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ORIGINAL CONTRIBUTION

Allicin up-regulates cellular glutathione level in vascular endothelial cells

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Abstract *Background* Allicin in garlic is the primary active compound known to rapidly interact with free thiols. *Aims of the study* To examine the effect of allicin on gene expression and glutathione cellular level in vascular endothelial cells. *Methods* Cultured endothelial cells were exposed to allicin; mRNA was prepared and subjected to Micro-array and Real-Time PCR. Glutathione cellular level was determined on cell lysates. *Results* Micro-array analysis demonstrated allicin-induced up- and down-regulation of 116 and 100 genes, respectively. Up-regulated genes included the phase II detoxifying enzymes thioredoxin reductase 1 and 2, heme oxygenase-1 and glutamate cysteine lygaze modifier subunit, the rate limiting enzyme in glutathione biosynthesis. Endothelial cells exposed to allicin and its derivatives containing glutathione or cysteine residues increased cellular glutathione. Allicin increased the glutathione level in a concentration and time-dependent manner up to 8-fold at a concentration of 10–20 μM after 28 h exposure. Furthermore, allicin derivative-treated cultures demonstrated a 50% decrease in tBuOOH cytotoxicity. *Conclusions* These results may suggest a putative role for allicin and its derivatives in preventing reactive oxygen species damage by up-regulating the phase II detoxifying enzymes and increasing the cellular glutathione level.

Key words endothelial cells – glutathione – oxidative stress – antioxidant capacity – thiols

Introduction

Garlic (*Allium sativum*) has been known throughout much of human history as a natural medicine. Allicin (diallyl thiosulfinate), responsible for the typical smell of garlic, is the best-known and most widely-studied compound [20]. Allicin is a small molecule that freely permeates through the phospholipids bilayers and immediately interacts with various compounds to form different thiol-contained metabolites, such as S-allylmercaptoglutathione (GSSA) or S-allylmercaptocysteine (CSSA) [25, 27]. Garlic, or garlic extracts containing allicin, exhibit beneficial effects in hyperlipidemic animal models by reducing fatty streak formations [9, 11]. The garlic compound S-allylcysteine (SAC) (the major compound of aged garlic) attenuates oxidized-low density lipoprotein-induced injury of vascular endothelial cells (EC) by preventing intracellular glutathione depletion and minimizing

the release of peroxides [14, 16]. Additionally, the anti-proliferative effect of several allium-derived compounds on colon cancer cells is accompanied by an increased glutathione cellular level [30]. Reactive oxygen species (ROS) can cause major damage to cells by oxidizing lipids and proteins, causing membrane damage. The ROS-induced oxidative stress is associated with pathological diseases, such as atherosclerosis, diabetes type 2, and neurodegenerative diseases [4, 24]. Under normal conditions, 99% of the total glutathione exist in cells in its reduced form (GSH). It is the most important molecule in the cell that offers protection against ROS by forming oxidized glutathione (GSSG) or other GSH conjugates [4, 8]. The formed GSSG can be enzymatically-reduced by glutathione reductase or alternatively exported out of the cell to maintain the high cellular GSH/GSSG ratio [8]. GSH synthesis in cells is normally regulated by feedback inhibition of the rate-limiting enzyme, glutamate-cysteine-ligase (GCL) [2]. The phase II detoxifying enzymes (including GCL) responsible for the cellular protective mechanism against oxidative stress contain cis-acting elements in their promoter region called antioxidant response elements (ARE) that are regulated by the nuclear factor erythroid 2 (NF-E2)-related factor-2 (Nrf2) [24]. The present study explores the hypothesis that allicin and its derivatives, GSSA and CSSA, up-regulate the expression of oxidative stress-related genes and the GSH cellular level in EC.

