CHAPTER 7. INSULIN INCREASES BLOOD FLOW RATE IN THE MICROVASCULATURE OF CREMASTER MUSCLE OF RATS (Study 4)

The objectives of the Study 4 were to investigate hemodynamic actions of insulin in skeletal muscle microvasculature. Therefore, the microvascular hemodynamic changes (vessel diameter and erythrocyte flow velocity) in the rat cremaster muscle in response to systemic insulin administration were evaluated by using of intravital real-time confocal laser-scanning microscope system in combination with selective fluorescent labeling.

Research methods and procedures

Animals and diets

Sixteen male Sprague-Dawley rats weighing from 210 to 300 g (Cler Japan, Tokyo, Japan) were used for the experiment. The rats were kept under controlled conditions of light (0800-2000) and temperature (23 ± 1 ℃). The rats were fed standard chow (CE-2; Cler Japan, Tokyo, Japan) ad libitum and fasted for 5 to 8 hours before experiments.

Preparation of the rats

Rats were anesthetized with sodium pentobarbital (100 mg/kg, intramuscular injection). A supplemental dose of anesthetic (40 mg/kg) was given 2.5 hours after the first injection. Body temperature of the rats were kept constant at 37 ± 1 °C with a heating lamp. Once anesthetized, animals were intubated with a polyethylene tube to facilitate spontaneous breathing. Polyethylene catheters were inserted into the right carotid artery for BP measurement and blood sampling and into the right jugular vein for injections of fluorescent dyes. Left cremaster muscles were prepared for intravital microscope system by a modified method originally described by Porter (51). Muscle was continuously superfused with saline at 37 °C during the experiment.

Injection of insulin and intravital microscopic observation

Rats were injected subcutaneously with insulin (1 U/kg, Humulin R U-40, Eli Lilly

Japan K.K., Kobe, Japan) or isotonic saline. Anesthetized rats were placed on the stage of an intravital microscope (BHWI, Olympus Optical, Tokyo, Japan) equipped with a real-time confocal laser-scanning optics (InSight plus, Meridian Instruments, Okemos, USA) as previously described (93). Fluorescent images of microvessels were visualized and recorded by a video system composed of silicon intensifier target tube camera (C2400-8, Hamamatu Photonics, Shizuoka, Japan), a video timer (VTG-33, For A, Tokyo, Japan), an S-VHS video cassette recorder (BR-S605B, Victor, Japan, Tokyo Japan) and a TV monitor (PVM-1445MD, Sony, Tokyo, Japan). A final magnification of microvasculature 550 or 1,100X was attained on the TV screen by use of a recording lens (NFK 5.0X, Olympus, Tokyo, Japan) and a long working distance objective (CDPIan 20X, numerical aperture (N.A.), CDPIan 40X, N.A., Olympus, Tokyo, Japan). Arterioles with diameters from 8.1 to 12.1 μm and capillaries (2.8 ~ 6.3 μm) were selected for microscopic observation. Parameters of microvasculature hemodynamics, i.e., erythrocyte flow velocity and vessel diameter, were measured before and at 15 and 30 min after injection of insulin or saline as follows.

To visualize microvessels and to measure vessel diameter, a solution of FITC-labeled dextran (FITC-Dx, MW 150,000; Sigma Chemical, St. Louis, USA) dissolved in saline solution was injected (15 mg/ml, 0.5 ml/kg) intravenously. This permitted imaging of bright fluorescence of the vascular lumen, and enabled mapping of the vascular architecture and accurate measurements of the luminal diameter. Diameter of microvessels in rats were measured carefully with a vernier caliper on individual frames of video-recorded images. Diameter values are averages of 6 measurements of each vessel.

To measure erythrocyte flow velocity, erythrocytes obtained from a donor rat were incubated with phosphate-buffered saline containing fluorescein isothiocyanate (FITC; ICN Pharmaceuticals, Cleveland, USA) at a concentration of 1 mg/ml according to the method of Homma et al. (94). Labeled cells were then washed twice with saline containing 1% BSA to remove unconjugated fluorescent dyes. The final percent of the labeled cells was adjusted to approximately 50% by adding isotonic saline. These suspensions (1 ml/kg) were injected intravenously to measure the centerline erythrocyte

flow velocity. Flow velocity was calculated from video-recorded images by frame-by-frame analysis, and averaged for 10 measurements.

