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Characterization of α-Terpineol as an Anti-inflammatory Component of Orange Juice by in Vitro Studies Using Oral Buccal Cells

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Epithelial buccal cells (KB) were exposed to orange juice or orange juice fractions containing either the dry matter (DM), the volatile compounds (aqueous distillate, AD), or individual nonvolatile or volatile components. Intracellular formation of the pro-inflammatory cytokine IL-6 was analyzed by flow cytometry. Exposure to whole orange juice resulted in an increase in IL-6 formation of 23% compared to nontreated control cells, whereas treatment of the cells with either DM or AD resulted in a 22 or 1% increase, respectively. Dose–response experiments revealed that exposure of the cells to a 2- or 4-fold concentrated AD resulted in an increased IL-6 formation, whereas an inhibiting effect was measured after treatment of the cells with an 8-fold concentrated AD. These results indicated the presence of pro- as well as anti-inflammatory compounds in the aqueous distillate. To identify the active principles, volatile compounds present in the AD-treated cells were analyzed by GC-MS. In particular, limonene, linalool, and α-terpineol were shown to be present in significant amounts. Subsequent studies on the IL-6 formation revealed that limonene had a stimulating effect and α-terpineol had an inhibiting effect, whereas linalool had no effect. This anti-inflammatory effect of α-terpineol on IL-6 formation was verified by quantitative real-time reverse transcription Polymerase Chain Reaction experiments in which α-terpineol inhibited the gene expression of the IL-6 receptor.

KEYWORDS: Orange juice; α-terpineol; interleukin-6; epithelial buccal cells; flow cytometry; immune modulatory effect

INTRODUCTION

Due to its high vitamin C content, orange juice is commonly believed to support resistance to common viral infections. In fact, drinking of two glasses (500 mL) of orange juice per day for two weeks has recently been shown to decrease the levels of pro-inflammatory markers in healthy adults (*1*). The active principle of orange juice is widely hypothesized to be ascorbic acid (*1, 2*), although results reported from controlled human trials are conflicting (*3*). This might be due to the fact that systematic, controlled dose–response trials in humans with a standardized nutritional and immunological status are still lacking.

Ascorbic acid and terpenes, such as limonene and perillic acid, are also assumed to act as immune-modulatory agents (*4, 5*). However, for volatile compounds in particular, knowledge about health effects is scarce because appropriate biological test systems suitable for measuring the structure-related bioactivity are missing.

The oral mucosa is the first tissue in contact with undigested, nonmetabolized food after oral ingestion and is, thus, exposed to all of its volatile and nonvolatile components. Moreover, one important feature of the mucosal surfaces is their function as a barrier protecting the underlying tissues from micro-organisms and toxic products. However, the epithelial structure covering these surfaces is also not simply a physical border. It also serves as a selective uptake and transport site for a variety of molecules and is, thus, part of the immune system. Epithelial cells from the oral cavity have been shown to be involved in the early inflammatory signal transduction pathways by producing cytokines in response to different stimuli (*6*). Particularly, the oral mucosa cell line "KB" has been reported to produce a variety of cytokines (*7*). Cytokines are part of the immune system that defends the human body against attacks by bacteria, viruses, and parasites. Besides the adaptive immune reactions involving antigen-specific activity and specific antibody production, the innate immune reactions are nonspecific, consisting of physical and chemical barriers such as skin, gastric acid, mucus, or tears, as well as active mechanisms by cells, for example, phagocytes. Phagocytes have the capacity to directionally extend cellular portions of their plasma membrane, engulfing and overtaking a foreign particle or microorganism. One group of phagocytes are macrophages, which release the pro- and anti-inflammatory cytokines at a potential infection site. Among the pro-inflammatory cytokines released in response to cell or tissue damage, interleukin-6 (IL-6) is the most important mediator of fever and induces the secretion of acute phase response proteins during

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inflammation. In addition, IL-6 is a pathophysiological factor in several hyperproliferative diseases and paraneoplastic syndromes often accompanying cancer (*8*).

Whereas an induction of IL-6 release is considered to be a pro-inflammatory event, its inhibition results in anti-inflammatory effects, which help to reduce inflammatory reactions associated with various infections, including that of the common cold.

So far, no systematic study has been reported on the influence of orange juice and its components on IL-6 formation, although KB cells have been demonstrated to produce IL-6 (*9*).

Therefore, the aim of the present work was to develop a cell culture model using buccal (KB) cells to characterize the immune-modulatory agents of orange juice by applying activity-guided fractionation techniques.

MATERIALS AND METHODS

Chemicals. Unless stated otherwise, reagents and chemicals were purchased from Sigma. Chemicals for quantitative real-time reverse transcription Polymerase Chain Reaction (QRT-PCR analysis) were ordered from Quiagen. Orange juice from concentrate was purchased from a local supermarket and stored in its original packaging at room temperature in the dark prior to use. Linalool, limonene, and α-terpineol were purchased from Fluka.

Carcinoma Buccal (KB) Cell Line. KB (ACC 136) is an epithelial cell line originally described as an epidermoid carcinoma established from the mouth of a Caucasian man in 1954, but recently reported to be a subclone of the human cervix carcinoma cell line HELA (German National Resource Centre for Biological Material). KB cells were maintained in 90% Dulbecco Eagle minimal essential medium with nonessential amino acids and Earle's balanced salt solution supplemented with 10% fetal calf serum, 2% L-glutamine, and 2% penicillin–streptomycin (PAA Laboratories' no. A15-151, M11-004, P11-010,) at 37 °C and 5% CO_2. For each assay, cells were exposed to medium without fetal calf serum at 37 °C and 5% CO_2 for 24 h.

