Clinical and Experimental Pharmacology and Physiology (2002) **29**, 84–91

GARLIC AND ITS ACTIVE METABOLITE ALLICIN PRODUCE ENDOTHELIUM- AND NITRIC OXIDE-DEPENDENT RELAXATION IN RAT PULMONARY ARTERIES

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SUMMARY

1. The aims of the present study were to investigate the effects of fresh garlic and one of its active metabolites, allicin, on rat isolated pulmonary arteries (RPA).

2. In endothelium-intact and phenylephrine-precontracted RPA, the addition of a water or a 5% ethanol extract of fresh garlic (1–500 μg/mL) resulted in a dose-dependent relaxation reaching a maximum (mean±SEM) of –91 ± 3 and –93 ± 2%, respectively, with an ED_{50} of 113 ± 12 and 106 ± 10 μg/mL, respectively. The vasorelaxation was readily reversible upon washing and no tachyphylaxis was noted.

3. An extract of the external garlic storage leaf produced a significantly greater relaxation than the inner stem. Microfiltration of extracts with a 10 000 molecular sieve did not attenuate relaxation. Inactivation of alliinase and allicin formation, with either boiling of the garlic clove for 30 min or 100% ethanol treatment, completely abolished relaxation. In contrast, similar treatment of crushed garlic with formed allicin retained the relaxation response.

4. Pure allicin produced a similar relaxation as garlic extract, with an EC_{50} of approximately 0.8 μg/mL. Disruption of endothelium or N^G-nitro-L-arginine methyl ester pretreatment attenuated the relaxation, whereas indomethacin had no effect.

5. Prior garlic (500 μg/mL) treatment enhanced acetylcholine relaxation by shifting the response curve to the left, but had no effect on nitric oxide (NO) donor-induced responses.

6. These results demonstrate that garlic and the active metabolite allicin are capable of eliciting a NO-dependent relaxation in RPA and that this response is likely to be mediated via garlic activation of NO formation rather than its stabilization.

Key words: allicin, alliinase, endothelium, garlic, nitric oxide, pulmonary, vasorelaxation.

INTRODUCTION

Considerable evidence has accumulated to suggest that garlic has medicinal properties. Several reports have shown that a daily supplement of garlic may lower blood pressure, decrease ischaemic injury, reduce serum cholesterol, inhibit platelet function and enhance thrombolysis.[1–9] Garlic has also been suggested to improve arterial oxygenation associated with pulmonary dysfunction in patients with hepatopulmonary syndrome.[10] However, concrete scientific data to substantiate many of these claims remain elusive. In a recent publication, it was reported that oral administration of a steam-distilled garlic oil preparation did not significantly lower serum cholesterol or other lipid levels.[11] This and other studies seriously question the validity of the many claimed beneficial effects of garlic.[11,12] Recently, we reported that long-term oral garlic feeding in rats was effective in preventing the development of hypoxic pulmonary hypertension when the animals were subjected to 90 min hypoxia.[13] The results of this preliminary study suggest that garlic and/or its metabolites may be uniquely effective in the pulmonary vasculature; however, the exact mechanism(s) of this effect of garlic remains undefined.

Thus, the aims of the present study were to investigate and characterize the effect of garlic on pulmonary vasomotion. Specifically, we used isolated pulmonary artery ring preparations from rats and characterized the vasoactive effects of fresh garlic, different components of a garlic clove, allicin (a major precursor and/or active metabolite) and the chemical stability of garlic in response to heat and/or alcohol inactivation. Finally, the role of endothelium, nitric oxide (NO) and vasodilatory prostanoids on the observed vascular effects of garlic, as well as its interactions with other vasodilators, were also examined. The results of our study demonstrate that extracts of fresh garlic clove are capable of eliciting an endothelium (NO)-dependent relaxation and that allicin formation is important for its vasodilatory effect. Our findings further suggest that these vascular effects of garlic may be related to its ability to activate the formation of NO from the endothelium rather than chemically stabilizing and/or prolonging the activity of the released NO.

METHODS

Pulmonary artery ring studies

Male rats weighing 250–350 g were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) and the thoracic cavity was opened and both left and right main lobes of the lungs were quickly excised. The intralobar pulmonary arteries, approximately 1.0 cm in length, were carefully dissected from the surrounding connecting tissues under a stereomicroscope. The arteries were cut into rings (2–3 mm), with the outside diameter ranging from 0.5 to 1.2 mm. Each ring was then mounted, as described previously,[14] using two triangular-shaped 30 gauge stainless-steel needles in jacketed tissue chambers

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Received 14 May 2001; revision 17 August 2001; accepted 28 September 2001.

containing Krebs'–Henseleit (K-H) solution (37°C) gassed with 95% O_2 and 5% CO_2. The K-H solution consisted of (in mmol/L): NaCl 118; KCl 4.6; $NaHCO_3$ 27.2; $MgSO_4$ 1.2; KH_2PO_4 1.2; $CaCl_2$ 1.75; Na_2EDTA 0.03; glucose 11.1. The upper needle of each arterial ring was attached, with a silk suture, to a force-displacement transducer (FT 0.03C; Grass Instrument Co., Quincy, MA, USA) and changes in isometric force were recorded on a Grass Polygraph (model 7C).

