

Citrus peel polymethoxyflavones nobiletin and tangeretin suppress LPS- and IgE-mediated activation of human intestinal mast cells

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Abstract

Purpose Allergic diseases with mast cells (MC) as main effector cells show an increased prevalence. MC also play an essential role in other inflammatory conditions. Therapeutical use of anti-inflammatory nutraceuticals directly targeting MC activation could be of interest for afflicted patients. Nobiletin and tangeretin are citrus peel polymethoxyflavones, a group of citrus flavonoids, possessing anticancer, antimetastatic, and anti-inflammatory activities. Here, we analyzed the effects of nobiletin/tangeretin on LPS- and IgE-mediated stimulation of human intestinal mast cells (hiMC).

Methods MC isolated from human intestinal tissue were treated with different concentrations of nobiletin or tangeretin prior to stimulation via LPS/sCD14 or IgE-dependently. Degranulation, pro-inflammatory cytokine expression and phosphorylation of ERK1/2 were examined.

Results Expression of CXCL8, CCL3, CCL4 and IL-1 β in response to LPS-mediated stimulation was inhibited by nobiletin/tangeretin. hiMC activated IgE-dependently showed a reduced release of β -hexosaminidase and cysteinyl LTC₄ in response to nobiletin, but not in response to tangeretin. Expression of CXCL8, CCL2, CCL3, CCL4 and TNF in IgE-dependently activated hiMC was decreased in a dose-dependent manner following treatment with

nobiletin/tangeretin. IL-1 β expression was only reduced by tangeretin. Compared to treatment with NF- κ B inhibitor BMS345541 or MEK-inhibitor PD98059, nobiletin and tangeretin showed similar effects on mediator production. Phosphorylation of ERK1/2 upon IgE-mediated antigen stimulation was significantly suppressed by nobiletin and tangeretin.

Conclusions Nobiletin and, to a lesser extent, tangeretin could be considered as anti-inflammatory nutraceuticals by reducing release and production of proinflammatory mediators in MC.

Keywords Mast cells · Allergy · Nutraceuticals · Flavonoids · Citrus fruits

Introduction

Citrus peel flavonoids are secondary plant compounds found in citrus fruits—in high levels concentrated in the peel of citrus fruits [1]. Nobiletin (3',4',5,6,7,8-hexamethoxyflavone) and tangeretin (5,6,7,8,4'-pentamethoxyflavone) belong to the family of citrus peel polymethoxyflavones and differ by one additional methoxy group in nobiletin [1]. Because of their methoxylation they have apolar characteristics and can be well absorbed in intestine [1]. Therefore, in context of nutraceuticals they could be of special relevance. It is known that the consumption of vegetables and fruits is inversely associated with the risk of several chronic diseases [2]. Citrus fruit-derived flavonoids such as nobiletin and tangeretin have been reported to exert diverse beneficial biological properties. They exert anticancer [3–6], antimetastatic [7–9], antidiabetic [10–12] and neuroprotective [13–16] effects in vivo and in vitro. Both nobiletin and tangeretin

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have known anti-inflammatory actions [17–22]. Anti-inflammatory effects on monocytes activated via LPS have been extensively analyzed, revealing an involvement of NF- κ B [19, 23–27]. First hints for antiallergic activities on RBL-2H3 cells concerning pro-inflammatory cytokine production and on murine skin and asthma models concerning scratching behavior and lung inflammation and eosinophilic infiltration are given as well [17, 28, 29] whereby the influence on primary MC has not been analyzed so far.

Mast cells (MC) are key effector cells of inflammatory processes, especially immediate-type allergic reactions, but their involvement is also discussed in diseases such as inflammatory bowel disease. After being activated via diverse stimuli, MC can release a wide range of inflammatory mediators. These are pre-stored mediators such as histamine and proteases, but also de novo synthesized cytokines and lipid mediators [30]. MC are also known of being LPS-sensitive cells, so being activated by LPS from bacteria which can be relevant, e.g., in IBD. However, the typical activation signal for MC is the IgE-dependent stimulation via the high-affinity IgE receptor, Fc ϵ RI, by cross-linking of surface-bound IgE by antigen [31, 32].

The prevalence of MC-associated diseases such as allergies increased in the last decades [33]. Conservative medications are available, but because of side effects and bad compliance among afflicted patients, development of new therapeutical strategies is very important. Nutraceuticals being—in contrast to pharmaceuticals—of natural origin could be a good alternative or additive therapy associated with a better acceptance. Here we analyzed the effect of citrus peel polymethoxyflavones nobiletin and tangeretin on MC activated by different stimuli.

Materials and methods

Isolation and culture of human intestinal mast cells

Human intestinal MC (hiMC) were isolated from macroscopically normal surgical tissue from bowel resection in cancer patients by a mechanic and enzymatic procedure [34]. Permission to conduct the study was obtained from the local ethical committee. Human mucosal tissue was shredded mechanically, and digested enzymatically with pronase (Roche Diagnostics GmbH, Mannheim, Germany), chymopapain (Sigma-Aldrich, Munich, Germany) and collagenase (Serva, Heidelberg, Germany). The gained cell suspension was cultured overnight in culture medium (RPMI 1640 with 10 % heat-inactivated fetal calf serum, 100 μ g/ml streptomycin, 100 U/ml penicillin, 100 μ g/ml gentamycin, and 2.5 μ g/ml amphotericin B, Gibco Invitrogen, Paisley, UK). An enrichment of hiMC was undertaken

by magnetic cell separation of c-Kit⁺ cells using *CD117 microbead kit* after *dead cell removal kit* (MACS™ system, Miltenyi Biotech, Bergisch-Gladbach, Germany). Pure hiMC were cultured with 25 ng/ml SCF (PeproTech, London, UK) in combination with 2 ng/ml IL-4 (PeproTech, London, UK) for at least 10 days before being used in experiments.

