Mechanisms involved in the vasorelaxant effects produced by the acute application of amfepramone *in vitro* to rat aortic rings

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Abstract

Amfepramone (diethylpropion) is an appetite-suppressant drug used for the treatment of overweight and obesity. It has been suggested that the systemic and central activity of amfepramone produces cardiovascular effects such as transient ischemic attacks and primary pulmonary hypertension. However, it is not known whether amfepramone produces immediate vascular effects when applied *in vitro* to rat aortic rings and, if so, what mechanisms may be involved. We analyzed the effect of amfepramone on phenylephrine-precontracted rat aortic rings with or without endothelium and the influence of inhibitors or blockers on this effect. Amfepramone produced a concentration-dependent vasorelaxation in phenylephrine-precontracted rat aortic rings that was not affected by the vehicle, atropine, 4-AP, glibenclamide, indomethacin, clotrimazole, or cycloheximide. The vasorelaxant effect of amfepramone was significantly attenuated by NG-nitro-L-arginine methyl ester (L-NAME) and tetraethylammonium (TEA), and was blocked by removal of the vascular endothelium. These results suggest that amfepramone had a direct vasorelaxant effect on phenylephrine-precontracted rat aortic rings, and that inhibition of endothelial nitric oxide synthase and the opening of Ca²⁺-activated K⁺ channels were involved in this effect.

Key words: Cardiovascular pharmacology; Amfepramone; Obesity; Rat aorta; Nitric oxide; Potassium channels

Introduction

Obesity is a major health problem associated with the lifestyle of modern society worldwide. It has been linked to cardiovascular disease, dyslipidemia, osteoarthritis, diabetes mellitus, cancer, and decreased longevity (1,2). Dietary changes and increased exercise are essential steps to counter this relatively recent tendency towards obesity. However, patients who are obese or overweight may also benefit from pharmacological therapy (2).

Currently, appetite-suppressant drugs, pancreatic lipase inhibitors, thermogenic agents and dietetic products are indicated for the treatment of obesity (3). Because adverse effects are associated with some obesity drugs (4), new drugs are urgently needed. Among the cardiovascular complications of appetite-suppressant drugs are hypertension, tachycardia, arrhythmias, valvular regurgitation and myocardial infarction (5-7). The mechanisms of these adverse effects include their serotoninergic agonist properties, their activity as combined norepinephrine and serotonin reuptake inhibitors in the peripheral and central autonomic system, and their ability to stimulate catecholamine release (6-9).

Amfepramone is an appetite-suppressant drug used for the treatment of overweight and obesity. Clinical evidence suggests that adverse reactions to oral amfepramone include cardiovascular effects such as transient ischemic attacks and primary pulmonary hypertension (10,11). These clinical observations are in line with experimental studies in which amfepramone administered intravenously to dogs produced i) a transient vasodepressor effect, followed by a vasopressor effect (12); ii) a dose-related depressor reaction (13); and iii) a marked pressor response when administered intracerebroventricularly (13). Increasing evidence suggests that systemic and central administration of amfepramone produces a number of different cardiovascular effects. Bispo
da Silva and Cordellini (14) found that treatment with amfepramone in vivo caused hyporeactivity to noradrenaline in the aorta, which was abolished by both endothelium removal and the presence of NG-nitro-L-arginine methyl ester (L-NAME), suggesting a role for nitric oxide (NO) in the vascular effects of amfepramone. To the best of our knowledge, the immediate vascular effects of amfepramone when applied in vitro to rat aortic rings and the mechanisms of its effects have not been evaluated. Therefore, the present study aimed to analyze the effect of amfepramone on phenylephrine-precontracted rat aortic rings with or without endothelium. The influence of 10⁻⁶ M atropine, 10⁻⁵ M L-NAME, 10⁻⁵ M tetraethylammonium (TEA), 10⁻³ M 4-AP, 3.1 × 10⁻⁷ M glibenclamide, 10⁻⁵ M indomethacin, 10⁻⁵ M clotrimazole, and 10⁻⁵ M cycloheximide on the effects of amfepramone was also evaluated.

