Evidence for Synergy Between α₂-Adrenergic and Nonadrenergic Mechanisms in Central Blood Pressure Regulation

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Background—Both α₂- and non-α₂-adrenergic mechanisms seem to be involved in the hypotensive effect of imidazoline-like drugs. This study aimed at investigating how these 2 mechanisms work together to modify blood pressure (BP).

Methods and Results—LNP 509, which appeared in this study to be devoid of α₂-adrenergic activity, was administered to anesthetized rabbits and wild-type (WT) mice into the cisterna magna and into the fourth ventricle, respectively. Mean arterial pressure decreased by a maximum of 46±4% and 16±2%, respectively. In D79N mice, which lack functional α₂A-adrenergic receptors, LNP 509 also reduced mean arterial pressure by 17±2%. The hypotension induced by LNP 509 (100 μg/kg intracisternally) was prevented by S23757 (1 mg/kg intracisternally), an antagonist highly selective for I₁-imidazoline binding sites (I₁BS). A synergy between LNP 509 and the α₂-adrenergic agonist α-methylnoradrenaline (α-MNA) was observed in rabbits (cisterna magna injection) and in WT mice (fourth ventricle injection) but not, as expected, in D79N mice. Similar to LNP 509 alone, rilmenidine (fourth ventricle injection), which binds both to α₂-adrenergic receptors and to I₁BS, decreased BP in D79N mice. In WT animals, rilmenidine had a significantly greater effect. Microinjections performed in rabbits showed that the synergism occurred at least in part in the nucleus reticularis lateralis of the brainstem.

Conclusions—These results demonstrate that a central imidazoline-sensitive, but non-α₂-adrenergic, mechanism can modify BP by itself. This mechanism, which may involve I₁BS, interacts synergistically with an α₂-adrenergic mechanism to decrease BP. (Circulation. 2002;105:XXX–XXX.)

Key Words: nervous system, sympathetic ■ hypertension ■ pharmacology ■ receptors, adrenergic, alpha

The central nervous system (CNS) plays a key role in the regulation of cardiovascular function. The nucleus tractus solitarii (NTS) and the nucleus reticularis lateralis of the rostral ventrolateral medulla (NRL/RVLM) are 2 nuclei that are highly involved in this control.1 α₂-Adrenergic receptors (α₂ARs) are distributed throughout the CNS, including the NTS and the NRL/RVLM.2,3 They are known to mediate many effects, such as sedation, anesthetic-sparing response, analgesia, and sympathoinhibition.4 Thus, α₂AR agonists administered centrally cause hypotension.5 α₂ARs have been classified into 3 subtypes: α₂AAR, α₂BAR, and α₂CAR.6 All of them are present in the CNS.7 Gene-targeted mice have been engineered for each α₂AR subtype to determine the functional relevance of subtype diversity. For example, D79N mice have a point mutation of the α₂AR gene. The mutation of Asp79 to Asn un couples the α₂AR from its transduction pathways in vivo.8 Thus, D79N mice constitute functional α₂A-knockout animals.9 Experiments performed in D79N and α₂A-knockout mice have confirmed that α₂A is the α₂AR subtype that mediates the central effects of α₂AR agonists, including their hypotensive action.9

One unique mechanism of action explains the central hypotensive effect of the α₂-adrenergic agonist, α-methylnoradrenaline (α-MNA). This catecholamine is the active metabolite of α-methyldopa, and it reduces blood pressure (BP) in inhibiting the vasomotor tone by activating α₂ARs in the NTS.10 The mechanism of the central antihypertensive effects of imidazoline-related compounds appears more complex. As a matter of fact, a large body of experimental data indicates that these drugs also activate α₂ARs to reduce BP.5,11,12 Nevertheless, several groups have reported that a nonadrenergic mechanism also contributes to their hypotensive action. This
assumption was based on structure-activity relationship studies and on antagonism experiments. The nonadrenergic component of the central hypotension induced by imidazoline-like drugs was afterward attributed to their ability to act at specific binding sites, insensitive to catecholamines, called imidazoline binding sites (IBS) and located in the NRI/RVLM region. Because relevant animal models and ligands selective for these nonadrenergic sites over α2-AR were lacking, it was impossible to clarify how (in the case that these 2 mechanisms should work together) they might affect BP regulation and also how they might account for the overall hypotensive action of imidazoline-like drugs. It may be interesting to note that after central injection, the onset of the hypotensive effect of these drugs is much faster than that of α2-MNA. Their maximal effect is also greater. These observations suggest that (imidazoline-specific) non-adrenergic and adrenergic mechanisms may cooperate when they are triggered by hybrid imidazoline drugs (which are capable of binding on both types of sites), such as clonidine, rilmenidine, and moxonidine. Taking advantage of the recent availability of selective tools and of genetically engineered mice with impaired α2-AR, the present study was designed to clarify all these aspects.