Determination of IL-6 Formation in KB Cells. For the determination of IL-6 formation, KB cells (2×10^6 cells/mL) were exposed to 1 μg/mL brefeldin A (Sigma, no. B-7651). Brefeldin A inhibits the Golgi-mediated cytokine transport, resulting in intracellular cytokine accumulation, which can be detected by flow cytometry (*10, 11*). As a reference, maximum IL-6 production was achieved by treatment of the cells with 10 ng/mL phorbol-12-myristate-13-acetate (PMA) and 1 μg/mL ionomycine (*12, 13*) in a total volume of 1 mL cell culture medium at 37 °C for 0, 3, 6, 9, 12, and 24 h. Each reference assay was performed in six replicates.

For sample treatments ($n \geq 4$), KB cells were exposed at 37 °C for 6 h either to whole orange juice (100 μL plus 900 μL of cell culture medium, corresponding to a 1:10 dilution), dry matter, sugars, organic acids, ascorbic acid, or aqueous distillate according to the quantified content in 100 μL of orange juice. In other sets of experiments, KB cells were exposed to the aqueous distillate (corresponding to 200, 400, or 800 μL of orange juice plus 800, 600, or 200 μL of cell culture medium, respectively), limonene, linalool, and α-terpineol according to the amount present in 800 μL of orange juice (**Figure 1**).

After the incubation period, 2 mL of FACS lysing solution (Becton and Dickinson Biosciences; no. 349202) was added to wash off the sample and to facilitate the subsequent permeabilization. Sample tubes were centrifuged at 500*g* for 5 min. Supernatants were discarded, and sample tubes were incubated with 500 μL of FACS permeabilizing solution 2 (Becton and Dickinson Biosciences; no. 340973) to permeabilize the membranes of the cells. Samples were incubated at room temperature in the dark for 10 min. KB cells were washed with 2 mL of buffer containing 1.2 g of KH_2PO_4/L, 8.22 g of Na_2HPO_4/L, 0.1% sodium azide, and 0.5% bovine serum albumin (PAA Laboratories; no. K41-001-100) and centrifuged at 500*g* for 5 min. The supernatants were discarded. A volume of 20 μL of a specific fluorescent antibody solution against human IL-6 (anti-Hu IL-6 FITC, Becton and Dickinson Biosciences; no. 340526) was added. Samples were mixed and incubated at room temperature in the dark for 30 min. Afterward, cells were washed with 3 mL of buffer and resuspended in 500 μL of FACS cell fix solution (Becton and Dickinson Biosciences). Afterward, a total amount of 10000 KB cells was analyzed by flow cytometry.

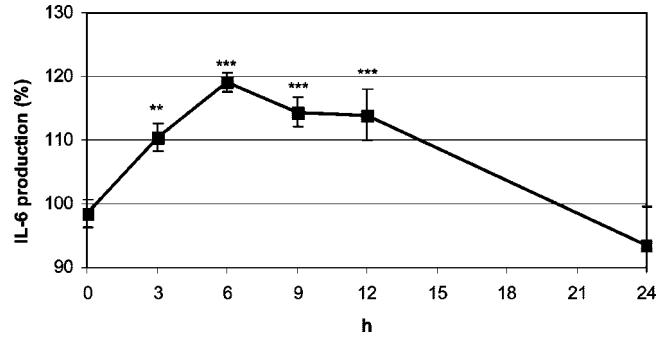

Figure 1. Intracellular production of interleukin-6 in carcinoma buccal (KB) cells after 3, 6, 9, 12, and 24 h of exposure to a mixture of 12-phorbol-13-myristate-acetate and ionomycin ($n = 5$) (control without additions = 100%). Student's *t* test versus nontreated control cells: **, $p < 0.01$; ***, $p < 0.001$.

Fractionation of Orange Juice. For the isolation of the dry matter (DM), orange juice (100 g) was freeze-dried. An aqueous distillate (AD) containing the volatile components of orange juice (100 g) was prepared by using the solvent-assisted flavor evaporation (SAFE) technique (*14*). The yields of both fractions were determined by weight.

Quantitation of Selected Nonvolatile Components. Sucrose, D-glucose, and D-fructose were determined enzymatically by using a test kit from R-biopharm (no. 10716260035). R-biopharm test kits 10139068035, 10139076035, and 10409677035 were used to quantitate L-malic acid, citric acid, and L-ascorbic acid, respectively.

Quantitation of Selected Volatile Compounds. The aqueous distillate was extracted three times with distilled diethyl ether. Organic layers were combined and dried over anhydrous sodium sulfate. After filtration, volatile compounds were concentrated at 42 °C by means of a Vigreux column.

Samples were analyzed by high-resolution gas chromatography (HRGC) and mass spectrometry. Important compounds were identified by comparing the retention indices and mass spectra with those of reference compounds. For quantitation, *p*-menth-1-ene was added as internal standard prior to sample extraction. Compounds were quantified by calculation of FID response factors and recovery studies (Maier and Schieberle, unpublished results).

Recovery of Volatiles in KB Cells. KB cells were cultured until confluency and incubated with a mixture of cell culture medium and AD for 6 h. The resulting concentration corresponded to that of 800 μL of orange juice. Also, KB cells were exposed to cell medium without any additions.

After 6 h, exponates were aspirated and collected. The cells were washed twice with 10 mL of phosphate buffer solution (PAA Laboratories; no. K41-001-100) and aspirated. Washing solutions were added to the particular exponates. Thereafter, KB cells were lysed with 2 mL of lysis buffer (20 mM HEPES buffer; PAA Laboratories; 150 mM sodium chloride, 5 mM EDTA, 0.5% Triton X-100, 1.5 mM magnesium chloride, 10 mM potassium chloride, 1 mM sodium vanadate, 10 mM sodium fluoride, and 100 mL of bidistilled water).

Lysates were aspirated and combined. To isolate the cell lysate, the suspension was centrifuged at 15000*g* for 1 h and divided into the pellet and lysate. Exponates, pellets, and lysates were extracted with diethyl ether and distilled by means of the SAFE technique (*14*). Identification and quantification of the target compounds were performed as described above.

