An In Vitro Study of Different Extracts and Fractions of *Allium sativum* (Garlic): Vascular Reactivity

Patricia Ganado¹, Mercedes Sanz¹, Eugenia Padilla¹, and Teresa Tejerina¹,*

¹Department of Pharmacology, School of Medicine, Universidad Complutense de Madrid, 28040 Madrid, Spain

Received July 3, 2003; Accepted February 11, 2004

**Abstract.** The aim of this study was to investigate the effect of different novel extracts and fractions obtained from *Allium sativum* (garlic) on in vitro vessel contraction in order to deepen our knowledge of their mechanism of action on vascular reactivity. The contraction induced by noradrenaline (NE, 10⁻⁶ or 10⁻⁵ M) or KCl (80 mM) was relaxed with all the extracts and fractions studied, but this effect was higher with RG 20-100 (raw garlic fraction) and FG 20-100 (frozen garlic fraction). To increase our understanding of the mechanisms of action of RG 200-100 and FG 200-100, we found their inhibitory actions were retained in the absence of endothelium, whereas inhibition of the entry of extracellular calcium and mobilization of intracellular calcium may play an instrumental role.

**Keywords:** *Allium sativum* (garlic), vascular reactivity, aorta, mesenteric artery (4th branch)

**Introduction**

It is widely accepted that dietary factors play a key role in the prevention of some human diseases, including cardiovascular diseases. *Allium sativum* (garlic) has been employed as an herbal medicinal agent for thousand of years (1). Among the beneficial effects attributed to garlic is its ability to relax vascular smooth muscle. However, a controversy exists about its mechanism of action. Several authors have proposed a mechanism of action mediated by the activation of nitric oxide synthase in vascular endothelium (2), but a direct effect on vascular smooth muscle has also been demonstrated, suggesting the occurrence of vasodilatation via smooth muscle cell membrane hyperpolarization and/or inhibition of the opening of calcium channels (3). Together these observations suggest that garlic might block the development of hypertension associated with atherosclerosis, the principal contributor to myocardial and cerebral infarction.

The aim of this study was to elucidate the in vitro mechanism of action of different extracts and fractions of garlic obtained in a novel way and to elucidate the differences between raw and frozen garlic. For the first time, raw and frozen garlic extracts have been differentiated. We determined the effects of garlic on aorta and mesenteric reactivity.

**Materials and Methods**

**General procedure**

Male Wistar rats (ANUC Complutense University, Madrid, Spain) (250 ± 20 g) were housed identically in an air-conditioned room under a 12-h light-dark cycle and were fed a standard laboratory diet (Panlab S.L., Barcelona, Spain) for 7 days before the beginning of the experiments. Tap water was freely available. All protocols concerning animals were approved by the Universidad Complutense of Madrid (EEC official registration 28079-15ABC).

The rats were anesthetized with ethyl ether and killed by exanguination from the common carotid arteries. The thoracic aorta and mesenteric arteries were rapidly removed and placed in Krebs Henseleit solution of the following composition: 119 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.0 mM MgSO₄, 11.1 mM glucose, 1.2 mM KH₂PO₄, and 2.5 mM CaCl₂.

Adherent fat and surrounding tissue were cleaned off. The aorta was cut into rings of approx. 2 – 3 mm in width, and the rings were suspended between two stainless steel hooks in organ baths containing 10 ml of Krebs Henseleit solution. The solution was kept at
36 ± 0.5°C and gassed continuously with a 95% O₂ – 5% CO₂ gas mixture. The rings were mounted under 1-g tension. Each preparation was allowed to equilibrate for 60 min. The isometric force was digitalized by a MacLab A/D converter (Chart v3.2, A.D. Instruments, Castle Hill, Australia) and stored and displayed on a Macintosh computer (4). Using a dissecting microscope, a segment of small mesenteric artery, approximately 2 mm in length, corresponding to a fourth order branch of the superior mesenteric artery, was carefully dissected free from its vein. The artery was mounted in a small vessel myograph (5). Two 40-μm tungsten wires were passed through the lumen of an isolated cylindrical segment (approximately 175-μm inside diameter), one wire was fastened with screws to a fixed tissue mount, and the other was pulled out by parallel hooks, which were attached to a strain-gauge force transducer (U-gauge; Shinko Co., Ltd., Tokyo), the position of which was adjusted with a micromanipulator. The vessel was set to a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100-mmHg transmural pressure (6). Vessels were allowed to equilibrate at 36 ± 0.5°C and gassed continuously with O₂. The isometric force was digitalized by a PC.

