Impaired voltage-gated K^+ channel expression in brain during experimental cancer cachexia

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Abstract **Cancer-induced cachexia affects most advanced cancer patients. It is characterized by anorexia, profound metabolic dysfunctions, and severe neurological disorders. Here we show that voltage-gated potassium channel (Kv) expression is impaired in the brain of tumor-bearing animals. Expression of both delayed rectifier (Kv1.1, Kv1.2, Kv1.3, Kv1.5, Kv1.6, Kv2.1, Kv3.1, Kv4.2) and A-type potassium channels (Kv1.4, Kv3.3, Kv3.4) was greatly down-regulated in brain from animals bearing a Yoshida AH-130 ascites hepatoma. The possible compensatory mechanisms (Kv1.4/Kv4.2), expression of redundant genes (Kv3.1/Kv3.3) and heteromultimeric channel formation (Kv2.1/Kv9.3) were also affected. The high circulating levels of TNFα and the reduced expression of the anti-apoptotic protein Bcl-XL found in the brain of tumor-bearing animals indicate that this response could be mediated by an increase in brain cell death due to apoptosis. The results suggest that brain function is impaired during cancer cachexia, and may account for the cancer-induced anorectic response and other neurological alterations.**

Key words: Potassium channel; Cancer cachexia; Apoptosis; Brain; Anorexia

1. Introduction

Cancer-induced cachexia is related to the presence and growth of a tumor and leads to malnutrition due to the induction of anorexia or decreased food intake. The cachexia-anorexia syndrome is invariably associated with chronic pathophysiological processes such as cancer progression and HIV infection and is also the cause of mortality in more than two thirds of patients with advanced cancer [1]. The condition is characterized by a profound wasting of adipose tissue and muscle, pain, depression, anxiety, taste and food aversions, and several other homeostatic perturbations [2]. Cancer progression is multifactorial and chemically complex, and involves cell-to-cell interactions induced by factors generated by the tumor and/or the host. In this pathophysiological context, cytokines act as autocrine, paracrine and intracrine effectors and induce important physiological changes. Cytokines such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and interleukin-6 (IL-6) induce most of the symptoms and biochemical features of cancer cachexia and lead to neurotoxicity and neurodegeneration [3,4]. In fact, abnormal levels of cytokines have been described in brain after prostate adenocarcinoma tumor progression [5]. Tumor implantation activates the cytokine production network within the brain and paracrine and autocrine interactions can sustain local cytokine production through positive-feedback systems [6]. In this situation the neuronal processes can be modulated as a result of direct ion channel regulation.

The electrical properties of nerve cells are determined mostly by the voltage-gated potassium channels (Kv). Kv play an essential role in regulating resting membrane potential, action potential duration and frequency, and neurotransmitter release in mammalian neurons. The Kv family is the most diverse and numerous group of channels including conducting and non-conducting subunits. The complexity of the functional protein may be further increased by the presence of associations between gene products. Studies in heterologous expression systems have highlighted the wide variety of biophysically and pharmacologically distinct Kv channels [7] carrying out diverse functions in neuronal physiology as key determinants of membrane excitability. Indeed, the differential expression of Kv channel subunits in the brain may be crucial to achieving the required neural response.

Though cancer-induced cachexia syndrome is invariably associated with abnormal behavior – anorexia – and neurological disorders [2,8,9], the effects of cancer-induced cachexia on Kv channel expression in the brain have not been assessed. The aim of the present study was to analyze the expression of representative members of various conducting Kv channel subfamilies (Kv1–4) and the non-conducting Kv9.3 modulatory subunit in brain from tumor-bearing animals. We show that Kv gene expression is impaired in brain from animals bearing a cachectic tumor. These results indicate that the numerous neuronal dysfunctions observed during cancer-induced cachexia may be due to changes in Kv channel expression, probably related to the induction of apoptosis in the brain cell.

2. Materials and methods

2.1. Animals and tumor implantation

Female Wistar rats (approx. 200 g) from Iffa-Credo (France) were fed ad libitum on a regular chow diet with free access to drinking

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water. A Yoshida AH-130 ascites hepatoma cell suspension (approx. 10^8 cells in 2 ml) was injected intraperitoneally, the control rats being injected with 2 ml of 0.9% (w/v) NaCl solution as previously described [10]. The Yoshida AH-130 is a rapidly growing tumor with a volume doubling time of 1 day. Total cell content was estimated using trypan blue staining. All extractions took place 7 days after tumor transplantation [10].

