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Research report

NOS isoenzyme content in brain nuclei as related to food intake in experimental cancer cachexia

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Abstract

Evidence implies that nitric oxide (NO) in the central nervous systems mediates anorexia in tumor-bearing hosts. We have therefore evaluated, by immunohistochemical image analyses, net alterations of nitric oxide synthases (nNOS, eNOS, iNOS) in brain nuclei [paraventricular hypothalamic nucleus (PVN), medial habenular nucleus (MHB), lateral habenular nucleus (LHB), paraventricular thalamic nucleus (PV), lateral hypothalamic area (LHA), ventromedial hypothalamic nucleus (VMH), nucleus of the solitary tract (NTS)] of tumor-bearing mice (TB) with prostanoid-related anorexia. Pair-fed (PF) and freely fed (FF) non-tumor-bearing mice were used as controls. c-fos was analyzed as indicator of neuronal activation. nNOS was significantly increased in VMH and PVN from TB mice, while eNOS was significantly increased in LHB and LHA. iNOS was significantly increased in LHA and PVN nuclei, but decreased in MHB, LHB and VMH from tumor-bearers. However, several of these alterations were similarly observed in brain nuclei from pair-fed controls. Provision of unspecific NOS-antagonists to TB mice increased nNOS, eNOS and iNOS in several brain nuclei (PVN, LHA, VMH), but left tumor-induced anorexia unchanged. c-fos was significantly increased in all brain nuclei in PF mice except for NTS, LHA and PVN compared to controls, while tumor-bearing mice had increased c-fos in LHA and PVN only compared to controls. Our results demonstrate a complex picture of NOS expression in brain areas of relevance for appetite in tumor-bearing hosts, where most changes seemed to be secondary to stress during negative energy balance. By contrast, NOS content in PVN and LHA nuclei remains candidate behind anorexia in tumor disease. However, nitric oxide does not seem to be a primary mediator behind tumor-induced anorexia. NO may rather secondarily support energy intake in conditions with negative energy balance.

Theme: Endocrine and autonomic regulation
Topic: Neuroendocrine regulation: other

Keywords: Anorexia; NOS; Cachexia; Brain

1. Introduction

Anorexia is well recognized in cancer cachexia as common denominator for development of weight loss in progressive cancer [32]. Regulation of food intake and energy balance is in part controlled by neurons converging on nuclei in hypothalamus, including serotonergic mechanisms [19,27], although the precise control mechanisms have not been defined. A number of reports suggest a role for cytokines and/or prostanoids in the brain of anorectic tumor-bearing (TB) hosts [35,36,49], but nitric oxide (NO) is also a possible mediator of anorexia. NO is derived from L-arginine by the action of three different isoforms of NO synthase (NOS). NO is a messenger molecule formed in both the central and the peripheral nervous system of mammals [4], with functions as a neurotransmitter/modulator or paracrine agent [16,39] being a possible link between synaptic and nonsynaptic transmission [16]. Food

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deprivation has thus been found to increase NOS activity in diencephalons [44], and intracerebroventricular injections of NOS inhibitor caused significant attenuation of food intake during re-feeding in rats, possibly by interference with central serotonergic mechanisms [43,44]. Similarly, provision of NOS antagonists in the drinking water to tumor-bearing mice caused reduction of both tumor growth and cachexia [5]. Thus, there is indirect evidence to support that nitric oxide and brain NOS may be involved in the control of energy homeostasis and perhaps appetite control.

Brain NOS occurs constitutively in two isoforms that are distinct gene products. Neuronal NOS (nNOS) is found in neurons as well as endothelium of certain blood vessels [2,52], while endothelial NOS (eNOS) is preferentially localized in endothelial cells of the vasculature and ependymal cells of the choroid plexus in the brain [38,41,45,48]. eNOS is occasionally also present in neurons [34]. Immunohistochemical analyses have confirmed a wide distribution of these NOS isoforms in brain tissue [9,41,45], while inducible NOS (iNOS) is not regularly expressed in the brain, but it may be induced in microglia in response to bacterial toxins, cytokines and sometimes in aging subjects even in the absence of disease [17,20,51].

The aim of the present study was to search for a possible link between anorexia, altered NOS content and thereby indirectly NO metabolism in brain areas relevant to the control of appetite in tumor-bearing mice.

