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Chemerin receptor blockade improves vascular function in diabetic obese mice via redox-sensitive- and Akt-dependent pathways

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Running title: Chemerin decreases vascular insulin signaling

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ABSTRACT

Chemerin and its G protein-coupled receptor (ChemR23) have been associated with endothelial dysfunction, inflammation and insulin resistance. However, the role of chemerin on insulin signalling in the vasculature is still unknown. We aimed to determine whether chemerin reduces vascular insulin signalling and whether there is interplay between chemerin/ChemR23, insulin resistance and vascular complications associated with type 2 diabetes (T2D). Molecular and vascular mechanisms were probed in mesenteric arteries and cultured vascular smooth muscle cells (VSMC) from C57BL/6J, non-diabetic lean db/m and diabetic obese db/db mice as well as in human microvascular endothelial cells (HMEC). Chemerin decreased insulin-induced vasodilatation in C57BL/6J mice, an effect prevented by CCX832 (ChemR23 antagonist) treatment. In VSMC, chemerin, via oxidative stress- and ChemR23-dependent mechanisms, decreased insulin-induced Akt phosphorylation, GLUT4 translocation to the membrane and glucose uptake. In HMEC, chemerin decreased insulin-activated nitric oxide signalling. AMPK phosphorylation was reduced by chemerin in both HMEC and VSMC. CCX832 treatment of db/db mice decreased body weight, insulin and glucose levels and vascular oxidative stress. CCX832 also partially restored vascular insulin responses in db/db and high fat diet (HFD)-fed mice. Our novel in vivo findings highlight chemerin/ChemR23 as a promising therapeutic target to limit insulin resistance and vascular complications associated with obesity-related diabetes.

New & Noteworthy: Our novel findings show that the chemerin/ChemR23 axis plays a critical role in diabetes-associated vascular oxidative stress and altered insulin signaling. Targeting chemerin/ChemR23 may be an attractive strategy to improve insulin signaling and vascular function in obesity-associated diabetes.

Keywords: Adipokines, insulin, vascular smooth muscle, endothelial cells, type 2 Diabetes.
INTRODUCTION

Obesity, characterized by hypertrophy and hyperplasia of adipose tissue, is a critical risk factor for hypertension, dyslipidaemia, cardiovascular disease, and type 2 diabetes (T2D) (33). Obesity is also linked to decreased sensitivity to the biological actions of insulin, a condition identified as insulin resistance (48). In addition to its central energy storage function, adipose tissue secretes several bioactive hormones and cytokines, called adipokines (51). Adipokines have autocrine/paracrine effects that influence not only adipose tissue development and function (21), but also energy homeostasis, glucose and lipid metabolism, food intake, inflammation, and vascular function (26, 51). Dysregulation of adipokine production and secretion contribute to the pathogenesis of obesity and its associated vascular complications (51).

Chemerin, also known as retinoic acid receptor responder protein 2 (RARRES2) or tazarotene-induced gene 2 protein (TIG2), is highly expressed in the placenta, liver, and white adipose tissue (WAT), with a lower expression in tissues such as lung, brown adipose tissue, heart, ovary, kidney, skeletal muscle and pancreas (4, 14). It is secreted as an 18-kDa inactive pro-protein and undergoes extracellular serine protease cleavage to generate the 16-kDa active chemerin (50). Chemerin is a chemoattractant protein that binds to the G protein-coupled receptor CMKLR1 (chemokine-like receptor 1), also known as ChemR23, which is expressed in macrophages, dendritic cells, adipocytes (50) as well as in endothelial and vascular smooth muscle cells (VSMC), as recently reported (46). Although chemerin also activates the G protein-coupled receptor 1 (GPR1) with similar affinity to CMKLR1 and is a ligand for a third receptor, chemokine receptor-like 2 (CCRL2), which does not seem to activate intracellular responses,
essentially all known responses to chemerin have been attributed to the activation of ChemR23 (1).

Chemerin is currently described as a biomarker for adiposity in humans. Circulating chemerin levels were shown to be strongly associated with multiple components of metabolic syndrome, including body mass index (BMI), triglycerides, high-density lipoprotein cholesterol (HDL-C) and hypertension (4), and are also linked to adipogenesis (15, 35). Circulating chemerin levels are increased in numerous diseases associated with chronic inflammation (47). Serum levels of chemerin correlate with levels of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and C reactive protein (CRP) (24, 47).

Importantly, ChemR23 knockout mice present reduced adiposity and body mass (9) and chemerin levels are reduced by weight loss and fat reduction (9). Chemerin expression is upregulated in adipocytes of diet-induced obese mice (10, 34). Increased chemerin expression in WAT, skeletal muscle, and liver has been reported in mouse models of obesity/diabetes and chemerin exacerbates glucose intolerance, lowers serum insulin levels, and decreases tissue glucose uptake in obese diabetic db/db mice (10, 35). More recently, it has also been demonstrated an important role for reactive oxygen species (ROS) in chemerin signalling in vascular cells. Particularly chemerin, through ROS, stimulates mitogenic and pro-inflammatory signalling pathways promoting vascular damage and remodelling (45).

