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Cachexia induced by Yoshida ascites hepatoma in Wistar rats is not associated with inflammatory response in the spleen or brain

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ABSTRACT

Recent data indicate that peripheral, as well as hypothalamic pro-inflammatory cytokines play an important role in the development of cancer cachexia. However, there are only a few studies simultaneously investigating the expression of inflammatory molecules in both the periphery and hypothalamic structures in animal models of cancer cachexia. Therefore, using the Yoshida ascites hepatoma rat's model of cancer cachexia we investigated the gene expression of inflammatory markers in the spleen along with the paraventricular and arcuate nuclei, two hypothalamic structures that are involved in regulating energy balance. In addition, we investigated the effect of intracerebroventricular administration of PS-1145 dihydrochloride (an Ikβ inhibitor) on the expression of selected inflammatory molecules in these hypothalamic nuclei and spleen. We observed significantly reduced food intake in tumor-bearing rats. Moreover, we found significantly decreased expression of IL-6 in the spleen as well as decreased NF-κB in the paraventricular nucleus of rats with Yoshida ascites hepatoma. Similarly, expression of TNF-α, IL-1β, NF-κB, and COX-2 in the arcuate nucleus was significantly reduced in tumor-bearing rats. Administration of PS-1145 dihydrochloride reduced only the gene expression of COX-2 in the hypothalamus. Based on our findings, we suggest that the growing Yoshida ascites hepatoma decreased food intake by mechanical compression of the gut and therefore this model is not suitable for investigation of the inflammation-related mechanisms of cancer cachexia development.

1. Introduction

Approximately 80% of cancer patients in the advanced stages of the disease develop cachexia that significantly decreases their quality of life and survival (Gullett et al., 2011). In general, cancer cachexia results from decreased energy intake, increased energy expenditure, as well as excessed catabolism and inflammation (Baracos et al., 2018). The main mediators of the cachexic process include molecules released by the host and/or tumor cells (e.g. cytokines TNF-α, IL-1β, IL-6, ciliary neurotrophic factor, and interferon γ) and catabolic products of tumor cells (e.g. lipid-mobilizing factor and proteolysis-inducing factor) (Porporato, 2016; Tisdale, 2002). These mediators might induce catabolic processes in several tissues and organs regulating energy intake and expenditure, including skeletal muscles, white and brown adipose tissue, liver, and heart (Argiles et al., 2014). However, recent data indicate that cancer also affects the brain's neuronal circuits regulating energy homeostasis by inducing inflammation in hypothalamic tissue (Burfeind et al., 2018; Lira et al., 2011; Michaelis et al., 2017).

In contrast to peripheral tissues and organs, the role of the brain in the development of cancer cachexia remains only vaguely described. In 2005, Konsman and Blomqvist discovered significantly increased neuronal activity in forebrain structures, including hypothalamic nuclei participating in regulating energy homeostasis in anorexic and cachexic rats injected with Morris hepatoma 7777 (Konsman and Blomqvist, 2005). Arruda et al. (2010) showed that central administration of TNF-α increased energy expenditure, whereas intracerebroventricular administration of infliximab (an antibody against TNF-α) to rats with Walker-256 tumors significantly increased food intake and prolonged their survival. In 2011, Braun et al. found that centrally administered IL-1β enhanced expression of *muscle ring-finger protein-1 (also known as* tripartite motif containing 63, MurF1), which mediates muscular atrophy in skeletal muscles (Braun et al., 2011). Based on the above-mentioned data it can be suggested that cancer induces changes in the activity of brain structures regulating energy homeostasis and that cytokines represent signaling molecules interconnecting peripheral and central mechanisms responsible for the development of anorexia and cachexia.

Yoshida ascites hepatoma represents a frequently used rat model of

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cancer cachexia. This tumor model is characterized by rapid and progressive loss of body weight and reduced food intake, along with protein and lipid hypercatabolic states in host tissues (Costelli et al., 1999; Tessitore et al., 1987). Even if several studies indicate that pro-inflammatory cytokines play a role in the development of cachexia, the role of inflammation-related signals in the development of cancer cachexia in rats with Yoshida ascites hepatoma remains unclear.

Therefore, to elucidate the mechanisms responsible for the development of cancer cachexia in the Yoshida ascites hepatoma model, we investigated the gene expression of selected markers for inflammation in the spleen as well as in the hypothalamic paraventricular (PVN) and arcuate (NARC) nuclei of Wistar rats injected with Yoshida ascites hepatoma cells. In addition, we investigated the effect of intracerebroventricular administration of PS-1145 dihydrochloride on the expression of selected inflammation markers in tumor-bearing rats. This compound inhibits activity of NF-κB by blocking IκB kinase phosphorylation. We expected that PS-1145 might attenuate hypothalamic inflammation and therefore affect the development of cachexia and eventually the growth of Yoshida ascites hepatoma.

