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Pioglitazone improves insulin sensitivity and reduces weight loss in Walker-256 tumor-bearing rats

Flaviane de Fatima Silva [a], Milene Ortiz-Silva [a], Winny Beatriz de Souza Galia [a], Priscila Cassolla [a], Maria Fernanda Rodrigues Graciano [a], Cassia Thaïs Bussamra Vieira Zaia [a], Dimas Zaia [b], Ângelo Rafael Carpinelli [c], Francemilson Goulart da Silva [c], Helenir Medri de Souza [a,*]

[a] Department of Physiological Sciences, State University of Londrina, 86051-990 Londrina, PR, Brazil
[b] Department of Chemistry, State University of Londrina, 86051-990 Londrina, PR, Brazil
[c] Department of Physiology and Biophysics, University of São Paulo, 05508-900 São Paulo, PR, Brazil

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ABSTRACT

Aim: The lipogenic effect of pioglitazone (PGZ), an insulin (INS) sensitizer, is well established. However, few studies have evaluated PGZ effects in preventing weight loss in cancer. We investigated PGZ effects, alone or associated with INS, on INS resistance, cachexia and metabolic abnormalities induced by Walker-256 tumor in rats.
Main methods: PGZ (5.0 mg·kg^{-1}, oral) or PGZ + INS (NPH, 1.0 UI·kg^{-1}, sc), were once-daily administered during 12 days, starting on the day inoculation of Walker-256 tumor cells. Rats were separated in small (about 17 g) and big (about 30 g) tumor-bearing.
Key findings: Big tumor-bearing rats showed greater cachexia, blood triacylglycerol and free fatty acids and INS resistance. PGZ and PGZ + INS treatments did not change tumor growth and food intake, but reduced several abnormalities such as INS resistance, increased blood free fatty acids, retroperitoneal fat wasting and body weight loss in small tumor-bearing rats. The prevention of retroperitoneal fat wasting did not involve reduction of tumor necrosis factor-α expression increased. In big tumor-bearing rats, PGZ and PGZ + INS treatments reversed the high blood triacylglycerol and free fatty acids levels, but had no effect on other parameters.
Significance: PGZ and PGZ + INS improved INS peripheral sensitivity, possibly by decreasing blood free fatty acids, and reduced fat tissue wasting and body weight loss in small tumor-bearing rats. The results suggest clinical benefits of PGZ in preventing INS resistance, adipose tissue wasting and weight loss when the tumor is small, *i.e.*, in less severe cachexia.

1. Introduction

Cancer cachexia is a syndrome characterized by severe catabolism, which results in muscle and adipose tissue loss marked and consequently of body weight [1–3]. Cachexia is the main cause of morbidity and mortality in advanced cancer patients [4], since effective clinical treatments to overcome this syndrome are not currently available.

The skeletal muscle and fat tissue wasting in cancer cachexia is caused by molecules known as mediators of cachexia, produced by tumor cells, such as proteolysis inducing factor (PIF) and lipid mobilizing factor (LMF), as well as by host tissues, such as tumor necrosis factor (TNFα), interleukin 1 (IL1) and 6 (IL6), which are capable to stimulate proteolysis and lipolysis [3,5].

However, it is possible that insulin (INS) resistance, often observed in patients [6–10] and animal models of cancer [11–13], contributes to exacerbation of catabolic processes in muscle and fatty tissue and therefore to aggravate cachexia, since INS exerts potent anabolic and anti-catabolic effects [14].

Accordingly, it was reported that INS resistance is present before the onset of weight loss in mice with colon-26 adenocarcinoma and rosiglitazone treatment, an INS sensitizer, improved INS sensitivity and attenuated early stages of cachexia, without changing tumor mass [11]. In addition, in later stages of cachexia, treatment with rosiglitazone attenuated fat and body mass loss in colon-26 adenocarcinoma bearing mice [15]. Although these findings [11,15] suggest a role for INS resistance in cachexia pathogenesis, few studies have investigated the effect of INS sensitizing agents on prevention of weight loss associated with cancer.