Flow Cytometry. Measurement of the intracellular IL-6 content was performed by using a Fluorescence Activated Cell Sorter (FACS) (Calibur Flow Cytometer, Becton and Dickinson Biosciences, Heidelberg, Germany). A beam of an argon laser of 488 nm frequency is directed onto a hydrodynamically focused stream of fluid. Each suspended single cell passes through the beam and scatters the light. Cells can be characterized by the forward scatter (FSC) in size and by the sideward scatter (SSC) in granularity. Cells labeled with the specific

fluorescent antibody against human IL-6 (anti-Hu IL-6 FITC) can be detected by one of the four fluorescent detectors (FL-1 $\lambda = 530$ nm). The intensity of the detected emission light is proportional to the intracellular IL-6 content. Per single measurement, 10000 cells were measured, and the concentration of IL-6 was determined for each control and treated cell. Results are expressed as percent relative to values obtained from nontreated control cells (nontreated cells = 100%). Linearity of the measurements was $r^2 = 0.9999$ for dilutions of the dry matter fraction ranging from 1:1 to 1:10. Day-to-day and day-within-day precisions were below 5 and 3%, respectively.

High-Resolution Gas Chromatography (HRGC). Identification of compounds was performed using a gas chromatograph type 8160 (Fisons Instruments, Mainz, Germany) equipped with a fused silica capillary column, FFAP, 30 m × 0.32 mm, film thickness = 0.25 μm (Phenomenex, Torrance, CA). The samples were injected at 40 °C. The oven was held at this temperature for 10 min and then raised at 6 °C/min to 240 °C. A flame ionization detector (FID) was used. Retention indices (RI) were calculated from the retention times of *n*-alkanes by linear interpolation (*15*).

For identification, mass spectra were generated by means of a mass spectrometer MAT 95S (Finnigan, Bremen, Germany) at 70 eV in the electron impact mode (MS-EI) and at 115 eV in the chemical ionization mode (MS-CI; reagent gas, isobutane) using the capillaries and oven programs mentioned above.

Influence of Selected Volatile Compounds on the Gene Expression of the IL-6 Receptor. Sample treatment was performed as described above. After 6 h of exposure, cells were washed with 4 °C phosphate buffer solution, and RNA was isolated using RNeasy MiniKit (Qiagen). Thereafter, RNA samples were quantitated by photometric methods, and cDNA was synthesized. For all cDNA samples, a QRT-PCR of 10 protein genes, including GAP-DH, β-actin, β-tubulin, PPIA, ACTB, YMHAZ, TBP, RPLP, GUSB, HPRT1, and UBC, was performed. β-Actin was used as the housekeeping gene as its expression was not influenced by either of the samples (data not shown). Therefore, amplification of the IL-6 receptor gene was normalized to β-actin gene amplification.

Statistical Analysis. The unpaired Student's *t* test was used to analyze constitutive and stimulated/inhibited intracellular IL-6 production in KB cells. Significant differences were defined as having a *p* value of at least <0.05. Data are presented as mean values ± standard deviation.

RESULTS AND DISCUSSION

For the characterization of anti-inflammatory compounds in orange juice, first, a screening model for compounds inhibiting the intracellular formation of the pro-inflammatory cytokine IL-6 in KB cells had to be developed. Formation of intracellular IL-6 was measured by flow cytometry. Compared to ELISA techniques, which are also widely applied for this purpose, flow cytometry analysis offers the advantage of very precisely measuring single-cell effects with a high number of technical replicates, as many thousands of cells can be measured in a single run.

Because it was necessary to exclude cytotoxic effects, the influence of orange juice on the viability of KB cells was tested in a preliminary experiment. Up to concentrations of 100 μL/mL (10% dilution) and an exposure time of 6 h, the viability of the orange juice treated cells did not significantly differ from nontreated cells (data not shown), whereas higher concentrations and longer treatment resulted in a significant decrease of living cells.

Next, a screening model for the inhibition of the intracellular IL-6 formation in KB cells was developed, based on the maximum stimulation mediated by a combination of the well-known IL-6 inducers phorbol-12-myristate-13-acetate (PMA) and ionomycine (*12, 13*). Cells were exposed to a mixture of both mediators for up to 24 h in a preliminary experiment, and the resulting IL-6 formation, measured by flow cytometry, reached a maximum of 19% higher than the control after 6 h of exposure (**Figure 1**).

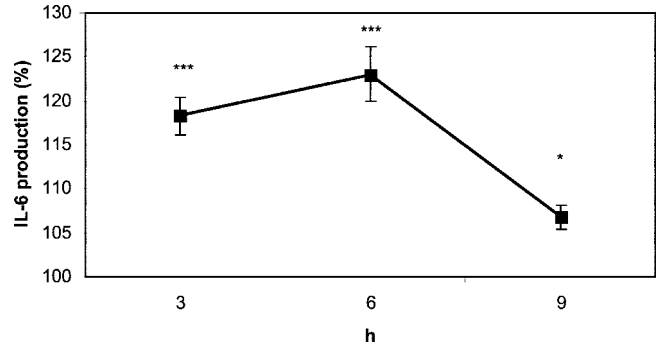

Figure 2. Intracellular production of interleukin-6 in carcinoma buccal (KB) cells after 3, 6, and 9 h of exposure to 1:10 diluted orange juice ($n = 5$) (control without additions = 100%). Student's *t* test versus nontreated control cells: *, $p < 0.05$; ***, $p < 0.001$.

Table 1. Concentration of Selected Compounds in Orange Juice

compound	concn[a] (mg/100 g)
ascorbic acid	34 ± 1
citric acid	862 ± 4
L-malic acid	188 ± 5
sucrose	3520 ± 11
D-glucose	2514 ± 13
D-fructose	2436 ± 17

[a] Data are mean values of triplicates ± standard deviation.

