Cold-Induced mRNA Expression of Angiogenic Factors in Rat Brown Adipose Tissue

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ABSTRACT. Brown adipose tissue (BAT) is the major site of non-shivering thermogenesis in rodents. Rapid angiogenesis is induced in association with adaptive hyperplasia of this tissue when the animal is exposed to cold. We demonstrated previously adrenergic activation of mRNA expression of vascular endothelial growth factor (VEGF) in rat BAT and its possible contribution to the cold-induced angiogenesis in this tissue. In the present study, we examined the effect of cold exposure on mRNA expression of other two angiogenic factors, VEGF-B and basic fibroblast growth factor (bFGF), in rats. Conventional Northern blot analysis revealed abundant mRNA expression of VEGF-B as well as VEGF, but not bFGF, in BAT. When rats were exposed to cold at 4˚C, the VEGF mRNA level was increased by 2.7-fold in 1–4 hr and returned to the basal level within 24 hr. In contrast, the VEGF-B mRNA level did not change throughout the course of cold exposure. A significant expression of bFGF mRNA was detected in BAT by reverse transcription-polymerase chain reaction (RT-PCR). To evaluate the tissue bFGF mRNA level quantitatively, a competitive RT-PCR method was developed using a shorter RNA fragment as a competitor. The bFGF mRNA level in BAT was found to increase by 2.3-fold in 4 hr and decreased to the basal level within 24 hr after cold exposure. These results suggest that cold exposure leads to induce VEGF and bFGF rapidly and transiently in BAT, which in turn stimulate the proliferation of vascular endothelial cells in this tissue.—KEY WORDS: angiogenesis, BAT, bFGF, VEGF family.

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Brown adipose tissue (BAT) plays a key role in non-shivering thermogenesis in rodents (for a review, see [10]). Thermogenic function of BAT depends on the uncoupling protein 1 (UCP1) which is specifically expressed in mitochondria of this tissue. It is known that cold exposure produces hyperplasia and growth of BAT associated with a marked increase of UCP1 synthesis, leading to increased activities of heat production and body temperature regulation. Autoradiographic studies have demonstrated that BAT hyperplasia results from a rapid proliferation not only of precursor cells of brown adipocytes but also of endothelial cells forming the blood vessels [3, 7]. 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 [3, 7, 20]. In contrast, little is known about the mechanism of angiogenesis associated with cold-induced BAT growth.

There may be numerous growth factors and cytokines possibly being involved in BAT angiogenesis. We reported previously a rapid and transient induction of vascular endothelial growth factor (VEGF) mRNA in BAT after cold exposure and adrenergic stimulation [1]. Since VEGF is known as a potent endothelial cell-specific mitogen *in vitro* [18] and an indispensable angiogenic factor *in vivo* [5, 6], it is quite likely that VEGF is involved in blood vessel formation associated with the cold-induced BAT growth. Recently, some VEGF homologs named as VEGF-B and VEGF-C have been identified [11, 13, 15, 19]. This VEGF gene family also have the endothelial cell mitogenic activity and share the binding activity to the VEGF receptors. In addition, there have been reported that brown adipocytes synthesize and secrete another growth factor basic fibroblast growth factor (bFGF, or FGF-2), which is also known to show the mitogenic activity to endothelial cells *in vitro* and angiogenesis *in vivo* [4]. Here we report that bFGF as well as VEGF, but not VEGF-B, is induced in rat BAT after cold exposure, suggesting that VEGF and bFGF work cooperatively in angiogenesis associated with the cold-induced BAT growth.

MATERIALS AND METHODS

Cold exposure of rats: Wistar female rats were kept at 24˚C with a 12-hr light-dark cycle and given free access to food and water. When cold exposure was performed, the rats were transferred to a cold room at 4˚C. The animal care and procedures were approved by the Animal Care and Use Committee of Hokkaido University.

