ORIGINAL PAPER

Chronic Exendin-4 Treatment Prevents the Development of Cancer Cachexia Symptoms in Male Rats Bearing the Yoshida Sarcoma

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Abstract Cancer cachexia is the syndrome of weight loss, loss of appetite, and wasting of skeletal muscle and adipose tissue experienced by many individuals with cancer. Currently, few effective treatment and prevention strategies are available for these patients, due in part to a poor understanding of the mechanisms contributing to cachexia. Insulin resistance has been associated with cancer cachexia in epidemiological, human, and animal research. The present experiment was designed to examine the ability of Exendin-4, a GLP-1 agonist and insulin sensitizing agent, to prevent the development of cachexia symptoms in male Sprague Dawley rats bearing the Yoshida sarcoma. Following tumor implantation or sham surgery, rats were treated daily with saline or Exendin-4 (3 µg/kg body weight/day) and were monitored for tumor growth and cachexia symptoms for 21–23 days. As a result of large variability in treatment effects, data were analyzed separately for animals with large and small tumors. Exendin-4 treatment reduced tumor growth and prevented the development of cancer cachexia symptoms in animals with small, but not large, tumors. In addition, insulin levels were preserved in Exendin-4-treated tumor-bearing animals. The results of this experiment demonstrate a novel preventative therapy for cancer cachexia and a novel use of Exendin-4. Further research is necessary to determine the mechanisms through which Exendin-4 exerts these potent effects.

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Introduction

Cancer cachexia, a syndrome of weight loss, loss of appetite, and wasting of body tissues, contributes significantly to both morbidity and mortality in individuals with cancer [10]. Those who experience cachexia have reduced survival time, as well as diminished psychological and physical health [3, 49]. In addition, cachectic cancer patients often display more negative side effects and a decreased therapeutic response during chemotherapy [15, 19, 42]. The majority of individuals with cancer will experience some degree of cachexia during the course of their disease [49, 50], making cancer cachexia a clinically relevant syndrome for which there are currently no effective methods of treatment or prevention.

One potential avenue of cachexia prevention involves a potent mediator of energy balance. Insulin function is often impaired in individuals with and animal models of cancer cachexia [1, 5, 6, 9, 11, 13, 14, 16, 20, 21, 24, 26, 29, 33, 36, 41, 45–48]. Given the ability of insulin dysfunction to result in altered body composition in other animal models of disease [31], there is interest in determining the role that insulin resistance may play in cachexia development [23]. Insulin sensitizing agents have been examined previously, with varying success, in the context of cancer cachexia treatment [11, 12, 30, 34, 37, 38], but few have been examined with the goal of prevention [1].

The majority of insulin release is dependent upon the action of incretin hormones, including GLP-1 [2, 22]. GLP-1 receptors are found throughout the central nervous system and the periphery, including skeletal and smooth muscle, and adipose tissue, and utilize receptor signaling pathways that include INSR, IRS-1, and IRS-2 [2, 17]. GLP-1 agonists, such as Exendin-4, have become attractive therapeutic options for

improving insulin sensitivity. Exendin-4 lowers blood glucose levels, increases insulin secretion, improves beta-cell function, and promotes peripheral insulin sensitivity [8, 52]. Exendin-4 increases insulin sensitivity in isolated myotubules and adipocytes [25]. Additionally, this peptide has potent anti-inflammatory effects [4] and has been shown to reduce cell proliferation in a number of cancer cell lines [27, 28, 32]. Exendin-4 has not previously been investigated for the treatment or prevention of cancer cachexia.

Currently, no therapies are effective in the treatment or prevention of cancer cachexia. Given the ability of Exendin-4 to exert insulin sensitizing effects, the present experiment investigated the effects of chronic Exendin-4 treatment on the development of cancer cachexia in rats implanted with the Yoshida sarcoma. It was hypothesized that Exendin-4 treatment would improve insulin sensitivity and prevent the development of cachexia symptoms, while reducing tumor growth.

Materials and Methods

Experimental Animals

Sixty-eight male Sprague Dawley rats (65–69 days old; Harlan Sprague Dawley, Indianapolis, IN, USA) served as experimental subjects. Rats were individually housed in hanging wire mesh cages in a temperature- and humidity-controlled room, with a 12:12 h light/dark cycle (lights on at 04:00). Following arrival in the laboratory, rats were allowed to acclimate to the laboratory environment for 1 week prior to the start of the experiment and received ad libitum access to purified rodent chow (AIN-76A; Dyets Inc., Bethlehem, PA, USA). Throughout the experiment, all rats received ad libitum access to tap water and purified rodent chow. All procedures were approved by the Purdue University Animal Care and Use Committee.

