Acute effects of a partially purified fraction from garlic on plasma glucose and cholesterol levels in rats: Putative involvement of nitric oxide

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Garlic has been extensively used as a medicinal plant. Most of its numerous beneficial effects such as antioxidant, antibacterial, antitumoral involve sulfur-derived amino acids. In the present work, we reevaluated the acute effects of aqueous extract of garlic on plasma glucose and cholesterol levels in normal rats. Control (vehicle H2O) or garlic extract-treated group at 100-120 mg protein/kg body wt were intraperitoneally injected (IP) and glucose, cholesterol, insulin and nitric oxide metabolites levels were determined after a short-term duration of 6 h. We confirmed that garlic contained an active fraction, exerting both glucose and cholesterol-lowering activity. The glucose-lowering effect was triggered by an increase in insulinemia. Preliminary study indicated that the active agent was different from S-allyl-cysteine-sulfoxide, the active principle implicated in hypoglycaemic and hypolipidemic effects of garlic or arginine. The mechanism of action seemed to involve nitric oxide (NO), which increased time and dose-dependently. The garlic effects were abolished by diphenyleneiodonium chloride (DPI = 1 mg/kg body wt), a specific inhibitor of NO production, suggesting the involvement of constitutive nitric oxide synthase.

Keywords: Garlic, Rat, Glucose, Cholesterol, Insulinemia, Nitric oxide.

Garlic is well known as a healing agent1. Its numerous beneficial effects include antioxidant2, antitumoral3, antibacterial4 and anti-atherosclerotic5,6 and most of its effects are believed to be triggered by sulfur-derived amino acids7. For instance, S-allyl-cysteine-sulfoxide (SACS) is considered as the active principle implicated in hypoglycaemic8 and hypoplipidemic9 effects. Although the mode of action of garlic or its derivatives is still uncertain, nitric oxide (NO) has been proposed as a putative mediator10, especially in anti-hypertensive effect11. NO is synthesized from L-arginine by NO synthase (NOS), which exists in three isoforms – neuronal, endothelial constitutive and an inducible isoform12. NO derived from constitutive NOS (cNOS) is reported to modulate vasomotor tone, inhibition of platelet or leukocyte aggregation and adhesion to the endothelium, suggesting its anti-atherogeneity properties13.

In the present study, we investigated for the first time the short-term effects of aqueous extract of garlic on glucose and cholesterol-lowering activity. In addition, attempts were made to identify a newly reported active component, which appeared different from SACS, based on physico-chemical properties and mechanism of action.

Materials and Methods

Preparation of garlic extracts

Garlic (Allium sativum L.) was purchased from local market, peeled, weighed and ground with an electric mincer. It was dissolved in double-distilled water at a concentration of 2 g/ml (on the basis of weight of starting material) and centrifuged at 10,000 g for 15 min and 4°C (Beckman J20). Supernatant was sonicated (Sonicator UP 400S) and centrifuged again. Clear supernatant was then aliquoted and stored at -80°C until use.

Aqueous extract was subjected to the extraction with ethanol as described previously14, with slight modification. Briefly, 1 vol of aqueous extract was precipitated twice with 7 vols of ethanol and centrifuged at 10,000 g for 15 min at 4°C. Supernatant was dried using a rotavapor, dissolved in double-distilled water and referred as ‘ethanol-soluble fraction’. After washing with ethanol-water [7: 1, v/v] and drying, pellet was dissolved in double-distilled water and referred as ‘ethanol-insoluble fraction’. Glucose-lowering activity was tested in both fractions.

Ethanol-soluble fraction was further subjected to chromatography on Sep-Pak C18 reverse phase cartridge. After extensive washing, first with ethanol and then with double-distilled water, loading of the cartridge with ethanol-soluble fraction yielded two fractions — polar fraction, a non-adsorbed one, eluted with double distilled-water and a non-polar fraction,
eluted with 10% ethanol. Concentrations of aqueous extract and non-polar fraction were evaluated by protein measurement according to the biuret method (Sigma kit, France).^{15}

**Animals and treatment**

Male and female Wistar rats weighting 180-220 g were obtained from Pasteur's Institute, Tunis, Tunisia and maintained under standard laboratory conditions at 22 ± 2°C, on a light/dark cycle (12 h) and supplied with standard pellet diet and tap water *ad libitum*. Procedures involving laboratory animals and their care were conducted in conformity with the institutional guidelines of Tunis University of Medical Sciences and in accordance with the NIH guidelines.

Animals were divided in two groups of 8 to 10 rats each. Group I received vehicle (H₂O) and served as control and Group II received either aqueous extract of garlic (100 or 120 mg protein/kg body wt) or purified non-polar extract (22.5 mg protein/kg body wt) or equivalent amount of arginine contained by garlic (90 mg/kg body wt).^{16} Garlic or extracts were acutely administered by a single IP injection at time = 0. The L-N-arginine methyl ester (L-NAME from Sigma, France) at 40 mg/kg body wt or DPI (Fluka-Aldrich, France) at 1 mg/kg body wt were dissolved in double-distilled water and IP injected 2 h, prior to garlic injection. Experimental duration never exceeded 6 h (see results section), after which rats were anaesthetized with urethane and sacrificed by decapitation. The plasma was used for determination of glucose, cholesterol, insulin and NO metabolites.

