Role of sympathetic activity in controlling the expression of vascular endothelial growth factor in brown fat cells of lean and genetically obese rats

Cristina Tonello[a], Antonio Giordano[b], Valeria Cozzi[a], Saverio Cinti[b], Michael J. Stock[c], Michele O. Carruba[a], Enzo Nisoli[a,*]

[a] *Centre for Study and Research on Obesity, Department of Pharmacology, School of Medicine, LITA Vialba, L. Sacco Hospital, Milan University, Via G.B. Grassi, 74, 20157 Milan, Italy*
[b] *Institute of Normal Human Morphology, School of Medicine, Ancona University, 60131 Ancona, Italy*
[c] *Department of Physiology, St George's Hospital Medical School, Tooting, London SW17 0RE, UK*

Received 14 November 1998; received in revised form 27 November 1998

Abstract The thermogenic activity of brown adipose tissue (BAT) is heavily dependent on high perfusion, through its dense vascular system. Angiogenesis must go hand-in-hand with BAT functions, but little is known about the factors controlling it. In the present study we demonstrate that: (a) vascular endothelial growth factor (VEGF) is synthesised and released in brown adipocytes in culture; (b) VEGF mRNA isoforms and protein appear in dispersed mature brown adipocytes and whole tissue; (c) VEGF expression is increased in BAT from cold-exposed rats, and in cultured brown adipocytes exposed to noradrenaline and the β_3-adrenoceptor agonists; (e) BAT from genetically obese (*falfa*) rats exhibits reduced expression of VEGF as well as a change in the ratio of mRNA isoforms. It is concluded that sympathetic control of VEGF expression via noradrenaline acting on β_3-adrenoceptors plays a major role in developmental and adaptive angiogenesis, and defects in this contribute to the reduced thermogenic capacity of BAT in genetic obesity.

Key words: Vascular endothelial growth factor; Obesity; Noradrenaline; Brown fat; Angiogenesis

1. Introduction

Brown adipose tissue (BAT) is an important site of energy expenditure, which is functionally quiescent in obesity [1]. Chronic exposure to cold, by increasing the sympathetic output, induces the recruitment process of BAT [2], which consists of a series of events that transiently transform a proliferatively dormant into a hyperplastic organ. The mechanisms by which brown fat atrophy is mediated in obesity are not completely known, although we have recently reported that tumour necrosis factor-α (TNF-α) induces apoptosis of brown adipocytes and that noradrenaline counteracts it [3]. The thermogenic activity of brown fat requires a very high perfusion rate, both for the supply of oxygen and substrate and for the export of the heat and carbon dioxide produced. BAT hyperplasia is accompanied by angiogenesis and results from a rapid activation of mitosis in brown fat precursor cells and endothelial cells forming the capillaries [4].

The process of new vessel generation from a preexisting microvascular network implies the production of several angiogenic factors [5], but little is known about the mechanisms of angiogenesis in BAT and their modulation by factors operating during development and recruitment. Asano et al. [6] have recently shown that vascular endothelial growth factor (VEGF), one of the most potent angiogenic factors, is expressed abundantly in rat brown fat. VEGF is a dimeric 46 kDa protein that acts as a potent and specific mitogen for vascular endothelial cells and possesses a leader sequence which allows it to be secreted [7]. In vitro and in vivo studies established that VEGF acts as a mitogen for endothelial cells [8], and as a promoter of endothelial cell migration [9]. Multiple forms of VEGF mRNA, encoding peptides of different lengths, are the result of alternative splicing of the gene product [10]. The three major VEGF mRNA forms, which encode proteins of 188, 164 and 120 amino acids, were observed in various rat tissues [11]. VEGF 165/164 (human/rodent homologue) is often assumed to be the predominant form, although truly quantitative assessments are lacking [11]. The various VEGF isoforms have strikingly different secretion patterns, which suggest multiple physiological roles for this family of polypeptides.

Given the limited understanding of factors affecting BAT angiogenesis, the aims of the present study were: (i) to investigate the expression of the VEGF isoforms in rat brown fat cells differentiated in culture; (ii) to analyse the in vitro and in vivo noradrenergic modulation of their expression; (iii) to verify any relevant changes in this molecular system in genetically obese animals. We conclude that VEGF is synthesised in and released from brown adipocytes, that its production appears to be under the stimulatory control of noradrenaline, mainly through β_3-adrenoceptors, and that this noradrenergic control is modified in genetically obese animals.