After acclimation to the laboratory environment, rats were then weight-matched (starting body weights 291–355 g) and divided into two experimental groups, which differed in tumor status. The control group ($n=20$) received a sham surgery. The tumor-bearing group (TB, $n=48$) was subcutaneously implanted with the Yoshida sarcoma. Surgery was performed on experimental day 0.

Tumors and Tumor Implantation

Yoshida sarcoma ascites tumor cells were purchased from the Development Therapeutics Program at the National Cancer Institute (Bethesda, MD, USA). A detailed description of the tumor implantation process has been described previously [24]. Briefly, donor animals were used to perpetuate the tumor line in the laboratory. Following an overdose of sodium pentobarbital, the tumor tissue was quickly dissected from the donor animal,

divided into fragments, and placed on ice. The animal receiving the tumor was anesthetized under isoflurane anesthesia, and a tumor fragment (approximately 6 mm^3) was subcutaneously implanted on the right flank between the upper and lower limbs. All TB animals received tumor tissue from the same donor animal. Sham surgeries were performed in control animals. Surgeries were performed on experimental day 0.

Tumor volume was determined through measurement of tumor size by external digital caliper, with the longest longitudinal diameter (length, L), greatest transverse diameter (width, W), and the great vertical diameter (height, H) recorded daily. Tumor volume was calculated using a standard ellipsoid formula [$V = \pi/6 \times (L)(W)(H)$] [51].

Chronic Exendin-4 Treatment

On day 1, animals were further divided into two treatment groups ($n=10$, 24 for control saline and TB saline; $n=10$, 24 for control Exendin-4 and TB Exendin-4). One group received a daily i.p. injection of Exendin-4 (3 μg/kg body weight; American Peptide Company, Sunnyvale, CA, USA), while the other received a similar volume of sterile, physiological saline. In a pilot experiment, this dose of Exendin-4 was found to increase insulin sensitivity in non-tumor-bearing animals after 14 days of treatment, with minimal decreases in body weight or food intake. Daily injections were performed 3 h prior to the start of the dark cycle.

Analysis of Body Composition

Body composition was analyzed on day 20 in conscious rats using the EchoMRI system (Echo Medical Systems, Houston, TX, USA). The EchoMRI system has been validated in mice and rats, and its results are well correlated with chemical carcass analysis [40]. Data are presented as a percentage of body fat. Hind leg diameter was utilized as a measure of skeletal muscle mass. The contralateral hind leg, relative to the tumor, was extended and the diameter of the upper leg was measured via external digital caliper and recorded.

Sacrifice

After 21–23 days, rats were sacrificed under ether inhalation anesthesia followed by rapid decapitation approximately 4 h prior to the start of the dark cycle. Trunk blood was collected for analysis of non-fasting blood glucose and plasma insulin levels. Blood glucose levels were measured using the Precision Xtra Glucose Monitoring System (Abbott Laboratories, Abbott, IL, USA). The remaining blood samples were centrifuged at 2,000 rpm for 15 min at 4 °C, and plasma was removed and stored at −80 °C for subsequent analysis of plasma insulin levels by radioimmunoassay. Exendin-4 or saline injections were not administered on the day of sacrifice.

The epididymal fat pads were removed from each animal and weighed, as a measure of terminal fat mass. Additionally, hind leg diameter, both contralateral and ipsilateral to the tumor, was recorded, as a measure of terminal skeletal muscle mass. In TB rats, the tumor was also removed and weighed. Small samples of epididymal fat and quadriceps muscle were collected and placed in RNAlater for analysis of mRNA expression by QPCR.

Radioimmunoassay (RIA)

Plasma insulin levels were determined using a commercial Rat Insulin RIA kit (Millipore, Billerica, MA, USA), with upper and lower detection limits of 0.1 and 10 ng/mL, respectively. All samples were run in duplicate and per manufacturer's instructions. Unknown concentrations of insulin were calculated based on a standard curve generated for each kit.

