Involvement of Bradykinin in Acute Exercise-Induced Increase of Glucose Uptake and GLUT-4 Translocation in Skeletal Muscle: Studies in Normal and Diabetic Humans and Rats

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Acute exercise induces glucose uptake in skeletal muscle in vivo, but the molecular mechanism of this phenomenon remains to be identified. In this study, we evaluated the involvement of bradykinin in exercise-induced glucose uptake in humans and rats. In human studies, plasma bradykinin concentrations increased significantly during an ergometer exercise (20 minutes) in 8 healthy normoglycemic subjects and 6 well-controlled type 2 diabetic patients (mean hemoglobin A_{1c} [HbA_{1c}], 6.4% ± 0.6%), but not in 6 poorly controlled type 2 diabetics (mean HbA_{1c}, 11.6% ± 2.6%). In rat studies, plasma bradykinin concentrations also significantly increased after 1 hour of swimming in nondiabetic and mildly diabetic (streptozotocin [STZ] 45 mg/kg intravenously [IV]) rats, but not in rats with severe diabetes (STZ 65 mg/kg IV). Glucose influx (maximum velocity [V_{max}]) and GLUT-4 translocation in skeletal muscle of nondiabetic rats significantly increased after 1 hour of swimming, but these increases were abrogated by subcutaneous infusion of bradykinin B_2 receptor antagonist HOE-140 (400 $\mu g \cdot kg^{-1} \cdot d^{-1}$). Insulin-stimulated tyrosine phosphorylation and phosphatidylinositol (PI) 3-kinase activity in response to insulin injection (20 U/kg IV) in the portal vein were significantly attenuated in exercised rats pretreated with HOE-140 compared with saline-treated exercised rats. Our results suggest that plasma bradykinin concentrations increase in response to acute exercise and this increase is affected by blood glucose status in diabetic patients. Moreover, the exercise-induced increase in bradykinin may be involved in modulating exercise-induced glucose transport through an increase of GLUT-4 translocation, as well as enhancement of the insulin signal pathway, during the postexercise period in skeletal muscle, resulting in a decrease of blood glucose.

PHYSICAL EXERCISE increases the rate of glucose uptake in contracting skeletal muscle, a process with important clinical implications, especially in patients with reduced glucose uptake into peripheral tissues such as those with type 2 diabetes mellitus.[1] This effect of exercise on glucose uptake is similar to the action of insulin, since both stimuli increase glucose uptake in skeletal muscle through the translocation of glucose transporter type 4 (GLUT-4) from the intracellular pool to the plasma membrane.[1-3] Insulin stimulation is known to induce a rapid phosphorylation of the insulin receptor and its substrates including insulin receptor substrate-1 (IRS-1) on their tyrosine residues, followed by activation of phosphatidylinositol 3-kinase (PI 3-kinase).[4,5] On the other hand, little is known about the mechanism of exercise-induced glucose transport, although bradykinin action is thought to be a possible candidate.[6]

Bradykinin is a nonapeptide involved in various biological processes such as pain, inflammation, vascular permeability, hypotension, edema, and smooth muscle contraction,[7] and also modulates glucose metabolism in vivo.[8,9] Bradykinin is known to be released from human skeletal muscle during physical exercise,[10,11] as well as contracting cat skeletal muscle during electrical stimulation.[12]

We have previously demonstrated that bradykinin infusion increased glucose uptake into dog peripheral tissues, and that infusion of a bradykinin antagonist abolished this effect.[8] Furthermore, we identified the presence of bradykinin B_2 receptors in dog adipocytes, skeletal muscle, and L6 myotubes.[13,14] We also demonstrated that bradykinin could increase insulin-stimulated phosphorylation of insulin receptor and IRS-1, which in turn potentiated the translocation of GLUT-4, resulting in increased glucose uptake.[13,14] However, other studies have also shown that bradykinin influenced glucose uptake into myotubes through an insulin-independent pathway. In the absence of insulin, bradykinin triggered GLUT-4 translocation in L6 myotubes transfected with bradykinin B_2 receptors and c-*myc* epitope-tagged GLUT-4 (GLUT-4 *myc*).[15] Therefore, it is possible that bradykinin released locally from contracting muscles might be involved in GLUT-4 translocation through potentiation of the insulin signal transduction system and/or by triggering it directly, subsequently resulting in increased glucose uptake during exercise.

