RESEARCH ARTICLE

Garlic components inhibit angiotensin II-induced cell-cycle progression and migration: Involvement of cell-cycle inhibitor $p27^{Kip1}$ and mitogen-activated protein kinase

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Garlic has been used for prevention and treatment of hypertension; however, the molecular mechanisms of garlic's effects remain to be elucidated. In this study, the mechanisms of the *in vitro* effect of organosulphur compounds derived from garlic on growth and migration of cultured aortic smooth muscle cells isolated from spontaneously hypertensive rats were investigated. We demonstrated that allyl methyl sulphide (AMS) and diallyl sulphide (DAS) inhibited aortic smooth muscle cell angiotensin II-stimulated cell-cycle progression and migration. Neither cell viability nor annexin-V-binding analysis revealed cytotoxic effects of both organosulphur compounds at the used concentrations. Instead, their inhibitory effects were associated to the prevention of the cell-cycle inhibitor $p27^{Kip1}$ (p27) downregulation and the reduction of extracellular signal-regulated kinase 1/2 phosphorylation. When we assessed the antioxidant activity of AMS and DAS, we found that both organosulphur compounds inhibited angiotensin II-reactive oxygen species generation. Our findings show that AMS and DAS, compounds derivate from garlic, could be effective antioxidants targeted at the arterial remodelling seen in hypertension.

Keywords:
Angiotensin II / Cell cycle / Garlic / Hypertension / Migration

1 Introduction

Remodelling of large and small arteries by excessive proliferation and migration of vascular smooth muscle cells (SMCs) contributes to the development and complications of hypertension [1]. Several vasoactive agents have been implicated in this process, of which angiotensin II (Ang II) is one of the most important [2]. Ang II induces contraction, migration and proliferation, and is a potent progression factor that stimulates the transition from G0/G1 phase in the cell cycle [3]. Ang II-mediated actions involve cell-cycle inhibitors downregulation [4] activation of mitogen-activated protein kinases (MAPKs) [2] and stimulation of reactive oxygen species (ROS) generation [5]. Therefore, modulation of Ang II events has a critical therapeutic implication for vascular disease. Garlic (*Allium sativum* L.), among the oldest cultivated plants is used both as food and as medicinal plant. In fact, this common food plant is a rich source of several phytonutrients used in the treatment and prevention of a number of diseases, including cancer [6], hypercholesterolemia [7], diabetes [8] and hypertension [9, 10]. However,

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Abbreviations: AMS, allyl methyl sulphide; **Ang II,** angiotensin II; **AoSMC,** aortic smooth muscle cell; **CM-H_2DCFDA,** 5-(and 6)-chloromethyl-2′7′-dichloro dihydrofluorescein diacetate; **DAS,** diallyl sulphide; **ERK,** extracellular signal-regulated kinase; **FCS,** fetal calf serum; **MAPK,** mitogen-activated protein kinase; **MTT,** 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; **PI,** propidium iodide; **ROS,** reactive oxygen species; **SHR,** spontaneously hypertensive rat; **SMC,** smooth muscle cell

evidence based on rigorous clinical trial about garlic use is not convincing, and certain issues regarding the proper use of garlic, *i.e.* employ of different available preparations, dose, duration and interaction with generic drugs should be optimized [11]. Many studies have shown the antioxidant activity and cardiovascular disease-protective properties of organosulphur compounds [12–17] but the mechanisms implicated are far from being clarified. Further research should also be carried out to identify garlic-specific compounds that are responsible for most of their biological effects. Recently, there has been an increasing scientific attention on the effects of organosulphur compounds derived from garlic, thiosulphinates and volatile sulphur compounds which are also responsible for the pungent of the vegetable [18] *In vitro* studies have confirmed the ability of some of these compounds to reduce endothelial cell proliferation, migration, invasion and tube formation [19]. They also modulate the expression of adhesion molecules [20] and are capable of having prophylactic effects on cerebral injury in stroke-prone spontaneously hypertensive rats (SHRs) [21]. The purpose of the current investigation was to assess the role of two organosulphur compounds derived from garlic, allyl methyl sulphide (AMS) and diallyl sulphide (DAS) on AngII-induced proliferation and migration of aortic smooth muscle cell (AoSMC) SHR, and to elucidate the underlying cellular signalling transduction events.