Materials and methods

Materials

Bovine serum albumin (regular and tissue culture grade) (BSA), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 5-sulfosalicylic acid (SSA), GSSG, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), glutathione reductase, collagenase II, DL-buthionine-(S,R)-sulfoximine (BSO), 2-vinylpyridine, N-acetyl-cysteine (NAC) and tBuOOH were from Sigma (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM), fetal calf serum, L-glutamine, antibiotics solution (penicillin, streptomycin and nystatin), fibronectin, phosphate buffered saline (PBS), EZ-RNA 2 kit and trypsin-EDTA solution were from Biological Industries (Beit Haemek, Israel). Calf serum was from Gibco BRL (NY, USA). Fibroblast growth factor-2 (FGF-2), a generous gift from Amgen Inc. (Boulder, CO, USA), was dissolved in DMEM supplemented with 0.5% tissue culture grade BSA. EGM™-2 was from Cambrex (Walkersville, MD, USA). [^{3}H]Proline was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Reverse IT first strand, absolute SYBR green ROX mix, absolute QPCR adhesive seals and water RNAse-free were from ABgene (Surrey, UK). Microamp 96-well reaction plates for real time PCR and human genome U133A 2.0 array were from Applied Biosystems (Foster City, CA, USA). DNAse was from Ambion (Austin, TX, USA). Tissue culture dishes were from Nunc (Roskilde, Denmark).

Cell culture

Human umbilical vein endothelial cells (HUVEC) were prepared from human umbilical cord according to the method described by Jaffe et al. [18]. Cells were cultured in EGM™-2 at 37°C in a humidified 10% CO_2 atmosphere. Experiments were conducted on confluent cultures at passages 2–4. Bovine aortic EC were prepared from bovine aorta as previously described [12], cultured in DMEM supplemented with 5% fetal calf serum, 5% calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 12.5 U/ml nystatin, and FGF-2 (3 ng/ml; added every 48 h) at 37°C in a humidified 10% CO_2 atmosphere. Passages 5–20 were used for experiments.

Preparation of allicin and its derivatives

Pure allicin was produced by reacting synthetic alliin with immobilized allinase as previously described [26]. Allicin derivatives, GSSA and CSSA, were synthetically produced [25].

Array processing

Affymetrix Hu133A 2.0 oligonucleotide array was used as described [35]. Total RNA was used to prepare biotinylated-target RNA, with minor modifications from the manufacturer's recommendations [36]. The target cDNA generated was loaded on the array and scanned according to the manufacturer's recommendation using an Affymetrix GeneChip Instrument System [36]. Ratios for GAPDH and beta-actin (3′/5′) were confirmed to be within acceptable limits (0.97–0.96 and 1.14–1.2) and BioB spike controls were present on all chips with BioC, BioD and CreX also present in increasing intensity. When scaled to a target intensity of 150 (using Affymetrix MAS 5.0 array analysis software), scaling factors for all arrays were within acceptable limits (1.355–1.655), as well as the background, Q values and mean intensities.

Array analysis

The oligonucleotide arrays were filtered using MAS 5. Treated and control samples were compared generating a list of "active genes" representing probe sets increased by at least twofold (as calculated from the MAS 5 Log Ratio values) or decreased by at least 50% (*P*-value 0.0025) in the treated sample. In the control samples, up-regulated genes with signals lower than 20 or detected as absent, and down-regulated genes with baseline signals lower than 20 and detected as absent were excluded.

Real-Time PCR

Total RNA was collected by EZ-RNA 2 kit, treated with DNAse, converted to cDNA, assayed in triplicate by real-time quantitative PCR using an ABI Prism 7700 Sequence Detector System (Applied Biosystem, Foster City, CA, USA) with Absolute SYBR Green ROX mix, and analyzed (SDS 2.1 software). Primers: GCL modifier subunit (GCLM)—Forward: 5′-GGC ACAGGTAAAACCAAATAGTAAC-3′ and Reverse: 5′-CAAATTGTTTAGCAAATGCAGTCA-3′ [17], and heme oxygenase-1 (HO-1)—Forward: 5′-TTCTCCGA TGGGTCCTTACACT-3′ and Reverse: 5′-GGCATAA AGCCCTACAGCAACT-3′ [17]. Results were normalized based on the quantity of β-actin using the forward (5′-CCTGGCACCCAGCACAAT-3′) and reverse primers (5′-GCCGATCCACACGGAGTACT-3′) [7].

Determination of protein synthesis

Confluent HUVEC cultures exposed to ^{3}[H]proline (1 μCi/ml) were washed, TCA precipitated proteins collected, lysed in 0.2 N NaOH, mixed with Ultima Gold, and radioactivity determined.