Although chemerin has been shown to impair insulin signalling and to induce insulin resistance in skeletal muscle cells (41) and cardiomyocytes (52), the role of chemerin in vascular insulin resistance particularly in the context of diabetes, has not been fully elucidated. Therefore, the present study aimed to determine whether chemerin influences vascular insulin signalling and whether there is interplay between chemerin/ChemR23, insulin resistance and vascular
complications associated with T2D. We hypothesized that chemerin, through ChemR23, decreases vascular insulin signalling and that ChemR23 antagonism attenuates abnormal vascular responses to insulin in obese diabetic db/db mice.

**MATERIALS AND METHODS**

All experimental protocols on mice were performed in accordance with the Ethical Principles in Animal Experimentation adopted by the West of Scotland Research Ethics Service and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the National Council for Animal Experimentation Control (CONCEA) and were approved by the Ethics Committee on Animal Use (CEUA) from the University of Sao Paulo (USP) (protocol nº 12.1.1593.53.0).

**Animals**

Ten to twelve weeks-old male C57BL/6J, lean non-diabetic db/m and obese diabetic db/db mice were housed in individual cages in a room with controlled humidity and temperature (22°C - 24°C), and light/dark cycles of 12 hours (h). Animals had free access to food and tap water. Animals were treated with vehicle (PEG400/cremophor) or CCX832, a ChemR23 antagonist, (a gift from ChemoCentryx, Inc., Mountain View, CA, USA) (75 mg/kg/day, for 3 weeks, by oral gavage). Animals were separated into 4 groups: db/m + vehicle, db/m + CCX832, db/db + vehicle and db/db + CCX832. In initial experiments, in order to confirm that the vehicle had no effects itself, two additional groups were included and maintained for the same three week-period: db/m and db/db mice without any treatment (i.e. untreated db/m and db/db mice).
Since no differences were observed between the untreated and vehicle groups, the remaining protocols were performed in animals treated with vehicle or CCX832. In another set of experiments, six week-old male C57BL/6J mice were maintained either on a control diet (protein 22%, carbohydrate 70% and fat 8% of energy, PragSolucoes, Jau, Brazil) or on a high-fat diet [(HFD), protein 10%, carbohydrate 25% and fat 65% of energy, PragSolucoes] for 18 weeks. Insulin sensitivity was calculated using the HOMA-IR index (Homeostasis Model Assessment), which takes into account insulin and fasting blood glucose levels, using the following mathematical formula: HOMA-IR = fasting insulin × fasting glucose/22.5. Additional nutritional and metabolic information from the mouse models can be found in previous studies (6, 7, 42). At the end of treatment animals were maintained under anaesthesia with 2.5% isoflurane for blood collection and then culled by carbon oxide (CO₂) inhalation.

Cultured Vascular Cells

Vascular smooth muscle cells (VSMC) from mesenteric arteries of C57BL/6J mice were isolated and characterized as previously described (45). Sub confluent cell cultures were rendered quiescent by serum deprivation for 24 h before experimentation. Low-passage cells (passages 4–6) from different primary cultures were used in our experiments.

Human microvascular endothelial cells (HMEC) (Life Technologies, Carlsbad, CA, USA) were also studied. Endothelial cells were cultured in Medium 131 supplemented with Microvascular Growth Supplement (MVGS; 25 ml), Gentamicin (50 µg/ml) and Amphotericin B (0.25 µg/ml). For functional studies, confluent cells were quiescent for 4 hours in low-serum medium containing 0.5% FBS and subsequently stimulated according to the experimental protocol. Four to six different batches of endothelial cells were studied for each experiment.
Cells were stimulated with recombinant chemerin (R&D Systems - 0.5 ng/mL). When inhibitors were used in any protocol, parallel experiments were performed to determine the effects of the inhibitor itself. Data were included in the graphics only if the inhibitor by itself produced a significant effect.

**Plasma Biochemistry**

Mice were fasted for 12 h and blood was collected immediately prior to sacrifice in tubes containing heparin. After collection, plasma was separated by centrifugation (2,000 rpm, 10 min). Plasma was aliquoted, snap frozen and storage at -80 °C. Glucose, cholesterol and triglycerides were determined by an automated analyser (Roche/Hitachi cobas c systems - cobas c 311 Autoanalyser).

**Chemerin and insulin levels**

Chemerin and insulin plasma levels were determined by ELISA, according to instructions from the manufactures (Cat. No. MCHM00 - R&D Systems and 10-1247-01 – Mercodia, respectively).

**Functional studies in mesenteric arteries**

First- and second-order mesenteric resistance arteries from C57BL/6J, db/m and db/db mice, as well as from mice treated with a control or a HFD for 18 weeks, were cut into 2 mm ring segments and mounted in a wire myograph, as previously described (16). Myograph chambers were filled with 5 mL of physiological solution [(in mmol/L): 130 NaCl, 14.9 NaHCO₃, 4.7 KCl, 1.18 KH₂PO₄, 1.17 MgSO₄•7H₂O, 5.5 glucose, 1.56 CaCl₂•2H₂O, and 0.026
EDTA] and continuously gassed with a mixture of 95% O\textsubscript{2} and 5% CO\textsubscript{2} at a temperature of 37\degree C. After 30 min of stabilization, the contractile ability of the preparations was assessed by adding KCl (120 mmol/L) to the organ baths. Endothelial integrity was verified by relaxation induced by acetylcholine (1 µmol/L; ACh) in vessels pre-contracted with phenylephrine (2 µmol/L; PhE). Concentration-effect curves to human regular insulin (Eli Lilly®, Sao Paulo, Brazil) were performed in arteries from all animal groups. In some experiments, the vascular preparations were incubated with Tiron (ROS scavenger, 100 µmol/L), CCX832 (ChemR23 antagonist, 10 nmol/L) or YS-49 (PI3K activator, 1 µmol/L). For the HFD mice, CCX832 was added to the chamber 30 minutes prior the curves.

