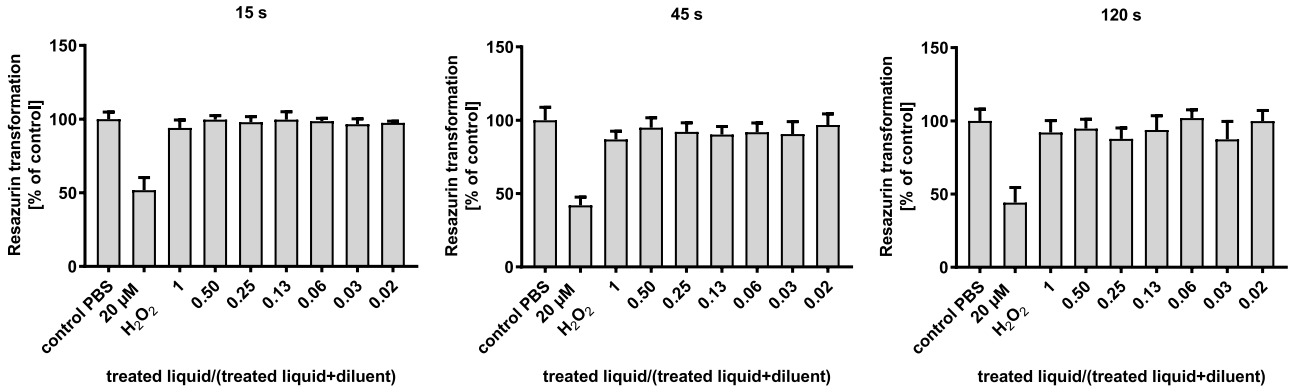

Fig. 3. Cell proliferation of human keratinocytes, comparison of HaCaT cell resazurin transformation 3 days after stimulation with plasma treated PBS + cysteine 2 mM, treatment time 15 s, 45 s, 120 s, addition of catalase, mean +SD of 2 experiment in sextuplicates.

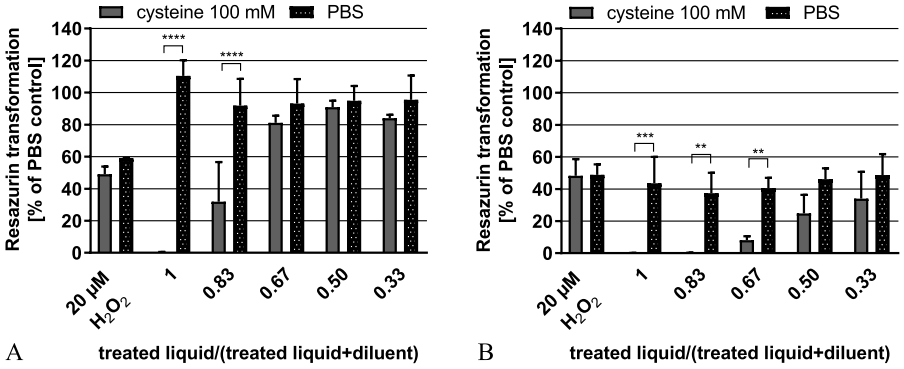

Fig. 4. Cell proliferation of human keratinocytes, comparison of HaCaT cell resazurin transformation 3 days after stimulation with plasma treated PBS +/- cysteine 100 mM, treatment time 10 min, dilution of treated liquid with PBS, addition of catalase and 5 min waiting (A), without catalase (B), mean +SD of 3 independent experiments.

