A new pyrroline compound selective for I₁-imidazoline receptors improves metabolic syndrome in rats

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Running title page

Sympathetic system, adiponectin and syndrome X

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Number of pages: 38

Number of tables: 2

Number of figures: 7

Number of references: 58

Number of words in Abstract: 222

Number of words in Introduction: 833

Number of words in Discussion: 1946
Abbreviations:

I₁Rs : I₁ imidazoline receptors

SHHF Spontaneous Hypertension and Heart Failure

RSNA : Renal Sympathetic Nerve Activity

PIC : Para-IodoClonidine

SPR Surface Plasmon Resonance

SD : Sprague Dawley

NHS : N-hydroxysuccinimide

EDC : N-ethyl-N0-(3-diethyl-aminopropyl)carbodiimide

HBS : HEPES Buffer Saline
Abstract

Symptoms of the metabolic syndrome (MetS), such as insulin resistance, obesity and hypertension have been associated with sympathetic hyperactivity. In addition, 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 non-adrenergic I₁-imidazoline receptors (I₁Rs) may represent a new concept in MetS pharmaco-therapy. LNP599, 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. Acute administration of LNP599 (10 mg/kg i.v.) in anesthetized SD rats, markedly decreased sympathetic activity, causing hypotension and bradycardia. Chronic treatment of spontaneously hypertensive heart failure rats with LNP599 (20 mg/kg p.o.) had favorable effects on blood pressure, bodyweight, insulin resistance, glucose tolerance, lipid profile and 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), who defined it 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, 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; Rumantir et al., 1999; Grassi et al., 2007; Parati and Esler, 2012). In the literature, there are experimental arguments which support the fact that sympathetic overactivity may have some beneficial effects, such as stimulation of lipolysis and of thermogenesis and a consequent reduction of body weight. Nevertheless, there is a general agreement among clinicians 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. Severity and duration of sympathetic overactivity could also tip the scale on one way or the other. Feinstein and Julius summarized this point, indicating that, while short term sympathetic activation is useful for survival, it is deleterious (hypertension, overweight) when it becomes permanent (Feldstein and Julius, 2009).

So far the role of sympathetic hyperactivity in the pathogenesis of MetS has never been demonstrated (Straznicky et al., 2012). Nevertheless, Grassi et al. have proposed that sympathetic hyperactivity could play a causal role in the development of MetS (Grassi et al., 2005).
Furthermore, 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). Adiponectin pathway has been recently proposed as an interesting target for the treatment of MetS (Mancia et al., 2007; Gu and Li, 2012).

Many studies have investigated centrally acting antihypertensive agents, particularly sympatho-inhibitory compounds acting on both $\alpha_2$-adrenergic and non-adrenergic 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; Velliquette and Ernsberger, 2003b; Velliquette et al., 2006). However, so far, the lack of selectivity of the ligands did not permit determination of the respective contributions of $\alpha_2$-adrenergic- and I$_1$R-mediated effects.

Imidazoline receptors were first described as pharmacological targets which 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., 2012b). For the latter reason, they were named imidazoline receptors. Later on, binding experiments have 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; Bricca et al., 1989). Based on affinities of a variety of ligands, 2 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 imidazoline receptors (I_1Rs) 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_1Rs, we made use of a chemical library of pyrroline analogues of sympatho-inhibitory drugs that do not bind to \( \alpha_2 \)-adrenergic receptors (Schann et al., 2012a). From among 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 i) to test whether a drug selective for I_1Rs, with sympatho-inhibitory and adiponectin secretion stimulating effects was capable of beneficial effects on both cardiovascular and metabolic parameters in a model of MetS ii) to investigate mechanisms of its actions.

In this purpose, we used 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_1Rs, LNP 599 caused sympathetic inhibition and stimulated adiponectin secretion.