**Immunoblotting**

Western blotting was developed in cultured VSMC from C57BL/6J and aorta from db/m and db/db mice treated with vehicle or CCX832 as previously described (45). The mesenteric arteries were not further explored for other experiments due to the limitation of the amount of tissue. Briefly, tissues were homogenized in lysis buffer [(in mmol/L) sodium pyrophosphate 50, NaF 50, NaCl 5, EDTA 5, EGTA 5, HEPES 10, Na\textsubscript{3}VO\textsubscript{4} 2, PMSF 50, Triton 100 0.5%, and leupeptin/aprotinin/pepstatin 1 mg/mL] and then sonicated for 5 sec. Proteins (50 – 60 µg) extracted from each lysate were separated by electrophoresis on SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% milk in Tris-buffered saline solution with Tween (TBS-T) for one hour at room temperature. Primary antibodies were diluted in TBS-T containing 3% of BSA and incubated overnight at 4\degree C. After incubation for one hour with secondary antibodies in room temperature, signals were revealed with chemiluminescence, visualized by autoradiography and quantified densitometrically with
open-source software ImageJ (http://imagej.nih.gov/ij/). Results were normalized by β-actin, α-tubulin or respective total proteins. Antibodies were used as follow: anti-eNOS [9572], anti-phospho eNOS [9571], anti-phospho Akt [9271], anti-Akt [9272], anti-phospho PI3K [4228], anti-phospho AMPK [2531], anti-AMPK [2532]; anti-GLUT4 [07-1404] (Millipore; 1:1000); anti Na⁺/K⁺ ATPase (3010 Cell Signaling; 1:10000); anti-α-tubulin [T6074] and anti-β-actin [A2228] (Sigma; 1:10000).

Isolation of membrane and cytosolic fractions

After stimulation, VSMC were treated with ice-cold hypotonic lysis buffer (10 mmol/l Tris, pH 7.4, 1.5 mmol/l MgCl₂, 5 mmol/l KCl, 1 mmol/l DTT, 0.2 mmol/l sodium vanadate, 1 mmol/l PMSF, 1 g/ml aprotinin, 1 g/ml leupeptin). After passing the lysate through a 25-gauge syringe needle with several rapid strokes, the samples were centrifuged at 2,000 g at 4°C for 5 min. The supernatant was recentrifuged at 100,000 g at 4°C for 60 min. The pellet was resuspended in lysis buffer containing 0.1% Triton X-100 and served as the membrane fraction. Proteins were measured by the Bradford method using BSA as the standard. Membrane fraction was used to perform western blot analysis for glucose transporter-4 (GLUT4) translocation.

Immunofluorescence

Paraffin sections of aorta (4 µm) were deparaffinised in xylene, rehydrated through graded ethanol, and washed in water. All sections were incubated in EDTA (pH 8) and boiled for 15 min at 95°C for antigen unmasking. Slides were cooled to room temperature, permeabilized in 0.5% Triton X-100 in PBS for 5 min, and blocked with 10% donkey serum, 1% BSA in 1xTBS-T for one h at room temperature in a humidified chamber. For 8-hydroxyguanosine (8-
OHG) immunostaining, slides were incubated overnight with anti-8-OHG goat polyclonal antibody (Abcam ab10802, 1:200) in a humidified chamber. Alexa-fluor-488-conjugated donkey anti-goat secondary antibody (Molecular probes, A-11055, 1:300) was used after primary antibody incubation for 1 h at room temperature in the dark. The slides were treated with 0.1% Sudan Black B (Sigma Aldrich, 199664) in methanol for 10 min in order to remove lipofuscin-mediated autofluorescence. Nuclei were counterstained with 4-6-diamidino-2-phenylindole (DAPI at 100 µg/ml) for 10 min. Sections were mounted with a coverslip using ProLong Gold anti-fade mounting media containing DAPI (Molecular probes, P-36931) in the dark. Fluorescence images were captured using Axiovert 200M microscope with a laser-scanning module LSM 510 (Carl Zeiss AG, Heidelberg, Germany). Fluorescence quantification was performed using the open-source software ImageJ (http://imagej.nih.gov/ij/) and determined by the average of five different fields captured from each vessel. For negative controls, goat IgG-matched isotype controls were used (Santa Cruz, sc-2028).