3.4. Cell migration is altered by high concentrations of cysteine derivatives

The scratch assay was used to detect changes in motility of keratinocytes 12–24 h after treatment. For treated solutions with 2 mM cysteine, no influence on migration velocity was observed (see Fig. S6). For higher cysteine and cysteine derivatives concentrations (13 mM to 100 mM, in part diluted) changes in cell migration were observed for some conditions (Fig. 6). A non-negligible impact of cysteine itself was detected. Moderate concentrations of cysteine lead to a non-significant increase in migration velocity (13 mM, 25 mM). A possible explanation is the inhibition of N-Methyl-**D**-Aspartate (NMDA) receptors via cystine [41], which is occurring due to normal oxidation of cysteine via ambient oxygen. These receptors are known from neuronal cells but is also expressed in epithelial cells. NMDA receptor activation was shown to inhibit re-epithelialization by keratinocytes in acute wounds [42]. This suggests that when using mild or moderate plasma treatment intensities which produce significant amounts of cystine, an increase of epithelialization might be achieved. However, a narrow applicable treatment window seems to exist as for higher treatment intensity/higher cysteine derivative concentration a significant decrease of cell migration was observed (> 67 mM cysteine solutions). In this case, a possible mechanism is the activation of NMDA receptor by cysteine-S-sulfonate, leading to Ca^{2+} influx maybe causing cell death [36]. The activation or inhibition of this receptor seems to be depending on the present concentrations of different modulators. This prejudicial influence for concentrated cysteine derivatives is corresponding with the proliferation assay, where they lead to cell death in the long-term.

3.5. Keratinocytes encounter changes in metabolic and secretory activity

The short-term viability of cells directly after treatment was determined using a modified version of the proliferation assay (up to 6 h past challenge). Plasma treated PBS, showed only a minor effect (Fig. S4). The presence of 100 mM cysteine in the solution leads to increased

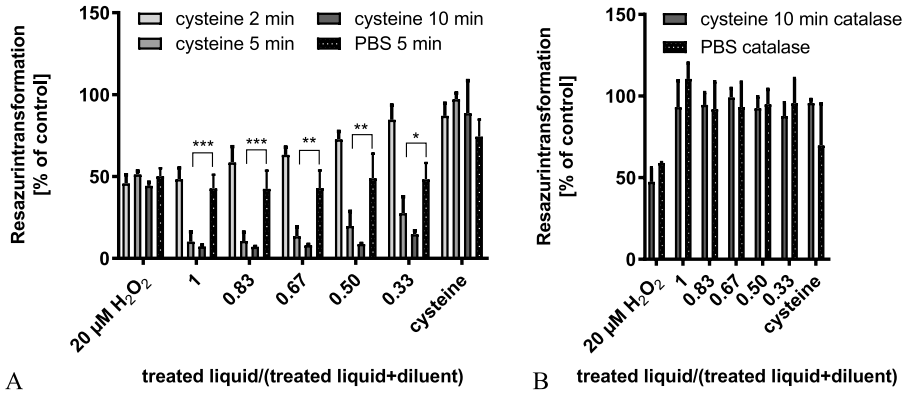

Fig. 5. Cell proliferation of human keratinocytes, comparison of HaCaT cell resazurin transformation 3 days after stimulation with plasma treated PBS +/- cysteine 10 mM, treatment time 2/5/10 min (A) and 10 min then addition of catalase before transfer on cells (B), dilution of treated liquid with PBS, mean +SD of 3 independent experiments.

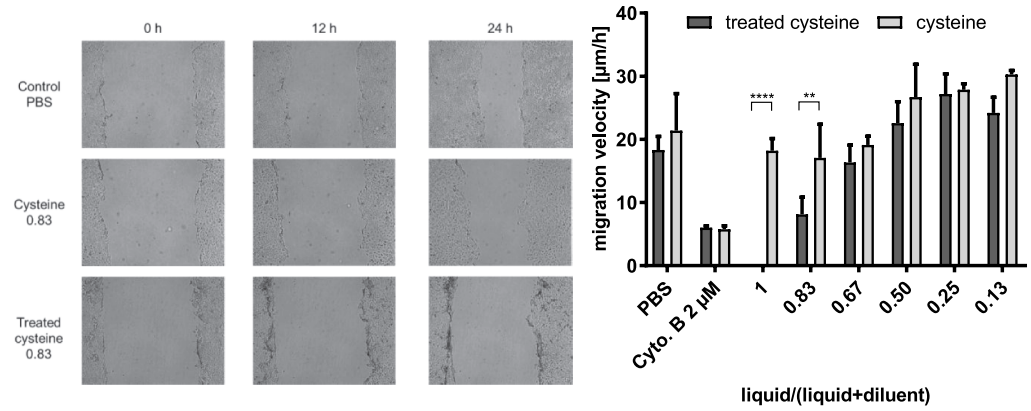

Fig. 6. Migration velocity of human keratinocytes is altered after cysteine derivative treatment. Scratch assay at different time points after stimulation with plasma treated/ untreated cysteine (100 mM, 10 min treatment, left), HaCaT migration velocity 12–24 h after stimulation, mean +SD of 3 independent experiments (right).