In the present study, we examined the changes in the plasma bradykinin concentration during exercise in humans and rats with normal glucose tolerance or diabetes. We also investigated the effect of an exercise-induced elevation of bradykinin on glucose transport, GLUT-4 translocation, and phosphorylation of the insulin receptor and IRS-1 in rat skeletal muscle.

SUBJECTS AND METHODS

Subjects

In experiments involving humans, we studied 8 healthy nondiabetic controls and 12 age- and weight-matched patients with type 2 diabetes mellitus. All subjects were euthyroid and without acute or chronic renal, hepatic, or cardiac disease. Diabetic patients were categorized as well-controlled type 2 diabetics (DM-1; n = 6) and poorly controlled type 2 diabetics (DM-2; n = 6) based on hemoglobin A_{1c} (HbA_{1c}) below 8.0% and above 8.0%, respectively. Diabetic patients were

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treated with diet therapy (n = 3), sulfonylurea (n = 3), or insulin therapy (n = 6). The study was approved by the Ethics Committee of Kumamoto University School of Medicine, and informed consent was obtained from each subject.

In animal studies, 6- to 8-week-old male Wistar rats weighing between 180 and 220 g were used in all experiments. Rats were allowed free access to food and water. The experimental protocol was also approved by the Ethics Review Committee for Animal Experimentation of Kumamoto University School of Medicine.

Human Experiments

Human experiments were performed on the ward of the Department of Metabolic Medicine, Kumamoto University Hospital. One week before the experimental day, we estimated the maximum heart rate of each subject during exercise by a stepwise loading test using an ergometer. On the experimental day, subjects fasted for more than 8 hours prior to the study and used no medication. Before exercise, a catheter was inserted into the antecubital vein and each subject rested for 30 minutes in a sitting position. The first and second baseline blood samples were obtained at the time of catheter insertion and immediately before exercise, respectively. After the second sample, each subject performed a moderate-load (up to 70% of maximum predicted heart rate) exercise for 20 minutes in the sitting position, using a computer-driven, electrically braked cycle ergometer (Aerobike 800; Combi, Tokyo, Japan). The workload was 25 W for 30 seconds initially, but was increased gradually by 20 to 30 W/min until the heart rate reached the selected target. Blood samples were drawn at 5-minute intervals, and the arterial blood pressure and pulse rate were monitored at 3-minute intervals during exercise. Venous blood samples were also obtained at 15 minutes after exercise (Fig 1A). The blood samples were immediately placed on ice and centrifuged at 1,800 × *g* at 4°C for 10 minutes. The plasma and serum were stored at −20°C until analysis.

Animal Experiments: Protocol 1

Rats were divided into 3 groups: nondiabetic (control, n = 12), severely diabetic (n = 12), and mildly diabetic (n = 12). The severly and mildly diabetic groups were defined as rats with plasma glucose higher than 400 mg/dL after treatment with STZ injection 65 mg/kg and rats with plasma glucose between 250 and 350 mg/dL after treatment with STZ 45 mg/kg, respectively.[16] STZ was injected as a bolus dose in the tail vein, and 3 days later, induction of diabetes was confirmed by measurement of blood glucose levels. All experiments were performed within 1 week after the confirmation of blood glucose levels. Six rats of each group were killed by decapitation, and the blood was collected for measurement of plasma glucose, insulin, and bradykinin concentrations. The remaining 6 rats of each group were submitted to a 60-minute bout of swimming exercise individually in a water bath filled to a depth of approximately 80 cm with water maintained at 35° to 37°C. Each rat was kept swimming. At the end of exercise, blood samples were collected for measurement of the above-mentioned parameters after decapitation (Fig 1B).

Animal Experiments: Protocol 2

Rats were divided into 3 groups: sedentary with saline infusion (S, n = 12), exercise with saline infusion (E, n = 12), and exercise with HOE-140 infusion (E-H, 400 $\mu g \cdot kg^{-1} \cdot d^{-1}$, n = 12).[17,18] To estimate a nonspecific effect of HOE-140, sedentary rats with HOE-140 infusion (S-H, 400 $\mu g \cdot kg^{-1} \cdot d^{-1}$, n = 6) were also evaluated. Under anesthesia with sodium pentobarbital (60 mg/kg body weight by intraperitoneal injection), a mini-osmotic pump was implanted subcutaneously in each rat. The pump was filled with either saline or HOE-140. After an overnight fast on the fifth day of implantation, half of the rats in each group were killed by decapitation, and blood samples were collected for measurement of plasma glucose, insulin, and bradykinin concentrations. The remaining 6 rats of the 2 exercise groups were loaded with 1 hour of swimming in a water bath maintained at 37°C. Rats of the S and S-H group were allowed to remain sedentary for 1 hour. Immediately after the exercise or sedentary period, rats were killed by decapitation and blood samples were collected in prechilled tubes for analysis of the parameters already outlined. Subsequently, the soleus and gastrocnemius muscles were then dissected out, trimmed of fat and connective tissue on ice, frozen in liquid N_2, and cryopreserved at −80°C until needed for membrane preparation (Fig 1C).