2 Materials and methods

2.1 Reagents

Antibodies were purchased from Santa Cruz Biotechnology: p27 (sc-1641), P-ERK1/2 (sc-7383), and extracellular signal-regulated kinase 2 (ERK2) (sc-154) or Sigma: Actin (A1978). 5-(and 6)-Chloromethyl-2′7′-dichloro dihydrofluorescein diacetate (CM-H_2DCFDA) was purchased from Molecular Probes (Eugene, OR, USA). All other reagents, except those mentioned in the text, were provided by Sigma (St. Louis, MO, USA).

2.2 Cell cultures

SMCs derived from thoracic aortas from 16-week-old male SHR were isolated according to a technique previously described [22].

2.3 Cell viability

In order to establish the effective concentrations on cell cultures of organosulphur compounds derived from garlic without affecting cell viability, this was measured by a CellTiter non-radioactive cell proliferation Assay (Promega). Cells were seeded in 96-well microtiter plates and exposed 48 h to 0.1% fetal calf serum (FCS)/DMEM to synchronize the culture. Cells were stimulated with Ang II (10^{-7} M) alone or Ang II plus 20 and 100 μM of AMS or DAS. DMEM with 0.1% FCS was used as control. After 24 h exposure, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mM) was added, and cells were incubated in the dark at 37°C for 4 h. Absorbance at 570 nm was measured in a Microplate Reader (BioRad).

2.4 Flow cytometry analysis

To further investigate the effect of AMS and DAS on cell-cycle progression, we performed cell-cycle analysis on Ang II-stimulated cells by flow cytometry. Cells were fixed with 70% ethanol (20 min, –20°C), treated with 20 μg/mL RNase A (Biodynamics, Buenos Aires, Argentina) and stained with 25 μg/mL propidium iodide (PI). The fluorescence signal was recorded using a FACSCalibur (Becton Dickinson) and analyzed with WinMDI and Cylchred softwares. Cells in G0/G1, S and G2/M phases were expressed as percentages of total events (10 000).

2.5 Annexin V assay

Annexin V-FITC apoptosis detection kit from Becton Dickinson was used by following the manufacture's recommendations. In brief, quiescent AoSMCs stimulated 24 h with Ang II (10^{-7} M) alone or Ang II plus 100 μM of AMS or DAS were washed twice with cold PBS and gently scraped and suspended in 400 μL of 1X Binding Buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were transferred to a tube, added 5 μL of FITC Annexin V and 5 μL PI, and incubated for 15 min at room temperature (25°C) in the dark. Flow cytometry analysis was performed within 1 h.

2.6 Cell migration assay

AoSMC migration was assessed by the scrape-wound migration assay described by Pukac [23] with some modifications. In a few words, cells were cultured in 24-well tissue culture plates until confluence and then rendered quiescent for 24 h before the experiments. All the experiments were performed in the presence of hydroxyurea to prevent cell proliferation [24]. Before stimulation, a gap was made in confluent cultured AoSMC in 24-well plates, using a sterile toothpick. Under inverted microscopy video images of selected fields were obtained at the beginning of the assay (0 h) and after 24 h of incubation with Ang II (10^{-7} M) alone or Ang II plus 20 and 100 μM of AMS or DAS. DMEM with 0.1% FCS was used as control. Migration was determined by the difference between the cell-free area at 0 and 24 h at six marked fields on each well.

2.7 Western blot analysis

Cell lysates prepared with lysis buffer supplemented with protease inhibitors were electrophoretically separated on 12% SDS-PAGE and Western blot analysis was performed as described previously [25].