Pioglitazone (PGZ), as the rosiglitazone, is an INS sensitizer of the class of thiazolidinediones (TZDs) used for the treatment of type 2 diabetes. The INS sensitizing effect of TZDs occurs by activation of

* Corresponding author.
E-mail address: hmedri@uel.br (H.M. de Souza).

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peroxisome proliferator-activated receptor gamma (PPARγ) in adipose tissue [16,17]. PPARγ increases the expression of genes responsible for uptake, storage and metabolism of lipids, reducing blood free fatty acids and triacylglycerol, and consequently the negative interference of free fatty acids in muscle and liver INS signaling, improving insulin signaling in these tissues [18,19]. Free fatty acids, which are increased in cancer bearing blood [20], impair INS signaling by decreasing the proteins phosphorylation of INS signaling pathway, such as the protein kinase B (Akt or PKB) [21–24]. TZDs/PPARγ also increase INS sensitivity by reducing the release of adipokines that cause INS resistance, such as TNFα [25] and resistin [26], and by increasing adiponectin release [27], a known stimulator of fatty acid oxidation [18].

In addition to the enhancement of lipids uptake and storage (lipogenesis), TZDs also stimulate the differentiation of pre-adipocytes into adipocytes (adipogenesis), causing hyperplasia and hypertrophy of adipose tissue [28].

Thus, considering: a) the INS sensitizer, adipogenic and lipogenic effects of PGZ [28–31]; b) the INS anabolic and anti-catabolic effects [14] and c) that Walker-256 tumor-bearing rats exhibit cachexia, INS resistance and hypoglycemia [12,13,20,32], the aim of this study was to investigate the PGZ effects, isolated or associated with INS, on INS resistance, cachexia and metabolic disorders in this cancer animal model.

2. Materials and methods

2.1. Drugs and chemicals

PGZ (Actos) was purchased from Abbott Laboratories (Chicago, USA) and NPH INS (Humulin®) and regular INS (Humalog®) from Eli Lilly (Rio de Janeiro, Brazil). Primary and secondary antibodies were acquired from Cell Signaling Technology® (Danvers, USA), Abcam® (Cambridge, USA), Santa Cruz Biotechnology® (Dallas, USA), Cusabio Biotech Co. Ltd. (Wuhan, China) or Jackson ImunoReaserch Laboratories, Inc. (West Grove, USA). Protease inhibitors were acquired from AMRESCO® LLC (Solon, USA). Radioactive INS was purchased from PerkinElmer (Massachusetts, USA). The other reagents were obtained from Sigma Chemical Co. (St Louis), Merck & Co. (Darmstadt, Germany), Reagen (Rio de Janeiro, Brazil) or Laborclin (Pinhais, Brazil).

2.2. Animals and Walker-256 tumor inoculation

Male Wistar rats (220–230 g), kept in collective boxes, 23 ± 2 °C, light/dark cycle of 12 h, with free access to water and standard rodent chow (Nuvilab, CR-1 Nuvital®, Colombo, Brazil) were used in all experiments. Walker-256 carcinosarcoma cells were maintained as previously described [20,33]. For tumor implantation, 8×10^7 tumor cells were suspended in phosphate buffered saline (PBS: 16.5 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and inoculated subcutaneously in the right flank of rats (tumor-bearing rats). Healthy rats were injected with PBS in the same place.

2.3. Treatment protocol

Walker-256 tumor-bearing rats were treated with PGZ (5.0 $mg \cdot kg^{-1}$, oral gavage), alone or in association with INS (NPH, 1.0 $UI \cdot kg^{-1}$, sc), once a day (17:00 pm) for 12 days, since the day of tumor cells inoculation. Doses were based on previous study [34]. PGZ was daily dissolved in water and INS was diluted in saline (0.9% NaCl). Control rats (tumor-bearing and healthy) received vehicles. All experiments were performed on day 12 after tumor cells inoculation, 3 h after the last treatment, in fed rats. It is noteworthy that the mean survival of Walker-256 tumor-bearing rats is 13–15 days after tumor cells inoculation [35]. The experimental protocols were approved by the Ethics Committee for Animal Use of the State University of Londrina (CEUA/UEL, register number 09161).