Arterial and capillary blood flow rates were calculated from the measured values of vessel diameter and centerline velocity.

Blood analysis

Serum insulin and blood glucose were determined just prior to insulin or saline administration and at 15 and 30 min after injection. Arterial blood samples (200 μl) were collected in Eppendorf tubes for determination of blood glucose and serum insulin. Analysis of blood samples was conducted similarly Study 2.

Statistical analysis

Results are expressed as mean ± SEM. Between-group comparisons of metabolic parameters were performed using Mann-Whitney's U-test. Hemodynamic parameters were analyzed using repeated measure ANOVA to identify time and drug effects. When ANOVA showed a significant difference, Fisher's PLSD (Protected Least Significant Difference) test was used. $P<0.05$ was considered statistically significant.

Results

Alterations in blood glucose levels, serum insulin levels, MAP and HR are shown in Table 7. These values were not affected by the subcutaneous injection of physiological saline. With the injection of insulin, the serum insulin levels increased by 50% of the baseline value by 15 min, and nearly returned to the baseline value by 30 min. A significant decrease in the glucose levels was shown both at 15 and 30 min. MAP was unchanged, and HR showed a slight but statistically significant increase after insulin administration.

From the video-recorded images of microvessels and blood flow behavior, vessel diameter and erythrocyte velocity in arterioles and capillaries in cremaster muscles were measured. Initial diameters of the observed microvessels were 9.9 ± 0.4 μm (n=16) in arterioles and 4.8 ± 0.3 μm (n=16) in capillaries. The initial erythrocyte velocities in arterioles and capillaries were 1221 ± 116 and 603 ± 97 μm/sec, respectively.

As shown in Fig. 7-a, insulin administration caused arteriolar dilation at 15 and 30 min. In contrast, vasodilation of arterioles was not detected in the saline group. Erythrocyte flow velocity in arterioles was significantly increased by insulin compared with saline controls (Fig. 7-b). As the result, the blood flow rate, which was calculated from vessel diameter and centerline erythrocyte flow velocity, showed a marked increases in arterioles at 15 and 30 min (Fig. 7-c).

On the other hand, capillary diameter was not modified by insulin as shown in Fig. 8-a. Fig. 8-b shows that capillary erythrocyte flow velocity was significantly increased by insulin. Fig. 8-c shows that capillary blood flow rate was also increased by insulin at 15 and 30 min.

Table 7 Effect of injection of insulin or saline on blood glucose and serum insulin levels, mean arterial pressure and heart rate in rat (Study 4)

	Saline			Insulin, 1U/kg		
n	8			8		
Time after injection (min)	0	15	30	0	15	30
Blood glucose, mg/dl	121 ± 2	121 ± 4	121 ± 4	128 ± 9	104 ± 0.4*††	99 ± 4**††
Serum insulin, μU/ml	75 ± 12	87 ± 20	75 ± 12	77 ± 11	139 ± 12* †	88 ± 11
Mean arterial pressure, mmHg	78 ± 6	77 ± 6	77 ± 5	80 ± 8	82 ± 8	89 ± 7
Heart rate, beats/min	373 ± 11	375 ± 9	373 ± 6	378 ± 19	410 ± 15	422 ± 13†

Values are means ± SEM. *$p < 0.05$, ** $p < 0.01$ vs saline group, † $p < 0.05$, †† $p < 0.01$ vs time 0

