Characterization of the Eugenol Effects on the Bioenergetic Profile of SCC-4 Human Squamous Cell Carcinoma Cell Line

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Eugenol (EU), the active ingredient in clove oil, is commonly used as successful therapeutic compound in dentistry due to its antiseptic and anti-inflammatory effects. Recent research studies suggest that eugenol has also a potential anti-cancer effect. This study was thereby purported to assess the effects of EU on the bioenergetic profile of the SCC-4 human squamous cell carcinoma cell line. To this aim, SCC-4 cells were treated for 24 hours with free EU and EU incorporated in polyurethane structures (50 μM each). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XF-24e extracellular flux analyzer (Agilent Technologies Inc.). Analysis of the SCC-4 bioenergetic profile was performed in the presence of the classic modulators of the electron transport chain: oligomycin, FCCP, and antimycin A+rotenone. Our data showed that cells stimulated with free EU induced a decrease of OCR linked parameters and an increase of ECAR, effects that were abolished by the incorporation of EU in polyurethane structures. In conclusion, free eugenol elicits inhibitory effects on mitochondrial respiration in the SCC-4 cell line, a result that might be suggestive for its anti-tumoral effects.

Keywords: eugenol, SCC-4 human squamous cell carcinoma, mitochondrial function, cellular bioenergetics, glycolysis

In the past decades it was a tremendous effort in the research field in an attempt to identify the most effective therapeutic strategies in cancer [1-3]. Despite this intensive *battle*, cancer still remains the leading death cause worldwide with over 20 million new cancer cases predicted by 2025 [4]. Since mitochondria represents the key center in integrating energetics and cell death, in the last years novel pharmaceutical or phytochemical compounds able to modulate mitochondrial function as potential therapeutic target, have been intensively tested [5]. Accordingly, phytochemicals (i.e., biologically active plant compounds) able to exert anti-anticancer properties are highly investigated nowadays, due to their low toxicity and reduced side effects [5].

One of such agent is eugenol (4-allyl-2-methoxyphenol), a volatile phenolic active ingredient of clove oil from *Eugenia caryophyllata* buds and leaves. Due to its sedative, analgesic [6], and antibacterial effects [7], eugenol (EU) is commonly used in dentistry. Yet, there have been repeatedly reported side effects of EU, when used in high concentrations in the oral cavity, ranging from local irritative and cytotoxic effects to hypersensitivity reactions [8, 9]. Hence, there is a serious questioning regarding its toxicity since studies about cyto/genotoxicity of EU are limited and controversial [10]. Despite this, the US FDA (Food and Drug Administration) approved the use of clove oil as a natural analgesic and antiseptic in dentistry [11].

Recently, numerous studies have demonstrated the anti-tumoral effect of EU: antiproliferative effects in several tumour cell lines or in B16 melanoma xenograft model [12-14]; apoptotic efects in numerous malignant cells, such as mast cells [15], melanoma cells [13], or HL-60 leukemia cells [16].

To the best of our knowledge, there are no data describing the effects of EU on bioenergetic profile of human squamous carcinoma cells, thereby the present study was aimed to assess the effects of EU on mitochondrial and metabolic parameters, in SCC-4 human squamos cell carcinoma cell line.

Experimental part

Cell culture

SCC-4 human squamos cell carcinoma cell line (derived from squamous cell carcinomas of the tongue) was purchased from ATCC (ATCC® CRL-1624™, Virginia, USA). The cells were cultured in F12/Dulbecco's Modified Eagle Medium (DMEM) supplemented with 400 ng/mL hydrocortisone, 10% fetal bovine serum (FBS) and antibiotics to avoid contamination (100 U/mL penicillin and 100 μg/mL streptomycin). A controlled humidity

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atmosphere in the presence of 5% CO_2 at 37°C was used for cell growth. The medium was changed every 3 days. For cell number quantification, the Neubauer chamber was used in the presence of Trypan blue.