Quantitative Real-Time Polymerase Chain Reaction (QPCR)

RNA was isolated from tissue samples for determination of mRNA expression. Tissue samples (100 mg) were homogenized in 2 mL of Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from 0.5 to 3 μg of RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) and diluted in nuclease-free water for storage at −80 °C. Primers for housekeeping genes and genes of interest are listed in Table 1. QPCR was performed in duplicate using a BioRad iCycler and Maxima Sybr Green solution (Thermo Scientific, Barrington, IL, USA) with two-step (L32 and Atrogin-1) or three-step (β-actin and HSL) amplification for 40 cycles. L32 was amplified from

Table 1 Primers sequences for QPCR analysis of mRNA expression

Gene	Primer sequence
L32	Forward: 5′-CAG ACG CAC CAT CGA AGT TA-3′
	Reverse: 5′-AGC CAC AAA GGA CGT GTT TC-3′
β-Actin	Forward: 5′-CGT GGG CCG CCC TAG GCA CCA-3′
	Reverse: 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′
Atrogin-1	Forward: 5′-GTC CAG AGA GTC GGC AAG TC-3′
	Reverse: 5′-GTC GGT GAT CGT GAG ACC TT-3′
Hormone-sensitive lipase (HSL)	Forward: 5′-GAA TAT CAC GGA GAT CGA GG-3′
	Reverse: 5′-CCG AAG GGA CAC GGT GAT GC-3′

each sample for use as an endogenous control for Atrogin-1, while β-actin served as an endogenous control for HSL. The method of Pfaffl was used to determine expression of the genes of interest [43]. Expression was calculated based on normalization to the housekeeping gene, relative to the efficiency of the housekeeping and interest genes in the target tissue.

Statistical Analyses

Data are presented as mean ± standard error of the mean. The variance in tumor growth observed in saline- and Exendin-4-treated animals was significantly different during this experiment, with Exendin-4-treated rats having significantly greater variance than saline-treated rats. This led to the hypothesis that Exendin-4 treatment may have been more or less effective in some animals based on tumor size and growth rate. To better quantify the effects of Exendin-4 treatment on cancer cachexia symptoms, tumor-bearing rats in both treatment groups were divided into tertiles based on final tumor volume on the day of sacrifice (days 21–23). All data from TB animals from the upper (TBU) and lower (TBL) tertiles were analyzed, while data from TB animals falling in the middle tertile were excluded from the present analysis. Final groups sample sizes were (1) $n=9$ TBU saline, (2) $n=10$ for TBU Exendin-4, (3) $n=7$ for TBL saline, and (4) $n=8$ for TBL Exendin-4. Saline- and Exendin-4-treated control groups had group sizes of 10 animals each.

Tumor volume was analyzed in TBU and TBL rats in separate two-way ANOVAs, with treatment (saline or Exendin-4) and time serving as independent variables. Planned Bonferroni comparisons were performed when appropriate. Final tumor weight was analyzed via two-way ANOVA with tertile (upper or lower) and treatment (saline or Exendin-4) as independent variables, with planned Bonferroni comparisons as appropriate.

Terminal change in body weight, minus tumor weight in the TB group, was analyzed via two-way ANOVA, with treatment (saline or Exendin-4) and tumor status (control, TBL, or TBU) serving as independent variables. Planned Bonferroni comparisons were performed when appropriate. Average daily food intake data, presented as a mean of daily food intake throughout the 21–23 days of the study, as well as body adiposity, hind leg diameter, blood glucose levels, and plasma insulin levels, were similarly analyzed by two-way ANOVA, with planned Bonferroni comparisons when appropriate.

