Adrenergic activation of vascular endothelial growth factor mRNA expression in rat brown adipose tissue: implication in cold-induced angiogenesis

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Cold exposure produces adaptive hyperplasia and growth of brown adipose tissue (BAT), the major site of non-shivering thermogenesis in rodents, associated with increased angiogenesis in this tissue. Vascular endothelial growth factor (VEGF), one of the most potent angiogenic factors, was found to be expressed abundantly in BAT of the rat. When rats were exposed to cold at 4 °C, the VEGF mRNA level in BAT was increased by 2–3-fold in 1–4 h, but returned to the basal level within 24 h. VEGF expression in other tissues such as heart, kidney and lung did not change after cold exposure. The cold-induced increase in VEGF mRNA was abolished by surgical sympathetic denervation, but mimicked by administration of noradrenaline or a β_3-adrenoceptor agonist CL316,243, indicating the critical role of the β-adrenergic pathway in VEGF expression in BAT. Among three isoforms of VEGF, the mRNA of a short form (VEGF120) lacking heparin-binding activity was preferentially increased after cold exposure and treatment with the adrenergic agonists. These results suggest that cold exposure activates the sympathetic nerves and leads to a rapid increase in synthesis of VEGF in BAT, which in turn stimulates the proliferation of surrounding vascular endothelial cells.

INTRODUCTION

Brown adipose tissue (BAT) is the major site of non-shivering thermogenesis in rodents (for a review, see [1]). It is known that cold exposure produces adaptive hyperplasia and growth of BAT associated with an increase in synthesis of several proteins specific for this tissue, such as mitochondrial uncoupling protein. Autoradiographic studies have demonstrated that BAT hyperplasia results from a rapid activation of mitosis, not only of precursor cells of brown adipocytes, but also of endothelial cells forming the capillaries [2]. It is established that proliferation of brown adipocytes is directly controlled by sympathetic nerves distributed in this tissue, principally through the β-adrenergic action of noradrenaline [1–5]. However, little is known about the mechanism of the increased angiogenesis associated with BAT growth.

Vascular endothelial growth factor (VEGF) is a heparin-binding polypeptide secreted from various types of cells [6] and is a potent mitogen specific for vascular endothelial cells *in vitro* [7]. Numerous studies *in vivo* have demonstrated that expression of VEGF and its receptor is enhanced during angiogenesis under various physiological and pathological processes, such as development of the embryo [8–10], oestrous cycle [11], tumour growth [12,13] and wound healing [14]. Thus it is now accepted that VEGF is one of the most important angiogenic factors reported to date [15]. There has been no report, however, as to whether VEGF plays a role in angiogenesis associated with adaptive tissue growth, such as BAT growth after cold exposure and enlargement of fat depots in obesity.

Here we demonstrate high expression of VEGF mRNA in rat BAT, and a rapid, transient increase in VEGF mRNA after cold exposure and adrenergic stimulation, especially in the shortest product of alternative mRNA splicing (VEGF120) lacking the heparin-binding domain.

MATERIALS AND METHODS

Animals and treatments

Adult female Wistar rats were kept at 24 °C with a 12 h light–dark cycle and given free access to food and water. Under Nembutal anaesthesia, unilateral sympathetic denervation of the interscapular BAT was performed by severing five branches of the intercostal nerve on the right side of the BAT, as described previously [16]. Successful denervation was confirmed by reduced contents of noradrenaline in the tissue (less than 3 % of those in the left-side control). The rats were either transferred to a cold room at 4 °C or subcutaneously administered with noradrenaline (Sigma, St. Louis, MO, U.S.A.) and a highly selective agonist of β_3-adrenoceptor CL316,243, disodium (*R*,*R*)-5{[2-(3-chlorophenyl)-2-hydroxyethyl]-aminopropyl}-1,3-benzodioxole-2,2-dicarboxylate [17]. The rats were then killed 1 h–10 days after the treatments, and various tissues such as the interscapular BAT, retroperitoneal white adipose tissue, heart, kidney and lung were taken and stored at −80 °C. The care of animals and procedures used were approved by the Animal Care and Use Committee of Hokkaido University.