Analysis of cell morphology

To analyze the cell morphology of SCC-4 cells, a number of 10 K cells/well were cultured in 12-well plates and allowed to attach. The cells were further stimulated with different compounds (free EU, EU incorporated in polyurethane structures, and dimethyl sulfoxide-DMSO, the solvent used to dissolve EU) at a concentration of 50μM each. At 24 h post-stimulation, images of the tumor cells (10x magnification) were taken using the Olympus IX73 inverted microscope equipped with DP74 camera (Olympus, Tokyo, Japan).

Extracellular flux (XF) analysis

SCC-4 cells (10 K cells/well) were seeded in Seahorse 24-well cell culture plates (provided by Agilent Technologies Inc.) and allowed to attach overnight (experiments were performed in triplicate). Afterward, the cells were treated with the mentioned compounds for 24 h at 37 and 5% CO_2 at the specified concentration (50 μM of each compound: free EU, EU incorporated in polyurethane structure, and DMSO); the control group used in the study comprised cells incubated only with cell culture medium (i.e., untreated cells). Background correction wells were included in the experiment (i.e., wells with no seeded cells) for normalization of the data to background plate noise. In the day of the experiment, the incubation media was replaced by unbuffered XF Assay Media supplemented with 25 mM glucose.

The extracellular flux analyzer Seahorse XFe-24 (Agilent Technologies Inc.) was used to evaluate the oxygen consumption rate (OCR), as a measure of mitochondrial respiration rates and extracellular acidification rate (ECAR), the indirect measure of glycolysis, as previously described [17]. Analysis of mitochondrial function was performed in the presence of the classic modulators of the electron transport chain (ETC): oligomycin (1 μg/mL), FCCP (3 μM), and antimycin A+rotenone (5 μM each). The following OCR parameters were calculated: *Basal respiration* – OCR measured before starting the first automatic injection; *Proton leak* – the level of O_2 used to maintain the proton gradient, calculated as the difference in OCR after oligomycin and antimycin A+rotenone injections; *ATP turnover* - the amount of O_2 consumption needed for ATP production, calculated as the difference between basal respiration and proton leak; *Maximal respiration* - OCR measured after FCCP injection (i.e., the uncoupled respiration); *Reserve capacity* - the difference between maximal and basal respiration; *Non-mitochondrial respiration* - the amount of O_2 not consumed by mitochondrial process, measured after antimycin A+rotenone injection. For further data analysis, OCR parameters were corrected to instrumental background and non-mitochondrial respiration. OCR was reported in units of pmoles/minute and ECAR in mpH/min.

Reagents

Polyurethane structures containing EU were synthesized by our group of research from the Department of Analytical Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy Victor Babes Timisoara. Hexamethylene-diisocyanate, polyethylene glycol, MHH≈200, acetone and Tween®20 were purchased from Merck (USA), while ethylene glycol was purchased from Lach-Ner (Czech Rep.). XF Assay Media and XF Calibrant were provided by Agilent Technologies Inc. (USA). All the other chemicals were from Sigma-Aldrich.

Statistical analysis

Data are presented as mean ± SEM. Group comparisons were performed by one-way analysis of variance (ANOVA) and Dunnett's post-hoc multiple comparison test (GraphPad Prism version 5.0). Values for $p < 0.05$ were considered statistically significant.

Results and discussion

Cell morphology

In the present study, the morphology of SCC-4 human squamous cell carcinoma cells was evaluated at 24 h post stimulation with EU. Since our research group recently synthesized safety polyurethane structures containing EU in order to increase its therapeutic time of action [18], cells were randomized in 4 groups:

-The control group - untreated cells (CTRL)

-The DMSO group - cells treated with 50 μM DMSO, which was used to prepare the EU stock solutions (DMSO)

-the group treated with free EU, 50 μM (EU)

-The group treated with EU incorporated in polyurethane structures, 50μM (P)

In figure 1 are depicted the changes elicited by different compounds on cell morphology.

SCC-4 cells stimulated with DMSO did not show significant changes as compared to the untreated cells, displaying a normal epithelial-like morphology (fig. 1 a, b). Treatment with free EU elicited an alteration in tumor cells morphology (some cells changed to round shape and started to detach from the plate) and also reduced the number of viable cells (fig. 1 c), whereas, the cells stimulated with polyurethane structures containing EU (P group) showed minor changes and only fewer cells detached from the plate (fig. 1 d).