Drugs

The following drugs were used: noradrenaline bitartrate (NE), nitroprusside (SNP), N⁶-nitro-L-arginine methyl ester (L-NAME), indomethacin, ethylene glycol-bis(β-aminomethyl ether)N,N,N',N'-tetraacetic acid (EGTA) (Sigma Chemical Co., St. Louis, MO, USA); potassium chloride and calcium chloride (Merck, Darmstadt, FRG). Ascorbic acid (10⁻¹M) was added to the NE solution to avoid NE oxidation.

All extracts and fractions were provided by Dr. I. Matsura (The University of Illinois at Chicago, Chicago, IL, USA). Fresh garlic bulbs cultivated in California, USA in 1997 were used in this study.

The extracts and fractions were obtained using the following procedures:

Raw skinned garlic cloves (13.5 kg) were crushed at room temperature and extracted with hot methanol (25 L). After removal of the solvents by evaporation, the extract named RG-EXT was obtained at a yield of 16% (2.20 kg). The suspension of RG-EXT (1.69 kg) in 6 L of water was divided into three portions, and each was applied to a column packed with 1000 cc of a reversed-phase porous polymer, Diaion HP20 (Mitsubishi Chem. Ind. Co., Ltd., Tokyo) by a stepwise elution of water (3 L), 20% aqueous methanol (1 L), and 100% methanol (2.5 L) to afford fractions named RG-HP20-W (1.62 kg, 16% yield), RG-HP20-20 (17.5 g, 0.17% yield), and RG-HP20-100 (53.4 g, 0.5% yield), respectively, after the same procedure was repeated three times.

Frozen skinned garlic cloves (13.5 kg) were crushed with methanol and extracted with hot methanol (25 L). After removal of the solvents by evaporation, the extract named FG-EXT was obtained at a yield of 17% (2.34 kg). FG-EXT (1.82 kg) was fractionated in the same manner as RG-EXT, affording the three fractions named FG-HP20-W (1.76 kg, 17% yield), FG-HP20-20 (9.6 g, 0.1% yield), and FG-HP20-100 (49.6 g, 0.5% yield), respectively. RG/FG EXT and RG/FG 20-20 solutions were prepared in Krebs Henseleit solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 15.5 mM NaHCO₃, and 11.5 mM glucose. RG/FG 20-100 solutions were prepared in Krebs Henseleit solution plus dimethyl sulfoxide (DMSO).

Experimental procedure

Aorta arteries: After the equilibration period (90 min), different experiments were carried out: In order to investigate the direct effect of garlic on basal contraction (1 g), we added different garlic concentrations (30 – 750 μg/ml) or DMSO (RG/FG 20-100 vehicle) to the organ bath and allowed each dose to act for 15 min. Other aortic rings were contracted with NE 10⁻⁴ M or KCl (80 mM); and when the contraction reached a plateau, different concentrations of garlic (30 – 750 μg/ml) or DMSO were added. On the other hand, we tested the inhibitory effect of these extracts and fractions on contractions induced by NE or KCl. For this, we contracted the aorta with NE (10⁻⁴ M) or KCl (80 mM); and then the arteries were washed out, incubated in the presence of garlic (30 – 750 μg/ml) or DMSO for 15 min, and the previous procedure was repeated. DMSO concentration was equivalent to that used to dissolve each concentration of RG/FG 20-100.