The effect of orange juice on IL-6 formation in KB cells was then tested by applying orange juice in a concentration of 100 μL plus 900 μL of cell culture medium (1:10 dilution) for up to 9 h. The results (**Figure 2**) showed a maximum of IL-6 formation (22% higher than the control) after 6 h of exposure. However, at any time, IL-6 contents were above those of nontreated control cells, thereby indicating a pro-inflammatory effect of orange juice in KB cells.

The following experiments were, therefore, aimed at identifying the pro-inflammatory orange juice components. In these settings, orange juice was always applied to the cells in a dilution of 1:10 for 6 h.

To identify the most active orange juice components responsible for the increase in IL-6 formation in KB cells, orange juice was fractionated into an AD, containing the volatiles, and the DM. The yields of both fractions isolated from 100 g of orange juice were 87 g (AD = water + volatiles) and 11 g (DM), respectively.

Treatment of KB cells with 1:10 diluted orange juice for 6 h resulted in an increase of IL-6 formation of 23%, whereas the AD (concentration according to 10% orange juice) did not induce the IL-6 formation at a level of statistical significance compared to the nontreated control cells (**Figure 3**). In contrast, treatment with either the DM or the combination of DM and AD (concentrations according to 1:10 diluted orange juice) resulted in IL-6 increases of 22 and 23%, respectively. These results clearly demonstrated that components present in the dry matter of orange juice are responsible for its IL-6 enhancing effect. To identify the active components on IL-6 production, selected compounds were quantified and applied to the cells in concentrations corresponding to 1:10 diluted orange juice. The amounts quantified (**Table 1**) were well in line with published data on orange juice constituents (*16*). None of the quantified compounds affected the viability of KB cells significantly when used in concentrations corresponding to 1:10 diluted orange juice

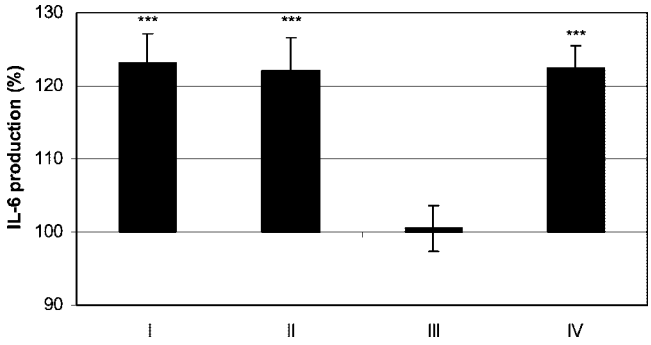

Figure 3. Intracellular production of interleukin-6 in carcinoma buccal (KB) cells after 6 h of exposure to 10% orange juice and to fractions isolated thereof: I, 1:10 diluted orange juice; II, dry matter; III, aqueous distillate; IV, dry matter + aqueous distillate ($n = 12$; control without additions = 100%). Student's t test versus nontreated control cells: ***, $p < 0.001$.

Table 2. Percent Increase of Intracellular Production of Interleukin-6 in Carcinoma Buccal Cells after 6 h of Exposure to Orange Juice Constituents

sample	increase[a] (%)
dry matter	22 ± 5***
glucose, fructose, sucrose	10 ± 4*
citric acid, malic acid	11 ± 4*
ascorbic acid	1 ± 4
recombinate of all constituents	22 ± 3***

[a] Data ($n = 9$) are mean values ± standard deviation; Student's t test vs nontreated control cells: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

(data not shown). To investigate the effect on IL-6 formation, cells were treated with either a mixture of sucrose, fructose, and glucose or a mixture of citric and malic acid, whereas ascorbic acid was applied singly. Again, all compounds were applied in "natural concentrations" according to their quantified amounts in 1:10 diluted orange juice. Treatment with either the sugar or the organic acid mixture resulted in clear increases in IL-6 to about 10 and 11%, respectively (**Table 2**). The effect of the organic acids was not based on a change in pH, because control cells exposed to a cell culture medium that was adjusted to the same pH as the medium with the mixture of acids (pH 4.2) did not show any increase in IL-6 formation compared to the nontreated control cells exposed to a cell culture medium at pH 7.2 (data not shown).

IL-6 contents of cells treated with ascorbic acid (250 μM) also did not differ from nontreated control cells. Therefore, no anti-inflammatory effect on IL-6 formation was found for ascorbic acid. In contrast, anti-inflammatory effects for ascorbic acid had earlier been reported in the literature (*2, 17*). However, a decreasing effect of ascorbic acid on IL-6-producing human monocytes has been reported for ascorbic acid concentrations of 20 mM (*2*), a concentration 80-fold higher than that applied in the present experiments. In a randomized, placebo-controlled cross-over human intervention trial in which 3 g of ascorbic acid per day (about 30 times the recommended daily intake) was administered for 2 weeks, no effect on the blood contents of pro-inflammatory cytokines such as IL-6 or IL-1ra was demonstrated (*18*). Therefore, anti-inflammatory effects of ascorbic acid in diet-representative concentrations may be based on mechanisms other than IL-6 inhibition.