2.4. Insulin tolerance test (ITT)

For the ITT, regular INS (0.25 $UI \cdot kg^{-1}$) was injected into inferior vena cava of rats anesthetized with thiopental (50 $mg \cdot kg^{-1}$, ip), on day 12 after tumor cells or vehicles inoculation. Blood samples for evaluation of glycemia were collected from inferior vena cava at 0 (basal), 5, 10, 15 and 30 min after INS injection. The constant of plasma glucose disappearance (KITT), an indicator of INS peripheral response, was calculated as previously described [13]. Decreased KITT is an indicator of INS resistance [11].

2.5. Assessment of cachexia-anorexia parameters and tumor growth

Food intake was measured (day 11) by the difference between the amount of feed supplied and the remainder after 24 h. On day 12, rats were weighed, anesthetized with thiopental (50 $mg \cdot kg^{-1}$, ip) and laparotomized to collect of blood samples from the inferior vena cava to evaluate the concentrations of plasma triacylglycerol, free fatty acids and INS. Thereafter, the rats were euthanatized and the retroperitoneal and mesenteric adipose tissue were carefully removed, weighed, quickly frozen in liquid nitrogen and stored at -80 °C for protein analysis. The tumor was dissected and weighed for assessment of tumor growth. The change in body mass was measured by the difference between the final body mass (day 12), discounted the tumor mass, and initial body mass (day 1).

2.6. Western blotting to assess p-Akt, total Akt, p-HSL, total HSL and TNFα

The retroperitoneal and mesenteric adipose tissues, stored at −80 °C, were homogenized in buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM $Na_4P_2O_7$, 10 mM NaF, Triton × 100 1%, glycerol 10%, 0.5 mM Na_3VO_4, 20 mM Tris, pH 7.8) containing 0.2 mM of protease inhibitors cocktails (PIC) and phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged (15,294 g, 40 min, 4 °C) and the total proteins of the supernatant were quantified [36]. Equal amounts of total protein (80 μg) diluted in Laemmli buffer, were applied to 10% polyacrylamide gel, submitted to electrophoresis (SDS-PAGE) and transference to nitrocellulose membrane. The membranes were incubated with primary antibody (1:1000) anti-Akt (Cell Signaling®, catalog n° 9272), anti-phospho-Akt^{Ser473} (Cell Signaling®, catalog n° 9271), anti-HSL (Santa Cuz®, catalog n° sc25843), anti-phospho-HSL^{Ser552} (Cusabio Biotech®, catalog n° csb-pa579109), anti-TNFα (Abcam®, catalog n° ab9755) or anti-γ-tubulin (Sigma®, catalog n° T5326) overnight at 4 °C, followed by incubation with secondary antibody, conjugate to peroxidase (1:5000) and finally with peroxidase substrate (ECL) for chemiluminescence detection (Amersham Image®). The density of the blots was analyzed in Image J software (National Institutes of Health, USA) and expressed in arbitrary units (AU) after normalization by constitutive protein γ-tubulin.

2.7. Assessment of secretion and insulin content in pancreatic islets

Pancreatic islets were isolated from rats by pancreas digestion with collagenase as previously described [37]. Groups of 5 islets were pre-incubated, at 37 °C during 30 min, in Krebs-Henseleit (KH) with 0.2% bovine serum albumin (BSA) in 5.6 $mmol \cdot L^{-1}$ glucose. Thereafter, islets were incubated, 37 °C during 1 h, in KH with 0.2% BSA in different concentrations of glucose (5.6, 11.1 or 16.7 mM). For total INS content, islets in each well were disrupted in acid ethanol solution (1.4% chloridric acid and 74% ethanol) and sonicated (3 pulses of 5 s). At the end of the experiments, the medium was collected and INS was measured.