In another group of experiments, we tested the mechanism of action of RG 20-100 in more detail. In order to elucidate any effect of RG 20-100 on nitric oxide (NO) or prostaglandins synthesis, aortic rings were incubated with L-NAME (3 x 10⁻⁴ M) or indomethacin (10⁻⁶ M) for 20 min. After this incubation, a dose-response curve to RG 20-100 (30 – 750 μg/ml) was determined in arteries previously contracted with NE (10⁻⁶ M). In another group of arteries, the endothelium was removed by inserting a stainless-steel rod into the rings (7) and the same dose-response curve to RG 20-100 was repeated. We also studied the effect of RG 20-100 on the SNP curve. For this, the arteries were contracted with NE (10⁻⁴ M) and then accumulative doses of SNP (10⁻⁸ – 10⁻⁴ M) were added. This protocol was repeated in arteries previously incubated with...
RG 20-100 (100 and 300 μg/ml).

The aim of this experiment was to elucidate the effect of RG 20-100 on an NO donor (SNP), which is endothelium-independent. Finally, we investigated the effects of RG 20-100 on calcium influx/efflux. The rings were washed out in a Ca\(^{2+}\)-free solution for 2 h [Ca\(^{2+}\)-free Krebs Henseleit solution (0Ca\(^{2+}\)-Krebs) + EGTA (10\(^{-5}\) M)]. At the end of this washing, the arteries were contracted with 80 mM KCl for 10 min in order to depolarize the cell membranes and open the calcium channels. Cumulative concentration-response curves for calcium were obtained by increasing the calcium concentration in the bath from 1 to 5 mM stepwise over the next 45 min (8, 9). When the maximum response was obtained, the arteries were washed out and the rings were re-incubated in Ca\(^{2+}\)-free Godfraind solution for 1 h. The high K\(^+\) depolarizing procedure was repeated, but RG 20-100 (100, 300, or 500 μg/ml) was added to the bath 15 min before the first addition of Ca\(^{2+}\). The results were expressed as percentages of the maximal contractile response induced by 5 mM Ca\(^{2+}\). The aim of this experiment was to study the effect of RG 20-100 on extracellular calcium influx. On the other hand, we studied the effect of RG 20-100 on sarcoplasmatic calcium. We contracted the rings with NE (10\(^{-6}\) M) in Krebs Henseleit solution in order to obtain a contraction in which extracellular calcium and intracellular calcium were implicated; the arteries were washed out in 0Ca\(^{2+}\)-Krebs + EGTA (10\(^{-5}\) M) for 1 h, and a second contraction and a third contraction with NE were repeated. In this case, only intracellular calcium was implicated. This protocol was repeated, incubating with RG 20-100 (500 μg/ml). Mesenteric arteries: After the equilibration period, similar experiments on aorta were carried out: In order to investigate the direct effect of garlic on basal contraction, we added different garlic concentrations (30 – 750 μg/ml) to the organ bath and let each dose act for 15 min. In another group of experiments, the mesenteric arteries were contracted with NE (10\(^{-6}\) M) or KCl (80 mM); and when the contraction reached a plateau, different concentrations of garlic (30 – 750 μg/ml) or DMSO were added. We also tested the inhibitory effect of these extracts and fractions on contractions induced by NE or KCl. For this, we contracted the aorta with NE (10\(^{-5}\) M) or KCl (80 mM), and then the arteries were washed out, incubated in the presence of garlic (30 – 750 μg/ml) or DMSO for 15 min, and the previous procedure was repeated.

Statistic analyses

All values used in the analyses represent the mean ± S.E.M. of 15 rats in each group of experiments. Comparisons among the different groups were performed by the two way ANOVA test (line graphics) or one way ANOVA test (bar graphics), and differences were considered significant when P < 0.05. We used Statgraphics Plus for Windows v.4.0.

Results

Aorta: effect of the extracts and fractions of garlic on basal contraction

No significant effect was found when garlic was added to aorta preparations (data not shown).