2. Materials and methods

2.1. Brown adipocytes isolation and cell culture treatment

Brown fat precursor cells were isolated from the interscapular brown fat of male Sprague-Dawley rats (150–160 g body weight; Harlan Nossan, Correzzana, Italy), as previously described [12,13].

Three million cells seeded in 6-well culture plates (Costar, Milan, Italy) were cultured under a water-saturated atmosphere of 6% CO_2 in air at 37°C in 2.0 ml of a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM glutamine, 10% newborn calf serum, 4 nM insulin, 4 nM triiodothyronine, 10 mM HEPES, and 50 IU of penicillin, 50 μg of streptomycin, and 25 μg of sodium ascorbate per ml (all from Sigma, Milan, Italy). Different concentrations of the β_3-adrenoceptor agonist SR58611A

*Corresponding author. Fax: (39) (2) 38210381.
E-mail: csro@unimi.it

PII: S0014-5793(98)01627-5

(*N*-[(2*S*)-7-carbethoxy-1,2,3,4-tetrahydronaphth-2-yl]-(2*R*)-2-hydroxy-2-(3-chlorophenyl)ethanamine hydrochloride) (generous gift from L. Manara, Sanofi Winthrop, Milan, Italy) were added to cultured fat cells at day 8 (i.e. at the time of confluence and differentiation) for 18 h for enzyme immunoassay (EIA) experiments. Noradrenaline (Sigma, Milan, Italy) (1 μM) or SR58611A (1 μM) was added to cultured fat cells at day 8 for 4 h for reverse transcriptase-polymerase chain reaction (RT-PCR) experiments.

2.2. PCR assay

Total cytoplasmic RNA was isolated from 1×10^6 cultured cells or from 20 mg of tissue using the RNazol method (TM Cinna Scientific, Friendswood, TX, USA). For PCR analysis, performed as previously described [13], an aliquot (10% vol) of the cDNA was amplified using the specific primers for rat VEGF: 5′-CCA TGA ACT TTC TGC TCT CTT G-3′ (nucleotides 2–24) and 5′-GGT GAG AGG TCT AGT TCC CGA-3′ (nucleotides 610–630) [14] by *Taq* DNA polymerase (Promega, Milan, Italy) in 25 μl of standard buffer (10 mM Tris-HCl, pH 9, 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, and 200 μM dNTPs). After 35 cycles a final 10-min incubation at 72°C was carried out. Another aliquot (2% vol) of the same cDNA was amplified, using the specific primers for rat β-actin [15], for 20 cycles to normalise PCR VEGF results in obese animals and noradrenaline- or SR58611A-treated cells. 10 μl of each PCR reaction product was separated by electrophoresis (2.0% agarose gel in Tris-acetate-EDTA buffer, containing 0.1 mg/ml of ethidium bromide), revealed with a QuickImage-D (Camberra Packard, Milan, Italy) and densitometrically analysed with Phoretix 1D version 3.0. The specificity of PCR products was confirmed by sequence analysis, performed by M Medical (Florence, Italy).

2.3. VEGF immunoassay

The VEGF concentrations in the medium of cultured cells were measured by means of Cytokit Red TM VEGF (Cyt Immune Sciences Inc., College Park, MD, USA) using a polyclonal rabbit antibody that recognises rat VEGF [16]. Medium of each well was collected and concentrated by means of Centrisart I (Sartorius, Göttingen, Germany). EIA was performed according to the procedures suggested by the manufacturer. The range of detection of this assay is 0.195–200 ng/ml. The assay was performed in triplicate and the intra-assay coefficient of variation was ± 7.0%.

2.4. Western blotting

Western blotting was performed as previously described [13]. Aliquots corresponding to 50 μg of protein were applied to 15% SDS-polyacrylamide gels. Western blotting consisted of overnight electroblotting the proteins from the polyacrylamide gel onto nitrocellulose filter paper, blocking with 3% non-fat dried milk (2 h at room temperature), incubating the filter at room temperature for 2 h with the primary antibody (polyclonal rabbit antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and for 1 h with secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Boehringer Mannheim, Milan, Italy) at room temperature. As little as 15–25 ng of VEGF protein can be detected by this method. The coefficient of variation between the different experiments was lower than 5%.

