Flow-Cytometric investigation of Cellular Metabolism during Oxidative Stress and the Effect of Tocopherol

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Abstract: Many studies and scientific publications report on potentially beneficial effects of the lipophilic antioxidant vitamin E on cellular metabolic pathways. The present work presents data on the influence of tocopherol on different intracellular parameters of intact and living human skin fibroblasts by flow-cytometric measurements. The parameters analysed were the intracellular pH, representing cell metabolism and cell function, intracellular glutathione, representing one of the cell's own radical scavenger enzyme systems, membrane potential and cell viability. In order to cause large numbers of free radicals cells were UVB-irradiated prior to measurement. The results of the flow-cytometric measurements indicate that vitamin E has significant protecting effects on the measured biochemical parameters during oxidative stress. In the presence of the lipophilic radical scavenger a significant stabilizing effect on pH, intracellular glutathion levels and membrane potential could be observed. Furthermore, vitamin E administration was associated with increased cell viability after UVB irradiation.

Key words: Vitamin E, flow cytometry, human skin fibroblasts, cellular viability, intracellular pH, intracellular glutathione, membrane potential, UVB

Introduction

At present, many epidemiological and intervention studies report on the protective effect of the lipophilic antioxidant vitamin E against pathophysiological alterations on metabolism, such as arteriosclerosis, cataract, cancer or signals of pre-ageing and other degenerative processes in general. Nevertheless information on the exact mode of action within the biochemical pathways of the cell is still poor and often based on in-vitro experiments with lysed cell material which may cause unpredictable changes in the cellular biochemistry. The purpose of this work consists in measuring the vitamin E influence on different biochemical parameters within intact and living human cells as a modell for topical application. All investigations were carried out with a primary cell line of human skin fibroblasts. The parameters analysed were the intracellular pH, representing cell metabolism and cell function, intracellular glutathione, one of the cell's own antioxidant defending system, membrane potential, representing cell ac-

tivity and finally cellular viability. These biochemical parameters were determined by a non invasive method called flow-cytometry. This method is based on staining monodispersed cell suspensions with fluorescent dyes, which are characteristic for certain cell structures or cell functions. Fluorescent intensity and the simultaneously measured electrical cell volume allow the calculation of the exact intracellular dye concentration and enable the quantitative determination of each parameter. With this method effects of vitamin E are measurable under physiological conditions, where vitamin E is allowed to cooperate with all its usual antioxidative partners. Considering the mode of action of vitamin E as free radical scavenger, the skin fibroblasts were UVB-irradiated in order to cause oxidative stress by a large number of free radicals within the cell system. Treatment with dl-α-tocopheryl-acetate took place in advance to study the preventive effect of the antioxidant.

Materials and Methods

Cell material and cell preparation: Primary human skin fibroblasts of praeputio origin were used in all experiments. Cells were cultured in six different petridishes ($\varnothing$ 9 cm) at 37°C with 5% CO_2 for three days in DMEM/ Nutrient Mix F12 (1:1) with L-Glutamin and HEPES (Gibco BRL, CH-Basel), 10% fetal calf serum (Gibco BRL, CH-Basel), 2% L-glutamine 200 mM (Sigma, CH-Buchs), 1% antibiotic-antimycotic-solution (Gibco BRL, CH-Basel) consisting of 10000 μg/ml penicilline G-sodium, 10000 μg/ml streptomycine-sulfate and 25 μg/ml amphotericine B. As a further antimycoticum 0.5% of nystatine 10000 μg/ml (Gibco BRL, CH-Basel) was added.

After three days the cell monolayer reached confluence and two of the six dishes were treated for 18.5 hours with 15 ml of cell culture medium containing 1.64% dl-α-tocopheryl-acetate, corresponding to 1.5% dl-α-tocopherol (F. Hoffmann-La Roche, CH-Basel) solubilised in cell culture medium with 1% lutrol F127 (BASF, D-Ludwigshafen). The solubilization procedure is described below. In parallel another two dishes were treated with 15 ml of cell culture medium containing 1% lutrol F127 in order to study the influence of the solubilizer. The last two dishes were treated only with 15 ml of cell culture medium.