2.8 Measurement of ROS in intact cells

Intracellular ROS levels were measured with the fluoroprobe CM-H_2DCFDA in unstimulated cells and in cells exposed to Ang II (10^{-7} M) in the absence and presence of AMS or DAS (20 and 100 μM; 1 h pre-treatment). Apocynin (50 μM), an inhibitor of NADPH oxidase, was also used. Cells were loaded with CM-H_2DCFDA (6 μmol/L), dissolved in DMSO and incubated 45 min at room temperature. Fluorescence was measured continuously for 20 min on a microplate fluorometer (Fluoroskan Ascent, Labsystems) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

2.9 Statistical analyses

Data are presented as mean ± SEM of (n) independent experiments. The statistical significance was assessed by one-way analysis of variance (ANOVA). p-Value < 0.05 was considered significant.

3 Results

3.1 Effect of garlic organosulphur compounds on AoSMC viability

AMS and DAS were studied on Ang II-stimulated AoSMCs from SHR. AMS and DAS had no significant effect on MTT convention to formazan compared with that in Ang II-stimulated group, discarding any detrimental effect of these organosuphur compounds on cell cultures (Fig. 1).

3.2 Effect of garlic organosulphur compounds on cell-cycle progression

Incubation of AoSMCs for 30–40 h with 0.1% FCS culture media led to the accumulation of the cells in the G1 phase (Fig. 2). After 24 h stimulation with Ang II (10^{-7} M), the amount of cells in S phase was increased and those in G1 phase was decreased. Ang II-treated AoSMCs incubated with AMS or DAS (100 μM) showed that both organosulphur compounds markedly blocked Ang II-induced cell-cycle progression by arresting the cells in G0/G1 phase (Fig. 2). Lower concentrations of AMS or DAS did not cause any effect on Ang II-induced cell-cycle progression (data not shown). Importantly, we did not detect any significant increase in the percentage of sub G0/G1 cells after incubation with AMS or DAS, which indicates a specific interference with the progression of G1 phase without inducting apoptosis. This result was corroborated by measuring Annexin-V binding, which revealed that AMS and DAS, at the used concentrations, did not induce an apoptotic response (Fig. 3).

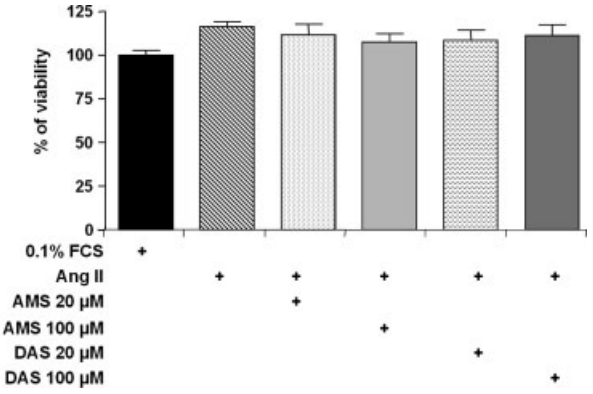

Figure 1. AMS and DAS do not affect cell viability. AoSMC were incubated with Ang II 10^{-7} M containing 0, 20 and 100 μM either AMS or DAS for 24 h. Cell viability was measured by MTT assay. Data, mean ± SEM, from three different experiments represent the percentage of viability *versus* 0.1% FCS stimulation (assigned as 100%).

3.3 Effect of garlic organosulphur compounds on AoSMC migration

Ang II induced a significant increase of AoSMC migration after 24 h stimulation (Fig. 4). Ang II-stimulated AoSMC migration was affected when cells were co-incubated with AMS or DAS. DAS markedly inhibited Ang II-stimulated cell migration in a dose-dependent manner; but only 100 μM of AMS decreased the effect of Ang II on AoSMC migration (Fig. 4).