2.5. Immunohistochemistry

Genetically obese male Zucker rats (*fa/fa*) and their lean littermates (+/*fa*), 6 weeks of age, were obtained from Harlan Nossan (Correzzana, Italy). Some rats were kept at room temperature (20–22°C; referred to as control rats), some were acclimated at 4°C for 2 weeks (cold-acclimated rats) and some were treated (1 mg/kg/day) intraperitoneally with CL 316,243 (disodium *R,R*-5([2-[3-chlorophenyl]-2-hydroxy ethyl]-1,3-benzodioxole-2,2-dicarboxylate) (a generous gift from Kurt Steiner, Wyeth-Ayerst, USA), a selective β_3-adrenoceptor agonist, for 2 days. The animals were anaesthetised with 0.36 M chloral hydrate (400 mg/kg) and perfused intra-aortically with a 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4). Interscapular brown adipose tissue was carefully dissected and immediately postfixed overnight at 4°C in the same fixative, before being washed, dehydrated in ethanol and embedded in paraffin. Immunoreactivity for VEGF was assessed in 3 μm thick serial sections using the avidin-biotin-peroxidase method (ABC method). Negative controls were obtained in each instance by omitting the primary antibody and using preimmune serum instead of the primary antiserum.

2.6. Data analysis

The comparisons were made using one-way ANOVA followed by Student-Newman-Keuls post-hoc comparisons; $P<0.05$ vs controls was considered significant.

3. Results

Expression of VEGF mRNA was revealed by RT-PCR in rat cultured preconfluent and confluent cells (Fig. 1A). Two VEGF mRNA isoforms were detected under our experimental conditions: one of 628 bp and corresponding to the VEGF 164 isoform, and the other of 496 bp and corresponding to the VEGF 120 isoform. In addition, both the rat preconfluent and confluent cells contained a protein species of approximately 23

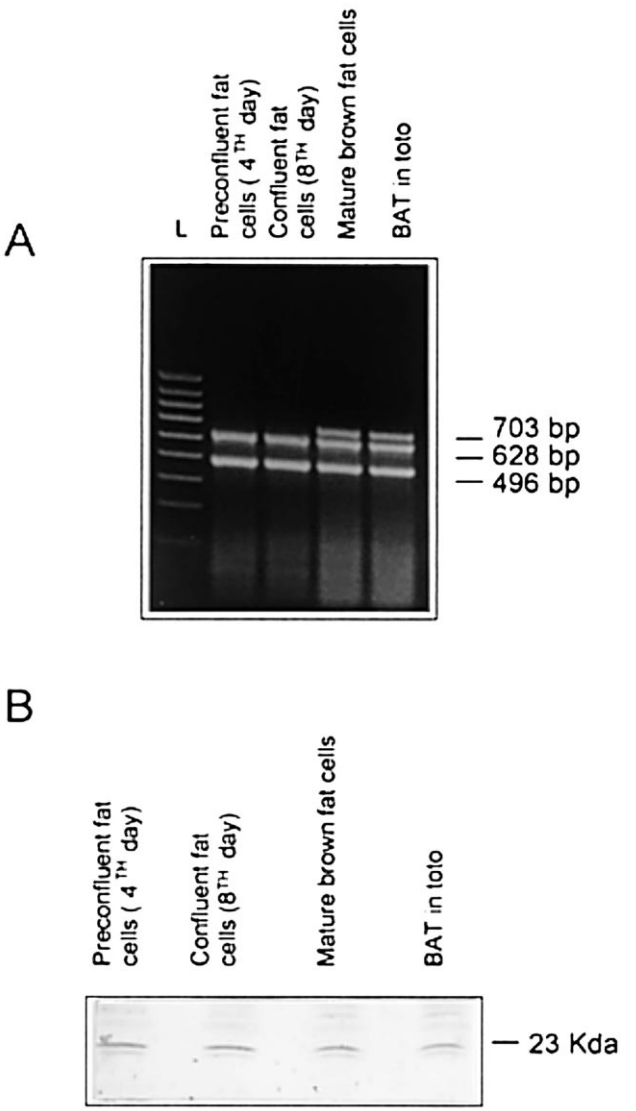

Fig. 1. RT-PCR and Western blot analysis of RNAs and proteins isolated from preconfluent and confluent brown adipocytes, mature brown adipocytes and rat BAT. A: Ethidium bromide agarose gel electrophoresis pictures of VEGF cDNA fragments amplified by PCR from the various preparations; L, 100 bp DNA ladder (M Medical, Florence, Italy). The PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 56°C for 1 min and polymerization at 72°C for 1 min). B: Immunoblot obtained by separating 50 μg of protein on 15% SDS-polyacrylamide gel under reducing conditions.

