



DIFFERENTIAL EXPRESSION OF NGF AND BDNF IN RAT ADIPOSE DEPOTS DURING EARLY DEVELOPMENT AND ADULTHOOD

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Abstract

According to the current paradigm, the white adipose tissue (WAT) not only releases fatty acids, cholesterol, retinoids and other lipids stored in the adipocytes, but also produce and secrete numerous signaling proteins collectively termed adipokines. Among them are various cell growth factors such as nerve growth factor (NGF) and brain-derived growth factor (BDNF). While literature around obesity and its related diseases is abundant, we still do not know how WAT behaves during normal development. In the present study, we explored the expression of NGF and BDNF in the peripancreatic fat tissue (Ppf) and the epididymal fat tissue (EpF) during a critical period for the metabolic maturation of male Wistar rats. At birth almost no abdominal WAT was observed in the rats. From day 15 to adulthood (2 months), it increased in weight. However, the adipocyte diameter was not different during the first month, compared to adulthood, indicating that the tissue growth in this stage was mostly hypertrophic, while in the second month we also observed hyperplasia of the adipocytes. We measured the production of the neurotrophins NGF and BDNF by the abdominal WAT depots. We observed a significant reduction in the expression of NGF in EpF at 28d and two months. However, NGF expression in Ppf remained stable from postnatal 15d to adulthood, except for a nadir at 28d. On the other hand, we failed to detect BDNF during the first 2 months of life in Ppf, but it was expressed at detectable amounts in EpF, peaking at day 20. We do not know how these secretory changes influence the development of the cells and the surrounding tissues. However, in obesity the amount of WAT is increased, and it is necessary to understand the mechanisms of WAT growth in physiological state to identify with the unbalance in this tissue present in obesity.

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Introduction

White adipose tissue (WAT) plays an important role in nutrient metabolism, increasing the triglycerides storage during caloric intake, and releasing fatty acids when energy intake exceeds the energy expenditure (1). WAT is an endocrine and paracrine organ, which synthesizes and releases different pro- and anti-inflammatory cytokines known as adipokines or adipocytokines (reviewed in 2).

Neurotrophins, such as nerve growth factor (NGF) (3) and brain-derived neurotrophic factor (BDNF) (4), are synthesized in many cell types, including adipocytes. These neurotrophins exert multiple actions on multiple cells from the neural, endocrine, immune and cardiovascular systems (5-7). It has been shown that NGF may function as a guide for the sympathetic fibers and blood vessels to the pancreatic islets in early developmental stages of the pancreas, and also maintain beta-cell innervation in later stages of life (6). Neurotrophins also induce metabotropic changes in glucose and lipids homeostasis (7). Actually, the changes in NGF and BDNF secretion by WAT, and their possible physiological roles during develop-

ment are not known. Interestingly, our group has also described a physiological hyperinsulinemic and hyperglycemic state during the perinatal development of rats just before weaning, which precedes the metabolic maturation of these animals (8).

Here, we aimed to elucidate the expression profiles of NGF and BDNF in peripancreatic fat tissue (Ppf) and epididymal fat tissue (Epf) at different stages, comprising the above mentioned temporal window during the metabolic development of male Wistar rats.

Materials and Methods

Experimental animals and sample collection. Wistar rats were obtained from the local animal facility, maintained in a facility with a 12:12 h light-dark cycle (0600-1800), and allowed free access to standard laboratory rat diet and tap water. All methods used in this study were approved by the Animal Care Committee of the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. Animal care was kept according to the International Guiding Principles for Biomedical Research Involving Animals, Council for International Organizations of Medical Sciences, 2010.

Ppf and Epf were obtained from fasted male Wistar rats of 15, 20 and 28 days post birth (dpb), and 2 months old animals. Prior to surgery, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/Kg) and weighted. Adipose depots were immediately removed, washed with ice-cold phosphate buffer saline (PBS, 0.1M pH 7.4), weighted and submerged in liquid nitrogen for subsequent isolation of total protein. Alternatively, adipose tissue samples were fixed during 24 h at 4 °C, in buffered solution of paraformaldehyde 4%.

Morphometric analysis. Previously fixed Ppf and Epf adipose tissues were submerged 24 h in 30% sucrose, mounted in tissue-tek (Sakura finetek USA Inc) and stored at -70 °C until use. Tissue samples were cut in 20 μ m thick sections using a cryostat. Images were obtained and digitalized using a microscope Leica Micro Dissection System DM6000 B/DM6000 M, coupled to a Hitachi HV-D20 camera. Fifteen to 25 random fields were taken for each adipose depot and the diameter of 400 to 500 adipocytes per animal were measured using the ImageJ (1.38X) software.