Chronic treatment of spontaneously hypertensive, heart failure rats with LNP599 had favorable effects on all the symptoms of MetS, i.e. blood pressure, bodyweight, insulin resistance, glucose tolerance, lipid profile and increased plasma adiponectin.
Methods
1. LNP599

(3-Chloro-2-methyl-phenyl)-(4-methyl-4,5-dihydro-3H-pyrrol-2-yl)-amine hydrochloride has been described elsewhere (Fig 1) (Bousquet et al., 2011).

2. In vitro experiments

Cell culture

Murine 3T3-L1 pre-adipocytes (Green and Kehinde, 1975) were grown until confluence at 37°C in Dulbecco’s modified Eagle medium (DMEM) containing 4.5 g/l D-glucose, 10% fetal calf serum (FCS), and antibiotics. At confluence, 3T3-L1 adipocyte differentiation was initiated by the addition for 48 h of a cocktail containing 100 µM methyl-isobutylxanthine, 100 nM dexamethasone, and 175 nM insulin. Cells were then re-fed every 2-3 days by DMEM containing 10% FCS and 175 nM insulin. At day 10 following confluence, more than 95% of the cells had the phenotype of mature adipocytes.

PC-12 cells obtained from Dr. G. Rebel (IRCAD, Strasbourg, France) were cultured in 75-cm² flasks in DMEM (1 g/l glucose) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. When the cells reached confluence (3 to 4 days after plating), they were harvested by a 2-min exposure to 0.25% trypsin at 37°C. For binding assays, after removing the medium, cells 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 cold PBS, harvested, and homogenized in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA. Homogenates were centrifuged at 20 000g for 15 min 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 protein content (BC Assay Uptima kit, Interchim, Montluçon, France), using bovine serum albumin as a standard.

Frozen PC-12 cells were scraped into cold Tris-HEPES buffer (5 mM Tris-HEPES, pH 7.7, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5 mM MgCl₂) and homogenized and lyzed with a Potter homogenizer. After centrifugation at 75 000g for 20 min, the pellet was washed twice in cold Tris-HEPES buffer and centrifuged. Pellets were resuspended in the same buffer at a concentration of 1 to 2 mg of protein/ml. Membrane preparations were stored at -80°C until use.

**Binding experiments**

Saturation binding assays were performed using six [¹²⁵I]-PIC concentrations ranging from 0.5 to 11 nM in the presence of 10 µM rauwolscine to prevent any α₂-adrenergic receptor binding. [¹²⁵I]-PIC (2200Ci/mmol) was purchased from Perkin Elmer (USA).

Incubation was initiated by the addition of 3T3-L1 cell membranes (20-30 µg of protein) in a final volume of 250 µl of Tris–HEPES buffer (5 mM Tris–HEPES, pH 7.7, 0.5 mM EDTA, 0.5 mM EGTA and 0.5 mM MgCl₂) and was carried out at 25°C for 45 min. The reaction was stopped by rapid vacuum filtration through GF/B glass fiber filters treated with 0.3% polyethyleneimine (PEI) with a Brandel harvester, followed by three rapid washes of the filters with 3 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.4. Radioactivity retained on the dried filters was determined in a Minaxi gamma counter (Packard, Meriden, CT, USA).

Nonspecific binding was defined as [¹²⁵I]PIC binding in the presence of 10 µM BDF 6143 according to the method previously described (Separovic et al., 1996; Greney et al., 2000).

Competition binding assays were performed in the same way as the saturation experiments but using 0.5 nM [¹²⁵I]-PIC in the presence of 10 µM rauwolscine and seven different concentrations of the unlabelled ligand under investigation, ranging from 10⁻¹⁰ to 10⁻⁴ M. Incubation was initiated by the addition of 3T3-L1 (10-30 µg of protein) or PC12 cell
membranes (10–25 µg of protein). As in saturation assays, nonspecific binding was defined as 

\[^{125}\text{I}]\text{PIC}\] binding in the presence of 10 µM BDF 6143. Each point is the mean of three to five experiments performed in triplicate using different membrane preparations.