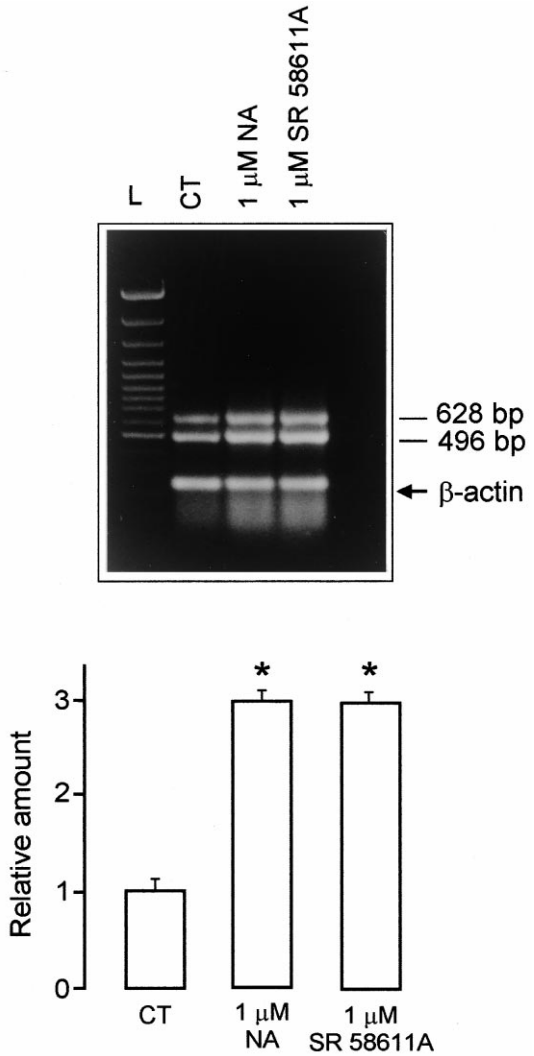

Fig. 2. RT-PCR analysis of VEGF in confluent cells treated with noradrenaline (NA) or β_3-adrenoceptors agonist (SR58611A). Upper: Representative agarose gel showing the PCR analysis for the VEGF and β-actin mRNA content; L, DNA ladder; CT, untreated cells. Lower: Densitometric analysis of RT-PCR experiments is reported. The bars represent the mean values ± S.E.M. of three separate experiments plotted in relation to the area under the curve for the VEGF/β-actin ratio of control cells taken as one. $^*P < 0.05$ vs control cells.

kDa, which cross-reacted with polyclonal anti-VEGF antibodies (Fig. 1B). Both VEGF mRNA isoforms and protein appeared to be present also in enzymatically dispersed rat mature brown fat cells and in rat BAT in toto (Fig. 1A,B). In the latter two mRNA preparations a third isoform of 703 bp, corresponding to the VEGF 188 isoform, was detected.

Since cold exposure, by inducing sympathetic noradrenergic stimulation, activates BAT thermogenesis, VEGF mRNA expression was measured in BAT taken from animals exposed either to a cold environment (4°C for 4 h) or to room temperature. The VEGF mRNA levels were significantly higher in BAT of cold-exposed than room-temperature-acclimated animals (data not shown), confirming previously reported results [6]. These results were also confirmed in vitro using noradrenaline treatment (1 M for 4 h) to induce VEGF mRNA expression in cultured brown adipocytes (Fig. 2). To study the role of the β_3-adrenoceptor in this noradrenaline-mediated effect, the cultured brown fat cells were treated for 4 h with 1 M SR58611A, a selective β_3-adrenoceptor agonist, and total RNA was harvested for RT-PCR analysis. RT-PCR products were run and the quantities of minor and major mRNA VEGF isoforms of each sample were densitometrically analysed, their values were summated and normalised with β-actin values. Fig. 2 shows that SR58611A was able to increase 3-fold the VEGF mRNA expression. Neither noradrenaline nor SR58611A was able to significantly change the β-actin mRNA levels in cultured brown adipocytes over a wide concentration range (from 1 nM to 100 μM, data not shown).