The effect of HOE-140 treatment on insulin-stimulated phosphorylation of the insulin receptor β-subunit and IRS-1 and PI 3-kinase activity was examined in 3 rats of the S, E, E-H, and S-H groups. After exercise, these rats were anesthetized, the abdominal cavity was opened, and 20 U/kg insulin was injected into the portal vein. Two minutes after injection, the hindlimb muscles were quickly excised and homogenized as already described.

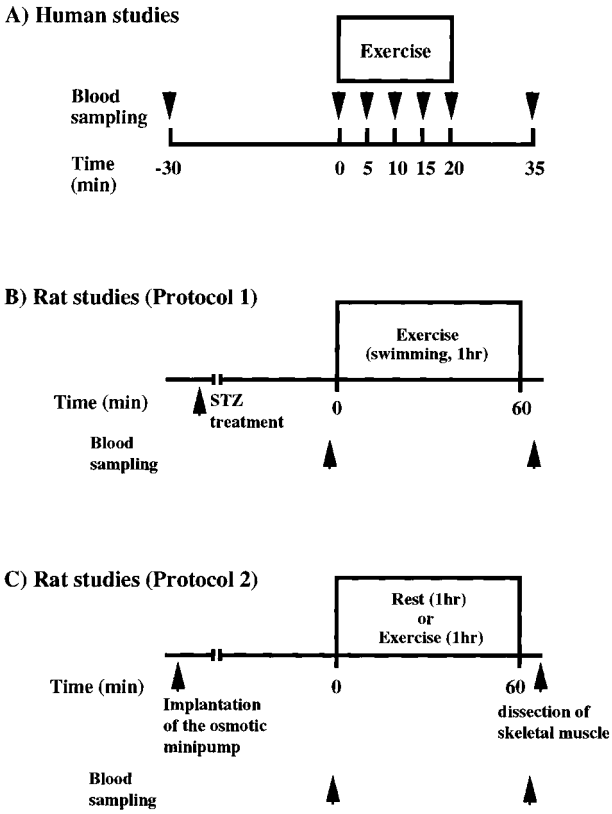

Fig 1. Study protocols. (A) Protocol for submaximal graded exercise and blood sampling in humans. Arrows indicate blood sampling for measurement of plasma glucose, insulin, and bradykinin concentrations. (B) Protocol 1: exercise and blood sampling in normal and STZ-induced diabetic rats. Rats were injected with STZ 45 mg/kg (mild diabetes) or 65 mg/kg (severe diabetes) 3-4 days before exercise. Blood sampling (↑) was performed before and after exercise. (C) Protocol 2: exercise and blood sampling in sedentary (S), sedentary and HOE-140–treated (S-H), exercise (E), and exercise and HOE-140–treated (E-H) rats. An osmotic minipump filled with saline or HOE-140 (400 $\mu g \cdot kg^{-1} \cdot d^{-1}$) was implanted subcutaneously in each rat. Five days after implantation, rats were fasted overnight and blood samples (↑) were taken, followed by 1-hour exercise (E and E-H groups). Rats in the S and S-H groups were allowed to remain sedentary for 1 hour. Immediately after the exercise or sedentary period, blood samples were collected in tubes for subsequent analysis of plasma glucose, insulin, and bradykinin (↑). After euthanasia, the soleus and gastrocnemius muscles were dissected out and cryopreserved at −80°C until needed for membrane preparation.