**Biacore experiments**

Label-free kinetic interaction analysis was performed by Surface Plasmon Resonance (SPR) using the Biacore X instrument (Biacore, Uppsala, Sweden) at 25°C to investigate the kinetic of LNP599 association and dissociation to adipocyte membranes in real time. Purified membranes were immobilized on a CM1 sensor chip via amine coupling. The system was equilibrated with HBS (10 mM HEPES, pH 7.4, with 0.15 M NaCl, and 0.005% surfactant P20) and maintained at a flow rate of 5 µl.min\(^{-1}\). The experimental flow cell dextran was activated with 35 µL of a fresh mixture of EDC (N-ethyl-N0-(3-diethyl-aminopropyl)carbodiimide) and NHS (N-hydroxysuccinimide) (1:1; v/v). Membranes were diluted to 10 mg/ml in 10 mM sodium acetate (pH 3.5), and 50 µl of this mixture was injected over the activated chip surface. Any remaining active CM dextran was blocked by 35 µl of ethanolamine-HCl. A reference surface was prepared without membranes in a separate flow cell on the same chip treated with 35 µl of NHS/EDC, then 35 µl of ethanolamine-HCl. Non-specifically bound membranes were removed by injecting 25 µl of regeneration solution (10 mM NaOH, 1 M NaCl).

For binding assays, 20 µl of ligand dissolved in HBS was injected at a flow-rate of 10 µl.min\(^{-1}\) followed by a 30-s regeneration step. Results are expressed in resonance units (RU); 1 RU corresponds to a surface mass change of 1 pg protein.mm\(^{-2}\). Sensorgrams were fitted to the titration kinetics 1:1 binding with drift model using the BIA evaluation 3.2 software to determine the on and off rate constants (k\(_{\text{on}}\) and k\(_{\text{off}}\)) from which the dissociation constant K\(_d\) was calculated (K\(_d\) = k\(_{\text{off}}\)/k\(_{\text{on}}\)). Results are given as mean values from six experiments ± SEM.
Adiponectin production

To determine adiponectin secretion by mature adipocytes, day-10 post confluent 3T3-L1 adipocytes differentiated in 12-well plates were washed three times with DMEM, then cultured for 6 h in DMEM alone, in the absence or presence of 3 µM LNP599. When indicated, the I1R antagonist efavoxan (100 µM) was added 30 min before LNP599 exposure. Culture medium was then collected, centrifuged for 3 min at 10 000 g to remove cell contaminants, and the supernatant was stored at -80°C until use. After two washes with ice-cold PBS, adipocytes were collected in PBS, homogenized, and stored at -80°C. An aliquot of adipocyte homogenate was kept for protein determination. Adiponectin was measured by an ELISA kit (R&D Systems, Lille, France) according to the manufacturer’s recommendations. Adiponectin concentration in the culture medium was normalized by cell protein content.

Quantitative real-time PCR

Total RNA was extracted by the RNeasy mini kit, according to the manufacturer’s recommendations (Qiagen SAS, Courtaboeuf, France). One µg of total RNA was digested with DNase I (Invitrogen), then reverse-transcribed with Superscript reverse transcriptase (Invitrogen) as recommended. After heat-denaturation, and 10-fold dilution, 1/20 of the reverse transcription reaction 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 SybrTM Green I (Eurogentec, Angers, France), in the presence of 300 nM of each sense and adiponectin specific antisense oligonucleotides, 200 µM dNTP, 2.5 mM MgCl2, 1.25 unit of Hot Gold Star DNA polymerase in its recommended buffer. The sequences of the sense and antisense oligonucleotides were 5’-AGGCCGTGATGGCAGAGATG-3’ and 5’-CTTCTCCAGGTTCTCCTTTCCTGC-3’ for adiponectin, 5’-AAGCGCGTCCTGGCATTGTCT-3’ and 5’-CCGCAGGGCAGCAGTGGT-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, RT-PCR products were analyzed in a post-amplification 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.