Results

Change in Body Weight and Food Intake

Terminal change in body weight was calculated by subtracting the weight of the tumor at the time of sacrifice from the total weight gained during the experiment for each rat (Fig. 1a). In
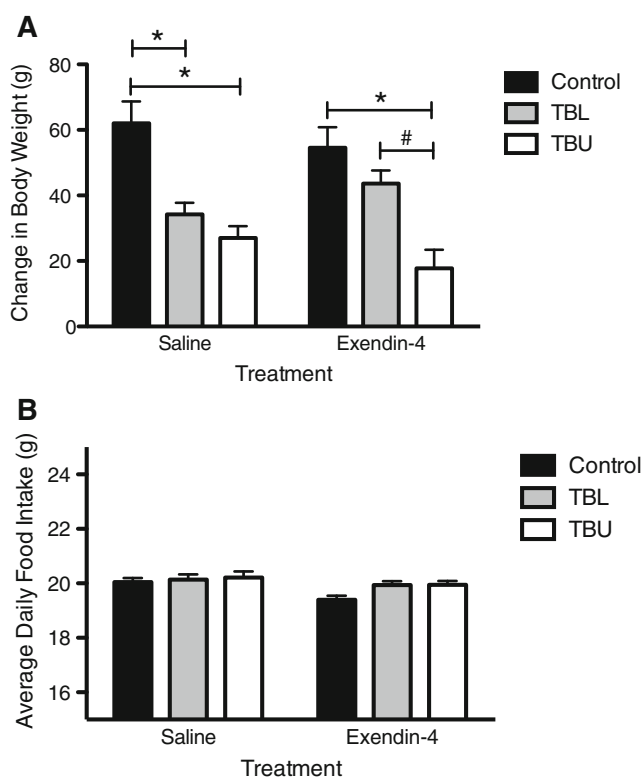
Fig. 1 Change in body weight and average daily food intake in control and tumor-bearing rats (*TBL*, lower tertile; *TBU*, upper tertile) following chronic saline or Exendin-4 treatment. **a** Terminal change in body weight, minus tumor weight, was significantly lower in saline-treated TBL and TBU rats, as compared to controls ($p < 0.05$). In contrast, terminal change in body weight was significantly lower in Exendin-4 treated TBU rats, as compared to both control and TBL rats. No significant differences were found between control and TBL Exendin-4-treated rats. **b** Food intake was significantly lower in Exendin-4-treated rats, as compared to saline-treated rats, but no differences were observed based on tumor status. *$p < 0.05$ compared to control, #$p < 0.05$ compared to TBL

the saline-treated groups, TB rats gained significantly less body weight as compared to controls ($p < 0.01$ for TBL, $p < 0.001$ for TBU). However, in rats treated with Exendin-4, only rats in the upper tertile of tumor volume experienced less weight gain as compared to controls and TBL rats ($p < 0.05$ for TBU). In contrast, TBL rats gained a comparable amount of weight to control rats ($p > 0.05$).

No differences in food intake were observed between groups based on tumor status (Fig. 1b).

Tumor Volume and Final Tumor Weight

During the experiment, no significant difference in tumor volume was observed between saline and Exendin-4 treated TBU rats (Fig. 2a). In contrast, among TBL rats, significant differences between treatment groups were observed. While tumor volume in both groups increased over time, Exendin-4-treated TBL rats exhibited a smaller tumor volume starting at
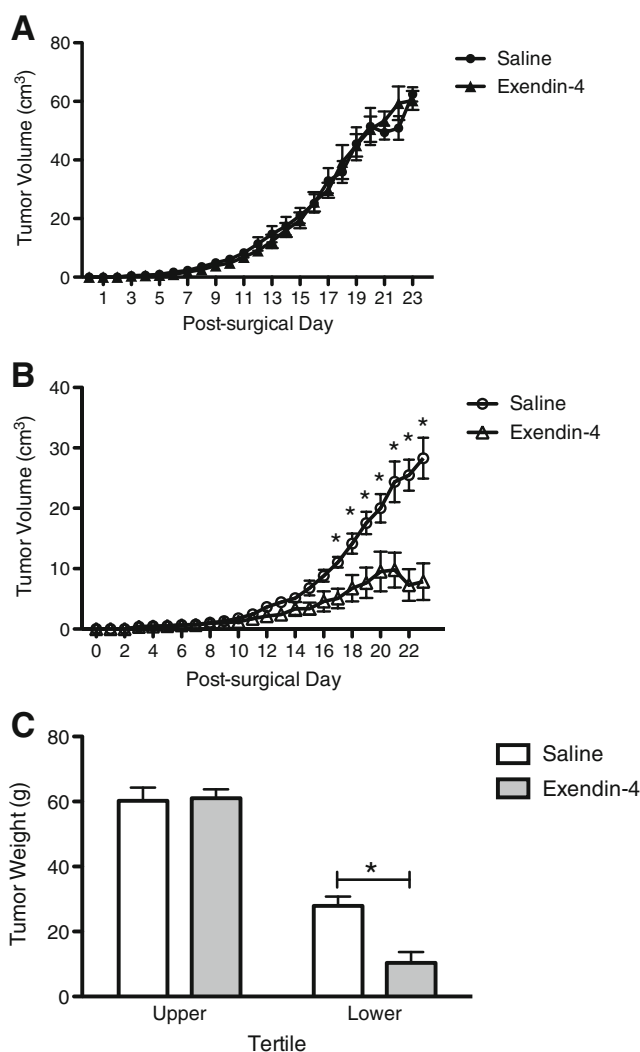

Fig. 2 Tumor volume and final tumor weight in saline- and Exendin-4-treated rats bearing the Yoshida sarcoma. **a** In TBU rats, tumor volume was comparable between saline- and Exendin-4-treated groups. **b** In TBL rats, tumor volume was significantly lower in Exendin-4-treated animals from days 17 to 23, as compared to saline-treated animals ($p < 0.05$). **c** On the day of sacrifice, tumors were removed and weighed. While no differences were observed between saline- and Exendin-4-treated TBU rats, tumors isolated from Exendin-4-treated TBL rats weighed significantly less than those isolated from saline-treated TBL rats. *$p < 0.05$

day 17 and continuing to the end of the experiment (day 17–23, $p < 0.05$ for all) (Fig. 2b).