2. Materials and methods

2.1. Animals

Twenty four adult male Wistar rats (175–200 g; Charles River, Germany) were used. Animals were housed 3 per cage in standard conditions (22 ± 1 °C, 12 h light/dark cycle, humidity 55 ± 10%) with ad libitum access to tap water and regular pellet diet. All experimental procedures were approved by the Animal Care Committee of the Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava and State Veterinary and Food Administration of the Slovak Republic. The rats received care in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

2.2. Experimental protocol

At the beginning of the experiment, 24 animals were randomly divided into 4 groups:

- control animals without tumor cell inoculation (C; $n = 6$)
- rats injected intraperitoneally (i.p.) with Yoshida AH-130 tumor cells (Y; n = 6)
- rats injected i.p. with Yoshida AH-130 tumor cells and given a physiological solution via intracerebroventricular (i.c.v.) cannula (Y + SAL; n = 6)
- rats injected i.p. with Yoshida AH-130 tumor cells and treated with PS-1145 dihydrochloride via i.c.v. cannula (Y + PS1145; n = 6)

At the beginning of the experiment (day 0), Yoshida AH-130 tumor cells were injected i.p. into animals of the Y, Y + SAL, and Y + PS1145 groups. Subsequently (day 4 and 5), osmotic minipumps were implanted in animals from the Y + SAL and Y + PS1145 groups. On the 19th day of the experiment, all animals were sacrificed (Fig. 1). Food and water intake were determined as the weight difference of water and food over the following two days: 8–9, 11–12, and 15–16.

2.2.1. Administration of Yoshida AH-130 cells

Yoshida AH-130 tumor cells were administered i.p. to the right lower quadrant of the abdominal cavity of the rats as a dose of 5×10^6 cells in 2 mL of 0.1 M phosphate buffer solution. No anesthesia was used for tumor cell injection. The presence of ascites was detected by palpation of the rats' abdomen as previously described (Bauer et al., 2002; Corradi et al., 2012; Lopeznovoa et al., 1980). This approach allowed us to determine the timing of tumor occurrence after injection of Yoshida AH-130 tumor cells.

2.2.2. Implantation of osmotic minipumps

Coordinates used for i.c.v. implantation of an osmotic mini-pump cannula (Brain Infusion Kit 2, Alzette, Durect Corporation, Cupertino, USA) were identified using a stereotactic rat brain atlas (Paxinos and Watson, 1997). Rats were anesthetized via intramuscular (i.m.) application of ketamine-xylazine solution: 1.2 mL/kg ketamine (Narkamon 5%, Spofa, Prague, Czech Republic) and 0.4 mL/kg xylazine (Rometar 2% Spofa, Prague, Czech Republic). Their heads were then fixed into the stereotactic apparatus (David-Kopf Instruments, California, USA) and bregma was identified as a reference point. An infusion cannula was inserted targeting the 3rd lateral ventricle (stereotaxic coordinates from bregma: AP -1.4 mm, ML +2.2 mm, DV -4.0 mm), fixed by cyanoacrylate glue and connected to a subcutaneously positioned minipump (Paxinos and Watson, 1997) via polyethylene tubing. The minipumps were filled with vehicle (saline, pH 7.4, Y + SAL group, $n = 6$) or PS-1145 dihydrochloride (10 μg/day/animal, Y + PS1145 group, n = 6). The dosage of PS-1145 dihydrochloride was chosen based on previous studies (Oh-I et al., 2010; Posey et al., 2009). After surgery, the rats were housed individually in cages until recovery.

2.3. Tissue processing and microdissection of the brain areas

After decapitation of animals, brains and spleen were promptly removed, frozen on dry ice, and then stored at −70 °C until processing. Brain coronal sections (300 μm) were prepared using a cryostat (Richter Jung, Budapest, Hungary) at −12 °C. Sections containing the hypothalamus were placed on microscope slides. The two investigated hypothalamic nuclei (NARC, PVN) were then isolated from the sections using a micropunch (Palkovits, 1973) and immediately stored at −70 °C for further analysis.

2.4. RNA isolation and real-time polymerase chain reaction (PCR)

RNA was isolated using the TRI Reagent RT (Molecular Research Center, Inc., Cincinnati, OH, USA) following the manufacturer's instructions. The concentration of RNA was determined using a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Subsequently, RNA was transcribed into cDNA using the RevertAid H minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific) using an oligo dT primer in accordance with the manufacturer's instructions. The total volume of the real-time PCR was 10 μL/sample, containing 20 ng of template cDNA (2 μL) mixed with 6 μL of FastStart Universal SYBR Green Master Rox (Roche Diagnostics, Basel, Switzerland), 1 μL of specific primer pair set, and 1 μL of water. We determined the expression of the following genes (Table 1); splice variant of FBJ (murine osteosarcoma viral oncogene homolog B; ΔFosB), AP-1 transcription factor subunit (c-fos), cytochrome *c* oxidase subunit 2 (COX-2), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), nuclear factor kappa B (NF-κB), and tumor necrosis factor alpha (TNF-α). Samples were analyzed on ABI7900HT Fast Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA) using the following temperature template: (1) 2 min at 50 °C, (2) 10 min at 95 °C, (3) 40 cycles of 15 s at 95 °C, and (4) 1 min at 60 °C. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels and were expressed as the relative fold change, as determined via the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Finally, specificity of the amplified products was determined via a melting curve analysis. In the spleen samples, we determined the expression of TNF-α, IL-1β, IL-6, NF-κB, and COX-2. In the brain samples, we determined the expression of TNF-α, IL-1β, NF-κB, c-Fos, COX-2, and ΔFosB.

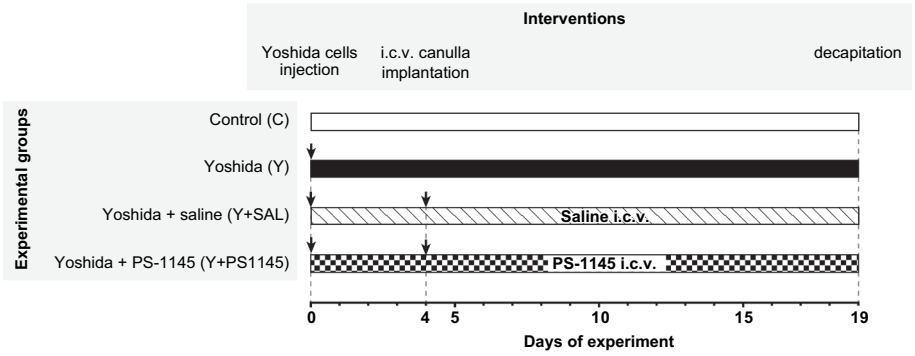

Fig. 1. Design of experiment.