Treatment of MC with nobiletin or tangeretin

hiMC were cultured for 1, 4 or 18 h in culture medium containing 5–100 μ M nobiletin or tangeretin (Indofine Chemical Co., Inc., Hillsborough, NJ, USA) dissolved in DMSO (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Dead cell counting by trypan blue staining showed that treatment of hiMC with 45 and 100 μ M nobiletin or tangeretin for 18 h had no effect on cell viability. Controls of hiMC contained the same concentration of the vehicle DMSO. For IgE-dependent stimulation, during this incubation hiMC were loaded with 0.1 μ g/ml myeloma IgE (Merck Millipore, Darmstadt, Germany). After incubation, cells were stimulated with 0.1 μ g/ml polyclonal antihuman IgE (provided by Dr. U. Blank, INSERM Unite 699, Paris, France) for 10 min to detect phosphorylation status of signaling molecules, or for 90 min to analyze cytokine mRNA expression and release of β -hexosaminidase. For LPS-mediated stimulation, after the incubation period hiMC were stimulated with lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma-Aldrich, Munich, Germany) and sCD14 (PeproTech, London, UK) in a concentration of 1 μ g/ml each for 3 h [35] to analyze mRNA expression of relevant mediators.

Release of β -hexosaminidase

Degranulation of MC was quantified by determining the amount of released β -hexosaminidase in the supernatant by color enzyme assay [36].

RNA preparation and real-time RT-PCR

Total RNA was prepared by using a high pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). Real-time RT-PCR was performed as described [37, 38]. Primer sequences of the specific human sense and antisense primers used are listed in Table 1.

Western blot analysis

Western blot was performed as described [38]. Membranes were probed with phospho-ERK (MAPK)-1/2 (12D4) mouse mAb (Cell Signaling Technology®, Frankfurt, Germany). Visualization was performed by the secondary

Table 1 Specific human sense and antisense primers

Primer	Sense sequence	Antisense sequence	Length (bp)
Human			
<i>GAPDH</i>	5'-TGG TCT CCT CTG ACT TCA AC-3'	5'-CCT GTT GCT GTA GCC AAA TT-3'	128
<i>CXCL8</i>	5'-CTG AGA GTG ATT GAG AGT GG-3'	5'-ACA ACC CTC TGC ACC CAG TT-3'	113
<i>CCL3</i>	5'-CTC TGC ATC ACT TGC TGC TGA CAC-3'	5'-CAC TCA GCT CCA GGT CGC TGA C-3'	212
<i>CCL4</i>	5'-GCT AGT AGC TGC CTT CTG CTC TCC-3'	5'-CAG TTC CAG CTG ATA CAC GTA CTC C-3'	238
<i>IL-1β</i>	5'-CCT GTC CTG CGT GTT GAA AG-3'	5'-CTG CTT GAG AGG TGC TGA TG-3'	179

antibody antimouse IgG, HRP-linked (Cell Signaling Technology®, Frankfurt, Germany) and Super Signal® West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA). The obtained signals were measured by bioimaging analyzer (Alpha Innotech Corporation, San Leandro, CA, USA). The membranes were stripped, and ERK1/2 (137F5) rabbit mAb (Cell Signaling Technology®, Frankfurt, Germany) was used as secondary probe for normalization of the obtained signals.

Statistics

Data expressed as mean $\pm$ SEM. One-way ANOVA with Tukey's multiple comparison as post hoc test was used to analyze differences between groups. Post hoc tests were performed when the mean values were significantly different in one-way ANOVA. A value of $p < 0.05$ was considered to be statistically significant.

Results

Nobiletin and tangeretin strongly reduce LPS/sCD14-activated pro-inflammatory cytokine expression in hiMC

Nobiletin and tangeretin show anti-inflammatory effects on monocytes activated via LPS [19, 23–27]. The influence of these citrus flavonoids was analyzed on LPS-sensitive MC. Treatment of hiMC with LPS/sCD14 led to an up-regulation of the proinflammatory cytokines and chemokines IL-1 β , CXCL8, monocyte chemoattractant protein-1 CCL2, macrophage inflammatory protein-1 α CCL3 and macrophage inflammatory protein-1 β CCL4 [35]. Figure 1 representatively shows the results of LPS/sCD14-mediated stimulated hiMC treated with nobiletin (Fig. 1a) or tangeretin (Fig. 1b) for CXCL8, IL-1 β , CCL3 and CCL4. Treatment with nobiletin or tangeretin resulted in a significant dose-dependent down-regulation of CXCL8, IL-1 β , CCL2, CCL3 and CCL4. The down-regulatory effect especially of nobiletin was very strong with an inhibition near control values for concentrations of 45 or 100 μ M. Nobiletin

showed stronger inhibitory effects on CXCL8 and CCL4 expression than tangeretin. Expression of tryptase and TNF was not influenced by tangeretin, but was significantly down-regulated by nobiletin—although less than the cytokines mentioned above (not shown). For all mediators analyzed, a dose-dependent effect could be observed for both nobiletin and tangeretin.

Nobiletin but not tangeretin inhibit degranulation of hiMC stimulated IgE-dependently

The typical activation signal for MC, especially in context of allergy, is the IgE-dependent activation via the IgE receptor Fc ϵ RI. We analyzed the effect of nobiletin on Fc ϵ RI-dependent degranulation of hiMC and found a slight reduction in release of β -hexosaminidase (Fig. 2a, b) and cysteinyl LTC₄ (Fig. 2c, d). 15 μ M of nobiletin reduced β -hexosaminidase release only to 74.1 ± 8.4 % of the control. The effect on release of cysteinyl LTC₄ was stronger. Noteworthy, no corresponding down-regulatory effects on mediator release of hiMC could be detected for tangeretin. Even the highest concentration of 100 μ M showed no reduction in release of β -hexosaminidase or cysteinyl LTC₄.

Nobiletin and tangeretin reduce pro-inflammatory cytokine expression in IgE-dependently activated hiMC

Induced cytokine mRNA expression following stimulation by Fc ϵ RI cross-linking was strongly reduced by nobiletin and tangeretin treatment. We found a down-regulation of mRNA levels of CXCL8, CCL2, CCL3, CCL4 and TNF. IL-1 β expression was significantly reduced by tangeretin but not by nobiletin treatment. Figure 3 representatively shows the results for CXCL8, IL-1 β , CCL3 and CCL4. Different concentrations of nobiletin resulted in a dose-dependent down-regulation of mRNA expression. Tangeretin showed almost the same inhibitory influence for concentrations of 15 μ M or higher. A lower concentration of 5 μ M (not shown) resulted in no significant inhibition. For CXCL8 and CCL4 expression, nobiletin treatment resulted in a stronger down-regulation compared to tangeretin treatment.

