Central effects of leptin on cardiovascular and neurohormonal responses in conscious rabbits

KIYOSHI MATSUMURA, ISAO ABE, TAKUYA TSUCHIHASHI, AND MASATOSHI FUJISHIMA
Second Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812-8582, Japan

Matsumura, Kiyoshi, Isao Abe, Takuya Tsuchihashi, and Masatoshi Fujishima. Central effects of leptin on cardiovascular and neurohormonal responses in conscious rabbits. Am J Physiol Regulatory Integrative Comp Physiol 278: R1314–R1320, 2000.—We determined the cardiovascular and neurohormonal responses to intracerebroventricular injection of leptin in conscious rabbits. Intracerebroventricular injection of leptin elicited dose-related increases in mean arterial pressure and renal sympathetic nerve activity while producing no consistent, significant increases in heart rate. Peak values of mean arterial pressure and renal sympathetic nerve activity induced by intracerebroventricular injection of 50 µg of leptin (+ 17.3 ± 1.2 mmHg and + 47.9 ± 12.0%) were obtained at 10 and 20 min after injection, respectively. Plasma catecholamine concentrations significantly increased at 60 min after intracerebroventricular injection of leptin (control vs. 60 min; epinephrine: 33 ± 12 vs. 97 ± 27 pg/ml, P < 0.05; norepinephrine: 298 ± 39 vs. 503 ± 86 pg/ml, P < 0.05). Intracerebroventricular injection of leptin also caused significant increases in plasma vasopressin and glucose levels. However, pretreatment with intravenous injection of pentolinium (5 mg/kg), a ganglion blocking agent, abolished these cardiovascular and neurohormonal responses. On the other hand, intravenous injection of the same dose of leptin (50 µg) as used in the intracerebroventricular experiment failed to cause any cardiovascular and renal sympathetic nerve responses. These results suggest that intracerebroventricular injection of leptin acts in the central nervous system and activates sympathoadrenal outflow, resulting in increases in arterial pressure and plasma glucose levels in conscious rabbits.

catecholamines; central nervous system; glucose; renal sympathetic nerve activity

LEPTIN, the product of the ob gene, is a 16-kDa protein monomer that has been identified as a blood-borne factor regulating appetite (27, 30). Leptin is secreted by peripheral adipocytes in response to stimuli that include food intake and insulin administration and appears to serve as a signal of satiety (21). Various lines of evidence suggest that leptin participates not only in negative feedback control of body adiposity but in cardiovascular and sympathetic regulations (11, 16). Intravenous injection of leptin increased sympathetic nerve activity to the kidney, the adrenal, and the brown adipose tissue without significant change in blood pressure in anesthetized Sprague-Dawley rats (10). On the other hand, intracerebroventricular injection of leptin has been shown to increase lumbar and renal sympathetic nerve activity (RSNA), resulting in an increase in blood pressure in anesthetized Wistar rats (4). In addition, intracerebroventricular injection of leptin causes increases in arterial pressure and heart rate (HR) in food-deprived normotensive conscious rabbits (2). These previous studies suggest that leptin acts at the central nervous system to augment sympathoadrenal outflow and to increase blood pressure.

However, some previous studies were conducted on anesthetized rats (4, 10). Because the sympathetic nervous system and baroreceptor reflex are extremely influenced by anesthesia (12, 17), the responses of sympathetic nervous system and blood pressure might not have been assessed precisely. Furthermore, Dunbar et al. (4) demonstrated that intracerebroventricular injection of leptin increases sympathoadrenal outflow and blood pressure without significant change in plasma glucose. We anticipated that activation of sympathoadrenal outflow induced by intracerebroventricular injection of leptin might elicit an increase in plasma glucose level in conscious animals, although intracerebroventricular (14) or hypothalamic (20) injection of leptin has been shown to stimulate glucose uptake in various peripheral tissues, including skeletal muscle and brown adipose tissue. Moreover, we considered that central leptin might stimulate the secretion of vasopressin, because one of the putative brain regions where leptin acts is the hypothalamus (11, 23). We hypothesized that intracerebroventricular injection of leptin stimulates the secretion of vasopressin and activates sympathoadrenal outflow, resulting in an increase in plasma glucose levels in conscious animals. Thus the present study was designed particularly to investigate the central effect of leptin on blood pressure, sympathetic nervous system, and blood variables, including plasma catecholamines, vasopressin, and glucose levels in conscious rabbits with direct recording of the RSNA.