Final tumor weight followed a similar pattern. Exendin-4 treatment reduced final tumor weight in TBL rats as compared to saline treatment, but there was no difference in tumor weight between saline-treated and Exendin-4-treated TBU rats (Fig. 2c, $p < 0.05$).

Body Composition

On day 20, saline-treated tumor-bearing animals exhibited a lower percentage of body fat, as compared to saline-treated controls ($p < 0.01$ for both TBU and TBL) (Fig. 3a). In contrast,
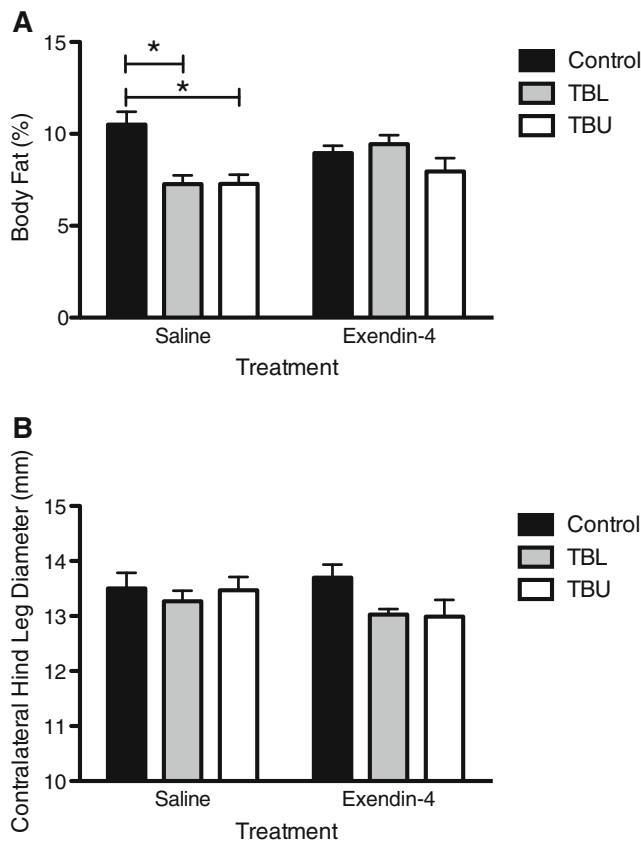
Fig. 3 Body composition on day 20. **a** Percent body fat, as measured by EchoMRI, was reduced in saline-treated TBU and TBL rats, as compared to controls ($p < 0.05$). In contrast, no differences were observed among Exendin-4-treated rats. **b** No significant differences in contralateral hind leg diameter were observed between groups

this effect was not observed in Exendin-4-treated rats. Analysis of contralateral hind leg diameter revealed no significant differences based on tumor status or treatment on day 20 (Fig. 3b).

Terminal analyses were performed to further quantify body composition. Analysis of epididymal fat pad weight by two-way ANOVA revealed no significant effect of tumor status or treatment (Fig. 4a). On the day of sacrifice, lower contralateral hind leg diameter was observed in TBU and TBL animals, as compared to control animals (Fig. 4b). This effect was observed in both saline-treated and Exendin-4-treated animals. Similar results were observed with ipsilateral hind leg diameter; however, this effect was only observed in TBU, but not TBL, rats. Again, this effect was observed in both treatment groups.