All vessels were passively stretched to generate a resting tension of 2.0 g for isometric contraction recording. After 40 min equilibration, pulmonary rings were exposed to a maximum depolarizing concentration of KCl (70 mmol/L). When contractile responses plateaued, ranging 0.4–0.7 g active tension, pulmonary rings were rinsed with K-H solution and allowed to equilibrate for an additional 40 min prior to the start of garlic extract testing. At appropriate times, submaximal tone was elicited with 0.1–1 μmol/L phenylephrine, during which time increasing concentrations of garlic extracts were added to the bathing medium. In all cases, only one dose–response curve of garlic extract was determined in each pulmonary segment, except for cases when repeated testings of garlic extract were indicated to determine tachyphylaxis development and following various pharmacological interventions, such as pretreatment with the NO synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), indomethacin and superoxide dismutase. Data are expressed as a percentage relaxation or percentage decrease in phenylephrine-induced constrictory tone.

Prior to garlic extract testing, the functional integrity of the intimal endothelium was first determined in all rat isolated pulmonary artery rings by the presence or absence of acetylcholine (ACh)-induced endothelium-dependent relaxation. The addition of 0.01–1 μmol/L ACh in endothelium-intact pulmonary arteries precontracted with 0.1–0.3 μmol/L phenylephrine resulted in a dose-dependent relaxation reaching a maximum of –82 ± 3% (n = 28 rings). To determine the role of the endothelium in the observed response, the intimal surface of some pulmonary arteries was denuded by mechanical rubbing with a wooden applicator before the vascular reactivity studies. The effectiveness of endothelial disruption was assessed in each vessel by a near-complete loss of the ACh (1 μmol/L)-induced relaxation (–9 ± 2%). To determine specifically the role of endothelium-derived NO, we pretreated some vessels with a specific inhibitor of endothelial NOS (0.3 mmol/L L-NAME) for 20 min prior to the studies. In contrast, to determine the role of endothelium-derived vasodilatory prostanoids in the garlic extract-induced relaxation, responses of a separate series of rat pulmonary arteries to garlic extract were determined in the presence or absence of 5 μmol/L indomethacin. We have shown previously that this concentration of indomethacin results in complete inhibition of cyclo-oxygenase and the production of prostanoids in isolated coronary arteries.[15]

Preparation of garlic extracts

Cloves of fresh garlic, purchased from the local supermarket and weighing approximately 1.0–3.0 g wet weight, were mixed with either pure distilled water, 5% ethanol or 100% absolute ethanol in a 1 : 9 weight to volume ratio to produce a stock concentration of 100 mg/mL. Homogenization of garlic cloves was accomplished by mortar and pestle and the resulting homogenates were allowed to stand at room temperature for 10–20 min prior to transfer to microcentrifuge tubes and centrifugation at 17 000 g for 5 min to separate the supernatant from the pellet. Various concentrations of garlic extract were then diluted from this initial stock solution with the same solvent used initially in each preparation. All garlic extracts were prepared fresh each day and within 1 h prior to testing. Additional internal control testings of different solvents did not show any significant relaxant response.

Reagents

Acetylcholine chloride, indomethacin, phenylephrine and L-NAME were purchased from Sigma Chemical Co. (St Louis, MO, USA), whereas Cu/Zn superoxide dismutase was purchased from DDI Pharmaceuticals (Mountain View, CA, USA) and S-nitroso-*N*-acetylpenicillamine (SNAP) was obtained from Research Biochemicals (Natick, MA, USA). All other reagents were purchased from Fisher Scientific (Norcross, GA, USA). Pure allicin was a generous gift from Dr Larry Lawson (Murdock, Madaus, Schwabe Group, Springville, UT, USA). Acetylcholine was prepared in sodium acetate buffer (pH 4.0) and stored at 4°C to ensure stability. All other solutions were prepared immediately prior to use.

Statistics

All measurements are expressed as the mean ± SEM of a minimum of eight to 16 different pulmonary artery ring preparations obtained from a minimum of four to eight rats. Data were analysed using ANOVA and multiple comparisons between groups with Bonferroni correction. The ED_{50} values for garlic extract and EC_{50} values for allicin, ACh and SNAP were determined using the mid-point of the log–log dose– (garlic extract) and concentration (allicin, ACh and SNAP)-responses of their respective relaxation. Statistical significance was designated as $P < 0.05$.

RESULTS

Fresh garlic extracts on isolated pulmonary arteries

In all rat freshly isolated, endothelium-intact pulmonary arteries precontracted with 0.01–1 μmol/L phenylephrine, the addition of either water or 5% ethanol extracts of whole fresh garlic (1–500 μg/mL) resulted in a slow-developing dose-dependent relaxation reaching a maximum of –91 ± 3% (n = 8 rings) and –93 ± 2% (n = 16 rings), respectively (Fig. 1a). The average lag time for the development of garlic relaxation was 1–1.5 min and it required 3–4 min to reach the maximum relaxation effect. The ED_{50} of the relaxations induced by the water and 5% ethanol extracts of garlic were 113 ± 12 and 106 ± 10 μg/mL, respectively. The addition of an absolute ethanol (100%) extract of garlic failed to produce a marked vascular response (E_{max} = –13 ± 3% with 500 μg/mL; n = 16 rings; Fig. 1a). A similar lack of vasorelaxant response was also noted in a pure polyethylene glycol extract of garlic (–3 ± 3%; n = 8 rings; data not shown).