3. In vivo experiments

**Animals**

Twelve-week old male Sprague Dawley rats (SD) (n= 20) and 12-week-old male SHHF (spontaneously hypertensive, heart failure) 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 24 November 1986 (86/609/EEC) and the French Department of Agriculture (License no. 67–337 to P. Bousquet). The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath et al., 2010).
Renal sympathetic nervous activity (RSNA) recording

The 12-week-old male Sprague-Dawley 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 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 (7–8 ml/kg x 72 cycles/min) with a mixture of oxygen and room air (~80–20%). AP was measured by connecting the arterial catheter to a pressure transducer (TNF-R; Ohmeda, Bilthoven, The Netherlands) coupled to an amplifier (model 13-4615-52; Gould, Cleveland, OH). RSNA was amplified (x50 000), band-pass filtered (300–3000 Hz: Model P-511J; Grass, Quincy, MA), and rectified by an analog home-made rectifier including a low-pass filter with a cut-off 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 software (National Instruments), the AP and RSNA signals were sampled at 500 and 5000 Hz, respectively (Orea et al., 2007). AP and RSNA were continuously recorded before (baseline) and after administration of LNP599 (10 mg/kg) given as an i.v. 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 i.v. overdose of pentobarbital sodium.
**Chronic drug treatment of SHHF rats**

LNP599 was administered in the drinking water for 12 weeks at a dose of 20 mg/kg/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). Bodyweight, 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 pentobarbitone 50 mg/kg, ip (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 iv pancuronium bromide (Organon SA, France). Blood pressure was monitored after stabilization, using a pressure transducer (Gould P23XL) and recorder (Gould electronics BS 272, Longjumeau, France). Mean arterial blood pressure (MBP) was calculated as diastolic pressure plus one third of the differential blood pressure. HR was also monitored from the pressure signal with a Gould Biotach amplifier (Gould electronics 13-4615-66, Longjumeau, France).

**Plasma biochemical measurements**

Blood samples were obtained from the tail veins of anesthetized rats (isoflurane 2.5%, Abbott, Rungis, France) following an 18-h fast. Blood samples were centrifuged for 15 min 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 ELISA kits (insulin: Mercodia, Uppsala, Sweden; adiponectin: B-Bridge International, Mountain View, USA; glucagon: Gentaur, Kampenhout, Belgium) according to
the manufacturer’s recommendations. Insulin resistance was evaluated by calculating the Homeostasis Model Assessement (HOMA-IR): fasted insulinemia (µU/ml) x fasted glycemia (mM) / 22.5.

**Glucose tolerance test (IVGTT)**

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, 45 min after the glucose load (Accu Check Go, Roche Diagnostics, Meylan, France). Areas under the curve (AUC) were determined to compare groups.

4. Statistical analysis

Data are presented as mean ± SEM. Comparisons between groups were made using Student's t-test or two-way analysis of variance (ANOVA) followed by a Bonferonni post-hoc test (GraphPad Software Inc., San Diego, USA). P-value less than 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., San Diego, CA). Ki values were calculated according to the Cheng and 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 I1R binding sites

In PC12 cell membranes, clonidine displaced the I1R specific binding of [125I]-PIC with two affinity sites (high affinity sites: $K_i = 6.0 \pm 0.9$ nM (37%) and low affinity sites: $K_i = 1309 \pm 99$ nM (n=3)).

LNP599 also exhibited two affinity sites displacement of the [125I]-PIC specific binding [high affinity: $K_i = 15 \pm 1$ nM (37%) and the low affinity: $K_i = 1407 \pm 89$ nM (n=3)] as shown in Figure 2.

Binding properties of LNP599 on a large set of receptors/transporters and enzymes (55 receptors and transporters and 20 enzymes) were investigated in specific competition experiments. Based on the above-mentionned $K_i$ value of LNP599 for its high affinity sites, the $10^{-7}$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 I1Rs and its lipophilic properties, it has been further used for all the cardiovascular and metabolic investigations.

Expression of I1Rs 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 bound to an
homogeneous population of sites with a Kd value of $2.3 \pm 0.7$ nM and a Bmax $= 803 \pm 105$ fmol/mg protein ($n = 3$) (Figure 3A). The Hill coefficient (nH) of the saturation binding data has been calculated using the GraphPad software. nH $= 1.1 \pm 0.2$ was very close to 1 indicating that there was probably no cooperativity.

