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Imidazoline-like drugs improve insulin sensitivity through peripheral stimulation of adiponectin and AMPK pathways in a rat model of glucose intolerance

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Imidazoline-like drugs improve insulin sensitivity through peripheral stimulation of adiponectin and AMPK pathways in a rat model of glucose intolerance. *Am J Physiol Endocrinol Metab* 309: E95–E104, 2015. First published May 26, 2015; doi:10.1152/ajpendo.00021.2015.—Altered adiponectin signaling and chronic sympathetic hyperactivity have both been proposed as key factors in the pathogenesis of metabolic syndrome. We recently reported that activation of I1 imidazoline receptors (I1R) improves several symptoms of the metabolic syndrome through sympathoinhibition and increases adiponectin plasma levels in a rat model of metabolic syndrome (Fallmann L, Regnaut V, Greney H, et al. *J Pharmacol Exp Ther* 346: 370–380, 2013). The present study was designed to explore the peripheral component of the beneficial actions of I1R ligands (i.e., sympathoinhibitory independent effects). Aged rats displaying insulin resistance and glucose intolerance were treated with LNP599, a peripherally acting I1R agonist. Glucose tolerance, insulin sensitivity, and adiponectin signaling were assessed at the end of the treatment. Direct actions of the ligand on hepatocyte and adipocyte signaling were also studied. LNP599 reduced the area under the curve of the intravenous glucose tolerance test and enhanced insulin hypoglycemic action and intracellular signaling (Akt phosphorylation), indicating improved glucose tolerance and insulin sensitivity. LNP599 stimulated adiponectin secretion acting at I1R on adipocytes, resulting in increased plasma levels of adiponectin; it also enhanced AMPK phosphorylation in hepatic tissues. Additionally, I1R activation on hepatocytes directly enhanced AMPK phosphorylation. To conclude, I1R ligands can improve insulin sensitivity acting peripherally, independently of sympathoinhibition; stimulation of adiponectin and AMPK pathways at insulin target tissues may account for this effect. This may open a promising new way for the treatment of the metabolic syndrome.

adiponectin and AMPK signaling; glucose tolerance; I1 imidazoline receptors; insulin resistance; metabolic syndrome; sympathetic tone

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**METABOLIC SYNDROME** (MetS) is defined as a cluster of interrelated risk factors that promotes the development of cardiovascular and metabolic diseases. The most prominent risk factors, also used as diagnosis criteria, are abdominal obesity, elevated blood pressure, atherogenic dyslipidemia, and insulin resistance associated or not with elevated fasting plasma glucose (16, 17, 31).

Adiponectin, a major adipocyte-derived hormone, can assemble into several oligomeric isoforms, including low, middle, and the most physiologically active, high-molecular-weight isoforms, and acts via two receptors, AdipoR1 and AdipoR2 (23, 36). Adiponectin resistance, consecutive to decreases in both adiponectin level (hypoadiponectinemia) and adiponectin receptor expression, may account for the initial loss of insulin sensitivity of target cells, evolving into premetabolic, then MetS, and, finally, overt cardiovascular disease (40, 42). Based on these observations, promoting adiponectin pathway has emerged as a potential therapy for the treatment of MetS (18, 22, 45).

Several components of MetS, i.e., hypertension (8, 12), obesity (6, 14), insulin resistance (44), and diabetes (37), have been associated with increased sympathetic nerve activity. Hence, sympathetic hyperactivity has been proposed as another key factor in the pathogenesis of MetS (13, 27). Pharmacological inhibition of sympathetic activity could therefore also represent an interesting strategy for the treatment of the MetS.

