Biochemical and Molecular Actions of Nutrients

Garlic (*Allium sativum* L.) Modulates Cytokine Expression in Lipopolysaccharide-Activated Human Blood Thereby Inhibiting NF-κB Activity[1]

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ABSTRACT Garlic is proposed to have immunomodulatory and anti-inflammatory properties. This paper shows that garlic powder extracts (GPE) and single garlic metabolites modulate lipopolysaccharide (LPS)-induced cytokine levels in human whole blood. GPE-altered cytokine levels in human blood sample supernatants reduced nuclear factor (NF)-κB activity in human cells exposed to these samples. Pretreatment with GPE (100 mg/L) reduced LPS-induced production of proinflammatory cytokines interleukin (IL)-1β from 15.7 ± 5.1 to 6.2 ± 1.2 μg/L and tumor necrosis factor (TNF)-α from 8.8 ± 2.4 to 3.9 ± 0.8 μg/L, respectively, whereas the expression of the anti-inflammatory cytokine IL-10 was unchanged. The garlic metabolite diallydisulfide (1–100 μmol/L) also significantly reduced IL-1β and TNF-α. Interestingly, exposure of human embryonic kidney cell line (HEK293) cells to GPE-treated blood sample supernatants (10 or 100 mg/L) reduced NF-κB activity compared with cells exposed to untreated blood supernatants as measured by a NF-κB-driven luciferase reporter gene assay. Blood samples treated with extract obtained from unfertilized garlic (100 mg/L) reduced NF-κB activity by 25%, whereas blood samples treated with sulfur-fertilized garlic extracts (100 mg/L) lowered NF-κB activity by 41%. In summary, garlic may indeed promote an anti-inflammatory environment by cytokine modulation in human blood that leads to an overall inhibition of NF-κB activity in the surrounding tissue. J. Nutr. 133: 2171–2175, 2003.

KEY WORDS: • *garlic* • *cytokines* • *nuclear factor-κB*

Since ancient times garlic (*Allium sativum* L.) has been used as a medicinal plant. Garlic was listed in the Codex Ebers, an Egyptian medical papyrus, for the treatment of a diversity of disorders including heart disease, headache and tumors (1). Although the medical properties of garlic have been intensively studied during the last 100 years, it remains largely unclear how garlic mediates its effects. To date, antithrombotic, lipid-lowering, antitumoral and antioxidant properties of garlic have been demonstrated (1–4). In addition, there is some evidence for an immunomodulatory effect of garlic or selected garlic constituents showing increased T-lymphocyte blastogenesis and phagocytosis, as well as modulation of cytokine production in vitro and in vivo (5–8). *S*-Allylcysteine, the major compound of aged garlic extract (AGE), seems to mediate an inhibitory effect on the transcription factor nuclear factor κB (NF-κB)[3] (9,10), an important player in the regulation of the immune system. NF-κB is a central transcription factor in adaptive immunity and is one central regulator of proinflammatory gene expression. It is strongly involved in inflammatory diseases such as arthritis, inflammatory bowel disease and atherosclerosis (11).

Therefore, the aim of the present study was to examine whether garlic powder extracts (GPE) and selected garlic constituents affect the production of pro- and anti-inflammatory cytokines in a human whole-blood ex vivo model, and whether these altered cytokine levels result in decreased NF-κB activity in cells exposed to GPE-treated cell-free blood sample supernatants.

Because there is an ongoing debate concerning whether organosulfur compounds or other constituents, such as flavonoids, mediate the protective effects attributed to garlic, we investigated the effect of sulfur fertilization on the immunomodulatory effects mediated by GPE.

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[3] AGE, aged garlic extract; allyl-Cys, allyl-cysteine; DADS, diallyl disulfide; DLR, dual luciferase reporter-gene assay; DMSO, dimethyl sulfoxide; GPE, garlic powder extract; γ-Glu-allyl-Cys, γ-glutamyl-allyl-cysteine; HEK 293, human embryonic kidney cell line 293; IL, interleukin; INF-γ, interferon-γ; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; SAC, *S*-allylcysteine; TNF-α, tumor necrosis factor-α.