Determination of total and oxidized glutathione (GSSG)

Washed cultures were collected into 10 mM HCl and further lyzed by freezing and thawing 3 times. Proteins were precipitated by addition of SSA (10%) followed by centrifugation (10,000×*g*) and the supernatants collected for glutathione determination. Total glutathione (GSH + GSSG) was determined by the Anderson recycling method [1]. To determine the GSSG level, 2-vinylpyridine was used to conjugate GSH and remove it from the mixture as described [1]. For normalization, the protein pellets of cellular samples were lyzed in 0.5 N NaOH and quantified by the Lowry method [23].

Neutral red (NR) cytotoxicity assay

EC cultures in fibronectin-coated (5 μg/ml) 96 wells plates treated with CSSA or NAC were washed with serum free DMEM and treated with tBuOOH 0.1 mM for 3 h. The quantity of viable cells was determined by the NR staining method: cells were incubated in NR working solution (1:100 in DCCM) for 2 h at 37°C, washed twice with PBS and the dye extracted with Sorrenson solution (0.07 M trisodium citrate, 0.03 M citric acid and 0.1 N HCl) at room temperature. The optimal density (OD) of the dye was determined at 550 nm in an ELISA reader. Cell cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity}(\%) = [1 - \text{NR uptake in treated wells} / \text{NR uptake in control wells}] \times 100$$

Control (untreated wells) was determined as 0% cytotoxicity. The results are presented as mean ± SD of three experiments performed in triplicate.

Statistical analysis

Two-tailed Student's *t* test and ANOVA were used for statistical analysis. Differences at $P < 0.05$ were considered statistically significant.

Results

Effect of allicin on cellular protein biosynthesis in HUVEC

Confluent HUVEC cultures incubated with allicin (35 μM) for 48 h demonstrated normal morphology (data not shown). HUVEC cultures were incubated in the absence or presence of allicin (35 μM) up to 21 h and [^{3}H]proline was added to the cultures for 3 h intervals as indicated in Fig. 1a. Proline incorporation increased in cultures exposed to allicin for 3–6 but not for 10–13 and 18–21 h time intervals. This increase was concentration-dependent with a maximal effect at 35–40 μM (Fig. 1b).

HUVEC micro-array analysis

Micro-array analysis was performed under the conditions in which protein synthesis was increased by allicin (35 μM for 5.5 h). mRNA was extracted from cultures treated with and without allicin and subjected to micro-array analysis. The human genome GeneChip including 22,277 genes was used. The expression of

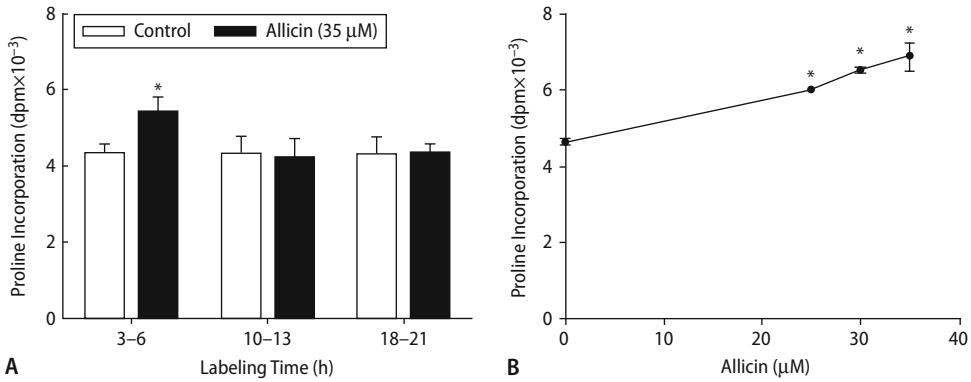

Fig. 1 Allicin affect on protein synthesis in HUVEC. HUVEC cultures were incubated in the absence or presence of allicin (35 μM) and exposed to [^{3}H]proline for the indicated time periods (**a**), or in the presence of increasing concentrations of allicin and [^{3}H]proline for 5.5 h (**b**). The amount of [^{3}H]proline incorporation was determined and presented as mean ± SD of three independent experiments performed in triplicate. *$P < 0.05$

116 genes was up-regulated by more than twofold and that of 100 genes down-regulated by at least one-half. The affected genes were divided into biological process categories according to the Gene Ontology Consortium (Table 1). The percentage of genes in the various categories suggested that: (1) signal transduction genes (pro-proliferation) were mostly down-regulated; (2) response to stress genes were mostly up-regulated; (3) genes involved in cell adhesion, cell death (pro-and anti-apoptosis), oxygen and ROS metabolism were all up-regulated; and (4) cell–cell signaling genes were all down-regulated. The up-regulated genes included genes of the phase II detoxifying enzymes group (Table 2). The significant up-regulation of HO-1 and GCLM observed in the micro-array experiment was further validated by Real Time PCR.