It has recently been reported that dicyclopropylmethyl-(4,5-dimethyl-4,5-dihydro-3H-pyrrol-2-yl)-amino hydrochloride (LNP 509), a new pharmacological tool, had neither affinity for α2-ARs and α1-ARs nor activity at α2-ARs. This drug has been shown to bind selectively to the I1 subtype of IBS (I1/BS) over the I2 subtype of IBS (I2/BS). In the present study, we used LNP 509 and α-MNA to trigger nonadrenergic and α2-adrenergic mechanisms, respectively. We looked at their effects on BP when given alone and when given in combination in rabbits and mice. We also studied the response to rilmenidine after central injection in mice.

Methods

[^35S]GTPγS Binding Assay

The method of Jasper et al was modified as described previously. Membranes of CHO cells (clone 1E5) transfected with human α2-ARs were incubated with 0.2 nmol/L[^35S]GTPγS and drugs for 1 hour at 25°C. Nonspecific binding was defined by 10 µmol/L cold GTPγS. Reactions were stopped by vacuum filtration over Whatman GF/B filters. Filters were washed with ice-cold buffer (30 mmol/L HEPES/NaOH, 100 mmol/L NaCl, and 3 mmol/L MgCl2, pH 7.4 at 4°C), and incorporated radioactivity was determined by using liquid scintillation counting.

Animals and Hemodynamic Measurements

The present study was conducted in compliance with institutional guidelines and those formulated by the European community for use of experimental animals (L358-86/609/EEC). Studies were performed on normotensive male rabbits (Zika strain, Wendling, Was-selonne, France), weighing 2.5 to 3.5 kg, prepared as described previously, and on 10- to 20-week-old B6.129S2-Adra2a tm1Lel mice, ie, D79N mice. These mice were kindly provided by Dr L. Limbird (Vanderbilt University, Medical Center, Nashville, Tenn). C57BL/6N mice were the wild-type (WT) control strain (Charles River, St Aubin les Elbeuf, France). Mice were anesthetized with pentobarbítone (50 mg/kg IP) and tracheotomized. The right carotid artery and the left external jugular vein were catheterized to measure arterial pressure and to administer anesthetic and curare, respectively. The mice were then ventilated with room air (tidal volume 170 µL, 120 cycles/min) by means of a MiniVent type 845 mouse ventilator (Hugo Sachs Elektronik) and paralyzed with pancuronium bromide (250 µg/kg IV).

Mean arterial pressure (MAP) was calculated as diastolic pressure plus one third of the differential pressure. The heart rate (HR) was continuously monitored from the pressure signal with a Gould Biotach amplifier (model 13-4615-66).

Central Administration of Drugs

Rabbits

The head of each rabbit was placed after surgery in a stereotaxic frame (La Précision Cinématographique Française). Drugs were administered either into the cisterna magna (intracisternal injections) or microinjected into the NRI/RVLM (2 mm lateral to the midline, 4 mm rostral to the obex, and 8 mm from the dorsal surface), as described previously. At the beginning of each intracisternal experiment, a volume of cerebrospinal fluid, equal to the volume injected during the experiment, was withdrawn. The volume of injection (per dose) was 100 µL for intracisternal experiments and 0.5 µL for microinjections. However, for intracisternal experiments, (+)-(2-fluoro-5-methylphenyl)-4,5-dihydro-1H-imidazole (S23757) was given at 400 µL. When synergy was investigated, 10 minutes separated the 2 intracisternal injections. For microinjections, both drugs (each in a volume of 0.5 µL) were drawn up successively in the same needle and were administered very slowly in 1 injection. The site of injection was confirmed histologically as described previously.