2. Materials and methods

2.1. Experimental procedures

The experimental protocol was approved by the Committee for Ethics at the Göteborg University. Adult, female age-matched C57BL/6 mice (20–23 g) (M&B, Ejby, Denmark) were housed in plastic cages in a temperature-controlled room with a 12-h dark/light cycle. The animals received laboratory rodent chow (B&K Universal, Sollentuna, Sweden) and tap water ad libitum for 14 days before the experiments. Mice were separated into groups of three to four mice per cage with a wire floor. Animal groups were: tumor-bearing, pair-fed (PF) and freely fed (FF) controls. All groups were allowed 3 days for adaptation to wire floors in the cages before the start of experiments. The experimental procedures started on day 0 with either tumor or sham implantations under general anesthesia (a combination of Ketalar® [Ketamine Hydrochloride, Warner Larbert Nordic, Solna, Sweden], 100 mg/kg i.p., and Rompun® [Xylazine Hydrochloride, Bayer Sverige, Göteborg, Sweden], 5 mg/kg, ip). In some experiments, tumor-bearing mice were daily provided unspecific NOS-inhibitors in the drinking water (L-NAME, L-NoArg; 100 μg/g bw/day) during 14 days as described elsewhere [5].

Tumor-bearing mice were implanted s.c. bilaterally in the flank with 3–5 mm^3 of a transplantable MCG-101 methylcholanthrene-induced sarcoma [23]. Non tumor-bearing mice were sham implanted, with a time-lag of 3 days vs. tumor-bearing mice and subsequently either pair-fed to match decreasing food intake in the tumor-bearing mice or were freely fed [24]. Water was provided ad lib to all animals. Daily food intake and body weight were registered between 0800 and 0900 a.m. as total food intake divided by the number of animals in a cage. All mice were sacrificed on day 14 upon implantation. Blood samples were obtained by cardiac puncture during general anesthesia. Plasma and serum were removed immediately for PGE_2 and IL-6 determinations as described below. The brains were immediately fixed in situ by transcardiac perfusion. The vascular bed was rinsed with 20 ml saline through the left cardiac ventricle followed by perfusion with 20 ml room-temperatured, 4% paraformaldehyde in phosphate buffer. Brains were rapidly removed and placed for 20–24 h of post-fixation in paraformaldehyde at room temperature. Tissue specimens were paraffin embedded and cut into 8-μm sections for immunohistochemical analysis. The sections for analysis of paraventricular hypothalamic nucleus (PVN) were taken between interaural line 3.10 and 2.86 mm, bregma −0.70 and −0.94 mm, and the other hypothalamic nuclei were taken between interaural line 2.46 and 1.86 mm, bregma −1.34 and −1.94 mm; analysis of brain stem nuclei was performed between interaural line −2.68 and −3.16 mm, bregma −6.48 and −6.96 mm, according to Franklin and Paxinos [11]. The following structures were investigated: paraventricular hypothalamic nucleus (PVN), medial habenular nucleus (MHB), lateral habenular nucleus (LHB), paraventricular thalamic nucleus (PV), lateral hypothalamic area (LHA), ventromedial hypothalamic nucleus (VMH), and nucleus of the solitary tract (NTS). Brain nuclei were verified by light microscopy of sections stained with Wright's hematoxylin in sequential sections, as shown by location in Fig. 1.

2.2. Immunohistochemical staining

Immunohistochemical staining for NOS was optimized. Antigen retrieval was accomplished by trypsination (NOS isoenzymes staining) or by microwave-radiation treatment in acid conditions (c-fos staining). Primary antibodies were diluted in 1% TBS–BSA containing 0.1% Saponin (1:200; Multiclonal Rabbit anti-rat NOS1, SC-648; Multiclonal Rabbit anti-mouse NOS2, SC-650; Multiclonal Rabbit anti-human NOS3, SC-654, 1:100; Multiclonal Goat anti-human c-fos, SC-52-G), and incubated overnight (20 h) at room temperature after blocking non-specific protein binding sites with 5% TBS-BSA for 30 min. The remainder of the immunohistochemical procedures were performed as described [49]. All primary antibodies were from Santa Cruz Biotechnology, CA, USA. Secondary antibodies and developing system were from Dakopatts, Älvsjö, Sweden.