A New Pyrroline Compound Selective for I1-Imidazoline Receptors Improves Metabolic Syndrome in Rats

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ABSTRACT

Symptoms of the metabolic syndrome (MetS), such as insulin resistance, obesity, and hypertension, have been associated with sympathetic hyperactivity. In addition, the adiponectin pathway has interesting therapeutic potentials in MetS. Our purpose was to investigate how targeting both the sympathetic nervous system and the adipose tissue (adiponectin secretion) with a drug selective for nonadrenergic I1-imidazoline receptors (I1Rs) may represent a new concept in MetS pharmacotherapy. LNP599 [3-chloro-2-methyl-phenyl)-(4-methyl-4,5-dihydro-3H-pyrrol-2-yl)-amine hydrochloride], a new pyrroline derivative, displaced the specific [125I]para-iodoclonidine binding to I1R with nanomolar affinity and had no significant affinity for a large set of receptors, transporters, and enzymes. In addition, it can cross the blood-brain barrier and has good intestinal absorption, permitting oral as well as intravenous delivery. The presence of I1Rs was demonstrated in 3T3-L1 adipocytes; LNP599 had a specific stimulatory action on adiponectin secretion in adipocytes. Short-term administration of LNP599 (10 mg/kg i.v.) in anesthetized Sprague-Dawley rats markedly decreased sympathetic activity, causing hypotension and bradycardia. Long-term treatment of spontaneously hypertensive heart failure rats with LNP599 (20 mg/kg PO) had favorable effects on blood pressure, body weight, insulin resistance, glucose tolerance, and lipid profile, and it increased plasma adiponectin. The pyrroline derivative, which inhibits sympathetic activity and stimulates adiponectin secretion, has beneficial effects on all the MetS abnormalities. The use of one single drug with both actions may constitute an innovative strategy for the management of MetS.

Introduction

“Syndrome X” was first described in 1988 (Reaven, 1988) as a combination of glucose intolerance, insulin resistance, dyslipidemia, and hypertension. Subsequently, other criteria, such as abdominal obesity, were added to give what is now more widely known as metabolic syndrome (MetS).

Several symptoms of the MetS, such as insulin resistance, obesity, and hypertension, have been individually associated with sympathetic hyperactivity (de Champlain et al., 1976; Coote and Sato, 1977; Vollenweider et al., 1993; Morgan et al., 1995; Grassi et al., 1998, 2007; Rumanit et al., 1999; Parati and Esler, 2012). The literature contains experimental arguments that support the fact that sympathetic overactivity may have some beneficial effects, such as stimulation of lipolysis and thermogenesis and a consequent reduction of body weight. Nevertheless, clinicians generally agree that the symptoms of MetS, namely, hypertension, insulin resistance, abdominal obesity, and hyperlipidemia, are associated with sympathetic overactivity. Contradictory influences of sympathetic hyperactivity, such as lipolysis and thermogenesis activation, might not be precluded, but the final result, at least in humans, appears in favor of detrimental cardiovascular and metabolic actions. The severity and duration of sympathetic overactivity could also tip the scale one way or the other. Feldstein and Julius (2009) summarized this point, indicating that although short-term sympathetic activation is...
useful for survival, it is deleterious (hypertension, overweight) when it becomes permanent.

So far, the role of sympathetic hyperactivity in the pathogenesis of MetS has never been demonstrated (Straznicky et al., 2012). Nevertheless, Grassi et al. (2005) proposed that sympathetic hyperactivity could play a causal role in the development of MetS.

Furthermore, the adiponectin production system appears to be another key element in the pathophysiology of metabolic disorders; a decrease in adiponectin is associated with human metabolic syndrome, obesity, type 2 diabetes, and dyslipidemia (Mancia et al., 2007). The adiponectin pathway has been proposed as an interesting target for the treatment of MetS (Mancia et al., 2007; Gu and Li, 2012).

Many studies have investigated centrally acting anti hypertensive agents, particularly sympathoinhibitory compounds acting on both \( \alpha_2 \)-adrenergic and nonadrenergic imidazoline receptors (I\(_1\)Rs), such as rilmenidine or moxonidine, which derive from clonidine (Bruban et al., 2002). It has been suggested that the beneficial metabolic effects of these compounds are related to their action on I\(_1\)Rs, whereas targeting \( \alpha_2 \)-adrenergic receptors is thought to be rather detrimental in this context (Ernsberger et al., 1999; Velliquette and Ernsberger, 2003a,b; Velliquette et al., 2006). However, to date, the lack of selectivity of the ligands has not permitted determination of the respective contributions of \( \alpha_2 \)-adrenergic- and I\(_1\)R-mediated effects.

Imidazoline receptors were first described as pharmacological targets that are triggered by clonidine and related compounds within the brainstem to induce hypotension (Bousquet et al., 1984). They were shown to be insensitive to catecholamines (as such, different from adrenergic receptors) but sensitive to drugs bearing an imidazoline moiety or chemically related structures, such as oxazolines or pyrrolines (Feldman et al., 1990; Schann et al., 2012). For the last reason, they were named imidazoline receptors. Later, binding experiments confirmed the presence of binding sites insensitive to catecholamines but sensitive to imidazoline drugs in several tissues, including brain (Ernsberger et al., 1987; Bricca et al., 1988, 1989). Based on the affinities of a variety of ligands, two different subtypes of imidazoline binding sites were described, and functional experiments led to a third subtype, in particular, in the pancreatic gland. Pharmacological investigations using selective agonists and antagonists have shown that only I\(_1\)Rs are involved in the hypotensive effects of imidazoline-like compounds (Bruban et al., 2001).