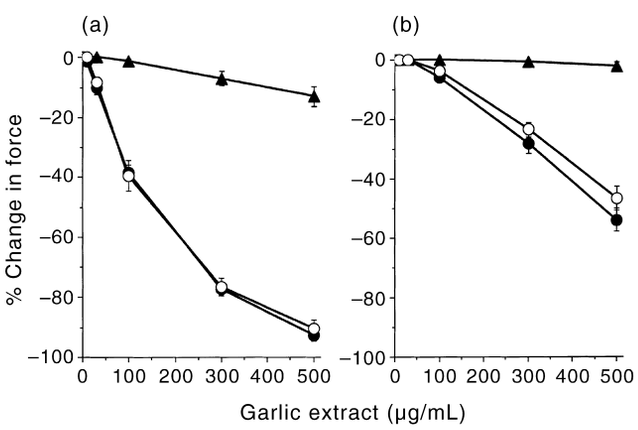

Fig. 1 Vasorelaxation effects of water and ethanol extracts of fresh garlic on rat isolated pulmonary arteries in the (a) presence and (b) absence of intimal endothelium. Pulmonary arteries were treated with 5 μmol/L indomethacin and precontracted with 0.1–0.3 μmol/L phenylephrine prior to garlic extract testing, as described in Methods. The averaged phenylephrine contractile tone for the intact endothelium group was 0.68 ± 0.03 g (n = 36), whereas that for the disrupted endothelium group was 0.69 ± 0.05 g (n = 25). All water (○), 5% ethanol (●) and 100% ethanol (▲) extracts of garlic were prepared fresh and added cumulatively into the incubation medium. Only one dose–response garlic study was performed with each pulmonary ring preparation. For the endothelium-disrupted group, the intimal endothelium was denuded with a wooden applicator before being tested. Data are the mean ± SEM of 12–16 different pulmonary ring preparations obtained from five to six different rats.

Mechanical disruption of intimal endothelium markedly attenuated the observed relaxation effects of garlic (Fig. 1b). The 100 μg/mL water and 5% ethanol garlic extract-induced relaxation responses were reduced to –4 ± 1 and –6 ± 1% (n = 10–22 rings), respectively, compared with the control endothelium-intact vessels (–40 ± 5 and – 39 ± 3%; n = 16 rings; respectively). The absolute ethanol extract once again failed to elicit a reproducible effect (Fig. 1b) in endothelium-denuded rings. Thus, for all subsequent garlic studies, unless otherwise noted, only water extracts of garlic were used to investigate the mechanism(s) of garlic-induced pulmonary vasorelaxation.

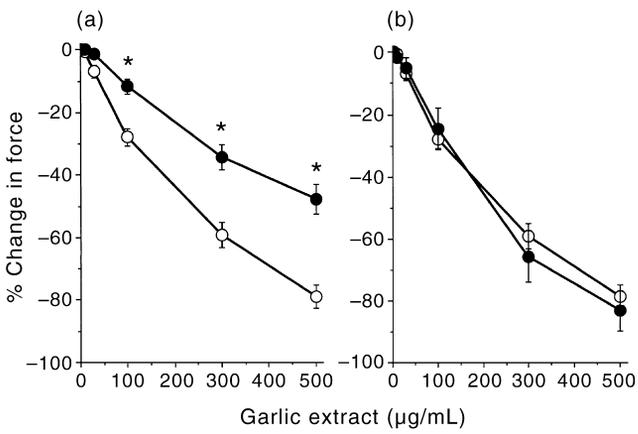

Fig. 2 Characteristics of garlic extract-induced relaxation on rat isolated pulmonary arteries. Pulmonary arteries were treated with 5 μmol/L indomethacin and precontracted with 0.1–0.3 μmol/L phenylephrine prior to garlic extract testing, as described in Methods. (a) Regional differences in the vasorelaxation response between extracts from either outer storage leaf (○) or inner stem (●) of the fresh garlic clove. (b) Results of microfiltration of whole garlic extract with a 10-K molecular sieving device (●) compared with the control, unfiltered garlic extract (○). The averaged phenylephrine contractile tone prior to the regional differences study in parts (a) and (b) was 0.78 ± 0.03 (n = 24) and 0.70 ± 0.03 g (n = 30) for the molecular sieving studies, respectively. Data are the mean ± SEM of 12–16 different pulmonary ring preparations obtained from four to five different rats. *P < 0.05 compared with the respective controls.

To determine whether there was a regional difference in relaxation potency between the outer storage leave of fresh garlic clove from its inner stem, we carefully separated these two garlic components and prepared them for use in vasorelaxant studies. Figure 2a shows that, on a wet weight to volume basis, the outer storage leaf produced a significantly greater relaxation response (–79 ± 4%; n = 12 rings with 500 μg/mL) than the inner stem (–48 ± 5%; n = 12 rings; P < 0.05). Microfiltration of the water extract of garlic with a 10 000 MW sieving device (Centricon; Amicon, Danvers, MA, USA) did not alter the observed relaxation response compared with the control extract without filtration (Fig. 2b). Similar filtration of these garlic extracts with a 1000 MW sieve also did not alter the observed relaxation (data not shown). These findings suggest that the active ingredient(s) in garlic extract responsible for the vasorelaxant effect concentrates mostly in the storage leaf of the garlic clove and has a molecular weight less than 1000.