METHODS

Preparation of animals. The experiments were conducted on male Japanese White rabbits weighing 2.5–2.8 kg. All experiments were carried out according to the institutional guidelines for animal experimentation at Kyushu University. Rabbits were anesthetized with pentobarbital sodium (30 mg/kg iv). Three days before experimentation, bipolar electrodes were implanted on the left renal sympathetic nerve, the right femoral artery, and the right common carotid artery.
and a stainless steel cannula was placed in the right lateral cerebral ventricle. RSNA was recorded as described previously (18, 19). Briefly, under aseptic conditions, the left kidney was exposed retroperitoneally, and a branch of the renal nerve was separated from the renal plexus and the surrounding connective tissues with the use of a dissecting microscope. RSNA was recorded by a pair of electrodes made from Teflon-insulated seven-stranded steel wire (Medwire, Mt. Vernon, NY). The area of the nerve and wire interface was embedded in silicone cement (Elastisol RT 604A and B cement, Wacker Chemicals, München, Germany).

A 23-gauge stainless steel cannula was implanted into the right lateral cerebral ventricle, 4 mm lateral to the bregma and 6 mm below the cerebral surface. The position of the cannula in the lateral cerebral ventricle was confirmed by the staining of all four ventricles after injection of 0.1 ml dye at the end of the experiments. The cannula was fixed to the skull with three jeweler’s screws and dental cement. A 27-gauge obturator was used to seal the cannula. After surgery, disodium sublenticillin (200 mg iv) was given to the rabbits to prevent postoperative infections.

At least 3 days after the surgical procedures, the following experiments were carried out on conscious rabbits placed in the box, which was made to just fit the rabbits’ size. On the day of the experiment, polyethylene catheters (PE-50) were inserted into the central ear artery and marginal ear vein under 1% lidocaine local anesthesia. The arterial catheter was connected to a pressure transducer (model P50, Gould, Oxnard, CA) to measure arterial pressure. HR was monitored using a cardiotachometer (model 1332, NEC San-ei, Tokyo, J apan).

RSNA was amplified (model DPA-100E, Dia Medical Sys-
tem, Tokyo, J apan) and filtered (100–3000 Hz), and the wave-
forms were integrated after a full-wave rectification using an
integrator amplifier (model 1322, NEC San-e) with the
sample-hold function reset to baseline by an internal timer set at 5 s. The residual integrated RSNA that existed after
intravenous administration of hexamethonium bromide (30
mg/kg iv) was taken as the noise level associated with nerve
recording. This value was subtracted from absolute values of
integrated RSNA before performing further data analysis.

A thoracic-intracerebroventricular injection of 0.1 ml saline
was injected intracerebroventricularly (aCSF; in mM: 133.3
NaCl, 3.4 KCl, 1.3 CaCl2, 1.2 MgCl2, 0.6 NaH2PO4, 32.0
NaHCO3, and 3.4 glucose).

Relationship between dose of intracerebroventricular leptin and cardiovascular responses. To determine the dose of murine leptin (Pepro Tech, London, UK) needed to increase arterial pressure, 5, 10, and 50 µg of leptin were injected intracerebroventricularly in ascending concentration order (n = 5 for each). These doses of leptin were dissolved in 80 µl of aCSF. The administration of each dose of leptin was separated by 60 min. Data for mean arterial pressure (MAP), HR, and RSNA were collected at 5-min intervals, and peak responses of these variables were determined after injection of each dose of leptin.

