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Leucine modulates the effect of Walker factor, a proteolysis-inducing factor-like protein from Walker tumours, on gene expression and cellular activity in C_2C_{12} myotubes

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ABSTRACT

Cancer-cachexia causes severe weight loss, particularly from the wasting of skeletal muscle, which occurs due to increased protein catabolism and/or decreased protein synthesis. The muscle protein degradation observed in cancer patients is mediated by a specific cytokine, proteolysis-inducing factor (PIF), which is produced by the tumour. This protein increases the ubiquitin–proteasome pathway activity, and the synthesis of muscle protein in these patients can be affected by several factors, including nutrient-related signalling. Some nutrients, such as leucine, can decrease the ubiquitin–proteasome pathway activity and increase the skeletal muscle protein content in cachectic animals. In this study, we investigated the effects of leucine on cell viability, morphology, functional proteasome activity, enzymatic activity, and protein synthesis and degradation in C_2C_{12} myotubes exposed to the proteolysis-inducing factor (PIF)-like protein purified from Walker tumour-bearing rats. Walker factor (WF) had no cytotoxic effects on myotube cells and morphological characteristics were not altered in the presence of WF and/or leucine. However, increased alkaline phosphatase activity was observed. At higher WF concentrations, chymotrypsin-like activity, cathepsin B activity and 20S proteasome gene expression increased. Treating myotubes with leucine before exposure to WF causes leads to a decrease in proteasome activity as well as the activity of chymotrypsin and cathepsin enzymes. Total protein synthesis decreased in WF-treated cells concomitantly as protein degradation increased. After leucine exposure, the observed effects of WF were minimal or even reverted in some cases. Taken together, these results suggest an important modulatory effect for leucine on the effects of WF in C_2C_{12} myotube cells.

1. Introduction

Cancer cachexia is a metabolic syndrome directly caused by cancer in which complex metabolic and neuro-endocrine modifications occur, resulting in anorexia, wasting, marked weight loss, especially the body skeletal muscle mass, weakness and poor performance statistics [1,2]. Depending on the circumstances, the mobilisation of muscle protein may provide substrates for both energy production and gluconeogenesis, for synthesis of acute phase reactants, and for other synthesis processes such as tumour growth [3]. These processes could lead to reduced response to chemotherapy and also to a decrease in survival and life quality [4,5]. Loss of skeletal muscle due to cancer cachexia reflects an imbalance between the rate of protein catabolism and the rate of protein synthesis [6], with a progressive reduction of skeletal muscle mass, although the visceral protein reserves are preserved [7]. The cachexia in cancer patients was believed to be the result of a nutritional deficit caused by the combination of increased energy consumption by the tumour mass and decreased energy intake, secondary to tumour-related factors, which modified the satiety centre's response from the central nervous system [8]. However, cachexia induced by tumours increases the production of catabolic factors that cause breakdown of host lipid and skeletal muscle protein stores, suggesting that the mobilised products of host tissues are used by the tumour to maintain its growth and integrity [9]. In this case, proteolysis in skeletal muscle is mediated by a tumour-generated, sulphated glycoprotein, referred as proteolysis-inducing factor or PIF [10]. In skeletal muscle, the effect of PIF on protein degradation is mediated by an increase in the expression and activity of the ubiquitin–proteasome proteolytic pathway. Thus, circulating PIF specific to the cachectic state is associated with up-regulation of the ubiquitin–proteasome proteolytic pathway in vitro and in vivo [11]. Understanding the induction and mechanism of cancer cachexia might lead to the development of therapeutic agents capable of modulating the steps in this process. Coadjutant therapy is currently being studied as an alternative treatment to improve the quality and survival rate of cancer

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patients. The branched-chain amino acid leucine has been investigated as an important cell signalling molecule in muscle cells and is the most important nutrient for protein synthesis and muscle energy. Many studies have shown that this amino acid can inhibit the expression of the ubiquitin–proteasome pathway in the muscles of cachectic rats [12,13] and can improve the protein content of muscle in tumour-bearing rats [14,15]. Based on these previous studies, we investigated the effect of leucine on cell activity and protein synthesis and degradation in C_2C_{12} skeletal muscle cells exposed to a proteolysis-inducing factor (PIF)-like protein that was purified from a tumour-inducing cachexia in rats, the Walker 256 carcinoma.

2. Material and methods

Biochemical reagents were purchased from Labsynth (Diadema, SP, Brazil), Merck KgaA (Darmstadt, Germany), Millipore Corporation (Jaffrey, NH, USA), Promega (Madison, WI, USA), Riedel-de Haën (Hanover, Germany), and Sigma Chemical Company (St. Louis, MO, USA).

2.1. Animals and tumour implants

Adult male Wistar rats were obtained from the Multidisciplinary Centre for Biological Investigation (CEMIB) at UNICAMP. The rats received intraperitoneal injections of Walker 256 tumour cells (approximately 1×10^6 cells in 1.0 mL saline solution) and were maintained with a 12 h light/dark cycle at 23 °C and fed standard chow. Ascitic fluid was obtained from the tumour-bearing rats peritoneal cavity as described by Gomes-Marcondes et al. [16]. The UKCCCR (United Kingdom Coordinating Committee on Cancer Research 1988) general guidelines for animal welfare were followed and all of the experimental protocols were reviewed and approved by the Institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, protocol 034-2 and 386-1).

