CHAPTER 4. HIGH-FAT DIET-INDUCED BLOOD PRESSURE ELEVATION IN RATS RELATED TO METABOLIC, HORMONAL AND NEURAL ALTERATIONS IN RATS (Study 1)

The objectives of the Study 1 were the investigation of BP and SNA in FAT or CHO-fed rats. In addition, what kinds of alteration of metabolic, hormonal and neural pressor factors were aggregated in short-term or long-term FAT or CHO-fed rats.

Research methods and procedures

Animals and diets

Thirty-two male Sprague-Dawley rats aged 4 wk were purchased (Cler Japan, Tokyo, Japan) and housed individually in stainless steel cages in a room maintained at 23 ± 1 ℃ with a 12h-12h light-dark cycle. After one week of acclimatization, the rats were randomly divided into two dietary groups and given the experimental diets for 3 (FAT: n=8, CHO: n=8) and 16 wk (FAT: n=9, CHO: n=7). The diet compositions were shown in Table 1. FAT provided 45, 35 and 20% of energy as fat, carbohydrate and protein, respectively, and CHO provided 5, 75 and 20%, respectively. The rats were allowed free access to food and water during the experimental periods.

Blood pressure measurements

After acclimation to the tail cuff apparatus (BP-98A, Softron, Tokyo, Japan), BP was measured on conscious restrained animals by tail cuff methods. Thereafter, BP was recorded between 1700 and 2000 h after at least 5 h fasting. BP data were obtained by averaging six successive measurements on each rat.

Urinary catecholamines analysis

Urine was collected for catecholamines analysis over a period of 24 h. Five days before the collection of urine, the rats were moved to metabolic cages. Urine was collected in the presence of 2 ml of 2 N HCl, and pH was adjusted to between 2 and 3

before storage at -30 ℃. NE and Epi analysis was performed by liquid chromatography (LC-10AS, Shimadzu, Kyoto, Japan) with a degasser (DG-300, Eicom, Kyoto, Japan), a column heater (CTO-6A, Shimadzu, Kyoto, Japan), a reverse-phase column (CA-50ODS, Eicom, Kyoto, Japan) and electrochemical detection (ECD-100, Eicom, Kyoto, Japan). After adding 0.1M phosphate buffer (pH 8.5), urinary samples were added 3,4-dihydroxybenzylamine (10 μg; DHBA, Sigma-Aldrich Chemical, Tokyo, Japan) and activated alumina (50 mg; Wako, Oosaka, Japan). By the preliminary purification cation exchange resin, catecholamines were concentrated by passage over alumina. The mixed buffer was stirred for 5 min and centrifuged at 3,000 rpm for 3 min. After the supernatant was aspirated, catecholamines absorpted alumina were washed by distilled water. The catecholamines were released from the alumina by 2% acetic acid (200 μl). The solution was centrifuged at 5,000 rpm for 5 min and the supernatant was filtered by filter. NE and Epi were then analyzed using a reverse-phase column and an electrochemical detector with glassy carbon electrode.

Euglycemic clamp test

Twelve male Sprague-Dawley rats aged 3 wk were purchased (Cler Japan, Tokyo, Japan) and all of the experimental conditions were same as described above. This test was conducted only at 3 wk . The rats were fasted 5-8 h before the euglycemic clamp study. The rats were anesthetized for the test with an intraperitoneal injection of urethane (1.1 g/kg). Catheters were placed in the jugular vein for infusion of glucose, femoral vein for infusion of insulin and femoral artery for blood sampling, respectively. At least 30 min after the end of surgery, the initial blood sample (0.5 ml) was obtained. A continuous intravenous infusion of insulin was then started at a rate of 8.3 mU/kg/min and continued for 90 min. Glucose (20%) was infused at variable rates to maintain blood glucose at a steady state. Blood samples were taken at 10-min intervals for measurement of plasma glucose using a glucose analyzer (Dry-Chem 100, Fuji-film, Tokyo, Japan). Ninety minutes after the commencement of infusions, an additional 0.5 ml of blood was withdrawn for determination of plasma insulin levels. The data for SSPG and GIR were

shown by the averaged plasma glucose levels and GIR from 60 to 90 min, respectively. Then, the plasma insulin levels at 90 min were shown as SSPI.