After this incubation procedure one petridish of each sample was UVB-irradiated (105 min., 312 nm, 1.78 J/cm² [UVP Inc. San Gabriel, CA 91778 USA]). Each second dish served as a non-irradiated control. After UVB-irradiation cells were trypsinated for 12 min. (trypsine/EDTA 0.5%/0.2%, Gibco BRL) and trypsine was inactivated by

15 ml cell culture medium and 35 ml 10 mM HEPES buffered saline (HBS ph 7.4). After centrifugation (1200 rpm = 720 g, 4°C), cell pellets were resuspended in 30 ml of HBS (→ = cell suspension with 10⁴ cells/ml for flow-cytometric measurements).

Solubilization of dl-α-tocopheryl-acetate: Mixture A: 1000 mg lutrol F127 were dissolved in 19 ml cell culture medium.

Mixture B: 494.1 mg dl-α-tocopheryl-acetate (F. Hoffmann-La Roche, CH-Basel) were stirred during 6 hours at 1200 rpm on a magnetic plate at 40°C in 5.93 g of mixture A and 23.58 g of cell culture medium to get a final concentration of 1.64% dl-α-tocopheryl-acetate and 1% lutrol F127 in the solubilizate. During the solubilization procedure the vessel was light protected with aluminum foil. The solubilizate was then UV-analysed at 284 nm to determine the final tocopherol concentration. The droplet size of the micelles was determined with a photon-correlation-spectrometer (Malvern-Instruments Ltd., England, Range 3-3000 nm). For solubilization of 3% of dl-α-tocopherol the amounts of tocopherol and lutrol F127 were doubled.

Cell staining

Viability and pH (ADB/PI-staining) [1, 2]: 500 μl cell suspension were incubated with 10 μl of a dye cocktail, consisting of 1 mg/ml 1,4-diacetoxy-2,3-dicyanobenzene (= ADB, Calbiochem Art. Nr. 266707) indicating the pH in living cells and 2 mg/ml propidiumiodide (PI, Sigma, CH-Buchs) binding to DNA-structures of dead cells. Both dyes were dissolved in dimethylformamide (= DMF, Fluka CH-Buchs). Staining occurred for 5 minutes at room temperature. All flow-cytometric measurements were standardized by adding monosized latex particles (2.5× 10⁵/ml) that had a well defined fluorescence.

Glutathione (OPT/OPTH-staining) [3]: 500 μl cell suspension were incubated with 10 μl each of two dyes: 6.70 mg/ml ortho-phthaldialdehyd (= OPT, Sigma, CH-Buchs) in dimethylformamide to determine simultaneously intracellular glutathione by fluorescence light emission between 390–440 nm and free protein SH-groups (FP-SH) between 500–750 nm as well as 2 mg/ml propidiumiodide in HBS to unequivolly identify dead cells as separate cell clusters by their comparatively increased red stain in the 500–700 nm light emission channel of the flow cytometer. The cell suspension was stained for 10 min. at 0°C.

In order to eliminate interference of non-specific OPT fluorescence, it is necessary to use 10 μl of 27.15 mg/ml mercury-dichloride (= $HgCl_2$, Sigma, CH-Buchs) in HBS as thiol-blocking agent to reduce the OPT-specific fluo-

rescence to zero [3]. This so-called OPTH-staining which lasts 5 minutes at 0°C is followed by a OPT- and PI-staining as described above. Subtraction of the OPTH-fluorescence from the OPT-fluorescence leads to the desired OPT/glutathione-fraction.

Membrane potential (DIOC6-staining) [4]: 500 µl cell suspension were incubated with 10 µl of a dye cocktail, consisting of 10 µg/ml 3,3'-dihexyl-oxacarbocyanine-iodide (= DIOC6, Eastman Kodak, CH-Baar) as indicator for the transmembrane potential as well as 2 mg/ml propidiumiodide (= PI, Sigma, CH-Buchs) to stain the DNA of dead cells. Both dyes were dissolved in dimethylformamide (= DMF, Fluka, CH-Buchs). Incubation conditions were 5 min. at room temperature. Monosized latex particles were used as internal standard.

Flow-cytometry

After staining, cells were measured in a flow cytometer (PAS III, Partec GmbH, D-Münster). The fluorescence of the dyes was excited with a HBO-100 high pressure mercury lamp and collected in two different emission channels F1 and F2.