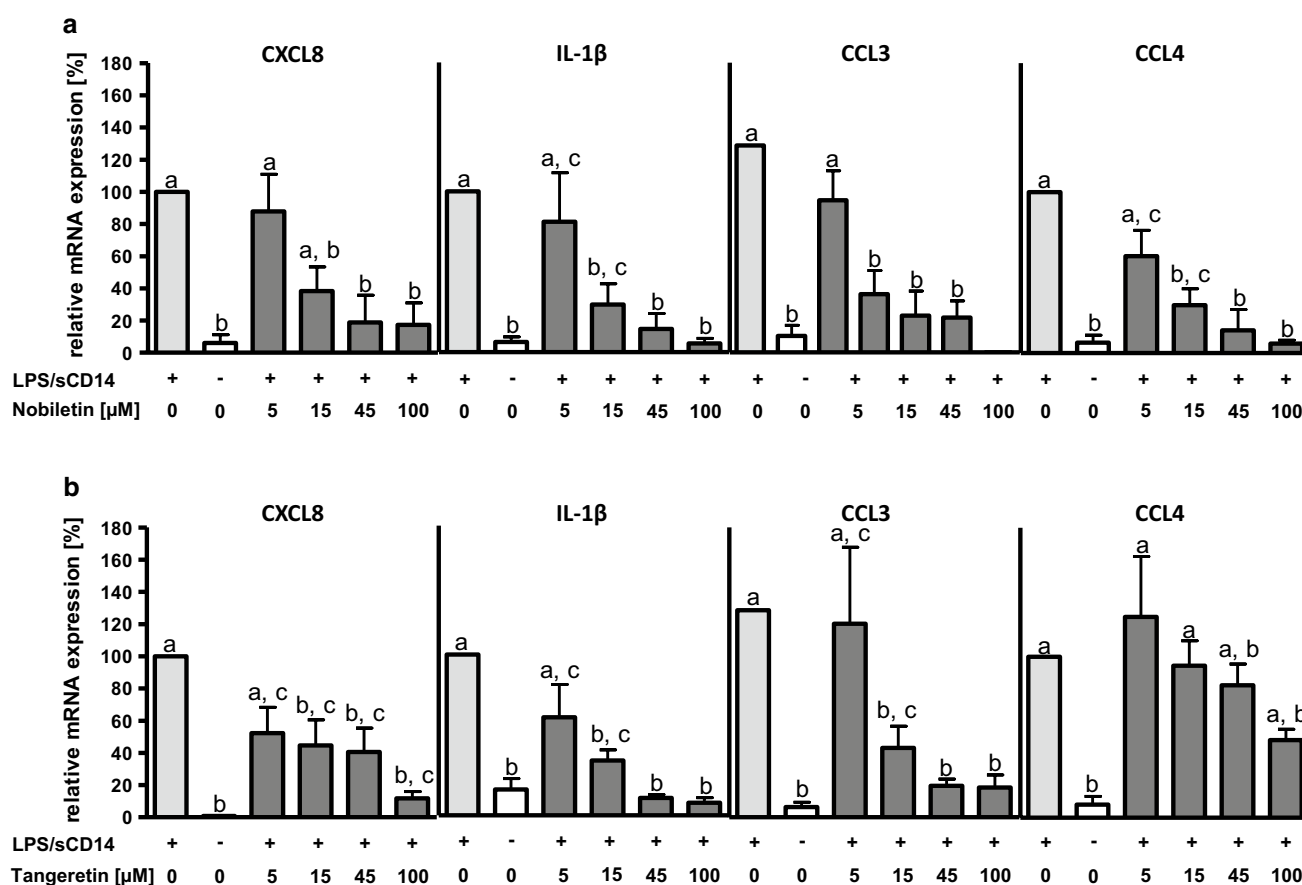


Fig. 1 Effect of nobiletin (**a**) or tangeretin (**b**) on expression of pro-inflammatory cytokines in LPS-mediated stimulated human intestinal mast cells (hiMC). Representatively mRNA expression of CXCL8, IL-1 β , CCL3 and CCL4 in hiMC is shown. Mean Ct values for 100 % are 27, 28, 31 and 28, respectively. hiMC were incubated with nobiletin (**a**) or tangeretin (**b**) or with a corresponding concen-

tration of vehicle DMSO as control for 18 h and stimulated via LPS/sCD14 for 3 h. Mean concentrations with 50 % inhibition rate (IC₅₀) of cytokine expression each were for nobiletin 47 ± 15 μ M and for tangeretin 48 ± 17 μ M. Values are mean $\pm$ SEM. Means without a common letter differ, $p < 0.05$ (nobiletin: $n = 4$, tangeretin: $n = 4-5$)

LPS-stimulated expression of pro-inflammatory cytokines in hiMC was reduced to control values following nobiletin or tangeretin treatment, whereas inhibitory effects on IgE-stimulated hiMC were lower. Therefore, we analyzed if a combination of nobiletin and tangeretin could have additive effects on IgE-dependently stimulated hiMC. Figure 3c shows the results for this combinatory treatment for expression of CXCL8, IL-1 β , CCL3 and CCL4. For all cytokines analyzed, the combinatory treatment resulted in a down-regulation to control values.

Tangeretin shows similar, nobiletin stronger inhibitory influences on pro-inflammatory mediator expression as NF- κ B inhibitor BMS345541