Effect of pentolinium on cardiovascular responses induced by intracerebroventricular injection of leptin. After a control period, the rabbits were injected with pentolinium (Sigma Chemical, St. Louis, MO; 5 mg/kg in 0.3 ml/kg iv), a ganglion blocking agent. In our preliminary experiments, this dose of pentolinium has been confirmed to suppress RSNA completely until at least 70 min after injection in conscious rabbits. Five minutes later, a blood sample (3.0 ml) was drawn from the arterial catheter to measure plasma catecholamines (epinephrine and norepinephrine), plasma vasopressin, plasma glucose, plasma osmolality, and hematocrit. Ten minutes after intravenous injection of pentolinium, leptin (50 µg) was injected intracerebroventricularly (n = 5). An additional blood sample was drawn at 60 min after intracerebroventricular injection of leptin. The blood samples were replaced by the same volume of 0.9% saline. Arterial pressure, HR, and RSNA were monitored continuously.

Effect of intravenous injection of leptin on cardiovascular and sympathetic responses. To evaluate the leakage of intrace-
broventricular injection of leptin into the systemic circula-
tion, the same dose of leptin (50 µg) used in the intracerebro-
ventricular injection experiment was injected intravenously
(n = 5). Arterial pressure, HR, and RSNA were monitored continuously.

Blood collection and analysis. Blood samples for measure-
ment of plasma catecholamines and plasma vasopressin were
centrifuged at 4°C. Plasma for catecholamines was stored at
−80°C, and other plasma was stored at −20°C until assay.
The plasma catecholamine concentrations were measured by
high-performance liquid chromatography (29), and plasma
vasopressin levels were measured by radioimmunoassay (18,
19). The assay sensitivities for vasopressin and catechol-
amines (epinephrine and norepinephrine) were 0.45 and 10
pg/ml, respectively. Plasma glucose levels were measured by
a Glucose Analyzer 2 (Beckman Instruments, Fullerton, CA).
Plasma osmolality was measured with a freezing-point os-
ometer (Osmotron-20, Orion Riken, Tokyo, J apan).

Statistics. All values are expressed as means ± SE. To
determine the effects of intracerebroventricular and intrave-
nous injections of leptin on cardiovascular and RSNA re-
sponses, one-way ANOVA with repeated measurements was
performed, followed by Duncan’s multiple range test to
determine which means differ from the control means. Stud-
ent’s t-test was used to determine the effects of intracerebro-
ventricular injection of leptin on blood variables. A value of
P < 0.05 was considered significant.

RESULTS

Relationship between dose of intracerebroventricular leptin and cardiovascular responses. Table 1 summarizes the baseline values for MAP, HR, and RSNA before intracerebroventricular injections of aCSF and each dose of leptin.

Table 1. Baseline values for MAP, HR, and RSNA before intracerebroventricular injections of aCSF and each dose of leptin

<table>
<thead>
<tr>
<th>Drug</th>
<th>MAP, mmHg</th>
<th>HR, beats/min</th>
<th>RSNA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF</td>
<td>85.2 ± 5.0</td>
<td>224 ± 15.1</td>
<td>100.0 ± 0</td>
</tr>
<tr>
<td>Leptin</td>
<td>5 µg</td>
<td>85.6 ± 4.4</td>
<td>227.0 ± 20.6</td>
</tr>
<tr>
<td>10 µg</td>
<td>86.0 ± 3.6</td>
<td>223.0 ± 20.6</td>
<td>98.9 ± 2.8</td>
</tr>
<tr>
<td>50 µg</td>
<td>86.8 ± 4.4</td>
<td>231.0 ± 23.3</td>
<td>95.4 ± 4.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. MAP, mean arterial pressure; HR, heart rate; RSNA, renal sympathetic nerve activity; aCSF, artificial cerebrospinal fluid.
before intracerebroventricular injections of aCSF and each dose of leptin. Baseline values of these variables did not change significantly. Intracerebroventricular injection of leptin elicited dose-related increases in MAP, HR, and RSNA (Fig. 1). The results shown in Fig. 1 illustrate peak responses for MAP, HR, and RSNA obtained during 60-min recording period. It should be noted that although MAP and RSNA increased during the first 10–20 min after leptin injection, the increase in HR was delayed and inconsistent. As a result, intracerebroventricular leptin did not significantly increase HR for the 90-min recording period (see Fig. 3). Because 50 µg of leptin caused significant increases in MAP and RSNA, we used this dose of leptin in the following experiments.