2.2. Samples and TNFα measurements

Blood was collected from killed animals, and the plasma was obtained by centrifugation and quickly frozen. Tissues were removed and weighed. The brain was immediately frozen with liquid nitrogen and maintained at −80°C until use. All animal handling was approved by the ethics committee of the University of Barcelona.

Plasma TNF was measured by using an enzyme-linked immunosorbent assay test (Genzyme Corp., Cambridge, MA, USA) following the manufacturer's instructions.

2.3. RNA extraction and Northern blot analysis

Total RNA from brain was isolated using the Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany). Up to 20 μg of total RNA was size-fractionated through 1% agarose, 3% formaldehyde gels in 20 mM 3-[*N*-morpholino]-propane sulfonic acid and 1 mM EDTA, pH 7.4. Application of equal amounts of RNA to each lane was confirmed by the addition of ethidium bromide to the samples before electrophoresis. The gel treatment and the high stringency hybridization conditions are described elsewhere [11]. Filters were hybridized overnight with 10^6 cpm/ml of the selected cDNA probe and washed once for 30 min at 65°C with 3×SSC and 1% SDS, once with 1×SSC and 1% SDS, and once with 0.2×SSC and 1% SDS before autoradiography. RNA from at least four independent animals was analyzed for each group and a representative Northern blot is shown. All filters were further hybridized to the 18S ribosomal band, and values were subtracted from the background, corrected, and standardized. The densitometric analysis of the blots was performed by using the Phoretix software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Results are the means ± S.E.M. of each experimental group. Where indicated, statistical analysis by Student's *t*-test was performed.

2.4. cDNA probes, constructs and reverse transcription-polymerase chain reaction (RT-PCR)

The use of Kv1.1, Kv1.2, Kv1.4, Kv2.1, Kv4.2 and Kv9.3 cDNA probes has been documented before [12,13]. The murine Kv1.3 (741–1459 bp, accession number: M30441), Kv1.5 (3003–3337 bp, accession number: AF302768), Kv1.6 (1–3219 bp, accession number: NM013568) and the rat 18S (494–706 bp, accession number: M11188) cDNA probes were PCR generated in our laboratory from brain (Kv1.3 and 18S) and heart (Kv1.5 and Kv1.6) total RNA using selected primers. The PCR fragments were cleaned up by means of a GFX PCR DNA and gel purification kit (Amersham Pharmacia Biotech, Piscataway, WI, USA) and subcloned into the cloning site of the pSTBlue-1 vector using the pSTBlue-1 acceptor vector kit (Novagen, Madison, WI, USA) following the manufacturer's instructions. The Kv3.3 and Kv3.4 constructs were kindly provided by Dr. A. Castellano and Dr. J. López-Barneo (University of Seville, Spain). The Bcl-XL and Bax cDNAs were generated by PCR in our laboratory (accession numbers: U34963 and U49729, respectively).

In accordance with the mRNA expression of Kv1.5 and Kv3.1 in brain [14,15], our Northern blot analysis gave a very low signal (not shown). We therefore analyzed their relative mRNA expression by semiquantitative RT-PCR. One μg of total RNA from brain was used on the Ready to go RT-PCR beads (Amersham Pharmacia Biotech) for a semiquantitative analysis as previously described [16]. The oligonucleotides selected to generate PCR products were: Kv1.5 (accession number: AF302768): F, 5′-GGATCACTCCATCACCAG-3′, bp 3003–3020; R, 5′-GGCTTCCTCCTCTTCCTTG-3′, bp 3337–3320; and Kv3.1 (accession number: Y07521): F, 5′-CTTCTTCATC CTGGTCTCCATCAC-3′, bp 727–750; R, 5′-GTAGTAGAT-GGTGGCAAAGAT-3′, bp 1231–1208. The RT reactions were incubated at 94°C for 5 min to inactivate the reverse transcriptase. The PCR conditions were 0.5 min, 92°C; 1 min, 58°C; 2 min, 72°C for 30 cycles. Ten μl from the final RT-PCR reactions were electrophoresed in a 1% agarose TBE gel (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The specific amplicons in the exponential phase were quantified by means of the Phoretix software. The r18S rRNA was used as an internal control; 100 ng of total RNA from brain was used in the RT-PCR reactions as described previously [16].

