Mol. Nutr. Food Res. 2005, *49*, 431 – 442 DOI 10.1002/mnfr.200400099

Effects of resveratrol, piceatannol, tri-acetoxystilbene, and genistein on the inflammatory response of human peripheral blood leukocytes

Nathalie Richard, Debora Porath, Alexander Radspieler and Joseph Schwager

DSM Nutritional Products, Kaiseraugst, Switzerland

Inflammatory processes are involved in the etiology of diseases. We analyzed the effect of resveratrol, piceatannol, synthetic tri-acetoxystilbene (TAS), and genistein (Bonistein™) on the production of inflammatory mediators including prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), interleukins, and chemokines, which participate in the progression of inflammation. In order to induce inflammatory responses, human peripheral blood mononuclear and/or polymorphonuclear leukocytes were stimulated with lipopolysaccharide (LPS) and interferon-γ (IFNγ) and the production of PGE_2, interleukin-8 (IL-8), and TNF-α was determined. In response to the stimuli, genes were substantially activated within <2 h (*e.g.*, TNF-α, IL-1α), or at a later stage, (*e.g.*, COX-2, IL-6, IL-8). Unlike genistein, resveratrol and related compounds dose-dependently reduced PGE_2 production. Genistein, piceatannol, and TAS diminished secretion of TNF-α, and IL-8. TAS reduced mRNA levels of COX-2, TNF-α, IL-8, IL-6, and IL-1α, while resveratrol impaired early expression of IL-8 and TNF-α. Piceatannol out-performed resveratrol, yet without matching TAS. Genistein downregulated TNF-α and IL-8 expression. These substances altered the LPS/IFNγ-induced gene expression in mononuclear cells rather than in polymorphonuclear leukocytes. Immunoblot analyses corroborated the distinct activity pattern of resveratrol and genistein. In conclusion, resveratrol and their derivatives attenuated the inflammatory response of PBLs at several levels, whereas genistein acts on cytokines and pro-inflammatory interleukins.

Keywords: Cytokines / Gene expression / Inflammatory response / Peripheral blood leukocytes / Phenolic compounds / Prostaglandin E_2

Received: December 10, 2004; revised: January 14, 2004; accepted: January 26, 2004

1 Introduction

Inflammatory processes are involved in the etiology of, *e.g.*, atherosclerosis, diabetes, and arthritis. Acute inflammation encompasses three phases – initiation, progression, and resorption – which are tightly controlled by mediators. Inflammatory stimuli, such as injury, mechanical stress, or pathogen-derived components, provoke a rapid release of mediators (histamin, bradykinin, complement) which trigger the onset of the inflammatory response. During the progression, there is a massive recruitment of cells to the inflamed tissue, mainly mediated by chemokines and cytokines [1, 2]. Eventually, inflammation is self-contained by the initiation of apoptotic events in recruited cells [3, 4]. Macrophages and polymorphonuclear leukocytes (PMNLs) are the main cellular substrates, which participate in all three phases of inflammation. Interestingly, similar molecular mechanisms (*e.g.*, signalling pathways, eicosanoid metabolism) seem to be involved in the onset and resolution of inflammation [4].

The critical soluble mediators of inflammation are metabolites of arachidonic acid from which prostaglandins (PGs) and leukotrienes originate. PGE_2 is among the first mediator released from cells that are activated by bacterial constituents or specific cytokines. Concomitantly, activated cells produce cytokines (*e.g.*, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)) which participate in cell activation and recruitment, where leukotrienes and chemokines play a dominant role.

Inflammatory responses can be attenuated at different steps of the arachidonate metabolism (*e.g.*, inhibiting biosynth-

Correspondence: Dr. Joseph Schwager, DSM Nutritional Products, Würmisweg, P.O. Box 3255, CH-4303-Kaiseraugst, Switzerland
E-mail: Joseph.Schwager@dsm.com
Fax: +61 688 1640

Abbreviations: IL, interleukin; **LDH**, lactate dehydrogenase; **LPS/IFNγ**, lipopolysaccharide/interferon-γ; **MNC**, peripheral blood mononuclear cell; **PBL**, peripheral blood leukocyte; **PGE_2**, prostaglandin E_2; **PMNL**, peripheral blood polymorphonoculear leukocyte; **TAS**, tri-acetoxystilbene; **TNF-α**, tumor necrosis factor α

esis of PGs or lipoxygenase-dependent metabolites). Recently, potent inhibitors of COX-2 and therefore PGE_2 synthesis that belong to the group of nonsteroidal anti-inflammatory drugs (NSAIDs) were identified (for a review see, *e.g.*, [5]). Similarly, some natural substances such as stilbenes or catechins also affect PG biosynthesis. For example, resveratrol or piceatannol, which are found in grapes and red wine, are known to have a wide range of biological activities (anti-oxidant, anti-viral, anti-inflammatory, cardioprotective, and chemopreventive properties) [6–10]. Most of these studies have been done with appropriate cell lines, macrophages, or animal models. Yet, because effector cells of inflammation (*i.e.*, PMNL and macrophage/monocytes) are blood-borne, it is of particular interest to monitor inflammatory events in cells of the peripheral blood. Although inflammation does not build up in the dynamic environment of the blood, all necessary cellular ingredients for inflammation are contained in the blood, from which they are recruited to the sites of insults. In addition, blood cells offer a convenient source to test substances with anti-inflammatory properties *ex vivo*. In this study, the putative anti-inflammatory effect of resveratrol, piceatannol, and the isoflavone genistein on chemokine and PGE_2 production was tested in peripheral blood leukocytes (PBLs) and associated to changes in the expression of genes of the inflammatory pathway.

2 Materials and methods

2.1 Reagents

Escherichia coli LPS (serotype 055 : B5) was from Sigma, (St. Louis, MO, USA). Ficoll-Isopaque was from Nycomed Pharma AS (Oslo, Norway). RPMI medium 1640, PBS, and fetal calf serum (FCS) were from GIBCO™ (Grand Island, NY, USA). Human recombinant interferon-γ (IFNγ) was from Peprotech (London, UK). Resveratrol was purchased from Sigma or synthesized by Dr. D. Burdick, Roche Vitamins Ltd. Tri-acetoxystilbene (TAS) was synthesized by DSM Nutritional Products. Piceatannol and NS398 were purchased from Cayman Chemicals (Ann Harbor, MI, USA). Genistein (Bonistein™) was a gift of Dr. I. Bendik, DSM Nutritional Products. Primers and probes were designed with the Primer Express program (Applied Biosystems, Foster City, CA, USA) and synthesized by Genosys or by Qiagen. Antibodies were purchased from Cayman Chemicals, Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Sigma.

2.2 Isolation of peripheral blood cell populations

Human blood from healthy volunteers was sterilely collected into heparinized tubes and diluted with one volume of sterile PBS. Mononuclear cells (MNCs) were isolated by Ficoll-Isopaque gradient centrifugation (at $400 \times g$ for 30 min at room temperature) and resuspended in culture medium (RPMI medium 1640, 0.25% fetal bovine serum (FBS), nonessential amino acids (Life Technologies, Gaithersburg, MD, USA), 50 U/mL penicillin, 50 μg/mL streptomycin, 5×10^{-5} M 2-mercaptoethanol). In some cases, cell viability was determined by the Trypan Blue exclusion test. Cells were adjusted to 1×10^6 cells/mL. Alternatively, human blood was sterilely collected into heparinized tubes and mixed with one volume of dextran/NaCl solution (3% dextran in 0.9% NaCl). The diluted blood was kept at room temperature for 30 min. PBLs were recovered from the upper layer into an excess volume of PBS. Cells were centrifuged at $200 \times g$ for 10 min at 4°C. In order to lyse residual erythrocytes, the cell pellet was resuspended in 0.2% NaCl for 30 s and isotonic osmolarity re-established by adding one volume of 1.6% NaCl. Cells were washed twice, resuspended in culture medium, and adjusted to 1×10^6 cells/mL. PBLs were further separated into MNCs and PMNLs by Ficoll gradient centrifugation (at $400 \times g$ for 30 min at room temperature). MNCs were recovered from the interface and PMNLs from the cell pellet. Each cell population was washed 3 times with PBS, resuspended in culture medium, and adjusted to 1×10^6 cells/mL.