In an attempt to identify new pharmacological compounds highly selective for I\(_1\)Rs, we made use of a chemical library of pyrroline analogs of sympathoinhibitory drugs that do not bind to \( \alpha_2 \)-adrenergic receptors (Schann et al., 2012). From these compounds, we selected LNP599 for its appropriate binding properties and because it is lipophilic enough to cross the blood-brain barrier (Bousquet et al., 2011).

Therefore, our aims were 1) to test whether a drug selective for I\(_1\)Rs, with sympathoinhibitory and adiponectin secretion–stimulating effects was capable of beneficial effects on both cardiovascular and metabolic parameters in a model of MetS and 2) to investigate the mechanisms of its actions.

For this purpose, we used spontaneous hypertension and heart failure (SHHF) rats (McCune et al., 1990; Heyen et al., 2002). This model appears to be relevant to the human disease state since it gathers all the cardiovascular and metabolic disorders constituting the human MetS. In addition, it is interesting to note that most drugs used to treat patients with MetS have been shown to be active in such animals (McCune et al., 1990).

This study reports that the imidazoline-like derivative selective for I\(_1\)Rs, LNP599 (3-chloro-2-methyl-phenyl)-(4-methyl-4,5-dihydro-3H-pyrrol-2-yl)-amine hydrochloride) caused sympathetic inhibition and stimulated adiponectin secretion. Long-term treatment of SHHF rats with LNP599 had favorable effects on all the symptoms of MetS (i.e., blood pressure (BP), body weight, insulin resistance, glucose tolerance, lipid profile, increased plasma adiponectin).

Materials and Methods

LNP599

LNP599 has been described elsewhere (Fig. 1) (Bousquet et al., 2011).

In Vitro Experiments

Cell Culture. Murine 3T3-L1 preadipocytes (Green and Kehinde, 1975) were grown until confluence at 37°C in Dulbecco’s modified Eagle medium (DMEM) containing 4.5 g/l glucose, 10% fetal calf serum, and antibiotics. At confluence, 3T3-L1 adipocyte differentiation was initiated by the addition for 48 hours of a cocktail containing 100 mM methylisobutyloxanthine, 100 nM dexamethasone, and 175 nM insulin. Cells were then refed every 2 to 3 days by DMEM containing 10% fetal calf serum and 175 mM insulin. At day 10 after confluence, more than 95% of the cells had the phenotype of mature adipocytes.

PC-12 cells obtained from Dr. G. Rebel (Institut de Recherche sur l’Appareil Digestif, Strasbourg, France) were cultured in 75-cm\(^2\) flasks in DMEM (1 g/l glucose) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin. When the cells reached confluence (3 to 4 days after plating), they were harvested by a 2-minute exposure to 0.25% trypsin at 37°C. For binding assays, after removing the medium, at confluence were frozen in the flasks at –20°C until used to prepare membranes.

Cell Extracts. For binding experiments, 3T3-L1 adipocytes were washed twice with ice-cold phosphate-buffered saline (PBS), harvested, and homogenized in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA. Homogenates were centrifuged at 20,000 \( g \) for 15 minutes at 4°C, and the supernatant was kept at –80°C until use. Pellets were resuspended in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and stored at –80°C. Aliquots of the homogenates and supernatants were used to determine the protein content (BC Assay Uptima kit; Interchim, Montluçon, France) using bovine serum albumin as a standard.

Frozen PC-12 cells were scraped into ice-cold Tris-HEPES buffer (5 mM Tris-HEPES, pH 7.7, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5 mM MgCl\(_2\)) and homogenized and lysed with a Potter homogenizer (Potter, Wheaton, Millville, NJ). After centrifugation at 75,000 \( g \) for 20 minutes, the pellet was washed twice in ice-cold Tris-HEPES buffer and centrifuged. Pellets were resuspended in the same buffer at a concentration of 1 to 2 mg/ml protein. Membrane preparations were stored at –80°C until use.

Binding Experiments. Saturation binding assays were performed using six \(^{125}\)Ipara-iodoclonidine (PIC) concentrations ranging from 0.5 to 11 nM in the presence of 10 \( \mu \)M rauwolscine to prevent any \( \alpha_2 \)-adrenergic receptor binding. \(^{125}\)I PIC (2200 Ci/mmol) was
Quantitative Real-Time Polymerase Chain Reaction. Total RNA was extracted by the RNeasy mini-kit, according to the manufacturer's recommendations (Qiagen SAS, Courtaboeuf, France). One microgram of total RNA was digested with DNaseI (Invitrogen, Carlsbad, CA) and then reverse-transcribed with Superscript reverse transcriptase (Invitrogen) as recommended. After heat denaturation and 10-fold dilution, 1 in 20 of the reverse transcription reactions was used for the real-time PCR performed on an ABI 7000 (Applied Biosystems, Foster City, CA). The PCR reaction was carried out in a final volume of 25 μl using the qPCR Mastermix Plus for Sybr'TM Green I (Eurogentec, Angers, France) in the presence of a 300 nM concentration of each sense and adiponectin-specific antisense oligonucleotide, 200 μM dNTP, 2.5 mM MgCl₂, and 1.25 unit of Hot Gold Star DNA polymerase in its recommended buffer. The sequences of the sense and antisense oligonucleotides were 5'-AGGCCGCTAGTGGCAGATG-GAGAT-3' and 5'-CTTCCTCCAGGT-TCTCCTTTTCCGTC-3' for adiponectin and 5'-AAGCCGCTGTCGATGGTTGCT-3' and 5'-CCGCAGGACGAGCTGGT-3' for 36B4, respectively. To ensure that subsequent amplification did not derive from contaminant genomic DNA and/or from primer dimer formation, a control without reverse transcriptase was included in parallel for each RNA sample. Moreover, reverse transcription-PCR products were analyzed in a postamplification fusion curve to ensure that a single amplicon was obtained. Standard curves were generated using serial dilutions of a reference cDNA, spanning five orders of magnitude and yielding correlation coefficients >0.98 and efficiencies of at least 0.95. Standard and sample values were tested in duplicate in each experiment. Relative expression for a given sample was normalized to that of the 36B4 gene.