Isolation of RNA and analyses by Northern hybridization

Total RNA was extracted from 0.1–0.3 g of tissue using TRIzol (Gibco–BRL, Tokyo, Japan) and its concentration was determined from the absorbance at 260 nm. Total RNA (30 μg) was separated on a 1 % (w/v) agarose/formaldehyde gel and transferred to and fixed on a nylon membrane. A VEGF cDNA probe corresponding to nts 3–619 of the published rat cDNA sequence [18] was synthesized by the PCR method. An 18 S ribosomal RNA cDNA probe corresponding to nts 390–551 of the published rat DNA sequence [19] was synthesized by the PCR method. The

Abbreviations used: BAT, brown adipose tissue; FGF, fibroblast growth factor; RT-PCR, reverse transcription-polymerase chain reaction; VEGF, vascular endothelial growth factor.

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cDNA probes were labelled with [α-^{32}P]dCTP using a multiprime DNA labelling kit (Amersham, Tokyo, Japan). The blots were hybridized on to the labelled probes at 42 °C for 20 h in the presence of 0.2 mg/ml salmon sperm DNA, washed twice at room temperature for 15 min with 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1 % (w/v) SDS and subsequently at 65 °C for 15 min with 0.1 × SSC/0.1 % (w/v) SDS, and then analysed with a BAS1000 bioimage analyser (Fuji Photo Film, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR)

VEGF mRNA was also analysed by RT-PCR using forward (5′-CGCGAATTCCATGAACTTTCTGCTCTCT-3′) and reverse (5′-TGAGAATTCTAGTTCCCGAAACCCTGA-3′) primers. These primers were designed on the basis of the sequences corresponding to the signal peptide and the 3′-untranslated region of rat VEGF cDNA, respectively. Extracts of the total RNA (2 μg) were incubated at 80 °C for 5 min, cooled immediately and reverse-transcribed by 60 units of Moloney murine leukaemia virus reverse transcriptase (Gibco–BRL), 50 pmol of reverse primer and 20 nmol of dNTPs in a total vol. of 10 μl at 37 °C for 1 h. After heating to 94 °C for 5 min, PCR amplification was performed with 0.75 units *Taq* DNA polymerase (Takara, Tokyo, Japan) and 50 pmol of forward primer in a total volume of 50 μl. PCR cycles used were 94 °C for 3 min, 50 °C for 2 min and 72 °C for 3 min in the first round, 94 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min in the next 35 rounds, and 94 °C for 1 min, 50 °C for 2 min and 72 °C for 10 min in the last round. Three PCR products (711 bp, 636 bp, 504 bp) were digested by *Eco*RI and ligated to the cloning vector pBluescriptKS+. Nucleotide sequences of the PCR products were analysed with an ABI 373A system (Perkin–Elmer, Tokyo, Japan). PCR products both after and in the absence of treatment with *Eco*RI and *Pst*I were separated by 2 % (w/v) agarose-gel electrophoresis and visualized by ethidium bromide staining. The density of each band was quantified using NIH Image analysis software. Preliminary experiments confirmed that the ratio of the three PCR products obtained was almost constant between 0.5–4.0 μg of initial RNA used and with cycle numbers from 25–40.

Data analysis

All values are presented as means ± S.E.M. Statistical analysis was performed by analysis of variance with *post-hoc* testing by Fisher's PSLD (protected least significant difference) multiple-range test.

RESULTS

High expression of VEGF mRNA in BAT and effects of cold exposure

The expression of VEGF mRNA in various tissues of rats, including white and brown adipose tissues, was examined by Northern-blot analysis. As shown in Figure 1, high expression of VEGF mRNA was found in lung, heart and BAT, probably reflecting abundant vascularization in these tissues. The mRNA level in white adipose tissue was much lower than that found in BAT. When rats were exposed to cold at 4 °C, the VEGF mRNA level in BAT was increased by 2–3-fold in 1–4 h, but returned to the basal level in 1 day and remained low for at least 10 days (Figure 2). In contrast with BAT, VEGF mRNA levels in heart, kidney and lung did not change significantly throughout cold exposure (Table 1).