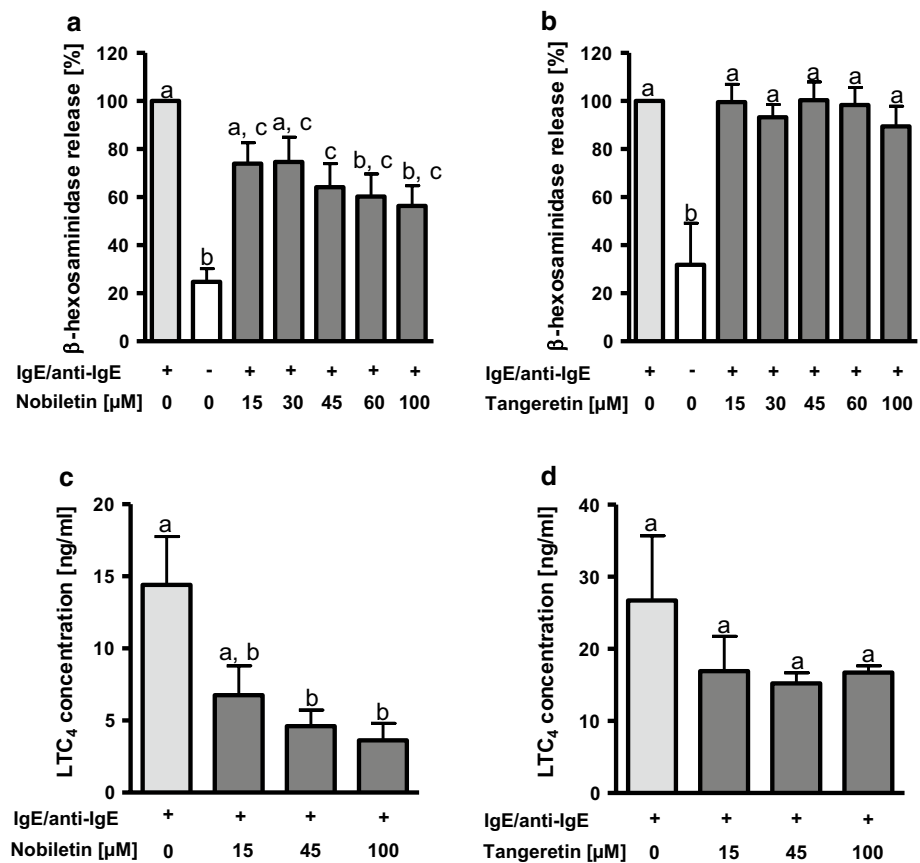
mRNA expression of the pro-inflammatory mediators CXCL8, CCL2, CCL3, CCL4 and IL-1 β after LPS/sCD14-mediated stimulation of hiMC was analyzed. As NF- κ B is a relevant transcription factor in LPS signaling, hiMC

were treated with NF- κ B inhibitor BMS345541 showing a significant reduction in expression of CCL2, CCL3, CCL4 and IL-1 β ; CXCL8 was not significantly inhibited. Figure 4a representatively shows the expression of CXCL8, IL-1 β , CCL3 and CCL4. Tangeretin showed similar effects, whereby nobiletin treatment resulted in stronger down-regulatory influences. Noteworthy, the co-treatment of BMS345541 with both nobiletin or tangeretin led to the strongest inhibition almost reaching control values for all mediators analyzed.

Inhibitory effects of nobiletin or tangeretin are similar to effects of MEK-inhibitor PD98059

IgE-dependently stimulated hiMC treated with the MEK-inhibitor PD98059 showed a significantly reduced expression of the pro-inflammatory cytokines CXCL8, IL-1 β and CCL2; CCL4 was not significantly down-regulated. Treatment with nobiletin or tangeretin led to a significant and similar

Fig. 2 Release of β -hexosaminidase (**a, b**) and cysteinyl LTC₄ (**c, d**) by human intestinal mast cells (hiMC) following treatment with nobiletin (**a, c**) or tangeretin (**b, d**). hiMC were incubated with nobiletin or tangeretin or with a corresponding concentration of vehicle DMSO as control for 18 h and stimulated by Fc ϵ RI cross-linking for 90 min. Values are mean $\pm$ SEM. Means without a common letter differ, $p < 0.05$ (**a, b** $n = 7$ – 8 ; **c, d** $n = 4$ – 5)



down-regulation of all mentioned cytokines. Figure 5 representatively shows expression of CXCL8, IL-1 β , CCL3 and CCL4 following nobiletin (**a**) and tangeretin (**b**) treatment. For CXCL8 and IL-1 β expression, where PD98059 also showed a significant down-regulation, this down-regulatory effect was the same compared to nobiletin or tangeretin. Co-treatment of the citrus flavonoids with PD98059 showed almost the same inhibitory effect as nobiletin or tangeretin alone.

Nobiletin strongly inhibits ERK activation

To identify the signaling pathways being involved in the down-regulatory effects of nobiletin and tangeretin on IgE-dependently induced cytokine expression, we measured the phosphorylation status of the signaling molecule ERK1/2. Previous studies showed that ERK is one of the main signaling molecules involved in cytokine expression of hiMC [37]. Moreover, the well-known MEK-inhibitor PD98059 is also a synthetic methoxyflavone with similar molecular structure to nobiletin and tangeretin [37, 39]. Western blot analyses showed that in IgE-dependently activated hiMC, the increased phosphorylation status of ERK was clearly down-regulated by nobiletin treatment (Fig. 6a). The down-regulation was

similar to the effect of MEK-inhibitor PD98059 and showed a reduction of about 86 % reaching control values. Co-treatment of PD98059 with nobiletin revealed a similar inhibitory effect compared to the single substance effects. Tangeretin treatment resulted in a lower but still significant reduction in ERK1/2 phosphorylation of about 60 % (Fig. 6b). Co-treatment of PD98059 with tangeretin as well as single PD98059 led to a lower ERK1/2 phosphorylation level compared to tangeretin treatment alone.

As nobiletin and tangeretin could be identified as ERK1/2 inhibitors showing a similar down-regulation of ERK1/2 phosphorylation as the MEK-inhibitor PD98059, we examined if also a short-term incubation for 1 h—as used for treatment with PD98059—would be sufficient for the inhibitory effects. Figure 6c–f shows time dependency of nobiletin and tangeretin treatment on cytokine expression of IgE-mediated stimulated hiMC. CXCL8 (Fig. 6c, d) and CCL3 (Fig. 6e, f) expressions are representatively shown. Figure 6 demonstrates that treatment for 1 h already resulted in a significant down-regulation of cytokine expression, underlining the hypothesis that nobiletin and tangeretin could act as an ERK1/2 inhibitor similar to PD98059.