SR58611A also dose-dependently increased VEGF concentrations in the medium of treated cells. Fig. 3 summarises the results of EIA experiments showing that the VEGF concentrations are markedly increased in the medium of cultured brown fat cells after 18 h treatment with different concentrations of SR58611A, indicating that stimulation of β_3-adrenoceptors not only increases the expression of VEGF, but also causes its release.

Obesity is generally associated with a reduction in BAT sympathetic nervous system activity, and hence with a relatively quiescent, atrophied and poorly vascularised brown fat [17]. To verify the VEGF expression in this condition, total RNA, extracted from BAT of genetically obese *fa/fa* Zucker rats and lean *+/fa* littermates, was used for RT-PCR. RT-PCR products were run and the quantities of three mRNA VEGF isoforms of each sample were densitometrically analysed, summated and normalised with β-actin values. Fig. 4 shows that total VEGF mRNA is decreased in obese animals. In particular, the ratio between the shortest isoform and the major one was decreased 2-fold in *fa/fa* rat compared to lean controls, suggesting the possibility of a different modulation of alternative splicing in obese animals.

In order to confirm the previous results and to identify the cytotypes expressing VEGF in vivo, we evaluated the VEGF

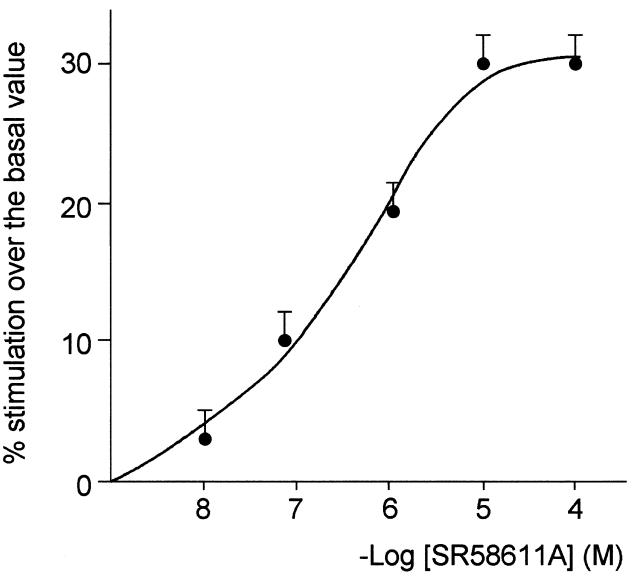

Fig. 3. Effect of various SR58611A concentrations on VEGF release from brown fat cells in EIA experiments. Results represent the mean ± S.E.M. of three different experiments, each performed in triplicate.