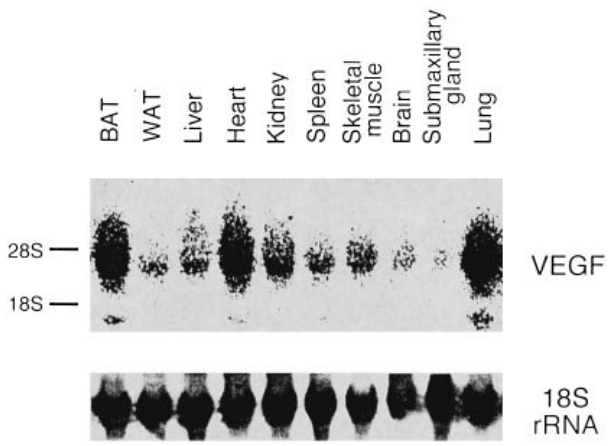

Figure 1 Northern-blot analysis of VEGF mRNA in various tissues of the rat

Total RNA (30 μg) extracted from each tissue was subjected to Northern-blot analysis. WAT, retroperitoneal white adipose tissue.

β-Adrenergic activation of VEGF mRNA expression

The effects of sympathetic denervation and noradrenaline treatment on VEGF mRNA expression in BAT were examined. As shown in Table 2, surgical severing of the sympathetic nerves did not affect the VEGF mRNA level when rats were kept at 24 °C, whereas this procedure blocked the rise induced by cold exposure. Noradrenaline administration produced an increase in the VEGF mRNA level similar to that found after cold exposure, in both intact and denervated tissues. VEGF mRNA expression was also increased when rats were administered the selective β_3-adrenergic agonist CL316,243. Thus the cold-induced increase in VEGF mRNA expression was abolished by sympathetic denervation, but mimicked by treatment with either noradrenaline or the β_3-adrenergic agonist.

Preferential expression of VEGF isoforms

There are several isoforms of VEGF translated from alternatively spliced mRNAs [20]. In rats, the presence of three isoforms, VEGF188, VEGF164 and VEGF120, is reported [21]. The effects of cold exposure on the mRNA expression of each isoform were examined by RT-PCR analysis. Since the primer set for the RT-PCR analysis was designed on the basis of the sequences corresponding to the signal peptide and the 3′-untranslated region of rat VEGF cDNA (see the Materials and methods section), all of the isoforms of VEGF mRNA were expected to be amplified with this primer set. As shown in Figure 3, three PCR products were detected in BAT using total RNA. Nucleotide-sequence and restriction-enzyme (*Eco*RI and *Pst*I)-digestion analyses of these PCR products confirmed that they were derived from mRNAs of VEGF-188, -164 and -120, respectively (results not shown). When rats were kept at 24 °C, the major isoform was VEGF188, and the shortest one (VEGF120) accounted for 11 % of the total VEGF mRNA (Figure 3 and Table 3). After 4 h of cold exposure, however, mRNA expression of VEGF120 was increased markedly, being about 30 % of the total VEGF mRNA (Table 3). Since the total VEGF mRNA was increased 2.66-fold (Table 1 and Table 3), the VEGF120 mRNA level was found to increase more than 9-fold from 9 at 0 h to 85 at 4 h. In contrast, the VEGF188 mRNA level was increased only 1.4-fold from 53