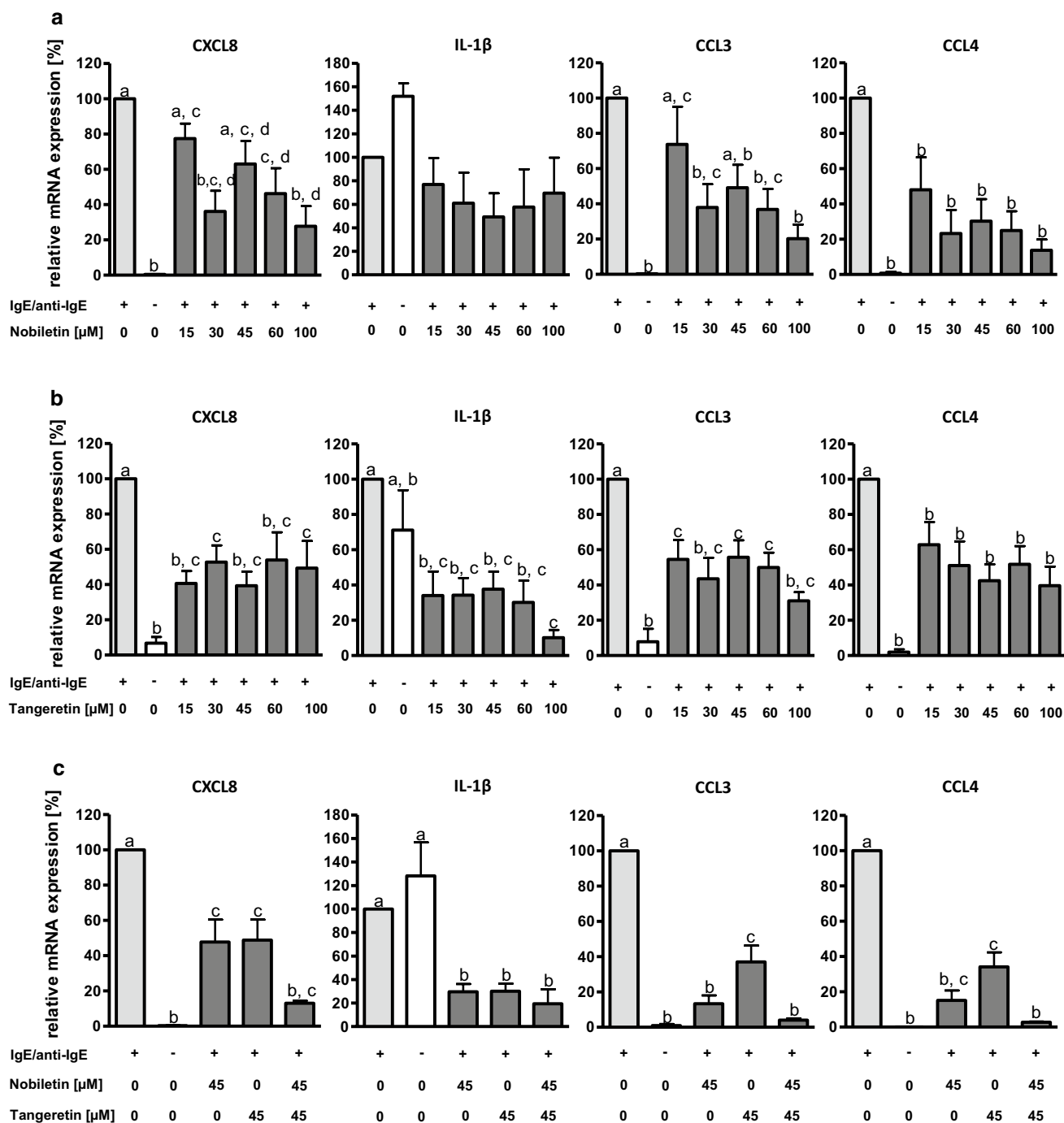
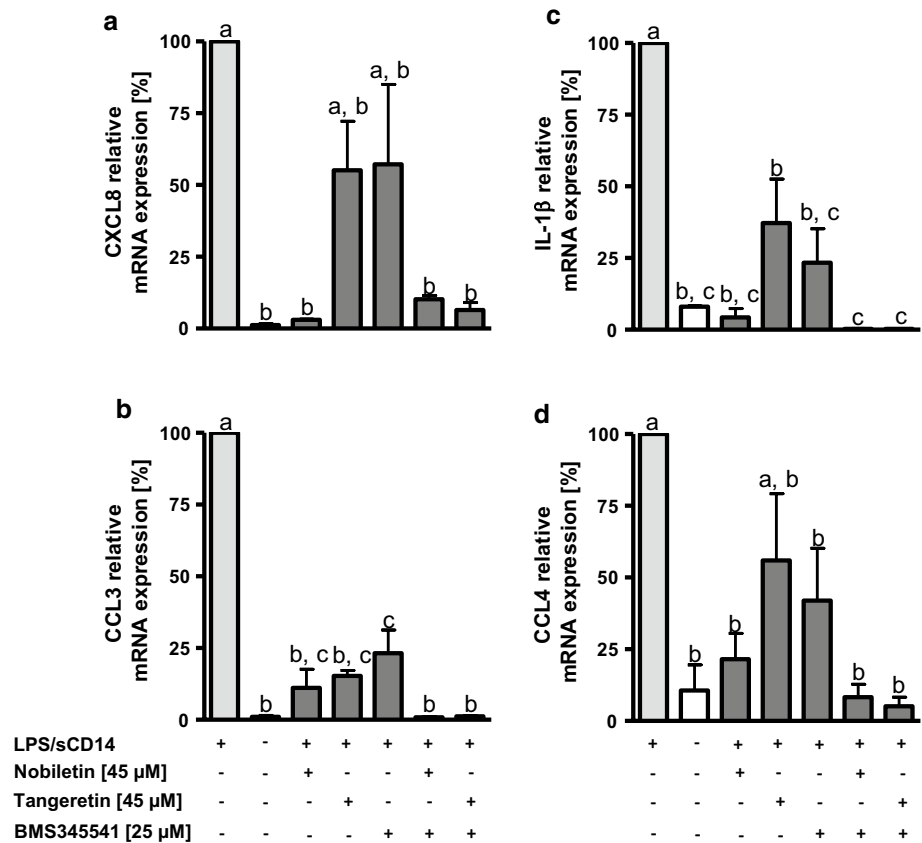


Fig. 3 Effect of nobiletin (a), tangeretin (b) or a combination of nobiletin and tangeretin (c) on expression of pro-inflammatory cytokines in IgE-mediated stimulated human intestinal mast cells (hiMC). Representatively mRNA expression of CXCL8, IL-1 β , CCL3 and CCL4 in hiMC is shown. Mean Ct values for 100 % are 28, 31, 29 and 27, respectively. hiMC were incubated with nobiletin (a), tangeretin (b) or nobiletin and tangeretin (c) or with a cor-

responding concentration of vehicle DMSO as control for 18 h and stimulated by Fc ϵ RI cross-linking for 90 min. Mean concentrations with 50 % inhibition rate (IC₅₀) of cytokine expression each were for nobiletin 37 $\pm$ 6 μ M and for tangeretin 32 $\pm$ 7 μ M. Values are mean $\pm$ SEM. Means without a common letter differ, $p < 0.05$ (nobiletin: $n = 4-5$; tangeretin: $n = 5-6$; nobiletin and tangeretin: $n = 4-5$)