In competition experiments, clonidine, an I1R reference ligand, displaced $[^{125}\text{I}]$PIC specific binding in 3T3-L1 cell membrane preparations with two affinity components ($K_i = 10.2 \pm 6$ nM (49 % of total sites) and $3010 \pm 678$ nM, n=4) demonstrating the presence of I1Rs in 3T3-L1 membrane extracts. LNP599, a competitor, also exhibited a two affinity sites displacement ($K_i = 8.5 \pm 5.9$ nM (32 % of total sites) and $1540 \pm 761$, n=4) at the I1R specific binding of $[^{125}\text{I}]$-PIC (Figure 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 Figure 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 Figure 3D. Similar dissociation rate constants ($k_{off}$) were obtained ($4.54 \pm 0.12 \times 10^{-3}$ and $7.46 \pm 0.16 \times 10^{-3}$ s$^{-1}$ for LNP599 and isoproterenol, respectively) while the association rate constant ($k_{on}$) of LNP599 was significantly higher ($73.3 \pm 1.6 \times 10^{3}$ M$^{-1}$ s$^{-1}$) than that of isoproterenol ($8.43 \pm 0.21 \times 10^{3}$ 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-h exposure to LNP599 significantly increased adiponectin secretion by 3T3-L1 adipocytes ($378 \pm 38$ vs $213 \pm 19$ ng/ml/mg of 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, due to 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. Pre-incubation with the I₁R antagonist, efaroxan, prevented the LNP599-induced increase in adiponectin secretion (237 ± 19 vs 378 ± 38 ng/ml/mg of protein, P<0.001), whereas efaroxan alone, at the same dose, had no significant effect as compared to controls (175 ± 16 vs 213 ± 19 ng/ml/mg of protein, P>0.05) (Figure 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-h exposure to the drug (Figure 4B).

Acute effects of LNP599 on hemodynamic parameters and sympathetic activity

In a series of pilot experiments, 3 different doses of LNP599 (1, 5, and 10 mg/kg) were injected i.v into anaesthetized SD rats to look at the dose–effect relationship. Mean blood pressure decreased by 14.8±3.7 (n=3), 33.4±3.4 (n=4) and 38.1±3.5% (n=3) respectively (see figure in supplemental data). Hypotensive effects were accompanied by bradycardia (10.1±3.1, 22.1±2.5, 28.3±3.6% respectively).

LNP599, at a dose of 10 mg/kg given intravenously to anaesthetized Sprague-Dawley rats provoked a 50% decrease in RSNA (Figure 5A). In parallel, LNP599 provoked a marked decrease in systolic, mean and diastolic AP (SBP: 105 ± 3 vs 141 ± 2 mmHg, P<0.001; MBP: 77 ± 4 vs 108 ± 3 mmHg, P<0.0001; DBP: 59 ± 3 vs 83 ± 4 mmHg, P<0.001) and HR (288 ± 10 vs 331 ± 14 bpm, P<0.01) (Figure 5B,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 on table 1, except heart rate and fasting glucose, all the cardiovascular and lipid and carbohydrate metabolic parameters dealing with metabolic syndrome were significantly altered in SHHF rats. Compared to SD 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 IVGTT); bodyweight was only moderately but significantly increased.

Effects of chronic administration of LNP599 on hemodynamic and metabolic parameters in SHHF rats