Effect of intracerebroventricular injection of leptin on cardiovascular and neurohormonal responses. Figure 2 shows the typical responses of MAP, HR, and RSNA that were elicited by intracerebroventricular injection of leptin. Intracerebroventricular injection of 50 µg of leptin provoked increases in MAP and RSNA, and peak values of these variables were obtained after 10 and 20 min, respectively (Fig. 3). After peak values were obtained, MAP and RSNA decreased and returned to the baseline levels within 60–90 min. HR, however, did not show any significant changes. Table 2 shows the effects of intracerebroventricular injection of leptin on plasma catecholamine and vasopressin concentrations and other variables. Plasma epinephrine, norepinephrine, vasopressin, and glucose concentrations significantly increased at 60 min. Plasma osmolality and hematocrit did not show any changes.

Effect of pentolinium on cardiovascular and sympathetic responses induced by intracerebroventricular injection of leptin. After pentolinium administration, MAP fell from 91.2 ± 3.4 to 60.0 ± 1.3 mmHg and HR increased from 191.0 ± 19.3 to 218.0 ± 19.3 beats/min. However, intracerebroventricular injection of leptin failed to cause any further responses in MAP or HR, and RSNA was almost completely suppressed until 60 min after injection of leptin (Fig. 4). Table 3 shows the effects of pentolinium on blood variables induced by intracerebroventricular injection of leptin. Plasma epinephrine, norepinephrine, and glucose levels did not show any significant changes. Intravenous injection of pentolinium increased plasma vasopressin concentrations; however, intracerebroventricular injection of leptin failed to cause further increase in plasma vasopressin concentrations.

Effect of intravenous injection of leptin on cardiovascular and sympathetic responses. The same dose of leptin (50 µg) used in the intracerebroventricular experiment was injected intravenously. After intravenous injection of leptin, arterial pressure, HR, and RSNA remained within 5% of their control values.

DISCUSSION
The present study demonstrated that intracerebroventricular injection of leptin caused significant increases in arterial pressure, RSNA, and plasma catecholamine concentrations. Intravenous injection of pentolinium, a ganglion blocking agent, abolished the responses of arterial pressure and of plasma catecholamine concentrations. These results suggest that the pressor response induced by the intracerebroventricular injection of leptin can be attributed primarily to enhanced sympathetic outflow. Furthermore, intravenous injection of the same dose of leptin used in the intracerebroventricular injection experiment failed to cause any cardiovascular and sympathetic responses, suggesting that the responses induced by intracerebroventricular injection of leptin were not caused by a leakage of leptin into the systemic circulation. To the best of our knowledge, this is the first study to demonstrate the responses of RSNA, plasma catecholamines, vasopressin, and other blood variables to central administration of leptin in conscious animals.

The role of leptin in cardiovascular regulations seems to be complicated. There are several potential depressor actions of leptin, such as renal natriuresis (13), improved insulin sensitivity (26), and increased endothelial nitric oxide (15). In contrast, leptin has a potential pressor action in that it activates the sympathetic nervous system. Intravenous injection of leptin
has been shown to activate the sympathetic nervous system without a significant change in blood pressure (10), suggesting that potential depressor actions may prevent the pressor responses induced by sympathetic activation. In contrast, chronic intravenous or carotid artery infusion of leptin increases arterial pressure (24). The effects of leptin on arterial pressure may vary depending on whether administration is acute or chronic. In the present study, intracerebroventricular injection of leptin activated the sympathoadrenal outflow, resulting in an increase in blood pressure. These results are consistent with previous findings demonstrated by Dunbar et al. (4). Because intracerebroventricular injection of leptin does not have potential peripheral depressor actions, activation of sympathetic nervous system might elicit an increase in arterial pressure. In addition, Casto et al. (1, 2) also reported that intracerebroventricular injection of leptin caused more increase in arterial pressure in food-deprived normotensive and hypertensive rats than that in ad libitum-fed rats. Cardiovascular effects of central leptin might be more evident in the food-deprived condition.