2.3 Cell stimulation and generation of supernatants

One mL of cell suspension was seeded into 12-well plates and stimulated with 100 ng/mL lipopolysaccharide (LPS) and 20 U/mL IFNγ for up to 24 h. Test substances, prepared in DMSO, were added concomitantly with LPS/IFNγ. Where appropriate, DMSO was added to adjust for the vehicle concentration. After different times of incubation cell suspension was collected and centrifuged. The cell culture supernatant was recovered and stored at –80°C until further analysis.

2.4 Cytotoxicity

Supernatants were harvested and lactate dehydrogenase (LDH) activity was determined by a spectrometric assay of unfrozen samples, using enzyme controls as standards (Sigma No. 2-E S1005). Briefly, 20 μL undiluted samples or standards was added to microtiter wells. A mixture containing β-NAD (172 mM) in Tris-acetate buffer (13.6 g/L Tris-base, 12.8 g/L KCl, 5.08 g/L L-lactate, 1 g/L NaN_2, pH 9.3) was added and the kinetics of the reaction was measured at 340 nm for 5 min. Results are expressed as mOD/min. In some cases, the total LDH contents of cells were determined by lysing cells with lysis buffer (Promega, Madison, WI, USA).

2.5 Immunoblots

Cells were lysed in NETT- C (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1% Triton X-100) and a cocktail of proteinase inhibitors (Complete™; Roche, Basel, Switzerland). Lysates were kept on ice for 15 min, sonicated (Branson Sonifier, 30% duty cycle, output control 3–4), cleared by centrifugation (20 800 × *g* for 2 min at 4°C) and stored at –80°C until use. Protein concentrations were determined with the bicinchoninic acid (BCA) reagents according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Protein samples were mixed with one volume Tricine-SDS sample buffer (Invitrogen, Carlsbad, CA, USA) containing 10% 2-mercaptoethanol and heated at 95°C for 5 min. Proteins (3–5 µg) were separated by electrophoresis in 10–20% Tricine gels (Novex, San Diego, CA, USA) and transferred to a nitrocellulose membrane. Membranes were incubated for 2 h in blocking reagent (BR) (Roche). Antibodies against actin, COX-1, COX-2, or TNF-α proteins were diluted in PBS-T/0.2 × BR (final dilution 1 : 1000). Membranes were incubated with antibodies for 2 h or overnight. Appropriate secondary antibody conjugated to horseradish peroxydase (*i.e.*, goat anti-rabbit immunoglobulin G (IgG) or donkey anti-goat IgG) was diluted 1 : 10 000 in PBS-T (PBS, 0.05% Tween 20). After incubation for 1 h, immune complexes were visualized by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ, USA).

2.6 RNA isolation and reverse transcription (RT)

RNA was extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). For reverse transcription, 1–1.5 µg total RNA was adjusted to a volume of 8 µL. One µL random hexamers (50 ng/µL) and 1 µL dNTP mix (10 mM) were added. The mixture was denatured at 70°C for 10 min and chilled on ice. cDNA synthesis was made by adding a mixture containing RT buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), DTT, $MgCl_2$, RNase OUT, and Superscript II™ RT. The final concentrations of these reagents were 20, 50, 0.1, 5 mM, and 2 U/50 µL and 2.5 U/50 µL, respectively. The samples were incubated at room temperature for 10 min, at 42°C for 50 min and at 70°C for 15 min. One µL RNase H (2 U/µL) was added per reaction followed by incubation at 37°C for 20 min. The cDNA solution was subsequently diluted to 100 µL in DEPC-treated water and stored at –80°C.

2.7 Quantitative RT-PCR

In a 50 µL PCR reaction, 3 µL cDNA (corresponding to 30–50 ng total RNA input) was amplified in a 7700 Sequence Detector, using the 2 × Universal Master Mix, 50 nM primers, and 100 nM probe (VIC-TAMRA-labeled) for the 18S rRNA internal control, and 300 nM primers and 100 nM probe (FAM-TAMRA-labeled) for the gene of interest. Primers and probe sets were designed with the Primer Express™ software. The cycle number at which the fluorescence exceeded the threshold of detection (C_T) for ribosomal RNA was subtracted from that of the target genes for each well (ΔC_T). Messenger RNA levels were then indicated as $2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T$ returns to the ΔC_T of unstimulated minus treated cells. Alternatively, the percentage change, relative to the (LPS/IFNγ)-stimulated cells, was defined as ($2^{-\Delta\Delta CT} \times 100$) where $\Delta\Delta C_T$ equaled the (LPS/IFNγ)-treated ΔC_T minus ΔC_T of the (LPS/IFNγ + compound)-treated cells.

2.8 Determination of PGE_2, interleukin-8, and TNF-α

The cell culture supernatant was harvested and the level of released prostaglandin E_2 (PGE_2) was determined with an EIA kit (Cayman Chemicals) according to the manufacturer's instructions. TNF-α concentrations in supernatants was measured with an ELISA kit purchased from R&D Systems (Wiesbaden, Germany). IL-8 was determined by ELISA (Porath *et al.*, in preparation). Briefly, Nunc immunosorb microtiter plates (Fisher Scientific, Wohlen, Switzerland) were coated with capture antibody (anti-IL-8) (Pharmigen, Basel, Switzerland). After blocking with PBS/BSA, culture supernatants were added at various dilutions for 4–12 h. Plates were then washed and incubated with biotin-conjugated anti-IL-8, followed by streptavidin-horseradish peroxidase (HRP) and the amount of immune complexes quantified using *p*-nitrophenyl phosphate as a substrate.

2.9 Statistical analysis

Two-group comparisons were performed by the Student's *t*-test for unpaired values.

3 Results

3.1 Evaluation of the impact of phenolic compounds on cell viability

In order to determine the cytotoxicity of the cell treatments and of the tested substances, cell culture supernatants were harvested and LDH activity was measured (Fig. 1). For PBLs, the amount of released LDH was similar in the different treatments (~9 to ~16 mU/mL). This reflects < 10% of total LDH activity released from lysed cells. A similar profile was obtained for PMNLs, although the amount of