Table I: Different fluorescent dyes with their wavelengths (nm) of excitation (Ex.) and emissions (Em.)

Dye	Exc.	Em. F1	Em. F2
ADB	307–383	422–438	513–750
OPT	307–383	422–438	513–750
DIOC6	328–477	516–547	587–750

According to the Coulter principle [5, 6] the cell volume was determined electrically in a hydro-dynamically focused cylindrical orifice of 100 µm diameter. This means that every cell was simultaneously characterized by three signals: two fluorescences F1, F2 and the accompanying electrical cell volume. The measured signals were amplified by a 3-decade amplifier and collected on line for the following evaluating step by the Classif 1 program [1, 7].

Intracellular α-Tocopherol

Intracellular α-Tocopherol has been measured by HPLC according to Hess et al [8]. In tocopheryl-acetate treated cells an average amount of 0.97 µg free tocopherol / 10^6 cells was found. In untreated cells the amount of tocopherol was below the detection limits.

Results and Discussion

All six differently pretreated cell monolayers were analysed for their intracellular pH, intracellular glutathione (= GSH), transmembrane potential and viability.

Reproducibility of every experiment was tested 4–6 times before calculating mean and standard deviation (s_x). All data refer to untreated, non-irradiated control cells (bar nr. 1 = 100%). In order to discover not only single effects of the factors like pretreatment, UVB-irradiation and cell passage, but also interactions between the different factors the data were analysed by three-dimensional analysis of variance (= Anova) which also allows testing of significance.

Intracellular pH

Several cellular regulation mechanisms keep the intracellular pH in a narrow physiological range. Therefore, already small shifts of pH values signalize changes in cellular metabolism and cell function. The ratio of blue to green fluorescence of the ADB-staining procedure (F1/F2) is a measure of intracellular pH [1, 2]. Reproducibility of the staining method is guaranteed with a relative standard deviation (s_{rel}) of 0.7%. Figure 1 shows that UVB-irradiation provokes a shift to lower intracellular pH, that could be prevented by 1.5% vitamin E.

The statistical evaluation shows a significant influence of both factors, vitamin-pretreatment and UVB-irradiation. Obviously there exists an interaction between these two factors confirming the common opinion, that vitamin
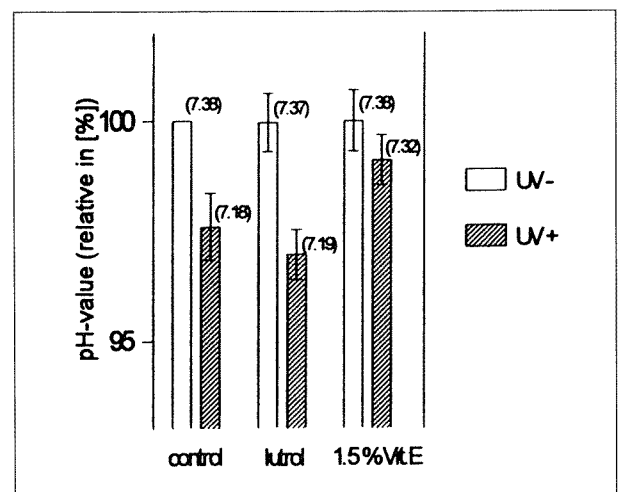
Figure 1: Intracellular pH (ADB-staining) as indicator of cell metabolism and cell function; UVB-irradiated, tocopherol-acetate treated cells (bar nr. 6) show a significant stabilization of the intracellular pH compared to the irradiated cells of the untreated control (bar nr. 2) and the irradiated lutrol treated cells (bar nr. 4). (Data of the absolute pH are shown in brackets).

Table II: Analysis of variance for the intracellular pH in primary human skin fibroblasts

Factor	F-Ratio	Prob > F
Treatment	12.2970	0.0002
UVB-irradiation	110.9380	0.0000
Passage	2.4320	0.1084
Treatment × UVB	11.3107	0.0003
Treatment × pass	0.6533	0.6300
UVB × passage	0.4174	0.6633

Table III: Analysis of variance for the intracellular glutathione in primary human skin fibroblasts

Factor	F-Ratio	Prob > F
Treatment	9.3700	0.0018
UVB-irradiation	83.3056	0.0000
Passage	3.5839	0.0503
Treatment × UVB	18.9076	0.0000
Treatment × pass	1.3711	0.2854
UVB × passage	0.8185	0.6633

E – as free radical scavenger – shows its protective effect especially in presence of oxidative stress existing after UVB-irradiation of the cells. In non-irradiated cells no influence of the different treatments could be detected.