Blood analysis and tissue samplings

Blood was collected by decapitation for analysis of glucose, insulin and leptin. Plasma glucose levels were determined using a glucose analytical kit by glucose oxidase method (Woko, Oosaka, Japan). Plasma insulin levels were determined using an ELISA kit (Morinaga Seikagaku, Kanagawa, Japan), and plasma leptin levels were determined using a Rat leptin RIA kit (IBL, Gunma, Japan). After decapitation, the retroperitoneal, mesenteric, epididymal and subcutaneous fat-pads and the hindlimb muscles were carefully dissected and weighed. The muscles were immediately frozen in liquid N_2, then kept frozen at -60 °C until the assay for IR contents.

Insulin receptor contents

IR content in soleus and gastrocnemius muscles was assayed by a modified method previously described by our laboratory (79). The frozen skeletal muscle tissues were powdered using a stainless steel mortar and pestle and homogenized for 45 s with a micro homogenizer (Physcotron NS-310E, Niti-on, Chiba, Japan) in ice-cold lysate buffer containing 50 mmol/l HEPES (pH 7.4), 100 mmol/l NaF, 10 mmol/l sodium pyrophosphate 4 mmol/l EDTA-2Na, 2 mmol/l sodium orthovanadate, 100 μg/ml aprotinin and 1% Triton X 100. The tissue homogenate was incubated for 60 min on ice with gentle stirring, then centrifuged at 14,000 rpm at 0 ℃ for 10 min. The supernatant was collected and assayed for protein concentration using the BCA protein assay kit (Pierce, Rockford, USA.) with BSA as a standard. For *Western blotting* studies, equal amounts of solubilized tissue proteins were mixed with lysate buffer and sample buffer containing 12.5% Tris-HCl (0.5 mmol/l , pH 6.8), 10% glycerol, 20% SDS (10%), 5% 2ß-mercaptoethanol and 5% bromophenol blue (0.05%). The solubilized proteins in the buffers were boiled for 4 min, then resolved to 8% SDS-PAGE (Tefuko, Tokyo, Japan) directly. The resolved proteins were electrophoretically transferred to nitrocellulose

membranes (Millipore, Tokyo, Japan) using a transfer buffer containing 25 mmol/l Tris-base, 192 mmol/l glycine, 20% methanol and 0.02% SDS. The membranes were incubated in blocking buffer (3% BSA in wash buffer containing 20 mmol/l Tris base, 150 mmol/l NaCl 0.01% Tween20) at room temperature for 60 min, then incubated overnight at 4 ℃ with an anti-insulin receptor {insulin Rß (C-19), Santa Cruz Biotechnology, Heidelberg, Germany}. After overnight incubation, the membranes were well washed in buffer and then incubated in 1% [I^{125}] protein A (IM144, Amersham, London, England) in blocking buffer at room temperature for 60 min. The membranes were washed at room temperature for 60 min with agitation, changing the wash buffer every 15 min. They were then dried and exposed to isotopic activity in an imaging-plate (BAS-SR 2025, Fuji-film, Tokyo, Japan) in a cassette (BAS-Cassette 2025, Fuji-film, Tokyo, Japan). Band intensities were quantified by a laser image analyzer (BAS 5000, Fuji-film, Tokyo, Japan).

Statistical analysis

Data are shown as the mean ± SEM. Statistical evaluation was performed with Student's *t*-test. $P<0.05$ was considered statistically significant.

Table 1 Composition of experimental diets

Ingredient	Diet	
	CHO	FAT
	(g/100 g diet)	
Casein	17.3	22.9
Corn starch	68.1	41.4
Soybean oil	1.42	1.87
Beef tallow	-	18.0
Mineral mix§	7.0	9.2
Vitamin mix§	0.70	0.92
Cellulose	5.0	5.0
Choline bitartrate	0.30	0.39
L-Methionine	0.26	0.35
	(% of calorie)	
Fat	5.0	45.0
Carbohydrate	75.0	35.0
Protein	20.0	20.0

§ Purchased from Cler, Japan.

Results

Weekly average energy intakes for 3 wk (FAT: 613 ± 14, CHO: 558 ± 20 kcal/wk, $P<0.05$) and 16 wk (619 ± 17 and 565 ± 17, respectively, $P<0.05$) were both significantly higher in the FAT group than in the CHO group. Body weight and tissue weights are shown in Table 2. Both at 3 and 16 wk on the experimental diets, total adipose tissue weight was significantly higher in the FAT group. Body weight was also higher in the FAT group, but the difference between the groups was not significant.

Hormonal, neural and hemodynamic data were shown in Table 3. After consuming the experimental diets for 16 wk, plasma glucose, insulin and leptin levels were higher in the FAT group. Urinary NE and Epi were also significantly higher in the FAT group. Systolic, mean and diastolic BP were significantly higher in the FAT group. Heart rate was the same in both groups. On the other hand, after 3 wk on the experimental diets, the plasma leptin levels were already significantly higher in the FAT group, but other parameters were not different between the two dietary groups.

