Garlic constituents and metabolites fail to inhibit the activation of the transcription factor NF-kappaB and subsequent expression of adhesion molecules in human endothelial cells

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Abstract

The transcription factor NF-κB is suggested to play a pivotal role in the atherogenesis by regulating the expression of certain gene products such as adhesion molecules. Garlic is consumed as food or dietary supplement worldwide in order to benefit from its positive cardiovascular effects. This study therefore addresses the question whether the garlic metabolites diallyldisulfide (DADS) and allylmercaptan (AM) (up to 100 µM) influence the TNF-α (1-10 ng/ml)-induced activation of NF-κB and the NF-κB-regulated endothelial gene product E-selectin in human umbilical endothelial cells (HUVECs). We show that neither DADS nor AM inhibit TNF-α-induced NF-κB binding activity as shown by electrophoretic mobility shift assay nor NF-κB transactivation activity as examined by a luciferase reporter gene assay. The TNF-α-induced expression of the endothelial adhesion molecule E-selectin was not reduced in response to DADS or AM as measured by flow cytometry. This study suggests that NF-κB is not a major target of garlic metabolites such as DADS or AM.

Keywords: garlic, organosulfur compounds, NF-κB, adhesion molecules. ICAM, E-Selectin
1. Introduction

Garlic (Allium sativum) is used as medicinal plant since centuries. Despite this long medicinal tradition and extensive research activities during the last 100 years the knowledge about its pharmacology remained largely elusive [1]. The pharmacological effects that are attributed to garlic are primarily an antithrombotic, a lipid-lowering and chemopreventive or antitumor effect [1]. Furthermore, some garlic compounds seem to have antioxidant properties that may lead to the inhibition of the transcription factor nuclear factor kappaB (NF-κB) and thus to the prevention of atherogenesis [2].

Indeed, there are several observation that suggest that NF-κB is a pivotal mediator in atherogenesis: Activated NF-κB is present in atherosclerotic lesions but not in normal vessels. NF-κB-regulated genes are expressed during atherogenesis. Furthermore, atherosclerotic lesions develop in response to diverse stimuli that are able to create oxidant stress and activate the NF-κB system and agents that inhibit lesion formation act often as antioxidant [reviewed in 3].

Since reports about NF-κB-inhibitory effects of garlic and garlic constituents are limited so far mainly to aged garlic extract (AGE) and its major compound S-allylcysteine (SAC) and to dosages in the milimolar-range [2,4,5], the aim of the present study was a) to examine also other garlic constituents, especially compounds that are described as metabolites after garlic application, such as diallysdisulfide and allylmercaptane [reviewed in 6], and b) to employ dosages that may be more relevant to be reached in plasma.

2. Methods
2.1. Materials

Used plasmids: pNF-κBluc, pFC-Mekk (Stratagene, Netherlands), pEGFP-N1 (Clontech, Germany) and pRL-TK (Promega, Germany). The garlic constituents/metabolites diallyldisulfide (DADS) and allylmercaptane (AM) were obtained from Prof. J. Auger (Universite de F. Rabelais, Tours, France) with a purity of ≥ 90%. γ-Glutamylcysteine (≥ 98% purity) and garlic powder was supplied by Dr. T. Haffner (Lichtwer Pharma, Germany). S-allylcysteine (SAC) (≥ 98% purity) was from Wakunaga (Madero Mission Viejo, USA). Garlic constituents were dissolved in DMSO and diluted in media. Garlic powder was suspended in water for 30 min. The insoluble residue was separated by centrifugation (24,000xg; 30 sec) and the supernatant used either directly or further diluted to the concentration needed. The final concentration of DMSO in the supernatants was ≤ 0.1%, a concentration that was shown not to interfere with the test system.

