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Insulin, not glutamine dipeptide, reduces lipases expression and prevents fat wasting and weight loss in Walker 256 tumor-bearing rats

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ABSTRACT

Cachexia is the main cause of mortality in advanced cancer patients. We investigated the effects of insulin (INS) and glutamine dipeptide (GDP), isolated or associated, on cachexia and metabolic changes induced by Walker 256 tumor in rats. INS (NPH, 40 UI/kg, sc) or GDP (1.5 g/kg, oral gavage) was once-daily administered during 11 days after tumor cell inoculation. GDP, INS or INS+GDP treatments did not influence the tumor growth. However, INS and INS+GDP prevented retroperitoneal fat wasting and body weight loss of tumor-bearing rats. In consistency, INS and INS+GDP prevented the increased expression of triacylglycerol lipase (ATGL) and hormone sensitive lipase (HSL), without changing the expression of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) in the retroperitoneal adipose tissue of tumor-bearing rats. INS and INS+GDP also prevented anorexia and hyperlactatemia of tumor-bearing rats. However, INS and INS+GDP accentuated the loss of muscle mass (gastrocnemius, soleus and long digital extensor) without affecting the myostatin expression in the gastrocnemius muscle and blood corticosterone. GDP treatment did not promote beneficial effects. It can be concluded that treatment with INS (INS or INS+GDP), not with GDP, prevented fat wasting and weight loss in tumor-bearing rats without reducing tumor growth. These effects might be attributed to the reduction of lipases expression (ATGL and LHS) and increased food intake. The results show the physiological function of INS in the suppression of lipolysis induced by cachexia mediators in tumor-bearing rats.

1. Introduction

Approximately 40% of deaths in patients with cancer are attributed to cachexia, since there is no effective treatment to combat this syndrome. Cancer cachexia is characterized by a progressive loss of body weight and skeletal muscle, as well as fat mass wasting due to the predominance of catabolism caused by disorders in the proteins (proteolysis) and lipids (lipolysis). Anorexia and changes in the carbohydrate metabolism (hypoinsulinemia and insulin resistance) and in the immune system are also associated with cachexia (Argilés et al., 2014; Mueller et al., 2016; Porporato et al., 2016).

Several factors produced by tumor cells, such as the lipid mobilizing factor (LMF) and the proteolysis-inducing factor (PIF), and cytokines produced by the host immune system, such as the tumor necrosis factor α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6), play an important role in the cancer cachexia and are recognized as cachexia mediators (Tisdale, 2010). It is possible that hypoinsulinemia and insulin (INS) resistance associated with cancer (Asp et al., 2010; Miksza et al., 2013) also contribute to the development of cachexia, since INS exerts potent anabolic and anti-catabolic effects (Carvalheira et al., 2002).

In healthy animals, administration of INS results in hyperphagia, stimulation of the synthesis and inhibition of fat and protein breakdown and weight gain. Based on this, therapy with INS has been proposed to decrease cachexia in cancer patients. INS treatment reverted anorexia and preserved the body weight in 3-methylcholanthren (MCA) tumor-bearing rats, without affecting tumor growth (Moley et al., 1985). However, in this study, body composition was not assessed remaining uncertain whether the prevention of weight loss promoted by INS occurred by reduction of adipose or muscle wasting, or both.

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INS treatment also prevented anorexia and weight loss in Walker 256 tumor-bearing rats, but reduced tumor growth (Fernandes et al., 1991), impairing the evaluation of the INS anti-cachectic effect, since a reduction in tumor size decreases cachexia.

Supplementation with glutamine also appears to be related to improvements in cachexia. This suggestion is based on the fact that glutamine acts as a source of energy for rapidly proliferating cells, such as immune system cells and enterocytes, as a precursor to synthesis of antioxidant enzymes (Fürst et al., 2004; Roth et al., 2002) and seems to improve the sensitivity and secretion of INS in patients with chronic diseases (Bakalar et al., 2006; Brennan et al., 2003; Dechelotte et al., 2006). Furthermore, in situations of an increased demand of glutamine, as in cancer, the proteolysis is activated (Griffiths et al., 2001; Yoshida et al., 2001).

Glutamine is commonly utilized to prevent gastrointestinal damage induced by chemotherapy or radiotherapy in cancer patients (Klimberg et al., 1989; Yoshida et al., 2001). However, the beneficial effects of glutamine are controversial due to the fact that glutamine is highly used as an energy source by tumor cells (Roth et al., 2002) and thus could enhance tumor growth. However, it is not established if the glutamine treatment, in fact, enhances tumor growth.

Considering that: a) Walker 256 tumor-bearing rats exhibit hypoinsulinemia (Fernandes et al., 1991) and INS resistance (Miksza et al., 2013; de Souza et al., 2015), which may contribute to metabolic disorders and cachexia in these animals (Cassolla et al., 2012; de Morais et al., 2012; de Souza et al., 2015); b) glutamine seems to improve the INS sensibility and secretion, and c) INS and glutamine effects on various cancer-induced metabolic abnormalities have not yet been evaluated, particularly their associated effects, this study examined the isolated and combined effects of treatments with INS and glutamine dipeptide (GDP) on the cachexia and metabolic changes induced by Walker 256 tumor in rats.