Nitric oxide production

Production of nitric oxide (NO) was determined with the NO fluorescent probe diacetate 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-2AM - Life Technologies, Carlsbad, CA, USA) as previously described (45). Endothelial cells were incubated with DAF-FM diacetate (5 µmol/L, 30 min) in serum free media, kept in the dark, and maintained at 37ºC. Cells were washed to remove excess probe and an additional incubation for 10 min was performed to allow complete de-esterification of the intracellular diacetates. Cells were stimulated with chemerin (R&D Systems - 0.5 ng/mL) for one hour and insulin (100 nmol/L), CCX832 (10 nmol/L), N-acetyl-cysteine (NAC; 10 µmol/L) and 740Y-P (PI3K/Akt signalling
activator; 1 µmol/L) for 30 min. After stimulation, cells were washed with PBS and harvested by mild trypsinization (trypsin - 0.025%). Trypsin was inactivated with soybean trypsin inhibitor (0.025%) in PBS (1:1). Additional PBS (37°C) was added to final volume of 10 mL. Cells were centrifuged for 3 min at 3,000 g. Following centrifugation, the supernatant was discarded, and the cell pellet reconstituted in PBS (250 µl). 200 µl of the cell suspension was transferred to black 96 well microplates (Nunc® 436034). The DAF-FM nitrosylation was assessed by a fluorimeter at excitation/emission wavelengths of 495/515 nm. Fluorescence intensity was adjusted to protein concentration.

**Glucose uptake measurement**

VSMC were exposed to chemerin or vehicle, CCX832, Tiron, AICAR (AMPK signalling activator; 1 mmol/L) or 740Y-P in the presence of insulin stimulation and then incubated with buffer Krebs-Ringer-Hepes (15 mmol/L Hepes [pH 7.4], 105 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L CaCl₂, 1 mmol/L KH₂PO₄, 1.4 mmol/L MgSO₄ and 10 mmol/L NaHCO₃) for 2 hours. The cells were incubated with insulin (100 nmol/L) for 30 min and 0.2 mmol/L glucose 2-deoxy-D-glucose containing 1 µCi/ml of 2-deoxy-D- [³H] -glucose was added over 30 min. The buffer was rapidly removed, followed by three washes with ice-cold PBS. Cells were lysed with 500 µL of 0.4 mol/L NaOH for 5 min and neutralized with 500 µL of HCl. The amount of radiolabeled glucose associated with the lysed cells was determined by liquid scintillation counting. To control for changes in osmotic pressure, the effect of insulin on 14C-mannitol uptake was also determined.

**Data and statistical analysis**
All data are expressed as mean±SEM. Relaxation responses are expressed as a percentage of contraction in response to PhE. The individual concentration–response curves were fitted into a curve by non-linear regression analysis. pD2 (defined as the negative logarithm of the EC$_{50}$ values) and maximal response (Emax) values were compared by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. The Prism software, version 5.0 (GraphPad Software Inc., San. Diego, CA, USA) was used to analyze these parameters as well as to fit the sigmoidal curves. To analyze the difference between the two groups an unpaired t-test was used. N represents the number of animals or experiments used and p values less than 0.05 were considered significant.

RESULTS

Chemerin decreases insulin-induced dilatation of mesenteric resistance arteries

As shown in Figure 1A, chemerin decreased insulin-induced relaxation of mesenteric arteries from C57BL/6 mice, an effect blocked by the ChemR23 antagonist CCX832 (Figure 1B). An activator of PI3K signalling (YS-49) and a ROS scavenger (Tiron) reversed chemerin effects on insulin-induced vasodilatation (Figures 1C and 1D).

Chemerin decreases insulin-induced NO signalling activation in endothelial cells

To verify whether chemerin affects NO signalling, HMEC were exposed to chemerin (0.5 mg/ml) and probed for eNOS phosphorylation. Chemerin decreased basal and insulin-induced eNOS phosphorylation of the active site (Ser$^{1177}$) (Figure 1E). These effects were attenuated both
by a ROS scavenger (N-acetyl-l-cysteine – NAC) and a PI3K activator (740Y-P) (Figure 1E), indicating that oxidative stress mechanisms and insulin-downstream signalling are involved in chemerin-induced decreased NO bioavailability, leading to endothelial dysfunction.

To determine whether reduced eNOS phosphorylation was associated with decreased NO production, levels of NO were estimated in insulin and chemerin-stimulated HMEC. Chemerin decreased NO levels in HMEC. Insulin increased NO levels in these cells and, in the presence of chemerin, insulin-induced NO production was significantly reduced. This effect involves ChemR23-, PI3K signalling- and ROS-dependent mechanisms since CCX832, 740Y-P and NAC partially inhibited chemerin effects (Figure 1F).

**Chemerin decreases insulin signalling in VSMC**

In addition to its effects on endothelial cells, chemerin decreased insulin signalling in VSMC. Insulin increased Akt phosphorylation in VSMC and chemerin decreased insulin-induced Akt phosphorylation via ChemR23 and ROS-dependent mechanisms (Figure 2A).

To further investigate chemerin effects on insulin signalling, GLUT4 translocation from the intracellular membrane compartments to the membrane was determined. In VSMC, chemerin decreased insulin-induced GLUT4 translocation to the membrane (Figure 2B), which was blocked by CCX832 and 740Y-P (Figure 2B). This suggests that ChemR23 and PI3K activation is contributing to the chemerin effects on GLUT4 translocation in VSMC.

**Chemerin reduces insulin-stimulated glucose uptake by VSMC**

We evaluated the effects of chemerin on AMPK phosphorylation, another mechanism for glucose uptake. Chemerin decreased AMPK phosphorylation (Thr\(^{172}\)) both in VSMC and HMEC
(Figures 2C and 2D). To evaluate whether chemerin also affects insulin-induced glucose uptake, 2-deoxyglucose uptake was measured in VSMC exposed to 2 μCi/ml 2-[³H]DG in the presence of unlabelled 2-DG. In VSMC, chemerin decreased insulin-induced glucose uptake (Figure 3) through ROS generation, AMPK and PI3K/Akt signalling since Tiron, AICAR, CCX832 and 740Y-P reversed the effects of chemerin on glucose transport (Figure 3).