3. Results and discussion

Cancer-induced cachexia is associated with loss of body mass together with adipose and muscle tissue wasting (Table 1). In tumor-bearing animals the brain is also affected. Freitas et al. [17] reported a substantial induction of oxidative stress in rat brain during tumor burden. This may explain the association of human cancer cachexia with a variety of undefined neurological disorders [2–4].

Numerous central nervous system dysfunctions are related with abnormal ion channel expression [18]. Previous studies have shown a relation between anorexia and alterations in potassium channels [19,20]. Voltage-dependent potassium channels are a complex family of proteins engendered by many members. Functional Kv channels are formed by either a homo- or a heteromeric structure [7]. So, the variety of currents generated could be the result of several Kv subunit associations forming the pore structure, and the complexity of the ion current could be further enhanced by the presence of auxiliary beta subunits [21]. In terms of activity, Kv channels can be divided into delayed rectifiers, with slow or non-inactivating current, and A-type channels, with a rapidly activating and rapidly inactivating current. Each type has a specific role in the membrane potential duration and propagation in nerve and muscle.

We analyzed the mRNA expression for various conducting and non-conducting Kv channel isoforms in brain during cancer-cachexia syndrome in rats. Fig. 1 shows the expression of the *Shaker* family (Kv1). All members, not only delayed rectifier channels Kv1.1, Kv1.2, Kv1.3 and Kv1.6 but also A-type channels such as Kv1.4, showed a decrease in mRNA expression (Fig. 1A), ranging from 30 to 60% (Table 2). Kv1.5 has been described as a classical striated muscle channel [22] and its expression in brain is very low [14], but it can be detected by PCR in nerve [23]. Our RT-PCR results showed that Kv1.5 mRNA expression also fell in brains of tumor-bearing animals, by about 42% (Fig. 1B, Table 2).

The *Shab* family (Kv2) is represented by Kv2.1 and Kv2.2.

Table 1
Body and tissue weights

	Control	Tumor
Body mass parameters (g)		
Initial body weight	128 ± 9	134 ± 8
Final body weight	190 ± 10	205 ± 15
Tumor weight	–	67 ± 8
Body weight gain	62	4
Tissues (g/100 initial body weight)		
Skeletal muscle	0.69 ± 0.05	0.47 ± 0.03**
Heart	0.54 ± 0.03	0.36 ± 0.04*
WAT	0.45 ± 0.03	0.21 ± 0.03**
Brain	0.98 ± 0.08	0.74 ± 0.05*

Animals bearing Yoshida AH-130 ascites hepatoma were killed after 7 days, tissues were extracted and body and tissue mass were measured. Eight animals weighing 131 ± 8 g were homogeneously distributed before tumor inoculation. Skeletal muscle: gastrocnemius; WAT: perirenal white adipose tissue. The results are means ± S.E.M. of four animals. *$P < 0.05$; **$P < 0.01$ vs control Student's *t*-test.