Fig. 4 Effect of nobiletin, tangeretin or NF- κ B inhibitor BMS345541 on expression of pro-inflammatory cytokines in LPS/sCD14-mediated stimulated human intestinal mast cells (hiMC). Representatively, mRNA expression of CXCL8 (a), CCL3 (b), IL-1 β (c) and CCL4 (d) in hiMC is shown. hiMC were incubated with 25 μ M BMS345541 for 1 h, and with 45 μ M nobiletin or tangeretin or with a corresponding concentration of vehicle DMSO as control for 18 h and stimulated via LPS/sCD14 for 3 h. Values are mean $\pm$ SEM. Means without a common letter differ, $p < 0.05$ ($n = 3-4$)



Discussion

In the present study, we demonstrate that citrus peel polymethoxyflavones nobiletin and tangeretin show inhibitory effects on expression of pro-inflammatory mediators and cytokines in MC. The effects could be found for both activation with IgE/anti-IgE and stimulation via LPS/sCD14 with stronger effects on LPS-dependent stimulation. Nobiletin showed stronger inhibitory effects than tangeretin with an inhibitory influence on ERK1/2 phosphorylation similar to MEK-inhibitor PD98059. Thus, nobiletin and—to a lesser extent—tangeretin might be potential therapeutic agents for treatment of LPS- or IgE-mediated diseases such as allergies.

The effect of nobiletin and tangeretin on LPS-mediated stimulated cells such as monocytes has been analyzed extensively. LPS-mediated stimulation leads via toll-like receptor 4 (TLR4)-signaling to the activation of the transcription factor NF- κ B. Nobiletin and tangeretin treatment of different cell types resulted in an inhibited activation suggesting NF- κ B as possible signaling molecule. NF- κ B is a prominent transcription factor of production of pro-inflammatory mediators and can among others be activated by bacterial LPS [23]. Choi et al. showed anti-inflammatory effects of nobiletin on LPS-activated RAW 264.7 cells concerning ROS production [23], and for nobiletin and

tangeretin concerning NO production [27]. Effects were mediated by inhibiting transcriptional activation of NF- κ B by suppressing its DNA-binding activity [23]. Thereby no effect of nobiletin on LPS-induced phosphorylation of I κ B- α or on NF- κ B translocation to the nucleus could be observed [23]. Mouse macrophages stimulated with LPS and treated with nobiletin showed a reduced PGE₂ production and expression of IL-1 β , TNF and IL-6, as well as IL-1 expression in human synovial fibroblasts [24]. Nobiletin suppressed LPS-induced activation of AP-1 and NF- κ B in RAW 264.7 macrophages [25]. Tangeretin and nobiletin reduced LPS-induced NO production in rat primary astrocytes by suppressing NF- κ B activation [40]. MAPK signaling pathways including ERK1/2 can be involved in LPS signaling by activating transcription factor NF- κ B modulating production of inflammatory mediators. Tangeretin decreased LPS-induced TNF, IL-1 β NO and COX-2 production in microglial cells by reducing phosphorylation of I κ B- α , IKK- β and ERK as well as nuclear translocation of the p65 subunit [41]. Analyses for influence of nobiletin or tangeretin on LPS-mediated activation of MC have not been done so far. Our data clearly indicate that nobiletin and tangeretin treatment resulted in a lower expression of inflammatory mediators in MC stimulated via LPS. Based on the known effects of nobiletin and tangeretin on NF- κ B in context of other cells, it was clear that this