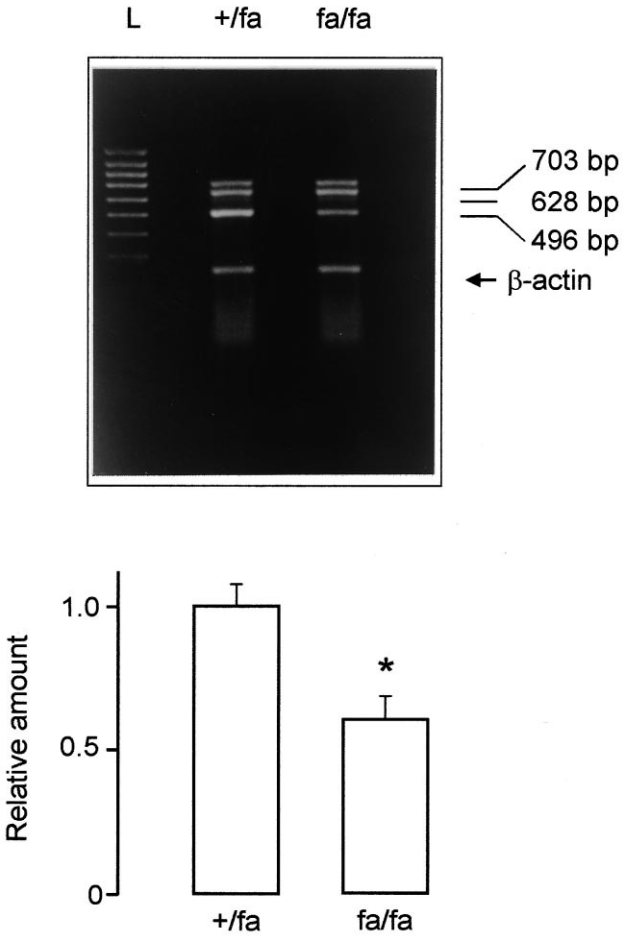

Fig. 4. RT-PCR analysis of VEGF in the BAT of obese (*fa/fa*) and lean (+/*fa*) Zucker rats. Upper: Representative agarose gel showing the PCR analysis for the VEGF and β-actin mRNA content; L, DNA ladder. Lower: Densitometric analysis of RT-PCR experiments is reported. The bars represent the mean values ± S.E.M. of three separate experiments plotted in relation to the area under the curve for the VEGF 120/VEGF 188 ratio in the BAT of lean (+/*fa*) rats taken as 1. $*P < 0.05$ vs (+/*fa*) rats.

immunoreactivity in rat interscapular BAT using the same polyclonal antibody used for the in vitro and in vivo Western blot evaluations. In control rats, the smooth muscle cells of the extra- and intralobular arteries and veins and the endothelial cells of capillaries were positive, while the brown adipocytes were only weakly positive or, most frequently, completely negative (Fig. 5A). After 2 weeks of cold acclimation, the immunoreactivity for VEGF increased both in the media of the blood vessels and in the brown adipocytes (data not shown). A very high degree of specific cytoplasmic immunostaining was also noted in brown adipocytes of rats treated with the β_3-adrenoceptor agonist CL 316,243 for 2 days (1 mg/kg/day), thus confirming the involvement of β_3-adrenoceptors in the noradrenaline-induced increase of VEGF expression in brown adipocytes (Fig. 5B). In the genetically obese (*fa/fa*) Zucker rats, the immunohistochemical expression of VEGF appeared very low, both in the blood vessel and in the brown adipocytes. No other cytotypes were found to be positive for VEGF in the interscapular brown adipose tissue in the experimental conditions examined.

4. Discussion

The possibility that VEGF could be produced in brown fat cells had been suggested by Asano et al. [6], who observed that it was expressed in the rat BAT. Until now, however, synthesis and release of VEGF had not been demonstrated directly as shown here for rat brown adipocytes. Among the different VEGF isoforms that are produced by alternative splicing in most of cells, only two (VEGF 164 and VEGF 120) were present in brown adipocytes differentiated in culture, while a third isoform (VEGF 188) is expressed in enzymatically dispersed cells or in BAT in toto, suggesting that brown adipocytes differentiated in culture do not completely differentiate. Alternatively, a different regulation of the alternative splicing in cells differentiated in culture or in vivo could explain these differences. The presence of VEGF protein in cultured brown adipocytes or BAT in toto was demonstrated by means of both Western blotting and immunohistochemistry.

It is known that cold exposure produces adaptive hyperplasia and growth of BAT that results from a rapid activation of mitosis, not only of precursor cells of brown adipocytes, but also of endothelial cells forming the capillaries [4]. Asano et al. [6] have shown that when the rats were exposed to low temperature for 1–4 h, the VEGF mRNA was increased 2–3-fold in BAT. This increased expression of VEGF in BAT is not surprising, because it is a tissue with a large capacity for adaptive hypertrophy and thermogenesis, and its increase after cold exposure could explain the intense neovascularisation in this condition. Using immunohistochemistry increased VEGF expression was seen to occur not only in the media of the vessels but even in brown adipocytes of animals exposed to cold. The in vitro and in vivo (immunohistochemistry) observation that β_3-adrenoceptor agonists were able to induce markedly the expression of VEGF in brown fat cells and to stimulate its release indicates that these effects of sympathetic activation are mediated, at least in part, by the β_3-adrenoceptors largely expressed in these cells.

Recently, we have demonstrated that brown fat cells can express an inducible form of nitric oxide synthase, ensuring NO production and release. This is stimulated by sympathetic activity in physiological conditions, and NO may represent an important mechanism through which noradrenaline can modulate different BAT functions, in addition to vasodilatation [18]. Since NO has been suggested to mediate the VEGF effects on endothelial cells [19], a reciprocal relationship between VEGF and NO could play a role in the regulation of endothelial integrity. Thus, sympathetic nervous system activation could simultaneously induce vasodilatation and neovascularisation through NO production and VEGF expression, ensuring an increased blood flow to BAT. Further studies are in progress to clarify the physiological link between VEGF and NO in brown adipocytes.