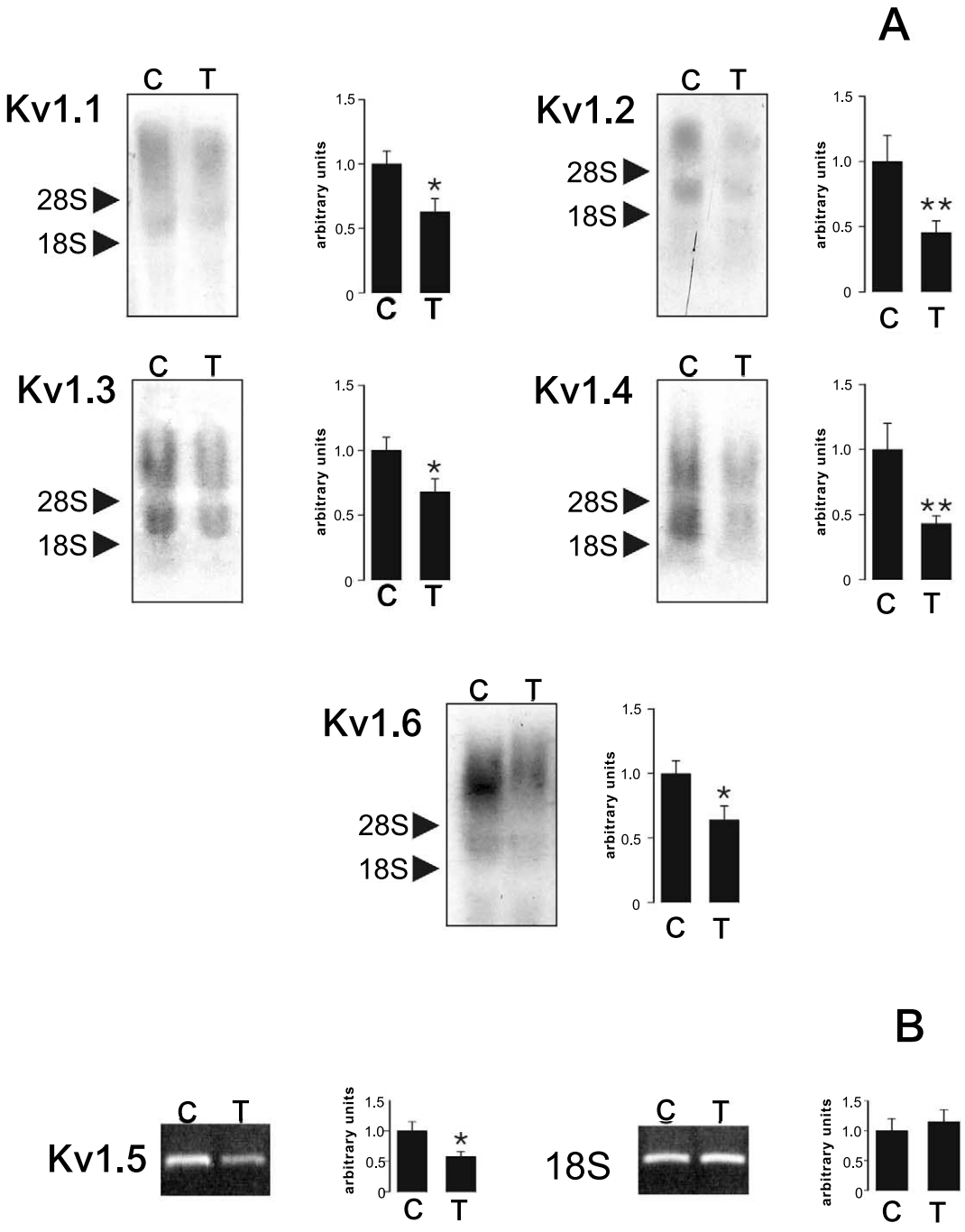

Fig. 1. RNA expression of Kv1 family members in the brain from animals under experimental cancer cachexia. At least four animals were inoculated with the Yoshida AH-130 ascites hepatoma cell suspension as described in the materials and methods section and total RNA was analyzed for Kv1 expression after 7 days of tumor growth. A: Kv1 Northern blot results. Values are means ± S.E.M. once corrected with the 18S rRNA expression. B: Kv1.5 RT-PCR results. Representative amplicon results from the Kv1.5 and 18S RT-PCR reactions in exponential phase are shown. C: control animals, T: tumor-bearing animals. $*P < 0.05$; $**P < 0.01$ vs C.

The Kv2.1 channel, which was first cloned from brain, is expressed in all mammalian brain neurons and it accounts for the major component of delayed rectifier current in nerve [24]. In addition, Kv2.1 has been identified as a major Kv channel in the heart, and it also forms an oxygen-sensitive Kv channel in vascular musculature [25]. Our results showed that Kv2.1 expression is decreased (~40%) in brain of animals under cancer-cachexia syndrome (Fig. 2, Table 2). By overexpression of a dominant-negative Kv2 subunit in embryonic neurons, the I_{kv} is partially suppressed and action potentials are greatly prolonged, supporting the idea major role in brain physiology [26]. Thus, an impaired Kv2.1 expression could contribute to some neural malfunctions during cachexia.