Aorta: effect of the extracts and fractions of garlic on NE or KCl contraction

The contractions induced by NE (10\(^{-6}\) M) were rapidly relaxed by all extracts and fractions of garlic, but this effect was higher with the RG/FG 20-100 fractions (92.9 ± 2.2%, and 86.7 ± 4.4%, with FG 20-100 and RG 20-100 respectively) (Fig. 1A). On the other hand, different concentrations of garlic (30 – 750 μg/ml) were added to the bath when KCl contraction (80 mM) had reached its plateau. In this case, in general, the relaxation produced by garlic was smaller than in the case of NE, except with RG/FG 20-100, where the relaxation obtained was similar. The maximum effect was reached again with RG/FG 20-100 fractions (93.0 ± 2.6% and 83.4 ± 2.2% with RG 20-100 and FG 20-100, respectively) (Fig. 1B).

Aorta: inhibitory effect of the extracts and fractions on contractions induced by NE or KCl

The inhibitory effect of these fractions and extracts on NE-induced contraction (10\(^{-6}\) M) was already observed with the first concentration added (30 μg/ml) and progressively increased in a concentration-dependent manner, with the maximum effect at 750 μg/ml. Once again, the maximum effect was induced by the RG/FG 20-100 fractions, but RG/FG EXT also inhibited the contractions in a marked manner (95.3 ± 1%, 93.3 ± 2.4%, and 79.8 ± 5.2% with RG 20-100, RG EXT, and FG 20-100 respectively) (Fig. 2A). The inhibitory effect of garlic on KCl contractions was again higher with the RG/FG 20-100 fractions (88.4 ± 3.5% and 83.1 ± 4.5% with RG 20-100 and FG 20-100, respectively). The rest of the extracts and fractions produced only minor effects (Fig. 2B).

In view of all these results, there was clear evidence of the major effect on vascular reactivity of the FG 20-100 and RG 20-100 fractions. Thus, we decided to investigate more in detail the mechanism of action of RG 20-100. We chose this fraction instead of FG 20-100 because RG 20-100 displayed greater and reproducible
activity, and raw garlic, rather than frozen garlic, is the more frequently consumed form.

Aorta: mechanism of action of RG 20-100

The relaxation induced by RG 20-100 in aortas previously contracted with NE in the presence of L-NAME (3 \times 10^{-6} \text{M}) or indomethacin (10^{-6} \text{M}) was not significantly different from that in the control group (only RG 20-100) (Fig. 3). In order to confirm this effect, we tested the relaxation induced by RG 20-100 in arteries without endothelium. The maximum relaxation obtained in arteries with endothelium (86.7 \pm 4.4\%) or in denuded arteries (83.7 \pm 2.8\%) was not significantly different (Fig. 4). These results confirmed an endothelium-independent mechanism of action of RG 20-100. Then, we focused on the action of RG 20-100 on vascular smooth muscle. The SNP curve in the control arteries was not affected when the aorta was previously incubated with RG 20-100 (100 and 300 \mu g/ml). The same results were obtained with DMSO incubation (Fig. 5). After this, we focused on the effect of RG 20-100 on calcium channels. For this, extracellular calcium entrance into the cell was tested. Considering the maximum contraction induced by 5 mM in the control group as 100\%, the maximum contraction induced by 5 mM in the groups treated with RG 20-100 decreased in a dose-dependent manner: 83.9 \pm 5.3\%, 81.8 \pm 10.1\%, and 56.6 \pm 5.8\% in groups treated with 100 \mu g/ml, 300 \mu g/ml, and 500 \mu g/ml of RG 20-100, respectively. These results produced an inhibition of extracellular calcium entrance into the cell (Fig. 6A). We also found an inhibition of intracellular calcium mobilization: Considering the 10^{-6} \text{M} NE contraction in normal Krebs solution as 100\%, we compared the contraction with NE in 0Ca^{2+} Krebs before and after incubating with RG 20-100. Thus, a decrease in the contraction (46.8 \pm 5.0\% to 19.9 \pm 2.9\% (NE 0Ca1) was observed in the control group and RG 20-100-
treated group). In addition, with the second contraction with NE in 0Ca\(^{2+}\) Krebs solution (NE 0Ca\(^{2+}\)), we found a residual contraction that diminished from 29.8 \(\mu\text{g}\) to 15.9 \(\mu\text{g}\) in the control group to 15.9 \(\mu\text{g}\) in the RG 20-100 treated group (Fig. 6B).