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MATERIALS AND METHODS

Materials. The plasmids used were pNF-κBluc, pFC-Mekk (Stratagene, Heidelberg, Germany), pEGFP-N1 (Clontech, Heidelberg, Germany) and pRL-TK (Promega, Heidelberg, Germany). Dried garlic powders of unfertilized and sulfur-fertilized (SO_4^{2-} 2.0 × 10^{-2} U/m^2 ≈ 3.4 × 10^{-2} kg/m^2 $CaSO_4$) *Allium sativum* cv Printanor were supplied by INRA, Dijon, France. Fertilization and cultivation of garlic plants as well as sampling was performed as described earlier (12). Dried garlic powder was suspended in dimethyl sulfoxide (DMSO) for 30 min. The insoluble residue was separated by centrifugation (24,000 × g, 30 s) and the supernatant was either used directly or further diluted to the concentration needed. Diallyldisulfide (DADS) was supplied by Sigma-Aldrich (Taufkirchen, Germany) and purified by distillation. Allicin (86% purity) was synthesized by oxidation of diallyldisulfide with peracetic acid as described earlier (13). DADS and allicin were stored at −87°C until use. Allicin was diluted in water to appropriate concentrations and DADS was diluted in DMSO.

Cell culture. The human embryonic kidney cell line 293 (HEK293; DSMZ-German collection of microorganisms and cell cultures, ACC 305) was grown in DMEM (Biowitthaker, Heidelberg, Germany) supplemented with 10% fetal calf serum (Biochrom KG, Berlin, Germany), 2 mmol/L glutamine (Merck, Munich, Germany) and penicillin/streptomycin (PAN-Biotech; Aidenbach, Germany). Cells were split 1:10 when they reached ~85–90% confluence using 0.05% trypsin/0.02% EDTA in PBS.

Analysis of total sulfur content of garlic powder. After the conversion of organic sulfur to sulfate, total sulfur was determined (14) as follows: a 1-g sample of dried garlic powder was ashed at 450°C in the presence of 10 mL of 2.7 mol/L $Mg(NO_3)_2$. The ash was then boiled for 2 min in 10 mL of concentrated hydrochloric acid (36 mol/100 mol), diluted in distilled water to dissolve the sulfate, filtered to remove insoluble material and the volume corrected to 100 mL. A 3-mL aliquot was analyzed for sulfate content using an inductively coupled plasma optical emission spectrophotometer (Jobin Yvon, Middlesex, UK), calibrated using a 574 μmol/L K_2SO_4 standard stock solution.

HPLC-UV analysis of garlic powders. The quantitative and qualitative analysis of the sulfur compounds in garlic dry powders were performed by ion-pair HPLC and UV detection. For sample preparation, 0.4 g of the powder was carefully ground and suspended in 20 mL of a 20 mmol/L phosphate buffer at pH 6.8 for 5 min in a shaker (noninhibiting condition for the alliinase), and 0.4 g of the powder was carefully ground and suspended in 20 mL of a mixture of methanol/water (80:20, v/v) additionally containing 0.05% of formic acid for 5 min in a shaker (inhibiting condition for the alliinase). The suspensions were centrifuged (3000 × *g* for 10 min) and 10 μL from the supernatant was injected onto the HPLC. The chromatography was performed on a Waters system (pump model 616, DAD model 966) using a Hypurity Elite C_{18} column (150 × 3 mm, 3 μm; ThermoQuest, Runcorn, Cheshire, UK). The gradient used was a mixture of 20 mmol/L sodium dihydrogen phosphate + 10 mmol/L heptane sulfonic acid (adjusted to pH 2.1 with 85% orthophosphoric acid) = eluent A and eluent A/acetonitrile 50% (v/v) following the subsequent gradient: 0 min 100% eluent A, 5 min 70% eluent A, 25 min 46% eluent A, 26 min 0% eluent A, and 30–40 min 100% eluent A. The reference standards used for quantification were alliin (purity >98%) and γ-glutamyl-allylcysteine (γ-Glu-allyl-Cys). All sulfurous-containing compounds were quantified by calculating them as γ-Glu-allyl-Cys. The remaining sulfur compounds were calculated as alliin. For purposes of identification and confirmation of compounds, a liquid chromatography/mass spectrometry electrospray ionization system was used (ThermoFinnigan LCQdeca ion trap quadrupole) with the following parameters: positive electrospray ionization; 0.1% ammonia formiate buffer, pH 3; 10 μL/min flow rate direct injection.