Table 1
Primers used in this study and their sequences. ΔFosB - splice variant of FBJ (murine osteosarcoma viral oncogene homolog B), c-fos - AP-1 transcription factor subunit, COX-2 - cytochrome *c* oxidase subunit 2, GAPDH - glyceraldehyde 3-phosphate dehydrogenase, IL-1β - interleukin 1 beta, IL-6 – interleukin 6, NF-κB - nuclear factor kappa B, and TNF-α - tumor necrosis factor alpha.

Gene	Primer sequence
ΔFosB	For. 5′-AGGCAGAGCTGGAGTCGGAGAT-3′
	Rev. 5′-GCCGAGGACTTGAACTTCACTCG-3′
c-fos	For. 5′-GGCAGAAGGGGCAAAGTAGA-3′
	Rev. 5′-AGTTGATCTGTCTCCGCTTGG-3′
COX-2	For. 5′-TGGGCCATGGAGTGGACTTA-3′
	Rev. 5′-ACGTGGGGAGGGTAGATCAT-3′
GAPDH	For. 5′-TGGACCACCCAGCCCAGCAAG-3′
	Rev. 5′-GGCCCCTCCTGTTGTTATGGGGT-3′
IL-1β	For. 5′-CAGCTTTCGACAGTGAGGAGA-3′
	Rev. 5′-TGTCGAGATGCTGCTGTGAG-3
IL-6	For. 5′- CTCTCCGCAAGAGACTTCCA −3′
	Rev. 5′- GGTCTGTTGTGGGTGGTATCC -3
NF-κB	For. 5′-AATATTCACCTGCACGCCCA-3′
	Rev. 5′-GTTTGCAAAGCCAACCACCA-3′
TNF-α	For. 5′-GATCGGTCCCAACAAGGAGG-3′
	Rev. 5′-GTTTGCTACGACGTGGGCTA-3′

2.5. Statistical analysis

Data were analyzed using a *t*-test, one-way ANOVA (factor group) or two-way ANOVA (factor time and group), followed by Bonferroni post-hoc test (GraphPad Prism 5, version 8.0.0 GraphPad Software, Inc. San Diego, CA, USA; Sigmaplot, version 11.0, Systat Software Inc., Chicago, IL, USA). Results were considered significant if $p \leq .05$. Analyzed data are expressed as mean ± SEM.

3. Results

3.1. Tumor incidence, ascites weight and volume

All animals from groups Y and Y + SAL developed tumors. In group Y + PS1145 tumor development was observed in 5 out of 6 animals (83.3%) injected with tumor cells. The one animal that did not develop a tumor after Yoshida AH-130 cell injection was excluded from analysis.

We did not observe any significant differences in the weight ($F_{(2,16)} = 0.0722$, $p = .931$, Fig. 2A) or volume ($F_{(2,16)} = 0.0550$, $p = .947$, Fig. 2B) of ascites between untreated tumor-bearing rats and tumor-bearing rats administered i.c.v. saline or PS-1145 dihydrochloride.

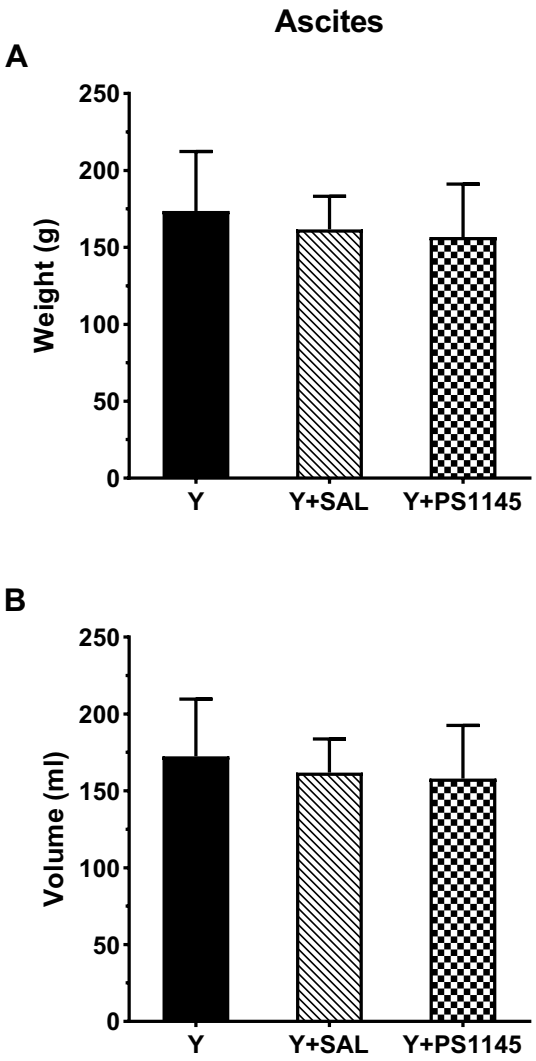

Fig. 2. Weight (A) and volume of ascites (B) within tumor-bearing rats (Y), tumor-bearing rats treated with saline (Y + SAL), and tumor-bearing rats treated with PS-1145 dihydrochloride (Y + PS1145). The weight and volume of ascites were measured on the day of decapitation (19th day of the experiment). Each value is expressed as the mean ± SEM and represents an average of 5–6 rats (analyzed via one-way ANOVA).

