Alteration of Leptin-Induced STAT3 Activation in the Brain of Senescence-Accelerated Mouse (SAM) P8

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We used senescence-accelerated mouse prone 8 (SAMP8), a useful model of accelerated aging, to investigate the responsiveness to leptin with aging. The state of leptin-induced STAT3 phosphorylation in the hypothalamus was found to be higher in SAMP8 than in SAMR1, a control mouse showing normal aging, at 14—18 months of age but not at 2 months of age. Moreover, leptin receptor Ob-Rb expression in the hypothalamus was up-regulated in SAMP8. The results indicate that leptin sensitivity increases with aging in the SAM mouse brain.

Key words senescence-accelerated mouse (SAM); SAM prone 8 (SAMP8); leptin; aging; STAT3

Leptin, a 16-kDa protein encoded by the *ob* gene,[1] is known to be an important regulator of energy balance through its actions in the brain on appetite and energy expenditure.[2,3] Leptin is mainly secreted by adipose tissue and is released into circulation to act both in the peripherally and the brain.[4—6] Leptin receptors (Ob-R) are found in many tissues in several alternatively spliced forms such as Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re.[7—9] Ob-Rb, one form of the receptor, has a long cytoplasmic region and activates JAK tyrosine kinase and STAT3, which is responsible for leptin signaling.[6,10—14]

Recent studies using rat models have suggested that aging causes leptin resistance in the brain.[15—20] However, the correlation between aging and leptin resistance has not been elucidated. The senescence-accelerated mouse (SAM) has been established as a murine model for accelerated aging, and SAM shows various age-associated disorders as observed in humans.[21,22] SAM consists of senescence-accelerated prone mouse (SAMP) and senescence-accelerated resistant mouse (SAMR), the latter showing normal aging characteristics.[23] Thus, in the present study, we used SAM to investigate the responsiveness to leptin with aging in the central nervous system (CNS).

SAMR1 (SAMR1/TA) and SAMP8 (SAMP8/Ta) strains were originally obtained from Takeda Chemical Industries, Ltd. (Osaka, Japan). Mice were maintained in a conventional room at 22—24 °C under a constant day–night rhythm and given food and water *ad libitum*. At 14—18 months of age, the weight of SAMP8 were significantly lower than that of SAMR1 (31.3 ± 0.8 g *versus* 40.0 ± 1.1 g). All animal experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Hokkaido University.

Murine leptin (Pepro Tech, London) was dissolved in saline, and all injections were administered intravenously *via* the tail vein and delivered at an injection volume of 5 ml/kg. Mice were sacrificed by decapitation and the brains were quickly removed. The hypothalamus of each brain was rapidly dissected out on an ice-cold plate. Then the samples were snap-frozen in liquid nitrogen and stored at −80 °C. For Western blotting, tissue samples were sonicated in a buffer containing 10 mM HEPES–NaOH (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF and 1% NP-40 for 30 s. The samples were centrifuged at 30000 ***g*** for 30 min at 4 °C, and the supernatants were collected. The samples were boiled with Laemmli buffer for 3 min, and total protein was fractionated by 8% SDS-PAGE and transferred to nitrocellulose membranes at 4 °C. After having been blocked with TBST (20 mM Tris–HCl (pH 7.6), 137 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk for 3 h at room temperature, the membranes were incubated with phospho-specific STAT3 (Tyr 705) antibody (Cell Signaling Technology, Inc.; diluted to 1 : 1000 in TBST containing 5% BSA) at 4 °C overnight. The filter was then washed with TBST and incubated with horseradish peroxidase-labeled anti-IgG antibody (Cell Signaling Technology, Inc.; diluted to 1 : 2000 in TBST containing 5% skimmed milk) at room temperature for 1 h. After washing with TBST, HRP-labeled antibodies were detected by chemiluminescence (Amersham).