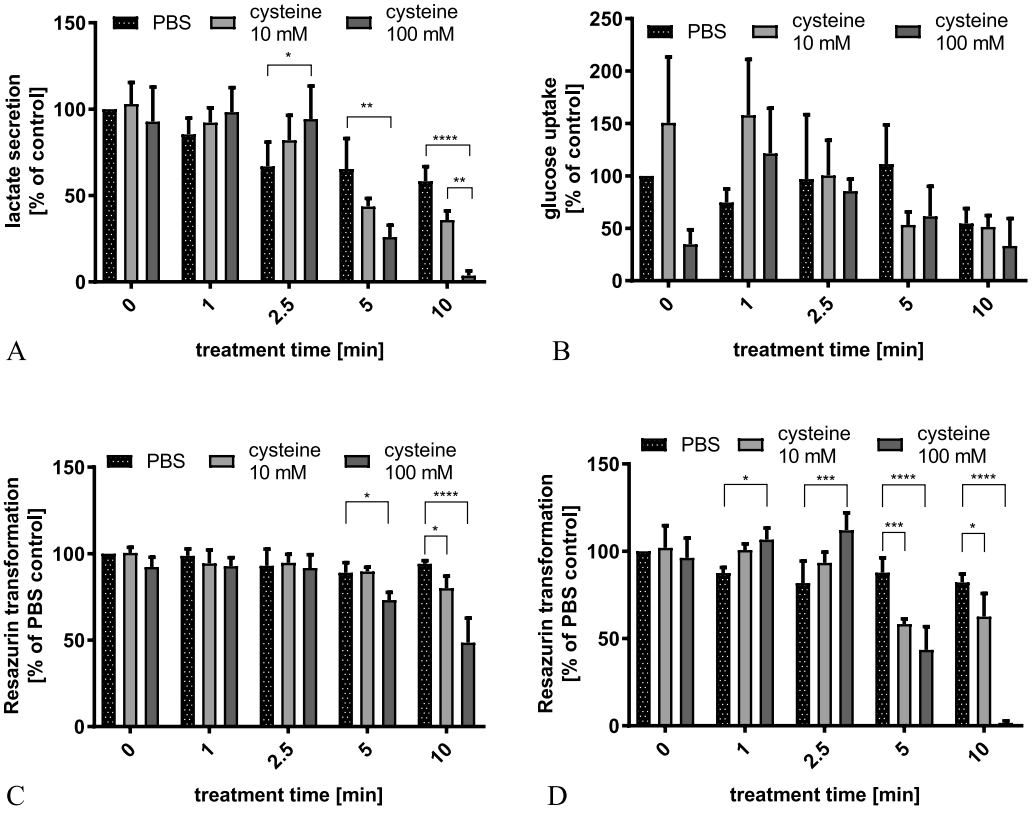

Fig. 7. Lactate secretion (A), glucose uptake (B) and cell metabolism of human keratinocytes (0–8 h, C and 24 h, D) after stimulation with cysteine derivatives, details see text, mean +SD of 3 independent experiments.

metabolic activity during the first hours strongest at the beginning. A similar impact had plasma treated cysteine solution 100 mM during the first hour when residual cysteine or derivatives were presumably metabolized. With time, the difference to control decreased, suggesting cysteine derivatives in combination with further oxidative challenges like hydrogen peroxide cause a breakdown of cell metabolism. To further investigate changes in metabolic activity, lactate secretion was measured (Fig. 7A). In contrast, CAP derived cysteine derivatives led to a strong reduction of lactate secretion, surpassing the effects of CAP treated PBS which supposedly is induced by deposited H_2O_2. Changes in glucose metabolism (Fig. 7B) showed a non-linear behavior, with cysteine concentration and treatment time both modulating the outcome. Some combinations led to a strong increase of glucose uptake (10/100 mM cysteine, 1 min CAP) while others did not change it. Long treatments led to a reduction of glucose uptake independent from cysteine concentration (10 min, 0/10/100 mM cysteine). Overall metabolic activity did reflect this in the case of 100 mM CAP treated cysteine (Fig. 7C) after 8 h, and more pronounced 24 h after treatment with CAP derived cysteine derivatives. CAP treated PBS did not alter the cellular metabolic activity after 8 h or 24 h.