Mice

The mice were anesthetized as described above. The head of each mouse was placed in a mouse stereotaxic frame (Cunningham Mouse Adaptor, Stoelting). A stainless-steel guide cannula (external diameter 0.4 mm, internal diameter 0.3 mm; Cortial SA) was placed in the fourth ventricle (4°C, 5.88 mm caudal to the bregma and 2.8 mm from the surface of the skull; coordinates are according to Franklin and Paxinos) and fixed with dental cement. The catheterization of vessels and the central drug injections (1 to 2 µL) were performed 48 hours after the stereotaxic procedures. After each experiment, the injection site was checked by an injection of 1 µL Evans blue through the guide cannula, so that to validate the experiment, the walls of the 4°C ventricle and the ventral surface of the medulla had to be stained in blue. Experiments in which the 4°C ventricle was not stained were considered. Intracisternal ventricular injections were not conclusive in our experimental conditions. The Evans blue injected into the lateral ventricle was confined to this cavity and did not diffuse to the 4°C or to the medullary ventral surface.

Statistics and Calculations

Data are given as mean±SEM or mean±SE. Homogeneity of the initial cardiovasculard parameters between groups was checked with an ANOVA. Mean values were compared by ANOVA (for repeated measures or intergroup) or by Student t tests (for paired or unpaired observations) as appropriate. Significance was set at P<0.05, and n indicates the number of experiments.

Results

[^35S]GTPγS Binding Assay

In CHO cells transfected with human α2-ARs, noradrenaline (NA) induced[^35S]GTPγS binding to G proteins (pEC50, 6.18±0.03, Figure 1A). MK912, an α2-AR antagonist, had no effect by itself but antagonized the effect of NA (10^-5).
Cardiovascular Effects of LNP 509

Given intracisternally in cumulative doses (10 to 1000 μg/kg) to rabbits, LNP 509 dose-dependently decreased MAP (55±2 versus 102±3 mm Hg, ED<sub>50</sub> 184±18 μg/kg) and HR (221±9 versus 310±7 bpm) (values at dose of 1000 μg/kg). These effects were significant for doses ranging from 100 to 1000 μg/kg (P<0.05, n=5). Repeated intracisternal injections of vehicle did not significantly modify the hemodynamic parameters (Figure 2). The hypotensive response to 100 μg/kg LNP 509 was prevented by an intracisternal injection of 1 mg/kg S23757, an antagonist highly selective for 1,BS<sup>22</sup> MAP decreased from 102±3 to 90±3 mm Hg in the control group (P<0.05, n=5), whereas it was stable at 97±4 mm Hg in the animals pretreated with S23757 (n=5).

Administered into the 4<sup>th</sup>V of mice at a dose of 1 mg/kg, LNP 509 induced a significant change in MAP in WT as well as D79N mice (59±2 versus 71±2 mm Hg, P<0.05 [n=13], and 60±2 versus 72±2 mm Hg, P<0.05 [n=13], respectively; Figure 3). The HR was not affected in either strain: 382±22 versus 407±25 bpm (WT mice) and 423±25 versus 433±24 bpm (D79N mice). In both WT and D79N mice, the

Synergy Between LNP 509 and α-MNA Effects

To examine a possible synergism between the nonadrenergic and the α-adrenergic mechanisms, we selected a subthreshold dose of LNP 509 and a threshold dose of α-MNA. At an intracisternal dose of 30 μg/kg, LNP 509 did not affect MAP in rabbits (95±2 versus 96±2 mm Hg, n=6) (Figure 4A). In 6 other rabbits, an intracisternal dose of 0.5 μg/kg α-MNA decreased MAP maximally from 100±2 to 90±1 mm Hg (P<0.05) (Figure 4A). In 6 other animals, α-MNA (0.5 μg/kg intracisternally) was given 10 minutes after LNP 509 (30 μg/kg intracisternally). MAP decreased immediately, and the maximal effect was reached within 5 minutes of injection (73±3 versus 101±2 mm Hg, P<0.05) (Figure 4A). This reduction in MAP was significantly different from that obtained with α-MNA or LNP 509 given alone (P<0.05). Moreover, LNP 509 injected twice at the same dose (30 μg/kg intracisternally) had no effect on MAP (−1±2%, n=4). Finally, we switched the order of injections; ie, α-MNA was injected first. The same maximal effect as when LNP 509 was administered first was observed, but it was delayed; it appeared 15 minutes after the injection of LNP 509 (Figure 4A). We obtained similar results when both drugs were microinjected together into the NRM/RVM of the rabbits. LNP 509 (4 μg/kg) was ineffective on MAP (±2%, P>0.05; n=5); 1 μg/kg α-MNA was also ineffective (0.5±2%, P>0.05; n=9). However, when LNP 509 was
injected first and α-MNA was subsequently injected into the NRL/RVLM, MAP decreased by 15±2% (77±6 versus 90±5 mm Hg, P<0.05; n=5) (Figure 4B). Interestingly, when α-MNA was microinjected first, we did not observe any MAP variation.