We (2, 7) recently described an original compound (LNP599) selective for I1 imidazoline receptors (I1R), with both sympathoinhibitory and adiponectin secretion-stimulating effects. I1R were first described in 1984 by Bousquet and coworkers (3) as part of the medullary receptors targeted by the centrally acting anti-hypertensive drug, clonidine, and its derivatives. LNP599 is a pyrroline analog of clonidine, with nanomolar affinity and high selectivity for I1R and no detectable affinity for the other pharmacological target of clonidine and related compounds, the α2-adrenergic receptors (2). Chronic treatment with LNP599 had favorable effects on all the symptoms of MetS, i.e., blood pressure, body weight, glucose tolerance, and lipid profile, and increased plasma adiponectin levels, in a rat model of MetS (spontaneously hypertensive, heart failure rats, SHHF). Additionally, the drug was shown to promote adiponectin synthesis and secretion

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from cultured 3T3-L1 adipocytes through an I1R-dependent mechanism of action (7). These data demonstrated for the first time that imidazole-like drugs may prove beneficial for the treatment of MetS.

The objective of the present study was to further explore the peripheral, direct (i.e., sympathoinhibitory independent) actions of I1R ligands on insulin resistance and glucose homeostasis. To this end, experiments were carried out in aged rats, a model of spontaneously developing glucose intolerance and insulin resistance ([11] and present data). In order to target only peripheral I1R and to rule out centrally mediated changes in cardiovascular parameters and glucose regulation, animals were treated intraperitoneally with LNP509, an analog of LNP509 that has been previously shown not to cross the blood-brain barrier (33). Moreover, the LNP509 ligand displays a good affinity for I1R and no detectable affinity for the α2-adrenergic sympathoinhibitory receptors (33). We established that 1) imidazole-like drugs can reduce insulin resistance acting peripherally, independently of any sympathoinhibition, and 2) the drug-induced improvement of insulin sensitivity is associated with an increase in circulating adiponectin levels and a stimulation of AMPK signaling in hepatocytes.

**MATERIAL AND METHODS**

**Animals and treatments.** Young (1–2 mo old, body weight 360 ± 6 g) and aged (12–14 mo old, body weight 633 ± 6 g) male Wistar rats (Elevage Janvier, St Berthevin, France) were housed in a temperature- and light-controlled room and allowed ad libitum access to standard diet and water. All procedures conformed to the French and European guidelines and were approved by the Animal Ethics Committee of the Strasbourg University.

The I1R ligand LNP509 [[cis-trans-dicyclopropylmethyl-(4,5-dimethyl-4,5-dihydro-3H-pyrrol-2-yl)-amine] (33) was administered daily by intraperitoneal (ip) injection for 7 days; control rats were injected in parallel with vehicle (NaCl 0.9%). At the end of the treatment period, cardiometabolic parameters were assessed in overnight-fasted, pentobarbital sodium-anesthetized animals. Rats were then administered an intravenous lethal dose of pentobarbital sodium. Tissues were removed and immediately fixed in 4% paraformaldehyde for histological examinations or flash-frozen and stored at −80°C for quantitative RT-PCR, Western blot or binding analysis, or ELISA assay.

**Arterial blood pressure and heart rate recording.** The femoral artery was catheterized, and arterial pressure signal was monitored continuously for 15 min with a pressure transducer coupled to a computerized data acquisition and analysis system (EMKA Technologies, Paris, France). At the end of the measurements, plasma was sampled and stored at −20°C until biochemical assay.

**Biochemical measurements.** Concentrations of plasma catecholamines were measured by HPLC (Plateau technique de Biochimie, NHC Strasbourg, France). Glucose levels were evaluated in a blood drop using a glucometer (AccuCheck, Roche Diagnostics, Meylan, France). Plasma insulin and adiponectin were quantitated by ELISA kits (insulin: Mercodia, Uppsala, Sweden; adiponectin: B-Bridge, Paris, France) according to the manufacturers’ instructions. The HOMA index of insulin resistance (IR) was calculated as: [fasting insulin (mU/l) × fasting glucose (mmol/l)]/22.5.

**Glucose and insulin tolerance tests.** Glucose (0.5 g/kg iv) was administered at t = 0 min. Plasma glucose and insulin concentrations were then determined at the indicated times; the area under the curve (AUC), peak glycemia, and rate of glucose clearance were calculated from these values. Insulin (1.5 U/kg ip) was administered at t = 0 min; plasma glucose was then determined after 15, 30, 45, and 60 min. The AUC was calculated from these values.