Northern blot analysis of RNA: The hybridization cDNA probes were obtained by the reverse transcription-polymerase chain reaction (RT-PCR) method: VEGF and 18S rRNA cDNAs were amplified as described previously [1]. VEGF-B cDNA corresponding to nucleotides 1–567 [19] was amplified from mouse BAT RNA with the primer set of 5'-CGCGAATTCATGAGCCCCCTGCTCCGTCG-3' and 5'-AGCCTTAAGTCACTTTCGCGGCTTCCGGC-3', and VEGF-C cDNA corresponding to nucleotides 330–674 [12] from mouse heart RNA with the primer set of 5'-GTTGCGGTCTGTGTCCAGCG-3' and 5'-GTGCTGG TGTTCATGCACTG-3'. PCR products were cloned in the pCR2.1 vector (Invitrogen, U.S.A.) and identified by sequencing with ABI 373A DNA sequencer (Perkin Elmer, Japan). Total RNA of BAT was isolated by TRIzol (Gibco BRL, Japan), separated on a 1% agarose/formaldehyde gel and transferred to and fixed on a Hybond N nylon membrane (Amersham, Japan). The cDNA probes described above

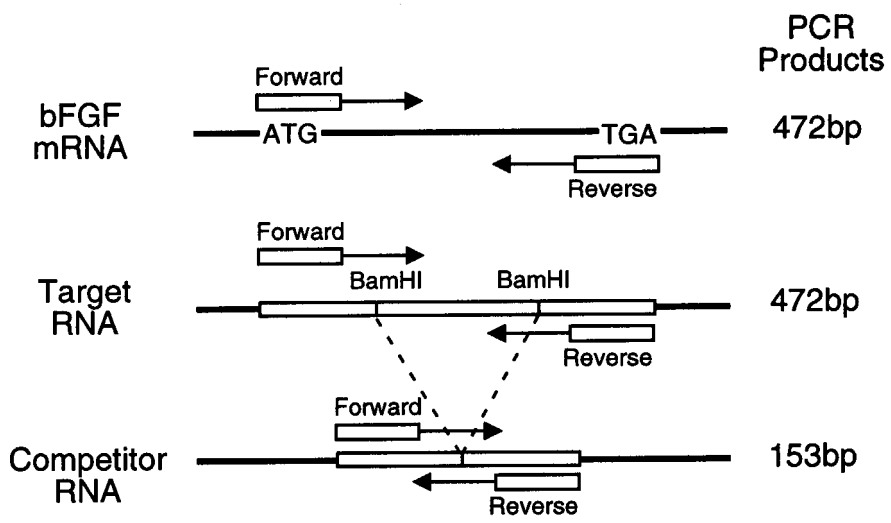


Fig. 1. bFGF mRNA, target RNA and competitor RNA, and their expected PCR products. The upper panel indicates the binding sites of the primers in rat bFGF mRNA and the expected size of PCR product (472 bp). The middle and lower panels represent the construction of synthesized standard target and competitor RNA and their expected PCR products, respectively. The PCR product from competitor RNA (153 bp), which lacks the fragment digested by *Bam*HI, is shorter than those from bFGF mRNA and standard target RNA (472 bp).

were labeled with [α-^{32}P] dCTP using multiprime DNA labeling kit (Amersham). The blots were hybridized on to the labeled probes at 42˚C for 24 hr in a hybridization buffer (50% formamide, 5X SSPE, 0.1% SDS, 0.2 mg/m*l* salmon sperm DNA), washed twice at room temperature for 15 min with 2X SSC/0.1% SDS and subsequently at 60˚C for 15 min with 0.1X SSC/0.1% SDS, and then analyzed with a BAS1000 bioimage analyzer (Fuji film, Japan).