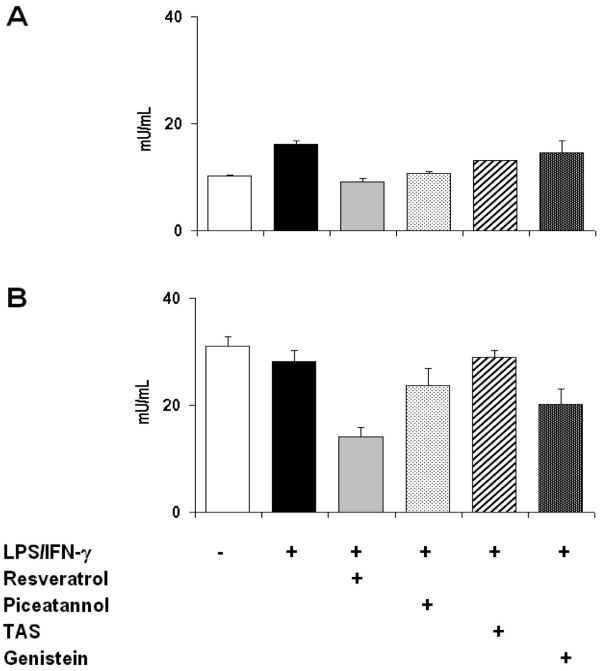

Figure 1. Effects of tested substances on the LDH release by (A) PBL and (B) PMNL after 24 h of culture. PBL (2.1×10^6 cells/mL) and PMNL (3.8×10^6 cells/mL) were stimulated with LPS (100 ng/mL) and IFNγ (20 U/mL). The amounts of released LDH are expressed in mU/mL. Where indicated, substances were added at 25 μM.

released LDH was higher. When normalized on cell numbers, cytotoxicity of the treatments was comparable for PBLs and PMNLs. The data indicate that treating cells with inflammatory stimuli and selected natural substances did not significantly alter LDH release and therefore cell viability.

3.2 PGE_2 production is markedly reduced by stilbenes

PGE_2 production by PBLs was measured to assess the *in vitro* inflammatory response. To this aim, PBLs were isolated by dextran sedimentation; this cell population was further separated into MNCs and PMNLs (see Section 2). Blood cells were stimulated with LPS and IFNγ for 24 h and the secreted PGE_2 was determined by ELISA. LPS/IFNγ-stimulated MNCs produced 5033 ± 656 pg/mL (two independent determinations), whereas PBLs, which contained also PMNLs, produced significantly less PGE_2 (Fig. 2A). Under the chosen experimental conditions, PMNLs did not produce PGE_2. Furthermore, we compared the production of PGE_2 by PBLs and MNCs from four different donors (not shown). LPS/IFNγ induced PGE_2 secretion by MNCs and PBLs; MNCs produced ~5-fold more PGE_2 than did PBLs. Since MNCs contain mono-

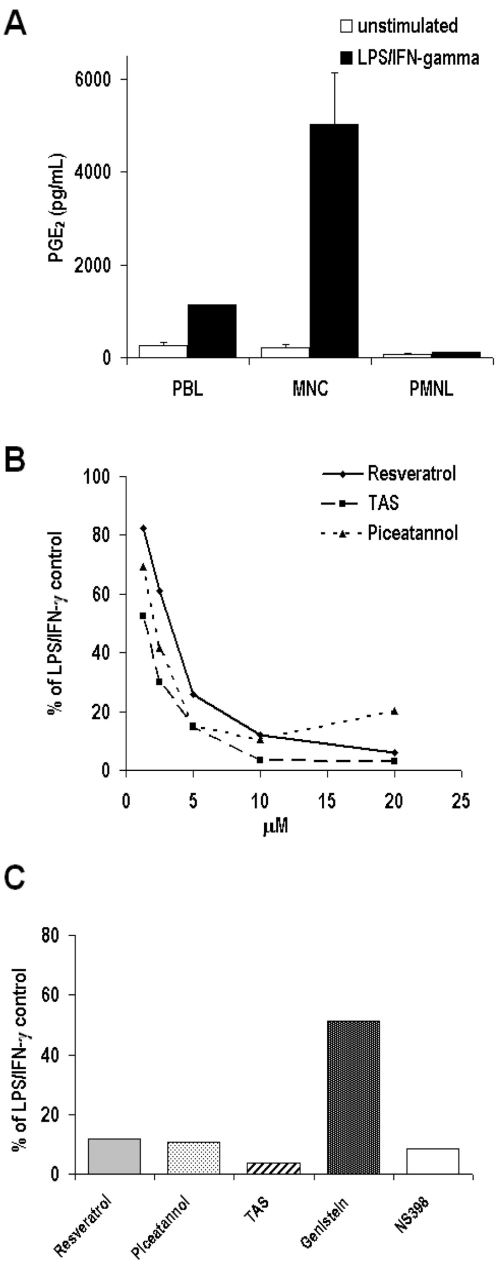

Figure 2. (A) PGE_2 production by unstimulated and LPS/IFNγ stimulated PBLs, MNCs, and PMNLs. Cells were cultured for 24 h and the amount of PGE_2 in supernatants determined by enzyme immoassay (EIA). Results from one of five independent experimental series are shown. (B) Phenolic compounds dose-dependently inhibit PGE_2 production by MNCs. Cells were cultured for 24 h without or with LPS/IFNγ in the presence of indicated concentrations of compounds. The amount of PGE_2 was determined in supernatants by EIA. Values are expressed as a percentage of PGE_2 produced by LPS/IFNγ-stimulated cells (4139 ±163 pg/mL). (C) Influence of various substances on PGE_2 production by MNCs. Cells were stimulated for 24 h without or with LPS/IFNγ in the presence of various compounds (at 10 μM). The amount of PGE_2 was determined in supernatants by EIA. The values are expressed as a percentage of PGE_2 produced by LPS/IFNγ-stimulated cells.

cytes/macrophages and thus LPS/IFNγ-responsive cells, which are the major cellular source of PGE_2, subsequent experiments focused mainly on this cell population.

In order to test whether PGE_2 production could be modulated by natural substances such as resveratrol, two related stilbenes, and isoflavones, blood cells were stimulated with LPS/IFNγ in the presence of candidate substances at various concentrations and their impact on secreted PGE_2 was determined. LPS/IFNγ-induced PGE_2 production was dose-dependently inhibited by resveratrol, piceatannol, and a synthetic derivative of resveratrol, TAS although to various degrees (Fig. 2B). IC_{50} values varied between 1.0 and 3.0 μM (TAS > piceatannol > resveratrol), indicating that TAS was the most potent inhibitor. In contrast, the IC_{50} for genistein was >10 μM. Additional substances were tested at 10 μM (Fig. 2C). Like NS398 (*i. e.*, a specific COX-2 inhibitor), stilbenes reduced significantly the PGE_2 production (to 3.5 – 16% of LPS/IFNγ control), whereas the isoflavone genistein had a less pronounced effect (maximal reduction to 51% of LPS/IFNγ control).

3.3 Phenolic compounds impair the production of IL-8 and TNF-α

Human MNCs responded to LPS/IFNγ stimulation by a burst of secreted TNF-α (Fig. 3A), while unstimulated cells did not secrete this cytokine. At 10 μM, resveratrol inhibited TNF-α production by only 20%, whereas piceatannol or TAS had significant effects. In marked contrast to its consequence on PGE_2 production, genistein abrogated cytokine production. Using PBLs as cellular substrate, similar results were obtained (data not shown). Upon activation, PBLs (not shown) and MNCs secreted large amounts of IL-8 (Fig. 3B). IL-8 production was unaltered by resveratrol, whereas piceatannol and TAS (at 10 μM) inhibited IL-8 production by >60%. Unlike its effect on TNF-α, genistein only moderately impaired the levels of secreted IL-8. Collectively, stilbene compounds and genistein exerted different and even opposed effects on the production of PGE_2, chemokines, and cytokines.