RT-PCR was done as described previously.[24] Total RNA was isolated using TRI REAGENT™ (SIGMA). cDNA was synthesized from 2 μg of total RNA by reverse transcription using 100 U of Superscript Reverse Transcriptase (GIBCO BRL) and Oligo $(dt)_{12—18}$ primer (GIBCO BRL) in a 20-μl reaction mixture containing 1×Superscript buffer (GIBCO BRL), 1 mM dNTP mix, 10 mM DTT, and 40 U of RNase inhibitor. Total RNA and Oligo $(dt)_{12—18}$ primer were incubated at 70 °C for 10 min prior to the reverse transcription. After incubation for 1 h at 42 °C, the reaction was terminated by denaturing the enzyme for 15 min at 70 °C. For PCR amplification, 1.2 μl of cDNA was added to 12 μl of a reaction mixture containing 0.2 μM of each primer, 0.2 mM of dNTP mix, 0.6 U of Taq polymerase, and 1×reaction buffer. PCR was performed in a DNA Thermal Cycler (Perkin-Elmer 2400-R). The primers used were as follows: Ob-Ra and Ob-Rb common upstream, 5′-aca ctg tta att tca cac cag ag-3′; Ob-Ra downstream, 5′-agt cat tca aac cat agt tta gg-3′; Ob-Rb downstream, 5′- tgg ata aac cct tgc tct tca-3′; GAPDH upstream, 5′-aaa ccc atc acc atc ttc cag-3′; GAPDH downstream, 5′-agg ggc cat cca cag tct tct-3′. The PCR products (10 μl) were resolved by electrophoresis in an 8% polyacryl-

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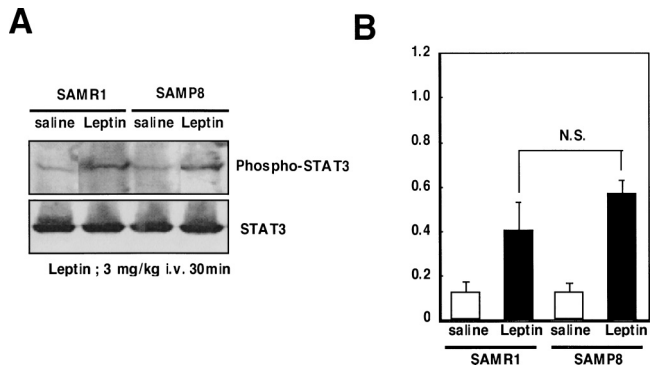

Fig. 1. Leptin-Induced STAT3 Phosphorylation in 2-Month-Old SAMR1 and SAMP8

(A) Leptin (3 mg/kg, i.v., 30 min) was administered to SAMP8 and SAMR1, and hypothalami were removed. Western blotting was performed using a phospho-STAT3 specific antibody. (B) Amounts of phospho-STAT3 are expressed as ratios of densitometric measurements of the samples to the STAT3 internal standard. No significant difference was found between leptin-induced STAT3 phosphorylation in SAMR1 and that in SAMP8. Values are presented as means±S.E. (n=6 per group).

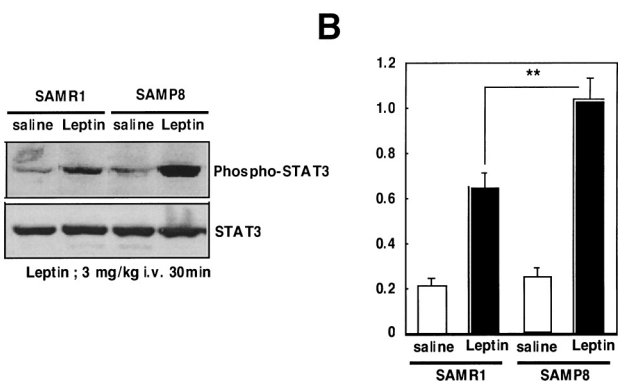

Fig. 2. Leptin-Induced STAT3 Phosphorylation in 14—18-Month-Old SAMR1 and SAMP8

(A) Leptin (3 mg/kg, i.v., 30 min) was administered to SAMP8 and SAMR1, and hypothalami were removed. Western blotting was performed using a phospho-STAT3 specific antibody. (B) Amounts of phospho-STAT3 are expressed as ratios of densitometric measurements of the samples to the STAT3 internal standard. A significant difference was found between leptin-induced STAT3 phosphorylation in SAMR1 and that in SAMP8. Values are presented as means±S.E. (n=6 per group). **p<0.01 (statistically significant difference between SAMR1 and SAMP8).