Effect of allicin on cellular glutathione level

The significant up-regulation of GCLM suggested the potential increase in glutathione in response to allicin.

Table 1 Distribution of the allicin up- and down-regulated genes determined by the gene-array among the "biological process" categories

Group	Up-regulated genes (%)	Down-regulated genes (%)
Protein and amine metabolism	19	11
General—nucleobase, nucleoside, nucleotide and nucleic acid metabolism	9	12
Pro-proliferation and development—nucleobase, nucleoside, nucleotide and nucleic acid metabolism	4	12
Cell growth and/or maintenance	10	19
General—signal transduction	8	4
Pro-proliferation—signal transduction	1	13
Response to stimulus	10	6
Response to stress	8	2
Cell adhesion	13	0
Cell death (pro- and anti-apoptosis)	7	0
Oxygen and reactive oxygen species metabolism	6	0
Cell–cell signaling	0	4
Others	5	17

Table 2 Allicin induced up-regulation of oxidative stress-related genes

Gene	Micro-array (fold increase)	Real time PCR (fold increase)
HO-1	11.3	14.6 ± 0.6
GCLM	2.8	3.9 ± 0.3
DUSP1	3.2	
SLC7A11	3.0	
Thioredoxin reductase 1	2.5	
Thioredoxin reductase 2	2.1	

The real time PCR results are presented as mean ± SD ($n = 3$)

HUVEC exposure to allicin (35 μM for 5 h) caused a 30% increase in the cellular glutathione level (data not shown). This hypothesis was further studied using bovine aortic EC since these cells are easier to grow and demonstrate higher sensitivity to allicin (a lower concentration of allicin was used in the following experiments). EC cultures exposed to allicin (15 μM) for various time periods demonstrated a slight decrease in the cellular glutathione level in the first 30 min of incubation followed by a significant increase (up to fourfold) after 24–48 h, which gradually decreased to the basal level after 72 h (Fig. 2a). Allicin demonstrated a concentration-dependent effect on the cellular glutathione level, reaching a maximal increase (up to eightfold) at a concentration of 15–20 μM after 28 h of incubation (Fig. 2b). Repetitive additions of allicin, every other day up to 10 days, demonstrated the capacity of allicin treatment to maintain a stable elevated glutathione level of about fivefold higher than the basal glutathione level (Fig. 3). The addition of BSO (15 μM), an inhibitor of the enzyme GCL, which is responsible for glutathione biosynthesis [2], reduced the allicin-induced increase in the glutathione level below the basal glutathione level. The addition of NAC (2 mM), following deacetylation may provide free cysteine to be used as a substrate for glutathione biosynthesis. This resulted in an increase in the cellular glutathione level, which also decreased to basal level in the presence of BSO (Fig. 4). The effect of allicin derivatives [GSSA or CSSA (0.3 mM)] as a function of time on the gluta-

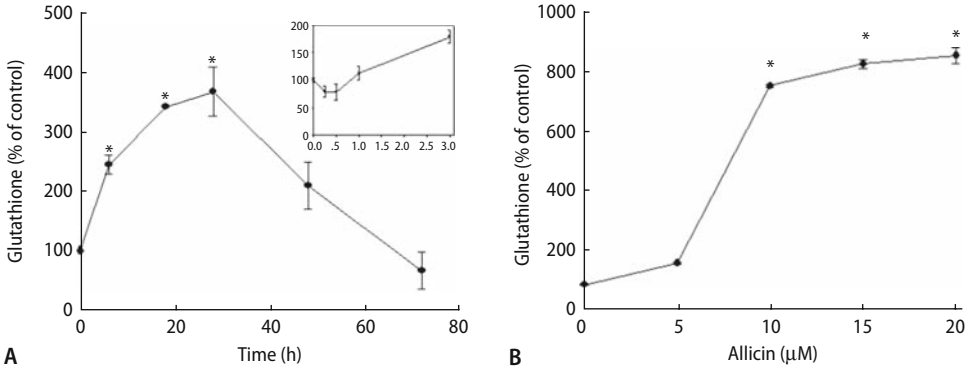

Fig. 2 Effect of allicin on the glutathione level. EC cultures were incubated: (**a**) with allicin (15 μM) for the indicated time periods (the insert represents incubation time up to 3 h); (**b**) with allicin at the indicated concentrations for 28 h. The glutathione level was measured and presented as mean ± SD of three independent experiments performed in duplicate. *$P < 0.05$

