

Verena M. Dirsch  
Hans-Peter Keiss  
Angelika M. Vollmar

## Garlic metabolites fail to inhibit the activation of the transcription factor NF-kappaB and subsequent expression of the adhesion molecule E-selectin in human endothelial cells

■ **Summary** *Background* The transcription factor NF- $\kappa$ B is suggested to play a pivotal role in atherogenesis by regulating the expression of proinflammatory gene products such as adhesion mole-

cules. Garlic is consumed as food or dietary supplement in order to benefit from its antiatherogenic effect. *Aim of the study* This study addresses the question whether the garlic metabolites diallyldisulfide (DADS) and allylmercaptane (AM) influence the TNF- $\alpha$ -induced activation of NF- $\kappa$ B and the NF- $\kappa$ B-regulated endothelial gene product E-selectin in human umbilical endothelial cells (HUVECs). *Methods* A putative effect of DADS and AM on NF- $\kappa$ B activity was examined by measuring NF- $\kappa$ B DNA binding activity by an electrophoretic mobility shift assay (EMSA) and on the NF- $\kappa$ B transactivation activity by a luciferase reporter gene assay. In addition, an effect of DADS and

AM on the expression of the NF- $\kappa$ B-regulated gene product E-selectin was investigated by flow cytometry. *Results* We show that neither DADS nor AM (up to 100  $\mu$ M) inhibit TNF- $\alpha$  (1–10 ng/ml)-induced NF- $\kappa$ B DNA binding nor NF- $\kappa$ B transactivation activity. The TNF- $\alpha$ -induced expression of the endothelial adhesion molecule E-selectin was not reduced in response to DADS or AM. *Conclusions* This study suggests that NF- $\kappa$ B is not a major target of garlic metabolites such as DADS or AM.

■ **Key words** garlic – organosulfur compounds – NF- $\kappa$ B – adhesion molecules

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V. M. Dirsch, Ph. D. (✉) · H.-P. Keiss ·  
A. M. Vollmar  
Dept. of Pharmacy,  
Center of Drug Research  
Butenandtstr. 5–13  
81377 Munich, Germany  
Tel.: +49-89/2180-7161  
Fax: +49-89/2180-7173  
E-Mail:  
verena.dirsch@cup.uni-muenchen.de

### Introduction

Garlic (*Allium sativum*) has been used as a medicinal plant for centuries. Despite this long medicinal tradition and extensive research during the last 100 years, the molecular mechanisms underlying the pharmacological effects attributed to garlic remained largely elusive [1]. Pharmacological effects attributed to garlic are primarily antithrombotic, lipid-lowering and chemopreventive or antitumor effects [1]. Furthermore, some garlic compounds seem to have antioxidant properties that may lead to the inhibition of the transcription factor nuclear factor kappaB (NF- $\kappa$ B) [2] or NF- $\kappa$ B-regulated genes [3] and thus to the prevention of atherogenesis.

Indeed, there are several observations that suggest that NF- $\kappa$ B is a pivotal mediator in atherogenesis: acti-

vated NF- $\kappa$ B is present in atherosclerotic lesions but not in normal vessels. NF- $\kappa$ B-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- $\kappa$ B system, and agents that inhibit lesion formation often act as antioxidants (reviewed in [4]).

The aim of the present study therefore was to examine whether garlic constituents, especially compounds that are described as metabolites after garlic application, such as diallyldisulfide and allylmercaptane (reviewed in [5]) are able to inhibit the TNF- $\alpha$ -induced activation of the transcription factor NF- $\kappa$ B and the expression of the NF- $\kappa$ B-regulated adhesion molecule E-selectin in endothelial cells.