Nevertheless, the results presented herein suggest carbohydrates and malic and citric acid as mediators for IL-6 production in the cells. This result was also confirmed by a recombinate experiment in which the mixture of the sugars and these acids

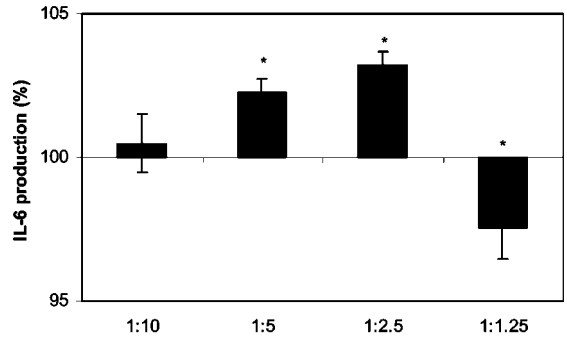

Figure 4. Intracellular production of interleukin-6 in carcinoma buccal (KB) cells after 6 h of exposure to increasing amounts of volatiles isolated from orange juice of different dilutions ranging from 1:10 to 1:1.25 ($n = 6$; control without additions = 100%). Student's t test versus nontreated control cells: *, $p < 0.05$.

induced the same IL-6 formation as the entire fraction (**Table 2**). Because the tested components making up 85% of the DM are responsible for the pro-inflammatory effect, the remaining 15% of the DM components are not likely to have a predominant effect on the formation of IL-6.

The AD had shown no significant effect on IL-6 content in KB cells when applied in concentrations representative of their content in 1:10 diluted orange juice (**Figure 3**). To investigate whether this effect was dose-dependent, KB cells were treated with increasing concentrations up to those corresponding to a 1:1.25 diluted orange juice (800 μL of orange juice plus 200 μL of cell culture medium).

None of the concentrations of the AD applied showed cytotoxic effects (data not shown). However, in this set of experiments, a dose-dependent effect on IL-6 formation was observed (**Figure 4**). Again, treatment of KB cells with the AD in the "natural" amounts present in the 1:10 diluted juice did not show any effect, whereas the application of 2- and 4-fold higher concentrations resulted in increased IL-6 contents compared with the untreated control cells. Interestingly, treatment of the cells with the 8-fold amount (corresponding to 1:1.25 diluted orange juice) led to a significant decrease in IL-6 formation. This anti-inflammatory effect of the AD was further proven in another set of experiments in which KB cells were exposed to either a mixture of the stimulants phorbol-12-myristate-13-acetate/ionomycine (PMA/I), the AD of 1:1.25 diluted orange juice, or the combination of PMA/I and the respective AD. Treatment of the KB cells with PMA/I stimulated the production of IL-6 in the cells by 19% compared to the nontreated control cells (**Figure 5**). Exposure of the cells to PMA/I plus AD led to a significant decrease of this effect ($p < 0.05$), as the total increase of IL-6 formation was 12% compared to nontreated control cells (II in **Figure 5**).

One possible explanation for this dose-dependent effect of the AD may be that volatile compounds present herein bind to the IL-6 receptor, leading to an internalization of the entire ligand–receptor complex. Although the effect of volatile compounds on the cellular production of IL-6 or its receptor has not been studied so far, this hypothesis is supported by mechanistic studies. It was demonstrated that IL-6 exerts its action via the IL-6 cell surface receptor complex, which internalizes upon ligand binding. The internalization is carried out by endocytic pathways and results in an increased IL-6 production and IL-6 receptor protein expression via a positive feedback mechanism (*19*). However, this positive feedback depends on the intracellular IL-6 concentration (*20–22*). On the

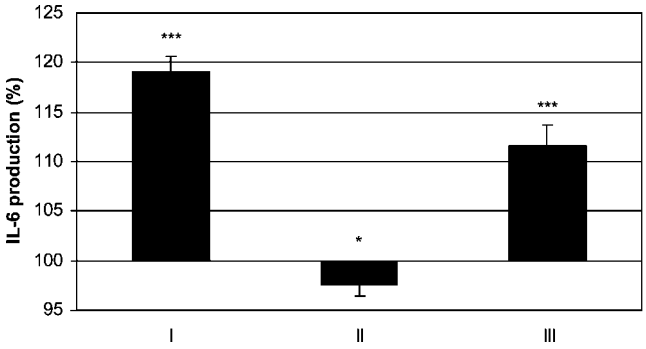

Figure 5. Intracellular production of interleukin-6 in carcinoma buccal (KB) cells after 6 h of exposure to the positive control in combination with the aqueous distillate corresponding to the concentration of 1:1.25 diluted orange juice: I, phorbol-12-myristate-13-acetate + ionomycin; II, aqueous distillate; III, phorbol-12-myristate-13-acetate + ionomycin + aqueous distillate ($n = 4$; control without additions = 100%). Student's t test versus nontreated control cells: *, $p < 0.05$; ***, $p < 0.005$; I vs III, $p < 0.05$.

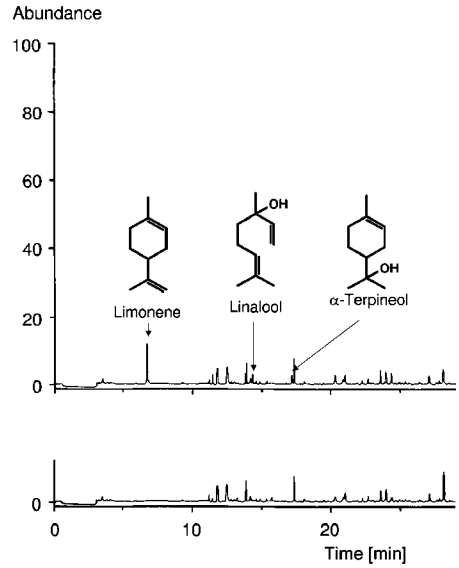

Figure 6. Gas chromatogram of the volatiles isolated from a cell lysate, which had been exposed to the distillate for 6 h ($n = 4$; upper trace) versus a gas chromatogram of a control cell lysate ($n = 4$; lower trace).

other hand, IL-6 formation decreases with low binding rates to the IL-6 receptor or by ligand-binding to other KB cell surface receptors (*19–22*).