Material and Methods

Animals
Experiments were performed on isolated thoracic aortic rings of adult male Wistar rats (body weight 250-300 g). Rats (n = 23) were purchased from the bioterium of the Escuela Superior de Medicina (México City). Animals were housed in plastic cages in a special temperature-controlled room (22 ± 2°C, 50% humidity) on a 12:12 h light/dark cycle (lights on at 7:00 am). The study was approved by the Animal Care Committee of the Escuela Superior de Medicina; the protocol was in agreement with the 1986 Animals (Scientific Procedures) Act of the British Parliament (http://www.legislation.gov.uk/ukpga/1986/14/contents, accessed February 10, 2015).

Preparation of aortic rings
Animals were euthanized by decapitation, and the aortas were immediately excised and placed in cold buffer before being cleaned and freed from surrounding connective tissue. The isolated arteries were cut into rings 4-5 mm long and placed in 10 mL tissue chambers filled with Krebs-Henseleit bicarbonate buffer (118 mM NaCl; 4.7 mM KCl; 1.2 mM KH₂PO₄; 1.2 mM MgSO₄·7H₂O; 2.5 mM CaCl₂·2H₂O; 25 mM NaHCO₃; 11.7 mM dextrose, and 0.026 mM calcium disodium EDTA). In some experiments the KCl concentration was increased to 80 mM and the 0.026 mM calcium disodium EDTA). In some experiments the KCl concentration was increased to 80 mM and the Na⁺ concentration reduced to maintain osmotic equilibrium. Tissue baths, maintained at 37°C and pH 7.4, were bubbled with a mixture of 95% O₂ and 5% CO₂.

To record isometric tension, aortic rings were mounted on two stainless steel hooks, one fixed to the bottom of the chamber and the other to a BIOPAC TSD125C-50g force transducer connected to a BIOPAC MP100A-CE data acquisition system (BIOPAC Systems, Inc., USA). The optimal tension, determined by preliminary experiments, was that which gave the greatest response to phenylephrine (10⁻⁶ M). Initially, a tension of 2 g (100%) was applied, and rings were allowed to equilibrate for 2 h. Thirty minutes after setting up the organ bath, contractile responses were tested with 10⁻⁶ M phenylephrine. Endothelium-denuded aortic strips were prepared by turning the rings gently several times on the distal portion of small forceps. Endothelial integrity was pharmacologically assessed by acetylcholine-induced vasodilatation (10⁻⁶ M). Segments that did not relax in response to acetylcholine were considered to be endothelium-denuded. After exposure to 10⁻⁶ M phenylephrine or 10⁻⁹ M acetylcholine, tissues were rinsed three times with Krebs solution to restore basal tension.

Drugs
All drugs except amfepramone, which was a generous gift from Productos Medix, Sociedad Anónima de Capital Variable (Mexico), were purchased from Sigma-Aldrich Co. (USA). All compounds were dissolved in distilled water. Fresh solutions were prepared for each experiment.

Experimental protocol
First set of experiments. Thirty minutes after the restoration of basal tension (see Preparation of aortic rings), 10⁻⁶ M amfepramone and 10⁻⁵-10⁻⁶ M acetylcholine, a positive control of vasorelaxation, were cumulatively added to intact rat aortic rings. Acetylcholine was added at intervals of approximately 2 min and amfepramone was added at intervals of approximately 10 min. Tension was expressed as a percentage of basal contraction (2.0 ± 0.16 g, 100%).

Second set of experiments. Thirty minutes after restoration of basal tension (see Preparation of aortic rings), 10⁻⁶ M phenylephrine was added to rat aortic rings with or without endothelium, which elicited a steady contraction after 20 min. Thirty minutes after adding phenylephrine, 10⁻⁵-10⁻⁶ M amfepramone and 10⁻⁵,10⁻⁶ M acetylcholine, a positive control of vasorelaxation, were cumulatively added. Acetylcholine was added at intervals of approximately 3 min and amfepramone was added at intervals of approximately 20 min. Tension was expressed as a percentage of phenylephrine-induced contraction (3.54 ± 0.25 g, 100%).