## Materials and methods

### ■ Materials

Plasmids used: pNF- $\kappa$ Bluc, 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 (Université de F. Rabelais, Tours, France) with a purity of  $\geq 90\%$ .  $\gamma$ -Glutamylcysteine ( $\geq 98\%$  purity) and garlic powder was supplied by Dr. T. Haffner (Lichtwer Pharma, Germany). S-allylcysteine (SAC) ( $\geq 98\%$  purity) was from Wakunaga (Madero Mission Viejo, USA). The NF- $\kappa$ B inhibitor parthenolide was purchased from Alexis (Germany). Garlic constituents were dissolved in DMSO and diluted in media. The final concentration of DMSO in the supernatants was  $\leq 0.1\%$ , a concentration that was shown not to interfere with the test system. Garlic powder was suspended in water for 30 min. The insoluble residue was separated by centrifugation (24,000  $\times$  g; 30 s) and the supernatant was used either directly or further diluted to the concentration needed.

### ■ Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by digestion with collagenase A (Roche, Germany). Cells were grown in ECGM (Promocell, Germany) in 25 cm<sup>2</sup> tissue culture flasks pre-coated with 0.25 % Collagen G (Biochrom KG; Germany) at 37°C/5 % CO<sub>2</sub>. 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 (Biowittaker, 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  $\sim 85$ – $90\%$  confluence using 0.05 % trypsin/0.02 % EDTA in PBS.

### ■ Cytotoxicity/cell viability

Cytotoxicity of test compounds towards HUVEC was judged by staining cell nuclei with crystal violet according to Gillies et al. [6]. Cell viability of HEK 293 cells was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan [7].

### ■ 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- $\alpha$  (10 ng/ml) for 1 h. Then cells were washed with PBS, collected (230 $\times$ g) and nuclear extracts prepared as described previously [8]. Protein aliquots were either frozen at  $-86^\circ\text{C}$  or immediately used for EMSA as described [8]. Gels were exposed to a storage phosphor screen (Packard, Germany) at  $-86^\circ\text{C}$ . Screens were read 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- $\kappa$ B oligonucleotide or unlabeled AP-2 oligonucleotide (Promega, Germany).

### ■ Dual luciferase reporter-gene assay (DLR)

Cells were seeded at a concentration of  $5 \times 10^5$  cells/60 mm dish. On the following day cells were transfected with the pNF- $\kappa$ Bluc and pRL-TK plasmid using the Ca<sup>2+</sup>-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 24 h, respectively, and subsequently stimulated with 1 ng/ml TNF- $\alpha$  for 6 h. Cells were washed with PBS, and NF- $\kappa$ B activity was measured with the DLR (Promega, Germany) according to the manufacturer's instructions using an AutoLumat plus luminometer (Berthold, Germany).

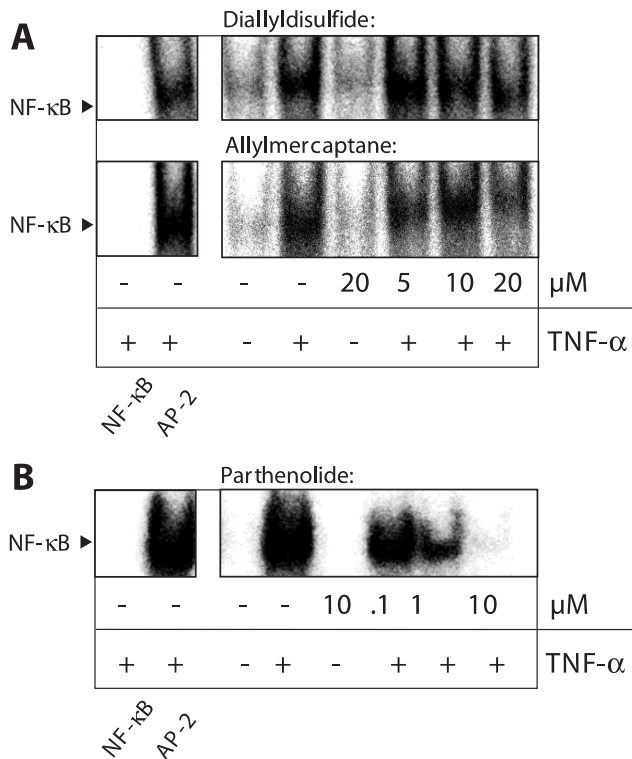
### ■ Quantification of E-selectin

HUVECs were grown to confluence in 24-well plates. Cells were preincubated with garlic constituents for 2 h and 24 h, respectively, and 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 mean value of the area covered by the curve by the CellQuest Software.