After 12 weeks of LNP599 at a dose of 20 mg/kg/day in the drinking water, mean blood pressure of SHHF rats was significantly decreased, but their HR was not modified (Table2). The average bodyweights of control and treated animals did not differ significantly in either group at t = 0 (12 weeks of age): 398 ± 8 g vs 418 ± 9 g (p > 0.05). Bodyweight continued to increase slightly in a similar way in both groups until week 8 of treatment (age: 20 weeks)(Figure 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 while that of the treated animals stabilized (587 ± 8 g 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 to non-treated control animals (fig. 6 B and C). Food intake and water intake related to body weight decreased over the 12 weeks-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 (Figure 7A), but fasted plasma insulin significantly decreased (14.3±3.1 vs 46.8±3.7 ng/ml, P<0.001) (Figure 7B). Calculation of the HOMA-IR index confirmed that SHHF rats treated with LNP599 had improved insulin sensitivity compared to control SHHF rats (128±28 vs 392±25, P<0.0001) (Figure 7C). LNP599 provoked a non-significant decrease in glucagon (Figure 7D). Moreover, glucose tolerance testing showed that LNP599 improved glucose tolerance of SHHF rats (AUC IVGTT: 574±30 vs 710±37 mmol.min/l, P<0.01) (Figures 7E,F). In addition, LNP599 provoked a marked increase in plasma leptin and adiponectin concentrations (Table 2). Following 12 weeks of LNP599, we observed a sharp decrease in plasma total cholesterol (2.6 ± 0.1 vs 3.8 ± 0.2 mM, P<0.0001) (Table 2). LNP599 was roughly neutral in its effects on plasma triglycerides and free fatty acids.

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 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 a very likely crosstalk 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; Grassi et al., 2007; Mancia et al., 2007). In addition, adiponectin has been shown to play a pivotal role in patho-physiology of MetS and its complications (Mancia et al., 2007). In this context, we selected an imidazoline derivative with a pyrroline moiety (LNP599) which displays sympatho-inhibitory effects and stimulates adiponectin secretion through selective activation of I$_1$R 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, highly selective for I$_1$Rs (Greney et al., 2002). We chose LNP599 in this study because of its appropriate binding properties on I$_1$R, 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 $[^{125}$I$] $ PIC specific binding by clonidine with high affinity confirmed that PC12 cell membranes represent a reference model for the study of I$_1$R (Edwards and Ernsberger, 2003). The binding results demonstrated that LNP599 is a selective and specific molecule for I$_1$R. Indeed, in PC12 cell membrane preparations, this molecule displaced the specific $[^{125}$I$] $ PIC binding to I$_1$R 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 table in supplemental data) and of α$_2$-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 directly fat cells to promote adiponectin synthesis and secretion, by an I1R-dependent mechanism. As a matter of fact, acute exposure of 3T3-L1 adipocytes to LNP599 provoked a marked increase in adiponectin secretion. This effect was blocked by the I1R-antagonist efaroxan (Edwards and Ernsberger, 2003; Wang et al., 2007). The presence of I1Rs on 3T3-L1 membrane was demonstrated in saturation binding experiments using [125I] PIC as the reference radioligand for I1R. Rauwolscine was used in order to mask α2-adrenergic receptors (Ernsberger et al., 1995). The [125I] PIC specific binding was displaced with high affinity by clonidine, the I1R reference ligand, in the presence of rauwolscine. In this I1R assay, LNP599 exhibited high affinity for I1R. Displacement of [125I] PIC by LNP599 fitted in a two compartment model, as in numerous other I1R binding studies (Greney et al., 2000; Urosevic et al., 2004). As shown previously, the biphasic competition curves on [125I] PIC binding sites obtained with clonidine or LNP599 could refer to either the coupled / uncoupled form of G protein-coupled receptors or 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 I1R sites, thus accounting for a 10-fold lower affinity compared to those obtained in competition experiments.

As far as the effect on sympathetic nervous activity was concerned, acute administration of LNP599 (10 mg/kg i.v.) in anesthetized SD 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 which markedly reduced blood pressure in pilot experiments. The sympathetic inhibitory effects of all the reference drugs acting centrally, such clonidine, moxonidine, and rilmenidine, were also demonstrated in SD 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 these compounds, including LNP599, also reduce sympathetic activity in rodent models of MetS. However, these acute 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. (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 is impaired. A further project on animals receiving high fat diet is already in progress in our laboratory to confirm the benefit of LNP599 in another model of metabolic syndrome and to look at the sympathetic effects of LNP599 during chronic 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 (SD), which leads to a spontaneous mutation, designated as fa^k for fatty (Koletsky, 1975a; Koletsky, 1975b). Therefore, in this study, we used SD male rats as control animals to highlight the metabolic and cardiovascular abnormalities exhibited by SHHF rats.