3.6. Intracellular redox state is modulated

Because reactive species, as well as cysteine and its derivatives, are expected to influence redox levels within cells, a fluorogenic probe (CellROX) was used to measure intracellular ROS (Fig. 8). Menadione, an inhibitor of electron transport chain in mitochondria led to a significant increased mean fluorescence (MFI) of the cells. CAP treated PBS increased the MFI not significantly. Untreated 100 mM cysteine led to a massive decrease of the MFI which is assumed to result from an

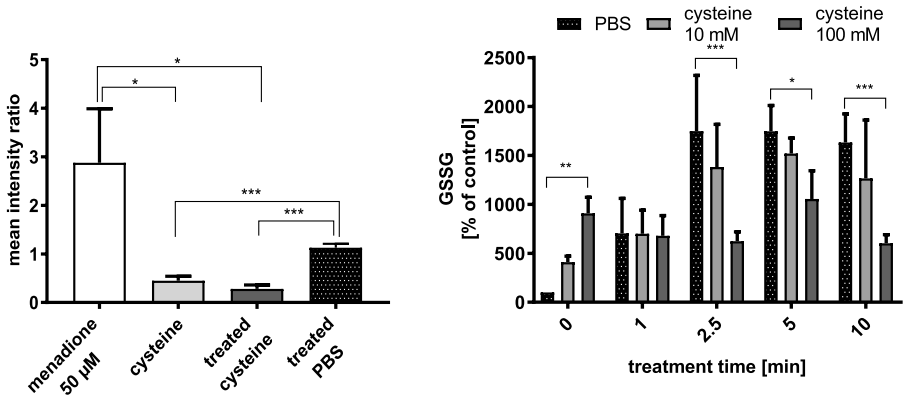

Fig. 8. Intracellular ROS levels in HaCaT cells after stimulation with plasma treated cysteine (100 mM) as CellROX Deep Red relative mean fluorescence intensity (untreated PBS = 1) (left) or total GSSG (untreated PBS = 100%, right), mean +SD of 3 independent experiments.