**Mesenteric artery: effect of the extracts and fractions of garlic on basal contraction**

As shown in the aorta, we did not find any significant change in basal contraction with the different extracts and fractions studied (data not shown).

**Mesenteric artery: effect of the extracts and fractions of garlic on NE or KCl contraction**

The relaxation of the contraction induced by NE (10\(^{-5}\) M) in mesenteric arteries was similar to that shown in the aorta: the maximum relaxation was again obtained with RG 20-100 and FG 20-100 (94.9 \(\pm\) 1.3\% and 94.6 \(\pm\) 0.8\% with RG 20-100 and FG 20-100, respectively) (Fig. 7A). The rest of the fractions and extracts relaxed in a minor manner: 80.2 \(\pm\) 0.4\%, 66.5 \(\pm\) 2.0\%, 56.0 \(\pm\) 5.6\%, and 41.9 \(\pm\) 5.6\% with RG EXT, FG EXT, RG 20-20, and FG 20-20, respectively. On the other hand, the contraction induced by KCl (80 mM) decreased when we added increasing concentrations (30 – 750 \(\mu\text{g/ml}\)) of the fractions and extracts of garlic to the organ bath. Once again RG 20-100 and FG 20-100 more greatly decreased this contraction (99.5 \(\pm\) 0.1\% and 97.6 \(\pm\) 0.9\% with RG 20-100 and FG 20-100, respectively) (Fig. 7B).

**Mesenteric artery: inhibitory effect of the extracts and fractions on contractions induced by NE or KCl**

Figure 8A shows the responses obtained when study-
ing the inhibitory effect of the fractions and extracts of garlic on NE or KCl contractions. Only RG 20-100 and FG 20-100 inhibited NE contractions, reaching the maximum effect with the 750 μg/ml dose: 99.32 ± 0.1% and 99.25 ± 0.1% of inhibition with RG 20-100 and FG 20-100, respectively. With respect to the inhibition of the contraction induced by KCl (80 mM) in the presence of these extracts and fractions, the following results were obtained: The most prominent inhibition was produced again with RG 20-100 and FG 20-100 fractions: 99.4 ± 0.1%, 98.8 ± 0.3% with RG 20-100 and FG 20-100, respectively. Likewise, the RG 20-20 fraction inhibited KCl contraction in a significant way (23.4 ± 2.0%) (Fig. 8B).

**Discussion**

Aside from its general use as a condiment, garlic (Allium sativum) is known for its pharmacological and nutritional properties (10). Among the pharmacological applications of Allium sativum, its properties on cardiovascular disorders have been studied over the years. The antilipidemic effects have been demonstrated in studies in humans (11 – 15). Garlic is also a potent antioxidant (16), it has an anticoagulant effect (17, 18), and decreases blood pressure (14, 19, 20). Numerous studies have demonstrated the vasodilatory effects of garlic (21, 22), but the mechanism of action remains unclear. While some people propose a mechanism of action in which an activation of NO synthase is implicated (2), others favor a direct effect on vascular smooth muscle.
Fig. 6. Measurement of calcium movements. Panel A shows the entrance of extracellular calcium (1 – 5 mM) into smooth muscle cells of control aortas and RG 20-100-treated aortas (100 μg/ml, 300 μg/ml, or 500 μg/ml). Panel B shows the effect of RG 20-100 (500 μg/ml) on intracellular calcium mobilization during NE (10^-6 M) contraction in aortic arteries. Each point represents the mean ± S.E.M. of 15 experiments. **P<0.01, ***P<0.001, with respect to the control. NE Krebs: contraction with NE in normal Krebs; NE 0Ca1: first contraction with NE in Ca²⁺-free Krebs; NE 0Ca2: second contraction with NE in Ca²⁺-free Krebs.