3.4 Inflammatory stimuli induced dramatic increases in mRNA levels of different genes in blood cell populations

During the onset of inflammation, a large panel of genes are activated. These changes were determined by quantifying mRNA levels with RT-PCR technology in unstimulated and stimulated cells. In order to evaluate basal gene expression in PBLs, MNCs, and PMNLs, mRNA levels relative to rRNA were determined in unstimulated cells (Fig. 4). Compared to PBLs and MNCs, PMNLs contained more COX-2

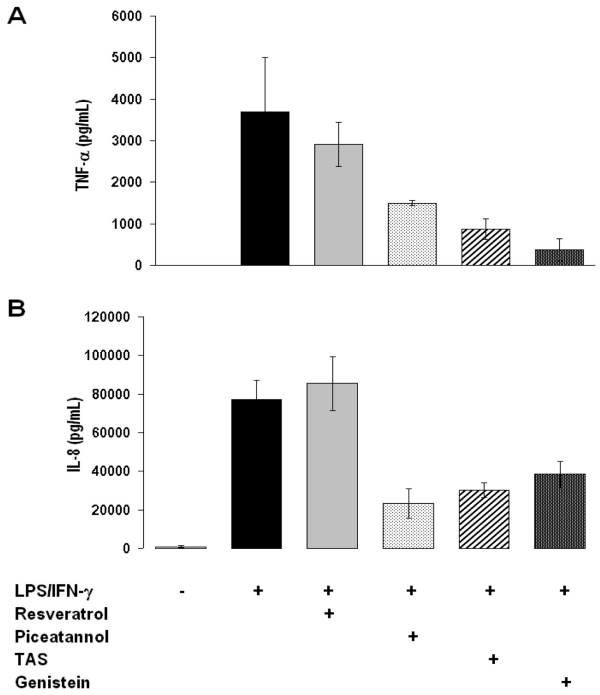

Figure 3. Effect of stilbenes and genistein on cytokine and chemokine production. (A) Levels of TNF-α in supernatants of unstimulated and stimulated MNC cultured for 24 h in the absence or presence of the indicated compounds (at 10 μM). (B) Levels of IL-8 in supernatants of unstimulated and stimulated MNCs cultured for 24 h in the absence or presence of the indicated compounds (at 10 μM). Similar results were obtained in an independent experimental series.

and IL-8 mRNA. In the case of IL-6, IL-1α, and IL-1β expression, MNCs and PBLs revealed almost identical amounts of mRNA but 10-fold more than did PMNLs. With regard to TNF-α and 5-lipoxygenase (5-LOX), expression was similar in all three cell populations.

Stimulation of cells with LPS/IFNγ upregulated numerous genes in MNCs and PBLs, but less in PMNLs (Fig. 5). The changes observed in PBLs roughly amounted to the sum of changes in MNCs and PMNLs (see, *e. g.*, IL-1α, IL-1β, or IL-6 in Fig. 5). The induction of COX-2 expression was comparable in MNCs and PBLs (~20-fold increase) but weak in PMNLs. Stimulated PBLs and MNCs produced similar amounts of TNF-α mRNA, which exceeded that of PMNLs 3-fold. Although the induction of IL-8 was low, PBLs had higher IL-8 mRNA than did MNCs and PMNLs. In comparison to these, stimulated PBLs contained more IL-6, IL-1α, and IL-1β mRNA. While we observed a decrease of the 5-LOX gene expression in all analyzed cell populations (Fig. 5), mRNA levels for other genes (*e. g.*, COX-1, PPAR-β) were unaffected by LPS/IFNγ stimulation (not shown).

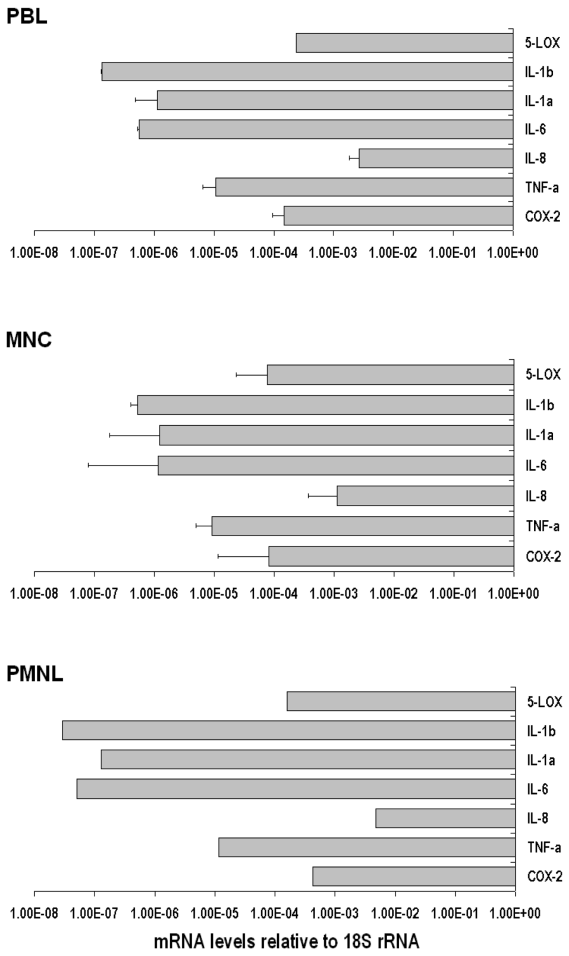

Figure 4. Expression of selected genes in leukocytes (*i.e.*, total peripheral blood leukocytes, MNCs, and PMNLs). RNA was isolated from cells which were cultured for 2 h and expression quantified by RT-PCR. The mRNA level in unstimulated cells is indicated relative to 18S rRNA.

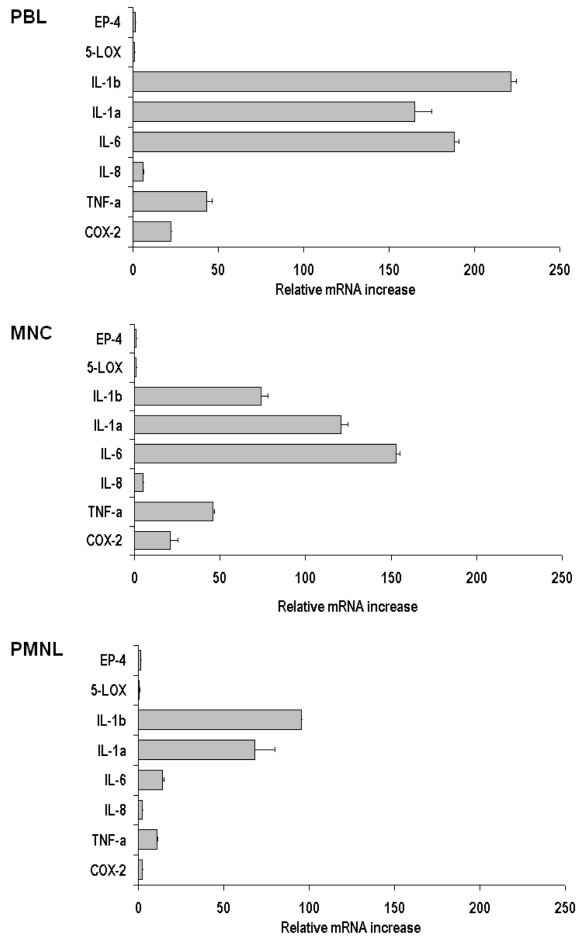

Figure 5. Effect of LPS/IFNγ stimulation on gene expression. The indicated cell populations were cultured without or with LPS/IFNγ for 2 h. The gene expression was determined by quantitative RT-PCR and the increase of mRNA quantities (relative to unstimulated cells) calculated as described in Section 2.