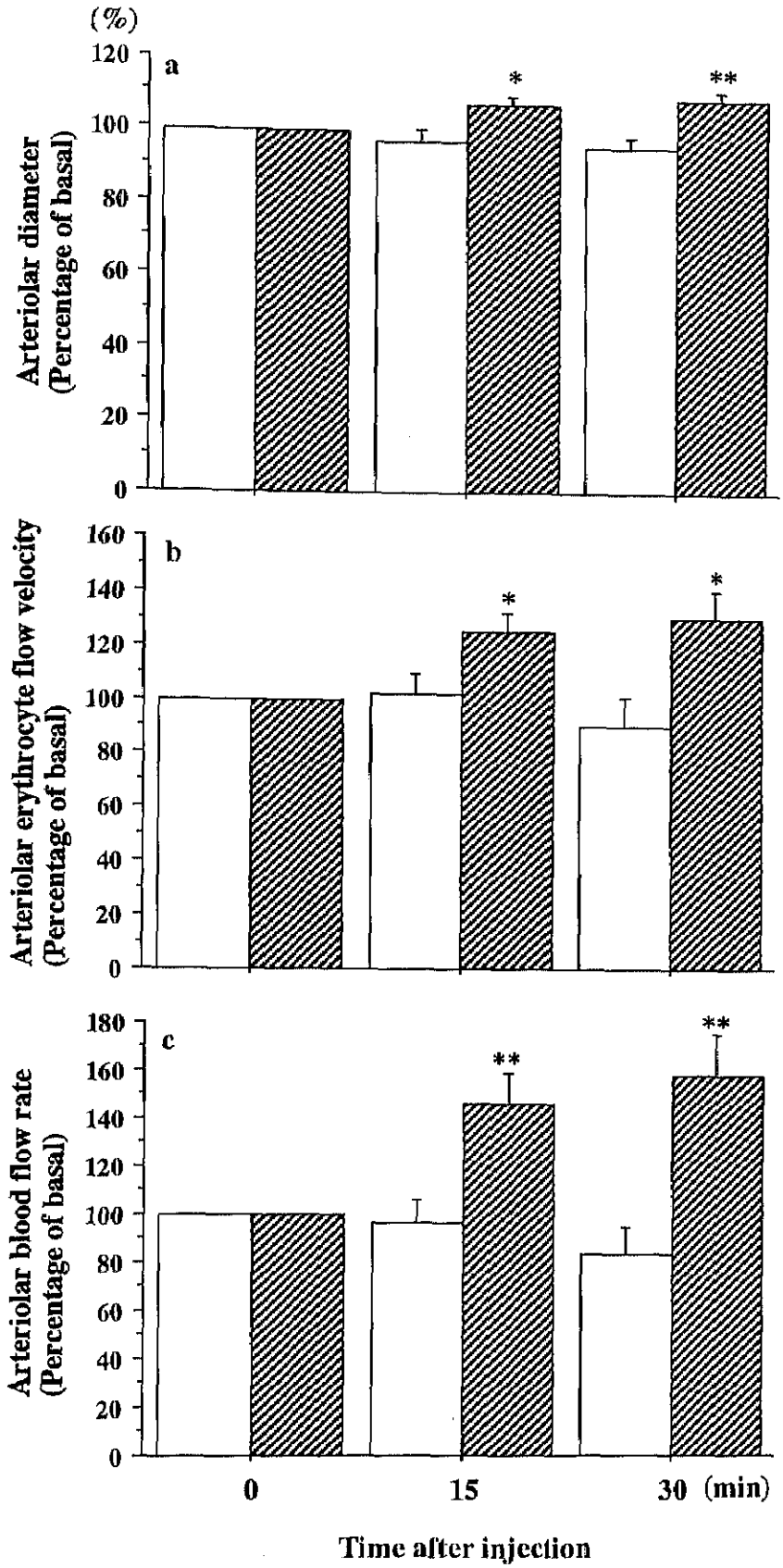

Fig. 7 Effects of subcutaneous injection of insulin (*slashed bar*) or saline (*open bar*) on microcircuration in the rats cremaster muscle arteriole. Each panels show changes of vessel diameter (a), erythrocyte flow velocity (b) and calculated blood flow rate (c). Values are means ±SEM (n=8). *$P<0.05$, **$P<0.01$ vs. saline group (Study 4).