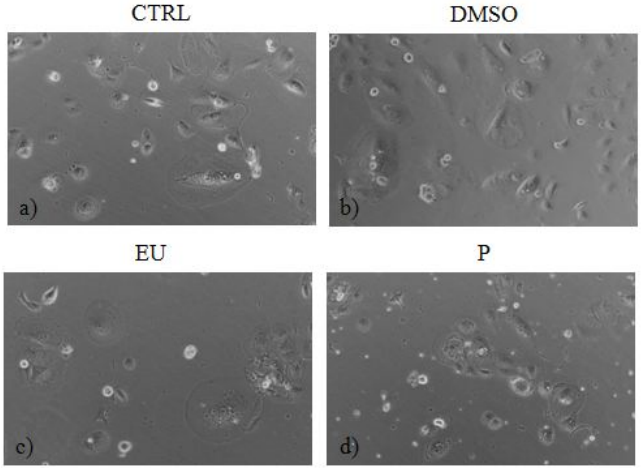

Fig. 1. Morphological aspects of SCC-4 malignant cells: a) CTRL (untreated cells); b) DMSO (cells stimulated with 50 μM DMSO); c) EU (cells stimulated with50 ìM free eugenol); d) P (cells stimulated with 50 μM eugenol incorporated in polyurethane structures). Images were taken at 24h post-stimulation (10x magnification)

Extracellular flux analysis

The main aim of this work was to evaluate the bioenergetic profile of SCC-4 human squamous cell carcinoma cells in response to EU (free or incorporated in polyurethane structures). Typical results of cells treated for 24 h with the mentioned compounds are presented in figure 2 and 3.

We firstly evaluated the bioenergetic profile of SCC-4 cell line according to the automatic injections that were performed. Thereby, the first compound used to challenge the metabolic activity was oligomycin (i.e., the inhibitor of

the mitochondrial ATP synthase binding to the F0 subunit, necessary for the ATP production from ADP) [19]. As a result of the ATP synthase *switch off*, we identified a decrease of OCR in all studied groups as compared to the corresponding basal states (fig. 2) and an increase in ECAR (*data not shown*), which demonstrates the switch of cell metabolism towards glycolysis, also identified in the case of tumour cells [20]. Following the blockage of ATP generation, FCCP was subsequently injected (a classic uncoupler of oxidative phosphorylation), which induces a disruption of the mitochondrial membrane potential where no ATP is produced and energy and oxygen are consumed. Physiologically, this is enhancing OCR via the uncoupling mechanism, and also the glycolysis, expressed by an increased ECAR [20]. In our study, the maximal respiration was lower than the corresponding basal respiration rates in all analyzed groups of cells (fig. 2). The rational for this finding is due to the *Warburg effect*, which states a preferential shift in cancer cells from oxidative phosphorylation to glycolysis with high lactate generation in the presence of oxygen [21], an observation also identified in our previous experiments on bioenergetic profile of malignant cells [22]. The final injection with antimycin A+rotenone (i.e., the classic inhibitors of mitochondrial complex III and I, respectively) induced an abrupt decrease of OCR, but not of ECAR (*data not shown*), since cells shifted to a glycolysis, in order to maintain their energy balance [23].

Secondly we evaluated the effects of EU (in free or incorporated in plyurethane structures) and we found that 50 μM free EU induced a significant decrease of basal respiration, ATP turnover, maximal respiration, and reserve capacity of SCC-4 cells *vs.* controls. On the contrary, all OCR linked parameters were increased in cells stimulated with EU incorporated in polyurethane structures (fig. 2). An important parameter in cell bioenergetics is the reserve capacity, which reflects the ability of cells to operate to its bioenergetic limit [20]. In our hands, since the maximal respiration was lower than basal state, the calculated reserve capacity presented negative values (fig. 2). Nevertheless, our data showed that free EU induced a significant decreased capacity to adapt to stress as compared to controls (fig. 2). On contrary, cells treated with EU incorporated in polyurethane structures induced an increased level of reserve capacity *vs.* controls (fig. 2).