3.4 Garlic organosulphur compounds impaired Ang II-induced p27 downregulation and ERK1/2 activation

The expression of the cyclin-dependent kinase inhibitor p27 was almost totally inhibited by Ang II and both AMS and DAS (100 μM) were able to avoid p27 downregulation (Fig. 5A). It has been previously demonstrated that Ang II activates MAPK within 5 min in a dose-dependent manner, with a maximal stimulation observed at 10^{-7} M [26]. Accordingly, we examined the effect of AMS and DAS on the Ang II-activated early signal-transduction pathway using phospho-ERK1/2-specific antibody. Ang II induced an increase in ERK 1/2 phosphorylation (Fig. 5B). Pre-treatment with AMS or DAS (100 μM) for 1 h before the addition of Ang II significantly reduced Ang II-mediated ERK1/2 activation ($p < 0.05$).

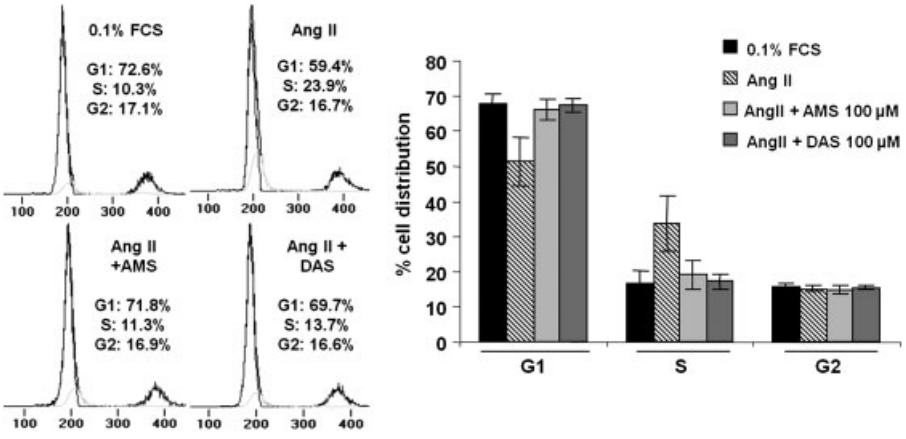

Figure 2. AMS and DAS induce G1 cell-cycle arrest in Ang II-stimulated cells. AoSMC were incubated in serum-free medium for 24 h to induce quiescence, followed by addition of Ang II 10^{-7} M plus AMS or DAS (100 µM), and cultured for 24 h. Flow cytometry analysis of the cell cycle was performed. The data are representative of three independent experiments.

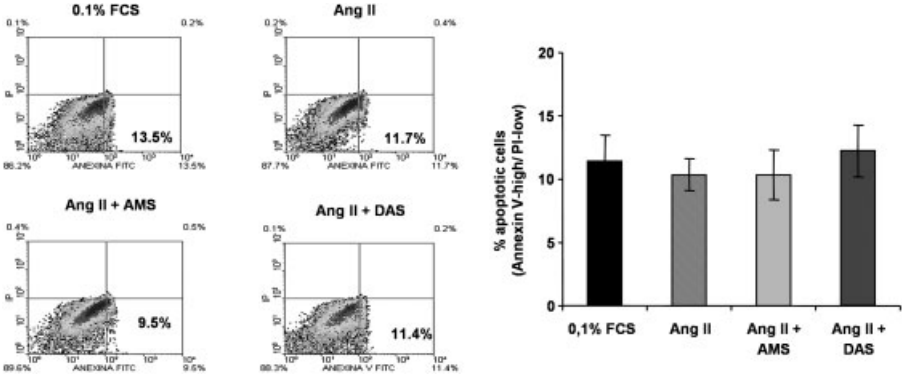

Figure 3. AMS and DAS do not induce apoptosis in Ang II-stimulated cells Annexin V-FITC binding analysis by flow cytometry was made on Ang II-stimulated AoSMC after AMS or DAS treatment. In these dot graphs, viable cells (Annexin V-low/PI-low) are found in the lower left quadrant, apoptotic cells (Annexin V-high/PI-low) in the lower right, postapoptotic secondary necrotic cells (Annexin V-high/PI-high) in the upper right and primary necrotic cells (Annexin V-low PI-high) in the upper left. Numbers in each quadrant are percentage of cells they contain. The data shown are representative of two independent experiments.