In animal models the initiation and development of genetic obesity are due at least in part to the low level of energy expenditure and consequent high efficiency of energy utilisation. Sympathetic activity in brown fat under normal environmental conditions is reduced in obese animals [20]. In adult *fa/fa* rats the interscapular BAT is functionally defective as well as its vascularisation [17,21]. Our results showed that the BAT production of the shortest VEGF isoform is lower in obese than in lean animals, and we have also seen that this differ-

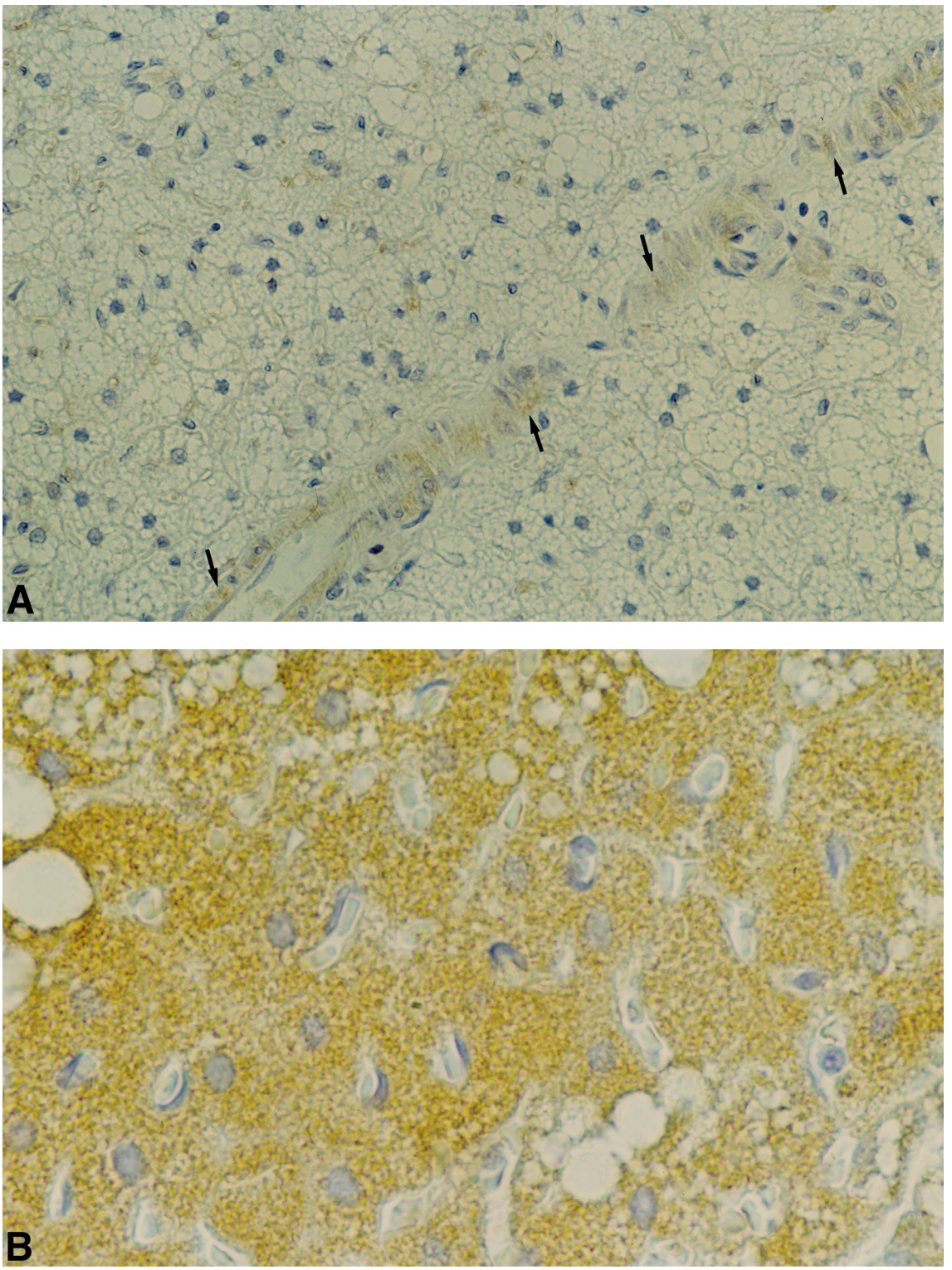

Fig. 5. Immunohistochemistry for VEGF (with a polyclonal antibody against the three isoforms, ABC method). A: BAT of a control rat kept at 20°C. B: BAT of a rat treated with the β_3-adrenoceptor agonist CL 316,243 (1 mg/kg/day, intraperitoneally, for 2 days). The brown adipocytes are intensely stained in the cytoplasm. A: 430×; B: 1075×.

ence increases with age (data not shown). This could explain the defective vascularisation seen in BAT of these animals, particularly as the shortest form of VEGF lacks heparin binding activity and is, therefore, more easily diffusible [6]. Thus, an impaired regulation of VEGF alternative splicing could explain the defective BAT vascularisation in obesity.