The characteristic odour from crushed fresh garlic is due to the interaction of alliinase with alliin and the formation of allicin. To determine whether allicin formation is absolutely necessary for the observed relaxation, we inactivated alliinase with either heating (boiling at 100°C for 30 min) or 100% ethanol treatment. Figure 3a shows that boiling of a fresh whole uncut garlic clove resulted in a complete loss of the relaxation effect. Similarly, absolute ethanol treatment of fresh whole garlic for 10 min prior to homogenization and centrifugation (ethanol extract-early) also resulted in a near-complete loss of garlic relaxation (Figs 1a,3b). In contrast, if fresh garlic cloves were crushed and allicin was allowed to form during a 10 min incubation at room temperature prior to heating or absolute alcohol treatment, a significant relaxation response was preserved. In boiled garlic extract (after homogenization and centrifugation) and whole garlic homogenates (boiled after homogenization but no centrifugation), the relaxation responses were –30 ± 3 and –25 ± 3% with 500 μg/mL (P < 0.05), respectively, compared with the control

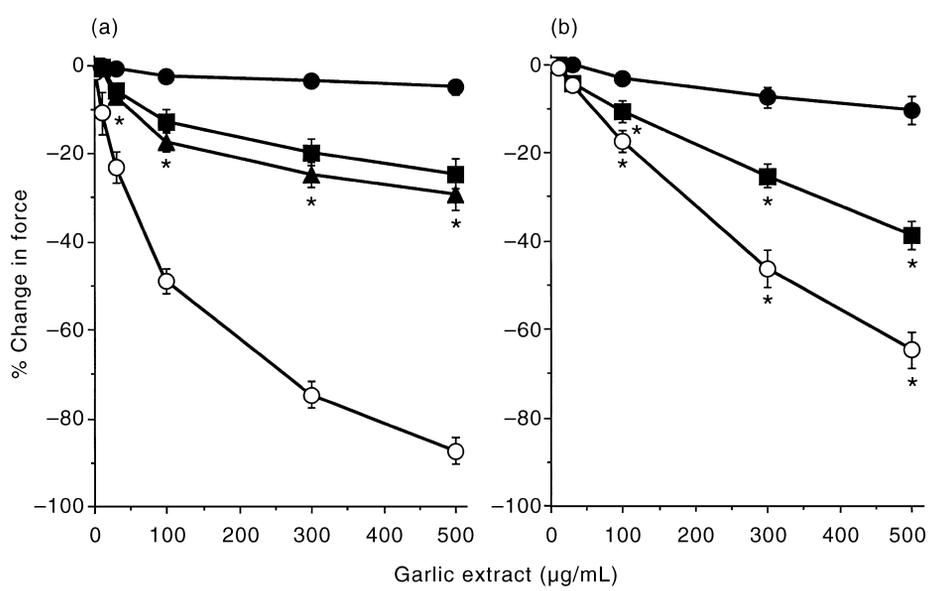

Fig. 3 Effects of boiling and absolute ethanol treatment on garlic extract-induced relaxation on rat isolated pulmonary arteries. Rat pulmonary arteries were prepared and pretreated as described in Methods. (a) Relaxation responses of control fresh garlic extract (○) and responses to garlic extract heated at 100°C for 30 min. (●), results of boiling the whole garlic clove without crushing or homogenization; (■), garlic homogenates that were allowed to equilibrate at room temperature for 10 min before boiling; (▲), results with boiling of the garlic supernatant after homogenization and centrifugation. (b) Effects of absolute ethanol treatment of garlic compared with the water extract (○). (●), (ethanol extract-early) mixing of ethanol with garlic during homogenization; (■), (ethanol extract-late) addition of absolute ethanol after the garlic clove was homogenized and equilibrated for 10 min prior to the addition of ethanol. The averaged phenylephrine contractile tone in parts (a) and (b) prior to the heat-inactivation study was 0.72 ± 0.03 (n = 35) and 0.70 ± 0.03 g (n = 30) for the ethanol inactivation studies, respectively. Data are the mean ± SEM of 12–16 different pulmonary ring preparations obtained from four to five different rats. *P < 0.05 compared with the respective controls.

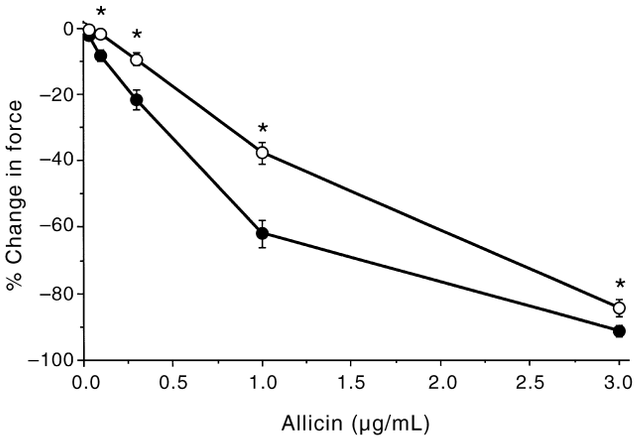

Fig. 4 Effects of pure allicin on rat isolated pulmonary arteries in the presence (●) and absence (○) of intimal endothelium. Pulmonary arteries were treated with 5 μmol/L indomethacin and precontracted with 0.1–0.3 μmol/L phenylephrine prior to allicin testing, as described in Methods. Only one dose–response allicin study was performed with each pulmonary ring preparation. For the endothelium-disrupted group, the intimal endothelium was denuded with a wooden applicator before being tested. The averaged phenylephrine contractile tone prior to allicin testing in the intact- and disrupted-endothelium groups was 0.50 ± 0.04 (n = 16) and 0.49 ± 0.03 g (n = 12), respectively. Data are the mean±SEM of 12–16 different pulmonary ring preparations obtained from four to five different rats. *P < 0.05 compared with the respective controls.

extract without boiling (–88 ± 3%; Fig. 3a). Similarly, following the addition of 100% ethanol 10 min after garlic was crushed and homogenized (ethanol extract-late), a significant relaxation response was preserved (–39 ± 3% with 500 μg/mL; P < 0.05) compared with the addition of ethanol 10 min prior to the crushing of garlic. In this same series of experiments, the control water extract of garlic resulted in a –63 ± 4% relaxation (Fig. 3b). These findings support the contention that activation of alliinase and the formation of allicin are important for garlic extract-induced pulmonary vasorelaxation.