Membrane Preparation of Rat Skeletal Muscle

The plasma membrane fraction (PM) and low-density microsomal fraction (LDM) were prepared according to the method of Hirshman et al[19] with slight modification. Approximately 4 to 5 g of the dissected muscle was homogenized by mincing, polytron, and homogenization in a Potter-Elvejhem homogenizer (Wheaton, Millville, NJ) using a Tris-sucrose buffer (255 mmol/L sucrose, 100 mmol/L Tris, and 0.2 mmol/L EDTA, pH 7.6). After measuring the total volume, a small aliquot of the homogenate was used for K^+-stimulated *p*-nitrophenol phosphatase (K*p*NPPase) assay and protein determinations for Western blotting. All of the following steps were performed at 4°C, unless otherwise indicated. After centrifugation of the homogenate at 34,000 × *g* for 20 minutes, the resulting supernatant and pellet were used to prepare LDM and PM fractions, respectively. PM and LDM fractions were isolated using the procedure recently described by Miyata et al.[14] An aliquot from each suspension was removed and used for K*p*NPPase and protein determinations, while the remaining sample was stored in liquid N_2 until needed for Western blot analysis and measurement of glucose transport activity.

Enzyme and Protein Determination

The activity of K*p*NPPase, marker enzyme for plasma membrane, was measured in the original homogenate and in the final PM and LDM preparations as described by Bers[20] and Bers et al.[21] The protein concentration was measured by the micro bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

Glucose Transport Measurement

Fragments of PM in the final preparation sample spontaneously seal to form vesicles in which it is possible to measure glucose uptake.[22,23] D-[^{14}C]glucose and L-[^{3}H]glucose uptake into membrane vesicles were measured under conditions of equilibrium exchange ($[glucose]_{in} = [glucose]_{out}$) at 25°C using a rapid-filtration technique as described by King et al.[22] The carrier-mediated transport was quantified by subtracting the initial rate of L-glucose influx from that of D-glucose, obtained from a graph of influx (in nanomoles per milligram of protein) versus time of L- and D-glucose. The rates of carrier-mediated influx were then plotted against the glucose concentration, and maximum velocity of glucose transport (V_{max}) and K_m were obtained by a nonlinear least-squares fit[24] of the data.

GLUT-4 and GLUT-1 Content in Muscle Homogenates and GLUT-4 and GLUT-1 Translocation

Aliquots of homogenate (50 μg), PM (30 μg), LDM (60 μg), and molecular-weight markers (Bio-Rad Laboratories, Hercules, CA) were applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide gels. After electrophoresis, the resolved proteins were transferred onto nitrocellulose filters. The membranes were immunoblotted with anti–GLUT-4 polyclonal antibody or anti–GLUT-1 polyclonal antibody and then visualized using [^{125}I] protein A. GLUT-4 and GLUT-1 levels were obtained by scanning the bands using Bio-Image Analyzer BA-100 (FUJIX, Tokyo, Japan) and are expressed as a percentage of the S group.

Tyrosine Phosphorylation of Insulin Receptor β-Subunit and IRS-1

The frozen hindlimb muscle was homogenized according to the method of Folli et al[4] with slight modification. After the extraction procedures, insoluble material was removed by centrifugation at 15,000 rpm in a Ti-100 rotor (Beckman, Palo Alto, CA) for 50 minutes. Equal amounts of 5 mg proteins were incubated overnight with either anti–insulin receptor β-subunit antibody or anti–IRS-1 antibody conjugated to protein A-Sepharose CL-4B at 4°C. Immunoprecipitates were solubilized in 50 μL sample buffer with 100 mmol/L dithiothreitol (DTT) by boiling for 4 minutes and then subjected to SDS-PAGE with 7.5% acrylamide gels. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane. The membrane was immunoblotted with anti–phosphotyrosine monoclonal antibody and then visualized using [^{125}I]anti–mouse IgG, or blotted with either anti–insulin receptor β-subunit or anti–IRS-1 antibody and then visualized using [^{125}I]anti–rabbit IgG. Quantification of specific bands was performed as already described.

PI 3-Kinase Activity

PI 3-kinase activity was measured according to the method of Folli et al[4] with minor modifications. The frozen muscle was homogenized in ice-cold solubilization buffer with a Polytron generator. Insoluble material was removed by centrifugation at 15,000 rpm for 50 minutes. Aliquots of the supernatant containing 5 mg protein were immunoprecipitated with anti–phosphotyrosine and anti–rat p85 PI 3-kinase antibodies, respectively. Following incubation with protein A-Sepharose CL-4B, the immunoprecipitates were subjected to a PI 3-kinase assay as described previously.[4] Radioactive spots were measured using a Bio-Image Analyzer.