NGF and BDNF determination in adipose depots. Quantification of both neurotrophins was carried out with total protein homogenates from Ppf and Epf adipose tissues. Briefly, 300 to 500 mg of fresh tissue were placed in TE buffer (250 mM Tris, 1 mM EDTA pH 8 with complete protease inhibitors cocktail; Roche, Inc), frozen in liquid nitrogen and dispersed by mechanical action. The homogenates were centrifuged at 12000 g and 4°C for 5 min, and the supernatants were used for analysis of NGF (DuoSet ELISA development system for rat beta-NGF,

R&D systems) and BDNF (BDNF Emax immunoassay system, Promega) by an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. Total protein concentration was determined spectrophotometrically using a Nanodrop (Thermo scientific) and measuring the absorbance at a wavelength of 280 nm. The levels of NGF were expressed as ng/mg of total protein and BDNF as pg/mg of total protein.

Statistical analysis. Data are expressed as means \pm standard error of the mean (SEM). An analysis of variance (ANOVA) was performed to assess the statistical differences amongst groups (Stat view 4.57; Abacus Concepts, Cary, NC). Threshold of significance was defined at $p \leq 0.05$.

Results

Morphometric analysis of peripancreatic and epididymal adipose tissue during the postnatal growth

The variation in the amount of mediators released by adipose tissues could impact on several physiological processes. A possible cause for a change in circulating neurotrophins could be for instance, an alteration of the proportion of adipose tissue with respect to the total weight of the animals. The morphometric properties of both adipose depots at different ages are shown in Table 1. At birth almost no abdominal WAT was observed in the rats. From day 15 to adulthood (2 months), it increased in weight, which was also reflected in a weight increase in Ppf and Epf. However, the adipocyte diameter was not different during the first month, compared to adulthood, indicating that the tissue growth in this stage was mostly hypertrophic, while in the second month we also observed hyperplasia of the adipocytes.

No differences were observed between Ppf and Epf during the first 28 days of life. The weights in both fat depots in young adult rats (2 months) were higher, compared to the other ages studied. Moreover, the weight of Epf depot was higher than that observed in Ppf depot ($p \leq 0.05$). This growth could arise from an increase in the number (hyperplasia) and/or the size (hypertrophy) of the adipocytes. Adipocyte diameter in Ppf and Epf remained constant amongst days 15, 20 and 28, but significantly increased in the adulthood ($p \leq 0.05$).

NGF and BDNF secretion in peripancreatic and epididymal adipose tissue during the postnatal growth

To explore the physiology of the adipocytes during the animal maturation, we studied the neurotrophin levels in these cells in the different developmental stages considered in the present study. Figure 1 shows that NGF levels peaked at days 15 and 20 in Epf and Ppf. Nevertheless, the amount of this neurotrophin decreased at day 28 and remained stable at 2 months of age in both cases. Noteworthy, Epf presented higher levels of NGF with

Table 1. Weights and morphological characteristics of peripancreatic and epididymal white adipose depots during rat development. * $p \leq 0.05$ with respect to previous age. ++ $p \leq 0.05$ with respect to peripancreatic fat at the same age.

		15 days	20 days	28 days	2 months
Body weight (g)		36.5 ± 0.6	51.1 ± 0.8*	78.7 ± 1.5*	269.9 ± 3.3*
Ppf weight (g)		0.025 ± 0.002	0.05 ± 0.002*	0.08 ± 0.005*	0.4 ± 0.02*
Epf weight (g)		0.037 ± 0.002	0.08 ± 0.003*	0.13 ± 0.005*	1.4 ± 0.11*++
Adipocyte diameter (mm)	Ppf	45.3 ± 0.7	47.5 ± 0.8*	46.48 ± 0.8*	50.9 ± 0.6*
	Epf	44.9 ± 1.0	44.0 ± 0.8	46.29 ± 0.7*	54.2 ± 0.7*++

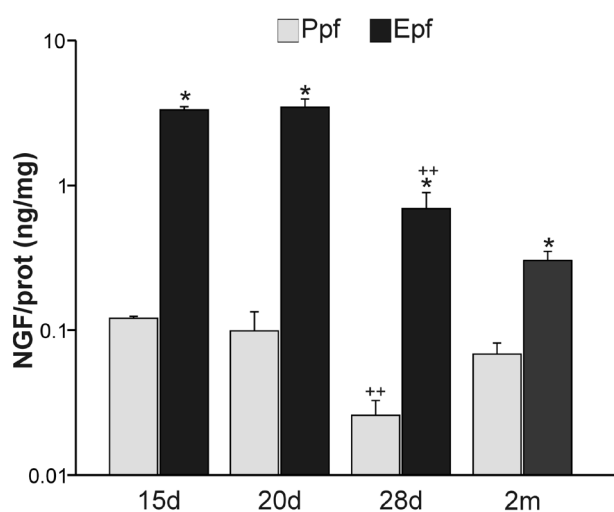


Figure 1. Amount of NGF derived from peripancreatic (Ppf) and epididymal (Epf) fat at different developmental stages. Total protein was extracted from both adipose depots at 15, 20 and 28 days post birth, as well as 2 months of age and were analyzed by ELISA method. The data represent the mean ± SEM and normalized with respect to total protein, from at least three independent experiments in duplicate. * $p \leq 0.05$ with respect to peripancreatic fat in the same age; ++ $p \leq 0.05$ compared to same adipose tissue of the previous age. $n=7$ for 15, 20, 28 days and 2 months.