**ChemR23 antagonism decreases body weight and insulin and glucose levels in db/db mice**

Since chemerin influences vascular insulin signalling, we investigated whether ChemR23 antagonism attenuates diabetes-related vascular dysfunction and insulin resistance. Plasma chemerin levels were significantly increased in diabetic obese db/db mice (Figure 4A). CCX832 treatment slightly reduced body weight of db/db mice when compared to vehicle-treated db/db mice (Figure 4B). In addition, CCX-treated db/db mice exhibited decreased plasma levels of glucose and insulin vs. vehicle-treated db/db mice (Figures 4C and 4D). The HOMA index indicated that CCX832 improves insulin sensitivity in db/db mice (Figure 4E). Increased cholesterol and triglyceride levels in db/db mice were reduced by CCX832. No differences in the lipid profile were observed in db/m mice treated with vehicle or CCX832 (Figures 4F and 4G).

**CCX832 partially reverses dysfunctional insulin responses in vessels from db/db mice**

As shown in Figure 5A, insulin-induced dilation was decreased in mesenteric arteries from vehicle-treated db/db mice vs. vehicle-treated db/m mice. CCX832 treatment partially improved insulin-induced vasodilation in db/db mice (Figure 5A). Moreover, Akt (Ser⁴⁷³) phosphorylation was reduced in aorta from vehicle-treated db/db mice vs. vehicle-treated db/m
mice and CCX832-treated db/db mice (Figure 5B), indicating beneficial effects of in vivo treatment with CCX832 on insulin signalling.

Additionally, as observed in Figure 5C, increased levels of chemerin were also observed in HFD-fed mice. The ChemR23 antagonist CCX832 attenuated impaired insulin-induced dilatation in mesenteric arteries from HFD-fed mice (Figure 5D).

**CCX832 reduces vascular oxidative stress in db/db mice**

Considering that chemerin increases ROS generation (45) and that oxidative stress accounted for most of the effects of chemerin upon vascular insulin signalling, we investigated whether ChemR23 antagonism attenuates ROS generation in db/db mice. Diabetic mice presented increased vascular levels of DNA oxidation products in comparison to vehicle- and CCX832-treated db/m mice (Figures 6A and 6B). This was reduced by treatment of db/db mice with CCX832 (Figures 6A and 6B).

**DISCUSSION**

Our results demonstrated that (1) chemerin decreases insulin-induced vasodilation by mechanisms involving ChemR23 activation, disruption of PI3K/Akt signalling and oxidative stress; (2) chemerin decreases insulin-induced eNOS phosphorylation and NO production in endothelial cells through mechanisms that affect ROS generation and PI3K/Akt signalling; (3) chemerin decreases activation of insulin signalling pathways via ChemR23 and oxidative stress in VSMC; (4) ChemR23 antagonism with CCX832 decreases glucose and insulin levels in db/db mice; (5) CCX832 partially restores insulin-induced vasodilatation and improves insulin
signalling in diabetic obese mice; (6) CCX832 decreases vascular oxidative stress in db/db mice. These novel findings show that the chemerin/ChemR23 axis plays a critical role in diabetes-associated vascular oxidative stress and altered insulin signalling.

Chemerin was first described as a chemoattractant agent, promoting chemotaxis of leukocyte populations that express ChemR23. Later on, chemerin was associated with regulation of key effectors of the glucose and lipid metabolism in mature adipocytes (11) as well as a regulator of adipogenesis in 3T3-L1 cells (14). Regarding vascular homeostasis, chemerin has been shown to promote proliferation of endothelial cells and release of MMP-2 and MMP-9, suggesting a contribution of the chemerin/ChemR23 system in angiogenesis. In co-cultures of fibroblast and endothelial cells, chemerin promotes the formation of endothelial tubules in a MAPK-dependent manner (8). On the other hand, in obesity, production of adipokines triggers chronic inflammation and leads to increased levels of proinflammatory cytokines (20), which can modify insulin signalling. TNF-α and IL-6, for example, are good predictors of the development of T2D (13). Of importance, chemerin has been associated with upregulation of IL-1β, TNF-α, IL-6 and MCP-1 (2, 45), an additional and potential mechanism whereby chemerin signals may modulate vascular oxidative stress and altered insulin signaling. TNF-α for example has been identified as a regulator of insulin sensitivity (40). In 3T3-L1 adipocytes, IL-6 reduces expression of insulin receptor substrate (IRS-1) and the glucose transporter GLUT4 (36).

Similarly, increased levels of TNF-α reduce IRS-1 phosphorylation and decrease insulin signal transduction in skeletal muscle and adipose tissue from obese rats (18). Accordingly, our data show that chemerin, a pro-inflammatory adipokine, decreases insulin-induced phosphorylation of Akt as well as reduces GLUT4 translocation to the membrane and insulin-induced glucose uptake in VSMC from control mice. Molecular effects of chemerin in vascular insulin signalling
are associated with a decrease in insulin-induced vasodilation, by mechanisms that affect Akt signalling and oxidative status. Together, these findings suggest that the adipokine chemerin confers an insulin resistant phenotype to VSMC.