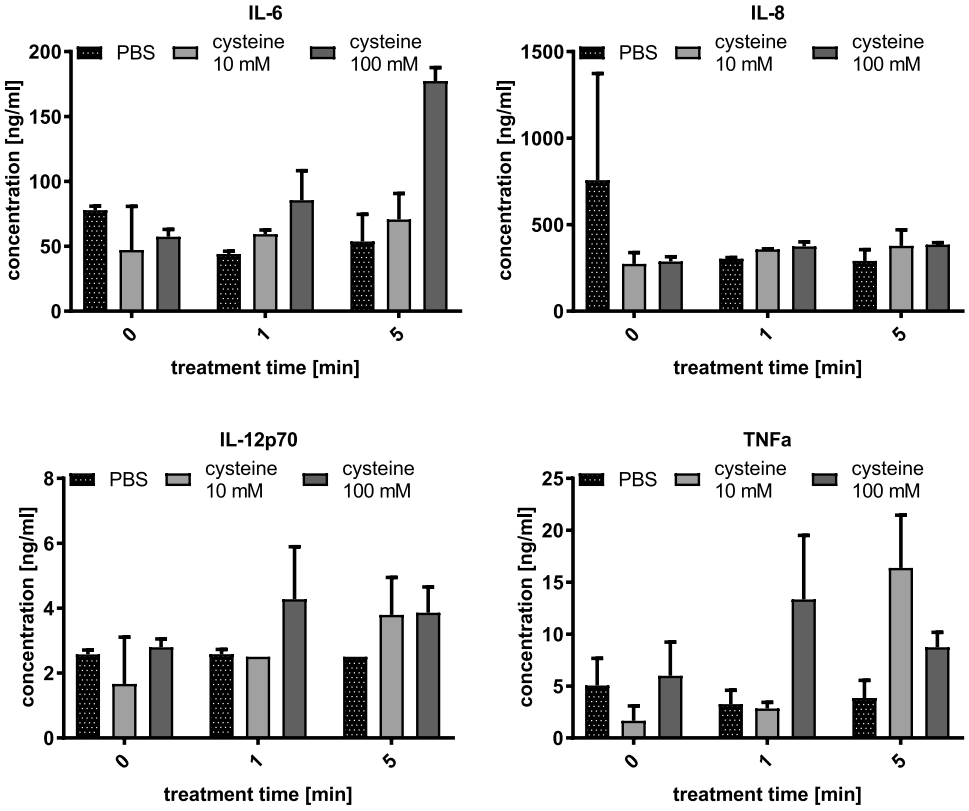

Fig. 9. Cytokines in keratinocyte supernatant, different cytokines measured with a multiplex assay in cell supernatant 24 h after stimulation with different plasma treated liquids, single experiment, pooled samples measured in triplicates, mean +SD.

increased GSH synthesis. Interestingly, CAP treated cysteine solution decreased the MFI further. Potentially, cystine formed during plasma treatment caused the decreased ROS level by scavenging cell derived reactive species such as mitochondria formed superoxide anion radicals [43]. Another possibility is the enhanced activation of reductive pathways like the NRF2-pathway in response to CAP derived cysteine derivatives [44].

Cellular GSH and GSSG were measured after stimulation (Fig. 8). For treated PBS the GSSG levels increased non-linearly with treatment time, likely due to plasma-derived hydrogen peroxide consuming GSH. A CAP treated 10 mM cysteine solution showed similar effects. A solution of 100 mM cysteine led to high GSSG levels for the untreated solution (10fold of control), but remained almost unchanged regardless of treatment time. The significant amount of cysteine and cystine delivered increases the intracellular GSH levels, which than scavenge both intra- and extracellular ROS to a level unmet in control as can be seen from CellROX MFIs. The possible efflux of GSH and formed GSSG is known in different cell types [45]. Potentially, a lack of reductive equivalents leads to efflux of GSSG instead of its reduction by glutathione reductase and would explain the smaller amounts of GSSG for longer treated cysteine in contrast to PBS [46].

3.7. Cytokine secretion increases

To get an impression of subsequent changes in cell-cell communication after treatment with cysteine derivatives, cytokine secretion was measured after 24 h (Fig. 9). Pro-inflammatory cytokines increased (IL-6, IL-12, TNF-α), while others, e. g. IL-8, were unaffected. The secretion of IL-6 is increased by NMDA receptor activation [47]. TNF-α is also related to NMDA receptor activation and blocking this receptor

decreased the transcription of the chemokine in a nerve lesion model [48]. As the receptor is present in keratinocytes and cysteine derivatives like S-nitrosocysteine or cysteine-S-sulfonate are discussed as agonists at this receptor [49,50], the NMDA receptor activation could be contributing to the increased secretion of cytokines.

As NMDAR controls cell motility and cell-cell signaling including pore formation, the interference with this target may present an important aspect of CAP impact in wound healing. As cysteine is a ubiquitous amino acid, its oxidation by CAP in situ during the treatment of a wound is likely. Downstream effects relayed via NMDAR could trigger immune response and activation of cell metabolism. In contrast, cysteine could be used as a modulator of plasma treated liquids, especially in the concept of cancer intervention.

4. Conclusion

Argon/oxygen plasma-derived reactive species lead to the formation of multiple oxidized cysteine and cystine derivatives in cysteine containing liquids. At levels related to physiologic conditions (2 mM), the resulting cysteine product portfolio does neither influence the proliferation of human keratinocytes nor the cells migratory or chemotactic ability. When using high concentrations of cysteine (100 mM) a reduction in cell proliferation and migratory ability was observed. This was accompanied by metabolic changes, e.g. decreased lactate secretion and a decrease in intracellular ROS levels. At the same time, the secretion of pro-inflammatory cytokines increased (IL12, TNF-α). These effects might be attributed to bioactive cysteine/cystine derivatives like cysteine-S-sulfonate, an NMDA receptor agonist, leading ultimately to energy depletion and reductive cell stress. Such, results indicate an improved cytostatic/cytotoxic impact of cysteine-containing solutions by plasma treatment suggesting a potential application as a “preserving agent” of the chemical energy of short-lived plasma-derived species.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cpme.2019.100086.

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