With respect to the increased IL-6 formation in KB cells after exposure to the 2- and 4-fold concentrated AD of orange juice (cf. **Figure 4**), one might speculate that the AD contains compounds which bind to the IL-6 receptor. The resulting decrease in IL-6 formation after exposure to an 8-fold concentrated AD might be explained by activation of IL-6 inhibiting signal transduction pathways due to a feedback mechanism initiated by high concentrations of AD components binding to the IL-6 receptor and internalized thereafter.

As this hypothesis is highly speculative at this point, KB cell lysates were analyzed for volatile components after being exposed to the AD. Because, after IL-6 receptor binding, the internalized receptor–ligand complex is degraded in the lysosomes (*23*), AD compounds must be detectable in cells. Although there might be other pathways for a cellular uptake of volatiles, the intracellular detection of these compounds would support the present hypothesis and is the basis for further studies elucidating the mechanism by which volatile compounds of orange juice act as anti-inflammatory agents.

KB cells were, therefore, treated again for 6 h with AD, corresponding to its "natural content" in 1:1.25 diluted orange juice. Afterward, the cells were washed and treated with a lysis buffer. The volatile fraction was isolated and separated by HRGC. Compared to nontreated control cells, the gas chromatogram obtained from the AD-exposed cells showed three additional peaks (**Figure 6**). On the basis of a comparison of retention studies and mass spectra with data of the respective reference compounds, these peaks were identified as linalool, limonene, and α-terpineol, which have been reported as orange juice odorants earlier (*24*, *25*).

To quantitate the recovery of limonene, linalool, and α-terpineol in the lysed cells in relation to the total amount present in the AD, the contents of these three volatiles were determined in the AD and in the orange juice. Limonene was analyzed at a concentration of 40108 μg/kg, linalool at a concentration of 950 μg/kg, and α-terpineol at a concentration of 3930 μg/kg. Particularly, the relatively high contents of α-terpineol concomitant with the rather low concentrations of limonene suggest a long period of storage as α-terpineol is known to be formed as an oxidative limonene degradation product (*25*).

The percent recovery of these AD components in the cells' exponate (cell culture medium + AD), the air space of the cell culture flask, and the cell lysate showed 104, 87, and 88% for limonene, linalool, and α-terpineol, respectively. The percentage recoveries found in the lysate of the cells for limonene, linalool, and α-terpineol were 10.3, 0.3, and 0.4%, respectively. Although these amounts might have originated from orange juice, it was not clear whether, for example, α-terpineol had been also formed by an intracellular metabolization of limonene as reported earlier (*26–28*). However, in another set of experiments, in which KB cells were exposed to limonene, no α-terpineol could be detected (data not shown). Thus, the amount of α-terpineol quantitated in the KB cell lysate after exposure to the AD is likely to be derived from α-terpineol present in the aqueous distillate.

On the basis of the idea of receptor internalization, the presence of the volatiles limonene, linalool, and α-terpineol in the KB cell lysate supports the hypothesis that these compounds might act as ligands of the IL-6 receptor. To further prove the IL-6 stimulating activity of the volatiles quantitated in the KB cell lysate, KB cells were exposed to limonene, linalool, and α-terpineol in concentrations according to their amounts in 1:1.25 diluted orange juice. Treatment with limonene resulted in a 5% increase of IL-6 production, whereas cells treated with linalool showed no significant effect in comparison to the nontreated control cells (**Figure 7**). In contrast, exposure of the cells to α-terpineol inhibited IL-6 formation by 3% compared to nontreated control cells (**Figure 7**).

The anti-inflammatory effect of α-terpineol was further proven in another set of experiments in which KB cells were treated with 1:10 diluted orange juice, α-terpineol in a concentration corresponding to 1:1.25 diluted orange juice, or the combination of both, orange juice and α-terpineol. Treatment of the KB cells with orange juice increased IL-6 formation by 23% compared to nontreated control cells (**Figure 8**). This effect was significantly reduced by exposing the cells to orange juice plus α-terpineol, resulting in a total IL-6 increase of 20% ($p < 0.05$).

Because recombination experiments in which IL-6 formation in KB cells was analyzed after treatment with a combination of limonene, linalool, and α-terpineol did not demonstrate the

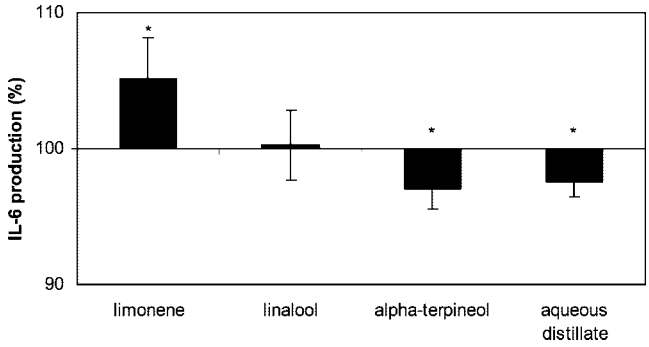

Figure 7. Intracellular production of interluekin-6 in carcinoma buccal cells after 6 h of exposure to limonene, linalool, and α-terpineol in concentrations corresponding to 1:1.25 diluted orange juice ($n = 9$) (control without additions = 100%). Aqueous distillate (1:1.25) was used for comparison. Student's t test versus nontreated control cells: *, $p < 0.05$.