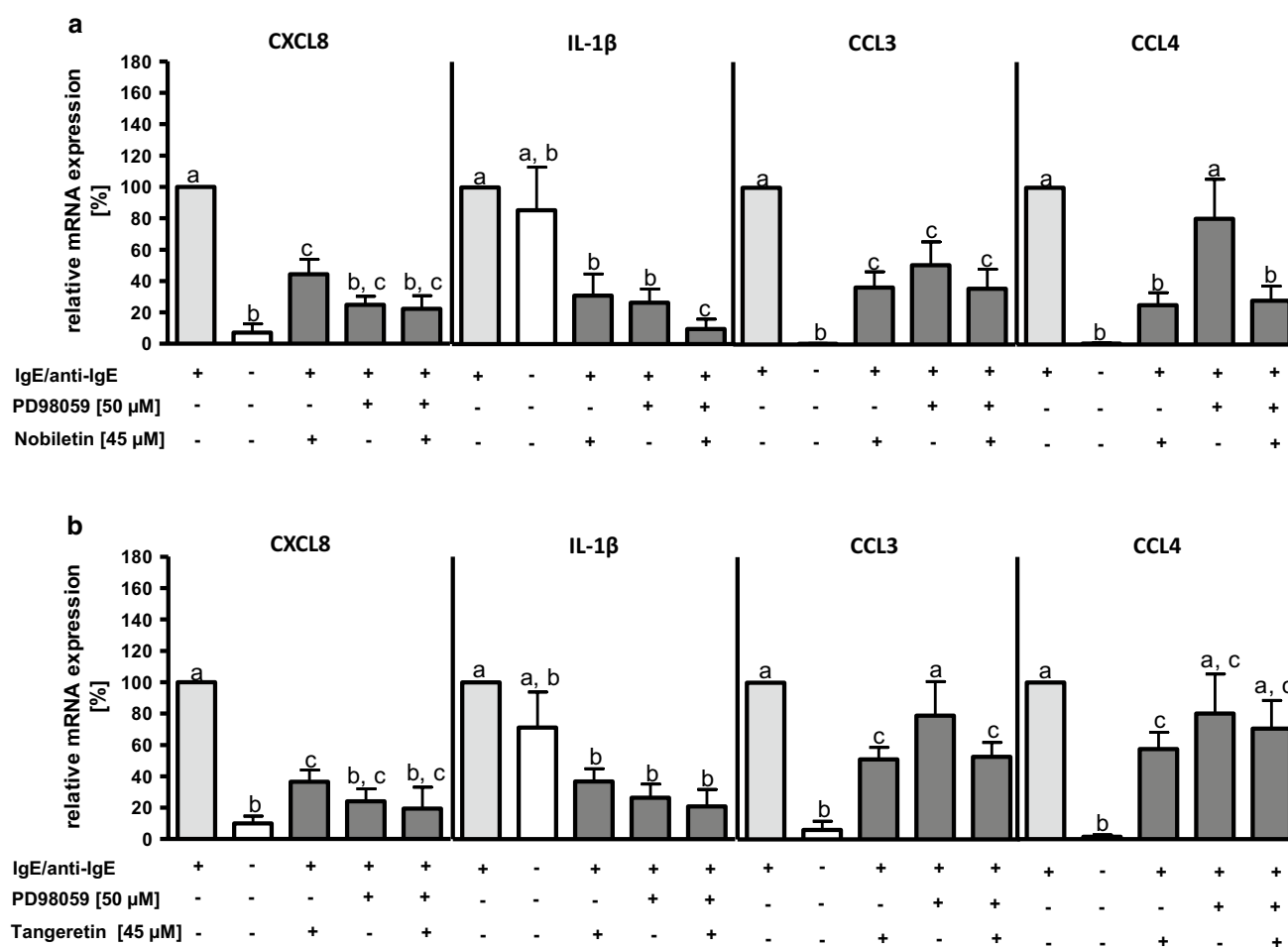


Fig. 5 Effect of nobiletin (**a**), tangeretin (**b**), or MEK-inhibitor PD98059 on expression of pro-inflammatory cytokines in IgE-mediated stimulated human intestinal mast cells (hiMC). Representatively, mRNA expression of CXCL8, IL-1 β , CCL3 and CCL4 in hiMC is shown. hiMC were incubated with 50 μ M PD98059 for 1 h, and with

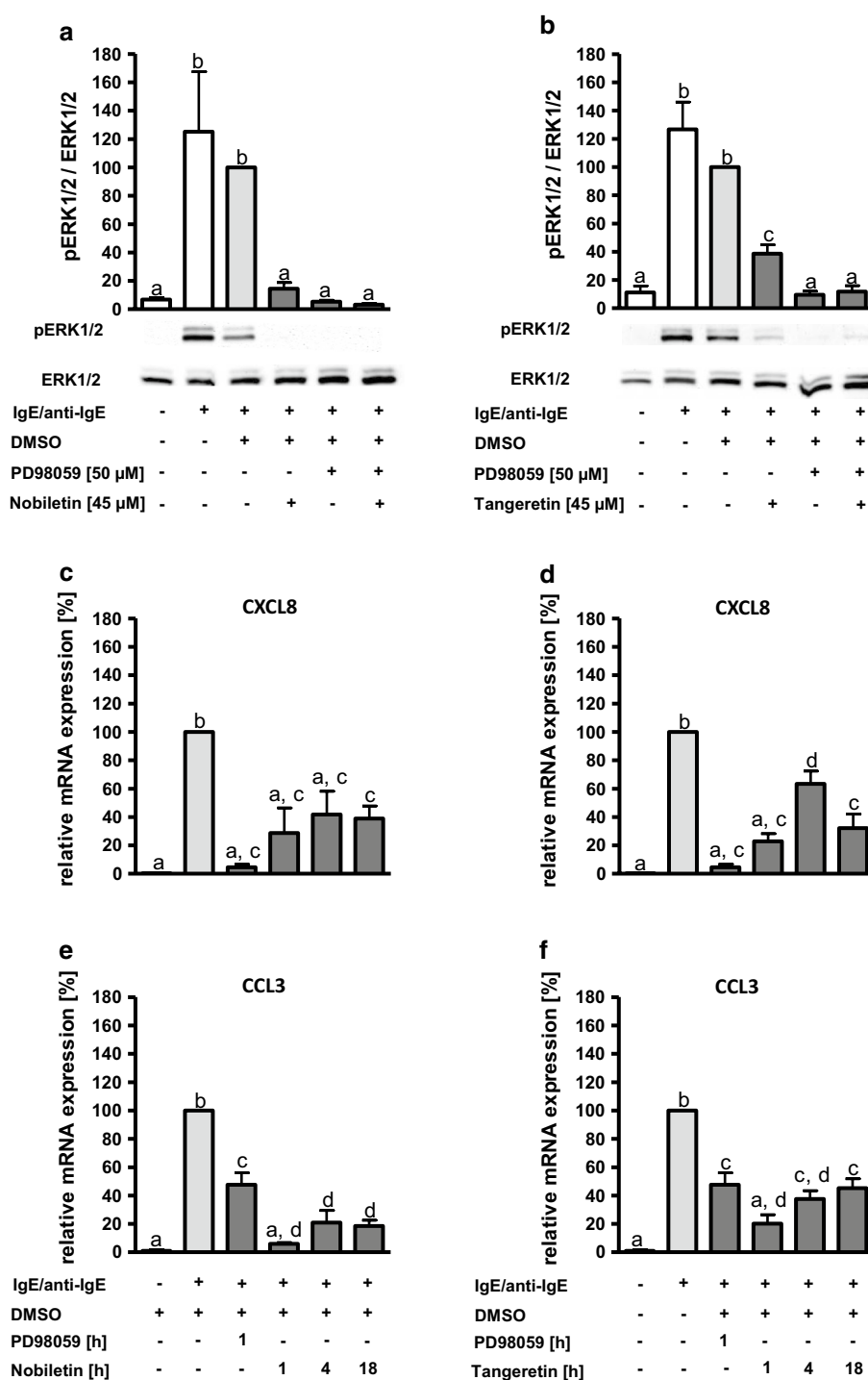
45 μ M nobiletin (**a**) or tangeretin (**b**) or with a corresponding concentration of vehicle DMSO as control for 18 h and stimulated by Fc ϵ RI cross-linking for 90 min. Values are mean $\pm$ SEM. Means without a common letter differ, $p < 0.05$ ($n = 4-5$)

could also be the relevant mechanism here. Therefore, MC stimulated with LPS/sCD14 were treated with nobiletin, tangeretin or the NF- κ B inhibitor BMS345541. As expected, nobiletin and tangeretin treatment of LPS-stimulated hiMC showed similar or even stronger effects as the NF- κ B inhibitor. Moreover, we found induction of TLR4 in hiMC by LPS-stimulation. Interestingly, analysis of mRNA expression levels of TLR4 of hiMC stimulated with LPS/sCD14 showed a significant down-regulation in response to nobiletin—however not in response to tangeretin—treatment (not shown). Thus, at least nobiletin could have some prolonged effects on hiMC which could explain the higher inhibition rate of nobiletin on cytokine expression in response to LPS- compared to IgE-dependent stimulation. Other in vitro studies dealing with LPS-stimulation in macrophages/monocytes showed inhibitory effects of nobiletin on cytokine and iNOS expression as well as on NF- κ B

signaling after long-term treatments of 18 or 24 h [19, 23, 24]. 24 h of nobiletin treatment in RAW264.7 cells led to reduction in iNOS protein expression, IL-1 mRNA expression and production [19] as well as expression of IL-6 and TNF [24]. These findings could imply a probable involvement of TLR4 expression and therefore a prolonged effect of nobiletin as well. Possibly prolonged effects could also be responsible for the successful treatments with nobiletin in inflammatory rodent models [3, 17, 29].