3.2. Food and water intake

We observed significantly reduced food intake in tumor-bearing animals over time ($F_{(2,60)} = 18.98$, $p < .001$) and between groups ($F_{(3,60)} = 7.792$, $p < .001$; Table 2). Similarly, water intake was significantly reduced in tumor-bearing animals ($F_{(3,60)} = 9.456$, $p < .001$). Moreover, we observed an interaction between investigated factors ($F_{(6,60)} = 2.847$, $p = .017$; Table 3).

Table 2
Food intake of control rats (C) in rats with induced Yoshida AH-130 ascites hepatoma (Y), rats with induced Yoshida AH-130 ascites hepatoma treated with saline (Y + SAL), and rats with induced AH-130 ascites hepatoma treated with PS-1145 dihydrochloride (Y + PS1145) on the 9th, 12th, and 16th day of the experiment. Each value is expressed as the mean ± SEM and represents an average of 6 rats. Results were considered significant when $p < .05$.

	Day 9	Day 12	Day 16
C	28.83 ± 0.39	28.16 ± 0.39	23.66 ± 1.88
Y	22.22 ± 1.38[a]	25.44 ± 3.23	18.11 ± 1.13
Y + SAL	25.38 ± 1.04[a,b]	21.38 ± 2.22	13.44 ± 1.42
Y + PS-1145	24.61 ± 1.10	23.77 ± 3.01[b]	17.38 ± 1.89 [b]

[a] vs. C.
[b] vs. Y (analyzed by two-way ANOVA).

Table 3
Water intake of control rats (C) in rats with induced Yoshida AH-130 ascites hepatoma (Y), rats with induced Yoshida AH-130 ascites hepatoma treated with saline (Y + SAL), and rats with induced AH-130 ascites hepatoma treated with PS-1145 dihydrochloride (Y + PS1145) on the 9th, 12th and 16th day of the experiment. Each value is expressed as the mean ± SEM and represents an average of 6 rats. Results were considered significant when $p < .05$.

	Day 9	Day 12	Day 16
C	47.83 ± 0.22	43.33 ± 1.49	39.16 ± 3.20
Y	60.77 ± 5.03 [a]	47.00 ± 2.16	49.33 ± 2.88
Y + SAL	34.44 ± 5.96[a,b]	40.83 ± 2.35	37.16 ± 4.09
Y + PS-1145	43.00 ± 1.60 [b]	34.55 ± 1.13 [b]	48.33 ± 5.96

[a] vs. C.
[b] vs. Y (analyzed by two-way ANOVA).

3.3. Free body weight and splenic weight

For the study of cancer cachexia, several animal models are used, including Yoshida ascites hepatoma (Bennani-Baiti and Walsh, 2011; Costelli et al., 1995; Tisdale, 1997). In this model, if the body weight of animals increases during the course of tumor development, this increase normally results from the accumulation of ascetic fluid in the abdominal cavity. Therefore, at the end of the experiment, we evaluated the free body weight of rats (free body weight = total body weight - ascites weight) rather than total body weight.

We observed significantly reduced free body weight in all groups of tumor-bearing rats when compared to control animals (between groups: $F_{(3,22)} = 20.212$, $p < .001$; C vs. Y: 335.33 ± 8.27 vs. 198.83 ± 25.65, $t = 5.934$, $p < .001$, C vs. Y + saline: 335.33 ± 8.27 vs.191.00 ± 15.92, $t = 6.274$, $p < .001$; C vs. Y + PS-1145: 335.33 ± 8.27 vs. 175.40 ± 6.22; $t = 6.629$, $p < .001$, Fig. 3A). Moreover, splenic weight was significantly reduced in tumor bearing rats treated with PS-1145 dihydrochloride when compared to controls (between groups: $F_{(3,23)} = 4.947$, $p = .010$; C vs. Y + PS1145: 0.9 ± 0.09 vs. 0.40 ± 0.10; $t = 3.659$, $p = .009$, Fig. 3B).

3.4. Cachexia index

For evaluation of cachexia development in tumor-bearing rats we used a cachexia index (CI) that was calculated according to the equation (Fracaro et al., 2016; Guarnier et al., 2010):

$$CI(\%) = [IBM–FBM + (WT) + GMC](IBM + GMC) * 100$$

with IBM = initial body mass, FBM = final body mass, WT = weight of the tumor, and GMC = mean body mass gain of the control animals. Although we observed cachexia (CI > 5%) in all groups with Yoshida ascites hepatoma, no significant differences between Y, Y + FS and Y + PS1145 group were found ($F_{(2,16)} = 1.222$, $p = .324$; Table 4).