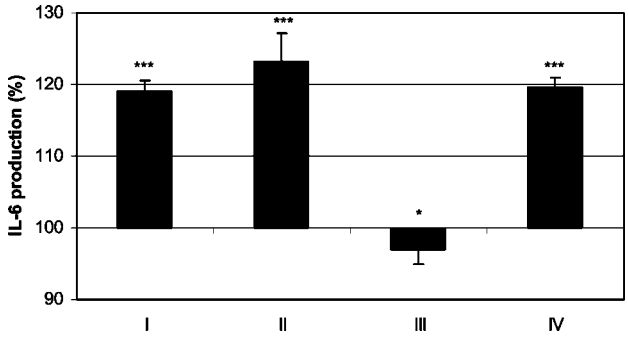

Figure 8. Intracellular production of interleukin-6 in carcinoma buccal (KB) cells after 6 h of exposure to the positive control (phorbol-12-myristate-13-acetate + ionomycin, I), orange juice (II, 1:10 dilution), α-terpineol (III, corresponding to its concentration in 1:1.25 diluted juice), or the combination of orange juice and α-terpineol (IV = II + III) ($n = 4$; control without additions = 100%). Student's t test versus nontreated control cells: *, $p < 0.05$; ***, $p < 0.001$; II versus IV: $p < 0.05$.

same IL-6 stimulation as experiments with the total AD (data not shown), additional IL-6 modulating compounds must be present in the AD of orange juice.

However, the immunomodulating effects on IL-6 formation in KB cells described for the volatiles limonene and α-terpineol gave rise to the question of whether these compounds might regulate the IL-6 receptor on a molecular level, such as affecting its gene expression. As KB cells were reported to express the IL-6 receptor protein (*29*), cells were again exposed to AD, limonene, or α-terpineol and IL-6 gene expression was analyzed by QRT-PCR

In this experiment, treatment of the KB cells with limonene resulted in a higher level of the IL-6 receptor gene expression compared to nontreated control cells (**Figure 9**), whereas treatment with α-terpineol resulted in a significant decrease. Interestingly, the aqueous distillate showed no significant effect on the IL-6 receptor gene expression, leading to the question of whether other active compounds present in the AD might compensate each other. However, the decreased IL-6 formation in KB cells after exposure to α-terpineol and the increased IL-6 formation after incubation with limonene support the hypothesis of both aroma compounds acting as ligands of the IL-6 receptor.

In summary, the presented results clearly support the hypothesis that orange juice does contain compounds able to inhibit the IL-6 formation in KB cells, such as α-terpineol. The cellular mechanism is suggested to be based on binding to the IL-6 receptor and subsequent internalization of the entire ligand–receptor complex.

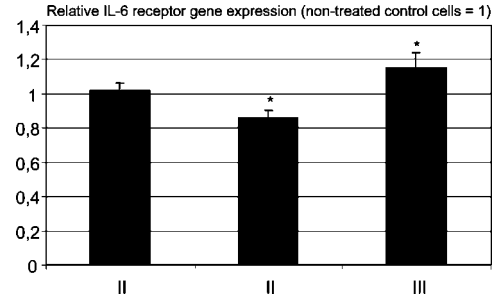

Figure 9. Influence on gene expression of the interleukin-6 receptor in carcinoma buccal (KB) cells after 6 h of exposure to an aqueous orange juice distillate (I), α-terpineol (II), and limonene (III) in concentrations according to the content in 1:1.25 diluted juice ($n = 7$; control without additions = 1). Student's t test versus nontreated control cells: *, $p < 0.05$.

The presented results suggest that addition of volatile compounds and, in particular, α-terpineol, can be a strategy to reduce the pro-inflammatory effect of orange juice in buccal cells. Fortification of orange juice with α-terpineol at 3144 μg/L, a concentration that effectively lowered IL-6 formation in buccal cells, will not cause any off-flavor, because the off-flavor threshold for α-terpineol in orange juice is 9060 μg/L (*30*). However, future controlled human intervention trials are needed to verify the presented results and to test whether a common exposure time of buccal cells to ingested orange juice leads to different effects compared to the long-term treatment of 6 h that was applied in the cell culture experiments presented herein.

LITERATURE CITED

(1) Sánchez-Moreno, C.; Pilar, M.; Begoña de Ancos, C.; Plaza, L.; Olmedilla, B.; Granado, F.; Martín, A. High-pressurized orange juice consumption affects plasma vitamin C, antioxidative status and inflammatory markers in healthy humans. *J. Nutr.* **2003**, *133*, 2204–2209.

(2) Härtel, C.; Strunk, T.; Bucsky, P.; Schultz, C. Effects of vitamin C on tracytoplasmatic cytokine production in human whole blood monocytes and lymphocytes. *Cytokine* **2004**, *27*, 101–106.

(3) Nieman, D. C.; Henson, D. A.; McAnulty, S. R.; McAnulty, L.; Swick, N. S.; Utter, A. C.; Vinci, D. M.; Opiela, S. J.; Morrow, J. D. Influence of vitamin C supplementation on oxidative and immune changes after an ultramarathon. *J. Appl. Physiol.* **2002**, *92*, 1970–1977.

(4) Souza, M. C.; Siani, A. C.; Ramos, M. F. S.; Menezes-De-Lima, J. R.; Henriques, M. G. M. O. Evaluation of anti-inflammatory activity of essential oils from two Asteraceae species. *Pharmazie* **2003**, *58*, 582–586.

(5) Raphael, T. J.; Kuttan, G. Immunomodulatory activity of naturally occurring monoterpenes carvone, limonene, and perillic acid. *Immunopharmacol. Immunotoxicol.* **2003**, *25*, 285–294.

(6) Sandros, J.; Karlsson, C.; Lappin, D. F.; Madianos, P. N.; Kinane, D. F.; Papapanou, P. N. Cytokine response of oral epithelial cells to porphyromonas gingivalis infection. *Res. Rep.* **2000**, *79*, 1808–1814.

(7) Steele, C.; Fidel, P. L., Jr. Cytokine and chemokine production by human oral and vaginal epithelial cells in response to *Candida albicans*. *Infect. Immun.* **2002**, *70*, 577–583.

(8) Barton, B. E. Interleukin-6 and new strategies for the treatment of cancer, hyperproliferative diseases and paraneoplastic syndromes. *Expert Opin. Ther. Targets* **2005**, *9*, 737–752.