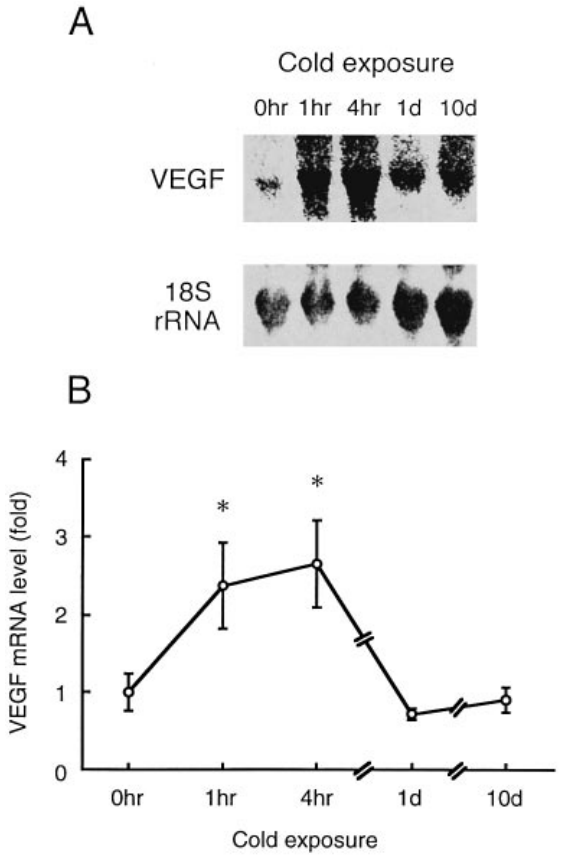

Figure 2 Effect of cold exposure on the VEGF mRNA level in BAT

Rats were kept at 24 °C (0 h) or exposed to cold at 4 °C for 1 h–10 days, and 30 μg of total RNA extracted from the interscapular BAT was analysed. (**A**) Typical autoradiograms of VEGF mRNA and 18 S rRNA. (**B**) The blots of VEGF mRNA were quantified with a bioimage analyser and normalized to 18 S rRNA blots. The values are expressed relative to the 0 h values, and are means ± S.E.M. for 4 rats. *$P < 0.05$ versus 0 h.

Table 1 Effects of cold exposure on the VEGF mRNA level in various tissues

The blots of VEGF mRNA were quantified and normalized by 18 S rRNA blots. The values are expressed relative to the respective 0 h values, and are means ± S.E.M. for 4 rats.

	Cold exposure				
Tissue	0 h	1 h	4 h	1 day	10 days
BAT	1.00 ± 0.24	2.37 ± 0.55*	2.66 ± 0.56*	0.71 ± 0.07	0.90 ± 0.17
Heart	1.00 ± 0.19	1.07 ± 0.18	1.37 ± 0.25	0.97 ± 0.17	0.89 ± 0.24
Kidney	1.00 ± 0.11	0.96 ± 0.14	1.16 ± 0.23	1.19 ± 0.03	1.30 ± 0.22
Lung	1.00 ± 0.28	1.32 ± 0.34	1.66 ± 0.16	1.47 ± 0.30	1.24 ± 0.45

* $P < 0.05$ versus 0 h.

to 72. Ten days after cold exposure, the expression patterns returned to those seen at 24 °C.

A similar preferential increase in the mRNA of the shorter isoforms of VEGF was also observed after administration of noradrenaline or CL316,243 (Table 4). For example, CL316,243 administration produced a 23-fold increase in VEGF120 from 3 to 68 and a 2-fold increase in VEGF188 from 70 to 141.

Table 2 Effects of sympathetic denervation and adrenergic-agonist treatments on the VEGF mRNA level in BAT

Seven days after surgical sympathetic denervation, rats were exposed to cold (4 °C) or administered noradrenaline or CL316,243 (3 μmol/kg, subcutaneously), and killed 2 h later. The drugs were given again 1h before killing. The values normalized by 18 S rRNA are expressed relative to those of the intact control and are means ± S.E.M. for 4 rats.

	Intact	Denervated
Control (24 °C)	1.00 ± 0.19	1.25 ± 0.12
Cold exposure (2 h)	2.49 ± 0.26*	1.48 ± 0.22
Noradrenaline	2.66 ± 0.27*	2.65 ± 0.27*
CL316,243	3.69 ± 0.81*	2.47 ± 0.48*

* $P < 0.05$ versus respective controls. $P < 0.05$ versus intact.