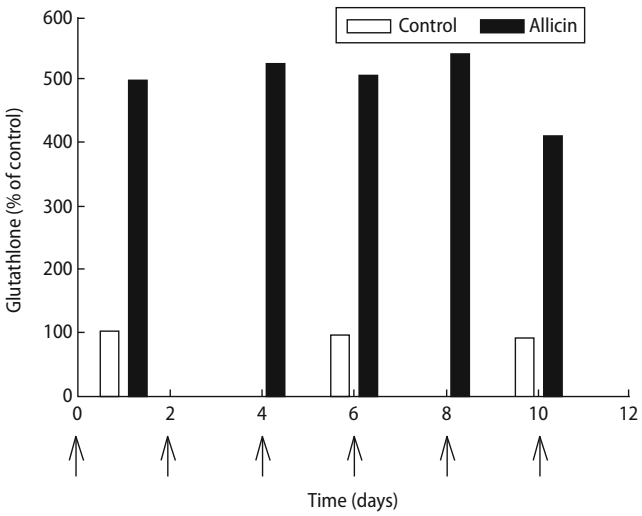

Fig. 3 Effect of repetitive additions of allicin on the glutathione level. EC cultures were incubated with repetitive additions of allicin (15 μM) every other day up to 10 days and the glutathione level measured. The experiment was performed twice in duplicate. ANOVA test showed significant differences between the control and treated groups

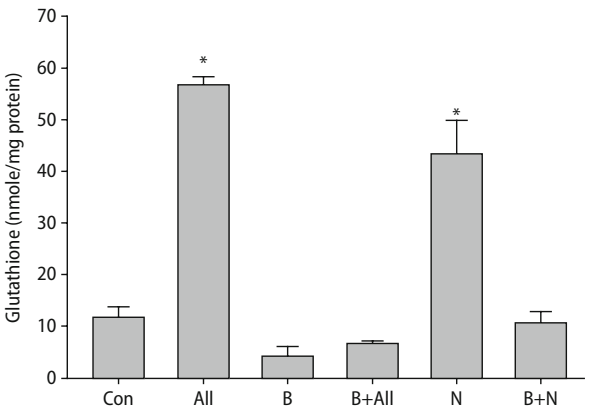

Fig. 4 Effect of BSO and NAC on the glutathione level. EC cultures were incubated with (All) or without (Con) allicin (20 μM), BSO (B; 15 μM) or NAC (N; 10 mM) for 24 h. The glutathione level was measured and presented as mean ± SD of three independent experiments performed in duplicate. ANOVA test showed significant differences between groups. *$P < 0.05$

thione level was studied in EC cultures. Treatment of EC cultures with the allicin derivative CSSA resulted in about a fivefold increase in the glutathione level after 5 h of incubation followed by a slight decrease after 24 h (Fig. 5). However, EC incubated with GSSA (another allicin derivative) demonstrated about a sixfold increase in the glutathione level after 24 h. The possible dissociation of glutathione from GSSA, which may contribute to the glutathione pool in the Anderson recycling assay, was tested. Less than 5% of the GSSA was dissociated and detected as glutathione in the assay and this value was deducted from the results. The glutathione values shown above represent total cellular glutathione (GSH plus GSSG). Therefore, both total glutathione and GSSG levels were further determined and demonstrated that in both control and treated EC the GSSG level was lower than 2% of the total cellular glutathione level. The above results demonstrating an increased glutathione level represent an increase in free glutathione.