Cytokine secretion of human whole blood. The secretion was measured as described before (15). Briefly, heparinized blood from healthy volunteers who were informed according to the Helsinki declaration was diluted 1:4 with RPMI 1640 (Biochrom KG, Berlin, Germany) supplemented with penicillin/streptomycin (Biochrom KG, Berlin, Germany). Lipopolysaccharide (LPS) from *Salmonella abortus equi* (Sigma, Heidelberg, Germany) in a final concentration of 10 μg/L and garlic powder extracts were added. The samples were incubated at 37°C and 5% CO_2 for 20 h. After incubation, the tubes were shaken, and blood cells were sedimented by centrifugation (16,000 × *g*, 2 min). The cell-free supernatants were stored at −80°C until cytokine measurement. Cytokines were quantified by sandwich ELISA. First and second antibodies for tumor necrosis factor (TNF)-α, interleukin (IL)-1β and interferon (IFN)-γ were purchased from Endogen (Bonn, Germany); the antibody pairs for IL-10 and IL-6 were purchased from PharMingen (Heidelberg, Germany) and R&D (Wiesbaden, Germany), respectively. Streptavidin-conjugated peroxidase (Dianova, Hamburg, Germany) and the chromogen tetramethylbenzidin were used for detection of the immune complexes. Absorption was measured at 450 nm using a reference wavelength of 690 nm. Due to the naturally occurring differences in human cytokine liberation, the value obtained for each volunteer was normalized to its LPS control. The LPS control was set as 100%.

Dual luciferase reporter-gene assay (DLR). HEK293 cells were seeded at a concentration of 5 × 10^5 cells/60-mm dish. On the next day, cells were transfected with the pNF-κBluc and pRL-TK plasmids using the Ca^{2+}-phosphate method. Transfected cells were seeded in 96-well plates at a concentration of 15,000 cells/well and grown for an additional 16 h. Then cells were either preincubated with garlic constituents for 2 h and subsequently stimulated with 1 μg/L TNF-α for 6 h or incubated with supernatants of garlic-treated as well as untreated and LPS-activated human whole blood for 6 h. Cells were washed with PBS and NF-κB activity was measured with the DLR (Promega, Heidelberg, Germany) according to the manufacture's instructions using a AutoLumat plus luminometer (Berthold, Bad Wildbad, Germany).

Statistics. All data are expressed as mean ± SEM, unless otherwise stated. Two-way ANOVA with Bonferroni post-test was used to test whether the concentration of the used garlic powder extracts affected cytokine liberation. A one-way ANOVA with Bonferroni post-test was used to test whether DADS or allicin affected cytokine liberation. Comparisons between different fertilization stages were performed by paired *t* tests. Differences with $P < 0.05$ were considered significant. Analyses of the data were performed using the software GraphPad PRISM, Version 3.02 (GraphPad Software, San Diego, CA).

RESULTS

Concentration of sulfur compounds in dried garlic powder. In powders of unfertilized garlic (*Allium sativum* cv Printanor), the total sulfur concentration was 96.4 mmol/kg (sulfur/dry weight). With sulfur fertilization ($CaSO_4$, 3.4 × 10^{-2} kg/m^2), the amount of sulfur in garlic powders rose to 190 mmol/kg. The distribution of sulfur in alliin, allicin, allyl-cysteine (allyl-Cys) and γ-glutamyl-cysteines was assessed by HPLC analysis of the same powders (**Fig. 1**). Sulfur-fertilization significantly increased the allicin, alliin, γ-glutamyl-1-propyl-cysteine and γ-glutamyl-phenylalanine levels detected. The amount of γ-Glu-allyl-Cys remained unchanged and the level of allyl-Cys was slightly reduced. Similar results were obtained with *Allium sativum* cv Morasol and *Allium sativum* cv Messidrome (data not shown). Thus, sulfur-fertilization of garlic seems to increase overall levels of sulfur constituents in garlic bulbs.

Garlic powder extracts, garlic metabolites and cytokine levels. Treatment of whole blood with 10 μg/L LPS led to a strong liberation of the pro-inflammatory cytokines IL-1β (15.7 ± 5.1 μg/L) and TNF-α (8.8 ± 2.4 μg/L) (**Fig. 2***A*,*B*). Co-treatment with 100 mg/L garlic powder extract significantly reduced liberated IL-1β (6.2 ± 1.2 μg/L) (Fig. 2*A*). Similarly, the amount of liberated TNF-α was significantly impaired (3.9 ± 0.8 μg/L) compared with human whole blood treated with LPS only (8.8 ± 2.4 μg/L) (Fig. 2*B*). GPE alone, in the absence of LPS, did not influence cytokine levels in human whole blood, indicating that it possesses a low endotoxin-like activity. Interestingly, an extract from sulfur-fertil-