3.5 Kinetics of the expression of genes during the inflammatory response and the influences of natural substances

The kinetics of expression of various genes in LPS/IFNγ-stimulated MNCs was determined and is shown in Fig. 6. Induction of TNF-α gene expression was vigorous and early; it peaked at 4 h of stimulation. For COX-2 and IL-6 genes, there was a continuous increment of mRNA levels during the 6 h period studied. In the case of IL-1α and IL-8 genes, expression augmented until 4 h followed by a decrease at 6 h. Comparable features were observed for at least ten additional genes (data not shown).

Next, we determined the impact of phenolic compounds on gene expression in MNCs at various time points (Fig. 6). Clearly, the TNF-α mRNA level was most affected by the tested phenolic compounds. In particular, TAS and genistein reduced the expression almost completely. In contrast, resveratrol, piceatannol, and genistein had only a small impact on COX-2 mRNA levels, while TAS significantly diminished the COX-2 expression at each time point. Concerning IL-8 expression, only TAS had a strong effect at each time point. For the IL-6 gene, substances (except TAS) increased IL-6 mRNA levels in stimulated MNCs. In the case of the IL-1α gene, the tested compounds (except TAS) reduced its expression only at early phases (up to 4 h) post stimulation.

3.6 Dose-dependent effects of resveratrol and TAS on gene expression

The effect of various concentrations of resveratrol and TAS on expression levels was tested during a 4 h incubation period (Fig. 7). For COX-2 gene, LPS/IFNγ stimulation

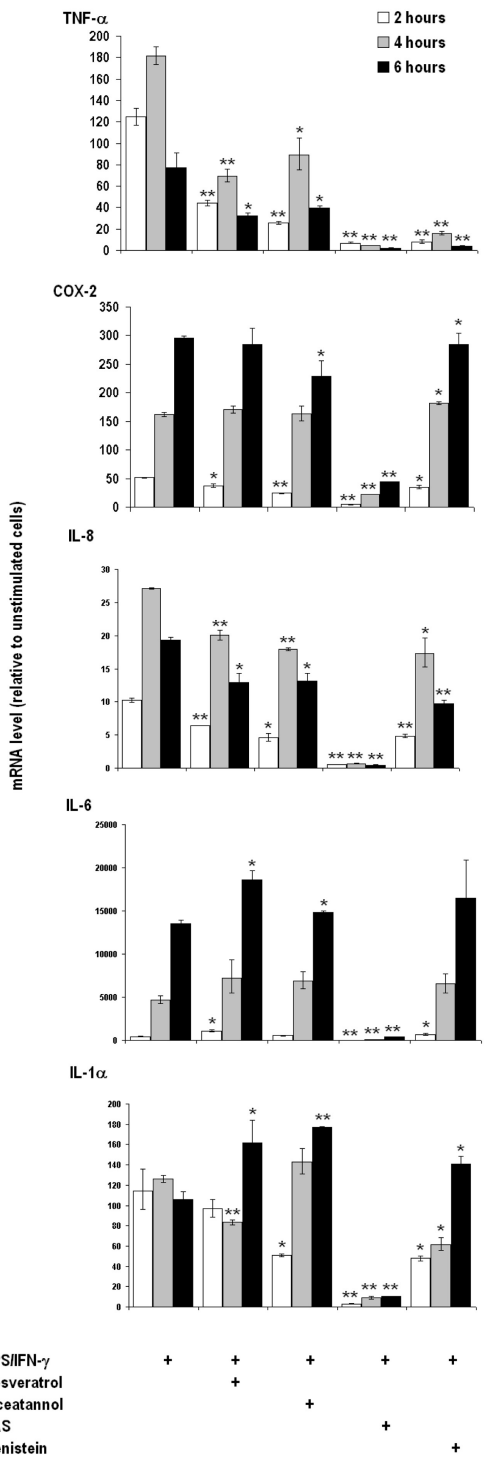

Figure 6. Kinetics of gene expression in LPS/IFNγ-stimulated blood leukocytes. The expression of different genes in MNC was determined by quantitative RT-PCR. After 2, 4, and 6 h of culture and the changes relative to time-matched unstimulated cells calculated as described. Where indicated, cells were stimulated in the presence of compounds (at 25 μM). *$p < 0.1$, ** $p < 0.01$ when compared to time-matched stimulated cells.

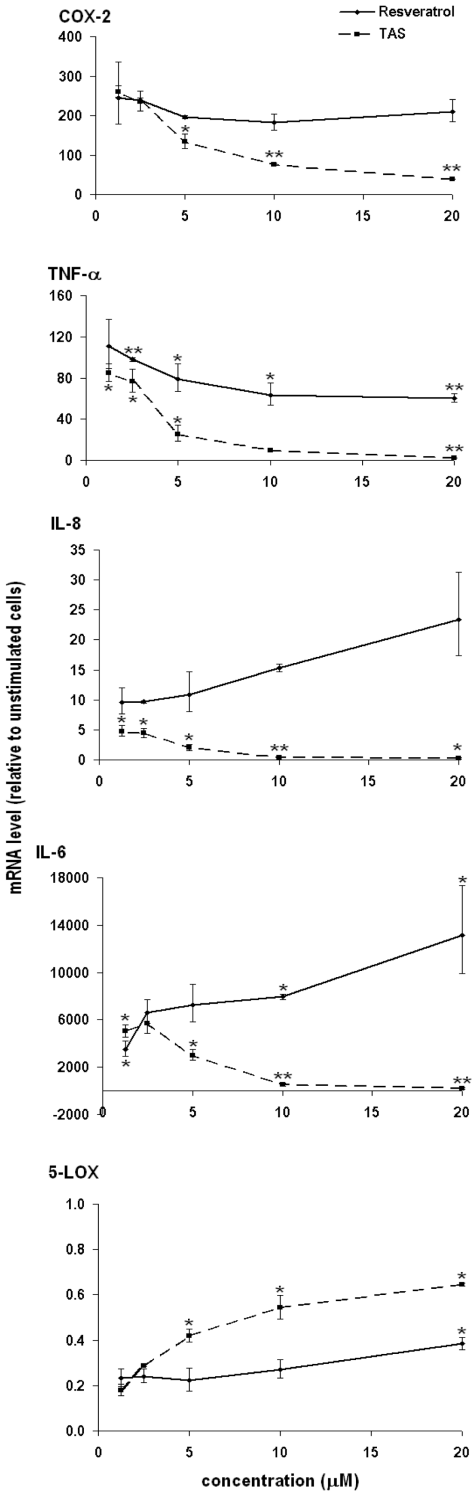

Figure 7. Dose-dependent effects of resveratrol and TAS on gene expression. MNCs were stimulated for 4 h with LPS/IFNγ in the presence of different concentrations of resveratrol and TAS. mRNA levels were quantified relative to unstimulated cells. See text for relative amounts of mRNA in LPS/IFNγ-stimulated cells. * $p < 0.1$, ** $p < 0.01$ when compared to stimulated cells.