This interaction was further investigated in mice. Administered to 5 WT mice, LNP 509 (1 mg/kg in 4th V) decreased MAP from 72±1 to 61±2 mm Hg (P<0.05) (Figure 4C). In 5 other WT animals, the 4th V injection of LNP 509 was followed by the 4th V injection of 10 μg/kg α-MNA (a dose that was ineffective on MAP, 70±4 versus 68±3 mm Hg; n=5). In that case, MAP decreased significantly from 72±5 to 49±4 mm Hg (P<0.05) (Figure 4C). This hypotension was significantly different from that induced by LNP 509 alone, whereas such a potentiation between the effects of LNP 509 and α-MNA was not observed when these drugs were administered in combination to D79N mice according to the same protocol: MAP changed from 71±1 to 60±2 mm Hg with LNP 509 alone (P<0.05, n=5) and from 74±3 to 65±3 mm Hg with LNP 509 plus α-MNA (P<0.05, n=5) (Figure 4C). These variations of MAP obtained in D79N mice were similar to those obtained in WT animals when LNP 509 was given alone.

Cardiovascular Effects of Rilmenidine in Mice
The effects of increasing doses of rilmenidine (3 μg/kg to 1 mg/kg, 4th V injection) were investigated in WT and D79N mice. Each animal received a single dose (5 animals per dose). In WT mice, rilmenidine lowered MAP in a dose-dependent manner, and the effect became significant from the dose of 30 μg/kg (Figure 5A): MAP decreased from 71±4 to 55±4 mm Hg at the highest dose (n=9). In transgenic mice, rilmenidine did not affect MAP, except at the dose of 1 mg/kg (65±3 versus 75±3 mm Hg, n=9) (Figure 5B). Although the hypotensive effect of 1 mg/kg rilmenidine was significantly weaker in D79N than in WT mice (P<0.05), it was very similar to the effect of LNP 509 given alone in both WT and transgenic mice.

Discussion
We used α-MNA to mimic the α2-adrenergic activity of hybrid drugs. This catecholamine binds to α2ARs with high affinity13,21 but does not recognize I1S at all.13 It is a full agonist at human and murine α2A ARs.21 Therefore, α-MNA can be considered a “pure” α2AR agonist.

LNP 509 was used in the present study to reproduce the non-α2-adrenergic component of imidazoline-like drugs for 2 reasons. First, it has been shown recently (Schann et al19) that
LNP 509 binds to I1 BS with a pKᵢ value of 5.98 ± 0.15, whereas it has no detectable affinity at α₂ ARs, α₃ ARs, and I1 BS; pKᵢ values are >5. Since that study, the binding characterization of LNP 509 has been extended to a large variety of other receptors (serotonin, dopamine, β₁ and β₂, histamine, adenosine, endothelin, Bradykinin, neuropeptide Y, cannabinoid, muscarinic, and nicotinic receptors), enzymes (monoamine oxidase A and B), and transporters (NA and serotonin). In all these systems, pKᵢ values are >5.

Second, LNP 509 is devoid of activity at α₂ ARs, because in the present study, it was unable to induce or prevent [³⁵S]GTPΓS binding to the G proteins associated to human α₂ ARs expressed in CHO cells. Our experiments were performed on the α₁ subtype because it has been shown that unlike the α₂̂ and α₃ AR subtypes, it is the subtype involved in the central hypotensive action of imidazoline-related compounds. Although LNP 509 was devoid of α₂ adrenergic activity, after intracisternal injection to rabbits, it lowered BP as much as hybrid drugs do. This hypotensive action was prevented by S23757, an antagonist highly selective for I1 BS. LNP 509 also caused hypotension when administered in the 4th V of WT and D79N mice. These in vivo data demonstrate that LNP 509 is capable of reducing BP in 2 different species and (2) that this activity is independent of any action at α₂ ARs. The recurrent and justified objection against previous studies concerning imidazoline-induced hypotension was that drugs selective for I1 BS over α₂ ARs could lose their selectivity at high doses and stimulate α₃ ARs to lower BP in animals with functional α₂ ARs. In the present work, thanks to the use of D79N mice, this objection is definitely forestalled. Therefore, although the involvement of other receptors cannot be definitively ruled out, the functional results reported in the present study, together with previous binding data, strongly suggest that the hypotensive action of LNP 509 involves I1 BS.