Competitive RT-PCR analysis of bFGF mRNA: Rat bFGF cDNA corresponding to nucleotides 533–1000 [17] was cloned by RT-PCR with the primer set of 5'-ATGGCTGCCGGCAGCATCACTT-3' (forward) and 5'-GAGTCAGCTCTTAGCAGACATT-3' (reverse) into *Eco*RI site of pBluescript KS+ vector. Standard target RNA was synthesized by T3 RNA polymerase (Gibco BRL) with this clone digested by *Xba*I as a template DNA (Fig. 1). RT-PCR product corresponds in size (472 bp) to that expected for rat bFGF mRNA. Competitor cDNA template was generated by cutting off the *Bam*HI site (nucleotides 615–934) of rat bFGF cDNA cloned into pBluescript KS+ vector lacking *Bam*HI site. Competitor RNA fragment was synthesized by T7 RNA polymerase (Gibco BRL) with competitor plasmid DNA digested by *Hind*III. RT-PCR product from competitor RNA (153 bp) is smaller than that from target (472 bp). For competitive RT-PCR analysis, BAT total RNA (1 μg) or standard target RNA (2–50 *p*g) with competitor RNA (0.1 *p*g) were incubated at 80˚C for 5 min, cooled immediately, and reverse transcribed by 60 U M-MLV reverse transcriptase (Gibco BRL), 50 pmoles of reverse primer and 20 nmoles of dNTP in a total volume of 10 μ*l* at 37˚C for 1 hr. After heating to 94˚C for 5 min, PCR amplification was performed by 0.5 U Taq DNA polymerase (Takara, Japan) and 50 pmoles of forward primer in a total volume of 50 μ*l*. PCR cycles were as follows: 94˚C 3 min, 56˚C 2 min, 72˚C 3 min (1 cycle), 94˚C 1 min, 56˚C 2 min, 72˚C 3 min (30 cycles) and 94˚C 1 min, 56˚C 2 min, 72˚C 10 min (1 cycle). Nucleotide sequence of the PCR product (472 bp) was analyzed and confirmed to be identical to that of bFGF cDNA. PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The density of each band was quantified using NIH Image analysis software.

Data analysis: All values are presented as means ± SE. Statistical analysis was performed by analysis of variance with post-hoc testing by Fisher's PSLD multiple range test.

RESULTS

High expression of VEGF and VEGF-B mRNAs in BAT: We reported previously a high expression level of VEGF mRNA in BAT of rats [1]. To determine the mRNA levels of other possible angiogenic factors, we performed Northern blot analysis of total RNA extracted from the interscapular BAT using the hybridization probes of VEGF, VEGF-B, VEGF-C and bFGF. As shown in Fig. 2, high expression of VEGF mRNA was observed in lung, heart and BAT. VEGF-B was also highly expressed in BAT, heart and spleen, but much less in other tissues. In contrast, VEGF-C and bFGF mRNAs were not detected in the same blots (data not shown).

VEGF and VEGF-B mRNA levels in BAT after cold exposure: The mRNA levels of VEGF and VEGF-B were measured in BAT 1 hr-10 days after rats were exposed to

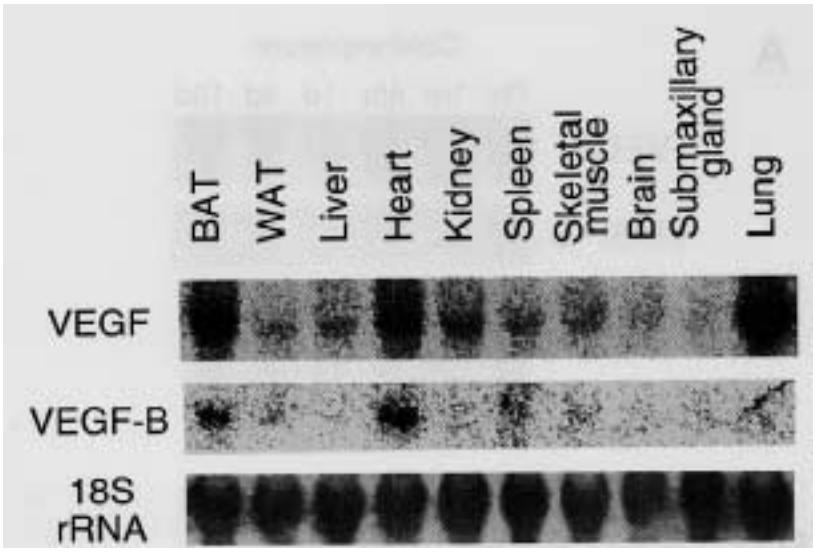


Fig. 2. Northern blot analysis of VEGF and VEGF-B mRNAs in various tissues of the rat. Thirty μg of total RNA extracted from each tissue was subjected to Northern blot analysis. BAT, brown adipose tissue; WAT, retroperitoneal white adipose tissue.