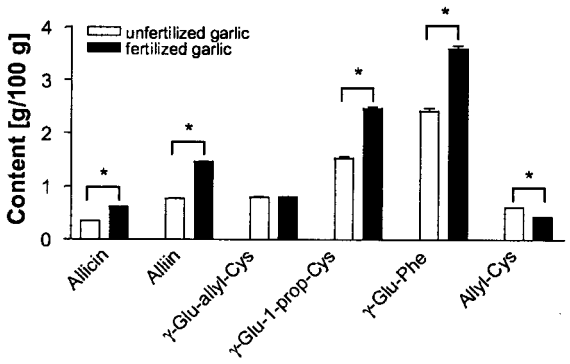

FIGURE 1 Sulfur fertilization increases the concentration of sulfur compounds in dried garlic powder. The following compounds in garlic powders of unfertilized or sulfur fertilized garlic, as indicated, were analyzed by HPLC-UV: allicin, alliin, γ-glutamyl-allyl-cysteine (γ-Glu-allyl-Cys), γ-glutamyl-1-propyl-cysteine (γ-Glu-1-prop-Cys), γ-glutamyl-phenylalanine (γ-Glu-Phe), allyl-cysteine (Allyl-Cys). g/100 g refers to g/100g garlic powder. Bars represent the mean ± SEM, $n = 2$. *Different from unfertilized garlic, $P < 0.05$.

ized garlic reduced IL-1β (to 4.4 ± 0.9 μg/L) and TNF-α (to 3.2 ± 0.6 μg/L) release more effectively than the extract from unfertilized garlic (IL-1β to 6.2 ± 1.2 mg/L and TNF-α to 3.9 ± 0.8 mg/L). The LPS-induced liberation of anti-inflammatory IL-10 was not affected by GPE (data not shown).

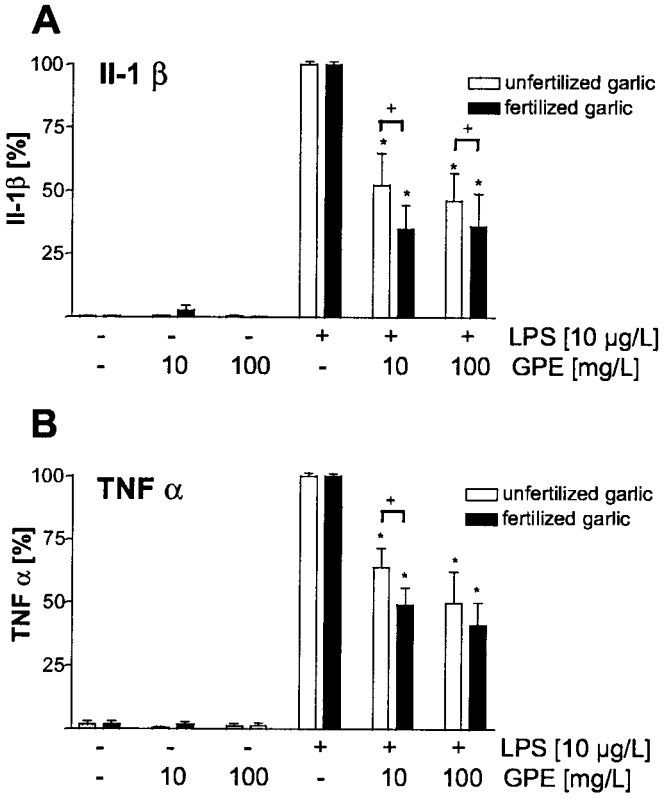

FIGURE 2 Garlic powder extracts (GPE) inhibit lipopolysaccharide (LPS)-induced liberation of interleukin (IL)-1β (*panel A*) and tumor necrosis factor (TNF)-α (*panel B*) in human whole blood. The mean amount of liberated IL-1β (15.7 ± 5.1 μg/L) and TNF-α (8.8 ± 2.4 μg/L) in LPS-treated blood samples was set as 100%. Bars represent the mean ± SEM, $n = 4$. *Different from LPS-activated cells, $P < 0.05$. +Different from unfertilized garlic, $P < 0.05$.