## Results

### ■ Garlic metabolites do not inhibit TNF- $\alpha$ -activated NF- $\kappa$ B binding activity

Fig. 1A demonstrates that TNF- $\alpha$  (10 ng/ml) activates DNA binding activity of NF- $\kappa$ B, and that this binding ac-



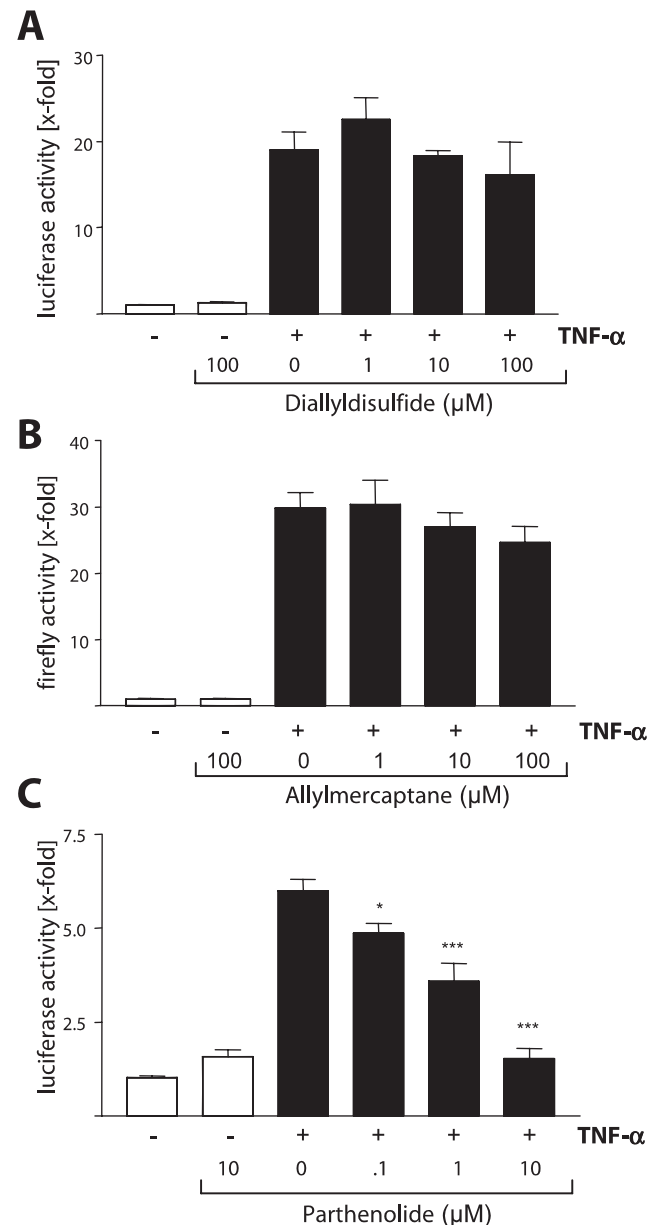
**Fig. 1** Garlic metabolites diallyldisulfide (DADS) and allylmercaptane (AM) do not inhibit NF- $\kappa$ B binding activity. HUVECs were either left untreated or were treated with DADS, AM (A) or as a positive control with parthenolide (B) as indicated. After 2 h TNF- $\alpha$  (10 ng/ml) was added for an additional hour. Equal amounts of nuclear extracts were employed to EMSA as described under *Methods*. The first two lanes of each gel show DNA binding of nuclear extracts of TNF- $\alpha$ -activated cells incubated with a 100-fold excess of an unlabeled NF- $\kappa$ B (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

