# Screening of Microbial Products Modifying the Action of Leptin (Obese Gene Product) by a Biosensor

Sir:

Leptin is the *ob* gene product synthesized in adipose tissue and plays an important role in body weight homeostasis and reproduction[1,2]. It acts by interacting with the receptor in hypothalamus known to be important for determining feeding behavior. Leptin receptor is abundantly expressed in hypothalamus. Administration of recombinant leptin to *ob*/*ob* (genetically obese and leptin deficient) or normal mice significantly suppresses food intake, body weight gain, and increase of energy expenditure[3,4]. These responses are accompanied by changes of several genes expression in the hypothalamus[5,6]. However the mechanisms by which leptin acts are still unknown and useful agonists/antagonists against leptin are not available.

In the purpose of getting tools for understanding leptin's biological actions, low molecular weight microbial metabolites, which interact with leptin, were screened using a BIAcore instrument (BIAcore1.000, Biacore), the surface plasmon resonance sensor. The principle of the BIAcore system is that one molecule involved in the interaction to be studied is covalently immobilized to a sensor chip, and the other interactant (analyte) is then passed across the sensor chip. The detection system measures and records a signal proportional to the mass of the analyte bound to the surface. In this way, the association phase can be visualized in real time as the analyte-containing solution flows over the surface, and the subsequent dissociation is similarly displayed after the flow switches back to buffer containing no analyte.

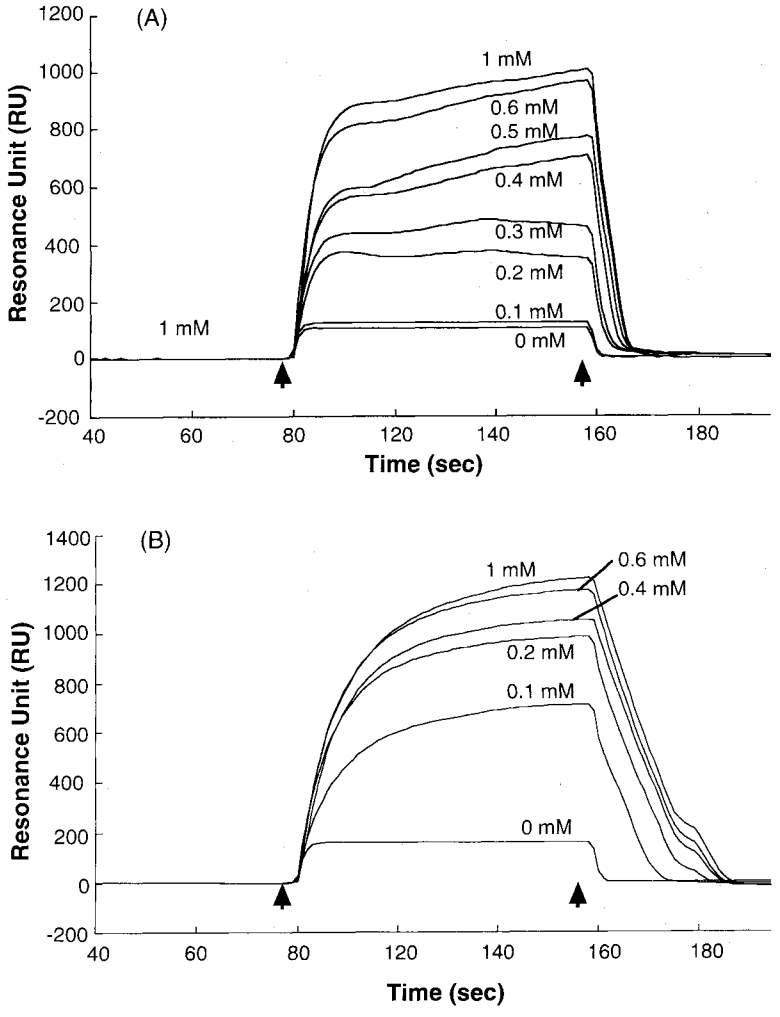
Mouse recombinant leptin (R & D systems, USA) was dissolved in coupling buffer (10 mM sodium acetate, pH 4.0) at a concentration of 62.5 mg/ml and was immobilized on the surface of CM5 sensor ship (Amersham Pharmacia Biotech) using the amine coupling method described in the BIAcore manual. Immobilized leptin with approximately 3400 RU was defined which corresponded to 4.5 ng protein. An analyte (99% HBS buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20), 1% MeOH solution of the test sample) was injected into the sensor chip in 10 $\mu$l quantity, and flow rate was set to 10 $\mu$l/minute with HBS buffer as the control flow solution. The sensorgrams were collected at 20°C. Screening samples were prepared by extraction of mycelial cakes of various microorganisms with acetone, and each extract was evaporated to dryness and dissolved in MeOH. The sensorgram was evaluated using BIA evaluation 3.0 software.