3.5 Garlic organosulphur compounds attenuate Ang II-induced generation of ROS

Ang II stimulation resulted in a significantly increase in CMH_2-DCFDA fluorescence (Fig. 6), and responses were sustained for up 20 min after Ang II addition. Apocynin, a selective inhibitor of NADPH oxidase, completely abolished Ang II-stimulated responses, indicating that NADPH oxidase is the source of intracellular ROS. Pre-exposure of AoSMC to AMS (100 µM) or DAS (20 and 100 µM) significantly reduced Ang II-mediated ROS generation as far as apocynin did (Fig. 6). These results suggest that garlic-derived compounds are capable of strong antioxidant activity.

4 Discussion

In vitro studies strongly suggest that garlic has the ability to reduce some events associated with cardiovascular disease [27–29]. Volatile organosulphur compounds, named thiosulfinates, are responsible for some of its bioactive actions [30]. These organosulphur compounds, mainly disulphides and trisulphides characteristic for garlic taxa, have shown to be chemically and pharmacologically active [31]. In this study, we investigated the effect of synthetic organosulphur compounds derived from garlic, AMS and DAS, on AoSMCs proliferation and migration, and evaluated the molecular mechanisms underlying these effects. The major findings of our study demonstrate that (i) AMS and DAS specifically block Ang II-induced cell-cycle progression, leading to the accumulation of cells in the G0/G1 phase; (ii) Ang II-induced migration of AoSMC was inhibited by AMS and DAS and (iii) avoidance of p27 downregulation and MAPK activation, and decrease of Ang II-induced ROS generation are some of the molecular mechanisms involved in AMS and DAS effect on Ang II-treated AoSMC. SMC proliferation is certainly important during vascular development, but it is clear that increased SMC growth and migration are important contributors to the pathogenesis of several important cardiovascular disease states including atherosclerosis, restenosis and hypertension. A large number of extrinsic cues (humoral factors, extracellular matrix, cell–cell

interactions among others) have been identified as regulators of SMC growth and migration [32]. However, the precise cellular signalling mechanisms involved are not completely understood, and very little is known about how (or if) these signalling pathways are integrated. In our study we found that Ang II-induced cell-cycle progression was almost completely inhibited by both organosulphur compounds. Our data also indicated that AMS and DAS led to G1 arrest. This is consistent with other studies showing that some garlic compounds (such as ajoene, Diallyl trisulphide, S-alk(en)yl cysteine sulfoxide) induce G1 cell-cycle accumulation in different cell lines [33, 34]. In addition, a double staining of annexin-V and PI showed that AMS and DAS did not induce cell apoptosis as other organosulphur compounds from garlic produce in cancer cells [35].