Corroborating our data, a recent study demonstrated that chemerin decreases glucose uptake and Akt phosphorylation in insulin-stimulated cardiomyocytes (52). Inhibition of ERK1/2 MAPK partially prevented chemerin-induced impairment of Akt phosphorylation and glucose uptake (52). In addition, Jager and colleagues have shown that interleukin-1β (IL-1β) reduces insulin-induced Akt phosphorylation (Thr\textsuperscript{308}). Similarly, IL-1β decreases IRS-1 and Akt protein expression in adipocytes, which leads to lower insulin-induced glucose uptake (19).

Furthermore, db/db mice treated with ChemR23 antagonist (CCX832) present a slight decrease in body weight, supporting the suggestion that chemerin regulates adipogenesis via ChemR23 receptor (34). However, the effects of depletion or inhibition of ChemR23 upon body weight are still controversial. While Rouger et al did not observe changes in body weight or fat mass in ChemR23 knockout (KO) mice (37), Ernst and colleagues showed that CMKLR1\textsuperscript{−/−} mice have lower food intake, total body mass, and percent of body fat compared with wild-type controls (9).

In addition to considering ChemR23 blockade effects on body weight, it is important to consider metabolic aspects, such as glucose and insulin levels. CCX832-treated db/db mice exhibited a significant decrease in plasma glucose and insulin levels and HOMA index, strengthening the notion that chemerin contributes to insulin resistance in this model of obesity related to T2D. In addition, cholesterol and triglyceride levels in db/db mice were also reduced by CCX832. Of importance, significantly increased levels of chemerin were observed both in db/db and HFD-treated mice, reinforcing the idea that this adipokine is linked to glucose
homeostasis and metabolic disorders in obesity and T2D (10). Ernst and colleagues demonstrated that CMKLR1<sup>−/−</sup> mice fed with high fat diet present reduced blood glucose and serum insulin. However, CMKLR1 loss is associated with glucose intolerance, which was linked to decreased glucose-stimulated insulin secretion (9). On the other hand, Rouger and colleagues verified that glucose tolerance was not affected in young ChemR23 knockout mice (37).

Classically, insulin metabolic signalling results in vasodilatation via increased NO production and production of prostaglandins and endothelium-derived hyperpolarizing factors (30). In addition to reducing insulin-induced vasodilatation in non-obese mice by mechanisms involving PI3K/Akt pathway and oxidative stress, chemerin also reduces eNOS phosphorylation (Ser<sup>1177</sup>) and decreases insulin-stimulated NO production in endothelial cells, also by oxidative stress-mediated events. These effects of chemerin may be directly involved in vascular insulin dysfunction associated with diabetes and obesity. It has been proposed that resistance to the vascular effects of insulin selectively involves the PI3K/Akt/NO signalling pathway (29). In endothelial cells, blockade of the PI3K pathway induces insulin resistance, which in turn blunts production of NO (28). As demonstrated in this study, most of chemerin effects are mediated by ChemR23 since its antagonism partially restores the decreased insulin-induced vasodilatation in both obesity models, db/db and HFD-fed mice, and also chemerin decreases vascular Akt phosphorylation in db/db mice.

Oxidative stress contributes to insulin resistance, particularly in skeletal muscle as well as dysfunction of pancreatic β cells (12). Cellular and systemic disorders that may contribute to overproduction of reactive oxygen species (ROS), including hyperglycaemia, dyslipidaemia, endoplasmic reticulum (ER) stress, advanced glycation end products (AGEs), nitric oxide synthase and lipid peroxides, can activate factors associated with insulin resistance (43). In
humans, there is a positive association between serum markers of oxidative stress and the degree of insulin resistance (32). In experimental animal models, oxidative stress interrupts IRS-1 and PI3K activation induced by insulin, and impairs the translocation of GLUT4 in 3T3-L1 adipocytes (44). In adipose tissue from healthy men with excessive caloric intake, oxidative stress induces insulin resistance partially via carbonylation and oxidation-induced inactivation of GLUT4 (3). Hence, increased ROS generation in vessels from db/db mice may be associated with decreased insulin signalling observed in those animals. In addition, chemerin/ChemR23 axis may contribute to insulin resistance since ChemR23 antagonism attenuates ROS generation and also restores insulin signalling in vessels from db/db mice. Furthermore, our findings clearly show the involvement of oxidative stress upon chemerin-induced insulin signalling impairment since antioxidants attenuate the reduced Akt phosphorylation as well as the decreased glucose uptake and insulin-induced vasodilatation observed in VSMC and arteries challenged with chemerin.

This study also demonstrates that chemerin decreases AMPK phosphorylation both in EC and VSMC. AMPK is a serine-threonine kinase that has emerged as an important mediator of glucose metabolism and AMPK-activating drugs are potentially useful in T2D treatment. It is already established that acute AMPK activation stimulates glucose uptake by skeletal muscle via both GLUT1 and GLUT4 (39). In cardiomyocytes, AMPK activation increases GLUT4 expression in a PKC-dependent manner (31). Moreover, AMPK activation by 5-amino-4-imidazole-1-β-D-carboxamide ribofuranoside (AICAR) increases heart and skeletal muscle glucose uptake (27, 38) possibly by increasing GLUT4 expression (17). Most importantly, overexpression of human GLUT4 gene protects db/db mice from insulin resistance and diabetes (5). Similarly, our findings show that AICAR (and also PI3K/Akt activation) reverses the effects
of chemerin on insulin-induced glucose uptake in VSMC from normal mice, in accordance with
the diminished AMPK phosphorylation in response to chemerin.