**Pancreas histology and function.** Fixed pancreatic tissues were embedded in paraffin, and 5-μm slices were cut and stained with hematoxylin-eosin. Microscopic images were captured using a video camera coupled to a computer (Leica Microsystems, Heerbrugg, Switzerland) and analyzed with Image J software. Total RNA was extracted from pancreas samples using the MagNA Pure Compact RNA Isolation Kit (Roche, Basel, Switzerland) and then reverse-transcribed into cDNA using the LightCycler Transcriptor First Strand cDNA Synthesis Kit (Roche). Insulin gene expression was measured by quantitative real-time polymerase chain reaction (qRT-PCR) using a LightCycler amplifier and a fluorescent Sybr Green I dye for detection (Roche) and using specific primers for the rat insulin gene (forward 5'-CAGCTACAATCATAGACCAT-3', reverse 5'-TA-AAGCTTGGATTGACCAA AAA-3'). The cycling conditions were: 95°C for 10 min, then 40 cycles at 94°C for 15 s, 63°C for 15 s, and 72°C for 20 s. Relative insulin gene expression in treated animals was standardized to the 18S housekeeping gene (26) and
transporter Glut2 was detected using a rabbit polyclonal anti-Glut2 antibody (Millipore); total AMPKα, AMPK phosphorylated on Thr172 (pAMPKα), total Akt, and Akt phosphorylated on Ser473 (pAkt) were probed using appropriate rabbit antibodies purchased from Cell Signaling. All antibodies were incubated overnight at 4°C with gentle shaking. Reactive proteins were detected and quantified using the Bio-Rad Chemidoc System. GAPDH expression level was used to normalize the Glut2 detection. AMPK and Akt activations were evaluated by the ratio pAMPKα/AMPKα and pAkt/Akt, respectively.

Insulin secretion by rin m5F pancreatic β-cells. Rin m5F pancreatic β-cells obtained from A. Langlois (Centre Européen d’Étude du Diabète, Strasbourg, France) were cultured in 24-well plates at 5 × 10^5 cells/well in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 2% penicillin-streptomycin. Insulin secretion was stimulated by 24-h exposure of the cells to high glucose (25 mM). The culture medium was then collected, and insulin levels were measured by ELISA. In some experiments, incubations were carried out in the presence of the calcium chelator EGTA-Na2 (0.5 mM). LNP509 was added at increasing concentrations (from 10^-7 to 10^-3 M) during the 24-h exposure to glucose.

Adiponectin secretion by isolated adipocytes. Adipocytes were isolated from the epididymal white adipose tissue of aged male Wistar rats according to a modified version of Rodbell’s adipocyte isolation method (32). Briefly, the adipose tissue was collected, minced, and incubated in collagenase solution (1 mg/ml collagenase type I-A in a Krebs-Ringer solution supplemented with 500 nM adenosine and 1% BSA) at 37°C for 45 min. The resulting suspension was filtered through a nylon mesh, centrifuged (500 g, 4 min), and washed three times to separate mature adipocytes from stromal cells. Adipocytes were plated in a 96-well plate (5,000 cells/well) in Krebs-Ringer buffer supplemented with 1% BSA and 1 U/ml adenosine desaminase in the absence or presence of LNP509 or insulin. After 3 h, the culture medium was collected and adiponectin content measured by ELISA (see above). In some experiments, efaroxan was added 30 min prior to LNP509.

normalized by the expression obtained in the control group. Expression level of the membrane glucose transporter Glut2 was determined by Western blot analysis (see below).