In the first part, SHHF and SD rats were compared at the same age (24 weeks) just to confirm that, in our hands, SHHF rats exhibit cardiovascular and metabolic abnormalities when SD rats are taken as references (table 1). After this confirmation has been 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 SD rats with LNP599. That is the reason why in vivo study was designed with only 2 groups: SHHF rats receiving vehicle and SHHF rats receiving LNP599.

Compared to SD 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 chronic treatment experiment was carried out.

For chronic treatments, the 20 mg/kg dose of LNP599 was given orally. Since bioavailability of LNP599 was assessed at 50-60% in rats, we used twice the dose which exhibited clear cut blood pressure effects after i.v administration.

Long term exposure of SHHF rats to 20 mg/kg of LNP599 prevented, to some extent, the age-related weight gain in treated rats compared to 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 on was not related to a fall in food and/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, acute and chronic administration of LNP599 significantly decreased mean blood pressure as a result of its sympatho-inhibitory action. Chronic oral treatment did not cause significant bradycardia and acute iv treatment reduced heart rate by only 13%.

Regarding glucose metabolism, it is noteworthy that SHHF rats had normal fasting glucose values compared to control SD rats; thus, it is not really surprising that LNP599 did not significantly change fasting glucose. However, IVGTT tests and HOMA-IR values show
SHHF rats exhibit severe glucose intolerance and insulin resistance, respectively. Chronic LNP599 treatment significantly improves glucose tolerance, and 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 sympatho-inhibitory 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 or not LNP599 could also modulate insulin synthesis and secretion, in particular during glucose challenge, remains to be investigated.

Interestingly, LNP599 tended to decrease plasma glucagon levels, a phenomenon that could also contribute to improve glucose tolerance.

The increased plasma adiponectin concentration may also represent a major determinant of increased insulin sensitivity of LNP599-treated rats. In rats, the effects of LNP599 on total plasma adiponectin concentrations appear to be comparable to that of thiazolidinediones, which act directly on fat cells through PPAR-γ receptors to promote the synthesis and secretion of adiponectin (Kato et al., 2008; Pita et al., 2011). Comparable effects of thiazolidinediones are also found in humans, with at least a two-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, chronic administration of LNP599 decreased plasma total cholesterol by about 30%. It is interesting to note that the cholesterol-lowering effect of LNP599 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 post-mortem macroscopic examination did not reveal any abnormality. In vivo as well as in vitro toxicology investigations, including ADME tests, remained negative so far (data not shown). Based on all these observations, one can assume that chronic treatment with LNP599 is quite well tolerated, at least in SHHF rats.

In conclusion, in a rodent model of MetS, our results 

**i)** show that an I$_1$R selective ligand reduce weight gain, mean blood pressure, and total cholesterol, and moreover improves glucose tolerance and insulin resistance 

**ii)** suggest that beneficial effects of the I$_1$ selective drug in metabolic syndrome might be due to sympathetic inhibition as well as to adiponectin secretion stimulation 

**iii)** 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 I$_1$R-selective drug, given as a single drug, might be a relevant strategy for the management of various components of the MetS.
Acknowledgments

None
Authorship Contributions

Participated in research design: Fellmann, Regnault, Greney, Niederhoffer, Julien, Lacolley, Fève, Bousquet.
Conducted experiments: Fellmann, Regnault, Greney, Muscat, Max, Oréa, Chetrite, Niederhoffer, Julien.
Contributed new reagents or analytic tools: Gasparik, Gigou.
Performed data analysis: Fellmann, Regnault, Greney, Pizard, Niederhoffer, Julien, Lacolley, Fève, Bousquet.
Wrote or contributed to the writing of the manuscript: Fellmann, Regnault, Greney, Pizard, Niederhoffer, Julien, Lacolley, Fève, Bousquet.
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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.