Treatment with DADS (100 μmol/L), an important garlic metabolite, significantly reduced IL-1β levels from 15.7 ± 5.1 μg/L (LPS only) to 7.7 ± 1.8 μg/L. TNF-α levels declined from 8.8 ± 2.4 μg/L (LPS only) to 4.5 ± 1.5 μg/L in the presence of 100 μmol/L DADS (**Fig. 3**A,B). Allicin (100 μmol/L), the major degradation product of alliin, inhibited the LPS-induced liberation of IL-10 from 680 ± 160 to 320 ± 82 μg/L (Fig. 3C).

Cytokine levels in human whole blood and activation of NF-κB in HEK293 cells. A crucial question was: would reduced levels of proinflammatory cytokines, such as TNF-α and IL-1β, and slightly increased amounts of anti-inflammatory IL-10 finally result in a reduced activation of NF-κB, and thus in a putative anti-inflammatory response? To examine this question, we used a NF-κB luciferase reporter gene assay. HEK293 cells were transfected with a NF-κB-driven firefly luciferase gene and a HSV-TK-driven renilla luciferase gene. Incubation of transfected HEK293 cells with cell-free LPS-

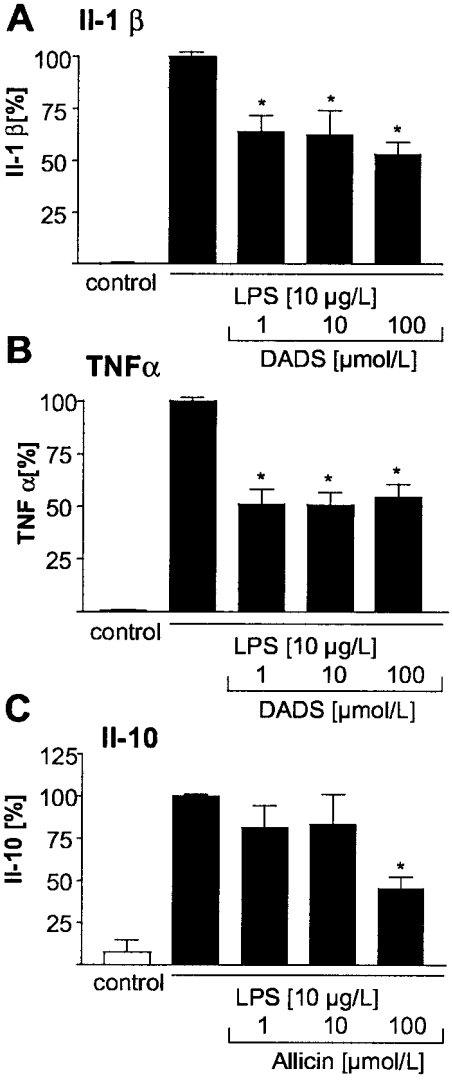

FIGURE 3 Lipopolysaccharide (LPS)-induced expression of interleukin (IL)-1β (*panel A*) and tumor necrosis factor (TNF)-α (*panel B*) is inhibited by diallyl disulfide (DADS); the expression of IL-10 (*panel C*) is inhibited by allicin. The mean amount of liberated IL-1β (15.7 ± 5.1 μg/L), TNF-α (8.8 ± 2.4 μg/L) and Il-10 (0.6 ± 0.1 μg/L) was set as 100%. Bars represent the mean ± SEM, $n = 4$. *Different from LPS-activated cells, $P < 0.05$.

activated blood sample supernatants of whole blood increased the luciferase activity about eightfold compared with the supernatants of untreated blood (**Fig. 4**). GPE-treated supernatants (10 and 100 mg/L) significantly reduced NF-κB activity, corresponding to their lowered proinflammatory cytokine levels. In agreement with the results obtained from cytokine measurements, blood sample supernatants treated with sulfur-fertilized garlic had a stronger inhibitory effect than samples treated with unfertilized garlic (Fig. 4). GPE alone had no direct inhibitory effect on TNF-α-induced expression of the NF-κB-driven luciferase reporter gene (data not shown). Together, these results suggest that garlic modulates inflammatory cytokines, leading to an overall reduction of NF-κB activity in a human cell model.

DISCUSSION

The major findings of the present study are that GPE inhibit NF-κB activity indirectly via a modulation of pro- and anti-inflammatory cytokines as seen in a human whole-blood ex vivo system, and that sulfur fertilization of garlic has a positive effect on this action.

Our results regarding the modulation of cytokines agree with a recent publication using similar concentrations of fresh garlic juice (6,7). However, in contrast to that study, we were unable to detect an influence on the cytokines INF-γ and IL-6. This may be due to the different garlic preparations used (fresh garlic juice vs. garlic powder extract) and different experimental set-ups. Studies using AGE (8) and the garlic constituent alliin (5) reported an increase of proinflammatory cytokines in response to LPS stimulation.