Immunoblotting. Pancreatic and hepatic tissues were homogenized in HEPES buffer containing protease and phosphatase inhibitors, then centrifuged 10 min at 10,000 g at 4°C. The membrane glucose transporter Glut2 was detected using a rabbit polyclonal anti-Glut2 antibody (Millipore); total AMPKα, AMPK phosphorylated on Thr172 (pAMPKα), total Akt, and Akt phosphorylated on Ser473 (pAkt) were probed using appropriate rabbit antibodies purchased from Cell Signaling. All antibodies were incubated overnight at 4°C with gentle shaking. Reactive proteins were detected and quantified using the Bio-Rad Chemidoc System. GAPDH expression level was used to normalize the Glut2 detection. AMPK and Akt activations were evaluated by the ratio pAMPKα/AMPKα and pAkt/Akt, respectively.

Table 1. Effects of 1-wk treatment with LNP509 on body weight, food and water intake, cardiovascular parameters, and plasma catecholamines in aged rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rats Receiving Vehicle (1 ml·kg⁻¹·day⁻¹ ip; n = 9)</th>
<th>Rats Receiving LNP509 (10 mg·kg⁻¹·day⁻¹ ip; n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 1</td>
<td>day 8</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>642 ± 15</td>
<td>635 ± 15</td>
</tr>
<tr>
<td>Food intake, g·kg⁻¹·day⁻¹</td>
<td>38 ± 4</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>Water intake, ml·kg⁻¹·day⁻¹</td>
<td>55 ± 4</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>Arterial blood pressure, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>111 ± 5</td>
<td>104 ± 6</td>
</tr>
<tr>
<td>Diastolic</td>
<td>98 ± 5</td>
<td>91 ± 8</td>
</tr>
<tr>
<td>Mean</td>
<td>102 ± 5</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>334 ± 9</td>
<td>355 ± 17</td>
</tr>
<tr>
<td>Plasma catecholamines, pmol/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>500 ± 49</td>
<td>750 ± 102</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>134 ± 50</td>
<td>199 ± 26</td>
</tr>
<tr>
<td>Dopamine</td>
<td>187 ± 86</td>
<td>195 ± 93</td>
</tr>
</tbody>
</table>

Data are means ± SE. Parameters were assessed before (day 1) and at the end (day 8) of the treatment period. There was no statistically significant difference between day 1 and day 8 within control and LNP509-treated groups.
LNP509 exposure; all other inhibitors were added 45 min prior LNP509 exposure.

**AMPK signaling in HepG2 cells.** Human hepatocarcinoma HepG2 cells were cultured in 24-well plates at 25,000 cells/well in DMEM-F12 without phenol red containing 10% heat-inactivated fetal calf serum and 2% penicillin-streptomycin. AMPK activation was triggered by exposing cells to increasing concentrations of LNP509 for 10 min. The ratio pAMPKα/AMPKα was evaluated by Western blot as described above and expressed relative to values obtained with AICAR-stimulated cells. In some experiments, efaroxan (100 μM) was added 30 min before LNP509 exposure.

**Binding experiments.** Membrane preparations were prepared from hepatic and adipose tissues of aged Wistar rats. Tissues were first homogenized in Tris-HEPES buffer with a Polytron homogenizer. The homogenate was then centrifuged at 20,000 g for 20 min at 4°C and washed twice. The final pellet was resuspended in Tris-HEPES buffer and stored at −80°C until binding assays. HepG2 cells were expended in T75 culture flasks as described above. At confluence, cells were harvested, centrifuged, and resuspended in 1 ml of Tris-HEPES buffer.

Competition binding assays were performed on membrane homogenates or cell suspension using 0.5 nM [125I]para-iodoclonidine (PIC; PerkinElmer Life and Analytical Sciences, Waltham, MA) (15) in the presence of 10 μM rauwolscine to prevent any α2-adrenergic receptor binding, and six different concentrations of the unlabeled ligand ranging from 10−9 to 10−3 M. Incubation was initiated by the addition of membrane preparation (hepatocyte membranes; 190–300 μg of protein; adipocyte membranes; 18–30 μg of protein) or cell suspension (20,000 cells/assay) in a final volume of 250 μl of Tris-HEPES buffer 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 with a Brandel harvester (Brandel, Gaithersburg, MD), 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). Nonspecific binding was defined as [125I]PIC binding in the presence of 10 μM BDF6143 [4-chloro-2-(2-imidazolin-2-ylamino)-isoindoline]. Each point is the mean of three to five experiments performed in triplicate using different membrane preparations.