**Measurement of glucose levels, insulinemia, cholesterol levels and NO metabolites**

Glucose levels and plasma insulin were determined enzymatically using commercially available glucose oxidase kit (Sigma, France) and RIA kit (Immunotech, France), respectively. Plasma total, HDL and LDL cholesterol levels were determined enzymatically using commercially available kits (Sigma, France), following the manufacturer's procedure. Plasma NO was measured by quantification of the NO metabolites nitrite and nitrate determined colorimetrically using a commercially available kit (Roche Diagnostics, France).^{17}

**Effect of heat treatment on glucose and cholesterol-lowering activity**

Thermostability of the glucose and cholesterol-lowering activities was determined by incubating aqueous extract during 15 min at 100°C. After centrifugation at 10,000 g for 15 min at 4°C, residual activity was tested in supernatant.^{18}

**Statistical analysis**

All data were expressed as mean ± SEM. Statistical analysis was carried out using student's test and one-way analysis of variance (ANOVA test). Statistical *P* value less than 0.05 was considered significant.

**Results**

Fig. 1 shows the time-course effect of an aqueous extract of garlic on NO metabolites of normal euglycaemic rats. Upon garlic injection, plasma NO levels increased rapidly and strongly (20-fold) from 15 min after treatment, reached a plateau at 1 h, which sustained till 2 h. In control animals, plasma NO levels remained low over the time. NO production was also tested in dose response experiments. Data from Fig. 2 showed a clear dose-related effect of garlic on plasma NO levels 30 min after injection. NO stimulation, which was significant from 20 mg protein/kg body wt, reached an optimum (15-20-fold

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Fig. 1—Effect of garlic on NO production: Time course study [Rats were IP injected with garlic at 120 mg protein/kg body wt (▲) or vehicle (△) and plasma NO metabolites determined at different times. Results were expressed as mean ± SEM of 8 rats. The arrow indicates start of injection. Data significance was assessed by ANOVA test with *p* < 0.01]

Fig. 2—Effect of garlic on NO production: Dose-response study [Rats were IP injected with increasing concentrations of garlic and plasma NO metabolites determined 30 min after injection. Results were expressed as mean ± SEM of 8 rats. Data significance was assessed by ANOVA test with *p* < 0.05 (20 mg) and *p* < 0.01 (80 and 120 mg)]
over basal) at the highest concentration used (120 mg protein/kg body wt).

To better characterize the effect of this active component contained by garlic, the non-polar fraction eluted from Sep-Pak C18 reverse-phase cartridge was compared to non-treated or heated garlic or to an equivalent dose of arginine found in garlic. Table 1 shows that all the garlic effects such as glucose and cholesterol lowering and plasma NO stimulation were mimicked by the active component, found in the non-polar fraction. These beneficial effects were lost upon heating and arginine was unable to mimic the non-polar fraction effects even on plasma NO.

To further characterize the NO metabolic pathway, we conducted pharmacological experiments using two constitutive NOS inhibitors L-NAME and DPI. Fig. 3 shows the effect of these inhibitors on garlic-induced NO production after 30 min of stimulation. Although these inhibitors had no significant effect on the basal levels, they clearly inhibited the garlic-induced one to roughly the same extent (2-fold). We further tested the ability of the NOS inhibitors to alter glucose and cholesterol levels. Data from Fig. 4 clearly showed that the DPI abolished both glucose and cholesterol lowering effect of garlic. Moreover, DPI alone had no effect.

**Discussion**

In the present paper, we studied the short-term effects of garlic (*Allium sativum* L.) on plasma glucose and cholesterol levels of normal wistar rats. We found that aqueous extract of garlic exerted an hypoglycaemic effect, which was in agreement with the previous studies. As this effect was mediated by an increase in insulinaemia (data not shown), garlic or its active fraction could be assimilated as a true

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**Table 1—Effect of garlic extracts on plasma glucose, cholesterol and NO levels**

[Rats were IP injected with vehicle (control), garlic, heated garlic, non-polar fraction or arginine. Glucose was determined after 2 h of incubation, cholesterol after 4 h and NO after 30 min. Results were expressed as mean ± SEM of 8 rats. Data significance was assessed by ANOVA test with p<0.05, except in (*) where p>0.05 which was not significant, when compared to control]