It remains that rather high doses of LNP 509 were required to lower BP. This might be interpreted in at least 2 ways: (1) LNP 509 may be weakly efficient on I1 BS, or (2) the α₂-adrenergic and non-α₁-adrenergic systems may work synergistically. Consequently, when one of them is impaired, the other is only weakly effective. It will not be possible to check the first hypothesis before a series of selective I1 BS ligands with various efficacy levels is available, but with LNP 509, we had the appropriate pharmacological tool to test the second hypothesis. A marked potentiation of the effect of α-MNA by LNP 509 was indeed observed both in rabbits and in WT mice. This synergy was nonexistent in D79N mice. In these animals, the effect of LNP 509 only was obtained, and its amplitude was similar to that of the effect induced by the injection of LNP 509 alone in WT and D79N mice. Thus, a synergy between an α₂-adrenergic and a non-α₁-adrenergic mechanisms occurs. If one assumes that LNP 509 and α-MNA are full agonists at α₂ ARs, the effect of the combination of the 2 drugs should be additive. If one compound were a partial agonist and the other one were a full agonist at α₂ ARs, one should observe a subadditive response. In the present study, a supra-additive effect is obtained, supporting the assumption that LNP 509 and α-MNA act by different mechanisms.

Rilmenidine decreased MAP in a dose-dependent manner when administered centrally to WT mice. It also lowered BP in D79N mice, but only at the highest dose. This effect was comparable to the effect triggered by LNP 509 when given alone both in WT mice and in D79N mice. Therefore, we assume that the effect induced by rilmenidine in transgenic mice is representative of the non-α₁-adrenergic mechanism that contributes to its hypotensive action. These data corroborate previous results showing the hypotensive action of moxonidine in D79N mice after central injection, but the data are in contrast to the data of the study reporting the lack of effect on BP of imidazoline-like drugs injected intravenously to such mice. The difference in the routes of drug administration or in the state of the animal (anesthetized versus conscious) might account for such a discrepancy. However, because rilmenidine is effective in WT mice whatever the route of injection (Zhu et al and present study), another hypothesis might be put forward. Considering the synergy aforementioned and the fact that rilmenidine is a hybrid drug, one could assume that in this strain, rilmenidine triggers a synergy between α₂-adrenergic and non-α₁-adrenergic mechanisms. Thus, a lower dose of rilmenidine might be sufficient to decrease BP in WT mice than in transgenic mice, in which no synergy can occur. This synergy could explain the rightward shift of the dose-response curve of LNP 509 compared with that of rilmenidine in rabbits (Feldman et al and present study). Thus, rilmenidine may trigger the synergy by itself, whereas LNP 509, which only binds to I1 BS, cannot do so.

In synergistic experiments, the sequence of injections was critical. In both species, the onset of hypotension was much faster when LNP 509 was given before α-MNA. This was even more striking when the drugs were microinjected into...
the NRL/RVLM of the rabbits. Hypotension was observed only when LNP 509 was injected before α-MNA. To explain this observation, one could assume that α-MNA is cleared rapidly after microinjection into the NRL/RVLM. We cannot rule out such a metabolic explanation, despite the fact that nothing is known about the NRL/RVLM region being capable of clearing catecholamines faster than other medullary regions, such as the NTS, where α-MNA has a marked effect on BP after microinjection.10 One can rather hypothesize that catecholamines are ineffective in the NRL/RVLM, unless I1BSs are previously occupied. Imidazoline-related compounds would make the hypotensive effects of catecholamines possible, at least in the NRL/RVLM. This assumption might explain why reasonable doses of catecholamines had no effect on BP when microinjected alone into this nucleus44 and why huge doses of α-MNA had to be injected to induce hypotension.20 Further study of the effect of treatment with imidazoline-like drugs on the expression of α2Rs on neuronal cells should further highlight our hypothesis.

The cell type on which such an interaction occurs remains unknown. It has recently been suggested that the sympatholytic action of imidazoline-like drugs is partly mediated by non-C1 bulbospinal neurons of the NRL/RVLM rather than by C1 adrenergic cells.31,32 Whether these non-C1 neurons are the site of the interaction between α2-adrenergic and non-α2-adrenergic (I1 subtype) mechanisms remains to be investigated.

In conclusion, (1) the combined use of LNP 509, a compound with no activity at α2ARs, and of mice with impaired α2ARs definitely confirmed that a central imidazoline-sensitive, but non-α2-adrenergic, mechanism is involved in the BP control; (2) this mechanism might be mediated by I1BS; and (3) α2-adrenergic and nonadrenergic mechanisms interact synergistically in the control of BP.

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