To further demonstrate that allicin is indeed the active metabolite in these garlic extracts responsible for the observed relaxation, we investigated the direct effects of pure allicin on rat isolated pulmonary arteries. Pure allicin was synthesized and purified by Dr Larry Lawson of the Murdock, Madaus, Schwabe Group. In rat endothelium-intact, phenylephrine-precontracted pulmonary arteries, the addition of 0.1, 0.3 and 1.0 μg/mL allicin resulted in –9 ± 2, –22 ± 3 and –62 ± 4% (n = 16 rings) relaxation, respectively, with an EC_{50} of approximately 0.8 μg/mL. However, in endothelium-disrupted rings, similar concentrations of allicin resulted in only –2 ± 1, –10 ± 2 and –38 ± 3% (n = 12 rings) relaxation, respectively, and shifted the EC_{50} to approximately 1.6 μg/mL (Fig. 4; P < 0.05). These findings are consistent with those of fresh garlic extract (Fig. 1a,b) and confirm that allicin or its metabolite is responsible for the relaxation effect.

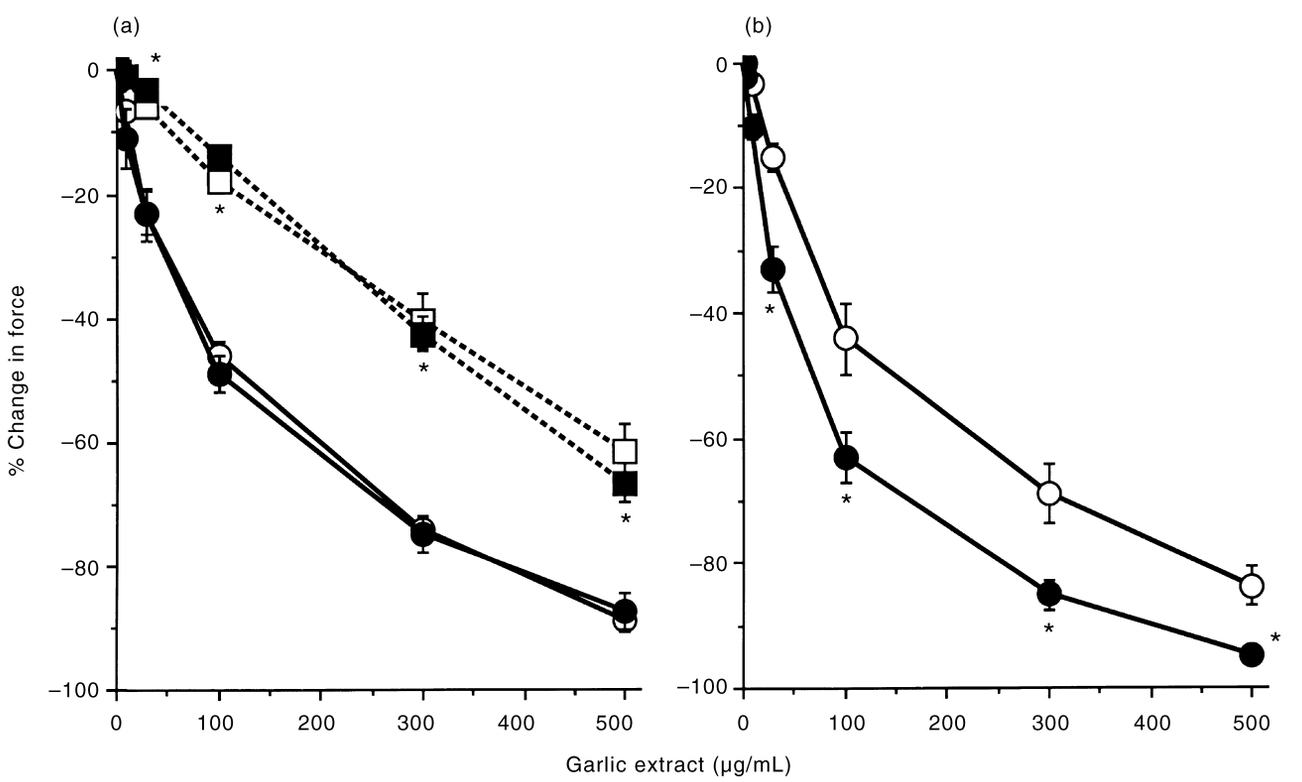

Fig. 5 Effects of (a) indomethacin and N^G-nitro-L-arginine methyl ester (L-NAME; ○, no indomethacin; ●, + indomethacin; □, + L-NAME; ■, indomethacin + L-NAME) and (b) superoxide dismutase (SOD; ○, control; ●, + SOD) on garlic extract-induced relaxation of rat isolated pulmonary arteries. Pulmonary arteries were prepared and precontracted with 0.1–0.3 μmol/L phenylephrine prior to garlic extract testing, as described in Methods. For indomethacin pretreatment, vessels were treated with 5 μmol/L indomethacin for 60 min prior to garlic extract testing, whereas 20 min pretreatment was performed for both 0.3 mmol/L L-NAME and 20 units/mL Cu/Zn SOD treatments. The averaged phenylephrine contractile tone prior to garlic testing in the indomethacin and L-NAME group was 0.73 ± 0.02 g (n = 80), whereas it was 0.73 ± 0.04 g (n = 32) in the SOD studies. Data are the mean±SEM of 12–16 different pulmonary ring preparations obtained from four to five different rats. *P < 0.05 compared with the respective controls.

Role of NO and prostanoids on garlic extract-induced relaxation

Differential vascular effects of garlic between endothelium-intact and -disrupted pulmonary arteries (Fig. 1a,b) suggest an important role for the endothelium and its production of vasoactive factors in the vasorelaxation effects of garlic. To determine the role of endothelium-derived NO and vasodilatory prostanoids in the garlic relaxation responses, we pretreated endothelium-intact pulmonary