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<thead>
<tr>
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<th>Plasma glucose (mg/dl)</th>
<th>Plasma cholesterol (mg/dl)</th>
<th>NO metabolites (µM)</th>
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<tbody>
<tr>
<td>Control (H₂O)</td>
<td>0.98 ± 0.04</td>
<td>60.50 ± 3.90</td>
<td>24.77 ± 6.39</td>
</tr>
<tr>
<td>Garlic (100 mg protein/kg body wt)</td>
<td>0.30 ± 0.03</td>
<td>25.60 ± 5.80</td>
<td>229.90 ± 15.65</td>
</tr>
<tr>
<td>Heated garlic (100 mg protein/kg body wt)</td>
<td>1.65 ± 0.04</td>
<td>94.80 ± 27.60</td>
<td>25.85 ± 4.69 (*)</td>
</tr>
<tr>
<td>Non-polar fraction (22.5 mg protein/kg body wt)</td>
<td>0.43 ± 0.05</td>
<td>34.10 ± 4.80</td>
<td>265.59 ± 4.22</td>
</tr>
<tr>
<td>Arginine (90 mg/kg body wt)</td>
<td>1.13 ± 0.06 (*)</td>
<td>52.90 ± 3.60 (*)</td>
<td>27.04 ± 1.36 (*)</td>
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**Fig. 3—Effect of NOS inhibitors on garlic induced NO production**

[Rats were IP injected with DPI (1 mg/kg body wt) or L-NAME (40 mg/kg body wt). After 2 h pre-incubation they were IP injected with garlic (100 mg/kg body wt) or vehicle and plasma NO metabolites determined after 30 min. Results were expressed as mean ± SEM of 8 rats. Data significance was assessed by ANOVA test with p<0.05 for G + DPI and G + L-NAME and p<0.01 for G. (*) indicated p>0.05 which was not significant when compared to control]

**Fig. 4—Effect of DPI on garlic induced glucose and cholesterol lowering-activity**

[Rats were pretreated with DPI (1 mg/kg body wt) during 2 h and injected with vehicle or garlic (100 mg/kg body wt). Plasma glucose levels were determined after 2 h and plasma cholesterol after 4 h. Results are expressed by mean ± SEM of 8 rats. Data significance was assessed by ANOVA test with p<0.01 for G. (*) indicated p>0.05 which was not significant when compared to control]
glucose-lowering agent\textsuperscript{21}. We also showed that this garlic contained fraction exerted cholesterol-lowering activity. These results were in accordance with previous studies in human\textsuperscript{22}, rat\textsuperscript{23} or mice\textsuperscript{24}. Our data confirmed that aqueous extract of garlic exerted both glucose and cholesterol-lowering activity\textsuperscript{25}. These effects had a rapid onset of action, which is being reported for the first time. The mode of administration appeared to be of great importance. We found that the IP or IV (results not shown) administration was more effective and rapid than gastric gavage or oral administration (unpublished data), because of differences in absorption and metabolism\textsuperscript{26}.

Moreover, such data might be explained by the high doses used in the present study. The average concentration of 100 mg protein/kg body wt corresponded to approximately 25 cloves or 80 g crude components glucose lowering agents as yet to be purified to induce increase in NOS activity was related to its atherosclerosis affected in pathological conditions, such as or DPI. Earlier studies showed that NO levels were molecular species (two major and two minor) with Rf values higher than the SACS, indicating a more non-polar nature\textsuperscript{28}. The mechanism of action involved nitric oxide. Indeed, all the presently described garlic effects i.e. glucose and cholesterol-lowering activities and NO production were abolished by NOS inhibitors L-NAME or DPI. Earlier studies showed that NO levels were affected in pathological conditions, such as atherosclerosis\textsuperscript{29} and hypertension\textsuperscript{30}. Also, garlic-induced increase in NOS activity was related to its antihypertensive effect\textsuperscript{11}.

To our knowledge, no previous study suggested a correlation between NO metabolites and hyperglycaemia or hypercholesterolemia in rats. This is the first report that linked garlic-induced glucose and cholesterol lowering activities with NOS activation in normal rats. Moreover, cNOS seemed to be involved, because all garlic effects were suppressed by DPI, a more selective cNOS inhibitor than L-NAME\textsuperscript{31}. Our data also confirmed that the active principle couldn’t be arginine nor allin-derived products, which had previously shown to act by NO-independent manner\textsuperscript{16,32}.

In conclusion, the present study showed that aqueous extract of garlic contained a newly described active principle, with a rapid onset of action, exhibiting glucose-lowering activity by its insulin secretagogue effect and anti-atherosclerotic property by its cholesterol-lowering activity. This molecule appeared to be different from arginine and sulfur-derived amino acids, based on physico-chemical properties and mode of action. Its mode of action seemed to involve NO synthesized by cNOS. However, further experiments are needed to assess: (i) the exact molecular nature of this active principle, which might be a saponin\textsuperscript{33}. Interestingly, saponins from garlic have shown to inhibit intestinal absorption of cholesterol\textsuperscript{34}, (ii) the implication of cNOS in insulin secretion process. Indeed, NOS inhibition has been shown to reduce glucose uptake during exercise in individuals with type II diabetes more than in control subjects\textsuperscript{35}. Further studies are also needed to assess the effectiveness of such new activity, as well as implication of NOS using hyperglycaemic and hypercholesterolemic animals.

References
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