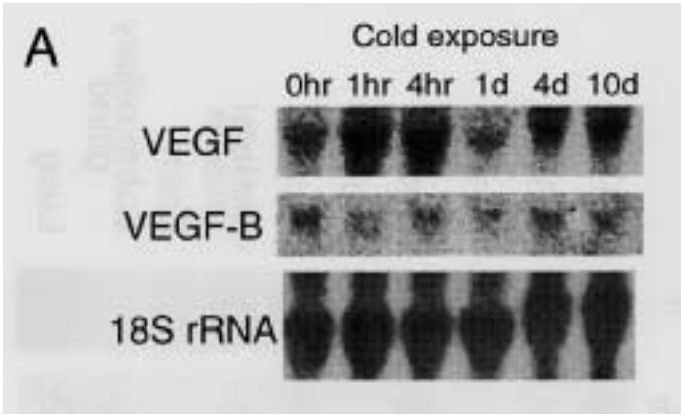
4˚C (Fig. 3). VEGF mRNA was transiently induced after 1 hr (× 2.3) and 4 hr (× 2.7), returned to a low level within 1 day and maintained at the basal level thereafter. In contrast, the VEGF-B mRNA level did not show any significant change during the course of cold exposure.

Competitive RT-PCR of bFGF: Although bFGF mRNA could not be detected in BAT by Northern hybridization, it was detected by applying the RT-PCR method to total RNA from BAT. Since the simple RT-PCR method is controversial for quantitation of the amount of bFGF mRNA, we tried to develop the competitive RT-PCR method using a competitor RNA which had the same primer binding sequences as bFGF mRNA as described in MATERIALS AND METHODS (Fig. 1). This was based on the widely accepted idea that the ratio of a target RNA to its competitor RNA would be equal to that of respective PCR products [2], and thus the competitor RNA can be used as an internal standard. As expected, a linear relationship was confirmed between the amount of synthesized target RNA (bFGF RNA) and the ratio of PCR products (Fig. 4). Then using this method, we quantified the bFGF mRNA level in BAT, and examined the effects of cold exposure. As shown in Fig. 5, the bFGF mRNA level was rapidly increased by 2.3-fold in 4 hr, decreased to a lower level in 1 day, and maintained at the basal level thereafter.

DISCUSSION

In adult mammals, physiological hyperplasia and angiogenesis are seen in restricted regions. Besides the gonadal tissue in estrous cycle, BAT is a typical organ that shows rapid tissue hyperplasia and angiogenesis under normal physiological conditions [3, 7]. In a previous report, we demonstrated abundant expression of VEGF in BAT and its likely role in angiogenesis associated with cold-induced hyperplasia in this tissue [1]. In the present study, we found that VEGF-B, a recently cloned VEGF homolog, was also expressed in BAT at a comparable level to those in heart and spleen. Since VEGF-B is known to have mitogenic activity of vascular endothelial cells [15], this homolog may also play a significant role in angiogenesis and/or tissue growth in BAT. However, unlike VEGF, VEGF-B mRNA expression in BAT did not change in response to cold exposure, which is the most effective physiological stimulus to induce BAT growth and angiogenesis. These results suggest a minor role of VEGF-B in the cold-induced angiogenesis. It is to be noted, however, this does not necessarily mean that VEGF-B is less important than VEGF in angiogenesis under other conditions such as embryonic development, although little information is available to date. The different responses between VEGF and VEGF-B expression imply that the gene expression of these two molecules are differently regulated. Our previous results demonstrated that mRNA expression of VEGF in BAT was activated by sympathetic nerves, largely through the β-adrenergic action of noradrenaline [1]. Further studies are needed to investigate the regulatory mechanism and/or factor controlling VEGF-B in BAT.