2.2. Walker factor purification and detection

Ascitic fluid obtained from rats with Walker 256 tumours was centrifuged, and high molecular weight proteins were precipitated with 40% ammonium sulphate. After 24 h of stirring, the solution was centrifuged and the supernatant was washed with PBS and dialysed for 5 days at 4 °C. WF was purified by affinity chromatography using an anti-PIF antibody and represented approximately 1.2% of the total protein in the ascitic fluid. The total protein concentration was measured using the Bradford colorimetric method [17]. The 24 kDa glycoprotein present in the concentrated Walker tumour ascitic fluid was resolved with 12% SDS–polyacrylamide gel electrophoresis and further quantified by western blotting using an antibody against PIF (purified by Cancer Research Laboratory, Aston University, Birmingham, UK, and generously donated by Prof. M.J. Tisdale) [18]. The bands were visualised using enhanced chemiluminescence detection and were analysed by optical densitometric analysis using Gel Pro-Analyser software [19] (data not shown). Walker factor (WF) has the same molecular weight as PIF (24 kDa) [19].

2.3. Leucine

Leucine (Sigma) was dissolved, diluted to 50 μM and tested using the ninhydrin method [20]. The experimental solution was filtered through a 0.22 μm membrane prior to use in cell culture.

2.4. Cell culture

C_2C_{12} myoblast cells from Cancer Research Laboratory (Aston University, Birmingham, UK) were grown in DMEM-high glucose medium (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 10% foetal calf serum (FCS, Sigma) and 1% penicillin/streptomycin (P/S, Sigma) at 37 °C in a humidified 5% CO_2 atmosphere. All experiments were initiated using cells grown to 80% confluence. To induce differentiation of myoblast cells to myotubes, the propagation medium was replaced with medium supplemented with 2% horse serum. Cells were assayed experimentally within 5 days of differentiation. Leucine-treated C_2C_{12} myotubes were incubated with leucine (50 μM, Sigma) for 24, 48 or 72 h. WF-treated C_2C_{12} myotubes were incubated for 24, 48 or 72 h with Walker factor at final concentrations of 1, 3, 5, 10, 15, 20 or 25 μg/mL. Leucine/WF-treated C_2C_{12} myotubes were first exposed to 50 μM leucine for 2 h, and then the cells were incubated with fresh DMEM medium supplemented with Walker factor at final concentrations of 1, 3, 5, 10, 15, 20 and 25 μg/mL.

2.5. Cytotoxicity assays

The viability of C_2C_{12} myotubes was assessed using the crystal violet method described by Murakami et al. [21]. After incubation, the culture medium was removed, and the C_2C_{12} myotubes were fixed in 10% formalin (Labsynth) and stained with 0.05% crystal violet (Riedel-de Haën, Hanover, Germany) in 20% methanol (Merck KgaA, Darmstadt, Germany). C_2C_{12} myotubes were washed in 0.1 M PBS (pH 7.4) and then incubated in 0.1 M sodium citrate (pH 4.2) and 50% ethanol (Labsynth) for 30 min. The absorbance was measured at 540 nm in a spectrophotometer microplate reader (Fusion–Packard Bioscience Company, USA).

2.6. Light and scanning electron (SEM) microscopy

C_2C_{12} myotubes were grown on coverslips and treated first with leucine, then with 5 or 25 μg WF. After 24 h of treatment, the myotubes were analysed with phase contrast microscopy (Leica Instruments, USA). Some myotubes were fixed in 2.5% paraformaldehyde/glutaraldehyde (Sigma) in 0.1 M PBS (pH 7.4) and then washed in PBS followed by post-fixation with 1% osmium tetroxide (Sigma) and dehydration in a graded ethanol series. The cells were subsequently dried to the critical point (CPDO030 – Balzers, BAL-TEC AG, Wiesbaden, Germany) and gold sputtered (SCD050 – Balzers) before being analysed in a scanning electron microscope (JSM-5800LV, JEOL, Peabody, MA, USA) operated at 10 kV.

2.7. Activity of proteolysis systems

2.7.1. Measurement of proteasome activity

Functional proteasome activity was determined by measuring chymotrypsin-like enzyme activity, the predominant proteolytic activity of proteasome β subunits, as determined using Orino et al. method [22]. After 24 h of incubation, C_2C_{12} myotubes were homogenised in cold 0.1 M PBS (pH 7.4), sonicated, and centrifuged at 10,000 rpm for 15 min at 4 °C. The resulting supernatant was analysed for total protein content [23]. Chymotrypsin-like activity was measured from aliquots of myotube homogenate supernatant (50 μL) by incubation with 100 μL of the fluorogenic substrate succinyl-Leu-Leu-Val-Try-7-amino-4-methylcoumarin (Suc LLVY-AMC) (0.167 μg/L in Tris–HCl, pH 7.4), which leads to the release of aminomethyl coumarin (AMC) from the substrate. The fluorescence was measured in a fluorometer (excitation: 360 nm, emission: 460 nm), and the results were expressed as units of fluorescence/μg protein/min.

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