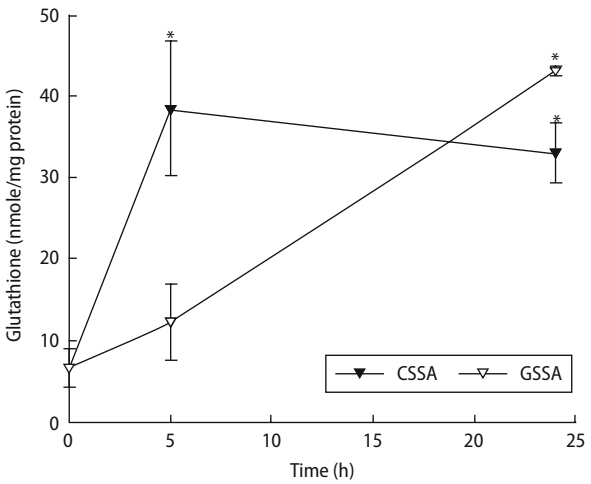

Fig. 5 Effect of CSSA and GSSA on the glutathione level. EC cultures were incubated with CSSA or GSSA (0.3 mM) for the indicated time periods. The level of glutathione was measured and presented as mean ± SD of three independent experiments performed in duplicate. *$P < 0.05$

Antioxidant effect of CSSA pretreatment in EC

EC cultures were incubated in the absence or presence of either CSSA (0.2 mM) or NAC (2 mM) for 18 h, washed and further exposed to tBuOOH (0.1 mM), a highly active oxidant, for 3 h followed by NR staining of viable cells, and the percentage of cytotoxicity determined. Treatment with tBuOOH resulted in 67.7 ± 8.7% cytotoxicity. However, pretreatment with CSSA (0.2 mM) and NAC (2.0 mM) reduced the cytotoxicity to 33.7 ± 9.5% (a significant reduction of 50%; $P < 0.05$) and to 50.3 ± 6.8% (a 25% reduction), respectively, indicating the significant capacity of allicin derivative compared to NAC to protect cells from oxidative stress. However, the total glutathione level observed in control, CSSA and NAC pretreated cultures as well as the GSH/GSSG ratio were not changed following tBuOOH treatment (data not shown).

Discussion

Free radicals and oxidative stress are involved in cellular injuries and subsequently in the initiation and progression of cardiovascular and neurodegenerative diseases [4, 24]. In the current study, the effect of allicin on gene expression was examined in general, and more specifically, on the expression of oxidative stress-related genes and glutathione level in vascular EC. The effect of allicin and its derivatives on both gene expression and the glutathione level was studied under optimal growth conditions in the presence of serum. It should be pointed out that the serum did not subdue the activity of allicin and its derivatives. This observation may reflect the hydrophobic nature of allicin and its derivatives resulting in rapid association with cellular membranes.

The allicin modulated genes (116 and 100 up- and down-regulated genes, respectively) were divided into biological process categories. In cell adhesion, cell death, and oxygen and ROS metabolism categories, only up-regulated genes were observed indicating the up-regulation of anti-oxidant cellular protective mechanisms in response to allicin treatment. The response included increased synthesis of adhesion molecules, expression of anti-apoptotic genes, and genes involved in ROS metabolism, probably aimed at replacing the cell surface damaged receptors and protecting the cells from oxidative stress. However, under allicin treatment, the signal transduction and cell-cell signaling genes were mostly down-regulated representing reduced proliferation potential of allicin treated cells.

The up-regulated genes included the following from the phase II detoxifying enzymes group: (1) GCLM, the modifier subunit of the rate-limiting enzyme of GSH synthesis; (2) HO-1, a cyto-protective enzyme, the rate limiting enzyme in the conversion of heme into biliverdin, followed by rapid metabolism of biliverdin to bilirubin, which is a potent antioxidant [3]. Generally, HO-1 acts as a key protective player in the cellular response to injury and oxidative stress; (3) Thioredoxin reductase 1 and 2, known to participate in thiol-dependent cellular reductive processes, regenerate thioredoxin, which serves as a reducing equivalent and may also directly reduce hydroperoxidized lipid [32, 33]; (4) SLC7A11 (also named xCT), induced by oxidative stress stimuli and belongs to the anionic amino acid transport system X_c^-, which mediates cystine influx, thus contributing to the maintenance of the intracellular GSH level [29]; and (5) DUSP1 (also named MKP1), a negative regulator of MAP kinases, induced in human skin fibroblasts by oxidative/heat stress and growth factors and identified as a hypoxia responsive gene [21].