Glucose, Insulin, and Bradykinin Analysis

The plasma glucose concentration was measured using the glucose oxidase method (Autoanalyzer; Technicon Instruments, Tarrytown, NY). Plasma insulin was determined by radioimmunoassay (Eiken, Tokyo, Japan). Plasma bradykinin concentrations were determined with a bradykinin enzyme immunoassay kit (Markit M bradykinin; Dainippon Pharmaceutical, Osaka, Japan).[25,26] In human experiments, plasma (1-5) bradykinin, a stable metabolite of bradykinin, was also measured using an enzyme immunoassay kit (Markit (1-5) bradykinin; Dainippon Pharmaceutical).[26]

Materials

Porcine insulin, D- and L-glucose, HEPES, Tris, phenylmethylsulfonyl fluoride, bovine serum albumin, DTT, DNase, and STZ were obtained from Sigma (St Louis, MO). D-[^{14}C]glucose and L-[^{3}H]glucose were purchased from DuPont-NEN (Boston, MA). [γ-^{32}P]ATP, [^{125}I]protein A, and [^{125}I]anti–mouse IgG were obtained from ICN Radiochemicals (Irvine, CA). Nitrocellulose membranes were purchased from Schleicher & Schuell (Dassel, Germany). Protein A-Sepharose CL-4B was purchased from Pharmacia Biotech (Uppsala, Sweden). Anti–GLUT-4 polyclonal antibody was purchased from Biogenesis (Sandown, NH). Anti–GLUT-1 polyclonal antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti–IRS-1 polyclonal antibody, anti–insulin receptor β-subunit polyclonal antibody, anti–phosphotyrosine monoclonal antibody (4G10), and anti–PI 3-kinase p85 polyclonal antibody were purchased from UBI (Lake Placid, NY). Alzet mini-osmotic pumps (model 2001) were purchased from Alza Pharmaceuticals (Palo Alto, CA). HOE-140, a bradykinin B_2 receptor antagonist, was kindly provided by Hoechst Marion Roussel (Tokyo, Japan).

Statistical Analysis

All data are expressed as the mean ± SEM. Differences between groups were examined for statistical significance using ANOVA. When only 2 groups were compared, data were analyzed by Student's *t* test for unpaired comparisons. Differences were considered statistically significant at a *P* level less than .05.

RESULTS

Human Experiments

Subject characteristics. Clinical characteristics and biochemical profiles of the normal subjects and well-controlled and

poorly controlled diabetic patients are summarized in Table 1. The age, body weight, and body mass index (BMI) were similar among the 3 groups. Fasting plasma glucose and HbA_{1c} were significantly ($P < .05$) higher in DM-1 and DM-2 versus normal subjects. Fasting plasma glucose and HbA_{1c} in DM-2 were significantly ($P < .05$) higher versus DM-1. Fasting plasma insulin in DM-1 was significantly ($P < .05$) higher versus normal subjects and DM-2.

Changes in plasma glucose, insulin, and bradykinin concentrations during exercise. Plasma glucose levels during exercise tended to decrease gradually in all groups, albeit insignificantly. Plasma insulin showed a slight increase at 5 minutes, and then slightly but nonsignificantly decreased during effort in normal and DM-1 subjects, while it remained unchanged throughout exercise in DM-2. Plasma bradykinin concentrations at baseline were not significantly different among the 3 groups. In normal and DM-1 subjects, plasma bradykinin significantly increased ($P < .05$) in response to exercise, reaching peak levels at 10 minutes. Then, they returned to basal after 15 minutes of exercise in DM-1, while remaining higher than basal in normal subjects. The peak value in normal subjects was significantly ($P < .05$) higher than in DM-1 (124 ± 31 *v* 60.2 ± 23 pg/mL). In contrast, plasma bradykinin levels in DM-2 did not change significantly throughout exercise (Fig 2).

Plasma (1-5) bradykinin concentrations were also measured in normal subjects and diabetic patients, and similar results were obtained (data not shown). Plasma (1-5) bradykinin significantly ($P < .05$) increased in normal and DM-1 subjects during exercise compared with basal values, but not in DM-2.

Animal Experiments

Protocol 1: changes in plasma glucose, insulin, and bradykinin concentrations after exercise in control and STZ-treated rats. Swimming significantly reduced plasma glucose in control rats (from 99 ± 14 to 82 ± 10 mg/dL, $P < .05$) and DM-1 rats (from 147 ± 15 to 122 ± 15 mg/dL, $P < .05$) but not in DM-2 rats (from 368 ± 26 to 365 ± 12 mg/dL). Plasma insulin levels before exercise were significantly ($P < .05$) lower in the order of DM-2 and DM-1 rats (6.5 ± 1.4 and 11.4 ± 1.9 μU/mL, respectively) compared with control rats (20.7 ± 2.6 μU/mL). There was no significant change in plasma insulin with exercise in all groups. Plasma bradykinin concentrations significantly increased in control rats (from 5.2 ± 0.9 to 13.9 ± 1.9 pg/mL, $P < .05$) and DM-1 rat (from 5.4 ± 1.4 to 9.6 ± 1.6 pg/mL, $P < .05$) but not in DM-2 rats (from 4.9 ± 1.4 to 5.8 ± 1.4 pg/mL) (Fig 3).