In Vivo Experiments

Animals. Twelve-week-old male Sprague-Dawley (S-D) rats (n = 20) and 12-week-old male SHHF rats (n = 20) were used in this study (Charles River Laboratories, L'Arbresle, France). Animals were housed in a temperature- and light-controlled room with free access to tap water and were fed ad libitum with standard diet (A04; SAFE, Augy, France).

At the end of in vivo experiments, rats were sacrificed with a bolus injection of a lethal dose of pentobarbital, and macroscopic examination was performed.

Ethics Statement. All animal care and experimental procedures complied with the rules of the European Communities Council Directive of November 24, 1986 (86/609/EEC) and the French Department of Agriculture (License no. 67-337 to P. Bousquet). The results of experiments involving animals (McGrath et al., 2010).

Renal Sympathetic Nervous Activity Recording. The 12-week-old male S-D rats were anesthetized with urethane (1.5 g/kg i.p., supplemented with 0.1 g/kg i.v. as needed) and placed on a heating blanket to maintain the rectal temperature at 37°C. Femoral arterial and venous catheters were inserted into the lower abdominal aorta and the inferior vena cava for arterial pressure (AP) measurement and drug administration, respectively. The left renal nerve was exposed via a flank incision. After careful isolation, a major branch of the nerve was placed on a bipolar platinum-iridium electrode and insulated with a silicone gel (604A and B; Wacker Chemie, Munich, Germany). Throughout the experiment, the rat was ventilated through a tracheal cannula (7 to 8 ml/kg x 72 cycles/min) with a mixture of oxygen and room air (~98/20%). AP was measured by connecting the arterial line to a pressure transducer (TNP-R; Ohmeda, Biltlaven, The Netherlands) coupled to an amplifier (model 13-4615-52; Gould, Cleveland, OH). The renal sympathetic nervous activity (RSNA) was amplified (~50,000), band-pass filtered (300–3000 Hz: Model P-511J; Grass, Quincy, MA), and rectified by an analog homemade rectifier including a low-pass filter with a cutoff frequency of 150 Hz. Using a computer equipped with an analog-to-digital converter (model AT-MIO-16; National Instruments, Austin, TX), and LabVIEW 5.1.
membranes, clonidine displaced the I1R specific binding of LNP599 (10 mg/kg) given as an intravenous bolus injection. At the end of the recording session, the ganglionic blocker chlorisondamine was administered (2.5 mg/kg i.v.) to assess the background noise level, which was then subtracted from all RSNA data for subsequent analyses. On completion of the experiment, the rats were euthanized with an intravenous overdose of pentobarbital sodium.

Long-Term Drug Treatment of SHHF Rats. LNP599 was administered in the drinking water for 12 weeks at a dose of 20 mg/kg per day (n = 17). Water intake was allowed ad libitum and monitored continuously so that drug concentration could be adjusted. Control untreated SHHF rats drank normal water (n = 10). Body weight, water, and food intake were monitored daily. After 12 weeks of LNP599 treatment, blood was sampled, blood pressure and heart rate (HR) were monitored, and a glucose tolerance test was performed.

Blood Pressure and Heart Rate Monitoring. Rats were anesthetized with 50 mg/kg i.p. pentobarbitone (Céva Santé Animale, Libourne, France) and tracheotomized. The femoral vein and artery were catheterized to administer anesthetics and curare and to measure AP, respectively. The rats were then ventilated with room air and paralyzed with 1.5 mg/kg i.v. pancuronium bromide (Organon SA, Puteaux, France). After stabilization, blood pressure was monitored using a pressure transducer (Gould P23XL) and recorder (BS272; Gould Electronics, Longjumeau, France). Mean arterial BP was calculated as diastolic pressure plus one-third of the differential BP. The HR was also monitored from the pressure signal with a Gould Biotech amplifier (13-4615-66; Gold Electronics, Longjumeau, France).

Plasma Biochemical Measurements. Blood samples were obtained from the tail veins of anesthetized rats (isoflurane 2.5%; Abbott, Rungis, France) after an 18-hour fast. Blood samples were centrifuged for 15 minutes at 2000 × g and the plasma was frozen at −80°C until assay for glucose, total cholesterol, triglycerides, and free fatty acids (Advia 2400; Bayer HealthCare). Insulin, adiponectin, leptin, and glucagon were measured with enzyme-linked immunosorbent assay kits (insulin; Mercodia, Uppsala, Sweden; adiponectin: B-Bridge International, Mountain View, CA; glucagon; Gentaur, Kampenhout, Belgium) according to the manufacturer’s recommendations. Insulin resistance was evaluated by calculating the homeostasis model assessment (HOMA-IR): fasted insulinemia (µU/ml) × fasted glycemia (mM)2/5.