2.2. Cell culture

Human umbilical endothelial cells (HUVECs) were isolated from umbilical veins by digestion with collagenase A (Roche, Germany). Cells were grown in ECGM (Promocell, Germany) in 25 cm² tissue culture flasks pre-coated with 0.25% Collagen G (Biochrom KG; Germany) at 37 °C/5% CO₂. Purity of endothelial cells was > 95% as judged by flow cytometry detecting “von-Willebrand”-protein. The human embryonic kidney cell line 293 (HEK 293) was grown in DMEM (Biowitthaker, Belgium) supplemented with 10 % FCS (Biochrom KG, Germany), 2 mM glutamine (Merck, Germany) and penicillin/streptomycin (PAN, Biotech; Germany). Cells were split 1:10 when reaching ~85-90% confluence using 0.05% trypsin/0.02% EDTA in PBS.
2.3. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Cells were incubated with the test compounds for 2 h and subsequently stimulated with TNF-α (10 ng/ml) for 1 h. Then cells were washed with PBS, collected (230xg) and nuclear extracts prepared as described previously [7]. Protein aliquots were either frozen at -86°C or immediately used for EMSA as described [7]. Gels were exposed to a storage phosphor screen (Packard, Germany) at –86°C. Screens were read out on a phospho-imager (Packard, Germany). The specificity of the binding reaction was determined by incubating control samples either with an excess of unlabeled NF-κB oligonucleotide or unlabeled AP-2 oligonucleotide (Promega, Germany).

2.4. Dual luciferase reporter-gene assay (DLR)

Cells were seeded at a concentration of 5x10^5 cells/60mm dish. On the following day cells were transfected with the pNF-κB luc and pRL-TK plasmid using the Ca^{++}-phosphate method. Transfected cells were seeded in 96-well plates at a concentration of 15,000 cells/well and grown for additional 16 h. Then cells were pre-incubated with garlic constituents for 2 h and subsequently stimulated with 1 ng/ml TNF-α for 6 h. Cells were washed with PBS and NF-κB activity was measured with the DLR (Promega, Germany) according to the manufactures instructions using a AutoLumat plus luminometer (Berthold, Germany).

2.5. Quantification of E-selectin

HUVECs were grown to confluence in 24-well plates. Cells were preincubated with garlic constituents for 2 h, subsequently stimulated with 10 ng/ml TNF for 4 h and expression of E-selectin quantified by flow cytometry (FACSCalibur, Becton Dickinson, Germany): Cells were incubated with 1% BSA/PBS to block nonspecific
binding, and immunostained with FITC-conjugated mouse monoclonal antibodies against E-selectin (Calbiochem, Schwalbach, Germany), diluted in 1% BSA/PBS. Quantification of expression levels was done by determining the geometric mean in the CellQuest Software, which represents the mean value of the area covered by the curve.

3. Results

3.1. Garlic metabolites do not inhibit TNF-α-activated NF-κB binding activity

Fig. 1A demonstrates that compared to nuclear extracts from TNF-α (10 ng/ml)-activated cells, nuclear extracts from cells pre-treated with DADS or AM (5-20 µM) showed no considerable decreased NF-κB binding activity. Similar results were obtained investigating γ-glutamylcysteine (1 nM-1 mM), a major constituent of whole and crushed garlic gloves [8], SAC (0.1-10 mM), a major sulfur constituent of aged garlic extract (AGE) [9] and a water garlic powder extract (0.01-1 mg/ml) (data not shown). To prove that the lack of inhibitory effects was not due to our experimental system HUVECs were treated with parthenolide, a well-established NF-κB inhibitor [10]. Parthenolide concentration-dependently (0.1-10 µM) inhibited NF-κB binding activity (Fig. 1B).

3.2. Garlic metabolites do not inhibit TNF-α-activated NF-κB transactivation activity

NF-κB may be inactive though bound to DNA. We therefore examined whether the garlic metabolites DADS and AM influence the transactivation activity of NF-κB by a NF-κB luciferase reporter gene assay. Neither DADS (1-100 µM) nor AM (1-100 µM) affected TNF-α (1 ng/ml)-induced luciferase activity (Fig. 2A/B). In contrast,
parthenolide concentration-dependently (0.1-10 µM) reduced TNF-α-mediated luciferase expression (Fig. 2C).

3.3. Garlic metabolites do not affect TNF-α-activated E-selectin expression

To examine whether the NF-κB-regulated endothelial adhesion molecule E-selectin is influenced by DADS or AM we analyzed E-selectin expression on the cell surface by flow cytometry. As shown in Fig. 3A/B neither DADS nor AM (5-20 µM) inhibited TNF-α (10 ng/ml)-induced E-selectin expression. DADS rather slightly increased E-selectin levels (Fig. 3A/B). The positive control parthenolide inhibited E-selectin expression at 10 µM (Fig. 3C).