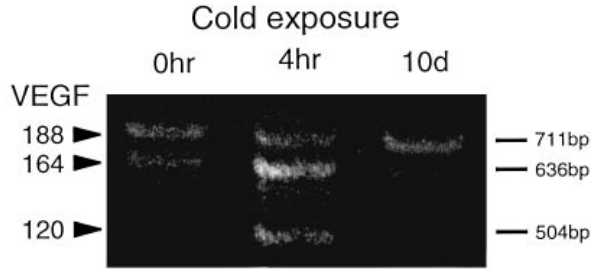

Figure 3 Cold-induced changes in the mRNA levels of VEGF isoforms in BAT

Total RNA (2 μg) used in Figure 2 was subjected to RT-PCR. The three PCR products correspond in size to those expected for VEGF-188 (711 bp), -164 (636 bp), and -120 (504 bp).

DISCUSSION

VEGF is a specific mitogen for vascular endothelial cells *in vitro* and induces angiogenesis *in vivo* [15]. VEGF mRNA is known to be expressed in various tissues of the rat, such as lung, brain, heart and kidney [22], and in epididymal white adipose tissue of the mouse [23]. However, there has been no report on VEGF expression in BAT. In this study, we found significant expression of VEGF mRNA in both white and brown adipose tissues. Particularly in BAT, the VEGF mRNA level appeared to be comparable with those found in highly expressed tissues, such as heart, kidney and lung. In contrast, the VEGF mRNA level was low in white adipose tissue. Such patterns of VEGF expression may reflect rich and poor vascularization in BAT and white adipose tissue respectively. The significant expression of VEGF in adipose tissues is not surprising, because they are tissues primarily having a large capacity for adaptive hypertrophy. It is not uncommon, for example, that the weight of white fat pads increases 2–3-fold after hyperphagia [24,25]. Marked hypertrophy of BAT with proliferation of pre-adipocytes and endothelial cells is also a well-known phenomenon seen during cold acclimation [1–5]. Being associated with such adaptive hypertrophy, angiogenesis and related events, such as the production of angiogenic factors, are expected to be stimulated. In fact, the results shown in this report reveal that the VEGF mRNA level in BAT is increased 2–3-fold after cold exposure in rats. Cold exposure has been known to increase the mRNA level of various BAT proteins, including mitochondrial uncoupling protein, lipoprotein lipase and glucose transporters [1,16,26]. The change

Table 3 Effect of cold exposure on the mRNA levels of individual VEGF isoforms

The band intensity of each RT-PCR product in Figure 3 was quantified with NIH Image analysis software and expressed as a percentage of the total intensity of the three bands. Values in parentheses are the mRNA levels of each isoform calculated as products of the percentage and total VEGF mRNA level in Table 1. Values are means ± S.E.M. for 4 rats. ND, not detectable.

Cold exposure	Total VEGF mRNA	% of isoform (calculated isoform level)		
		VEGF188	VEGF164	VEGF120
0 h	1.00 ± 0.24	51.1 ± 3.7 (53 ± 17)	37.9 ± 0.9 (38 ± 10)	11.0 ± 3.7 (9 ± 3)
4 h	2.66 ± 0.56	28.8 ± 2.9 (72 ± 11)	40.8 ± 1.7 (109 ± 23*)	30.4 ± 3.1 (85 ± 23*)
10 days	0.90 ± 0.17	66.5 ± 1.2 (60 ± 11)	33.5 ± 1.7 (30 ± 6)	ND

* $P < 0.05$ versus respective 0 h values.

Table 4 Effect of adrenergic agonists on the mRNA levels of individual VEGF isoforms

Total RNA (2 μg) used in Table 2 (Intact) was subjected to RT-PCR, and the band intensity of each RT-PCR product was quantified as in Table 3. Values in parentheses are the mRNA levels of each isoform calculated as products of the percentage and total VEGF mRNA level in Table 2. Values are means ± S.E.M. for 4 rats.