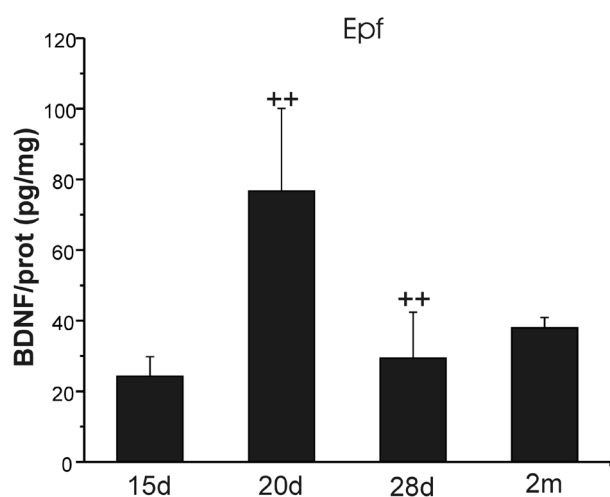


Figure 2. Amount of BDNF derived from peripancreatic (Ppf) and epididymal (Epif) fat at different developmental stages, same details and methods as in Figure 1. The data represent the mean ± SEM and normalized with respect to total protein, from at least three independent experiments in duplicate. ++ $p \leq 0.05$ with respect to the same adipose tissue of the previous age. $N = 7$ for 15, 20, 28 days and 2 months. BDNF was not detected in Ppf.

respect to Ppf at all stages. On the other hand, BDNF (Fig. 2) reached a maximum at day 20 in Epf, then decreased at day 28 and remained in the same level until 2 months. It seems that Ppf expresses very low levels or no BDNF, since its measurements were below the detection limit of the kit employed in this study.

Discussion

Adipose tissue is a heterogeneous organ with biochemical and metabolic differences that depend on its anatomical location (9-11). We found significant differences in the amount of NGF and BDNF in Ppf and Epf during development. Our findings show that NGF and BDNF levels were higher in Epf than in Ppf at all ages. This could be due to the metabolic status of each type of depot; for instance, rat Epf is rich in mitochondria and expresses a higher cytochrome-c oxidase activity compared, for example, to the inguinal adipose tissue (12).

Furthermore, we observed differences in the weight between Ppf and Epf. Thus, the amount of Epf was higher than the Ppf at all the studied ages. It has been observed that gonadal fat depots (such as epididymal, peritesticular, periovarian and periuterine) also increase with ageing (13).

Our results are in agreement with previous reports, which demonstrated that during the postnatal development, there is an increase in the number and size of adipocytes as a function of energetic balance (caloric intake and energy expenditure) (14). It has been documented that WAT growth is depot- and species-dependent (15), with visceral adipocytes generally larger than subcutaneous or intramuscular adipocytes (16). In humans, omental adipocytes are smaller than subcutaneous and other visceral adipocytes (17). It has been proposed that adipocyte size changes in relation to the cell lipid content, ranging from about 30 to 130 μm in diameter and the volume of an adipocyte is a determinant of cell functionality with larger adipocytes generally exhibiting higher metabolic activity and secreting more chemoattractants for immune cells (18). Our results demonstrate that during the first 28 days of life both depots grow, but the size of adipocytes remained quite stable, indicating hyperplasia. However in the adulthood both processes, hyperplasia and hypertrophy, are present.

NGF and BDNF are essential factors during the development of central and peripheral nervous system, playing roles in the control and regulation of different processes such as survival, apoptosis, proliferation, migration, differentiation, and synaptic plasticity, (reviewed in 5-7), also amyloid precursor protein processing and metabolism (see Viviana Triaca's review in this volume of *Adipobiology*). Nevertheless, numerous reports have established that the action of NGF and BDNF are not restricted to the nervous system and can affect an extensive range of non-

neuronal tissues and processes such as inflammation, immunity, and glucose and lipid metabolism (see 19). Ovary maturation (reviewed in 20) as well as the morphogenesis of the testis and epididymis (21) requires the participation of NGF and/or BDNF. In addition, NGF exerts a variety of effects in the cardiovascular system. For example, the levels of this neurotrophin are decreased in atherosclerotic coronary vascular tissue, whereas increased in the subepicardial adipose tissue (22). Moreover, NGF possess angiogenic effects (23-26). Furthermore, in a recent work Sornelli *et al* demonstrated that in animal models of stress and diabetes, the levels of NGF and BDNF produced by WAT and brown adipose tissue are significantly altered as compared to control animals (27).

We demonstrate that different amounts of NGF and BDNF are produced during postnatal development in Ppf and Epf depots. These differences were marked during the first 2-4 weeks of age and decrease in adult stages. Thus both molecules could be involved in maturation and/or in morphogenetic events during adipose tissue postnatal development. In addition, NGF (28) and/or BDNF could participate in the vascularization and/or sympathetic innervation of adipose tissue during development. The expression of these neurotrophins may contribute to differences in the amount, adipocyte size and functions of both adipose depots. These hypotheses require to be further analyzed in future works.

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