Fig. 7. Effect of the raw (RG) and frozen (FG) fractions and extracts of garlic and DMSO on NE (10^-5 M) (panel A) or KCl (80 mM) (panel B) contraction in the mesenteric artery. Each point represents the mean ± S.E.M. of 15 experiments. ***P<0.001, with respect to 100%; +++P<0.001, with respect to DMSO relaxation.
Garlic on Vascular Reactivity

The fractions and extracts used in this work were new and their mechanisms of action have never been studied. The aim of this study was to find this mechanism of action in isolated vascular arteries. The results presented have revealed that these fractions and extracts have a vasodilatory effect on both aorta and mesenteric arteries. Moreover, they also present an inhibitory effect on the contraction induced by NE or KCl. When we compare the relaxation of the contraction induced by NE or KCl to a similar extent. This indicates that besides hyperpolarization there are other mechanisms of action, probably calcium-dependent. Moreover, the results obtained in the inhibition of the contraction induced by NE or KCl suggest that in all cases there was an inhibition of both the entrance of extracellular calcium and mobilization of intracellular calcium.

In the second part of this study, we chose the RG 20-100 fraction to deepen our understanding of its mechanism of action because of its greater action on vascular reactivity. The results indicated an endothelium-independent mechanism of action. An inhibition of calcium channels (3).

The fractions and extracts used in this work were new and their mechanisms of action have never been studied. The aim of this study was to find this mechanism of action in isolated vascular arteries. The results presented have revealed that these fractions and extracts have a vasodilatory effect on both aorta and mesenteric arteries. Moreover, they also present an inhibitory effect on the contraction induced by NE or KCl. When we compare the relaxation of the contraction induced by NE or KCl in the presence of the extracts and fractions, let us conclude the following: FG 20-20 and RG 20-20 fractions relaxed the contraction induced by NE but not that by KCl. On the other hand, RG EXT and FG EXT relaxed the contraction induced by NE more than the KCl-induced contraction. This suggests a mechanism of action in which hyperpolarization but also other mechanisms of action, probably calcium-dependent, are implicated. Finally, RG 20-100 and FG 20-100 fractions relaxed the contraction induced by NE and KCl to a similar extent. This indicates that besides hyperpolarization there are other mechanisms of action, probably calcium-dependent. Moreover, the results obtained in the inhibition of the contraction induced by NE or KCl suggest that in all cases there was an inhibition of both the entrance of extracellular calcium and mobilization of intracellular calcium.

In the second part of this study, we chose the RG 20-100 fraction to deepen our understanding of its mechanism of action because of its greater action on vascular reactivity. The results indicated an endothelium-independent mechanism of action. An inhibition of calcium
entry in the muscle cell and an inhibition of the mobilization of intracellular calcium were implicated as shown in experiments realized in O\textsuperscript{Ca}\textsuperscript{2+} Krebs solution. In order to elucidate some differences between the behavior of aorta and mesenteric arteries in the presence of these extracts and fractions, we carried out similar experiments in the fourth branch of the mesenteric artery. However, similar results to those of the aorta were obtained in mesenteric arteries: The maximum relaxation of the contractions with NE and KCl was obtained with RG 20-100 and FG 20-100 fractions. These two fractions also inhibited the contractions induced by those agonists in an important manner. Endothelium-dependent relaxation is principally due to EDHF in mesenteric arteries (23), and extracellular calcium contributes to its contraction (5). Bearing this in mind, we can explain the data obtained in mesenteric arteries: FG 20-20 and RG 20-20 fractions similarly relaxed the contractions induced by NE and KCl. This suggests a vasodilatory mechanism of action related to an inhibition of extracellular calcium entry. Moreover, only RG 20-100 and FG 20-100 fractions inhibited the contractions induced by NE and KCl. This confirms the inhibition of the extracellular calcium entry produced by these fractions in mesenteric arteries.

Acknowledgments

We thank Dr. I. Matsura of The University of Illinois in Chicago for providing us with all the garlic extracts and fractions. This work was supported by a FISS grant (01/0815).

References