Treatment	Total VEGF	mRNA% of isoform (calculated isoform level)		
		VEGF188	VEGF164	VEGF120
Control (24 °C)	1.00 ± 0.19	68.5 ± 3.9 (70 ± 15)	28.3 ± 3.5 (27 ± 4)	3.3 ± 1.2 (3 ± 2)
Cold exposure (2 h)	2.49 ± 0.26	60.8 ± 4.9 (155 ± 25)	28.8 ± 1.5 (71 ± 7*)	10.4 ± 3.8 (23 ± 5*)
Noradrenaline	2.66 ± 0.27	51.8 ± 6.4 (133 ± 8)	35.0 ± 3.8 (96 ± 19*)	13.2 ± 3.1 (37 ± 10*)
CL316,243	3.69 ± 0.81	37.0 ± 2.7 (141 ± 36)	43.3 ± 0.7 (160 ± 36*)	19.7 ± 2.8 (68 ± 11*)

* $P < 0.05$ versus respective controls.

in the VEGF mRNA level was rapid and transient, but in other tissues, VEGF mRNA levels did not change. Thus cold exposure produced a rapid increase in VEGF mRNA expression specifically in BAT, implying a contribution made by VEGF towards the cold-induced proliferation of vascular endothelial cells and angiogenesis in this tissue.

The cold-induced increase in VEGF mRNA expression was suppressed by surgical sympathetic denervation, but mimicked by administration of noradrenaline. These results are quite consistent with the view that the cold-induced activation of gene expression and cell proliferation in BAT is triggered by noradrenaline released from sympathetic nerves, mostly through its β-adrenergic action [1]. More interestingly, VEGF mRNA expression was also increased when rats were given CL316,243, a highly selective agonist of the β_3-adrenoceptor. The β_3-adrenoceptor is believed to be present exclusively in adipocytes and smooth muscle cells in the gastrointestinal tract [27]. Our results, together with these previous findings, suggest that the primary cells for VEGF expression in BAT are adipocytes rather than vascular cells. In preliminary experiments using brown adipocyte primary culture, we confirmed an *in vitro* activation of VEGF mRNA expression by noradrenaline and CL316,243. Collectively, it seems likely that cold exposure activates sympathetic nerves and leads to an increase in both the synthesis and secretion of VEGF in brown adipocytes, which stimulates proliferation of the surrounding vascular endothelial cells.

It is known that there are three isoforms of VEGF (VEGF-188, -164 and -120) in rats which are translated from alternatively spliced mRNAs. Although there is no difference in endothelial cell mitogenic activity among them [28], they have different heparin-binding activities depending on the polycationic regions near the C-terminal ends, i.e. the shortest form, VEGF120, lacks heparin-binding activity, but VEGF164, formed by inclusion of a polycationic amino acid sequence, can bind to isolated heparin and probably to heparan proteoglycans on cellular surfaces and within extracellular matrices. Further addition of a very cationic 24-amino-acid-residue sequence in VEGF188 promotes tighter binding to these endogenous polyanions [29]. Therefore alternative mRNA splicing appears to modulate VEGF binding to endogenous heparan proteoglycans, thus controlling diffusion from cellular sites of synthesis and determining the extent of local storage. The results presented in this report demonstrate that both cold exposure and adrenergic stimulation produce a more marked increase in the shorter isoform mRNAs of VEGF, particularly VEGF120, in BAT. A similar preferential expression of the short isoform mRNAs of VEGF was also reported in the uterus in rats treated with progesterone [30]. The preferential increase in the shorter isoforms may be advantageous for rapid and widespread diffusion from the cellular sites of synthesis to target cells, i.e. from adipocytes to the surrounding vascular endothelial cells in BAT. Besides the physiological significance of such alternative splicing of the VEGF gene, the cellular and molecular mechanisms of its adrenergic control are intriguing subjects that should be clarified in further studies.

Recently, Yamashita et al. [31] reported that noradrenaline stimulates the expression of fibroblast growth factor (FGF)-2 in rat brown adipocyte primary culture. Because FGF2 has potent mitogenic activity for vascular endothelial cells *in vitro*, this mitogen, in addition to VEGF, may also contribute to the cold-induced angiogenesis in BAT. However, in preliminary experiments we observed that FGF mRNA expression in rat BAT is quite low *in vivo* and can be detected only after extensive

amplification by RT-PCR. Thus further studies are needed to determine the relative importance of VEGF and other factors, such as FGF2, in the cold-induced angiogenesis in BAT.

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