bFGF has been known as a potent mitogen of various types of cells including vascular endothelial cells [4]. Moreover, this cytokine induces protease activation and chemotaxis of endothelial cells and formation of a capillary-like structure *in vitro*, all of which are thought to be essential for angiogenic process [9, 14]. Yamashita *et al.* [21] reported that primary cultured brown adipocytes synthesized and secreted a noticeable amount of bFGF to stimulate capillary growth *in vitro*. In this *in vivo* study, we could not detect bFGF mRNA in BAT by the conventional Northern



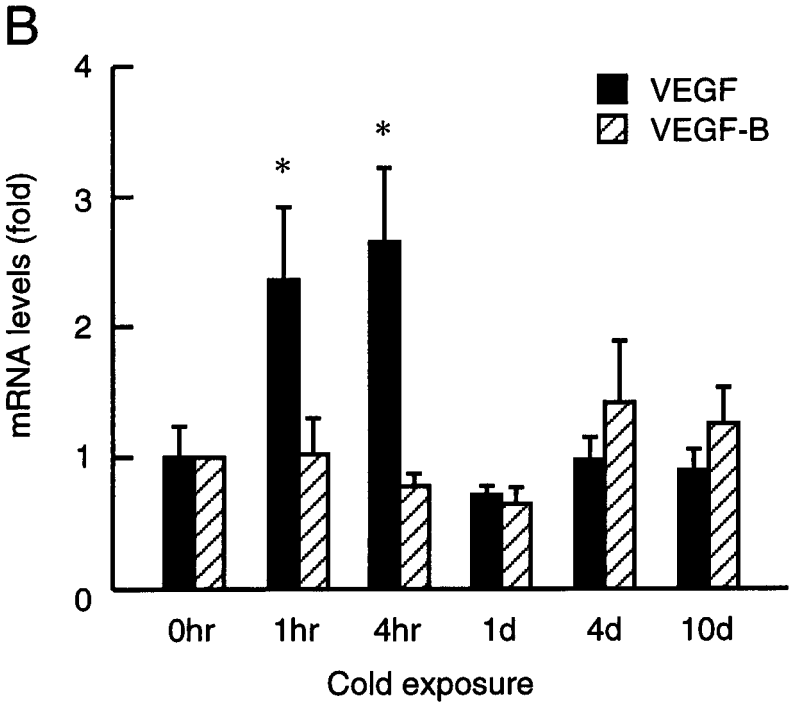


Fig. 3. Effect of cold exposure on the VEGF and VEGF-B mRNA levels in BAT. Rats were kept at 24˚C (0 hr) or exposed to cold at 4˚C for 1 hr-10 days, and 30 μg of total RNA extracted from the interscapular BAT was analyzed. A: Typical autoradiograms of VEGF and VEGF-B mRNAs and 18S rRNA. B: The blots of these mRNAs were quantified with a bioimage analyzer and normalized to 18S rRNA blots. The values are expressed relative to the 0-hr values, and are means ± SE for 4 rats. *$p<0.05$ vs 0 hr.

blot analysis, but confirmed its expression by the more sensitive RT-PCR analysis. In order to measure such low levels of bFGF mRNA quantitatively, we established a competitive RT-PCR method using an appropriate competitor RNA as an internal standard. Quantitative analysis of bFGF mRNA by this method revealed that its level in BAT increased rapidly and transiently after cold exposure. The bFGF mRNA response was almost similar to those of VEGF mRNA. *In vitro* studies have demonstrated adrenergic activation of bFGF expression in brown preadipocytes [21]. As mentioned above, mRNA expression of various genes including VEGF in BAT is under direct control of sympathetic nerves, which are activated by cold exposure. Collectively, it is likely that bFGF, in cooperation with VEGF, is also involved in the cold-induced angiogenesis in BAT. In fact, there have been several reports showing synergistic action of bFGF and VEGF to endothelial cell proliferation and capillary