Crosstalk between GPCRs and tyrosine kinase receptors (RTKs) is increasingly being
recognized as a major mechanism regulating complex signalling pathways (25). ChemR23 is an
G protein-coupled receptor that acts through G_i to activate various intracellular signalling
pathways, such as ERK1/2 (49). The insulin receptor (IR) belongs to the family of RTKs, which
play a critical role in development, cell division, and metabolism. Activation of RTK family
members results in autophosphorylation and phosphorylation of selective protein substrates
(IRs) exclusively on tyrosine residues. Recently, various likely mechanisms for the
transactivation between GPCR and RTK have emerged. Molecules such as PKC, Src and ROS as
well as adaptor/scaffold proteins such as Gab1, IRS-1, and GIT1 have been implicated as
mediators of GPCR-ligand induced RTK transactivation (23). This study shows that chemerin,
through ChemR23, modulates IRS-1 serine phosphorylation and the subsequent signalling
cascade. Recently, chemerin has been shown to stimulate bone marrow-derived mesenchymal
stromal cells migration via ERK1/2, p38MAPK and JNK-II kinases in a PKC-dependent manner
(22). A limitation of the present study is that mechanisms underlying ChemR23 and IR/IRS
crosstalk have not been explored. Thus, further studies are needed to understand and dissect
specific proteins linking ChemR23 and IR/IRS signalling.

In conclusion, our data indicate that the chemerin/ChemR23 system plays an important
role in diabetes-associated impaired vascular insulin signalling and oxidative stress, suggesting
its involvement in the pathogenesis of vascular insulin resistance. Antagonism of the system
ameliorates vascular dysfunction and normalizes insulin signalling in a T2D animal model.
Targeting chemerin/ChemR23 may be an attractive strategy to improve insulin signalling and vascular function in obesity-associated diabetes.

**PERSPECTIVES AND SIGNIFICANCE**

Adipokines participate in many physiological processes implicated in cardiovascular complications associated with obesity and diabetes. Advances in the understanding of the role of adipocyte-derived factors in obesity-associated vascular dysfunction may uncover mechanisms involved in adiposity-related diseases. Chemerin/ChemR23 system plays an important role in diabetes-associated impaired vascular insulin signalling and oxidative stress, suggesting its involvement in the pathogenesis of vascular insulin resistance. Antagonism of the system ameliorates vascular dysfunction and normalizes insulin signalling in a T2D animal model. We believe that targeting chemerin/ChemR23 may be an attractive therapeutic strategy to improve insulin signalling and vascular function in obesity-associated diabetes.

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DISCLOSURES

None.

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**FIGURES LEGENDS**

**Figure 1. Chemerin decreases insulin-induced vasodilatation via ChemR23, Akt/NO and oxidative stress-dependent mechanisms.** Endothelium-intact mesenteric arteries from C57BL/6J mice were incubated with chemerin (0.5 ng/mL) or vehicle for one hour. Relaxation response to insulin was evaluated in the absence (A) and in the presence of (B) CCX832 (ChemR23 antagonist), (C) YS-49 (PI3K activator) or (D) Tiron (ROS scavenger), which were added 30 min before the vehicle or chemerin. Vehicle and chemerin curves in A-D were originated from the same sets of experiments. (E) Phosphorylation of eNOS (Ser1177) was determined by western blot in endothelial cells stimulated with chemerin (0.5 ng/mL, one hour) and insulin (30 min) in the absence or presence of CCX832, NAC (ROS scavenger) or 740Y-P (PI3K activator). Values were normalized by total NOS expression. (F) Nitric oxide (NO)
production was determined by DAF2-DA fluorescence in endothelial cells and values were normalized by protein amount. Results represent the mean ± SEM of 5 to 6 experiments. Data were analyzed using one-way ANOVA followed by a post hoc Tukey test. * p<0.05 vs. vehicle; # p<0.05 vs. chemerin; σ vs. insulin, φ vs. chemerin + insulin.

Figure 2. Chemerin decreases vascular insulin signalling in VSMC. Phosphorylation of Akt (A), GLUT4 translocation to the membrane (B), phosphorylation of AMPK in VSMC (C) and HMEC (D) were determined by western blot. Cells were pre-treated with CCX832, NAC or 740Y-P, added 30 min before the stimulation with chemerin and/or insulin. Values were normalized by total protein or Na+/K+ ATPase. To keep the same sequence of experimental groups in all graphs, representative western blot figures for Akt were spliced as indicated (C). Data represent the mean ± SEM of 4 to 6 experiments. Data were analyzed using one-way ANOVA followed by a post hoc Tukey test or t-test. * p<0.05 vs. vehicle; # p<0.05 vs. chemerin, σ p<0.05 vs. insulin, φ p<0.05 vs. chemerin + insulin.