As a result of this screening, a fungus identified as *Chaetomium* sp. HB10 was found to produce an active substance. This strain was cultivated at 27°C for 14 days in 500-ml Erlenmeyer flasks each containing a medium consisting of 9 g of oatmeal and 30 ml of V8 juice. The mycelial cake from 10 cultured flasks was extracted with acetone. The extract was concentrated *in vacuo* and the residual aqueous suspension was extracted with EtOAc. The organic layer was evaporated and the residue was subjected to silica gel column chromatography packed with *n*-hexane. After washing with *n*-hexane and *n*-hexane - EtOAc (9 : 1), the column was developed with *n*-hexane - EtOAc (4 : 1). The combined active fraction was evaporated to dryness to yield a brown oil (550 mg). Based on the $^{1}H$ and $^{13}C$ NMR and FAB-MAS spectra, the active substance was identified as linoleic acid.

Then the interaction between leptin and authentic linoleic acid was analyzed. As shown in Fig. 1 (A), dose-dependent interaction was observed, and the association rate constant ($ka = 6.8 \times 104\,s^{-1}\,M^{-1}$) and the dissociation rate constant ($kd = 1.0 \times 10^{-2}\,s^{-1}$) were determined by the evaluation of the sensorgram. The high kd implied the rapid dissociation of linoleic acid from immobilized leptin. Since leptin (0.35 mM) injected together with linoleic acid completely inhibited the interaction between linoleic acid (1 mM) and immobilized leptin, the binding of linoleic acid to leptin appeared to be specific (data not shown). Although serum albumin is known to bind to free fatty acids (FFAs) in plasma, bovine serum albumin (BSA) did not work as a specific competitor to the interaction at the same concentration (data not shown).

Next we investigated the interaction between leptin and other fatty acids such as stearic acid, oleic acid, arachidonic acid and palmitic acid existing in animal plasma. Oleic acid showed the strongest affinity to the immobilized leptin among them (Fig. 1 (B)). The order of the affinity is oleic acid, 18: 1n-9 > linoleic acid, 18: 2n-6, arachidonic acid, 20: 4n-6 $\gg$ $\gamma$-linolenic acid, 18: 3n-6. However saturated fatty acids such as stearic acid, 18: 0 and palmitic acid, 16: 0 were inactive at the same concentration. It was also shown that methyl linoleate had less affinity to leptin than free linoleic acid (Fig. 2). Thus we conclude that the double bond and the carboxyl group of fatty acids are essential for the binding affinity.

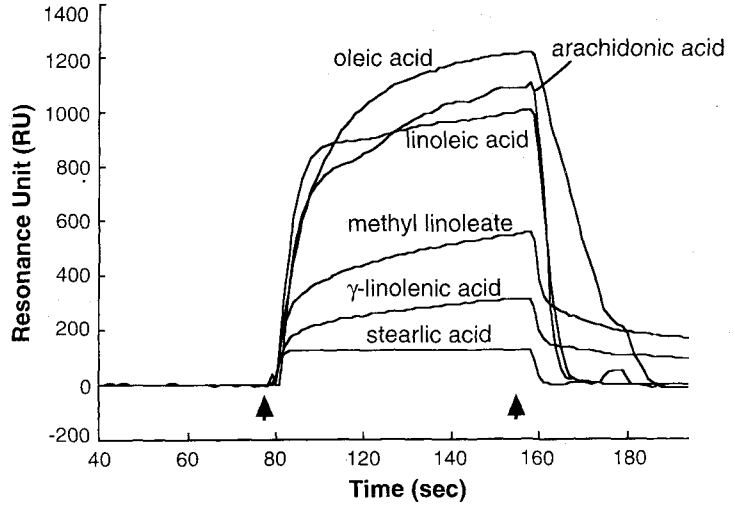
Fig. 1. Interaction of linoleic acid and oleic acid to immobilized leptin.




Linoleic acid (A) or oleic acid (B) was injected over leptin immobilized in the sensor chip at different concentrations (0~1.0 mM) at a flow rate of 10 $\mu$l/minute. Arrowheads indicate the initiation and termination of the injection.