Intracerebroventricular (14) or hypothalamic (20) injection of leptin has been shown to stimulate glucose uptake in various peripheral tissues, including skeletal muscle and brown adipose tissue. These studies suggest that central leptin potentially may decrease plasma glucose level, although it did not change after leptin administration (14, 20). In the present study, however, intracerebroventricular injection of leptin caused an increase in plasma glucose level. Hyperglycemia has been shown to be evoked by an increase in plasma epinephrine concentrations (3); thus this response of plasma glucose level was likely attributable to the increased plasma epinephrine concentrations. In contrast, Dunbar et al. (4) demonstrated that intracerebroventricular injection of leptin activates the sympathetic nervous system, although plasma glucose levels did not change in anesthetized Wistar rats. The advantage of the present study is that the experiments were conducted on conscious animals and plasma catecholamine concentrations were measured. Parallel changes of plasma glucose levels and plasma catecholamine concentrations suggest a relationship between these two variables. In addition, anesthesia extremely modulates sympathetic outflow (17), and these different responses of plasma glucose levels between the present and previous studies might be attributable to the state of consciousness of the animals used in the experiments.

Intracerebroventricular injection of leptin also elicited an increase in plasma vasopressin concentrations. In the present study, because neither plasma osmolality nor hematocrit changed after intracerebroventricular injection of leptin and because increased plasma levels of epinephrine and norepinephrine would be expected to cause an increase in venous return (6), the changes in the central venous pressure were not considered to stimulate the release of vasopressin. Leptin has been shown to act at the paraventricular nucleus of the hypothalamus (8) to alter the levels of mRNA for corticotrophin-releasing hormone (23) or to reduce food intake (28). Therefore, intracerebroventricular injection of leptin might directly stimulate the paraventricular nucleus of the hypothalamus, resulting in the
release of vasopressin into the systemic circulation. However, it seemed unlikely that this increase of vasopressin contributed to the increase in arterial pressure, because the change in plasma vasopressin concentration was slight. In the present study, intravenous injection of pentolinium markedly increased plasma vasopressin concentration. Then it decreased at 60 min after intracerebroventricular injection of leptin. Because a similar response of vasopressin has been observed after pentolinium administration in our previous experiment (19), this response might be explained by the effect of pentolinium by itself. Thus the effect of intracerebroventricular injection of leptin on plasma vasopressin concentration appeared to be masked by the effect of pentolinium.

Cardiovascular and sympathetic responses to leptin have been reported to be long lasting. Intravenous infusion of leptin slowly increased sympathetic nerve activity to the kidney, hindlimb, and adrenal gland without a significant change in arterial pressure (10). In addition, Casto et al. (2) reported that intracerebroventricular injection of leptin increased arterial pressure at 90 min after administration in food-deprived rabbits.

### Table 2. Effects of intracerebroventricular injection of 50 µg of leptin on blood variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Time, min</th>
<th>0</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine, pg/ml</td>
<td>33 ± 12</td>
<td>97 ± 27*</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine, pg/ml</td>
<td>298 ± 39</td>
<td>503 ± 86*</td>
<td></td>
</tr>
<tr>
<td>Vasopressin, pg/ml</td>
<td>1.08 ± 0.07</td>
<td>2.72 ± 0.30†</td>
<td></td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.349 ± 0.010</td>
<td>0.350 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.47 ± 0.32</td>
<td>7.71 ± 0.29†</td>
<td></td>
</tr>
<tr>
<td>Osmolarity, mosmol/l</td>
<td>286.5 ± 4.2</td>
<td>292.7 ± 6.0</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, †P < 0.01 compared with control (0 min) by paired t-test.

### Table 3. Effects of pentolinium on the responses of blood variables induced by intracerebroventricular injection of leptin