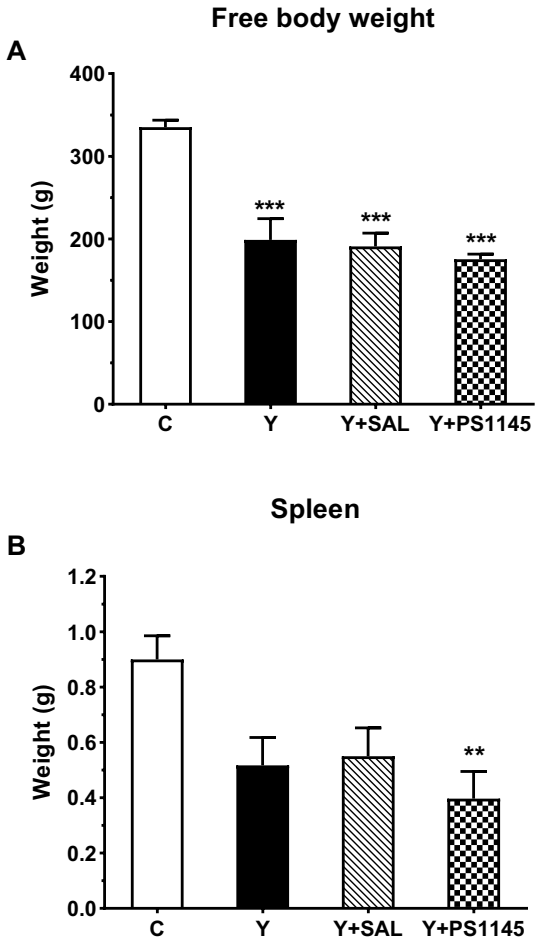

Fig. 3. Free body weight (A) and splenic weight (B) of control rats (C), tumor-bearing rats (Y), tumor-bearing rats treated with saline (Y + SAL), and tumor-bearing rats treated with PS-1145 dihydrochloride (Y + PS1145) on the day of decapitation (19th day of the experiment). Each value is expressed as the mean ± SEM and represents an average of 5–6 rats. * $p < .05$; ** $p < .01$; *** $p < .001$ (analyzed by one-way ANOVA).

Table 4
Cachexia index (CI) in rats with induced Yoshida AH-130 ascites hepatoma (Y), rats with induced Yoshida AH-130 ascites hepatoma treated with saline (Y + SAL), and rats with induced AH-130 ascites hepatoma treated with PS-1145 dihydrochloride (Y + PS1145) calculated on the day of decapitation (19th day of the experiment). Each value is expressed as the mean ± SEM and represents an average of 5–6 rats (analyzed by one-way ANOVA).

	CI
Y	37.86 ± 7.94
Y + SAL	44.54 ± 3.80
Y + PS-1145	50.53 ± 3.16

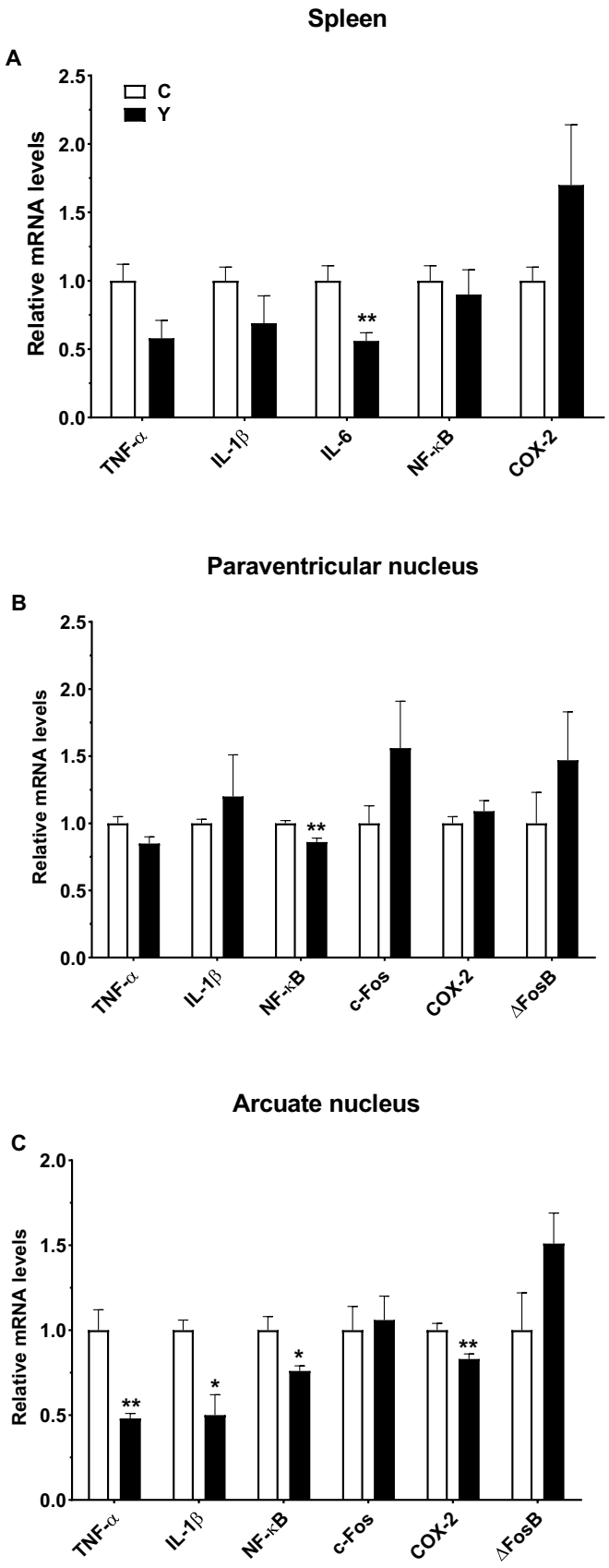

Fig. 4. Expression of selected markers in the spleen (A), paraventricular nucleus (B), and arcuate nucleus (C) of control and tumor-bearing rats. Each value is expressed as the mean ± SEM and represents an average of 5–6 animals. ΔFosB - splice variant of FBJ (murine osteosarcoma viral oncogene homolog B), c-fos - AP-1 transcription factor subunit, COX-2 - cytochrome *c* oxidase subunit 2, IL-1β - interleukin 1 beta, IL-6 – interleukin 6, NF-κB - nuclear factor kappa B, TNF-α - *tumor necrosis factor alpha.* * $p < .05$; ** $p < .01$; *** $p < .001$ (analyzed by *t*-test).