We further evaluated the EU effect on glycolysis and we found a significant increase of ECAR *vs.* controls, when cells were treated with free EU (fig. 3). Conversely, when EU was incorporated in polyurethane structures, it induced a significant decrease of ECAR as compared to untreated cells (fig. 2).

As expected, DMSO (the EU vehicle used at a constant final concentration of 50 μM throughout the experiments) did not influence the bioenergetic profile of studied cells (fig. 2, 3).

Our data are suggestive for an anticancer potential of EU, results which are in line with two studies which demonstrated that EU induces ROS-mediated mitochondrial permeability transition and resultant cytochrome c release in HL-60 cells [24], and an uncoupling effect induced by EU via an increase in F_0F_1 ATPase activity on isolated rat liver mitochondria [25]. Moreover, Wafai et al., recently demonstrated that EU induced a dose-dependent decrease in viability and proliferation of MCF-7 cells, an increase in reactive oxygen species, a decrease in ATP level and mitochondrial membrane potential, and a release of cytochrome-c and lactate dehydrogenase in the culture media [26].

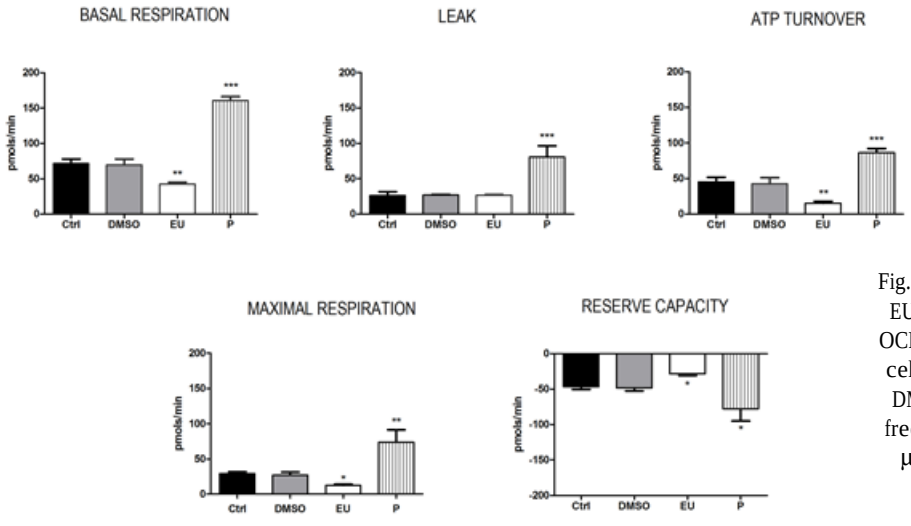

Fig. 2. The effects of 24-h treatment with EU and EU incorporated in polyurethane structure on OCR linked parameters. CTRL group: untreated cells; DMSO group: cells stimulated with 50μM DMSO; EU group: cells stimulated with 50 μM free eugenol; P group: cells stimulated with 50 μM eugeneol incorporated in polyurethane structures. (n = 6–8/group. *p < 0.05 *vs.* untreated controls).

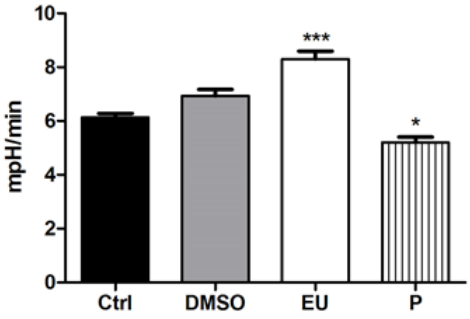

Fig. 3. The effects of 24-h treatment with free EU and EU incorporated in polyurethane structure on ECAR. (n = 6–8/group. *p < 0.05 *vs.* untreated controls)

Conclusions

In conclusion, in the present study we have shown that the *in vitro* treatment of SCC-4 human squamous cell carcinoma cell line with free eugenol exhibits inhibitory effects on mitochondrial respiration simultaneously with a switch to the glcolytic state, effects that were abolished by its incorporation in polyurethane structures. Accordingly, eugenol effects or type of adminstrations are clearly far from being elucidated, which is a rational for further investigations regarding its potential use as chemotherapeutic agent.

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