<table>
<thead>
<tr>
<th>Variables</th>
<th>Time, min</th>
<th>0</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine, pg/ml</td>
<td>14 ± 4</td>
<td>20 ± 6</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine, pg/ml</td>
<td>144 ± 97</td>
<td>182 ± 49</td>
<td></td>
</tr>
<tr>
<td>Vasopressin, pg/ml</td>
<td>85.6 ± 21.7</td>
<td>28.7 ± 11.2</td>
<td></td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.341 ± 0.012</td>
<td>0.340 ± 0.012</td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.78 ± 0.35</td>
<td>6.42 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>Osmolarity, mosmol/l</td>
<td>289.2 ± 3.0</td>
<td>291.6 ± 5.2</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE.
conscious Sprague-Dawley rats, although arterial pressure in ad libitum-fed rats did not change significantly. In the present study, however, intracerebroventricular injection of 50 µg of leptin provoked increases in MAP and RSNA, and peak values of these variables were obtained at 10 and 20 min, respectively. Then, MAP and RSNA decreased and returned to the baseline levels at 60–90 min. It seems difficult to interpret the time course difference between the present study and the previous ones; however, it may be explained by the experimental design, such as dose of leptin, route of administration, the species used in the experiments, and the state of consciousness of the animals. Rabbits may be resistant to increase arterial pressure compared with rats, because even intracerebroventricular infusion of angiotensin II caused only a small increase in MAP in conscious rabbits (7).

The present study did not provide the mechanisms of pressor response and activation of sympathetic nervous system induced by intracerebroventricular leptin. However, neurotransmitters, such as neuropeptide Y, corticotrophin-releasing factor, agouti-related protein, α-melanocyte-stimulating hormone (α-MSH), cocaine- and amphetamine-regulated transcript (CART), melanin concentrating hormone, and orexins, have been shown to be involved in feeding in the central nervous system and to interact with leptin (5, 22). Neurons in the medial part of the arcuate nucleus express both neuropeptide Y and agouti-related protein, and they are inhibited by leptin (5, 22). On the other hand, a separate population of neurons in the lateral arcuate nucleus expresses both α-MSH and CART, and these cells are activated by leptin (5, 22). Not only leptin but also neuropeptide Y and corticotrophin-releasing factor have been shown to influence sympathetic nerve activity in brown adipose tissue, thereby increasing thermogenesis (10, 11, 22). The interactions between leptin and these peptides in cardiovascular and sympathetic regulation remain to be investigated, although leptin increases RSNA through activation of hypothalamic melanocortin receptors (9). Further study is necessary to determine the interactions of these peptides in cardiovascular and sympathetic regulation.

The current study did not clarify the exact site where leptin acts in the central nervous system. However, the hypothalamus might be a candidate for the augmentation of the sympathetic nervous system and an increase in blood pressure, because leptin receptor immunoreactivity has been detected in the arcuate, paraventricular, and ventromedial nuclei of the hypothalamus and in the lateral hypothalamic area (25). In addition, in our recent experiments, microinjections of leptin (8 pmol/50 nl) into the nucleus of the solitary tract, rostral ventrolateral medulla, or area postrema failed to change arterial pressure in rats (unpublished observations), suggesting that the medulla oblongata does not play an important role in central cardiovascular regulation of leptin. A study focused on the microinjection of leptin into the hypothalamus will be necessary to clarify the role of leptin in the hypothalamus in cardiovascular and sympathetic regulations.

**Perspectives**

In summary, leptin exerts a central pressor action mediated primarily by enhanced sympathoadrenal outflow, and these effects are accompanied by increases in plasma vasopressin and glucose levels. Central leptin may interact with neurotransmitters, such as neuropeptide Y, corticotrophin-releasing factor, agouti-related protein, α-MSH, CART, melanin concentrating hormone, and orexins, to participate in cardiovascular and sympathetic regulation as well as in the regulation of appetite and energy expenditure, although physiological implications have not been determined. The hypothalamus, rather than the medulla oblongata, may be the putative brain region where leptin acts to regulate cardiovascular and sympathetic functions. However, further investigation is necessary to determine the exact site where leptin acts to augment the sympathoadrenal outflow in the central nervous system.

We thank M. Mihara and R. Imaizumi for expert technical assistance.

This work was supported in part by a grant from the Ministry of Education, J. an (No. 11670705) and a grant for research on sympathetic nervous system and hypertension from the Kimura Memorial Heart Foundation.

Address for reprint requests and other correspondence: K. Matsumura, Second Dept. of Internal Medicine, Faculty of Medicine, Kyushu Univ., Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan (E-mail: matsumura@ntmed2.med.kyushu-u.ac.jp).

Received 29 July 1999; accepted in final form 9 December 1999.

**REFERENCES**