Plasma Insulin and Blood Glucose Levels

Blood glucose and plasma insulin levels were measured at the time of sacrifice, in a non-fasted state. No significant differences in blood glucose levels were observed (Fig. 5a). In contrast, plasma insulin levels exhibited a different pattern (Fig. 5b). In saline-treated rats, both TBU and TBL had lower
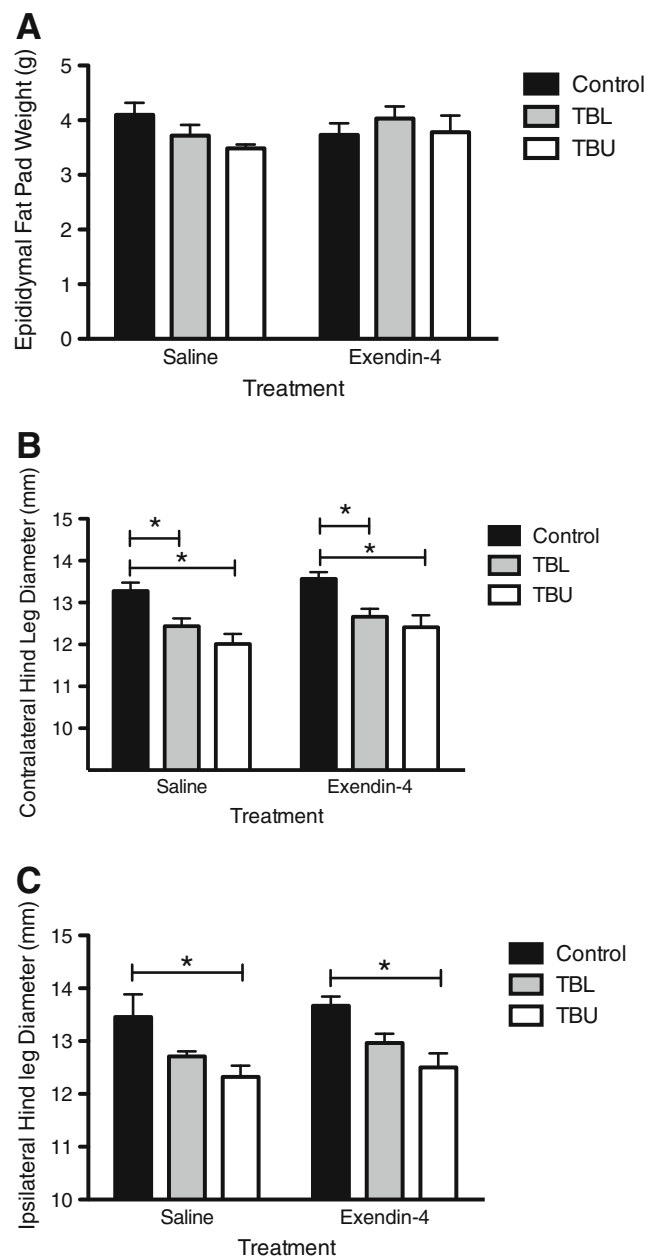

Fig. 4 Terminal body composition measurements. **a** No differences in epididymal fat pad weight were observed. **b** Contralateral hind leg diameter was lower in TBL and TBU animals in both treatment groups, compared to controls. **c** Ipsilateral hind leg diameter was lower in TBU rats in both treatment groups, as compared to controls. *$p < 0.05$

insulin levels, as compared to non-tumor-bearing controls. However, in Exendin-4-treated rats, insulin levels were comparable in control, TBU, and TBL rats ($p < 0.05$ for all).