Discussion

In the present study we addressed the important question whether garlic metabolites, such as DADS or AM are able to influence the activation of the transcription factor NF-κB and NF-κB-regulated endothelial gene expression. We found that neither DADS nor AM inhibit TNF-α-induced NF-κB binding and transactivation activity in HUVECs. They were also unable to suppress TNF-α-activated E-selectin expression in these cells. Studies addressing the bioavailability of garlic constituents and the identification of active garlic metabolites are scarce. There are no conclusive data that clearly identify main metabolites after garlic consumption in the blood stream. The few data available point to a potential role of DADS and AM as active metabolites [reviewed in 6]. Garlic preparations represent the most used dietary supplements in the US [11]. Intake of garlic is supposed to reduce the risk for cardiovascular diseases, such as atherosclerosis [11]. Atherosclerosis is a chronic inflammatory process in which the
transcription factor NF-κB seems to act as pivotal mediator [3]. Inhibition of activation of this transcription factor may thus be a reasonable approach to interfere with atherogenic processes. The results obtained from this study, suggests, that the garlic metabolites DADS and AM do not act via inhibition of NF-κB and NF-κB-regulated genes in human endothelial cells. We also were unable to detect an inhibitory effect of SAC on TNF-α-induced NF-κB-binding activity in concentrations of 0.1-10 mM. This is in contrast to studies that demonstrate that SAC inhibits oxidative stress and activation of NF-κB in various cell systems [2,4,5]. The concentrations used in these studies were, however, quite high (up to 20 mM SAC) and unlikely to be reached in plasma [6,12]. Our findings that NF-κB seems not to be a major target of garlic constituents are supported by in vivo data that show that supplementation of rabbits fed a cholesterol-enriched diet with AGE does not influence endothelial adhesion molecules or adherence of leucocytes [13].

In summary, we provide data that suggest that the garlic metabolites DADS and AM do not influence NF-κB and the endothelial NF-κB-regulated gene E-selectin. This does not exclude that garlic metabolites mediate health promoting effects via other mechanisms, such as an inhibitory effect on vascular smooth muscle cell growth as reported recently [13].

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References

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Figure legends

Fig. 1. Garlic metabolites diallyldisulfide (DADS) and allylmercaptane (AM) do not inhibit NF-κB binding activity. HUVECs were either left untreated or were treated with DADS, AM (A) or as a positive control with parthenolide (B) as indicated. After 2 h TNF-α (10 ng/ml) was added for an additional hour. Equal amounts of nuclear extracts were employed to EMSA as described under Methods. The first two samples of each gel contain nuclear extracts of TNF-α-activated cells incubated with a 100-fold excess of a unlabeled NF-κB (unlabeled competitor, line 1) or AP-2 (unlabeled noncompetitor, line 2) consensus oligonucleotide. All experiments were performed at least three times. Representative gel shift experiments are shown.

Fig. 2. Garlic metabolites diallyldisulfide (DADS) and allylmercaptane (AM) do not inhibit NF-κB transactivation activity. HEK 293 cells transfected with a firefly luciferase gene driven by a NF-κB-regulated promoter and as control with a renilla luciferase gene driven by a constitutively active HSV-TK promoter were either left untreated or were treated with DADS (A), AM (B) or as a positive control with parthenolide (C) as indicated. After 2 h TNF-α (1 ng/ml) was added for additional 6 h. Then luciferase reporter-gene assay was performed as described under Methods. Bars represent the mean ± SEM. of at least three independent experiments performed in triplicate. *** P < 0.001 (ANOVA/Bonferroni)

Fig. 3. Garlic metabolites diallyldisulfide (DADS) and allylmercaptane (AM) do not inhibit the induction of the adhesion molecule E-selectin. HUVECs were either left untreated or were treated with DADS (A), AM (B) or as a positive control with
parthenolide (C) as indicated. After 2 h TNF-α (10 ng/ml) was added for additional 4 h. Expression of E-selectin was quantified by flow cytometry as described under *Methods*. Bars represent the mean ± SEM of at least three independent experiments performed in triplicate. * P < 0.05, ** P < 0.01 *** P < 0.001 (ANOVA/Bonferroni). The insert shows a representative histogram obtained by flow cytometry.
Figure 1

A

Diallyldisulfide:

Allylmercaptane:

NF-κB

+ + - + - + + +

TNF-α

μM

20 5 10 20

NF-κB

B

Parthenolide:

NF-κB

AP-2

- - - - 10 .1 1 10

μM

+ + - + - + + +

TNF-α

NF-κB

AP-2
Figure 2
Figure 3