In conclusion, our results show that VEGF is synthesised in and released from brown adipocytes, and its production is directly modulated by sympathetic activity, at least in part via β_3-adrenoceptors. This sympathetic modulation ensures the supply of VEGF when functionally required and explains its deficit in genetically obese animals.

Acknowledgements: We wish to thank Prof. Paolo Mantegazza for his encouragement and support. This work has been partly supported by an Educational Grant from Knoll Farmaceutici Spa, Muggiò, Milan (Italy) to CSRO.

References

[1] Himms-Hagen, J. and Desaultels, M. (1978) Biochem. Biophys. Res. Commun. 83, 628–632.
[2] Cannon, B., Jacobsson, A., Rehnmark, S. and Nedergaard, J. (1996) Int. J. Obes. 20, (Suppl. 3) S36–S42.
[3] Nisoli, E., Briscini, L., Tonello, C., De Giuli-Morghen, C. and Carruba, M.O. (1997) Cell Death Different. 4, 771–778.
[4] Bukowiecki, L., Collet, A.J., Follea, N., Guay, G. and Jahjah, L. (1982) Am. J. Physiol. 242, E353–359.
[5] Folkman, J. (1982) Ann. NY Acad. Sci. 401, 212–227.
[6] Asano, A., Morimatsu, M., Nikami, H., Yoshida, T. and Saito, M. (1997) Biochem. J. 328, 179–183.
[7] Ferrara, N., Houck, K., Jakeman, L. and Leung, D.W. (1992) Endocr. Rev. 13, 18–32.
[8] Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V. and Ferrara, N. (1989) Science 246, 1306–1309.
[9] Senger, D.R., Ledbetter, S.R., Claffey, K.P., Papadopoulos-Sergiou, A., Peruzzi, C.A. and Detmar, M. (1996) Am. J. Pathol. 149, 293–305.
[10] Ladoux, A. and Frelin, C. (1993) Biochem. Biophys. Res. Commun. 194, 799–803.
[11] Bacic, M., Edwards, N.A. and Merrill, M.J. (1995) Growth Factors 12, 11–15.
[12] Néchad, M., Kuusela, P., Carneheim, C., Björntorp, P., Nedergaard, J. and Cannon, B. (1983) Exp. Cell Res. 149, 105–118.
[13] Nisoli, E., Tonello, C., Benarese, M., Liberini, P. and Carruba, M.O. (1996) Endocrinology 137, 495–503.
[14] Conn, G., Bayne, M.L., Soderman, D.D., Kwok, P.W., Sullivan, K.A., Palisi, T.M., Hope, D.A. and Thomas, K.A. (1990) Proc. Natl. Acad. Sci. USA 87, 2628–2632.
[15] Gaudette, M.F. and Crain, W.R. (1991) Nucleic Acid Res. 19, 1879–1880.
[16] Oliver, S.J., Cheng, T.P., Banquerigo, M.L. and Brahn, E. (1995) Cell Immunol. 166, 196–206.
[17] Himms-Hagen, J. (1986) in: Brown Adipose Tissue (Trayhurn, P. and Nicholls, D.G., Eds.), pp. 214–268, Edward Arnold, London.
[18] Nisoli, E., Tonello, C., Briscini, L. and Carruba, M.O. (1997) Endocrinology 138, 676–682.
[19] Morbidelli, L., Chang, C.H., Douglas, J.G., Granger, H.J., Ledda, F. and Ziche, M. (1996) Am. J. Physiol. 270, H411–415.
[20] Marette, A., Geloen, A., Collet, A. and Bukowiecki, L.J. (1990) Am. J. Physiol. 258, E320–E328.
[21] Trayhurn, P. and Nicholls, D.G. (1986) in: Brown Adipose Tissue (Trayhurn, P. and Nicholls, D.G., Eds.), pp. 299–338, Edward Arnold, London.