QPCR

Additional analyses examined expression of wasting-related genes. In epididymal fat samples, HSL expression was not significantly different between groups (Fig. 6a). In quadriceps
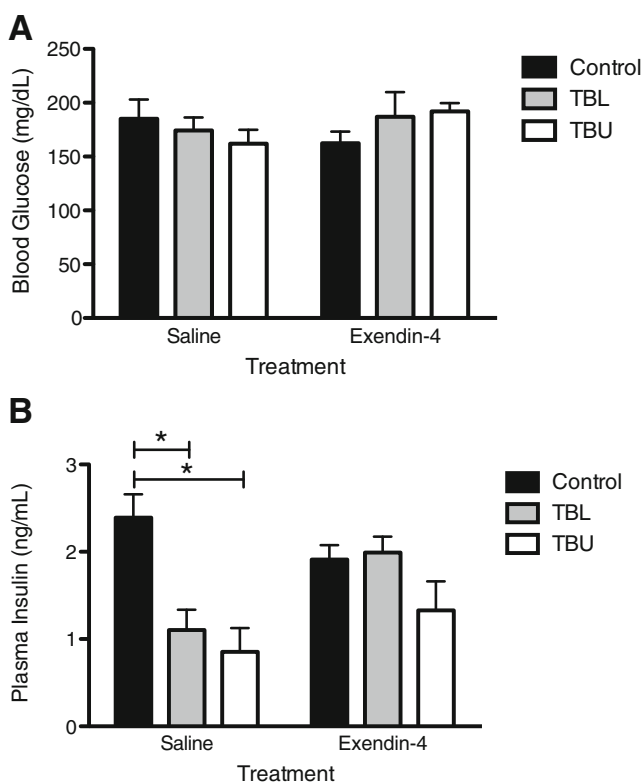
Fig. 5 Terminal blood glucose and plasma insulin levels. **a** No significant differences in blood glucose levels were observed. **b** Plasma insulin levels were reduced in saline-treated TBL and TBU rats, compared to controls. However, Exendin-4 treatment ameliorated this effect. *$p < 0.05$
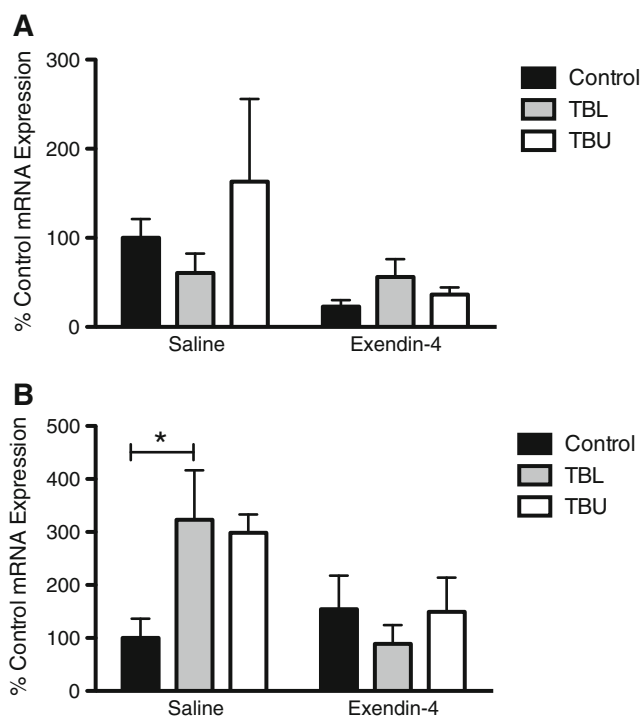
Fig. 6 Hormone-sensitive lipase (HSL) and Atrogin-1 mRNA expression in epididymal fat and quadriceps muscle, respectively. **a** No significant differences were observed in HSL expression. **b** Atrogin-1 expression was higher in saline-treated tumor-bearing rats, though this difference only reached statistical significance in TBL rats. Exendin-4 treatment resulted in Atrogin-1 expression that was comparable to controls in TBL and TBU rats. *$p < 0.05$

muscle samples, Atrogin-1 expression was increased in TBL rats, as compared to controls, in the saline-treated condition ($p < 0.05$) (Fig. 6b). This effect was not present in Exendin-4-treated animals.

Discussion

Currently, no effective treatments are available for patients with cancer cachexia and no prevention strategies exist. As a result, a large proportion of individuals with cancer will experience some degree of cancer cachexia [49, 50]. Cancer cachexia contributes significantly to both morbidity and mortality in individuals with cancer, making the prevention and treatment of cancer cachexia an important research focus. The present experiment was undertaken to determine the effects of chronic treatment with Exendin-4 on the development of cancer cachexia symptoms in rats bearing the Yoshida sarcoma.

Saline-treated animals bearing the Yoshida sarcoma experienced significant symptoms of cancer cachexia. TBL and TBU rats had lower levels of body weight gain, as well as lower levels of both body fat and skeletal muscle mass. A reduction in food intake was not observed in this experiment.

While minimal (approximately 5 %) differences have been observed previously in this model, differences of this magnitude are considered minimal [24]. Plasma insulin levels were significantly lower in TBL and TBU animals, as compared to controls. As further evidence of cachexia symptoms, expression of Atrogin-1 mRNA was higher in saline-treated TBL rats (non-significant elevation in TBU rats), as compared to control rats, suggesting an increase in skeletal muscle degradation via the ubiquitin–proteasome pathway. While TB rats were divided into upper and lower tertiles based on tumor growth, both TBL and TBU groups treated with saline developed the characteristic cachexia symptoms. This indicates that tumor burden itself may not be an important factor in determining the onset of cancer cachexia symptoms. These findings are consistent with the human literature [20, 26, 33, 41], and with previous experiments in the Yoshida sarcoma and other animal models [1, 9, 13, 16, 24, 29, 36, 46, 48].