arteries with either 5 μmol/L indomethacin, an inhibitor of cyclo-oxygenase, or 0.3 mmol/L L-NAME, an inhibitor of NOS, prior to garlic testing. Figure 5a shows that pretreatment with indomethacin did not alter garlic-induced relaxation, whereas pretreatment with L-NAME resulted in an approximate 50–75% inhibition of garlic extract-induced relaxation. The 100 μ and 500 μg/mL garlic extract-induced relaxation in L-NAME-pretreated rings was decreased to –14 ± 1 and –67 ± 3% ($P < 0.05$), respectively, compared with the control relaxation of –46 ± 2 and –89 ± 2%, respectively. The extent of L-NAME inhibition of garlic extract-induced relaxation was comparable with that observed in endothelium-disrupted pulmonary arteries (Fig. 1b), suggesting that NO, but not the vasodilatory prostanoids, accounts for all of the endothelium-dependent component of garlic-induced pulmonary vasorelaxation.

A key characteristic of the biological action of NO is its susceptibility to superoxide inactivation and treatment with superoxide dismutase (SOD) has been shown to potentiate and preserve the action of NO.[16,17] Figure 5b shows that pretreatment of rat pulmonary arteries with 20 units/mL SOD resulted in a similar potentiation of garlic extract-induced relaxation by shifting the dose–response curve to the left. The 100 and 500 μg/mL garlic-induced relaxation was increased from a control value of –44 ± 6 and –84 ± 3%, respectively, to –63 ± 4 and –95 ± 1% ($P < 0.05$), respectively, in SOD-pretreated pulmonary arteries. Similar SOD enhancement of ACh-induced relaxation was also noted in these rat isolated pulmonary rings (data not shown).

To further investigate a possible interaction between garlic extract- and NO-mediated relaxation, we compared ACh- and SNAP-induced relaxation in the presence and absence of garlic extract. Figure 6a shows that garlic treatment significantly potentiated the ACh-induced relaxation in endothelium-intact pulmonary arteries by shifting the dose–response curve to the left. In contrast, in endothelium-disrupted pulmonary arteries, garlic treatment did not alter SNAP-induced NO-mediated relaxation (Fig. 6b). These findings support our contention that garlic extract-induced endothelium-dependent relaxation is likely to be mediated via its stimulation of NO production rather than its stabilization.

DISCUSSION

The results of the present study demonstrate that water extract of fresh garlic is capable of eliciting an endothelium-dependent and