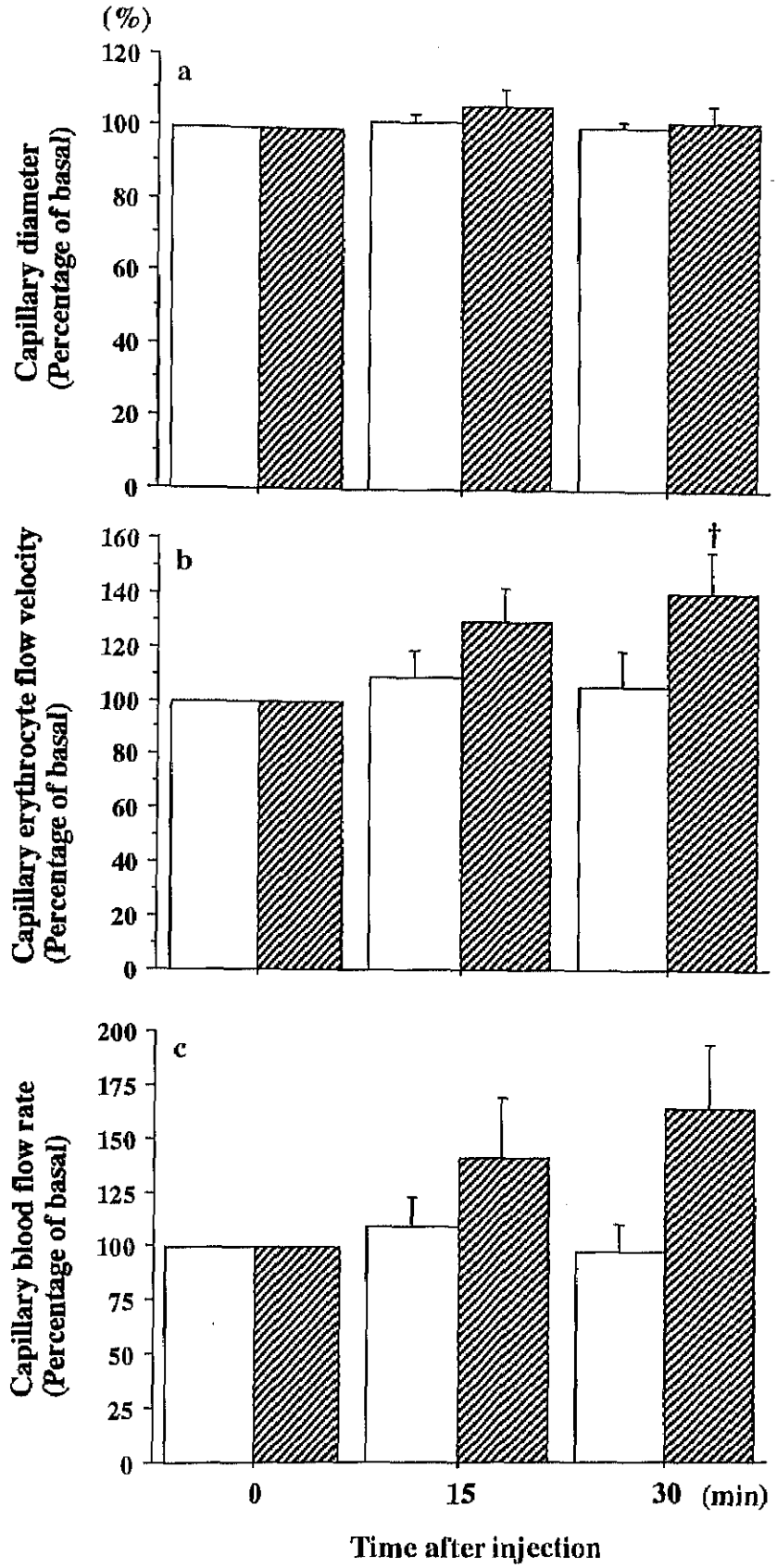

Fig. 8 Effects of subcutaneous injection of insulin (*slashed bar*) or saline (*open bar*) on microcircuration in the rats cremaster muscle capillary. Each panels show changes of vessel diameter (a), erythrocyte flow velocity (b) and calculated blood flow rate (c). Values are means ± SEM (n=8). †$P<0.05$ vs. time 0 (Study 4).

Discussion

The Study 4 is the first demonstration, using intravital real-time confocal laser scanning microscope system and by directly measuring diameter and erythrocyte flow velocity in arteriole and capillary, that the systemic insulin administration increases microvascular blood flow rate in the rat cremaster muscle microvasculature.