The data for the euglycemic clamp test at 3 wk are shown in Table 4. Basal glucose and insulin levels were similar in both groups. SSPG and SSPI levels were not different in both groups. GIR from 60 to 90 min after beginning of the insulin infusion was significantly lower in the FAT group than in the CHO group.

Skeletal muscle IR content was similar in both groups at 3 wk. At 16 wk, IR content in soleus and gastrocnemius muscles was significantly lower in the FAT group (Fig. 2).

Table 2 Body weight and tissue weights in rats after 3 or 16 weeks feeding of CHO or FAT (Study 1)

	3wk		16wk	
	CHO	FAT	CHO	FAT
n	8	8	6	8
Body weight, g	321±8.3	327±4.6	564±31.2	578±18.3
Heart, g	1.0±0.02	1.0±0.02	1.2±0.05	1.2±0.05
Liver, g	13.5±0.6	13.5±0.4	18.3±1.8	17.3±0.6
Kidneys, g	2.5±0.06	2.6±0.05*	3.1±0.2	3.2±0.1
Soleus muscles, g	0.23±0.01	0.23±0.01	0.39±0.02	0.36±0.01
Gastrocnemius muscles, g	3.2±0.06	3.2±0.09	5.4±0.2	5.3±0.1
Total adipose tissue§, g	20.7±2.0	25.7±1.2*	69.7±5.5	90.8±4.0*

§ The total weight of etroperitoneal, mesenteric, epididymal and subcutaneous fat-pads.

Values are means ± SEM. *P<0.05 vs. CHO group.

Table 3 Hormonal, neural and hemodynamic characteristics of rats after 3 or 16 weeks feeding of CHO or FAT (Study 1)

	3wk		16wk	
	CHO	FAT	CHO	FAT
n	8	8	7	9
Plasma glucose, mg/dl	136±3.3	131±3.8	155±2.5	164±2.3*
Plasma insulin, μU/ml	41.1±5.6	32.9±5.0	44.1±5.2	61.7±4.9*
Plasma leptin, ng/ml	25.8±2.6	45.0±3.4**	78.6±15.2	153.1±13.1**
Urinary norepinephrine, μg/day	808±242	963±189	1133±214	3042±675*
Urinary epinephrine, μg/day	253±68	282±48	381±109	639±59*
Systolic blood pressure, mmHg	130±2.4	129±2.5	153±1.5	166±3.6*
Mean blood pressure, mmHg	98±1.3	96±3.6	126±2.4	139±3.4*
Diastolic blood pressure, mmHg	83±1.5	81±4.2	109±2.5	120±2.2**
Heart rate, beats/min	399±8	402±10	356±8	363±13

Values are means ± SEM. *P<0.05, **P<0.01 vs. CHO group.

Table 4 Euglycemic clamp test in the rats at the 3wk feeding of CHO or FAT (Study 1)

	CHO	FAT
n	6	6
Body weight, g	248±10	240±9
Basal plasma glucose, mg/dl	169±4.3	180±14.1
Basal plasma insulin, μU/ml	63.5±6.9	62.7±8.3
SSPG, mg/dl	164±3.3	170±3.0
SSPI, μU/ml	214±22	200±24
$GIR_{60\text{-}90}$, mg/kg/min	29.5±1.3	17.3±1.1**

Values are means ± SEM. **P<0.01 vs. CHO group.