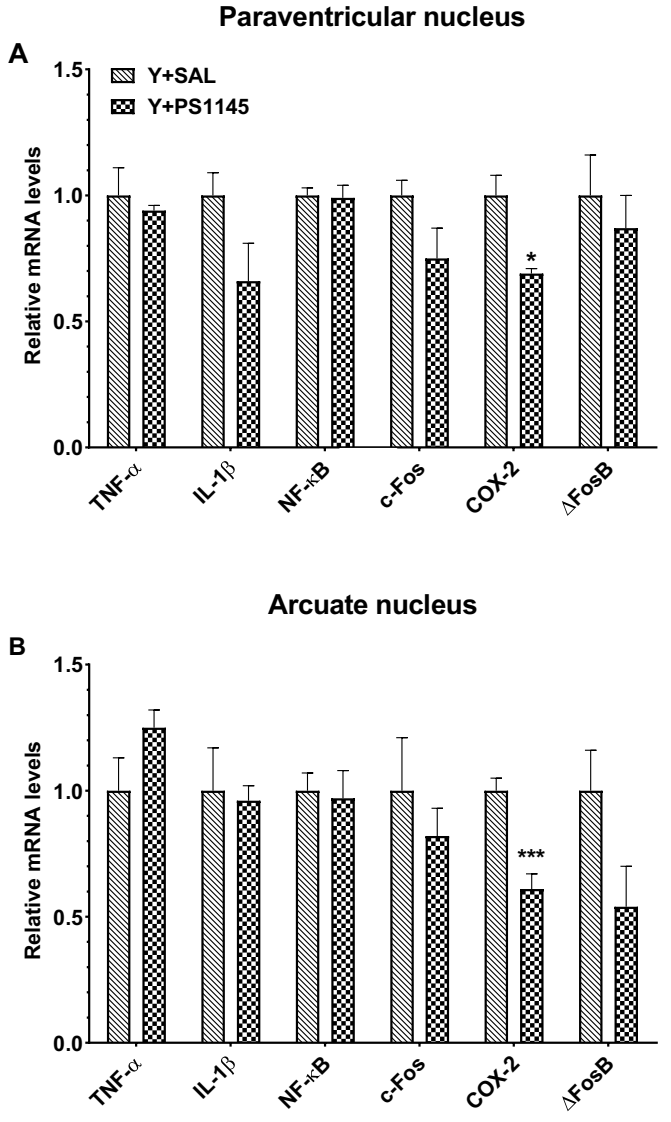

Fig. 5. Expression of inflammatory markers and markers of neuronal activity in the paraventricular (PVN, A) and arcuate nuclei (NARC, B) of tumor-bearing rats treated with saline (Y + SAL) and tumor-bearing rats treated with PS-1145 dihydrochloride (Y + PS1145). Each value is expressed as the mean ± SEM and represents an average of 5–6 animals. ΔFosB - splice variant of FBJ (murine osteosarcoma viral oncogene homolog B), c-fos - AP-1 transcription factor subunit, COX-2 - cytochrome *c* oxidase subunit 2, IL-1β - interleukin 1 beta, NF-κB - nuclear factor kappa B, TNF-α - .tumor necrosis factor alpha * $p < .05$; ** $p < .01$; *** $p < .001$ (analyzed by t-test).

3.5. Expression of markers of inflammation in the spleen and in the brain

We found significantly reduced expression of IL-6 in the spleen of tumor-bearing animals when compared to control rats (C vs. Y: 1.00 ± 0.02 vs. 0.86 ± 0.03; $t = 3.367$, $p = .008$, Fig. 4A). We also found significantly decreased expression of NF-κB in the PVN of tumor-

bearing rats compared to control rats (C vs. Y: 1.00 ± 0.02 vs. 0.86 ± 0.03; $t = 3.385$, $p = .008$, Fig. 4B). Similarly, in rats injected by tumor cells we found decreased expression of TNF-α (C vs. Y: 1.00 ± 0.12 vs. 0.48 ± 0.03; $t = 4.155$, $p = .002$), IL-1β (C vs. Y: 1.00 ± 0.06 vs. 0.50 ± 0.12; $t = 2.932$, $p = .017$), NF-κB (C vs. Y: 1.00 ± 0.08 vs. 0.76 ± 0.03; $t = 2.951$, $p = .015$), and COX-2 (C vs. Y: 1.00 ± 0.04 vs. 0.83 ± 0.03; $t = 3.415$, $p = .008$) in the NARC (Fig. 4C).

3.6. Effect of PS-1145 dihydrochloride on expression of markers of inflammation in the brain of tumor-bearing rats

PS-1145 dichloride administration significantly decreased expression of COX-2 in both the PVN and NARC in rats with Yoshida ascites hepatoma when compared to rats treated with saline (PVN: Y + SAL vs. Y + PS1145: 1.00 ± 0.08 vs. 0.69 ± 0.02; $t = 3.289$, $p = .011$, Fig. 5A; NARC: Y + SAL vs. Y + PS1145: 1.00 ± 0.05 vs. 0.61 ± 0.06; $t = 4.804$, $p < .001$, Fig. 5B).