Third set of experiments. Since amfepramone induced moderate concentration-dependent vasorelaxation on phenylephrine-precontracted rat aortic segments, an attempt was made to determine the mechanisms involved in this relaxant effect. Thirty minutes after adding 10⁻⁶ M phenylephrine (see Second set of experiments), aortic rings with endothelium were preincubated for 30 min with various compounds in order to explore the mechanisms involved in the relaxant effect induced by amfepramone on precontracted rat aortic rings. The compounds used for preincubation were: i) the vehicle (distilled water); ii) 10⁻⁶ M atropine, a competitive muscarinic acetylcholine receptor antagonist; iii) 10⁻⁶ M L-NAME, a direct inhibitor of NO synthase; iv) 10⁻² M TEA, a Ca²⁺-activated K⁺ channel blocker and non-specific voltage-activated K⁺ channel blocker; v) 10⁻³ M 4-aminopyridine (4-AP), a voltage-activated K⁺ channel blocker; vi) 3.1 × 10⁻⁷ M glibenclamide, an ATP-sensitive
K⁺ channel blocker, (KATP); vii) 10⁻⁵ M indomethacin, a prostaglandin synthesis inhibitor; viii) 10⁻⁵ M clotrimazole, a cytochrome P450 inhibitor; and ix) 10⁻⁵ M cycloheximide, a general protein synthesis inhibitor. After preincubation, the influence of vehicle and drugs on the vasorelaxant response to 10⁻⁵-10⁻⁵ M amfepramone was tested.

Data analysis and statistics
Data are reported as means ± SE. In all experiments, aortic segments were obtained from six animals. Statistical analysis was performed in two main data sets. In the first set, the vasorelaxant effects of amfepramone and acetylcholine on aortic rings, whether intact or precontracted with phenylephrine, were analyzed using a one-way repeated-measures analysis of variance (ANOVA). In the second set, the effects of endothelium, antagonist, inhibitors and blockers on the relaxant effect of amfepramone on phenylephrine-precontracted aortic segments were analyzed using a two-way repeated-measures ANOVA. Each analysis of variance was followed by the Student-Newman-Keuls post hoc test. Statistical significance was considered as P < 0.05 (15). The statistical analysis was performed with the SigmaPlot 12 program (Systat Software Inc., USA).

Results
Effect of amfepramone and acetylcholine on rat aortic rings
Figure 1 shows the effects of the cumulative addition of 10⁻⁶-10⁻⁵ M amfepramone and 10⁻⁰-10⁻⁵ M acetylcholine on intact rat aortic rings. Amfepramone and acetylcholine produced moderate concentration-dependent vasorelaxation in those aortic segments. The logEC₅₀ values for amfepramone- and acetylcholine-induced vasorelaxation were –7.91 and –6.87 M, respectively. Following the addition of amfepramone and acetylcholine, the maximum vasorelaxation (E₉⁰) was 17.34 ± 3.66% and 48.90 ± 8.75% of basal contraction, respectively. The vasodilator response produced by amfepramone appeared immediately on the addition of each concentration of this drug and reached a maximum value after 5 min. This vasodilator response was sustained and continued without change.

Effect of amfepramone and acetylcholine on phenylephrine-precontracted rat aortic rings with and without endothelium
Figure 2 shows the effects of the cumulative addition of 10⁻⁵-10⁻⁶ M amfepramone and 10⁻⁴-10⁻⁵ M acetylcholine on phenylephrine-precontracted rat aortic rings with and without endothelium. Amfepramone and acetylcholine elicited a concentration-dependent relaxation on aortic rings with intact endothelium, an effect that was blocked by the functional removal of the endothelium. The logEC₅₀ of amfepramone and acetylcholine for vasorelaxation in aortic rings with intact endothelium was –6.04 M and –7.67 M, respectively. The maximum vasorelaxation produced by amfepramone and acetylcholine in aortic rings with intact endothelium was 69.63 ± 5.53% and 98.51 ± 0.94%, respectively. The vasorelaxant response produced by amfepramone appeared immediately on the addition of each concentration of this drug and reached a maximum value after 15 min. This vasorelaxant response was sustained and continued without change.

Effect of atropine on the relaxant effect induced by amfepramone on phenylephrine-precontracted rat aortic rings
Figure 3 shows the effect of 10⁻⁶ M atropine on the vasorelaxation induced by 10⁻⁵-10⁻⁴ M amfepramone on phenylephrine-precontracted rat aortic rings. The maximum vasorelaxant effect produced by amfepramone was unaffected by the absence (86.65 ± 3.40%) or presence (90.72 ± 2.58%) of atropine.