tivity is not considerably decreased in cells pre-treated with DADS or AM (5–20  $\mu$ M). Similar results were obtained investigating  $\gamma$ -glutamylcysteine (1 nM–1 mM), a major constituent of whole and crushed garlic gloves [9], SAC (0.1–10 mM), a major sulfur constituent of aged garlic extract (AGE) [10] and a water garlic powder extract (0.01–1 mg/ml) (data not shown). As a positive control, HUVECs were treated with parthenolide, a well-established NF- $\kappa$ B inhibitor [11]. Parthenolide concentration-dependently (0.1–10  $\mu$ M) inhibited NF- $\kappa$ B binding activity (Fig. 1B).

#### Garlic metabolites do not inhibit TNF- $\alpha$ -activated NF- $\kappa$ B transactivation activity

A lack of effect on NF- $\kappa$ B binding activity does not necessarily mean that the transactivation activity of NF- $\kappa$ B remains unaffected. We therefore examined whether the garlic metabolites DADS and AM influence the transactivation activity of NF- $\kappa$ B by a NF- $\kappa$ B luciferase reporter

gene assay. Neither DADS (1–100  $\mu$ M) nor AM (1–100  $\mu$ M) affected TNF- $\alpha$  (1 ng/ml)-induced luciferase activity when preincubated for 2 h (Fig. 2A/B). Preincubation of HEK 293 cells with DADS (1–50  $\mu$ M) and AM (1–100  $\mu$ M) for 24 h also showed no effect (data not shown). Since preincubation of transfected HEK 293



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

cells with DADS 100  $\mu\text{M}$  for 24 h induced 30 % cell death, this concentration was not included in the analysis of the luciferase reporter gene assay. In contrast, parthenolide concentration-dependently (0.1–10  $\mu\text{M}$ ) reduced TNF- $\alpha$ -mediated luciferase expression (Fig. 2C).