The A-type K^+ current is mostly conducted in the brain by Kv1.4, Kv3.3, Kv3.4, Kv4.1, Kv4.2 and Kv4.3 [27]. As described above, Kv1.4 mRNA expression is lower in tumor-bearing animals. Several examples of compensatory behaviors in members that engender similar currents have been de-

Table 2
Summary of the altered voltage-dependent K^+ channel expression in brains from tumor-bearing rats

Channel	Percentage of inhibition	Channel	Percentage of inhibition
Kv1.1	32 ± 2	Kv2.1	37 ± 5
Kv1.2	54 ± 10	Kv3.1	68 ± 7
Kv1.3	37 ± 5	Kv3.3	57 ± 8
Kv1.4	57 ± 8	Kv3.4	52 ± 10
Kv1.5	42 ± 6	Kv4.2	35 ± 3
Kv1.6	36 ± 6	Kv9.3	34 ± 3

Results are means ± S.E.M. of at least three different animals. The percentage of inhibition is derived from Figs. 1 to 3. Values were normalized to 18S ribosomal band levels expressed as arbitrary units.

scribed, such as an isoform switching between Kv4.2 and Kv1.4 in rat ventricles from diabetic and myocardial infarction animals [28]. We did not find these compensatory mechanisms in brains from animals under cancer cachexia, since Kv4.2 expression was also diminished (Fig. 2). This result suggested major differences between the pathological situations studied, since only cancer cachexia patients display neurological disorders [2,9]. The Kv3 family (Shaw) also has several delayed rectifier (Kv3.1 and Kv3.2) and A-type channels (Kv3.3 and Kv3.4) distributed throughout the central nervous system [15]. The activation voltage and fast deactivation rates generated by Kv3 proteins help repolarize action potentials rapidly without affecting the threshold for action potential generation [15]. The Kv3 family may form functional channels by heterotetrameric constructions, and experimental evidence shows that they play critical roles in the generation of fast-