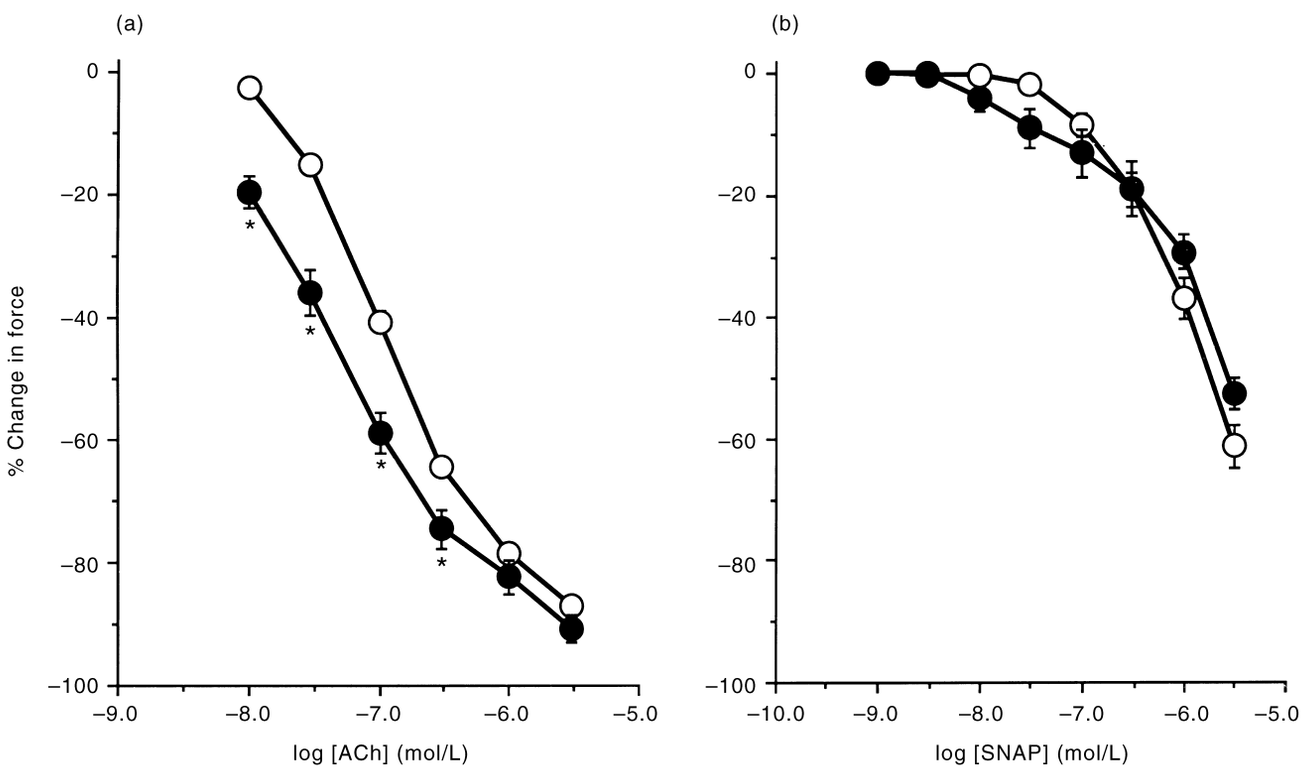

Fig. 6 Effects of garlic extract pretreatment on (a) acetylcholine (ACh) and (b) S-nitroso-*N*-acetylpenicillamine (SNAP)-induced relaxation of rat isolated pulmonary arteries. Pulmonary arteries were pretreated with 5 μmol/L indomethacin in the presence (●) or absence (○) of 500 μg/mL garlic extract and then precontracted with 0.3–1 μmol/L phenylephrine prior to either ACh (a) or SNAP (b) testing. Only two dose–response curves for either ACh or SNAP (before and after garlic treatment) were studied in each pulmonary ring. Timed control preliminary studies indicate no change in pulmonary artery responsiveness to ACh or SNAP during the experimental testing period. The averaged phenylephrine contractile tone prior to ACh testing in the absence and presence of garlic extract was 0.79 ± 0.03 ($n = 38$) and 0.36 ± 0.03 g ($n = 24$), respectively. Similarly, for the SNAP studies, the phenylephrine contractile tone in the absence and presence of garlic was 0.82 ± 0.08 ($n = 16$) and 0.36 ± 0.05 g ($n = 12$), respectively. Data are the mean±SEM of 12–16 different pulmonary ring preparations obtained from four to five different rats. *$P < 0.05$ compared with the respective controls.

-independent relaxation in rat isolated pulmonary arteries. This is consistent with our earlier reports with a commercial freeze-dried garlic powder preparation[13,18] as well as reports from other investigators working with rat isolated thoracic aorta and other visceral smooth muscles.[19,20] A similar vasorelaxation effect was also observed with a diluted (5%) ethanol extract of fresh garlic, whereas absolute ethanol (100%) extract was ineffective. The cause of failure of the absolute ethanol garlic extract to elicit a relaxant response is not well understood. It is possible that this may be related, in part, to the insolubility of the garlic active ingredients and/or an inhibition of the formation of these active garlic metabolites. Lawson has previously reported that an ethanol concentration above 99% results in a near-complete inhibition of the enzyme alliinase, which is vital for the conversion of the stable precursor, alliin, to the active intermediate allicin.[21] This finding suggests that our use of absolute ethanol to extract the active ingredients of garlic may have inadvertently inhibited this enzyme and the formation of allicin. Our finding that a significant relaxation response can be preserved if the conversion of alliin to allicin is permitted to occur for just 10 min prior to the addition of ethanol appears to support this contention and the importance of allicin formation.

It is well known that garlic contains many different sulphur-containing compounds, such as γ-glutamylcysteines and cysteine sulfoxides (alliin), which, upon activation of alliinase (by crushing the glove clove), transform to various thiosulfinates, such as allicin and ajoene.[21] A number of investigators have reported that the formation of allicin and its metabolites, which are responsible for the distinct garlic odour, is responsible for most, if not all, of the reported beneficial effects of garlic.[22–24] However, other investigators have disagreed and have suggested that elimination of all allicin formation, such as in aged garlic, also produces similar beneficial effects.[25–27] In the present study, we found that specific inhibition of alliinase, by boiling the whole garlic clove at 100°C for 30 min, completely abolished the relaxation effect, whereas if we were to allow the alliinase to interact with alliin (i.e. by crushing and homogenizing the fresh garlic clove for just 10 min) prior to boiling, significant vasodilation was preserved. Further support that allicin is an active ingredient in garlic extract is demonstrated by our experiments using pure allicin, which also produced a dual endothelium-dependent and -independent relaxation in the rat isolated pulmonary arteries. The EC_{50} for the pure allicin-induced relaxation was approximately 0.8 μg/mL, which was comparable with the projected allicin yield from the fresh garlic used in our studies. Specifically, it has been reported that the allicin yield from a fresh garlic clove can be as much as 6 μg/mg fresh weight[21] and, with our findings of the ED_{50} (113 ± 12 μg/mL) for the fresh garlic extract-induced relaxation, this translates to an allicin yield of 0.7 μg/mL. It is also of note that, in a separate series of experiments, we found that another garlic oil intermediate, namely diallyl trisulphide, formed primarily from either ethanol treatment or steam distillation of garlic, failed to elicit any relaxation response at a concentration as high as 1 mg/mL (data not shown). Similar findings of a vasodilatory effect with allicin and a lack of effect with diallyl disulphide have also been reported in a rat isolated blood-perfused lung preparation.[28] Taken together, these findings suggest that allicin formation from the crushing of fresh garlic cloves is probably the major active ingredient in the garlic extract that is responsible for the observed relaxation response in these rat pulmonary arteries.