Chronic treatment with Exendin-4 prevented the development of some cachexia symptoms in tumor-bearing animals, particularly in the lower tertile of tumor growth. Within the TBL group, Exendin-4-treated rats experienced weight gain and had fat mass comparable to their non-tumor-bearing controls. In addition, plasma insulin levels were comparable to those of controls. Reduced lean mass, as measured by hind leg

diameter, was observed in both Exendin-4-treated and saline-treated TBL and TBU rats, indicating that Exendin-4 treatment did not ameliorate muscle mass loss. Despite this, QPCR analysis indicated Exendin-4 treatment prevented the increase in Atrogin-1 expression associated with the tumor-bearing state, such that Atrogin-1 expression was not elevated above control levels in TBL or TBU groups. These results suggest that the ubiquitin–proteasome pathway may be less active, despite a decrease in lean mass. It is possible that the wasting associated with the cachexia syndrome has stopped, but muscle rebuilding has not yet occurred. Further experiments must investigate the potential effects of Exendin-4 in this avenue, possibly in conjunction with a high-protein diet or pharmacological treatment to increase muscle protein synthesis and rebuild lost muscle mass.

In addition to altering the development of cachexia symptoms in tumor-bearing animals, Exendin-4 treatment resulted in reduced tumor growth in animals in the TBL group. While the upper tertiles of tumor growth for each treatment were similar, tumors were significantly smaller in the lower tertile of Exendin-4-treated rats, as compared to the lower tertile of saline-treated rats. It is possible that treatment with Exendin-4 altered tumor growth through direct and/or indirect mechanisms.

Previous studies have demonstrated that application of Exendin-4 to certain cancer cell lines decreases cell proliferation. GLP-1 has been observed to exert independent effects on cell growth and proliferation, making this peptide and its receptor novel targets for cancer therapeutics. Exendin-4 treatment decreases cell proliferation in isolated breast [32] and colon cancer cells [28]. However, similar effects were not seen in pancreatic cancer cells [27], or primary mammary or liver cells [32]. GLP-1 receptors are expressed on a number of tumor cells [18, 28, 32], though GLP-1 receptor expression has not been measured in the Yoshida sarcoma. In addition, substantial overlap is presented between GLP-1 receptor signaling pathways and those known to affect tumor growth. For example, the Wnt/beta-catenin pathway controls cell growth and regulation in cancer cells and normal cells, and interacts with the GLP-1 receptor [18]. The nature of these interactions has not yet been well characterized. In future experiments, levels of circulating GLP-1, as well as expression of GLP-1 receptors on Yoshida sarcoma tumors, should be investigated to determine the involvement of GLP-1 and GLP-1 receptors in cancer and cancer cachexia.

Indirectly, Exendin-4 may improve the elements of insulin sensitivity, which may lead to an environment that is less conducive to tumor growth in general. While glucose tolerance and insulin sensitivity were not directly measured following Exendin-4 treatment, plasma insulin levels were comparable in control and TB rats treated with Exendin-4, while insulin levels were lower in saline-treated TB rats. Previous research has linked insulin dysregulation to increased tumor growth and cancer incidence in both humans and animals [44]. A preservation of proper insulin levels may indicate a preservation of insulin sensitivity in these animals and could lead to reduced tumor growth. Additionally, Exendin-4 is known to produce potent anti-inflammatory effects, which could indirectly result in a reduction in tumor growth in these animals. TNF-α, IL-1, and IL-6 are significant mediators of tumor growth and inflammation [39], as well as cachexia symptoms [35], and expression of these peptides is often increased in individuals with diabetes [7]. Daily treatment with a synthetic Exendin-4 analog reduced mRNA expression of TNF-α and IL-1, and reduced circulating levels of IL-6 in insulin resistant individuals with type II diabetes [4]. The influence of Exendin-4 on inflammation in the present model has yet to be investigated.

The present experiment demonstrates the potential of Exendin-4 treatment to act as a preventative agent in the development of Yoshida sarcoma-induced cancer cachexia. These data represent a novel use of Exendin-4, one of few compounds to prevent the development of cancer cachexia symptoms in any animal model. These results could have important implications for cachexia treatment in individuals with cancer, and the effects of Exendin-4 should be investigated in other models, including human xenograft models, to further explore these implications. Future experiments should attempt to determine if these effects are the result of a direct effect on tumor growth or indirect effects on insulin sensitivity and other parameters. In addition, the differential effects of treatment in animals which develop large or small tumors should be explored, as some inherent tumor-related factors, such as pro-inflammatory cytokines, may alter the effectiveness of Exendin-4 treatment. Such mechanistic knowledge would be helpful in determining the utility of this treatment in a clinical setting.

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