Footnotes

Financial support

This work was supported by French National Institute for Medical Research (INSERM) and the French Ministry of Health, (a grant of the National Program for Research on Diabetes) and the Universities of Strasbourg, Paris 6, and Nancy.

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Figure 1: Chemical structure of LNP599

Figure 2: Competition curves of clonidine and LNP599 on I1Rs in PC12 cell membrane preparations.
Total specific binding (100%) was between 2128 and 3407 cpm. Nonspecific binding (as defined by 10 µM BDF6143) represented 44% of the total binding. Competition curves with LNP599 (■) (n=3) and clonidine (▲) (n=3).

Figure 3: Binding and SPR studies in 3T3-L1 adipocyte cell membranes.
**Binding analysis:** (A), saturation binding studies with \[^{125}\text{I}]\text{PIC} (n=3). (B), competition experiments: Competition curves with clonidine (▲)(n=4) and with LNP 599 (■)(n=4)

**Biacore analysis:** (C), LNP599 (black line) and isoproterenol (grey line) (10\(^{-5}\) M) were injected at a flow-rate of 10 µl.min\(^{-1}\) into a flow cell of a CM1- sensor chip with 1850 RU of immobilized 3T3-L1 membranes. (D), kinetic titration analysis. Biacore analysis: (C), LNP599 (black line) and isoproterenol (grey line) both at 5×10\(^{-8}\) M were injected at a flow rate of 10 µl.min\(^{-1}\).

Figure 4: I1Rs mediate an inducing effect of LNP599 on adiponectin secretion and synthesis in mature 3T3-L1 adipocytes.
(A), adipocytes were exposed for 6 h to LNP599, without or with a prior 30 min exposure to efaroxan. Results represent the mean±SEM of 14 independent experiments. Black bar: control; white bar: efaroxan 100 µM; grey bar: LNP599 3 µM; striped bar: LNP599 3 µM + efaroxan 100 µM.
(B), adipocytes were treated with 3 µM LNP599 for the indicated times, then cell extracts were prepared and tested for adiponectin mRNA expression ($n=4$).

*, P<0.05; ***, P<0.001 LNP599 vs control; ###, P<0.001 LNP599 + efaxoxan vs LNP599.

Figure 5: Effects of acute administration of LNP599 (10 mg/kg, iv) on renal sympathetic nerve activity (A), arterial pressure (B) and heart rate (C) of Sprague Dawley rats ($n=6$) anesthetized with urethane (1.5 g/kg, ip).

Figure 6: Effects of 12 weeks of LNP599 (20 mg/kg/day in the drinking water) on body weight (A), and food and water intakes (B,C) in SHHF rats.

Black symbols: control ($n=10$); white symbols: LNP599 ($n=10$).* P<0.05, LNP599-treated vs control rats.

Figure 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.
Table 1: Cardiovascular and metabolic parameters of SD rats and SHHF rats at 24-weeks of age.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD (n=10)</th>
<th>SHHF (n=10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBP (mmHg)</td>
<td>111±6</td>
<td>166±5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
<td>119±6</td>
<td>176±6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SBP (mmHg)</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>Bodyweight (g)</td>
<td>547±11</td>
<td>587±9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glucose (mmol/l)</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>
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<tr>
<td>AUC IVGTT mmol.min/l</td>
<td>584±20</td>
<td>710±37</td>
<td>&lt;0.01</td>
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<tr>
<td>Total cholesterol (mmol/l)</td>
<td>1.97±0.11</td>
<td>3.84±0.18</td>
<td>&lt;0.0001</td>
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<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.09±0.18</td>
<td>4.59±0.23</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Free fatty acids (mmol/l)</td>
<td>0.48±0.06</td>
<td>0.94±0.03</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 2: Effects of LNP599 on cardio-metabolic parameters in SHHF rats

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

This work was supported by French National Institute for Medical Research (INSERM) and the French Ministry of Health, (a grant of the National Program for Research on Diabetes) and the Universities of Strasbourg, Paris 6, and Nancy.

Disclosures: none