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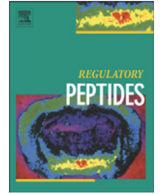

The insulin sensitizing effects of PPAR-γ agonist are associated to changes in adiponectin index and adiponectin receptors in Zucker fatty rats

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ABSTRACT

The adiponectin high molecular weight isoform (HMW-adp) and its relation with the other adiponectin isoforms (adiponectin index, S_A), have been identified as essential for the adiponectin insulin sensitizing effects. The objective of this study is to gain further insight on the effect of the insulin sensitizing agents, PPAR-γ agonists, on the distribution of the adiponectin isoforms and the adiponectin receptors, adipoR1 and adipoR2 in an animal model of obesity and insulin resistance.

To achieve the objective, Zucker fatty rats were treated with pioglitazone, rosiglitazone or placebo for six weeks. At the end of the treatment, total adiponectin, adiponectin isoforms and adiponectin receptors expression were measured. In order to see the possible relation with insulin sensitivity parameters, HOMA-IR, muscle insulin-stimulated glucose transport, muscle GLUT4 and plasma free fatty acids were also measured.

The two glitazones improved insulin sensitivity and both muscle insulin-stimulated glucose transport and GLUT4 total content. Total plasma adiponectin and visceral fat HMW-adp were increased only by pioglitazone. On the other hand, both glitazones changed the distribution of adiponectin isoforms in plasma, leading to an increase in the S_A of 21% by pioglitazone and 31% by rosiglitazone. Muscle adipoR1 expression was increased by both glitazones whereas liver adipoR2 expression was increased by rosiglitazone and tended to increase in the pioglitazone group.

The insulin sensitizing action of glitazones is mediated, at least in part, by their effect on muscle insulin-stimulated glucose transport and by their direct influence on the adiponectin index and the adiponectin receptors expression.

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1. Introduction

In recent years, the prevalence of obesity has increased around the world, and now affects approximately 300 million people (The World Health Report 2002: Reducing Risks, Promoting Healthy Life, World Health Organization, Geneva, 2002). Obesity is usually associated with insulin resistance and frequently leads to the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) [1], thus posing a substantial worldwide economic burden [2].

To date, adipose tissue has been known mostly for its energy storage functions. In recent years, it has also been recognized as an endocrine organ participating in the regulation of many different processes such as energetic metabolism and inflammatory and immune response [3]. These functions are accomplished by the secretion of adipokines, mostly hormones, which are able to act in a paracrine, autocrine and endocrine fashion. Changes in adipokine regulation observed in obese patients may be involved in insulin resistance, T2DM and CVD development [4].

Compared to other adipokines, adiponectin has attracted a great deal of attention due to the fact that it regulates glucose and lipid metabolism, thereby directly influencing insulin sensitivity [5]. The effects of adiponectin are mediated by its molecular forms and its receptors. Blood circulating adiponectin can be present in at least three full-length forms that have been differently classified and named in the last years: the adiponectin trimer (MW = 90 kDa), the medium-molecular weight adiponectin or adiponectin hexamer (MMW-adp, MW = 180 kDa) and the high-molecular weight adiponectin that contains 12 to 18 adiponectin trimers (HMW-adp, MW > 300 kDa) [6].

It seems that HMW-adp is the most biologically relevant molecular form in terms of insulin sensitivity regulation and protection against T2DM. HMW-adp may also be a good predictor of insulin resistance, metabolic syndrome (MS) and T2DM [6]. HMW-adp and its ratio to total adiponectin, defined as the adiponectin sensitivity index (S_A), are more likely to be associated to T2DM and MS than total adiponectin [7,8]

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The adiponectin receptors adipoR1 and adipoR2 are present in many tissues and have affinity for all full-length forms of adiponectin. AdipoR1 is ubiquitously expressed, though even more abundantly in muscle; adipoR2, on the other hand, is mainly expressed in liver [6]. AdipoR1 and adipoR2 mediate adiponectin action by different pathways including activation of the AMP-activated protein kinase (AMPK), stimulation of the PPAR-α and increase of fatty acid oxidation [6]. Both receptors have been implicated in insulin sensitivity. Adiponectin receptors are down-regulated in mouse models of obesity and insulin resistance, and after stimulation of those receptors an amelioration of insulin sensitivity has been shown [6].

Thiazolidinediones (TZDs), drugs with PPAR-γ agonist action, are known to improve insulin sensitivity and lipid profile, and to reduce many cardiovascular risk factors such as arterial hypertension, microalbuminuria, procoagulant and proinflammatory factors. Current treatment of T2DM with pioglitazone is based on the above facts [9]. The exact mechanism by which these compounds improve insulin sensitivity is unknown.

We hypothesize that this action could be mediated by the influence of TZDs on adiponectin production, adiponectin index (S_A) and/or on receptor-mediated action. To test this hypothesis, we conducted a study using the Zucker fatty rat evaluating the effect of TZDs on plasma and adipose tissues adiponectin molecular forms content, and receptor expression in muscle and liver.

2. Materials and methods

2.1. Type of study

An experimental study was performed on 30 male Zucker fatty rats that were randomly assigned to receive placebo, pioglitazone or rosiglitazone.

Sample size calculation: To obtain at least a 25% difference with a statistical power of 80% and an alpha level of 0.05 in plasma adiponectin levels between the TZD groups and the placebo group, a minimum of 8 animals were needed per group.