**Statistics.** Data are presented as means ± SE. Comparisons between treatments or groups were performed using one-way ANOVA followed by a Bonferroni post hoc test. P values < 0.05 were considered statistically significant.

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Fig. 3. LNP509 does not change pancreas morphology and function in aged rats and insulin secretion by Rin m5F pancreatic β cells. A–C: aged rats were treated for 7 days with vehicle (n = 9) or LNP509 (10 mg·kg−1·day−1 ip, n = 8). Pancreatic tissue was removed at the end of experiments and fixed in 4% paraformaldehyde for histological procedures or stored at −80°C for RT-PCR and Western blot analysis. A: total number and mean size of Langerhans islets (indicated by arrow) were evaluated on 9 randomly chosen tissue sections. B: relative insulin RNA expression was measured by qRT-PCR. C: expression level of membrane glucose transporter Glut2 was determined by Western blot analysis and corrected by GAPDH expression level (C, vehicle-treated; L, LNP509-treated). D: Rin m5F pancreatic β-cells were stimulated with 25 mM glucose in the presence or absence of calcium chelator EGTA (0.5 mM) or increasing concentrations of LNP509. Secreted insulin was measured in culture medium using ELISA. Data are means ± SE of 3 independent experiments.
RESULTS

Validation of aged rats as an experimental model of insulin resistance. Compared with young animals, aged rats displayed mild but significant hyperinsulinemia (1.44 ± 0.35 vs. 0.56 ± 0.09 μU/mL in young animals) and doubled HOMA-IR (Fig. 1A). Fasting glucose remained unchanged; however, the peak glycaemia following iv glucose (0.5 g/kg) was significantly increased (14.5 ± 0.7 and 18.9 ± 1.0 mmol/l in young and aged rats, respectively), and the glucose clearance was slower, so that the AUC iv glucose tolerance test (IVGTT) was 28% greater (Fig. 1B). These disorders in glucose regulation were not associated with hypertension (Table 1) or dyslipidemia (plasma total cholesterol = 1.76 ± 0.11 mmol/l and triglycerides = 0.67 ± 0.18 mmol/l).

Effects of LNP509 on cardiovascular parameters and plasma catecholamines. Aged rats were treated daily with LNP509 10 mg/kg ip. One week’s treatment did not significantly influence body weight and food and water consumption (Table 1). Cardiovascular parameters and catecholamine concentrations in plasma were also unchanged (Table 1), confirming the lack of sympathoinhibitory action of the drug under these conditions of administration.

Effects of LNP509 on glucose regulation and insulin resistance. Values for fasting glucose (7.0 ± 0.2 mmol/l), fasting insulin (3.1 ± 0.5 μU/mL), and HOMA-IR (22.9 ± 3.7) were not significantly different in rats treated with LNP509 compared with rats receiving the vehicle. Glucose tolerance was improved by LNP509, as shown by the 15% decrease in the AUC IVGTT after 10 mg·kg⁻¹·day⁻¹ LNP509 (Fig. 2A, left). Glucose-induced insulin secretion was similar in peak amplitude in both groups, but the secretory response was maintained for a significantly longer time in untreated animals than in LNP509-treated rats (Fig. 2A, right). Thus, in the treated animals, lower levels of plasma insulin were associated with more rapid decreases in glycaemia, suggesting a more efficient action of the hormone after LNP509 treatment.