Glucose Tolerance Test. After an overnight fast, a 0.5 g/kg glucose solution was injected intravenously. Plasma glucose concentration was evaluated using a glucometer at baseline and 3, 6, 10, 15, 30, and 45 minutes after the glucose load (AccuCheck Go; Roche Diagnostics, Meylan, France). Areas under the curve (AUC) were determined to compare groups.

Statistical Analysis

Data are presented as mean ± S.E.M. Comparisons between groups were made using Student’s t test or two-way analysis of variance followed by a Bonferroni post hoc test (GraphPad Software Inc., San Diego, CA). P value <0.05 was used as the criterion of significance.

Data from saturation and competition binding experiments were analyzed using the least-square fitting program Prism (GraphPad Software Inc.). K<sub>i</sub> values were calculated according to the Cheng–Prusoff equation (Cheng and Prusoff, 1973). The significance of the improvement of fit obtained by the two-site equation over the one-site equation was analyzed by F-statistics (partial F-test).

Results

Pharmacological Profile of LNP599: High Affinity and Selectivity for I<sub>1</sub>R Binding Sites. In PC12 cell membranes, clonidine displaced the I<sub>1</sub>R specific binding of [125I]PIC with two affinity sites [high-affinity sites: K<sub>i</sub> = 6.0 ± 0.9 nM (37%) and low-affinity sites: K<sub>i</sub> = 1309 ± 99 nM (n = 3)].

LNP599 also exhibited two affinity sites for displacement of the [125I]PIC-specific binding [high-affinity sites: K<sub>i</sub> = 15 ± 1 nM (37%) and low-affinity sites: K<sub>i</sub> = 1407 ± 89 nM (n = 3)] as shown in Fig. 2.

Binding properties of LNP599 on a large set of receptors or transporters and enzymes (55 receptors and transporters and 20 enzymes) were investigated in specific competition experiments. Based on the aforementioned K<sub>i</sub> value of LNP599 for its high-affinity sites, the 10<sup>−7</sup> M concentration of each competitor was tested. No significant displacement of the reference ligand for each target was achieved. This holds true in particular for the different subtypes of β-adrenergic receptors (see Supplemental Table). The SPR technique confirmed the presence of specific binding of LNP599 to immobilized PC12 cell membranes (9.8 ± 0.5 RU).

Because of the high selectivity of LNP599 for I<sub>1</sub>Rs and its lipophilic properties, it has been further used for all the cardiovascular and metabolic investigations.

Expression of I<sub>1</sub>Rs in 3T3-L1 Adipocytes. Cell extracts of murine 3T3-L1 adipocytes were used as a well characterized model of in vitro differentiated fat cells. [125I]PIC binding at 25°C in 3T3 cell membranes was saturable and of high affinity. Nonlinear regression analysis of saturation binding isotherms indicated that [125I]PIC binding to an homogeneous population of sites with a K<sub>d</sub> value of 2.3 ± 0.7 nM and a B<sub>max</sub> = 803 ± 105 fmol/mg protein (n = 3) (Fig. 3A). The Hill coefficient (n<sub>H</sub>) of the saturation binding data was calculated using the GraphPad software. n<sub>H</sub> = 1.1 ± 0.2 was close to 1, indicating that there was probably no cooperativity.

In competition experiments, clonidine, an I<sub>1</sub>R reference ligand, displaced [125I]PIC specific binding in 3T3-L1 cell membrane preparations with two affinity components (K<sub>i</sub> = 10.2 ± 6 nM (49% of total sites) and 3010 ± 678 nM, n = 4) demonstrating the presence of I<sub>1</sub>Rs in 3T3-L1 membrane extracts. LNP599, a competitor, also exhibited a two affinity sites displacement: K<sub>i</sub> = 8.5 ± 5.9 nM (32% of total sites) and
1540 ± 761 (n = 4) at the I1R specific binding of [125I]PIC (Fig. 3B).

The I1Rs in 3T3-L1 cell membrane were further characterized by SPR determinations. Sensorgrams for LNP599 or isoproterenol interactions with immobilized 3T3-L1 membrane proteins are shown in Fig. 3C. Isoproterenol that binds to β-adrenoceptors was used as a positive control. Both LNP 599 and isoproterenol bound to 3T3-L1 membrane proteins. Kinetic titration data are depicted in Fig. 3D. Similar dissociation rate constants (k_{off}) were obtained (4.54 ± 0.12 × 10^{-3} and 7.46 ± 0.16 × 10^{-3} s^{-1} for LNP599 and isoproterenol, respectively), whereas the association rate constant (k_{on}) of LNP599 was significantly higher (73.3 ± 1.6 × 10^{8} M^{-1} s^{-1}) than that of isoproterenol (8.43 ± 0.21 × 10^{8} M^{-1} s^{-1}). The dissociation equilibrium constants were therefore estimated to be 61.9 and 884.9 nM, respectively.

**Functional Implications of I1Rs in Adiponectin Secretion of 3T3-L1 Cells.** We then examined whether LNP599 could directly trigger adipocytes to modulate adiponectin secretion. A 6-hour exposure to LNP599 significantly increased adiponectin secretion by 3T3-L1 adipocytes (378 ± 38 compared with 213 ± 19 ng/ml per milligram protein, P < 0.001).