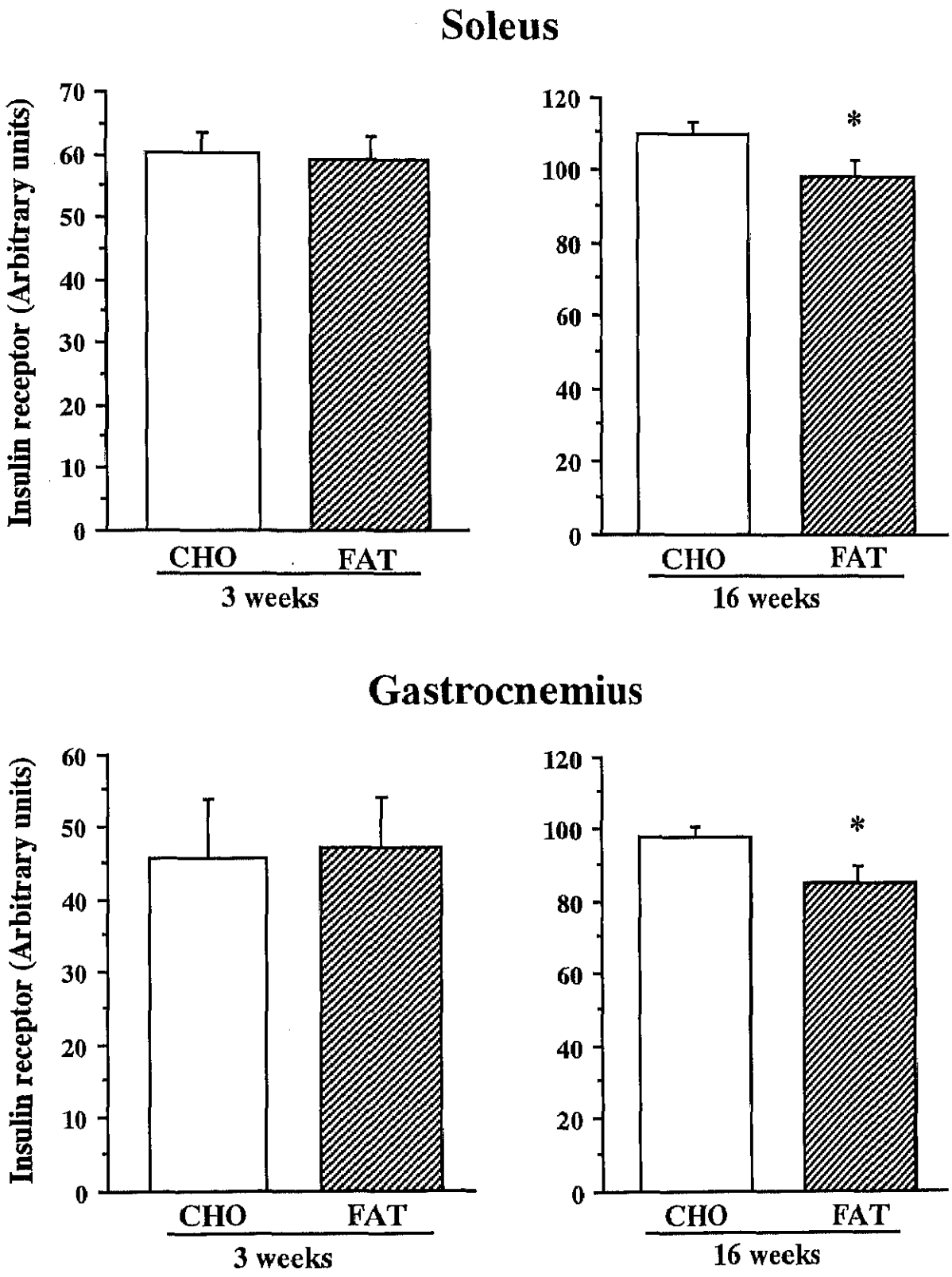

Fig. 2 Immunoreactive insulin receptor contents in soleus and gastrocnemius muscles in rats after 3 or 16 weeks feeding of CHO or FAT. Values are means ± SEM (n=8) and are shown with arbitrary units per an equal amount of protein. **P*<0.05 vs. CHO group (Study 1).

Discussion

In the Study 1, BP and metabolic, hormonal and neural factors of BP regulation were measured in rats fed the experimental diets for 3 or 16 wk. At 16 wk, BP was significantly higher in the FAT group than in the CHO group. The BP elevation in FAT-fed rats was concomitant with increase in body fat accumulation, plasma levels of insulin, glucose and leptin and urinary catecholamines, and decrease in insulin sensitivity. At 3 wk, on the other hand, BP did not increase although body fat store and plasma leptin levels were increased and insulin sensitivity was decreased in FAT group.

Chronic hyperinsulinemia increased plasma NE in rats (35). In addition, intracerebroventricular insulin administration increased lumbar SNA in rats (80). Edwards and Tipton (36) demonstrated that acute insulin infusion raised MAP. However, in chemically sympathectomized rats, insulin infusion did not produce an increase in MAP (36). In Dahl salt-sensitive rats, co-administration of an α-blocker and insulin abolished the insulin-induced elevation in MAP (33). However, several studies in human and animals failed to find a hypertensive effect of insulin (16, 37-42). Some of these studies did not also increase plasma NE levels or urinary NE excretion after insulin administration (41-43). The reason why BP or SNA responses to insulin administration were not consistent is not clear.