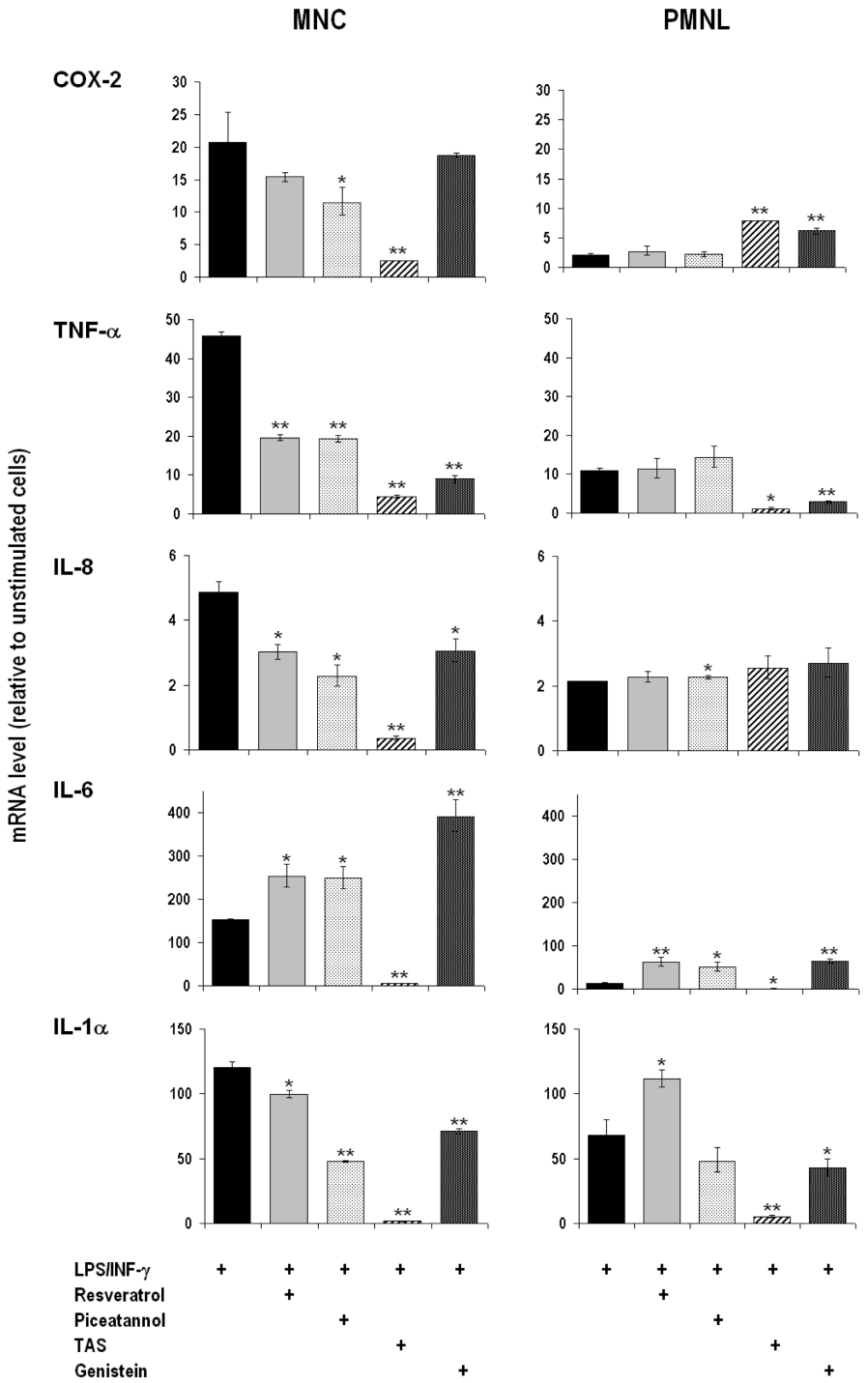

Figure 8. Effects of different substances on the expression of genes in MNCs and PMNLs. Cells were cultured for 2 h with or without LPS/IFNγ in the presence or absence of the indicated compounds (25 μM). The gene expression was quantified as described in the previous legend.
* $p < 0.1$, ** $p < 0.01$ when compared to stimulated cells.

resulted in >200-fold increase of mRNA relative to unstimulated cells. Consistent with the data presented above, resveratrol had no major effect on the COX-2 expression, even at high concentration. Conversely, TAS (at >5 μM) diminished this expression by >50%. As to TNF-α, stimulation of cells with LPS/IFNγ enhanced its expression (130-fold increase). Resveratrol and TAS reduced it dose-dependently, but the inhibition was more marked by TAS. IL-6 mRNA levels drastically rose by LPS/IFNγ stimulation (6400-fold increase). TAS impeded it in a dose-dependent way. Conversely, resveratrol modulated IL-6 expression at low and enhanced it at high concentration. For IL-8 gene, we observed a 16-fold mRNA increase by LPS/IFNγ stimulation. Unlike resveratrol, TAS lowered IL-8 expression at

each tested concentration. In the case of 5-LOX expression, LPS/IFNγ stimulation resulted in a downregulation of the gene (to ~20% of the expression level in unstimulated cells). The addition of either substance enhanced the gene expression towards prestimulation values.

3.7 MNCs and PMNLs are distinct target populations for anti-inflammatory substances

We further compared the effects of the selected substances on activated MNCs and PMNLs. To this aim, cells were stimulated without or with 25 μM of the compounds. Gene expression was analyzed after 2 h (Fig. 8). With regard to mRNA levels in stimulated MNCs, the impact of phenolic compounds matched the results obtained at other time points and concentrations (see Figs. 6 and 7), confirming the ranking of compounds according to their global impact: TAS > piceatannol > resveratrol. Genistein revealed an activity pattern similar to that of resveratrol or piceatannol, with the notable exception on TNF-α expression. In contrast, LPS/IFNγ stimulation of PMNLs upregulated the respective genes to a much lower extent (see also Fig. 5). This is best illustrated for COX-2 and IL-6 and is further indicative for efficient although not complete separation of mononuclear cells from polymorphonuclear cells. In PMNLs, the tested substances had no inhibitory effect on the expression of COX-2, or IL-8. Taken together, the data reveal that expression of inflammatory response genes in MNCs and PMNLs follows a cell-specific pattern and that PMNLs are rather inert to modulatory effects of anti-inflammatory compounds.

3.8 Effects of natural substances on the protein expression of COX-2 and TNF-α

In order to determine the expression of proteins related to the *in vitro* inflammatory response, lysates were prepared from cells cultured for 24 h and the amount of TNF-α and COX-2 determined by immunoblot analysis. Unlike unstimulated cells, LPS/IFNγ-stimulated cells contained readily detectable quantities of COX-2 and TNF-α proteins (Fig. 9). This corroborates that in PBLs, the LPS-induced PGE_2 production was COX-2 dependent. Cells stimulated in the presence of resveratrol, NS398, or genistein (at 10 μM) expressed comparable amounts of COX-2 protein; yet, at 50 μM genistein diminished cellular COX-2. Immunoblot analysis also revealed the presence of TNF-α protein in stimulated – but not in unstimulated – cells (Fig. 9). Resveratrol and genistein (at 30 μM and 50 μM) decreased the amount of TNF-α protein; unexpectedly, TNF-α protein expression was also affected by NS398.

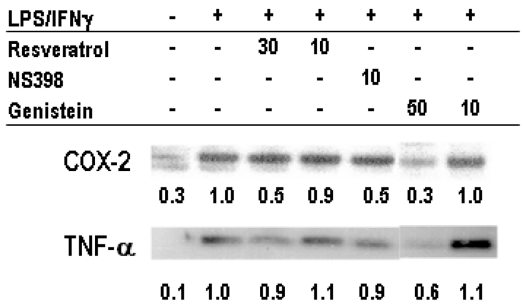

Figure 9. Immunoblot analysis of unstimulated and stimulated MNC. Cells were cultured for 24 h at the indicated conditions and lysates prepared. Total cellular proteins were analyzed for TNF-α (upper panel) and COX-2 (lower panel). Concentrations of the tested compounds are given in μM. The blot was further incubated with anti-actin (not shown) and the obtained signal used to normalize for the protein load. Numbers below the lanes indicate the ratio of TNF-α and COX-2 compared to the amount detected in LPS/IFNγ-stimulated cells.