In preliminary experiments, we performed dose-response curves with various LNP599 concentrations, which demonstrate that the maximal effect of the compound plateaued from 3 μM. Thereafter, because of the high affinity of LNP599 for the I1R site documented in the present study, we attempted to prevent LNP599 on adiponectin secretion with a 30-fold molar excess of efaroxan. Preincubation with the I1R antagonist efaroxan prevented the LNP599-induced increase in adiponectin secretion (237 ± 19 vs. 378 ± 38 ng/ml per milligram protein, P < 0.001), whereas efaroxan alone, at the same dose, had no significant effect compared with controls (175 ± 16 vs. 213 ± 19 ng/ml per milligram of protein, P > 0.05) (Fig. 4A). Interestingly, the LNP599-induced adiponectin secretion was associated by a 1.6- to 2.5-fold increase in adiponectin mRNA levels, which was already significant after a 3-hour exposure to the drug (Fig. 4B).

**Acute Effects of LNP599 on Hemodynamic Parameters and Sympathetic Activity.** In a series of pilot experiments, three different doses of LNP599 (1, 5, and 10 mg/kg) were injected intravenously into anesthetized Sprague-Dawley rats to look at the dose effect relationship. Mean BP decreased by 14.8 ± 3.7 (n = 3), 33.4 ± 3.4 (n = 4), and 38.1 ± 3.5% (n = 3), respectively (Supplemental Figure). Hypotensive effects were accompanied by bradycardia (10.1 ± 3.1, 22.1 ± 2.5, and 28.3 ± 3.6%, respectively).

LNP599, at a dose of 10 mg/kg i.v. to anesthetized Sprague-Dawley rats, provoked a 50% decrease in RSNA (Fig. 5A). In parallel, LNP599 provoked a marked decrease in systolic, mean, and diastolic AP (systolic BP: 105 ± 3 vs. 141 ± 2 mm Hg, P < 0.001; mean arterial BP: 77 ± 4 vs. 108 ± 3 mm Hg, P < 0.0001; diastolic BP: 59 ± 3 vs. 83 ± 4 mm Hg, P < 0.001) and HR (288 ± 10 vs. 331 ± 14 beats per minute, P < 0.01) (Fig. 5, B and C). The decreased sympathetic activity, arterial pressure, and HR persisted for more than 1 hour.
Comparative Metabolic and Cardiovascular Phenotypes of Sprague-Dawley and SHHF Rats. As shown in Table 1, with the exception of HR and fasting glucose, all of the cardiovascular and lipid and carbohydrate metabolic parameters dealing with metabolic syndrome were significantly altered in SHHF rats. Compared with S-D rats, SHHF rats exhibited hypertension, insulin resistance as indicated by the markedly increased HOMA-IR value and glucose intolerance as shown by the greater area under the curve of the intravenous glucose tolerance test [AUC intravenous glucose tolerance test (IVGTT)]; body weight was only moderately but significantly increased.

Effects of Long-Term Administration of LNP599 on Hemodynamic and Metabolic Parameters in SHHF Rats. After 12 weeks of LNP599 at a dose of 20 mg/kg daily in the drinking water, mean blood pressure of SHHF rats was significantly decreased, but their HR was not modified (Table 2).

The average body weights of control and treated animals did not differ significantly in either group at t = 0 (12 weeks of age): 398 ± 8 vs. 418 ± 9 g (P > 0.05). Body weight continued to increase slightly in a similar way in both groups until week 8 of treatment (age 20 weeks) (Fig. 6A). During the initial period of 8 weeks, the mean weight of the rats increased to 536 ± 10 g for control animals and 510 ± 9 g for treated animals (P > 0.05). This increase is obviously linked with growth. From week 9 to week 12 of treatment (age 24 weeks), the weight of the control animals continued to increase, whereas that of the treated animals stabilized (587 ± 8 and 492 ± 10 g at the age of 24 weeks, respectively P < 0.01).

Food and water intakes related to body weight before and at the end of the treatment compared with untreated control animals (Fig. 6, B and C). Food intake and water intake
related to body weight decreased over the 12-week treatment period, and this decrease was very similar in both groups of animals. This observation tends to preclude any unfavorable effect of the drug on taste.

Fasted glycemia was not affected by the treatment (Fig. 7A), but fasted plasma insulin significantly decreased (14.3 ± 3.1 vs. 46.8 ± 3.7 ng/ml, *P* < 0.0001) (Fig. 7B). Calculation of the HOMA-IR index confirmed that SHHF rats treated with LNP599 had improved insulin sensitivity compared with control SHHF rats (128 ± 28 vs. 392 ± 25, *P* < 0.0001) (Fig. 7C). LNP599 provoked a nonsignificant decrease in glucagon (Fig. 7D). Moreover, glucose tolerance testing showed that 7C). LNP599 provoked a nonsignificant decrease in glucagon (see above), it is tempting to speculate that sympathetic inhibition induced by LNP599 may account in plasma adiponectin (see Supplemental Table) and of enzymes, especially the different subtypes of β-adrenoceptors (see Supplemental Table) and of α2-adrenoceptors (Bousquet et al., 2011).