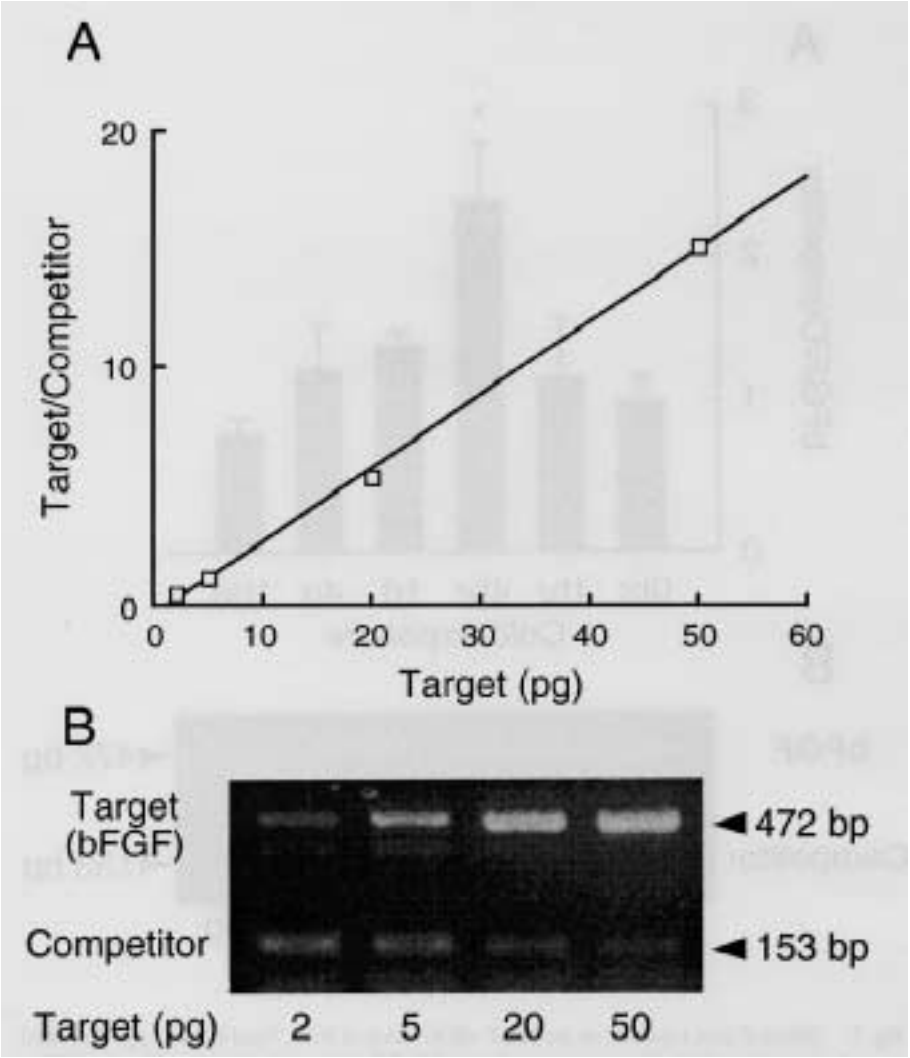


Fig. 4. Competitive RT-PCR method for quantitation of bFGF mRNA. Synthesized standard target RNA (2–50 *p*g) and competitor RNA (0.1 *p*g) were subjected to RT-PCR. A: A standard curve of the competitive RT-PCR. The band intensity in B was quantified with NIH image analysis software and the ratio of standard target RNA to competitor RNA was calculated. B: Typical ethidium bromide staining image of PCR products.

formation *in vitro* [8, 16]. Possible cooperative role of VEGF and bFGF is also suggested from the almost similar time-course of mRNA expression of these growth factors after cold exposure. It should be noted again that VEGF and bFGF mRNA were induced 1–4 hr after cold exposure, earlier than the proliferation of vascular endothelial cells in BAT, which was become apparent in 1 day [3]. To confirm the involvement of bFGF and VEGF in cold-induced angiogenesis in BAT, further studies are needed including the measurement of protein level of these factors, as well as of their mRNA level.