(9) Franke, A. A.; Cooney, R. V.; Henning, S. M.; Custer, L. J. Bioavailability and antioxidant effects of orange juice components in humans. *J. Agric. Food Chem.* **2005**, *53*, 5170–5178.

(10) Klausner, R. D.; Donaldson, J. G.; Lippincott-Schwartz, J. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **1992**, *116*, 1071–1080.

(11) Jung, T.; Schauer, U.; Heusser, C.; Neumann, C.; Rieger, C. Detection of intracellular cytokines by flow cytometry. *Immunol. Methods* **1993**, *159*, 197–207.

(12) Baran, J.; Kowalczyk, D.; Ozog, M.; Zembala, M. Three-color flow cytometry detection of intracellular cytokines in peripheral blood mononuclear cells. Commparative analysis of phorbol myritate acetate-ionomycin and phytohemagglutinin stimulation. *Clin. Diagn. Lab. Immunol.* **2001**, *8*, 303–313.

(13) Maden, J.; Howarth, P. H.; Godfrey, R. C.; Frew, A. J. The kinetics and stimulant dependence of cytokine production by blood and bronchoalveolar lavage T cells evaluated at the single cell level. *Cytokine* **1999**, *11*, 510–517.

(14) Engel, W.; Bahr, W.; Schieberle, P. Solvent assisted flavour evaporation–a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *Eur. Food Res. Technol.* **1999**, *209*, 237–241.

(15) Kováts, E. Gas chromatographic characterization of organic compounds: Part 1. Retention indices of aliphatic halogenides, alcohols, aldehydes and ketones (in German). *Helv. Chim. Acta* **1958**, *206*, 1915–1932.

(16) Souci, S. W.; Fachmann, W.; Kraut, H. *Food Composition and Nutrition Tables*, 6th ed.; Wissenschaftlich Verlagsgesellschaft mbH: Stuttgart, Germany, 2000.

(17) Schwager, J.; Schulze, J. Modulation of interleukin production by ascorbic acid. *Vet. Immunol. Immunopathol.* **1998**, *64*, 45–57.

(18) Lu, Q.; Bjorkhem, I.; Wretlind, B.; Diczfalusy, U.; Henriksson, P.; Freyschuss, A. Effect of ascorbic acid on microcirculation in patients with type II diabetes: a randomized placebo-controlled cross-over study. *Clin. Sci. (London)* **2005**, *108*, 507–513.

(19) Dittrich, E.; Rose-John, S.; Gerhartz, C.; Mullberg, J.; Stoyan, T.; Yasukawa, K.; Heinrich, P. C.; Graeve, L. Identification of a region within the cytoplasmic domain of the interleukin-6 (IL-6) signal transducer gp130 important for ligand-induced endocytosis of the IL-6 receptor. *J. Biol. Chem.* **1994**, *269*, 19014–19020.

(20) Castell, J.; Klapproth, J.; Gross, V.; Walter, E.; Andus, T.; Snyers, L.; Content, J.; Heinrich, P. C. Fate of interleukin-6 in the rat. Involvement of skin in its catabolism. *Eur. J. Biochem.* **1990**, *189*, 113–118.

(21) Thiel, S.; Sommer, U.; Kortylewski, M.; Haan, C.; Behrmann, I.; Heinrich, P. C.; Graeve, L. Termination of IL-6-induced STAT activation is independent of receptor internalization but requires de novo protein synthesis. *FEBS Lett.* **2000**, *470*, 15–19.

(22) Korolenko, T. A.; Heinrich, P. K.; Hemmann, U.; Weiergraber, O.; Dittrich, E.; Graeve, L. Cell endocytosis of the complex interleukin-6-soluble interleukin-6 receptor and its intralysosomal degradation. *Russ. Bull. Eksp. Biol. Med.* **1997**, *124*, 527–529.

(23) Uings, I. J.; Farrow, S. N. Cell receptors and cell signalling. *Mol. Pathol.* **2000**, *53*, 295–299.

(24) Büttner, A.; Schieberle, P. Evaluation of aroma differences between hand-squeezed juices from Valencia late and navel oranges by quantitation of key odorants and flavor reconstitution experiments. *J. Agric. Food Chem.* **2001**, *49*, 2387–2394.

(25) Averbeck, M. Zum Einfluss von Lagerung und Abfüllung auf qualitäts-bestimmende Aromastoffe in Orangensaft aus Konzentrat. Dissertation, Technical University, Munich, Germany, 2002.

(26) Crowell, P. L.; Lin, S.; Vedejs, E.; Gould, M. N. Identification of metabolites of the antitumor agent D-limonene capable of inhibiting protein isoprenylation and cell growth. *Cancer Chemother. Pharmacol.* **1992**, *31*, 205–212.

(27) Kodama, R.; Yano, T.; Furukwa, K.; Noda, K.; Ide, H. Studies on the metabolism of D-limonen (*p*-mentha-1,8-diene). IV Isolation and characterization of new metabolites and species differences in metabolism. *Xenobiotica* **1976**, *6*, 377–389.

(28) Miyazawa, M.; Shindo, M.; Shimada, T. Metabolism of (+)- and (−)-limonenes to respective carveols and perillyl alcohols by CYP2C9 and CYP2C19 in human liver microsomes. *Drug Metab. Dispos.* **2002**, *30*, 602–607.

(29) Robledo, O.; Chevalier, S.; Froger, J.; Barthelaix-Pouplard, A.; Pennica, D.; Gascan, H. Regulation of interleukin 6 expression by cardiotrophin 1. *Cytokine* **1997**, *9*, 666–671.

(30) Plotto, A.; Margaria, C. A.; Goodner, K. L.; Goodrich, R.; Baldwin, E. A. Odour and flavour thresholds for key aroma components in an orange juice matrix: terpenes and aldehydes. *Flavour Fragrance J.* **2004**, *19*, 491–498.

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