Protocol 2: changes in plasma glucose, insulin, and bradykinin concentrations after exercise or rest in saline- and HOE-140–treated rats. Plasma glucose decreased significantly after exercise in the E group (from 92 ± 10 to 82 ± 4 mg/dL, $P < .05$), while levels in the E-H group increased significantly (from 93 ± 12 to 111 ± 16 mg/dL, $P < .05$). Although plasma insulin did not change after exercise in both exercise groups, the plasma bradykinin concentration was significantly higher after

Table 1. Clinical and Biochemical Characteristics of Normal Subjects and Patients With Type 2 Diabetes

Characteristic	Normal Subjects	DM-1 (well-controlled type 2 diabetic)	DM-2 (poorly controlled type 2 diabetic)
No. of subjects	8	6	6
Sex, n (male/female)	6/2	4/2	5/1
Age (yr)	28 ± 2	27 ± 8	32 ± 6
Body weight (kg)	66.4 ± 7.4	67.3 ± 22.1	60.0 ± 18.0
BMI (kg/m^2)	23.0 ± 1.3	24.6 ± 6.0	21.7 ± 6.6
Fasting glucose (mg/dL)	73 ± 6	100 ± 16*	125 ± 24*†
Fasting insulin (μU/mL)	5.9 ± 2.0	11.1 ± 1.2*‡	7.3 ± 1.8
HbA_{1c} (%)	5.2 ± 0.8	6.4 ± 0.6*	11.6 ± 2.6*†
Treatment, (diet/SU/insulin)	0/0/0	3/2/1	0/1/5

NOTE. Data are the mean ± SEM.
Abbreviation: SU, sulfonylurea.
*$P < .05$ *v* normal subjects.
†$P < .05$ *v* DM-1.
‡$P < .05$ *v* DM-2.

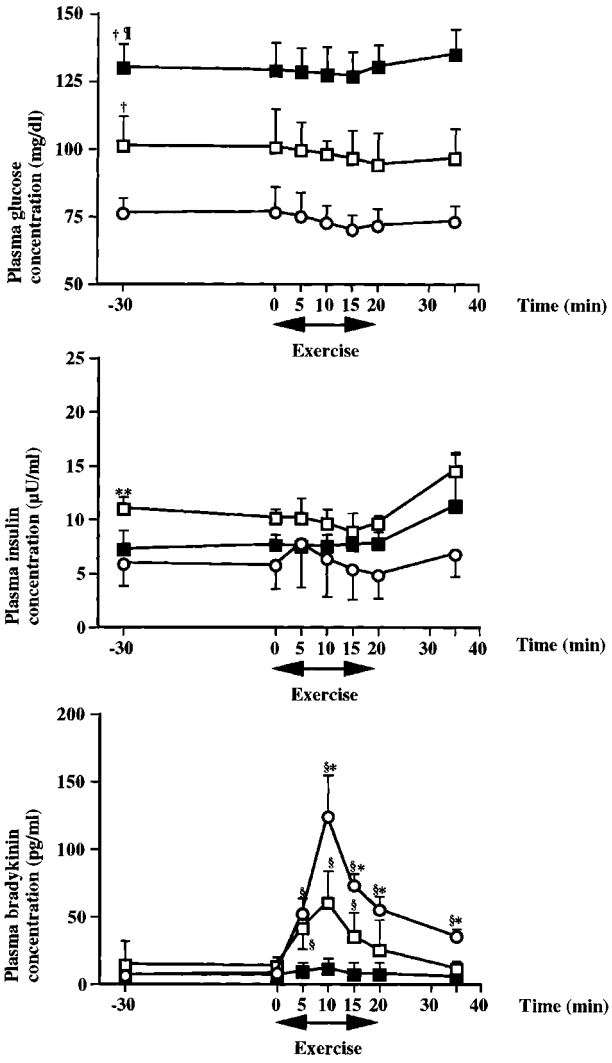

Fig 2. Changes in plasma glucose, insulin, and bradykinin concentrations in normal subjects and type 2 diabetic patients. Plasma glucose, insulin, and bradykinin were analyzed in normal subjects (○, n = 8), DM-1 (□, well-controlled type 2 diabetic patients, n = 6), and DM-2 (■, poorly controlled type 2 diabetic patients, n = 6) at the indicated time intervals (−30, 0, 5, 10, 15, 20, and 35 minutes). Values are the mean ± SEM. †$P < .05$ *v* normal subjects. ¶$P < .05$ *v* DM-1. **$P < .05$ *v* normal subjects. §$P < .05$ *v* normal subjects. *$P < .05$ *v* DM-1.