4. Discussion

It is suggested that development of cancer anorexia and cachexia is induced, at least partially, by cancer-related inflammatory molecules acting in both the periphery and the brain (Burfeind et al., 2016; Mravec et al., 2019). Surprisingly, even though we used the Yoshida AH-130 tumor model, which represents an established model of cancer anorexia and cachexia (Bennani-Baiti and Walsh, 2011), we did not find an increase in the investigated pro-inflammatory markers in the spleen, but instead found decreased splenic IL-6 mRNA levels. Similarly, in hypothalamic nuclei that play a central role in the maintenance of energy balance we found reduced levels of mRNA for pro-inflammatory molecules. Specifically, we found decreased gene expression of NF-κB in the PVN and decreased gene expression of TNF-α, IL-1β, NF-κB, and COX-2 in the NARC of tumor-bearing rats when compared to control animals. We propose several explanations for our findings.

Our data indicate that growth of Yoshida ascites hepatoma is not accompanied by elevated expression of inflammatory molecules in the periphery or brain. However, these results are counterintuitive, as the majority of the current studies have shown increased expression of inflammatory mediators in both the periphery and brain in response to peripheral tumor growth (Aoyagi et al., 2015; Porporato, 2016; Vaughan et al., 2013). Even using the same tumor model, Catalano et al. (2003) demonstrated elevated expression of TNF-α in several body tissues, including the spleen and brain of rats bearing Yoshida ascites hepatoma 7 days after inoculation of tumor cells. Moreover, the authors detected up-regulation of TNF-α receptor I. Differences between these findings and our study might be due to their use of a higher dose of injected tumor cells (10^8 vs. 5×10^6 in our model) and lower initial body weight of animals (50–75 g vs. 175–200 g in our study). Therefore, tumors induced by Catalano et al. (2003) might be accompanied by more progressive growth when compared to the tumors induced in our study. Thus, even if the authors used the same tumor cells, the two experiments might differ in the speed of tumor development as well as dynamics of immune responses (Catalano et al., 2003). Moreover, we hypothesize that the more progressive tumor growth led to damage of surrounding tissues, which consequently increased expression of the inflammatory factors investigated by Catalano et al. (2003).

Even if Yoshida ascites hepatoma is a widely-used model for cancer anorexia and cachexia (Bennani-Baiti and Walsh, 2011), the exact role of inflammation in this model remains questionable, as the inflammatory factors that might be responsible for anorexia and cachexia have yet to be determined, as documented by the below mentioned studies using Yoshida ascites hepatoma. Importantly, even if the study of Catalano et al. confirmed elevated expression of TNF-α and its receptor (Catalano et al., 2003), inhibition of IL-1 and TNF-α expression did not affect food intake and cachexia in tumor-bearing rats with Yoshida ascites hepatoma (Busquets et al., 2000). Similarly, while anti-TNF treatment attenuated catabolic processes in the liver, heart and skeletal muscle, it was not able to prevent body weight reduction (Costelli et al., 1993). In addition, Catalano et al. (2003) did not find any differences in the expression of IL-6 or its receptor in the brain, kidney, spleen, liver, or muscle of tumor-bearing rats. In another study, administration of an IL-1 receptor antagonist to tumor-bearing rats did not alter cachexia or tumor growth (Costelli et al., 1995). A positive effect of anti-inflammatory treatment was observed only after double-inhibition of both activator protein 1 (AP-1) and NF-κB, which reduced tumor growth of Yoshida AH-130 ascites hepatoma in rats (Moore-Carrasco et al., 2009). In addition, these authors also found a positive effect of double-inhibition of AP-1 and NF-κB on the weight of tibialis and gastrocnemius muscle as well as the heart and kidneys. Nonetheless, this treatment did not affect the overall body weight of animals (Moore-Carrasco et al., 2007). On the contrary, in our study inhibition of Ikβ did not affect tumor weight, volume, or food intake. However, we found that administration of PS-1145 dihydrochloride reduced the expression of COX-2 in both hypothalamic nuclei.

Importantly, it has to be noted that Yoshida ascites hepatoma is a tumor type with specific features when compared to other types of cancer. We suggest that this specificity may be one of the factors that might, at least partially, explain our findings. First of all, Yoshida ascites hepatoma is characterized by massive ascitic fluid accumulation in the abdominal cavity (Yoshida, 1956). Secondly, due to its features, Yoshida ascites hepatoma seems to be relatively hypovascularized when compared to other tumor types (Hori et al., 1990). This hypovascularization of the tumor has been demonstrated by several studies in a similar model, Yoshida sarcoma (Bakker et al., 2017; Luboldt et al., 2009). In general, inflammatory response to cancer growth is also triggered by inflammatory factors produced by tumor cells (Crusz and Balkwill, 2015). Cytokines and chemokines released by cancer cells attract leukocytes and further potentiate inflammatory responses of the organism to cancer (Coussens and Werb, 2002; Vaughan et al., 2013). We suggest that the limited vascularization of Yoshida ascites hepatoma might partially explain the low gene expression of investigated inflammatory factors in the spleen and brain. Based on these above-mentioned facts, we suggest that interactions between tumor and immune cells are less extensive in Yoshida ascites hepatoma when compared to other (solid) tumor models.