4 Discussion

In this study, we present evidence that stilbenes and genistein modulated the inflammatory response of peripheral blood leukocytes. The effects of these substances were determined at various levels: (i) expression of genes induced by inflammatory stimuli, (ii) effect on protein expression, and (iii) amount of produced metabolites that are associated with inflammation. Resveratrol, piceatannol, and TAS markedly diminished COX-2-dependent PGE_2 production. In contrast, the flavonoid genistein had only a marginal effect on the PGE_2 production; yet, it influenced the expression of genes involved in the cytokine/interleukin pathways. These observations suggest multiple modes of action of the substances on cellular metabolism.

Stimulation of peripheral blood leukocytes has been a versatile tool to evaluate effects of compounds on eicosanoid metabolites (see, *e.g.* [11, 12]), while various cell lines or macrophages were widely used to determine the effects on cell cycle and regulation of gene expression. Phenolic compounds inhibited COX-1 and COX-2 PGE_2 enzyme activities (Raederstorff, unpublished data; [6, 7]). Both cyclooxygenases are bifunctional and have cyclooxygenase and peroxidase activity [13]. Resveratrol is a cosubstrate for COX-2, while it is a potent inhibitor of the peroxidase activity in COX-1; tri-methoxystilbene, which is structurally closely related to TAS, had no inhibitory effect on COX-1 or COX-2 [14]. Under our experimental conditions, COX-1 could not be detected in activated PBLs after a 24 h culture period suggesting that produced PGE_2 was entirely due to COX-2 activity. Cellular COX-2 contents were not altered by the substances after a 24 h culture period. This contrasts

with data obtained from peritoneal macrophages that were stimulated for 2 h only [15] and where resveratrol markedly reduced the amount of cellular COX-2. It is possible that resveratrol solely slowed down the synthesis of the respective proteins and that at >12 h culture period the delay was compensated. Since after prolonged culture periods COX-2 gene and protein expression were not markedly affected by the phenolic compounds tested, we conclude that the inhibition of enzyme activity in PBLs largely accounts for the observed abrogation of PGE_2 secretion. Conversely, genistein did not inhibit PGE_2 production (Fig. 2 and [16]). Genistein's effect seems to be species-specific, since we observed that the eicosanoid production by RAW 264.7 cells, a murine macrophage line, was sensitive to genistein, whereas human THP-1 cells or PBLs were refractory to the isoflavone.

In PBLs, LPS/IFNγ induced the release of ILs and cytokines. Here, we show that activated MNCs produced TNF-α and IL-8, as did PMNLs which are recruited by IL-8 to the sites of injury [17 – 20, 21]. The production of these inflammatory mediators was impaired by piceatannol, TAS, and genistein but not by resveratrol. This reflects distinct effects of these compounds on cellular functions. Piceatannol and genistein are tyrosine kinase inhibitors [22]; this shared property might account for the inhibition of TNF-α and IL-8 [23, 24]. Phenolic compounds tested in this study also revealed cell-specific differences in modulating cytokine expression (Fig. 8). It is plausible that in MNCs and PMNLs the activation of cytokines is ruled by distinct mechanisms.

LPS/IFNγ stimulation induced rapid and vigorous expression of TNF-α and IL-1α, whereas COX-2 and IL-6 were activated later (Fig. 6). Concomitantly, early induced genes were more susceptible to the inhibitory activity of the tested substances than genes that were upregulated with a delay of some hours. As illustrated for TNF-α, the consequences of reducing mRNA levels are far reaching, since they affect the TNF-α protein production (Fig. 9). This observation could be explained by a short transient effect of compounds thus impeding early gene activation, followed by a rapid loss of their ability to influence gene transcription, that occurs relatively late after cell stimulation. Alternatively, delayed gene upregulation implies the involvement of multiple regulatory elements or IL-cytokine cross-talks that are elusive to the influence of one substance [25, 26].

Various studies have shown the impact of stilbenes and isoflavones on NF-κB signalling pathway (for a review see, *e. g.*, [27]). Antioxidants prevent the dissociation of NF-κB/I-κB complexes, and thus gene activation. In the human macrophage cell line U937, piceatannol acts on the nuclear transcription factor NF-κB and therefore on NF-κB dependent expression of, *e. g.*, COX-2 [28]. lt also impaired IKK activation, phosphorylation of I-κBα, NF-κBp65, and its subsequent nuclear translocation. In contrast, resveratrol inhibited the TNF-induced NF-κBp65 activation and prevented kinase activities including c-Jun and MEK activation [29]. In human monocytes, inhibition of the extracellular signal-regulated kinase (ERK)/MAP kinase pathway resulted in a coordinate reduction of cytokine, IL and PGE_2 secretion [30, 31]. Presumably, some of the substances tested in this study interfere in the cellular pathways of MNCs and PMNLs at a similar level. Conversely, since TAS lacks hydroxyl groups and therefore antioxidant properties, it is thought to affect transcription factors by a different mode of action. Collectively, these observations suggest that stilbenes and genistein modulate the inflammatory response in at least two ways: (i) the amplitude of inflammation is attenuated due to the reduced production of PGE_2 and cytokines; (ii) chronic inflammation due to excessive or uncontrolled cytokine and IL production is contained.

It is noteworthy that the regulation of IL-1α and IL-6 by the tested compounds is somehow controversial. IL-1α is a potent pro-inflammatory cytokine, as evidenced by its upregulation in LPS/IFNγ-activated PBL. Except resveratrol, all tested substances impaired its expression. In contrast, IL-6, which is a pro-inflammatory IL for, *e. g.*, hepatocytes, was the only gene, where all substances (with the exception of TAS) augmented mRNA levels in blood cells. Since IL-6 acts as a differentiation factor for B lymphocytes, its increased expression might be beneficial for an improved immune response by circulating B lymphocytes. This is reminiscent of the opposed effect of resveratrol on nitric oxide production in endothelial and macrophages ([32] and unpublished observations).

The physiological relevance of these data has to be examined in the light of achievable plasma concentrations of the tested phenolic compounds by dietary intake. Absorption of resveratrol in healthy human subjects revealed that nanomolar to micromolar plasma concentrations were achieved [33, 34]. In individuals that consumed 60 mg genistein, plasma levels of 2 – 3 μM were measured (our unpublished results). These values contrast with efficacious concentrations used in most *in vitro* experiments including those used in this study, where significant effects were observed at concentrations >10 μM. Nevertheless, *in vivo* effects like those suggested for resveratrol and isoflavones might result from the synergism of multiple subtle effects at sub-threshold concentrations.

In conclusion, we showed in this study that natural substances or derivatives thereof attenuate the inflammatory response of peripheral blood leukocytes by modulating the expression of pro-inflammatory genes and the PGE_2 production. Based on the obtained results, TAS, a synthetic derivative of resveratrol, is the most potent substance with a large panel of effects. Stilbenes appear to influence cell

metabolism at a relatively high hierarchical level and thus exert pleiotropic effects, whereas genistein, a representative of isoflavones, acts more specifically on cytokines and pro-inflammatory interleukins. We infer that the two groups of natural substances will unveil different potentials in treatment and prevention of inflammatory processes.

We are grateful to Drs. I. Bendik and D. Burdick for compounds and reagents, Dr. D. Raederstorff for continuous interest in the work and sharing unpublished data. We thank Drs. Bendik and Raederstorff for critical reading and commenting the manuscript.

5 References

[1] Libby, P., Inflammation in atherosclerosis. *Nature* 2002, *420*, 868 – 874.