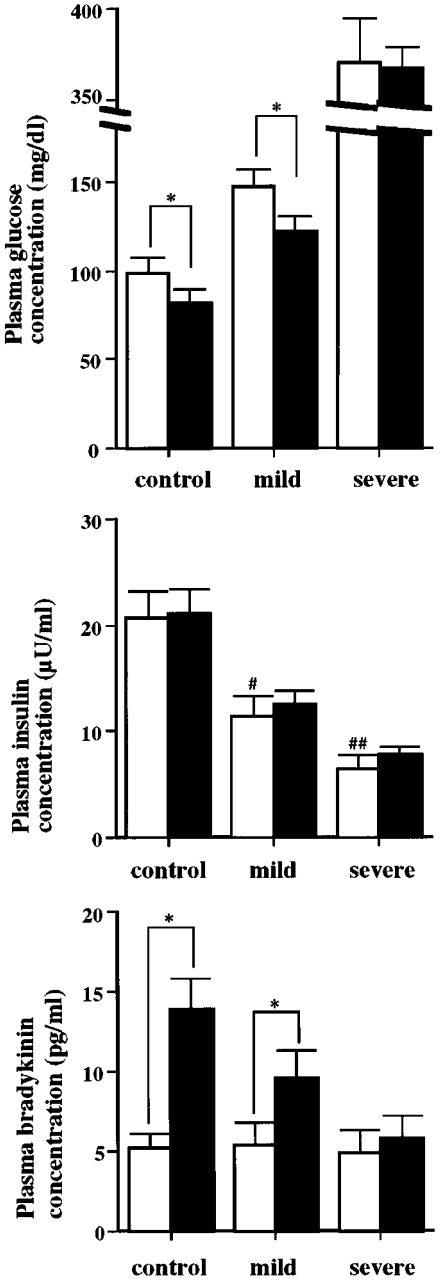

Fig 3. Changes in plasma glucose, insulin, and bradykinin concentrations in normal and STZ-induced diabetic rats before and after exercise. Rats were injected with STZ 45 mg/kg body weight (mild) or 65 mg/kg body weight (severe). Plasma glucose, insulin, and bradykinin were analyzed before (○) and after (■) exercise. Results are the mean ± SEM of 6 paired experiments. *$P < .05$ *v* before exercise. #$P < .05$ *v* control. ##$P < .05$ *v* mild.

exercise relative to baseline in the E group (from 5.3 ± 2.9 to 12.9 ± 4.9 pg/mL, $P < .05$) and E-H group (from 5.5 ± 3.1 to 12.4 ± 4.2 pg/mL, $P < .05$) (Fig 4).

In contrast, these parameters did not change after the resting period compared with basal values in both the S group and S-H group.

Rat body weight, muscle weight, and protein concentration in dissected muscles. Body weight and muscle weight were not different among the S, E, and E-H groups. Total homogenate protein represented 10% to 15% of the total muscle (data not shown) used for each experiment and was not significantly different among the 3 groups. Protein concentrations of PM and LDM fractions were similar among the 3 groups. Furthermore, there was no significant difference in body weight and muscle