An attempt to identify the effective constituents of GPE was made by employing allicin and DADS, two major conversion products of alliin after its interaction with alliinase. Allicin had no inhibitory effect on the production of IL-1β and TNF-α, but rather decreased IL-10 levels. DADS decreased the LPS-induced release of TNF-α and IL-1β even at low concentrations (1 μmol/L) and may thus contribute in part to the observed effect for GPE.

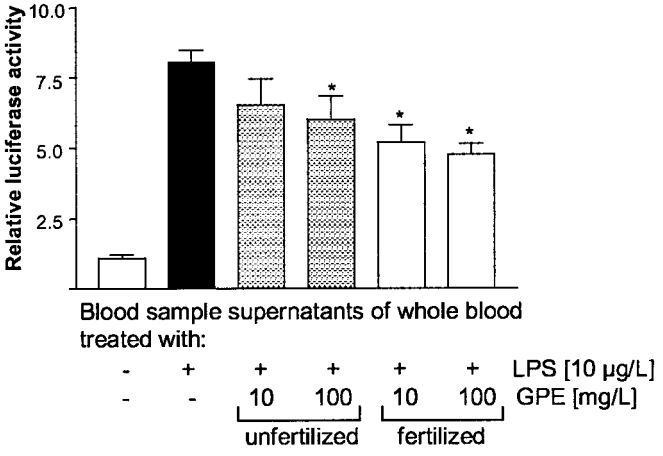

FIGURE 4 Garlic powder extract (GPE)-treated blood samples reduce NF-κB activity in HEK293 cells. Cells were incubated for 6 h with 5 μL of cell-free supernatant from whole-blood samples treated as indicated. NF-κB activity of HEK 293 cells stimulated with untreated blood supernatants was set as 1. The luciferase gene is regulated by a promotor that contains NF-κB binding sites only (pNF-κBluc plasmid). Thus, activation of NF-κB leads to an increased expression of the luciferase protein that is quantified by its activity. Bars represent the mean ± SEM, $n = 3$. *Different from LPS-activated cells, $P < 0.05$.

In vivo studies addressing the bioavailability of garlic constituents and the identification of active garlic metabolites are scarce. To date there are no conclusive data that clearly identify the main metabolites in the blood stream after garlic consumption. However, Germain et al. (16) demonstrated in a rat model that micromolar concentrations of DADS could be detected after oral administration of DADS in plasma and liver tissue, leading to the concentrations used in our ex vivo model. Further it has been shown that DADS can be metabolized by cytochrome P_{450} to allicin (17). However, allicin is highly unstable and is rapidly converted to DADS and allyl mercaptane (18).

Most interestingly, we showed that the modulation of LPS-induced cytokine levels resulted in lowered NF-κB activity in human HEK293 cells, which were used as a model for measuring NF-κB-driven gene expression by a luciferase reporter gene assay. NF-κB is a pivotal player in the inflammatory response (11) and is closely connected to atherosclerosis (19) and other inflammatory diseases (20).

Recently a direct inhibitory effect of AGE and its main constituent *S*-allyl cysteine on NF-κB was suggested (9,21). Our studies clearly exclude a direct inhibitory effect of GPE on NF-κB activity. The data presented rather suggest an indirect inhibitory effect on the transcription factor NF-κB by cytokine modulation.

Our study further reveals that sulfur-fertilization of garlic improves the beneficial effect of garlic. The amount of sulfur added to garlic plants was chosen according to two previous experiments as reported before (22,23). In earlier studies, the quality of the garlic powders used was often neglected, which could be one reason for the contradictory results concerning a health-promoting effect of garlic (24,25). Moreover, the data presented point to sulfur compounds as active principles of garlic with respect to the modulation of LPS-induced cytokine levels. This is an important finding because up until now, little attention has been paid to this aspect in the cultivation of garlic as a medicinal product. These findings are in line with recently published results of the sulfur-dependent antiplatelet activity of onions (26).

In summary, we showed that extracts of dried garlic powder and DADS reduce LPS-induced production of IL-1β and TNF-α in human whole blood. This modulation in cytokine pattern is correlated with the overall bulb sulfur content. Moreover, GPE-modulated cytokine levels may result in a reduction of the inflammatory response by reducing the proinflammatory activity of NF-κB in adjacent tissue.

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