Mechanical disruption of intimal endothelium or pretreatment with a specific inhibitor of NOS, but not inhibition of cyclo-oxygenase, significantly reduced the garlic extract- and allicin-induced relaxation by 60–75%. These findings suggest that approximately two-thirds of the garlic extract- and allicin-induced relaxation was probably mediated via the endothelial production and/or release of NO and not the vasodilatory prostanoids. Alternatively, allicin may act to stabilize and/or inhibit the breakdown of NO, because a key characteristic of the biological actions of NO is its rapid inactivation by superoxide free radicals. Indeed, Siegers *et al*. have recently reported that the active metabolite of garlic allicin was effective in scavenging oxygen radicals in a human granulocyte preparation.[29] We did not measure the concentration of either nitrite or nitrate, end-products of NO, in the incubation medium after garlic extract treatment; thus, we cannot resolve these two possibilities. The finding that garlic extract pretreatment was effective in potentiating ACh-induced relaxation may be used to support the hypothesis that garlic extract may act to stabilize or prolong the action of released NO. However, similar garlic extract pretreatment failed to potentiate the direct NO donor (SNAP)-induced relaxation, suggesting that the likely cellular mechanism of garlic extract mediated NO-dependent relaxation is via its direct stimulatory effect on endothelial synthesis and release of NO. This is also supported by our findings that pretreatment with SOD shifted the dose–response curve of garlic relaxation to the left in a manner similar to that found for NO- and ACh-induced relaxation.[16,17,30] These findings appear to be consistent with our contention that garlic extract produces a potent endothelium-dependent relaxation in rat pulmonary arteries by stimulating the endothelial production and release of NO and not via its effect on reducing oxidative stress. Further studies are needed to determine the molecular mechanism by which garlic extract enhances endothelial NO production.

The endothelium-independent component of garlic extract and allicin relaxation, which accounts for approximately 25–40% of the observed relaxation, is less well understood. Our findings that higher concentrations of allicin (> 3.0 μg/mL) or garlic extract (> 500 μg/mL) continue to produce a relaxation response in the endothelium-disrupted and L-NAME-pretreated pulmonary rings suggest a lower sensitivity for this endothelium-independent component of the garlic relaxation. A number of published reports suggest that garlic or its active metabolites could activate selective potassium channels and/or inhibit the opening of calcium channels in vascular smooth muscle cells to produce a vasorelaxation effect.[19,31] However, Kaye *et al*. have reported recently that the allicin-induced transient vasodepressor effect in their rat isolated blood-perfused lung preparation was not dependent on either the ATP-sensitive K^+ channels or on the formation of NO and vasodilatory prostanoids.[28] The cause of these conflicting findings is not well understood, but they are likely due to the differences in the model used and the duration of the experimental protocol. It is important to note that the vasodepressor response following bolus allicin injections in the blood-perfused preparation generally lasted less than 1 min,[28] whereas both garlic extract and allicin produced a rather stable and long-lasting relaxation response in our pulmonary rings bathed in physiological salt solution. Because the exact nature of allicin interactions with various cellular components in the blood, as well as the extent of allicin binding with the plasma proteins, is not clear, it is difficult to derive meaningful mechanistic information on the allicin-induced vasodepressor effect from the blood-perfused

preparations. Furthermore, the role of other K^+ channels, as well as allicin effects on the vascular calcium handling, have not been examined extensively. Studies are now underway in our laboratory to investigate the mechanisms of this garlic-induced endothelium-independent relaxation.

In a recent preliminary study with human isolated atherosclerotic coronary arteries, we noted that while the relaxation response of these arteries to the conventional endothelium and NO-dependent dilators, such as bradykinin and histamine, were diminished, the garlic extract-induced relaxation responses were only minimally altered.[32] These findings suggest that the dual mechanisms (endothelium-dependent and -independent) of garlic extract-induced vasodilation may be uniquely effective in certain pathological states where selective vascular (endothelial) dysfunction may have occurred. This may also account for the earlier reported protective effect of garlic against hypoxic pulmonary hypertension[13] and vasoconstriction.[18] Thus, in conclusion, the results of the present study demonstrate that the activation of alliinase and the formation of allicin are absolutely necessary for the observed pulmonary vasorelaxation effect of garlic and that this vascular effect of garlic may be the underlying mechanism for most of the reported beneficial effects of garlic in cardiovascular diseases.

ACKNOWLEDGEMENTS

The authors express their gratitude to Dr Larry Lawson (Murdock, Madaus, Schwabe Group, Springville, UT, USA) for his generous supply of pure allicin. This study was supported by grants from the National Institutes of Health, HL-50483 and the Pure-Gar Company (Tacoma, WA, USA).

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