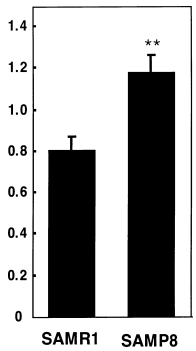

Fig. 3. Ob-Rb Leptin Receptor mRNA Expression in SAMR1 and SAMP8

Hypothalami were obtained from 17—20-month-old SAMP8 and SAMR1, and RT-PCR was performed. Amounts of Ob-Rb mRNA are expressed as ratios of densitometric measurements of the samples relative to the corresponding GAPDH internal standard. Values are presented as means±S.E. (n=5 per group). **p<0.01 (statistically significant difference between SAMR1 and SAMP8).

amide gel in 1×TBE buffer. The gel was stained with ethidium bromide, and the gels were photographed under ultraviolet light. Band densities were obtained using NIH Image 1.61 software. Results are expressed as means±S.E. Statistical analysis was performed using Student's t -test.

Leptin (3 mg/kg, i.v., 30 min) was administered to SAMP8 or SAMR1, and the level of STAT3 phosphorylation in the hypothalamus was measured. Up-regulation of STAT3 phosphorylation in SAM was observed (Figs. 1, 2). Leptin-induced STAT3 phosphorylation in SAMR1 and that in SAMP8 at 2 months of age were compared. As shown in Fig. 1, a significant difference was not found between of leptin-induced STAT3 phosphorylation in SAMR1 and that in SAMP8. However, in mice aged 14—18 months, significant up-regulation of STAT3 phosphorylation was observed in SAMP8 compared with that in SAMR1. Thus, leptin-induced STAT3 phosphorylation is up-regulated with aging in SAM. We next compared the expression levels of Ob-Rb leptin receptor. Hypothalami were obtained from 17—20-month-old SAMP8 and SAMR1, and RT-PCR analysis was performed. As shown in Fig. 3, significant up-regulation (1.5 fold) of Ob-Rb was observed in SAMP8 compared with that in SAMR1.

In this study, we investigated the effect of leptin on the aging brain using SAM. The levels of STAT3 phosphorylation at 2 months of age were not significantly different in SAMP8 and SAMR1. However, at 14—18 months of age, leptin-induced STAT3 phosphorylation was significantly higher in SAMP8 than in SAMR1. Thus, the results indicate that responsiveness to leptin is greater in SAMP8 than in SAMR1. The results suggest that leptin resistance does not occur with aging, but that, rather, leptin sensitivity is increased with aging. Body weights of SAMP8 were less than those of SAMR1. Thus, responsiveness to leptin corresponds with weight loss in SAM. It has been reported that leptin signal transduction in the CNS becomes impaired with age-related obesity in F-344xBN rats[16—18] and in Wister rats.[15] The reason for the greater responsiveness to leptin in SAMP8 is not known. However, rat models were used in previous studies and a mouse model was used in the present study, and it is therefore possible that these discrepancies may be due to species difference or the difference in animal models used. It is unknown whether leptin sensitivity will change with aging in SAMR1 mice and in normal mice, therefore, it is important to investigate these issues in the future. In this context, it is important subject to investigate aging and leptin resistance at the level of human subjects in the future. It has been reported that Ob-Rb leptin receptor mRNA is downregulated by aging in Wister rats.[15] However, in the present animal model, we observed significant up-regulation of Ob-Rb leptin receptor expression in SAMP8 compared with that in SAMR1. Stimulation of Ob-Rb leptin receptor activates STAT3 tyrosine kinase, which is responsible for leptin signaling.[6,10—14] Thus, it is possible that increased activation of leptin-induced STAT3 in SAMP8 may be due to up-regulation of Ob-Rb leptin receptor. Leptin has been shown to enter the brain through the blood–brain barrier (BBB) by a saturable transport mechanism.[25,26] Mice with diet-induced obesity exhibited resistance to peripherally administered leptin while retaining sensitivity to centrally administered leptin, indicating the possibility of impaired transport of leptin across the BBB.[27,28] On the other hand, BBB

function in SAMP8 was more deteriorated than that in SAMR1.[21] Thus, it is possible that increased activation of STAT3 in SAMP8 compared with that in SAMR1 is because of a difference in BBB permeability. The present findings would provide important aspect of leptin signaling in the aged brain.

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