Effect of L-NAME, TEA, 4-AP and glibenclamide on the relaxant effect induced by amfepramone on phenylephrine-precontracted rat aortic rings
Figure 4 shows the effect of 10⁻⁴ M L-NAME (A), 10⁻⁵ M TEA (B), 10⁻⁵ M 4-AP (D), and 3.1 × 10⁻⁷ M glibenclamide (E) on the vasorelaxation induced by 10⁻⁴-10⁻⁵ M amfepramone on phenylephrine-precontracted rat aortic rings. The maximum vasorelaxant effect produced by amfepramone was unaffected by the absence (74.63 ± 5.25%) or presence (83.35 ± 6.28%) of the distilled water vehicle and two other pretreatments: 4-AP (90.82 ± 2.47% vs 86.42 ± 4.79%), and glibenclamide (93.10 ± 2.73% vs 85.95 ± 3.35%). The maximum vasorelaxant effect produced by amfepramone was significantly (P < 0.05) attenuated in the presence vs absence of two compounds.
Effect of indomethacin and clotrimazole on the relaxant effect induced by amfepramone on phenylephrine-precontracted rat aortic rings

Figure 5 shows the effect of 10^{-5} M indomethacin on the relaxation induced by 10^{-9}-10^{-5} M amfepramone on phenylephrine-precontracted rat aortic rings. The maximum vasorelaxant effect produced by amfepramone was unaffected by the presence or absence of indomethacin (75.16±5.09% vs 83.16±6.69%) or clotrimazole (95.62±1.36% vs 90.34±3.09%).

Effect of cycloheximide on the relaxant effect induced by amfepramone on phenylephrine-precontracted rat aortic rings

Figure 6 shows the effect of 10^{-5} M cycloheximide on the relaxation induced by 10^{-9}-10^{-5} M amfepramone on phenylephrine-precontracted rat aortic rings. The maximum vasorelaxant effect produced by amfepramone was unaffected by the presence (95.08±1.54%) or absence (93.12±2.37%) of cycloheximide.

Discussion

The vasorelaxation produced by amfepramone on phenylephrine-precontracted rat aortic rings was: i) unaffected by vehicle, atropine, 4-AP, glibenclamide, indomethacin, clotrimazole and cycloheximide; ii) significantly attenuated by L-NAME and TEA; and iii) blocked by removal of the endothelium. These results suggest that the relaxant effect elicited by amfepramone on phenylephrine-precontracted rat aortic rings involved the inhibition of endothelial nitric oxide synthase and the opening of Ca^{2+}-activated K^{+} channels.

Effect of amfepramone and acetylcholine on rat aortic rings

Amfepramone and acetylcholine produced moderate, dose-dependent vasorelaxant responses in aortic rings with intact endothelium (Figure 1). The direct vasorelaxant effects produced in aortic rings by amfepramone reinforces the aforementioned findings of previous studies in dogs, in which the intravenous administration of amfepramone produced a dose-dependent depressor reaction (13) and a transient vasodepressor effect (12). However, the marked pressor response to intracerebroventricular administration of amfepramone (13), and the transient vasodepressor
effect produced by intravenous amfepramone, followed by a vasopressor effect (12), differ from our results as well as from the other results in those studies. Moreover, the vasorelaxant responses produced by amfepramone in aortic rings are not consistent with a clinical study in which oral administration of amfepramone produced ischemic attacks in the brain of an obese patient (11).

Discrepancies in the reported vascular effects of amfepramone may be related to differences in experimental conditions as well as routes of administration, because: i) the systemic (intravenous or oral) administration of amfepramone has been associated with transient vasodepressor and vasopressor responses in humans and dogs (11-13); ii) the central (intracerebroventricular) administration of...
channels in channel blocker (27), and channel blocker (28), but of K channels are involved in the response produced by amfepramone. Previously reported in other studies of acetylcholine (17,21,22), amfepramone and acetylcholine (Figure 2). That effect was previously reported in other in vitro studies of acetylcholine and other drugs with vasorelaxant effects (17,21,22).

Effect of amfepramone and acetylcholine on phenylephrine-precontracted rat aortic rings

Aortic segments were pretreated with phenylephrine 30 minutes before administration of amfepramone and acetylcholine because the latter compounds elicited moderate concentration-dependent vasorelaxation in the previous assay with rat aortic rings (Figure 1). Acetylcholine was used as a positive control of vasorelaxation. It should be pointed out that under our experimental conditions, phenylephrine enhances the vasorelaxant responses produced by amfepramone and acetylcholine (Figure 2). That effect was previously reported in other in vitro studies of acetylcholine and other drugs with vasorelaxant effects (17,21,22).