Kaufman et al. (17) investigated the effects of FAT on BP, insulin and SNA in rats. In that study, systolic BP was significantly higher in FAT-fed rats than in control-diet-fed rats. The elevation of BP in FAT-fed rats was concomitant with an increase in body weight, fasting plasma insulin levels and urinary NE excretion. In FAT-fed rats, when rats were fed a restricted FAT to avoid over weight, BP, plasma insulin levels and urinary NE excretion were similar to those in control-diet-fed rats. The normalization of BP by food restriction would be due to prevention of SNS hyperactivity (17).

Insulin resistance alone, even without hyperinsulinemia, also increases SNA (55). Insulin centrally activates SNS and increases circulating NE levels. Whereas, Shimosawa et al. have demonstrated that insulin inhibits NE release from peripheral nerve endings in the mesenteric arteries of rats (54). The inhibitory effect of insulin on NE

overflow was reduced in SHR having insulin resistance compared with WKY rats (55). Insulin-mediated skeletal muscle vasodilation was diminished in states of insulin resistance in human (56-59). In animal studies, a previous evidence demonstrated in cultured HUVEC that insulin-stimulated NO production was blocked by inhibitors of insulin signal transduction cascade, such as tyrosine kinase and PI 3-kinase (60). In addition, insulin-mediated vasodilation and elevation of erythrocyte flow velocity in arteriole were blunted by a tyrosine kinase inhibitor in hamster cheek pouch (61). These findings suggested that insulin resistance decreased insulin-mediated vasodilation by weakened NO production in skeletal muscles. It was suggested in a human study that diminished insulin-induced vasodilation associated with pathophysiology of hypertension (63).

A few studies reported that plasma leptin levels were higher in hypertension than not hypertension in human and animals (66, 69). Intravenous and central administrations of leptin increased plasma catecholamine levels in animals (70, 71). Leptin has also been shown to increase sympathetic activities in several tissues including kidney and hindlimb (11).

FAT-related BP elevation would be affected by fat content in an experimental diet and duration of the diet. When rats received diets consisting 60-67% fat for 4-8 wk, BP was higher in FAT groups than in low-fat control-diet groups (17-19, 23). When a fat content was relatively low (39.5%) in FAT, it would be likely to need longer experimental diet periods for BP elevation (21). On the contrary, when rats were maintained on diets consisting 40-45% fat for 4-8 wk, BP was not higher in FAT (31, 81). The studies would be insufficient fat content in the FAT or the experimental period. In the present study, 3 wk was too short to elevate BP by the FAT containing 45% fat. In addition, comparing 3 wk with 16 wk of the experimental diets, the degrees of difference on the pressor factors between FAT and CHO were similar or relatively small, although each data in the both diets was higher in 16 wk than in 3 wk. The aggregation of several pressor factors may be important for FAT-related BP elevation (9). Practically, BP elevation in FAT or fructose-rich diet feeding was preceded derangement of various pressor factors, such as obesity, increase in urinary catecholamines excretion, insulin resistance,

dyslipidemia and endothelial dysfunction (17, 18, 20, 21, 82).

This study first demonstrated decreased IR content in the skeletal muscles in FAT-related BP elevated rats. Previously, we measured IR mRNA in soleus muscle to compare FAT-fed with CHO-fed rats, and showed that FAT feeding did not affect IR mRNA levels (83). Unfortunately, we did not measure IR content in skeletal muscles and BP. Decreased insulin binding at maximal insulin concentrations in isolated soleus muscle in FAT-fed rats has been reported, suggesting that IR in skeletal muscle is decreased by FAT feeding, though these studies, too, did not measure BP (64, 65). The present study determined IR contents using *Western blotting analysis*, which is a more reliable method of protein assay than those in previous reports. The major factor regulating IR in obesity appears to be the levels of circulating insulin. The more insulin-resistant the animal, the higher the basal insulin and the greater the decrease in IR numbers (84). In the data for FAT-fed rats (study 1), plasma insulin levels were not higher at 3 wk, but significantly higher at 16 wk of feeding compared with CHO-fed rats, and IR content was lowered with FAT at only 16 wk. Thus, it is considered that the decreased IR content resulted in severe insulin resistance and deranged glucose homeostasis.

In conclusion of the Study 1, long-term FAT feeding aggravates multiple metabolic and hormonal pressor factors, such as excess body fat accumulation, hyperinsulinemia, insulin resistance, derangement of glucose homeostasis and hyperleptinemia, resulting in SNS activation and BP elevation. In addition, IR content in skeletal muscles was decreased by FAT. The decreased IRs are associated with marked insulin resistance and may be associated with FAT-related BP elevation.