Figure 3. Chemerin reduces insulin-stimulated glucose uptake in VSMC. 2-Deoxyglucose (2-DG) uptake was measured by exposing VSMC to 2 μCi/ml 2-[3H] DG in the presence of unlabelled 2-DG. Cells were treated with chemerin, Tiron, AICAR (AMPK activator), CCX832 and 740Y-P prior to the stimulation with insulin. Values represent the mean ± SEM of 5 to 6 experiments. Data were analyzed using one-way ANOVA followed by a post hoc Tukey test. * p<0.05 vs. vehicle, # p<0.05 vs. chemerin.
Figure 4. ChemR23 antagonism decreases body weight and insulin and glucose levels in db/db mice. (A) Plasma levels of chemerin were measured by ELISA in db/m and db/db mice. (B) Body weight of untreated, vehicle-treated and CCX832-treated db/m and db/db mice was measured along three weeks of treatment. Glucose (C) and insulin (D) levels in vehicle- and CCX832-treated db/m and db/db mice. (E) Insulin sensitivity, calculated using the HOMA-IR index, cholesterol (F) and triglyceride (G) levels in vehicle- and CCX832-treated db/m and db/db mice. Values represent the mean ± SEM of 5 to 8 experiments. Data were analyzed using one-way ANOVA followed by a post hoc Tukey test or t-test. * p<0.05 vs. db/m, \( \infty \) p<0.05 vs. db/db vehicle.

Figure 5. CCX832 attenuates vascular insulin dysfunction in db/db and HFD-fed mice. Concentration-effect curves to insulin (A) were performed in mesenteric arteries from vehicle- and CCX832-treated db/m and db/db mice. Phosphorylation of Akt (B) in aorta from vehicle and CCX832-treated db/m and db/db mice was determined by western blot. Values were normalized by total protein expression. (C) Plasma levels of chemerin were measured by ELISA in control diet or HFD- fed mice. (D) Concentration-effect curves to insulin were performed in mesenteric arteries from control diet- and HFD-treated mice. Curves were performed in the presence and absence of CC832 added 30 minutes prior curves. Results represent the mean ± SEM of 5 to 8 experiments. Data were analyzed using one-way ANOVA followed by a post hoc Tukey test or t-test. * p<0.05 vs. db/m, \( \infty \) p<0.05 vs. db/db vehicle; \( \Theta \) p<0.05 vs. control diet, \( \dagger \) p<0.05 vs. HFD.

Figure 6. CCX832 reduces vascular oxidative stress in db/db mice. (A) Representative images and (B) quantitative analysis of 8-OHG-positive nuclei in endothelium-intact aorta from vehicle and CCX832-treated db/m and db/db mice. Scale bar = 20 \( \mu \)m; 40X. Results represent
the mean ± SEM of 5 experiments. Data were analyzed using one-way ANOVA followed by a post hoc Tukey test or t-test. * p<0.05 vs. db/m, ∞ p<0.05 vs. db/db vehicle.
**Figure 2.**

(A) Western blot analysis of phospho-Akt (Ser473) (60 kDa), Total-Akt (60 kDa), and α-tubulin (50 kDa) in VSMC.

(B) Western blot analysis of GLUT4 (55 kDa) and Na+/K+ ATPase (113 kDa) in VSMC.

(C) Western blot analysis of phospho-AMPK (Thr172) (62 kDa), Total-AMPK (62 kDa), and α-tubulin (50 kDa) in VSMC.

(D) Western blot analysis of phospho-AMPK (Thr172) (62 kDa) in EC.

* indicates significant difference from Vehicle, # indicates significant difference from Insulin, σ indicates significant difference from CCX832, and φ indicates significant difference from NAC.
Figure 3.

[3H] 2-deoxy-D-glucose Uptake (Counts per minute)
Figure 4.

A. Plasma

- **Chemerin levels**
  - db/m
  - db/db

B. Body Weight (g)

- Untreated db/m
- db/m Vehicle
- db/m CCX832
- Untreated db/db
- db/db Vehicle
- db/db CCX832

C. Glucose

- db/m Vehicle
- db/m CCX832
- db/db Vehicle
- db/db CCX832

D. Insulin

- db/m Vehicle
- db/m CCX832
- db/db Vehicle
- db/db CCX832

E. HOMA-IR

- db/m Vehicle
- db/m CCX832
- db/db Vehicle
- db/db CCX832

F. Cholesterol

- db/m Vehicle
- db/m CCX832
- db/db Vehicle
- db/db CCX832

G. Triglycerides

- db/m Vehicle
- db/m CCX832
- db/db Vehicle
- db/db CCX832
Figure 5.

A

B

C

D

- Log [M] Insulin

Relaxation (%)

Vehicle

CCX832

p-Akt / t-Akt

HFD

Control Diet

HFD_CCX832

Chemerin levels

ng/mL

Control Diet

HFD

Chemerin levels

ng/mL

Control Diet_CCX832

HFD_CCX832

Relaxation (%) PhE

- Log [M] Insulin

p-Akt [Ser 473] (60 kDa)

Total Akt (60 kDa)

α-tubulin (50 kDa)
Figure 6.

<table>
<thead>
<tr>
<th>Condition</th>
<th>8-OHG Intensity (Folds of change)</th>
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<tbody>
<tr>
<td>db/m Vehicle</td>
<td>1</td>
</tr>
<tr>
<td>db/m CCX832</td>
<td>2</td>
</tr>
<tr>
<td>db/db Vehicle</td>
<td>3</td>
</tr>
<tr>
<td>db/db CCX832</td>
<td>4</td>
</tr>
</tbody>
</table>

B

![Bar chart showing 8-OHG intensity comparison](chart.png)