In a series of experiments, we confirmed the beneficial effect of the drug on glucose tolerance. The AUC IVGTT was reduced by 15 and 12% after 10 and 20 mg·kg⁻¹·day⁻¹ LNP509, respectively, compared with control (Fig. 2B). This beneficial action was related to an accelerated glucose clearance (0.238 ± 0.021 mmol·l⁻¹·min⁻¹ in control rats compared with 0.352 ± 0.015 and 0.314 ± 0.020 mmol·l⁻¹·min⁻¹ in rats treated with LNP 10 and 20 mg·kg⁻¹·day⁻¹, respectively), rather than changes in the peak glycaemia. A lower dose of LNP509 (5 mg·kg⁻¹·day⁻¹) was devoid of effect on glucose tolerance (Fig. 2B).

Improvement of glucose tolerance could result from enhanced insulin secretion, better insulin action on target cells (i.e., higher sensitivity of insulin target cells to the hormone), or both. We observed no increase in baseline (fasting) insulin and glucose-induced peak insulin secretion in treated rats compared with control animals (see above). In agreement with these data, insulin mRNA expression was unchanged by LNP509 treatment (Fig. 3B). There was also no difference in the morphology and function of pancreas: the number and size of Langerhans islets, as well as expression levels of the membrane glucose transporter Glut2, were similar in the two groups (Fig. 3A and C). Finally, the effect of LNP509 on secretory responses of RIN m5F pancreatic β-cells was explored (Fig. 3D). Insulin secretion was induced by exposure of the cells to high glucose; insulin release was reduced by the calcium chelator EGTA, indicating an exocytotic process. In agreement with the results obtained in vivo, there was no effect

Fig. 4. LNP509 enhances insulin action in aged rats. Aged rats were treated for 7 days with vehicle or LNP509 (10 mg·kg⁻¹·day⁻¹ ip). A: insulin (1.5 U/kg) was administered ip at t₀; plasma glucose concentration was then determined after 15, 30, 45, and 60 min, and AUC was calculated from these values. Data are means ± SE. *P < 0.05 vehicle (n = 7) vs. LNP-treated (n = 8). B: Akt activation was evaluated by the ratio Akt phosphorylated on Ser⁴⁷³ (pAkt)/total Akt determined by Western blot analysis in hepatic tissues from rats after glucose challenge (C, vehicle-treated; L, LNP509-treated; INS, positive control with cultured PC12 cells stimulated with 100 nM insulin for 30 min). Data are means ± SE. *P < 0.05 vehicle (n = 4) vs. LNP-treated rats (n = 5).
of the ligand on glucose-induced insulin secretion by pancreatic β-cells [82.2 ± 21.9 and 81.2 ± 11.2 μg insulin/l of the culture medium, in the absence and in the presence of LNP509 at the highest concentration tested (10^{-5} M), respectively]. All together, the collected data argued against the hypothesis of an LNP509-mediated augmentation of insulin secretion in aged rats.

To explore the hypothesis of an improvement of insulin action at target cells following LNP509 administration, an insulin tolerance test was carried out in another group of animals. Insulin injection produced marked and long-lasting hypoglycemia in all animals, but the effect was more pronounced in LNP509-treated rats (Fig. 4B). Thus, plasma glucose was reduced by 40–45% after 30 min and remained stable thereafter in vehicle-treated rats. In contrast, glycemia continued to decrease in rats receiving LNP509, to reach a 60% reduction after 60 min; consequently, the AUC was significantly decreased by LNP509 treatment (214 ± 7 vs. 247 ± 14; Fig. 4A). Activation of Akt, a key factor of the intracellular signaling pathways of insulin, was also significantly enhanced in hepatic lysates from treated animals after glucose challenge compared with control animals (Fig. 4A); AMPK activation was also enhanced in hepatic tissues of animals receiving LNP509 (+35% for the cells, i.e., adipocytes and hepatocytes. Animals treated with LNP509 10 mg·kg^{-1}·day^{-1} for 1 wk displayed significantly enhanced plasma adiponectin levels (2.04 ± 0.21 vs. 1.48 ± 0.11 mg/l; Fig. 5A); AMPK activation was measured in culture medium using ELISA. Data are means ± SE of 3–7 independent experiments. *P < 0.05 vs. nonstimulated cells. **P < 0.05 vs. nonstimulated cells.