### Discussion

Adiponectin is an adipokine that is specifically and abundantly expressed and secreted by adipose tissue and directly sensitizes the body to insulin (Kadowaki et al., 2006). Hypoadiponectinemia is generally observed during the course of obesity, and it likely plays a major role in the pathophysiology of insulin resistance, type 2 diabetes, and metabolic syndrome. Adiponectin receptors mediate adiponectin effects on energy homeostasis, more specifically by increasing glucose and fatty acid utilization in skeletal muscle and decreasing liver glucose production. Thus, adiponectin and its receptors represent potential therapeutic targets in the field of obesity-associated metabolic disturbances.

The sympathetic nervous system influences the synthesis and secretion of adiponectin by adipocytes. Therefore, there is likely a cross-talk between adiponectin and sympathetic hyperactivity in the pathophysiology of metabolic disorders observed in MetS.

Several works have suggested that sympathetic hyperactivity could be involved in the pathogenesis of the MetS (Morgan et al., 1995; Lee et al., 2001; Barnes et al., 2003; Huggett et al., 2004; Grassi et al., 2005, 2007; Mancia et al., 2007). In addition, adiponectin has been shown to play a pivotal role in pathophysiology of MetS and its complications (Mancia et al., 2007). In this context, we selected an imidazoline derivative with a pyrrole moiety (LNP599) that displays sympathoinhibitory effects and stimulates adiponectin secretion through selective activation of I1R sites. We tested the capability of LNP599 to prevent the worsening of the metabolic and cardiovascular disorders in a rodent model of MetS, SHHF rats.

Based on structure-activity relationship data, we were interested in aromatic aminopyrrolines, which are highly selective for I1Rs (Grenery et al., 2002). We chose LNP599 in this study because of its appropriate binding properties on I1R but also because it can cross the blood-brain barrier and has good intestinal absorption, permitting oral as well as intravenous delivery (Bousquet et al., 2011).

Displacement of [125I]PIC-specific binding by clonidine with high affinity confirmed that PC12 cell membranes represent a reference model for the study of I1R (Edwards and Ernsberger, 2003). The binding results demonstrated that LNP599 is a selective and specific molecule for I1R. Indeed, in PC12 cell membrane preparations, this molecule displaced the specific [125I]PIC binding to I1R with nanomolar affinity and had no significant affinity for a large set of receptors, transporters, and enzymes, especially the different subtypes of β-adrenoceptors (see Supplemental Table) and of α2-adrenoceptors (Bousquet et al., 2011).

As sympathetic hyperactivity is associated with a decrease in plasma adiponectin (see above), it is tempting to speculate that sympathetic inhibition induced by LNP599 may account for the increased plasma adiponectin concentration. Nevertheless, our results on 3T3-L1 adipocytes demonstrate that LNP599 targets fat cells directly to promote adiponectin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>S-D Rats (n = 10)</th>
<th>SHHF Rats (n = 10)</th>
<th><em>P</em> Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBP (mm Hg)</td>
<td>111 ± 6</td>
<td>166 ± 5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>119 ± 6</td>
<td>176 ± 6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>133 ± 6</td>
<td>203 ± 9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>338 ± 14</td>
<td>361 ± 8</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>547 ± 11</td>
<td>587 ± 9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>7.9 ± 0.3</td>
<td>8.04 ± 0.44</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.61 ± 0.29</td>
<td>46.8 ± 3.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>14.0 ± 2.9</td>
<td>534 ± 38</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AUC IVGTT mmol.min⁻¹</td>
<td>584 ± 20</td>
<td>710 ± 37</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>1.97 ± 0.11</td>
<td>3.84 ± 0.18</td>
<td>&lt;0.0001</td>
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<td>Triglycerides (mM)</td>
<td>1.09 ± 0.18</td>
<td>4.59 ± 0.23</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Free fatty acids (mM)</td>
<td>0.48 ± 0.06</td>
<td>0.94 ± 0.03</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

DBP, diastolic blood pressure; MBP, mean arterial blood pressure; NS, not significant; SBP, systolic blood pressure.

### Table 2

<table>
<thead>
<tr>
<th>Effect</th>
<th>Control (n = 10)</th>
<th>LNP599 (n = 10)</th>
<th><em>P</em> Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBP (mm Hg)</td>
<td>166 ± 5</td>
<td>146 ± 9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>176 ± 6</td>
<td>156 ± 9</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>203 ± 9</td>
<td>172 ± 10</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>361 ± 8</td>
<td>359 ± 6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>3.8 ± 0.2</td>
<td>2.6 ± 0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>4.6 ± 0.2</td>
<td>4.0 ± 0.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Free fatty acids (mM)</td>
<td>0.94 ± 0.03</td>
<td>0.96 ± 0.06</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Plasma leptin (μg/l)</td>
<td>14.4 ± 1.29</td>
<td>21.9 ± 2.20</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma adiponectin (mg/l)</td>
<td>5.54 ± 0.14</td>
<td>11.2 ± 0.81</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*bpm*, beats per minute; *DBP*, diastolic blood pressure; *SBP*, systolic blood pressure.
synthesis and secretion by an I\(_1\)R-dependent mechanism. In fact, acute exposure of 3T3-L1 adipocytes to LNP599 provoked a marked increase in adiponectin secretion. This effect was blocked by the I\(_1\)R-antagonist efaroxan (Edwards and Ernsberger, 2003; Wang et al., 2007). The presence of I\(_1\)Rs on 3T3-L1 membrane was demonstrated in saturation binding experiments using \([^{125}\text{I}]\)PIC as the reference radioligand for I\(_1\)R. Rauwolscine was used to mask \(\alpha_2\)-adrenergic receptors (Ernsberger et al., 1995). The \([^{125}\text{I}]\)PIC-specific binding was displaced with high affinity by clonidine, the I\(_1\)R reference ligand, in the presence of rauwolscine. In this I\(_1\)R assay, LNP599 exhibited high affinity for I\(_1\)R. Displacement of \([^{125}\text{I}]\)PIC by LNP599 fitted in a two-compartment model, as in numerous other I\(_1\)R binding studies (Greney et al., 2000; Urosevic et al., 2004). As shown previously, the biphasic competition curves on \([^{125}\text{I}]\)PIC binding sites obtained with clonidine, the I\(_1\)R reference ligand, and in the presence of rauwolscine. In this I\(_1\)R assay, LNP599 exhibited high affinity for I\(_1\)R. Displacement of \([^{125}\text{I}]\)PIC by LNP599 fitted in a two-compartment model, as in numerous other I\(_1\)R binding studies (Greney et al., 2000; Urosevic et al., 2004). As shown previously, the biphasic competition curves on \([^{125}\text{I}]\)PIC binding sites obtained with clonidine or LNP599 could refer to either the coupled/uncoupled form of G protein--coupled receptors or to a complex allosteric modulation of the receptors (Greney et al., 2002).