On the other hand, the fact that amfepramone and acetylcholine produced a concentration-dependent relaxation in phenylephrine-precontracted rat aortic rings with intact endothelium, but not after mechanical removal of this tissue (Figure 2), suggests that the vasorelaxant effects produced by these drugs are endothelium-dependent. This corroborates several reports suggesting that the endothelium plays an important role in the vasorelaxation produced by acetylcholine (23-25). Following the evidence-based conclusion that acetylcholine produces vasodilation though the stimulation of endothelium-dependent mechanisms, we decided to investigate whether the endothelium also participates in the vasorelaxant response produced by amfepramone.

Involvement of muscarinic receptors in the vasorelaxant response produced by amfepramone on phenylephrine-precontracted rat aortic rings

Atropine, an antagonist of muscarinic acetylcholine receptors (26), did not modify the direct vasorelaxation produced by amfepramone on rat aortic rings (Figure 3), which excludes the possible involvement of stimulation of muscarinic acetylcholine receptors in the vasodilator responses produced by amfepramone.

Involvement of the NO pathway and K+ channels in the vasorelaxant effect produced by amfepramone on phenylephrine-precontracted rat aortic rings

The fact that the vasorelaxant response induced by amfepramone in rat aortic rings was unaffected (P > 0.05) by 4-AP, a voltage-activated K+ channel blocker (27), and glibenclamide, an ATP-sensitive K+ channel blocker (28), but was significantly (P < 0.05) attenuated by L-NAME, a direct inhibitor of NOS (29) and TEA, a Ca2+-activated K+ channel blocker and non-specific voltage-activated K+ channel blocker (30), indicates the involvement of the release of NO by endothelium tissue and the activation by Ca2+ of K+ channels. This conclusion is supported by the fact that the mechanical removal of endothelium blocked the vasorelaxation produced by amfepramone and acetylcholine in phenylephrine-precontracted rat aortic rings, and by evidence that the NO pathway and K+ channels are involved in the endothelial-mediated control of vascular tone (24,31,32).

There is evidence suggesting that additional mechanisms are involved in endothelial control of vascular tone, such as prostacyclins (33,34) and endothelium-derived hyperpolarizing factor (EDHF), a cytochrome P450-derived arachidonic acid metabolite (35-37). Nevertheless, the vasorelaxation produced by amfepramone was unaffected by indomethacin, a prostaglandin synthesis inhibitor (38), and by clotrimazole, a cytochrome P450 inhibitor (37,39), thereby excluding the involvement of prostacyclins and EDHF in the endothelium-mediated vasodilation under the current experimental conditions.

On the other hand, the fact that the vasorelaxant response to amfepramone was not significantly (P > 0.05) different after preincubation with the distilled water vehicle indicates that vasorelaxation to amfepramone is highly reproducible. This excludes the possibility that attenuations produced by the
above inhibitors/blockers are tachyphylactic. Moreover, the fact that TEA, but not 4-AP, significantly (P<0.05) attenuated the vasorelaxant response induced by amfepramone suggests the involvement of Ca\(^{2+}\)-activated K\(^+\) channels and excludes the involvement of voltage-activated K\(^+\) channels. Admittedly, additional experiments that go beyond the scope of the present study will be required to further identify the specific subtype of Ca\(^{2+}\)-activated K\(^+\) channels involved in the vasorelaxant effects produced by amfepramone.

**Involvement of protein-synthesis in the vasorelaxant response produced by amfepramone on phenylephrine-precontracted rat aortic rings**

Cycloheximide, a general protein synthesis inhibitor (40), did not modify the direct vasorelaxation produced by amfepramone on rat aortic rings (Figure 6), which excludes the possible involvement of protein synthesis in the vasorelaxant effect produced by this appetite-suppressant drug.

In conclusion, our results suggest that the vasorelaxant responses produced by amfepramone in phenylephrine-precontracted rat aortic rings involved the inhibition of eNOS and the opening of Ca\(^{2+}\)-activated K\(^+\) channels.

**Acknowledgments**

We would like to express our deep gratitude to Dr. Carlos Castillo-Henkel for his unconditional friendship, professional guidance and life example.

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