**Effects of LNP509 on adiponectin secretion and AMPK signaling pathway.** Since adiponectin acts as a major insulin-sensitizing hormone, increased adiponectin plasma levels promote insulin action and AMPK is a major target of adiponectin intracellular signaling pathways, adiponectin and AMPK signaling were assessed in rats receiving LNP509 and in cultured cells.

![Fig. 5. LNP509 increases adiponectin secretion and AMPK activation in aged rats and primary cultured adipocytes.](image1)

![Fig. 6. LNP509 increases AMPKα phosphorylation in cultured human HepG2 hepatocytes.](image2)
ratio pAMPKα/AMPKα; Fig. 5B). In mature adipocytes isolated from aged rats, application of LNP509 elicited a concentration-dependent adiponectin secretion that was totally prevented by pretreatment of the cells with the I1R antagonist efaroxan (Fig. 5C). The LNP509-induced adiponectin secretion was not altered by inhibition of PI3K by wortmannin but was reduced after phosphodiesterase and FOXO1 blockade (−74% and −49%, respectively; Fig. 5D). Of note, the phosphodiesterase and FOXO1 inhibitors did not significantly influence basal (i.e., LNP509-independent) adiponectin secretion (Fig. 5D). Finally, the possibility of additional direct effects of LNP509 on AMPK was investigated in the human hepatocarcinoma cell line (HepG2 cells). Addition of LNP509 induced a concentration-dependent increase of the ratio pAMPKα/AMPKα, which was totally abolished by efaroxan (Fig. 6).

**DISCUSSION**

The present study was designed to explore the peripheral mechanisms involved in the beneficial actions of I1R ligands on glucose regulation and insulin resistance. Experiments were carried out in aged rats that displayed impaired glucose regulation and mild insulin resistance but no other cardiometabolic disorders. Thus, blood pressure and fasting glucose and lipid levels measured in aged animals were similar to those reported in young or healthy animals (7, 11, 19) and were much lower than those reported in rat models of diabetes or MetS (7, 19, 43). In order to target only peripheral I1R, the LNP509 ligand was administered intraperitoneally. LNP509 lowers blood pressure only after central administration,
indicating that it does not cross the blood-brain barrier, at least in its active form (33).

One-week treatment of aged rats with LNP509 improved glucose tolerance. This beneficial effect can result from increased glucose-induced insulin secretion and/or increased peripheral glucose utilization and clearance following an improvement of insulin action. In the present study, several observations argue for an LNP509-evoked enhancement of insulin sensitivity of target cells, at least hepatocytes, and rule out any enhancement in insulin secretion by pancreatic β-cells. First, there was no change in pancreas histology, insulin mRNA expression, and the Glut2 membrane expression, excluding a trophic or stimulatory action of the ligand on insulin-secreting cells; second, basal insulin release assessed both in vivo and in vitro was not modified by LNP509. Last, we found no detectable I1R-specific binding of LNP509 on RIN m5F membranes (data not shown). Inversely, lower levels in glucose-evoked insulin release provoked more rapid glucose clearance in the treated animals. Finally, direct measurements of 1) the hypoglycemic action of insulin administration and 2) the glucose-evoked activation of hepatic intracellular insulin signaling (pAkt/Akt ratio) confirmed the improved insulin sensitivity. Based on binding and/or functional experiments, three different subtypes of IR have been described to date (5). However, the metabolic effects of LNP509 were most likely mediated by I1R only. Thus, although I1R have been reported to increase insulin sensitivity (38), LNP509 displayed no detectable affinity for I1R in binding experiments (33). Similarly, the I1R has long been recognized as one of the targets regulating insulin secretion by pancreatic β-cells (5), but LNP509 was ineffective on insulin release.