LNP599 is able to bind to 3T3-L1 membrane preparations with a nanomolar-range affinity, both in competition binding experiments and in BIACore kinetic titration studies. Immobilization of membranes in SPR experiments likely masked or altered some I\(_1\)R sites, thus accounting for a 10-fold lower affinity compared with those obtained in competition experiments.

As for the effect on sympathetic nervous activity, short-term administration of LNP599 (10 mg/kg i.v.) in anesthetized S-D rats markedly decreased sympathetic activity, blood pressure, and heart rate. The dose of 10 mg/kg used in these experiments was selected as the one that markedly reduced blood pressure in pilot experiments. The sympathetic inhibitory effects of all the reference drugs acting centrally, such as clonidine, moxonidine, and rilmenidine, were also demonstrated in S-D rats under very similar technical conditions (Zhang and Johns, 1996; Vayssettes-Courchay et al., 2002; Peng et al., 2011). It would be preferable to determine whether all of these compounds, including LNP599, also reduce sympathetic activity in rodent models of MetS. However, these short-term experiments were carried out in normal animals rather than in SHHF rats. SHHF rats were described for the first time in 1990 (McCune et al., 1990). They exhibit hyperlipidemia with high plasma cholesterol and triglycerides; male SHHF rats exhibit glucose intolerance, whereas female SHHF rats do not. SHHF animals exhibit hypertension (McCune et al., 1990; Chen et al., 2011).

Moreover, according to Rahmouni et al. (2005), obesity is associated with leptin resistance but only for its metabolic effects, whereas sympathetic and blood pressure effects of leptin remain unaffected by obesity. Taking these observations into consideration, measurement of sympathetic activity in SHHF rats would not be relevant since the leptin pathway in SHHF rats would not be relevant since the leptin pathway is impaired. A further project on animals receiving a high-fat diet is already in progress in our laboratory to confirm the benefit of LNP 599 in another model of metabolic syndrome and to look at the sympathetic effects of LNP599 during long-term treatment in animals with intact leptin pathway.

In this work, we chose SHHF rats as a model of MetS. They display most of the human characteristics, including significant hypertension, suggesting increased sympathetic activity. They were generated from crossing a spontaneously hypertensive female rat and a Sprague-Dawley male rat, which leads to a spontaneous mutation, designated as fa\(^k\) for fatty (Koletsky, 1975a,b). Therefore, in this study, we used
S-D male rats as control animals to highlight the metabolic and cardiovascular abnormalities exhibited by SHHF rats.

In the first part, SHHF and S-D rats were compared at the same age (24 weeks) to confirm that, in our hands, SHHF rats exhibit cardiovascular and metabolic abnormalities when S-D rats are taken as references (Table 1). After this confirmation was obtained (i.e., in the second part of the study), our objective was to characterize the beneficial effects of LNP599 treatment in a rat model of metabolic syndrome; therefore, we did not treat S-D rats with LNP599. That is the reason why the in vivo study was designed with only two groups: SHHF rats receiving vehicle and SHHF rats receiving LNP599.

Compared with S-D animals, SHHF rats exhibited symptoms of MetS (high blood pressure, dyslipidemia, insulin resistance, and glucose intolerance). Based on the unique pharmacological properties of the selected compound and on the relevance of the animal model of MetS, a long-term treatment experiment was carried out.

For long-term treatments, the 20 mg/kg dose of LNP599 was given orally. Because the bioavailability of LNP599 was assessed at 50–60% in rats, we used twice the dose that exhibited clear-cut blood pressure effects after intravenous administration.

Long-term exposure of SHHF rats to 20 mg/kg LNP599 prevented, to some extent, the age-related weight gain in treated rats compared with controls. However, the body weight of treated animals never decreased under baseline value, suggesting that this compound is at least able to prevent the emergence of an excess in adiposity. Whether it can also reverse the phenotype of an established obesity remains an open issue.

Our data clearly show that the stabilization of body weight from 8 weeks of treatment and beyond was not related to a decrease in food or water intake. A decrease in weight gain has also been described in other MetS models treated with moxonidine (Henriksen et al., 1997; Ernsberger et al., 1999).