2. Material and methods

2.1. Chemicals

INS (NPH – Humulin®) was purchased from Eli Lilly of Brazil (Rio de Janeiro, Brazil). L-alanyl-L-glutamine, *i.e.*, GDP was purchased from Inter American Ajinomoto (São Paulo, Brazil). Enzymatic kits to determine the glucose and urea were purchased from Laborclin (Pinhais/PR, Brazil). The MILLIPLE® kit used to dose corticosterone was purchased from Merck Millipore. Primary antibodies were purchased from Abcam® (Cambridge, USA), Cell Signaling Technology® (Danvers, USA), Santa Cruz Biotechnology® (Dallas, USA) and Novus Biologicals® (Littleton, USA), secondary antibodies from Jackson Imuno Reaserch Laboratories, Inc. (West Grove, USA), protease inhibitors from AMRESCO® LLC (Solon, USA), nitrocellulose membranes from Perkin Elmer, INS labeled with iodine (^{125}I) from Genesis and other reagents from Sigma Chemical Co. (St Louis, USA), Merck & Co. (Darmstadt, Germany), Reagan (Rio de Janeiro, Brazil) or Laborclin (Pinhais/PR, Brazil).

2.2. Animals and inoculation of Walker-256 tumor cells

Male Wistar rats (220–230 g) fed on a standard laboratory diet (Nuvilab CR-1 Nuvital®, Colombo, Brazil) were used. Walker 256 tumor cells, kindly provided by the Cellular Metabolism Laboratory (Federal University of Parana, Brazil) (Fernandes et al., 1991) were maintained in our laboratory by weekly intraperitoneal passages in rats. For tumor implantation, Walker 256 tumor cells, collected from the intraperitoneal cavity of rats, were suspended in phosphate buffered saline (PBS: 16.5 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and inoculated subcutaneously (8.0×10^7 tumor cells/animal) on the right flank of the animals, as previously described (Cassolla et al., 2012; de Souza et al., 2015; Miksza et al., 2013). Healthy rats received subcutaneously the same volume of PBS.

In all experiments, except for the evaluation of corticosterone, the rats were submitted to 24 h fasting to prevent the influence of anorexia and lower hepatic glycogen content of tumor-bearing rats (Cassolla et al., 2012). Due to the fact that the rats survive for an average of 14 days after tumor inoculation, the experiments were carried out on day 13.

2.3. Treatments

Although glutamine is beneficial for health, its low solubility and stability in aqueous solutions limits its blood availability. Furthermore, approximately 50% of orally administered glutamine is extracted by the splanchnic bed (Newsholme et al., 2011). In contrast to glutamine, the GDP, a synthetic dipeptide composed by alanine and glutamine (Minguette-Camara et al., 2014) is soluble and stable in water and shows lower extraction by the splanchnic bed (Newsholme et al., 2003).

Tumor-bearing rats were treated with GDP (1.5 g/kg, oral gavage), INS (NPH, 40 UI/kg, sc) or INS+GDP. INS or GDP was administered once daily (5:00 p.m.) over 11 days after tumor cell inoculation. Moreover, control rats (tumor-bearing and healthy) received vehicle instead of GDP or INS. The GDP (Cruzat et al., 2010) and INS doses (Fernandes et al., 1996) were based on previous studies. No treatment was carried out on the day before the experiments (day 13). The purpose of this procedure was to prevent the severe INS induced hypoglycemia in 24 h fasted rats and evaluate the chronic effects of INS and GDP, excluding their acute effects. The experimental protocols were approved by the Ethics Committee for Animal Use from the State University of Londrina (CEUA/UEL, register number 07987).

2.4. Evaluation of tumor growth, blood parameters and cachexia-anorexia parameters

Food intake was measured (day 11) by the difference between the amount of food supplied and the remainder after 24 h. On day 13, after 24 h of food deprivation, the rats were weighed and blood samples were collected from the inferior cava vein for quantification of INS, glucose, lactate and urea. Thereafter, the rats were euthanized and the retroperitoneal adipose tissue, the gastrocnemius, soleus and extensor digitorum longus (EDL) muscle were removed and weighed. The retroperitoneal adipose tissue and gastrocnemius muscle were quickly frozen in liquid nitrogen and stored at 80 °C for protein analysis. The tumor was removed and weighed to evaluate the tumor growth. The change in body mass was calculated by the difference between the final body mass, discounted the mass of the tumor, and initial body mass.

In order to evaluate whether hypoglycemia induced by treatments with INS or INS+GDP influences corticosterone levels, blood and feces were collected from fed rats 4 h after the last treatment. Blood was collected from the inferior cava vein and feces were collected over 24 h. Corticosterone was measured in feces to evaluate whether there was a variation of corticosterone levels during the 24 h after the administration of INS or INS+GDP, i. e., during the period of hypoglycemia.

2.5. Western blotting to evaluate TNF-α, IL-6, hormone sensitive lipase (HSL), perilipin, triacylglycerol lipase (ATGL) and myostatin in gastrocnemius muscle or retroperitoneal fat

The tissues (gastrocnemius and retroperitoneal fat), previously stored at −80 °C, were homogenized in a lysis buffer containing: 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM $Na_4P_2O_7$, 10 mM NaF, Triton X100 1%, glycerol 10%, 0.5 mM Na_3VO_4, 20 mM Tris and 0.2 mM protease inhibitors (pH 7.8). The homogenate was centrifuged (13362*g*, 40 min, 4 °C) and the total proteins of the supernatant were quantified (Bradford, 1976). Equal amounts of total protein (80 μg), diluted in a Laemmli buffer, were applied to 10% polyacrylamide gel and submitted to electrophoresis

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