It has been reported that blood flow in muscle is increased by insulin (46, 47, 50, 95). Several investigators have reported the effects of insulin on arteriolar diameter in skeletal muscle, including data obtained by intravital microscope system that insulin increased arteriolar diameter during systemic administration (49) or topical application (51, 52). Furthermore, vasodilation of isolated arterioles in the presence of insulin was observed (53, 96). These data are consistent with the present findings regarding hemodynamics in arterioles. In contrast, several previous studies of systemic hyperinsulinemia showed no obvious effect of insulin on muscle tissue blood flow (48, 50) or skeletal muscle arteriole diameter (51, 52). The differences of the results in the previous studies are thought to be due to experimental conditions. Especially, circulating glucose levels are an impotant factor for vasodilation by insulin. Insulin-mediated vasodilation and/or change in blood flow volume were investigated with wide range of circulating glucose levels in rats (47-51). Systemic hyperinsulinemia increased blood flow volume or arteriolar diameter under conditions of steady-state euglycemia or hypoglycemia (47, 49, 50). It appears that glucose by itself induces vasoconstriction. Several reports showed that glucose constricted isolated artery (97) and impaired endothelium dependent vasodilation in arteriole (98) in rat intestinal arterioles. Our experimental condition which was hyperinsulinemia with moderate hypoglycemia conducted for reasonable evaluation of effects of insulin on microvasculature.

To investigate the effects of insulin on skeletal muscle microvasculature in detail, this study determined erythrocyte flow velocity in arterioles of rat cremaster muscle after insulin administration. The data show that arteriolar erythrocyte flow velocity was increased by insulin. Recent data provided an evidence that topical application of insulin on hamster cheek pouch arteriole increased blood flow rate concomitant with vessel dilation and

elevated erythrocyte flow velocity (61).

No study has investigated changes in capillary hemodynamics in skeletal muscles after insulin administration. Present data show that blood flow velocity is increased without vasodilation in the capillary. Consequently, the degree of increase in capillary blood flow rate was almost equivalent to that in arterioles. In capillary beds, insulin affects not only blood flow rate but also recruitment of vessels (47). The effect of insulin on capillary recruitment, however, was not determined in this study.

Insulin-mediated vasodilation may be caused by endothelium-dependent NO production (46, 52, 53, 60, 61, 96). Many studies conducted in rodent skeletal muscle arterioles demonstrated that insulin-mediated vasodilation was abolished by NOS inhibitors (46, 52, 53, 61, 96) or endothelium removal (53, 96). In addition, Zeng and Quon demonstrated that insulin stimulated NO production in a dose-dependent manner and the effects of insulin were completely blocked by inhibitors of NOS in human umbilical vein endothelial cells (60). It is thought that erythrocyte flow velocity can be one of indices of NO production. Because NO inhibits blood coagulation, increase in NO concentration in blood appear to elevate blood flow velocity. Mitchell and Tyml demonstrated that an NOS inhibitor reduced erythrocyte flow velocity and increased leukocyte adhesion, but not measured vessel diameter, in rat skeletal muscle capillaries (99). Another report showed that insulin-mediated elevation of erythrocyte flow velocity was abolished by an NOS inhibitor in hamster cheek pouch arterioles (61). Thus, the present study observed elevation of erythrocyte flow velocity after insulin injection, suggesting that insulin might increase NO production.

In the present study, systemic insulin administration did not elevate BP. It is thought that insulin has vasodilation of arterioles and diminishing vascular resistance in the skeletal muscle microcirculation of the rats. These depressor effects of insulin would contribute to normalize the BP after injection of the pressor hormone. On the other hand, many of human studies have suggested diminished skeletal muscle vasodilation in states of insulin resistance (56-59). In addition, previous study demonstrated in cultured HUVEC that insulin-stimulated NO production was blocked by inhibitors of tyrosine kinase and PI

3-kinase (60). In a few animal studies, insulin-mediated vasodilation and elevation of erythrocyte flow velocity in arteriole were blunted by a tyrosine kinase inhibitor in hamster cheek pouch (61). Another study demonstrated in isolated rat soleus muscle that generation of cGMP by NO donor SNP was decreased in obese Zucker compared with lean Zucker rats, suggesting impaired NO/cGMP signaling (62). Furthermore, the maximal activity of NOS was significantly decreased in insulin-resistant obese Zucker muscles (62). These findings suggested that insulin resistance decreased insulin-mediated vasodilation by weakened NO production in skeletal muscles. In the present study, the effect of insulin on microcirculation in FAT-fed rats was not investigated. To clarify that a lack of insulin-mediated vasodilation contribute to the FAT-related BP elevation, it is necessary to study in the future.

In conclusion of the Study 4, insulin induces increases in blood flow rate in rat cremaster muscle microcirculation. This effect is caused by insulin-mediated vasodilation in arterioles and elevation of erythrocyte flow velocity in arterioles and capillaries. The hemodynamic action of insulin may be a reason why hyperinsulinemia does not elevate BP in the present studies.