[2] Imhof, B. A., Aurrand-Lions, M., Adhesion mechanisms regulating the migration of monocytes. *Nat. Rev. Immunol.* 2004, *4*, 432 – 444.

[3] James, M. J., Penglis, P. S., Caughey, G. E., Demasi, M., Cleland, L. G., Eicosanoid production by human monocytes: does COX-2 contribute to a self-limiting inflammatory response? *Inflamm. Res.* 2001, *50*, 249 – 253.

[4] Lawrence, T., Willoughby, D. A., Gilroy, D. W., Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat. Rev. Immunol.* 2002, *2*, 787 – 795.

[5] Fitzgerald, G. A., Coxibs and cardiovascular disease. *N. Engl. J. Med.* 2004, *351*, 1709 – 1711.

[6] Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., *et al.*, Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 1997, *275*, 218 – 220.

[7] Subbaramaiah, K., Chung, W. J., Michaluart, P., Telang, N., *et al.*, Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J. Biol. Chem.* 1998, *273*, 21875 – 21882.

[8] Fremont, L., Biological effects of resveratrol. *Life Sci.* 2000, *66*, 663 – 673.

[9] Park, J. W., Choi, Y. J., Jang, M. A., Lee, Y. S., *et al.*, Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G2 phases of the cell cycle in U937 cells. *Cancer Lett.* 2001, *163*, 43 – 49.

[10] Wu, J. M., Wang, Z. R., Hsieh, T. C., Bruder, J. L., *et al.*, Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine (Review). *Int. J. Mol. Med.* 2001, *8*, 3 – 17.

[11] Brideau, C., Kargman, S., Liu, S., Dallob, A. L., *et al.*, A human whole blood assay for clinical evaluation of biochemical efficacy of cyclooxygenase inhibitors. *Inflamm. Res.* 1996, *45*, 68 – 74.

[12] Chan, C. C., Boyce, S., Brideau, C., Charleson, S., *et al.*, Rofecoxib [Vioxx, MK-0966; 4-(4′-methylsulfonylphenyl)-3-phenyl-2-(5*H*)-furanone]: a potent and orally active cyclooxygenase-2 inhibitor. Pharmacological and biochemical profiles. *J. Pharmacol. Exp. Ther.* 1999, *290*, 551 – 560.

[13] Smith, W. L., DeWitt, D. L., Garavito, R. M., Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.* 2000, *69*, 145 – 182.

[14] Szewczuk, L. M., Forti, L., Stivala, L. A., Penning, T. M., Resveratrol is a peroxidase-mediated inactivator of COX-1 but not COX-2: a mechanistic approach to the design of COX-1 selective agents. *J. Biol. Chem.* 2004, *279*, 22727 – 22737.

[15] Martinez, J., Moreno, J. J., Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. *Biochem. Pharmacol.* 2000, *59*, 865 – 870.

[16] Yamaki, K., Kim, D. H., Ryu, N., Kim, Y. P., *et al.*, Effects of naturally occurring isoflavones on prostaglandin E2 production. *Planta Med.* 2002, *68*, 97 – 100.

[17] Khansari, N., Chou, Y. K., Fudenberg, H. H., Human monocyte heterogeneity: interleukin 1 and prostaglandin E2 production by separate subsets. *Eur. J. Immunol.* 1985, *15*, 48 – 51.

[18] Wertheim, W. A., Kunkel, S. L., Standiford, T. J., Burdick, M. D., *et al.*, Regulation of neutrophil-derived IL-8: the role of prostaglandin E2, dexamethasone, and IL-4. *J. Immunol.* 1993, *151*, 2166 – 2175.

[19] Kimura, T., Iwase, M., Kondo, G., Watanabe, H., *et al.*, Suppressive effect of selective cyclooxygenase-2 inhibitor on cytokine release in human neutrophils. *Int. Immunopharmacol.* 2003, *3*, 1519 – 1528.

[20] Hessle, C. C., Andersson, B., Wold, A. E., Gram-negative, but not Gram-positive, bacteria elicit strong PGE2 production in human monocytes. *Inflammation* 2003, *27*, 329 – 332.

[21] Rotondo, S., Rajtar, G., Manarini, S., Celardo, A., *et al.*, Effect of trans-resveratrol, a natural polyphenolic compound, on human polymorphonuclear leukocyte function. *Br. J. Pharmacol.* 1998, *123*, 1691 – 1699.

[22] Foreback, J. L., Sarma, V., Yeager, N. R., Younkin, E. M., *et al.*, Blood mononuclear cell production of TNF-alpha and IL-8: engagement of different signal transduction pathways including the p42 MAP kinase pathway. *J. Leukoc. Biol.* 1998, *64*, 124 – 133.

[23] Oliver, J. M., Burg, D. L., Wilson, B. S., McLaughlin, J. L., Geahlen, R. L., Inhibition of mast cell Fc epsilon R1-mediated signaling and effector function by the Syk-selective inhibitor, piceatannol. *J. Biol. Chem.* 1994, *269*, 29697 – 29703.

[24] Muhl, H., Chang, J. H., Huwiler, A., Bosmann, M., *et al.*, Nitric oxide augments release of chemokines from monocytic U937 cells: modulation by anti-inflammatory pathways. *Free Radic. Biol. Med.* 2000, *29*, 969 – 980.

[25] Maloney, C. G., Kutchera, W. A., Albertine, K. H., McIntyre, T. M., *et al.*, Inflammatory agonists induce cyclooxygenase type 2 expression by human neutrophils. *J. Immunol.* 1998, *160*, 1402 – 1410.

[26] Poligone, B., Baldwin, A. S., Positive and negative regulation of NF-kappaB by COX-2: roles of different prostaglandins. *J. Biol. Chem.* 2001, *276*, 38658 – 38664.

[27] Surh, Y. J., Chun, K. S., Cha, H. H., Han, S. S., *et al.*, Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. *Mutat. Res.* 2001, *480–481*, 243 – 268.

[28] Ashikawa, K., Majumdar, S., Banerjee, S., Bharti, A. C., *et al.*, Piceatannol inhibits TNF-induced NF-kappaB activation and NF-kappaB-mediated gene expression through suppression of IkappaBalpha kinase and p65 phosphorylation. *J. Immunol.* 2002, *169*, 6490 – 6497.

[29] Manna, S. K., Mukhopadhyay, A., Aggarwal, B. B., Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *J. Immunol.* 2000, *164*, 6509 – 6519.

[30] Scherle, P. A., Jones, E. A., Favata, M. F., Daulerio, A. J., *et al.*, Inhibition of MAP kinase kinase prevents cytokine and prostaglandin E2 production in lipopolysaccharide-stimulated monocytes. *J. Immunol.* 1998, *161*, 5681 – 5686.

[31] Subbaramaiah, K., Dannenberg, A. J., Cyclooxygenase 2: a molecular target for cancer prevention and treatment. *Trends Pharmacol. Sci.* 2003, *24*, 96 – 102.

[32] Imamura, G., Bertelli, A. A., Bertelli, A., Otani, H., *et al.*, Pharmacological preconditioning with resveratrol: an insight with iNOS knockout mice. *Am. J. Physiol. Heart Circ. Physiol.* 2002, *282*, H1996 – 2003.

[33] Goldberg, D. M., Yan, J., Soleas, G. J., Absorption of three wine-related polyphenols in three different matrices by healthy subjects. *Clin. Biochem.* 2003, *36*, 79 – 87.

[34] Walle, T., Hsieh, F., Delegge, M. H., Oatis, J. E. Jr., Walle, U. K., High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos.* 2004, *32*, 1377 – 1382.