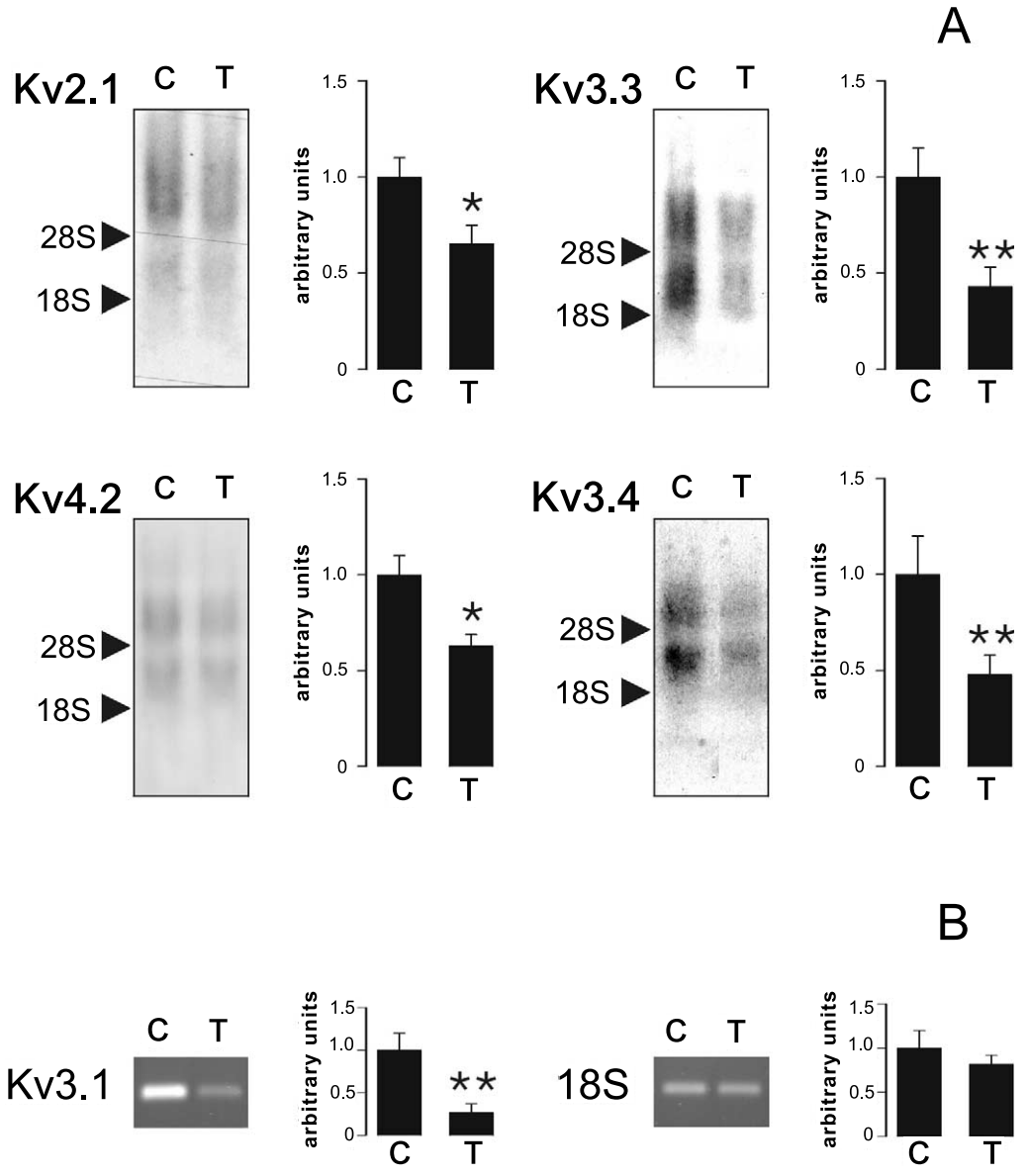

Fig. 2. Kv2, Kv3 and Kv4 family members in the brain from animals under experimental cancer cachexia. Legend is similar to Fig. 1. A: Kv Northern blot results. Values are means ± S.E.M. once corrected with the 18S rRNA expression. B: Kv3.1 RT-PCR results. Representative amplicon results from the Kv3.1 and 18S RT-PCR reactions in exponential phase are shown. C, control animals; T, tumor-bearing animals. *$P < 0.05$; **$P < 0.01$ vs C.

spiking properties in cortical GABAergic interneurons [15]. Our results showed that mRNA expression of both A-types Kv3.3 and Kv3.4 and the slowly inactivating Kv3.1 is lower in brains from tumor-bearing animals (57% and 52% for the two A-types and 68% for the delayed rectifier, Fig. 2, Table 2). So, although Kv3 channels may form a number of heteromeric and compensatory structures to maintain a fast deactivating current that generates rapid recovery after hyperpolarization [15], this activity seems to be deteriorated in brains from cachectic animals. In this context, certain compensatory mechanisms have been described and the expression of Kv3.1 and Kv3.3 seems to be redundant, since single deficient mice did not show any abnormal behavior. In contrast, double Kv3.1/Kv3.3 mutants display severe ataxia, tremulous movements, myoclonus, and hypersensitivity to ethanol [29].

Functional Kv channels may be formed by conducting and non-conducting subunits that modify the overall ion current kinetics [7]. Among non-conducting families, the Kv9 proteins are associated to Kv2 conducting members, and Kv2.1 is responsible for a major current in the central nervous system [24]. Kv2.1 and Kv9.3 channels have been implicated in oxygen sensitivity [13,25]. However, the Kv2.1 channel alone cannot account for the native O_2-sensitive K^+ current, since the heterologous expression of this protein is not activated in the voltage range of the membrane potential. Co-expression of Kv2.1/Kv9.3 generates currents activated at physiologically relevant potentials and the K^+ currents are inhibited by hypoxia [25]. We show that Kv9.3 mRNA expression is also decreased during cancer cachexia in brain (Fig. 3), as was the case for Kv2.1 (Fig. 2). These results suggest that the possible generation of heterotetrameric structures by these two genes may be greatly handicapped. The potential role of the Kv2.1/Kv9.3 channel in brain is not known, and further study is required to elucidate this point.

Kv channels have a dynamic regulation attributed to the relatively short half-lives of Kv mRNA and protein. Thus, we analyzed the abundance of several Kv proteins by Western blot (Kv1.3, Kv1.5 and Kv2.1) and found no relevant signal in brain from tumor-bearing animals (not shown). However, since in long-term conditions the Kv gene expression always parallels the protein expression [28,30,31] and since the cachectic syndrome is associated with an increased proteolytic metabolism [1] the timing of the protein expression impairment in the present study would be in agreement with previous observations.