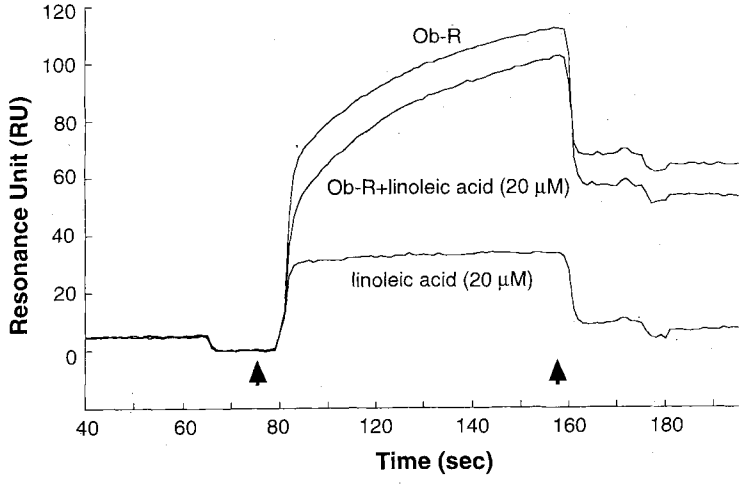
Then we investigated whether the leptin-FFA interaction has influences on the binding of leptin to its receptor. Rat leptin receptor cDNA coding the extra-membrane domain (nucleotide 1 to 2490) was reverse-transcribed from rat brain mRNA and amplified by the polymerase chain reaction. The cDNA was subcloned into the pET32b(+) expression vector (Novagen) and over-expressed in *E. coli* (AD494)pLys. After the expression, the leptin receptor fragment was purified using a commercial His-Tag affinity purification kit (Ni-NTA resin (QIAGEN, Germany)) according to its manual followed by cleavage of His-Tag and thioredoxin-Tag by thrombin. The recombinant protein was purified and dialyzed into phosphate buffered saline (PBS) (pH 7.4). Interaction of the recombinant receptor to the immobilized leptin was observed by the sensorgram and the association and dissociation rate constants for the interaction were calculated to be approximately $10^{-3}\,s^{-1}$ and $10^{-5}\,M^{-1}\,s^{-1}$, respectively, in agreement with reported values[7]. Linoleic acid (20 $\mu$M) injected simultaneously with the receptor (250 nM) showed decreased binding to the immobilized leptin (Fig. 3). Oleic acid also exhibited almost the same effect (data not shown). Physiological dose (100 $\mu$M) of BSA did not exert any effect on the inhibition (data not shown). Thus we have demonstrated for the first time that FFAs, which usually

Fig. 2. Interaction of various fatty acids to immobilized leptin.




Various fatty acids (1.0 mM) were injected over leptin surface at a flow rate of 10 $\mu$l/minute. Arrowheads indicate the initiation and termination of the injection.

Fig. 3. Effect of linoleic acid on the interaction between soluble leptin receptor and immobilized leptin.




Recombinant leptin receptor (Ob-R, 250 nM) was injected over leptin surface with or without linoleic acid (20 $\mu$M) at a flow rate of 10 $\mu$l/minute. Arrowheads indicate the initiation and termination of the injection.

exist in plasma of animals, specifically interfere the interaction between endocrine hormone and its receptor at concentrations less than their plasma concentrations.

Recently, CAMPBELL *et al.* described the interaction of radiolabelled FFA and human leptin[8]. However, the binding feature seemed not to be a one-to-one interaction and was co-operative in contradiction to our present results. The association curves from the BIAcore experiments analyzing the interaction between leptin and oleic acid or linoleic acid fit a single site model in BIA evaluation 3.0 software, indicating one-to-one interaction. This conflict may be caused by the difference of the

techniques employed by us and his group. It is to be noted that our method using the BIAcore instrument is more sensitive and effective for observation of the real time dissociation.

It was reported that diet-induced obesity and subsequential high concentration of plasma FFAs caused resistance to leptin, especially in its peripheral administration[9~11]. In addition, obese humans with high plasma FFA concentration and plasma leptin also showed a decreased sensitivity to leptin[12]. Based on our present results, it may be concluded that one of the reasons of leptin-resistance and appetite promotion caused by FFAs is due to the inhibition of the interaction between leptin and its receptor, perhaps in hypothalamus, and also in the saturable transport system of the blood-brain barrier. Further studies are needed to disclose the detailed mechanisms.

It is yet unknown how leptin regulates the appetite and energy expenditure of animals through its receptor and whether leptin is an important factor of human obesity. Our present studies have disclosed possibilities that FFAs and their derivatives with binding activity to leptin may be utilized for investigation of leptin's physiological significance, and that antagonists of FFAs for the binding to leptin may be useful for treatment of leptin-resistance caused mainly by a high fat diet. Our screening system using the BIAcore system may be useful to discover new compounds with such interesting biological activity.

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