(The results are independent of the cell passages [10th–12th passage] used.)

Intracellular glutathione

Intracellular glutathione as well as FP-SH are measured by the OPT-FP-SH are measured by the OPT-staining procedure, whereas the OPTH-staining represents the non-specific fluorescence in the OPT and FP-SH light channels. Substraction of the OPTH-fluorescence from the OPT-value leads to the desired glutathione fraction [3]. Reproducibility of this staining method is guaranteed with a relative standard deviation (s_{rel}) of 2.8%.

The statistical evaluation of Figure 2 shows a significant effect of the vitamin E-treatment (p < 0.0018), the
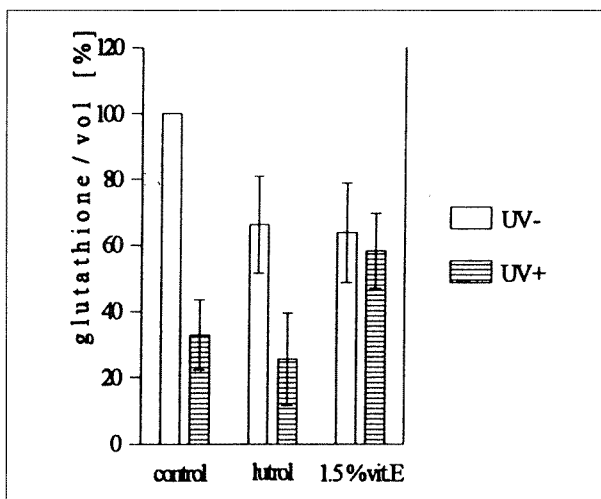
Figure 2: This figure shows a strong decrease in intracellular glutathione (= OPT-resp. OPTH-staining) in UVB-irradiated cells with and without lutrol treatment. In contrast, vitamin E-treated, irradiated cells show significant higher glutathione concentrations than the controle and lutrol-treated cells, which indicates a protective effect of vitamin E on the intracellular glutathione level during oxidative stress.

UVB-irradiation (p < 0.0001) and the interaction of these two factors (p < 0.0001). Thus, in agreement with the results of the intracellular pH, intracellular glutathione is protected by vitamin E only in presence of free radicals, that is during oxidative stress of the skin fibroblasts. Non-irradiated cells show no effect of vitamin E, emphasizing the mode of action of the antioxidant as free radical scavenger.

(The results were not influenced by the used cell passages [12th–14th passage].)

Membrane potential

Another interesting biochemical parameter to look at, is the cellular membrane potential, which depends on cell activity as well as the arrangement of the membrane structures. Alterations of these structures – caused by oxidation of the polyunsaturated fatty acids of the membraneous lipids or by free radical damage of the membrane proteins – lead to a change in cell permeability with an alteration in membrane potential. Vitamin E, as lipophilic antioxidant, is known to interact with cellular membranes, so that a protecting effect on membrane structures could be expected.

As shown in Figure 3, the membrane potential of the control as well as the lutrol-treated cells decreased significantly during UVB-irradiation, whereas in the presence of 1.5% vitamin E a complete stabilization of the cellular membrane during oxidative stress is observed. It should be pointed out, that the solubilizer lutrol F127 itself shows certain stabilizing properties. However, lutrol is not able to prevent the loss of the membrane potential during UVB-irradiation, which means that the results with vitamin E (solubilised with lutrol) are in fact caused by the antioxidant and not by lutrol. The DIOC6-staining showed a s_{rel} of 3.8%.