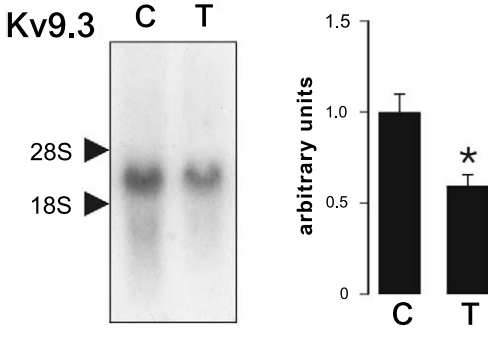

Fig. 3. Kv9.3 mRNA expression in the brain from animals under experimental cancer cachexia. Legend is similar to Fig. 1. Values are means ± S.E.M. once corrected with the 18S rRNA expression. C, control animals; T, tumor-bearing animals. *$P < 0.05$ vs C.

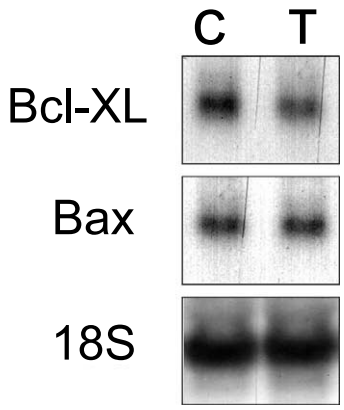

Fig. 4. Pro-apoptotic Bax and anti-apoptotic Bcl-XL mRNA expression in brain from animals under experimental cancer cachexia. At least four animals were analyzed and the result from a representative filter is shown. C, control animals; T, tumor-bearing animals.

Experimental cancer-induced cachexia is characterized by an increase in pro-inflammatory cytokines such as TNFα and IL-1β. In the tumor-bearing animals, plasma TNFα concentration was increased (10 ± 4 and 307 ± 16 pg/ml for controls and cachectic animals, respectively) as described elsewhere [10]. In addition, the IL-1β mRNA was up-regulated in brain from animals bearing prostate adenocarcinoma [6]. Both cytokines are clearly pro-apoptotic and this induction may lead to neuronal death by apoptosis. To investigate this hypothesis further, we analyzed the mRNA expression of several anti- or pro-apoptotic proteins in brain. Bcl-2 family proteins are divided functionally into death-inhibiting or death-inducing members. Bcl-XL is known to enhance cell survival, whereas Bax is pro-apoptotic [32,33]. Fig. 4 shows that the mRNA levels of Bcl-XL are about 60% lower in the brain of tumor-bearing animals. In contrast, the expression of Bax was unaltered. This unsurprising result agrees with previous studies showing that the levels of Bax may remain stable when apoptosis is mediated by TNFα [34]. Changes in brain levels of these two proteins, especially Bcl-XL, have been associated with either neuronal death or protection in a number of neurological disorders [32,33]. Previous investigations demonstrate an increase in cytokine expression – TNFα, in particular – in the brain of patients with a range of tumoral pathologies [35,36]. In addition, an increased brain TNFα expression has been observed in rats bearing the cachectic Walker 256 carcinoma (R. Curi, personal communication). Our data also suggest that the levels of TNFα, and possibly IL-1β [6], could be responsible for the massive neuronal death by apoptosis. This result is clearly in agreement with the loss of brain weight observed (Table 1). In this context, the down-regulation of Kv channels may be related with a neuronal dysfunction generated by cell brain apoptosis.

This study shows that Kv channel expression is impaired in brain from tumor-bearing animals under cancer cachexia. The possibility of homo- or heterotetrameric structures appears to be severely affected. We also show that possible compensatory mechanisms are blocked in our model. To what extent our results indicate a relationship between the neurological dysfunctions observed in cachectic patients and ion current disorders is not known, but clearly a lower Kv expression may modify not only overall brain activity but cognitive and behavioral responses as well. It is tempting to speculate that the

anorexia observed in cancer-cachexia patients could have some connection with impaired Kv channel expression. Indeed, Kv3.1/Kv3.3 double knock-out mice gained less weight than their litter mates; some began to lose weight gradually and died. However, no references to control of food uptake are available though surviving double mutants reached only 50–60% of the body weight of their control animals [39]. In another study, genetically deficient Kvβ animals had problems with learning and feeding conducts [37]. Changes in pro- and anti-inflammatory cytokine production have been observed in brain during cancer cachexia [2–6] and the potential role of these molecules in Kv channel regulation should be evaluated.

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