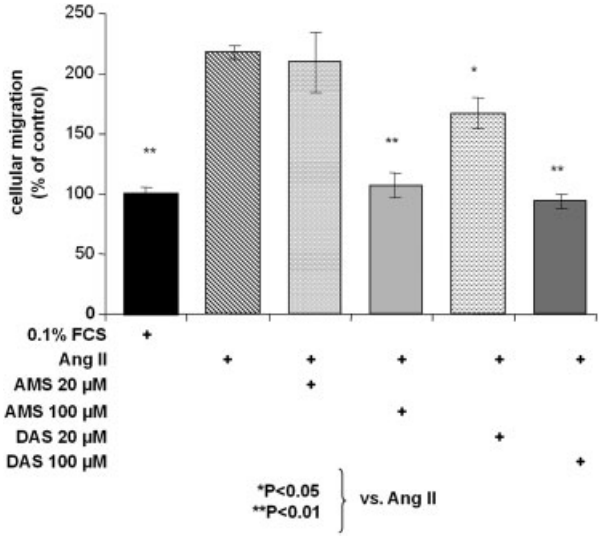

Figure 4. AMS and DAS inhibit Ang II-stimulated cell migration: AoSMCs were incubated in the presence of hydroxyurea with Ang II alone, or plus either AMS or DAS (20 and 100 µM) for 24 h. Cell migration was measured as described in Section 2. Data, expressed as the mean ± SEM ($n = 8$), are presented as percentage of area relative to control conditions (0.1% FCS). $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* Ang II.

Ang II is involved in the mechanisms of vascular remodelling during hypertension and it is well documented that this vasoactive substance stimulates SMC migration [36]. Our data showed that AMS and DAS inhibited Ang II-induced migration. It has been reported that garlic extract is a potent inhibitor of neutrophils migration through endothelial cell monolayers [37] and other studies clearly have demonstrated that administration of DAS significantly retarded endothelial cell proliferation and migration [19]. No evidence about the antimigratory effect of AMS and DAS on AoSMC has been reported until now. The above-mentioned results prompted us to investigate the underlying molecular mechanisms. Since the previous studies have demonstrated that suppression of both cell proliferation and migration is controlled by the cyclin-kinase inhibitor p27 [38, 39] and it has been suggested that this cell-cycle inhibitor coordinately regulates cell proliferation and migration [40], we investigated the effect of AMS and DAS on the expression of p27 in confluent cultures of AoSMC stimulated with Ang II (10^{-7} M). Diez-Juan *et al.* [40] reported that changes in p27 expression concomitantly regulate vascular SMC and fibroblasts growth and locomotion. A low level of p27 expression correlates with high proliferative and migratory capacity, whereas nuclear accumulation of p27 associates with a quiescent and static phenotype. Consistent with this, we demonstrated that AMS and DAS inhibited Ang II-induced proliferation and migration by avoiding p27 downregulation.