Summarizing the results from these three biochemical parameters pH, glutathione and membrane potential, vitamin E showed a significant stabilizing effect on the UVB-stressed skin fibroblasts. The relationship between these effects and the cellular viability was determined with the ADB/PI-staining (Fig. 4).
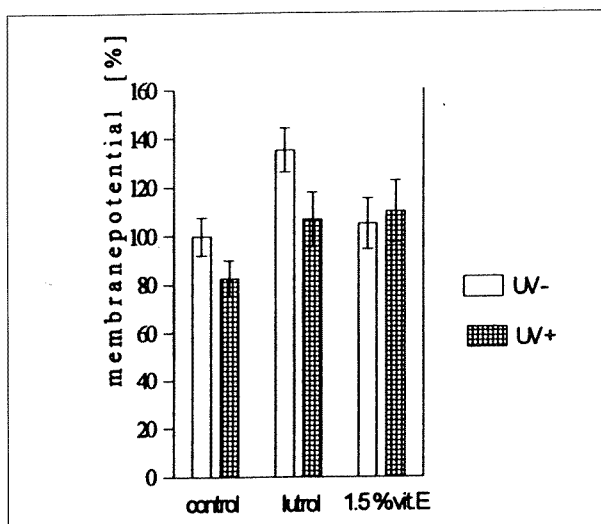
Figure 3: Cellular membrane stabilization by 1.5% vitamin E during oxidative stress due to UVB-irradiation.

Table IV: Analysis of variance for the cellular membrane potential in primary human skin fibroblasts

Factor	F-Ratio	Prob > F
Treatment	18.8245	0.0000
UVB-irradiation	11.0347	0.0032
Passage	0.3523	0.7879
Treatment × UVB	5.9313	0.0091
UVB × passage	0.1926	0.9003

Figure 4: Cellular viability cannot be significantly increased by 1.5% vitamin E during oxidative stress due to UVB-irradiation.

Table V: Analysis of variance for the cellular viability of primary human skin fibroblasts under a treatment with 1.5% vitamin E

Factor	F-Ratio	Prob > F
Treatment	0.3918	0.6795
UVB-irradiation	31.7397	0.0000
Passage	1.3774	0.2688
Treatment × UVB	1.3241	0.2822
Treatment × pass	0.5544	0.6975
UVB × passage	0.4111	0.6668

Cellular viability

The fluorescent dye ADB only permeates into living cells whereas propidiumiodide (PI) is highly specific for DNA structures of dead cells; therefore, this staining procedure allows a discrimination between dead and living cells [1, 2].

Figure 4 illustrates, that all non-irradiated cells in general (= white bars) show a very high viability of at least 96%, which means that neither lutrol nor vitamin E have toxic effects on the cells. In contrast, UVB-irradiation of the control as well as lutrol treatment of the cells lead to a significantly decreased viability due to the free radical damage. Although Vitamin E treatment shows the smallest loss of viability during UVB influence, the results demonstrate no significant protection compared with the control and the lutrol experiments (see Anova Fig. 4).

In conclusion, the results of all flow-cytometric measurements indicate that vitamin E has significant protecting effects on metabolic processes reflected by intracellular glutathione, intracellular pH and the cellular viability, particularly when the cells have been submitted to increased 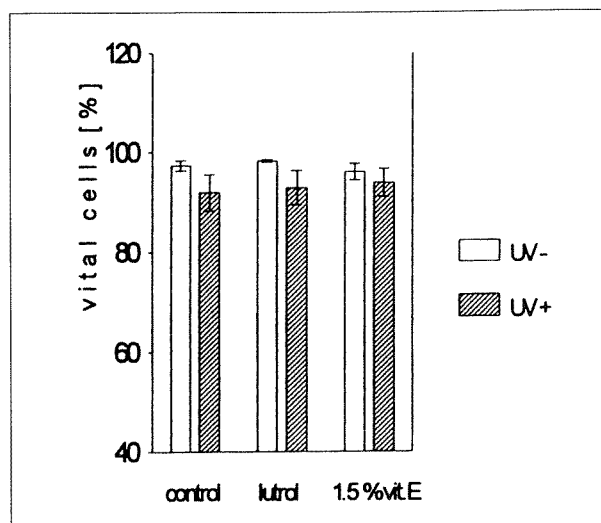oxidative stress by UVB-light. This can easily be explained by its mode of action as free radical scavenger. Based on these experiments not only can it be assumed that vitamin E protects skin fibroblasts of oxidative stress but can also be expected that Vitamin E could prevent preaging effects or malignomas due to UVB-influence in human skin.

Acknowledgements

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