As expected, short- and long-term administration of LNP599 significantly decreased the mean blood pressure as a result of its sympathoinhibitory action. Long-term oral treatment did not cause significant bradycardia, and short-term intravenous treatment reduced heart rate by only 13%.

Regarding glucose metabolism, it is noteworthy that SHHF rats had normal fasting glucose values compared with control S-D rats; thus, it is not surprising that LNP599 did not significantly change fasting glucose. However, IVGTT tests and HOMA-IR values show that SHHF rats exhibit severe glucose intolerance and insulin resistance, respectively. Long-term LNP599 treatment significantly improves glucose tolerance, decreases plasma insulin levels, and indicates that the drug improves insulin sensitivity, as assessed by the marked decrease in HOMA-IR. Since sympathetic hyperactivity is known to decrease insulin release, one could assume that the sympathoinhibitory effect of LNP599 could promote insulin secretion and could be involved in the more favorable metabolic profile. However, our results show that LNP599 markedly reduced insulin plasma levels and HOMA-IR values in rats with hyperinsulinemia and glucose intolerance; this result strongly suggests that an improvement in insulin sensitivity is implicated in the drug effect. Whether LNP599 could also modulate insulin synthesis and secretion, in particular during glucose challenge, remains to be investigated. It is noteworthy that LNP599 tended to decrease plasma glucagon levels, a phenomenon that could also contribute to improve glucose tolerance. The increased plasma adiponectin concentration

![Fig. 7. Effects of 12 weeks of LNP599 on glucose metabolism of SHHF rats. Plasma glucose (A), plasma insulin (B), HOMA-IR (C), plasma glucagon (D), glucose tolerance test (IVGTT) (E), and IVGTT area under the curve (F). Black bars or squares: control (n = 10); open bars or circles: LNP599 (n = 10). **P < 0.01; ***P < 0.001; ****P < 0.0001, LNP599-treated vs. control rats.](image-url)
may also represent a major determinant of increased insulin sensitivity of LNP959-treated rats. In rats, the effects of LNP959 on total plasma adiponectin concentrations appear to be comparable to that of thiazolidinediones, which act directly on fat cells through peroxisome proliferation-activated receptor-γ receptors to promote the synthesis and secretion of adiponectin (Kato et al., 2008; Pitsa et al., 2011). Comparable effects of thiazolidinediones are also found in humans, with at least a 2-fold increase in plasma adiponectin concentration (Yu et al., 2002; Phillips et al., 2003; Miyazaki et al., 2004).

Collectively, our findings strongly suggest that both the reduction in sympathetic activity and a direct effect on adiponectin production could be involved in the increased plasma adiponectin levels, which in turns improves insulin sensitivity.

In SHHF rats, long-term administration of LNP959 decreased plasma total cholesterol by about 30%. It is interesting to note that the cholesterol-lowering effect of LNP959 in rats with MetS was in the same range as seen with statins (Miller et al., 2004; Oltman et al., 2009).

Evolution of food intake and water intake related to body weight was similar in treated and in control animals. In treated animals, body weight itself did not decrease over time but plateaued after 8 weeks of treatment; vital parameters were preserved just before sacrifice, and postmortem macroscopic examination did not reveal any abnormality. In vivo as well as in vitro toxicity investigations, including absorption, distribution, metabolism, and excretion tests, remained negative so far (data not shown). Based on all these observations, one can assume that long-term treatment with LNP959 is quite well tolerated, at least in SHHF rats.

In conclusion, in a rodent model of MetS, our results 1) show that an I1R-selective ligand reduces weight gain, mean blood pressure, and total cholesterol and moreover improves glucose tolerance and insulin resistance; 2) suggest that the beneficial effects of the I1-selective drug in metabolic syndrome might be due to sympathetic inhibition, as well as to adiponectin secretion stimulation; 3) indicate that the adiponectin secretion–stimulating effect of the tested drug is mediated, at least in part, by a direct action of the drug on specific receptors located on fat cells. Thus, a well-tolerated I1R-selective drug, given as a single drug, might be a relevant strategy for the management of various components of the MetS.

Authorship Contributions

Participated in research design: Fellmann, Regnaut, Greney, Niederhofer, Julien, Lacolley, Fève, Bouquet.
Conducted experiments: Fellmann, Regnaut, Greney, Muscat, Max, Orea, Chetrite, Niederhofer, Julien.
Contributed new reagents or analytic tools: Gasparik, Gigou.
Performed data analysis: Fellmann, Regnaut, Greney, Pizard, Niederhofer, Julien, Lacolley, Fève, Bouquet.
Wrote or contributed to the writing of the manuscript: Fellmann, Regnaut, Greney, Pizard, Niederhofer, Julien, Lacolley, Fève, Bouquet.
Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.
Correction to “A New Pyrroline Compound Selective for I1-Imidazoline Receptors Improves Metabolic Syndrome in Rats”

In the article above [Fellmann L, Regnault V, Greney H, Gasparik V, Muscat A, Max J-P, Gigou L, Oréa V, Chetrite G, Pizard A, Niederhoffer N, Julien C, Lacolley P, Fève B, and Bousquet P (2013) *J Pharmacol Exp Ther* **346**:370–380], the reference linked to Bousquet et al., 2011 was inadvertently omitted. The missing reference is listed as follows:


The online versions have been corrected.

The printer regrets this error and any inconvenience it may have caused.