Previous studies have described peripheral inflammation as one of the crucial factors that contribute to development of cancer cachexia in many different tumor types. This effect is mediated either via direct action of pro-inflammatory cytokines on peripheral tissues or via induction of inflammatory changes in the central nervous system (Coussens and Werb, 2002; Langen et al., 2001; Li et al., 1998). In our experiment, we did not detect peripheral or central increases of selected inflammatory markers, yet animals with Yoshida ascites hepatoma developed cachexia. Moreover, animals with tumors showed significantly decreased food intake. Similar results were observed by Honors and Kinzig (2013) who showed decreased food intake and body weight in Yoshida sarcoma, a similar cancer rat model. In addition, the authors observed decreases in body fat and skeletal muscle mass as well as increased expression of Atrogin 1 (indicator of muscle atrophy) in the quadriceps muscle of tumor-bearing rats. Together with our results, these data indicate that reduced food intake might be the most dominant mechanism in the development of cachexia in Yoshida ascites hepatoma. We suggest that the massive volume of developed ascites in tumor-bearing rats caused significant pressure on organs of the gastrointestinal tract in the abdominal cavity and that this factor might be responsible for decreased food passage via the gut, followed by reduced food intake resulting into body weight loss.

Recent findings suggest that pathological body weight reduction might be associated with altered expression of inflammatory markers in the hypothalamus. These data indicate that hypothalamic inflammation triggers development of cancer cachexia (Coussens and Werb, 2002;

Langen et al., 2001; Li et al., 1998). However, in our study we observed reduced expression of pro-inflammatory cytokines in the NARC, a structure that plays a crucial role in the regulation of food intake (Gao and Horvath, 2008; Seoane-Collazo et al., 2015). We hypothesize that the pressure of ascites on the gastrointestinal tract within the abdominal cavity triggered reduction of food intake and that this state of caloric restriction participated in the observed reduction of pro-inflammatory cytokines levels in the NARC. This assumption is based on observations that calorie restriction attenuates the inflammatory response of the organism in various conditions (Chung et al., 2002; Seyfried et al., 2003).

We also investigated the effect of chronic intracerebroventricular administration of PS-1145 dihydrochloride (an Ikβ inhibitor) on tumor progression. Even though administration of PS-1145 dihydrochloride lowered COX-2 expression in both the NARC and PVN, it did not affect expression of any other inflammatory markers. In the brain, COX-2 plays an important role in inducing inflammatory processes associated with increased peripheral levels of cytokines, such as IL-1β. During peripheral inflammation, COX-2 is active in endothelial cells and perivascular macrophages and is responsible for increased expression of prostaglandin $E_{2,}$ which might in turn regulate further progression of central cytokines expression (Ek et al., 2001). Expression of COX-2 is regulated by the IκB kinase/nuclear factor kappa B (IKK/ NF-κB) pathway in several cancer types (Chen et al., 2013; Kim et al., 2009). Contrary to our results, another study showed a positive effect of double inhibition of AP-1 and NF-κB on reducing tumor growth and on the weight of muscles, heart, and kidneys in rats with Yoshida ascites hepatoma (Moore-Carrasco et al., 2007). Therefore, it is possible that other inflammatory pathways, such as c-Jun N-terminal kinase/activator protein 1 (JNK/AP1) might play a role rather than the IKK/NF-κB pathway, at least in the periphery. Regardless, further studies will be necessary to confirm the role of inflammation in Yoshida ascites hepatoma-associated cachexia.

Our data indicate that at least in some cancer types, the development of anorexia and cachexia is mediated via mechanisms other than inflammation. In support of this, Pourtau et al. (2011) found that anorexia could develop in the absence of increased plasma cytokine levels in a rat hepatoma model. In addition, these authors found blunted agouti-related peptide (AgRP) expression, which was attributed to increased leptin and decreased ghrelin plasma levels in tumor-bearing rats. However, the authors used a model with small tumors (1–2% of body weight), so it remains questionable if the expression of inflammation markers would remain the same after further tumor progression (Pourtau et al., 2011). In another study, Tsai et al. (2012) found that non-inflammatory pathways such as macrophage inhibitory cytokine-1/growth differentiation factor 15 (MIC-1/GDF15) might also be responsible for development of anorexia and cachexia. Both above-mentioned studies indicate that the development of cancer anorexia and cachexia might be mediated via non-inflammatory signaling molecules in certain cancer types. Whether non-inflammatory molecules also play a role in the development of cachexia in rats with Yoshida ascites hepatoma, or if cancer cachexia in these animals arises from mechanical obstruction of the gastrointestinal tract needs further investigation. From this point of view, experiments determining the effect of reduced intraabdominal pressure by removal of ascites in Yoshida ascites hepatoma might elucidate the mechanisms behind our observations.

5. Limitations of the study

It remains unclear whether anorexia and cachexia in rats with Yoshida ascites hepatoma was induced by obstruction of the gastrointestinal tract by the volume of ascites itself, or if signaling molecules in the fluid could have any role. These factors should be considered in further studies attempting to determine the mechanisms responsible for the development of anorexia and cachexia in rats with Yoshida ascites hepatoma. Removal of ascites and exchange of ascites to saline during the course of the experiment might elucidate the role of mechanical obstruction of the gut in the development of anorexia and cachexia in this cancer model.

In this study, we focused on the determination of hypothalamic inflammation and the effect of PS-1145 dihydrochloride-attenuated brain inflammation on tumor growth. However, we did not determine the effect of PS-1145 dihydrochloride in animals without tumors. Therefore, in further studies it will be necessary to also use groups of animals without tumors that will be administered intracerebroventricular saline or PS-1145 dihydrochloride.

6. Conclusions

Our data indicate that development of cachexia in rats with Yoshida ascites hepatoma might be related to mechanisms independent of the expression of investigated inflammatory markers in the spleen and central nervous system. We hypothesize that Yoshida ascites hepatoma decreased food intake by mechanical compression of the gut and therefore usage of this model for investigation of the inflammation-related mechanisms of cancer cachexia development must be reconsidered.

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