### ■ Garlic metabolites do not affect TNF- $\alpha$ -activated E-selectin expression

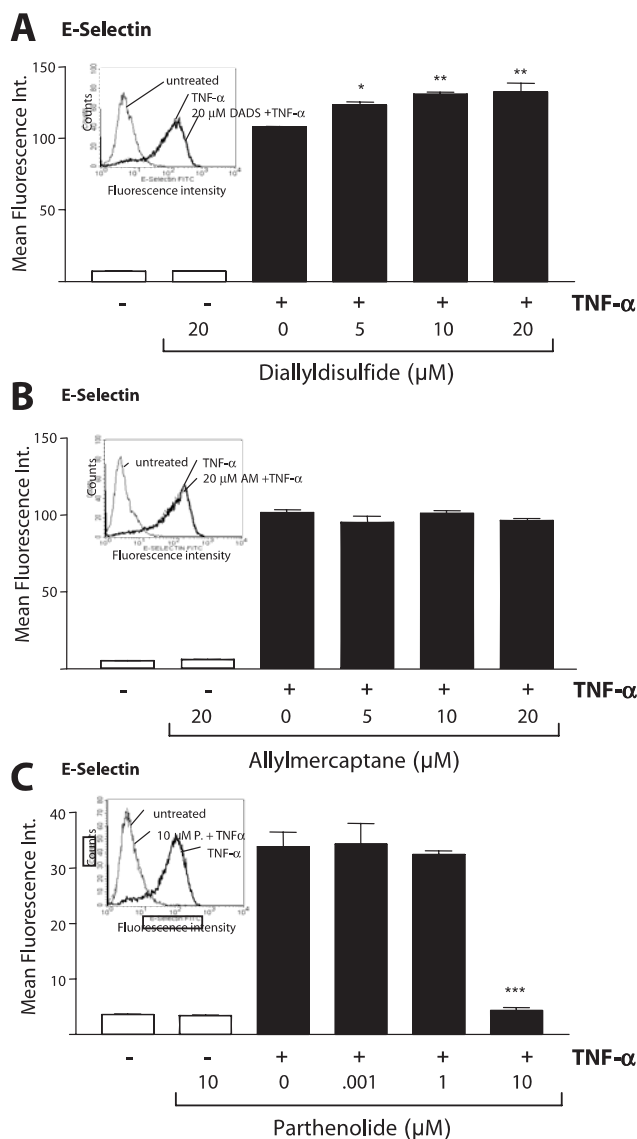
To examine whether the NF- $\kappa\text{B}$ -regulated endothelial adhesion molecule E-selectin is influenced by DADS or AM we analyzed E-selectin expression on the cell surface by flow cytometry. As shown in Fig. 3A/B neither DADS nor AM (5–20  $\mu\text{M}$ ) inhibited TNF- $\alpha$  (10 ng/ml)-induced E-selectin expression when preincubated for 2 h. DADS rather slightly increased E-selectin levels (Fig. 3A/B). Preincubation of DADS or AM (1–100  $\mu\text{M}$ ) for 24 h also showed no effect on E-selectin expression (data not shown). No cytotoxicity was detected on HUVEC up to 100  $\mu\text{M}$  DADS and AM (data not shown). The positive control parthenolide inhibited E-selectin expression at 10  $\mu\text{M}$  (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- $\kappa\text{B}$  and NF- $\kappa\text{B}$ -regulated endothelial gene expression. We found that neither DADS nor AM inhibit TNF- $\alpha$ -induced NF- $\kappa\text{B}$  binding and transactivation activity in HUVECs. They were also unable to suppress TNF- $\alpha$ -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 the main metabolites after garlic consumption in the blood stream. The few data available, however, point to a potential role of DADS and AM as active metabolites (reviewed in [5]).

Garlic preparations represent the most used dietary supplements in the US [12]. Intake of garlic is supposed to reduce the risk for cardiovascular diseases, such as atherosclerosis [12]. Atherosclerosis is a chronic inflammatory process in which the transcription factor NF- $\kappa\text{B}$  seems to act as pivotal mediator [4]. Inhibition of activation of this transcription factor may thus be a reasonable approach to interfere with atherogenic processes. The results obtained from this study suggest that the garlic metabolites DADS and AM do not act *via* inhibition of NF- $\kappa\text{B}$  and NF- $\kappa\text{B}$ -regulated genes in human endothelial cells. Even a preincubation time of 24 h for DADS and AM in order to allow sufficient uptake and metabolism did not lead to an effect on either NF- $\kappa\text{B}$  ac-



**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 pretreated with DADS (A), AM (B) or as a positive control with parthenolide (C) as indicated. After 2 h TNF- $\alpha$  (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  $\pm$  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

tivity or E-selectin expression. We were also unable to detect an inhibitory effect of SAC on TNF- $\alpha$ -induced NF- $\kappa\text{B}$ -binding activity at concentrations of 0.1–10 mM. This is in contrast to studies that demonstrate that SAC inhibits oxidative stress and activation of NF- $\kappa\text{B}$  in various cell systems [2, 13, 14]. The concentrations used in these studies were, however, quite high (up to 20 mM SAC) and unlikely to be reached in plasma [5, 15]. Our findings that NF- $\kappa\text{B}$  does not seem 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 [16].

In summary, we provide data that suggest that the garlic metabolites DADS and AM do not influence NF- $\kappa$ B and the NF- $\kappa$ B-regulated endothelial gene product E-selectin. This does not exclude that garlic metabolites mediate health promoting effects *via* other mechanisms. However, from the presented data it seems un-

likely that NF- $\kappa$ B-regulated pro-inflammatory genes in the endothelium are a major target of these garlic metabolites.

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