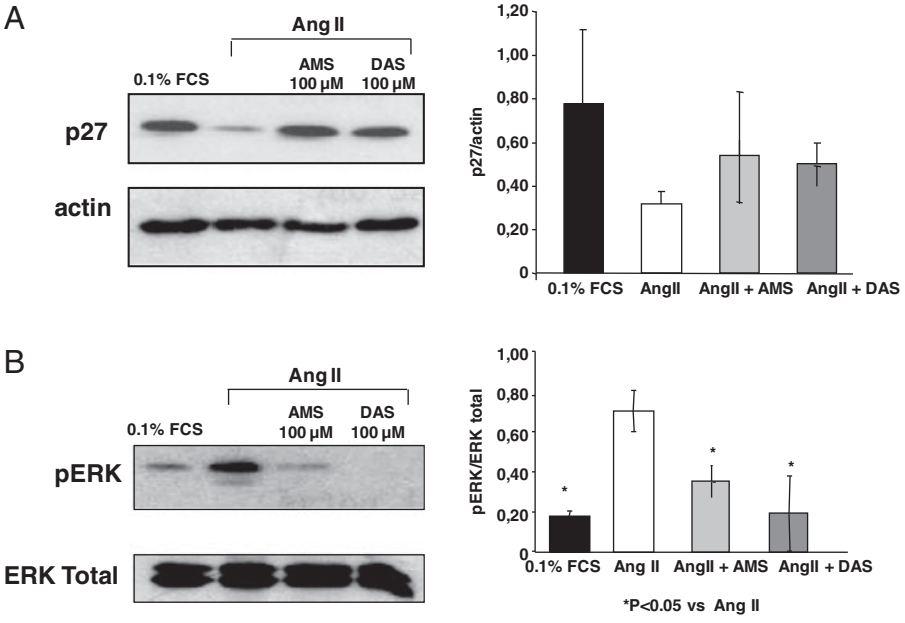

Figure 5. AMS and DAS avoid Ang II-induced p27 downregulation and MAPK activation. AoSMCs were treated with Ang II (10^{-7} M) in the presence or absence of AMS or DAS (100 µM, 1 h pre-exposed). Cells were separated by SDS-PAGE and analyzed by Western blot using specific antibodies. (A) After 24 h incubation, expression of cell-cycle inhibitor p27 was measured. Expression of actin was used as an internal control. Quantification of p27 expression is shown in the right panel and indicated values are means of three independent experiments. (B) After 10 min incubation, the levels of phosphorylation of ERK 1/2 were measured using phospho-specific or specific ERK 1/2 antibodies, and quantified by densitometry analysis. The right panel indicates the means of three independent experiments.

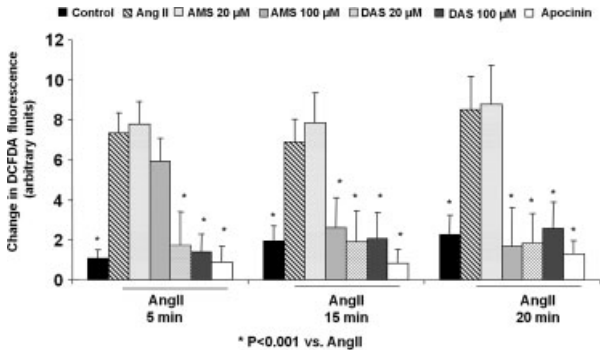

Figure 6. AMS and DAS inhibit generation of ROS in AoSMC. Bar graphs demonstrating the effect of AMS or DAS on CMH_2-DCFDA fluorescence in Ang II-stimulated AoSMC. Cells were pre-exposed to AMS, DAS (20 or 100 μM) or Apocynin (50 μM) for 1 h prior to Ang II addition. Results are presented as the Ang II -induced changes in CMH_2-DCFDA fluorescence, calculated as the difference between the stimulated response and the basal value. Experiments were repeated three times. $^*p<0.001$ *versus* Ang II.

Since the MAPK pathway plays a pivotal role in transducing environmental signals required for both cellular growth and migration, we examined the effect of both organosulphur compounds on the expression and activation of individual MAPKs in AoSMCs. Ang II-mediated activation of ERK1/2 was completely abolished by AMS and DAS.

Many of the organosulphur compounds properties are attributable to their antioxidant capacity [13, 41]. Garlic has been shown to contain antioxidant phytochemicals that prevent oxidative damage [10]. Ingestion of garlic leads to significantly lowered plasma and erythrocyte oxidant malondialdehyde levels and increased activities of some antioxidant enzymes, which indicates that consumption of garlic decreases oxidation reactions [42]. In addition, garlic exhibited protective effects against oxidative damage by reducing superoxide production [10], suppressing iNOS expression [13] and reversing the suppression of antioxidant enzymes (SOD, glutathione peroxidase) [21]. In our study, the production of ROS by Ang II was completely abrogated by DAS and in lesser extent by AMS. It has been reported that the intracellular redox state is a critical regulator of cell-cycle progression, and oscillations in intracellular redox state could play a central role in regulating progression from G0/G1 to S to G2 and M cell-cycle phases [43]. Our data allow us to speculate that the protection of AMS and DAS against vascular remodelling might be associated with modulation of both ROS overproduction and cell-cycle regulators expression. However, the exact mechanism of action by which AMS and DAS exert protection against oxidative stress and cell behaviour regulation remains to be elucidated and requires further examination. In conclusion, the findings of this study show that AMS and DAS have not only antioxidant effect but also have a protective effect against arterial remodelling seen in hypertension. This outcome is probably mediated *via* upregulation of the growth suppressor p27 and the attenuation of ERK 1/2 phosphorilation. Organosulphur compounds derived from garlic could be useful in preventing cardiovascular diseases associated with vascular remodelling.

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The authors have declared no conflict of interest.

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