In previous studies, the garlic oil product DATS was shown to up-regulate the ARE-dependent genes NQO1 and HO-1 via Nrf2 in HepG2 cells [6]. DAS, another garlic oil product, was shown to induce NQO1 in wild type mice but not in Nrf2 (-/-) mice [10], indicating a possible role for Nrf2 in mediating the response to garlic products. In the present study the direct activity of allicin induced a few ARE-regulated genes, but the involvement of Nrf2 was not explored. It should be noted that allicin does not induce all known ARE-regulated genes, such as NQO1 and glutathione S-trasferase, but the potential role of ARE in mediating the response to allicin is not excluded. For example, a group of four allicin up-regulated genes (HO-1, GCLM, Thioredoxin reductase 1, SLC7A11) were induced by laminar flow conditions in HUVEC, probably as part of the protective role of the steady laminar flow in vascular endothelial cells [31]. This process was inhibited by Nrf2 antisense, which suggests a Nrf2-regulated mechanism [5].

The group of regulated enzymes included the GCLM, which suggests the potential of allicin to up-regulate the glutathione cellular level. A significant increase (up to eightfold) was found in the glutathione (the major cellular antioxidant) in response to allicin. Therefore, the present observations could suggest the potential use of allicin or its derivatives to up-regulate the cellular antioxidant protective mechanisms. Allicin can easily penetrate the cell membrane and conjugate with GSH, forming GSSA [25, 28]. This may account for the short-term decrease in the intracellular GSH level observed following exposure to allicin (during the first 30 min of incubation). Allicin derivatives, CSSA and GSSA, up-regulated the glutathione level at a higher concentration (15-fold) than allicin. The effect of CSSA was evident after a shorter

time period than GSSA, probably due to either a higher cellular permeability or higher rate of dissociation of the former. It can be postulated that the allicin derivatives formed in the body on exposure to allicin may serve as a reservoir (buffer) for allicin-derived thiol-reactive molecules. Furthermore, the increased level of cellular glutathione represents an increase in its reduced (GSH) but not its oxidized (GSSG) form, indicating an increased antioxidant potential of the cells following exposure to allicin or its derivatives. Furthermore, cells pretreated with CSSA significantly protected the cells from tBuOOH-induced oxidative stress and cell death.

The enzyme GCL plays a critical role in maintaining GSH homeostasis under both physiological and pathological conditions [2, 22]. This enzyme is a heterodimer, which contains a catalytic subunit (GCLC) and a regulatory subunit (GCLM), although GCLC alone can catalyze GSH formation. Its binding with GCLM enhances the enzyme activity by lowering the Km for glutamate and ATP [13, 34]. In the present study the increase in GCLM level without concomitant increase in GCLC level was sufficient to up-regulate the glutathione level. The capacity of allicin to up-regulate the glutathione level through up-regulation of its biosynthetic pathway was further supported by the use of BSO, a GCL inhibitor that completely inhibited the allicin-induced increase in the glutathione level.

Hoekstra et al. [15] suggested that the glutathione redox cycle plays an important role as an endogenous antioxidant defense mechanism in cultured EC exposed to oxidative stress. The present study demonstrated the allicin-induced up-regulation of GSH in EC, which supports the results of the previous study. The higher level of glutathione in the CSSA and NAC pretreated cultures prior to the tBuOOH treatment may play a role in protecting the cells from oxidative stress. However, the expected reduction in glutathione level and increased GSSG level following exposure to tBuOOH were not observed. Therefore, the role of the increased glutathione in the protection of cells from oxidative stress is not clearly proved in the present study and it might be mediated by the induced phase II detoxifying enzymes. Further studies should be done to explore the mechanism(s) by which allicin and its derivatives protect cells from oxidative stress.

Fresh garlic clove contains 4–5 mg of allicin. Consumption of one-to-three cloves per day results in allicin blood concentration of 6–18 μM. In the present study, the allicin concentration was close to the level expected after garlic consumption.

The glutathione cellular level declines in numerous tissues as a result of aging. This reduced level is associated with various pathological processes, such as atherosclerosis [19] and Alzheimer's Disease [22]. Therefore, it is highly important to determine means to up-regulate the cellular glutathione level. The present study suggests that allicin and its derivatives can up-regulate the cellular glutathione level, possibly by up-regulating GCLM, the rate limiting enzyme in glutathione biosynthesis, thereby protecting cells from oxidative stress. This effect may attenuate the progression of diseases at which oxidative stress is involved.

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