On the basis of our previous data (7) and the present results, we propose an adiponectin-mediated improvement of insulin sensitivity as a possible mechanism involved in the peripheral favorable metabolic effects of I1R ligands (Fig. 8). In adipose tissue, binding of these ligands to membrane I1R increases adiponectin secretion and, consequently, adiponectin plasma levels; this could contribute to the stimulation of AMPK phosphorylation in insulin target tissues (at least in hepatic tissue). Additionally, I1R ligands also exert direct activating effects on AMPK-dependent transduction in target cells. Stimulation of adiponectin and AMPK pathways, in turn, would improve insulin signaling in hepatocytes, leading to higher insulin sensitivity and better glucose tolerance. In agreement with this hypothesis, HOMA-IR values significantly correlated with adiponectin plasma levels in LNP-treated animals (Fig. 8). Our observations are in good agreement with those of Pita et al. (30) and Phillips et al. (29), showing that the same ranges of adiponectin pathway stimulation (i.e., 35–50% stimulation) lead to significantly improved insulin sensitivity in Zucker rats and in humans. Of note also, LNP509, at the concentrations used, was at least as potent as insulin and AICAR in stimulating adiponectin secretion and AMPK phosphorylation, respectively.

How can I1R ligands modulate adiponectin levels acting peripherally? Our present data show that I1R ligands can stimulate adiponectin secretion by adipocytes independently of any sympathoinhibition, since LNP509 given intraperitoneally had no impact on cardiovascular parameters and circulating catecholamines. This direct stimulatory action at adipose tissue in vivo was confirmed in primary cultured adipocytes isolated from aged rats and is in agreement with our previous report on cultured 3T3-L1 differentiated adipocytes (7). Transcription of adiponectin is regulated by numerous and complex transduction pathways (35, 41, 42). For instance, a reduction in intracellular cAMP can oppose the endogenous SIRT1-FOXO1-dependent downregulation of adiponectin synthesis, resulting in increased adiponectin synthesis and secretion (10, 35). The LNP509-induced adiponectin secretion by adipocytes was almost abolished by enhancing cAMP levels and was largely reduced after FOXO1 inhibition. The direct transduction cascade can therefore be proposed: I1R → inactivation of adenylyl cyclase → ↓ cAMP → inactivation of SIRT1/FOXO1 → activation of PPARγ → ↑ adiponectin synthesis. The direct peripheral modulation of adiponectin and AMPK signaling highlighted in the present study probably contributes to the overall beneficial effect on insulin resistance of sympathoinhibitory antihypertensive agents (39, 43) and I1R ligands (7). Nevertheless, additional non-adiponectin mechanisms of action cannot be ruled out.

Adiponectin, through AMPK-dependent transduction pathways, is a well-established insulin sensitizer (20, 21, 45). In hepatocytes, AMPK phosphorylation could attenuate insulin resistance acting at lipid and/or glucose homeostasis by 1) decreasing triglycerides synthesis, 2) inhibiting hepatic glucose-neogenesis following modifications of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, and 3) increasing fatty acid oxidation, mitochondrial biogenesis, and glucose transport (4, 25, 34). A recent study also unraveled a new adipoR-independent but AMPK-dependent hepatic insulinsensitizing action of adiponectin related to upregulation of hepatic IRS-2 through an IL-6-dependent pathway (1). Future
studies should explore these mechanisms and determine whether insulin action is also enhanced in other target tissues, especially skeletal and cardiac muscles and adipocytes.

To conclude, our data show that activation of peripheral I1R stimulates adiponectin and AMPK pathways and that these effects are associated with improved glucose regulation and insulin action. Promoting adiponectin and AMPK pathways has emerged over the past decade as an appealing therapeutic strategy to restore insulin sensitivity (18). Recombinant forms as well as transgenic expression of adiponectin displayed beneficial metabolic effects in rodents (9, 24) and even protective against premature death in a mouse model of MetS (28). However, data are still limited, and such approaches may prove difficult to develop in human therapy. I1R ligands crossing the blood-brain barrier, with their dual actions on adiponectin pathway and sympathetic tone, open a promising new way for the overall treatment of MetS.

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