In summary, the present results show rapid and transient induction of VEGF and bFGF mRNAs expression in BAT by cold exposure, and suggest their role in the cold-induced angiogenesis in this tissue.

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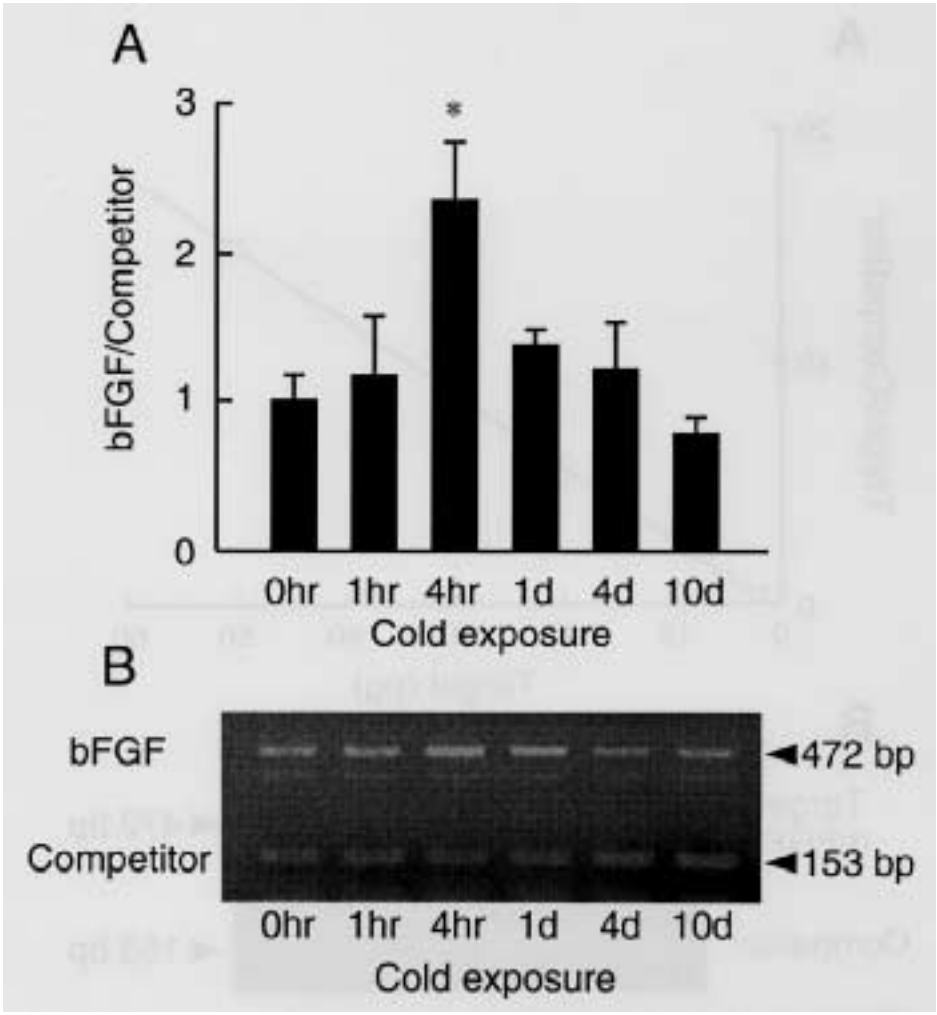

Fig. 5. Effect of cold exposure on the bFGF mRNA level in BAT. Total RNA (1 μg) from BAT and competitor RNA (0.1 *p*g) were subjected in RT-PCR, and the ratio of target PCR to competitor PCR products was calculated as in Fig. 4. In a graph of the time course of the bFGF mRNA levels (A), the values are expressed relative to the 0-hr values, and are means ± SE for 4 rats. *P<0.05 versus 0 hr. B: Typical ethidium bromide staining image of PCR products.

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