Several effects of nobiletin and tangeretin on allergic situations or cells are known. Used concentrations in vitro reached values of 100 μ M, in rodent models in vivo a daily administration of 5 up to 50 mg/kg body weight was reported, whereby already 5 mg/kg body weight/d showed significant effects in inflammatory animal models [42]. Translated to the human situation, the necessary and effective doses of nobiletin and tangeretin will probably not

Fig. 6 Effect of nobiletin (a) or tangeretin (b) on activation of ERK1/2, and time-dependent effect of nobiletin (c, e) or tangeretin (d, f) in comparison with MEK-inhibitor PD98059 on expression of pro-inflammatory cytokines in human intestinal mast cells (hiMC). Representatively pictures and densitometric analyses in % of stimulated vehicle control for Western blots of phosphorylated ERK1/2 and total ERK1/2 (a, b). Representatively, mRNA expression of CXCL8 (c, d) and CCL3 (e, f) in hiMC is shown. hiMC were incubated with 50 μ M PD98059 for 1 h, and with 45 μ M nobiletin or tangeretin or with DMSO as control for 1, 4 and 18 h. Cells were stimulated by Fc ϵ RI cross-linking for 10 min. Values are mean $\pm$ SEM. Means without a common letter differ, $p < 0.05$ ($n = 4-5$)



be reached by, e.g., consumption of orange juice in daily food, but by a concentrated administration as nutraceutical. Jang et al. [29] showed that nobiletin and tangeretin ameliorate histamine-induced scratching behavior in mice, and inhibited expression of IL-4, TNF as well as activation of NF- κ B, AP-1 and p38 in skin tissue. Nobiletin and tangeretin reduced expression of IL-4 and TNF in RBL-2H3 cells [29]. Nobiletin showed antagonistic effects in asthmatic rats

on eosinophilic airway inflammation [17]. Based on these inhibitory findings in LPS-mediated MC activation and first information from the literature concerning antiallergic properties of nobiletin and tangeretin, it was interesting to see if nobiletin or tangeretin show similar anti-inflammatory effects on MC activated by the typical activation signal via Fc ϵ RI. For MC degranulation, nobiletin and tangeretin only showed slight or no effects. The inhibitory

effects of nobiletin on release of β -hexosaminidase and LTC₄ were significant but mild, and tangeretin showed no significant reduction. Regarding expression of pro-inflammatory cytokines/chemokines, we observed that nobiletin and tangeretin reduced expression of CXCL8, IL-1 β , CCL2, CCL3, CCL4 and TNF. By down-regulation of such cytokines and by inhibition of degranulation, tangeretin and to an even higher extent nobiletin could act as an anti-inflammatory agent capable of attenuating allergic reactions. As on level of cytokine expression very strong effects could be observed for both nobiletin and tangeretin, our hypothesis was that the MAPK ERK1/2 known for relevance in cytokine expression in human MC [37] could be involved.

Analyzing the mechanism of action of nobiletin and tangeretin in inhibiting MC activation, we stimulated MC IgE-dependently and treated them with nobiletin, tangeretin or MEK-inhibitor PD98059. The MEK inhibitor showed a similar inhibitory rate compared to nobiletin or tangeretin treatment. Moreover, the phosphorylation status of ERK1/2 was down-regulated to control values by nobiletin or tangeretin. This could explain the reduced expression of chemokines like CXCL8 in response to preincubation with nobiletin/tangeretin [37]. Aoki et al. [42] revealed inhibitory effects of nobiletin treatment on C6 rat glioma cells by inhibiting ERK phosphorylation. The decreased phosphorylation status of ERK1/2 we observed for nobiletin is in accordance with Miyata et al. [43] showing an inhibitory activity of nobiletin on phosphorylation of MEK in human fibrosarcoma HT-1080 cells leading to an inhibition of phosphorylation of ERK, a downstream signaling molecule of MEK. Also for tangeretin, inhibitory effects on phosphorylation of ERK1/2 are known. Tangeretin-treated human T47D mammary cancer cells showed a time- and dose-dependent reduced phosphorylation status of ERK1/2 [44]. We found that the inhibitory capacity of nobiletin concerning ERK1/2 phosphorylation was on the same level as for the MEK-inhibitor PD98059. Therefore, ERK-regulated cytokines such as CXCL8 [37] and CCL2 were significantly and similarly down-regulated by nobiletin or tangeretin and PD98059 treatment. A co-treatment of citrus flavonoid and PD98059 resulted in no stronger inhibition. For CCL4, no inhibition of mRNA expression could be detected by PD98059, but tangeretin or nobiletin treatment led to a significantly reduced expression level which was in part also seen for the co-treatment with PD98059. This could give a hint that nobiletin and tangeretin not only act by inhibiting ERK activity but also show effects on other signaling molecules.

In summary, our results show a strongly reduced activation of hiMC after treatment with nobiletin and—to a lesser extent—tangeretin by reducing degranulation and/or expression of pro-inflammatory cytokines. The associated

signaling pathways involving ERK in IgE-dependent activation and NF- κ B in LPS/sCD14-mediated activation, which are known for being relevant in MC cytokine expression, seem to be affected. These data suggest citrus peel polymethoxyflavones nobiletin and tangeretin being potential new antiallergic components warranting further investigation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical standard Surgery tissue specimen from patients undergoing bowel resection served for isolation of mature human MC. This study has been approved by the local ethics committee and has therefore been performed in accordance with the ethical standards. All persons gave their informed consent prior to their inclusion in the study.

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