2.2. Animal studies

Zucker fatty rats (ZFR, fa/fa) were obtained from Harlan Iberica S.L at the age of 4 weeks. They were fed with standard chow and water ad libitum throughout the study and kept in sterile cages in a barrier animal facility with a 12-hour light/dark cycle.

At the age of 6 weeks they were randomly assigned to groups in which they received placebo, pioglitazone (Lilly S.A., Madrid, Spain) or rosiglitazone (GlaxoSmithKline, London, England).

Each treatment group (pio group or rosi group, n = 10) received 3 mg/kg·day of pioglitazone or rosiglitazone diluted in carboxymethylcellulose. The control group (n = 10) received the carboxymethylcellulose alone. Drug doses were adjusted every week and administrated daily for 6 weeks by oral gavage. Before the end of the treatment (week 5), one rat from the rosiglitazone group died, ending this group with only 9 rats.

Every 2 weeks blood was extracted from the tail vein after an overnight fast. Following a glucose determination (Glucocard, Menarini Diagnósticos, Barcelona, Spain), plasma was obtained by centrifugation and stored at −20 °C for future determinations. At the end of the treatment and after an overnight fast, the rats were sacrificed by exsanguination, blood was obtained from the heart and plasma was obtained and stored at −20 °C. Visceral and subcutaneous adipose tissue, liver tissue and the gastrocnemius muscle were removed and stored at −70 °C for further experiments. The soleus muscle was removed and immediately prepared for assessment of insulin-stimulated glucose transport.

Plasma insulin levels were determined by radioimmunoassay (RIA) (DRG Diagnostics, Mountainside, NJ) on samples obtained in weeks 0, 2, 4 and 6.

To evaluate the degree of insulin resistance we calculated the homeostasis model assessment index (HOMA-IR index, [insulin (μU/ml) × Glucose (mmol/L]/22.5) at 0, 2, 4 and 6 weeks of treatment.

Free fatty acids were determined at 0 and 6 weeks using the NEFA C ACS-ACOD commercial kit according to the manufacturer's instructions (Wako Chemicals GmbH, Neuss, Germany).

All animal protocols used in this study were previously approved by the animal ethics committee of the Fundación Jiménez Díaz – Capio.

2.3. Muscle insulin-stimulated glucose transport, GLUT4 total content and AMPK activity

Insulin-stimulated glucose transport in the soleus muscle was measured immediately after the animals were sacrificed. Briefly, the muscles were clamped to prevent contraction and preincubated at 37 °C under 95%O_2-5%CO_2 in glucose-free Krebs-Henseleit buffer (KHB) containing 0.1% BSA and 1 mM pyruvate for 1 h. During the last 10 min of the preincubation period, insulin (10^{-7} M) was added. After 1 h of preincubation, muscles were transferred into fresh identical medium containing 2-deoxyglucose (2 μCi/ml, 5 mM) and ^{14}C sorbitol (0.11 μCi/ml, 20 mM) and were incubated for 60 min in the absence and presence of insulin (10^{-7} M). Glucose uptake was terminated by washing the muscles in ice-cold KHB. Thereafter, the muscles were dissolved in solubilization buffer containing 0.3 M Cetyltrimethylammonium Bromide ($C_{19}H_{42}NBr$) and 0.3 M KOH. Sample-associated radioactivity was determined by scintillation counting. ^{14}C sorbitol was used as an extracellular space marker.

GLUT4, AMPKα1 and AMPKα phosphorylated on Thr172 (P-AMPKα) total content was measured in gastrocnemius muscle by Western blot. Briefly, after protein extractions following the trizol method (Sigma-Aldrich, St. Louis, MO), proteins were subjected to SDS-PAGE electrophoresis. For immunoblotting, proteins separated by SDS-PAGE were transferred to PVDF membranes. The membranes were blocked for 1 h at room temperature in a TBS buffer containing 10% of skim milk and then incubated with an anti-GLUT4 (Chemicon, Rosemont, IL), anti-AMPKα1 or Anti-P-AMPKα (Millipore, Billerica, MA) polyclonal antibodies. After washing, the membranes were incubated with the secondary antibody (Amersham Biosciences, Piscataway, NJ) and exposed to x-ray film (Amersham Biosciences, Piscataway, NJ) using the ECL Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ). After washing the membranes, α-Tubulin detection was used to correct differences in the protein loading for GLUT4. Finally, AMPK activity was calculated as following; P-AMPKα/AMPKα1.

2.4. Adiponectin plasma levels and adipose tissue content

At the end of the 6-week treatment, total adiponectin content in plasma and visceral and subcutaneous adipose tissue was determined by ELISA (Linco Research, Billerica, MA) following the manufacturer's instructions.

Adiponectin isoforms in plasma and adipose tissues were determined by Western blot analysis under non-reducing and non-heat denaturing conditions, as described by Waki *et al.* [10]. Briefly, 10 μl of plasma or 25 μg of proteins obtained after cellular lysis were subjected to SDS-PAGE in 4-15% acrylamide gradient gels. For non-reducing conditions, 2-mercaptoethanol and 1,4-Dithioerythritol (DTE) were excluded from the sample buffer. For non-denaturising conditions, samples were incubated for 1 hour at room temperature instead of the usual 95 °C for 10 min. For immunoblotting, proteins separated by SDS-PAGE in 4-15% acrylamide gradient gels were transferred to PVDF membranes. The membranes were blocked for 1 h at room temperature in a TBS buffer containing 10% of skim milk and then incubated with an anti-adiponectin polyclonal antibody (Chemicon,

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