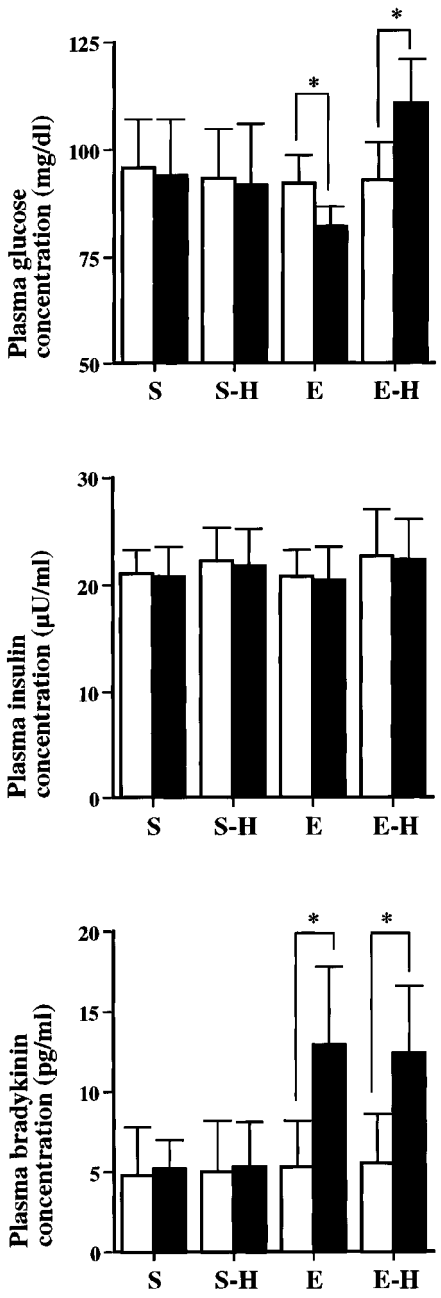

Fig 4. Changes in plasma glucose, insulin, and bradykinin concentrations in S, S-H, E, and E-H rats before and after exercise or rest. Plasma glucose, insulin, and bradykinin were analyzed before (□) and after (■) exercise or rest. Results are the mean ± SEM. *$P < .05$ *v* before exercise.

weight and protein concentrations of PM and LDM in the S-H group (Table 2).

KpNPPase activity in homogenate and membrane fractions. To confirm the purity of the membrane fractionation, we measured K*p*NPPase activity in homogenate and membrane fractions prepared from the test muscles. The activity of K*p*NPPase in the homogenate, PM, and LDM fractions was not different in muscles prepared from the 3 groups. The recovery of K*p*NPPase activity in the PM fraction (expressed as a percent of activity in the total homogenate) was not significantly different among the 3 groups. The plasma membrane K*p*NPPase enrichment relative to the enzyme activity of the total homogenate, an index of purity of the plasma membrane marker, was not significantly different among the 3 groups. Furthermore, K*p*NPPase activity in the homogenate, PM, and LDM of the S-H group was not significantly different compared with the 3 groups (6.4 ± 0.8, 115.6 ± 10.2, and 30.8 ± 2.8 nmol $\cdot$ mg^{-1} $\cdot$ min^{-1}, respectively).

Glucose transport measurements. The V_{max} of glucose transport in the exercise groups (E and E-H) was significantly ($P < .05$) higher versus the S group, and the increases were abolished by HOE-140 treatment (Fig 5, inset). In the S-H group, the V_{max} (3.8 ± 0.9 nmol $\cdot$ mg $\cdot$ protein^{-1} $\cdot$ s^{-1}) was not significantly different compared with the S group. On the other hand, the half-maximal dissociation constant ($K_{1/2}$) was not significantly different among the 4 groups ($K_{1/2} = 19 \pm 5.2$ mmol/L glucose in the S-H group). Therefore, HOE-140 treatment significantly suppressed the exercise-induced increase in V_{max} by 35% ($P < .05$) without changing the value of $K_{1/2}$.

GLUT-4 translocation after exercise. To investigate whether the increase in glucose transport paralleled the increase in GLUT-4 translocation in skeletal muscle, we examined GLUT-4 translocation from LDM to PM in muscles prepared from the S, E, and E-H groups. Exercise and/or HOE-140 infusion did not change the protein content of GLUT-4 in total homogenates prepared from the muscles (data not shown). A 50- to 55-kd band corresponding to GLUT-4 was observed in both LDM and PM fractions in all 3 groups (Fig. 6). Exercise alone (E) significantly decreased the GLUT-4 content in LDM by 45% ($P < .05$) and significantly increased it in PM by 94% ($P < .05$). Exercise with HOE-140 treatment (E-H) also decreased GLUT-4 content by 28% in LDM ($P < .05$) and increased it by 42% in PM ($P < .05$). However, the decrease of GLUT-4 content in LDM and its increase in PM in the E-H group were significantly ($P < .05$) smaller than those in the E group. The GLUT-4 content of PM and LDM in the S-H group was not significantly different compared with the S group (98% $\pm$ 4% in PM and 102% $\pm$ 6% in LDM *v* S group as 100%). On the other hand, there was no difference in the GLUT-1 content of the total muscle homogenate, LDM, and PM fractions in every group as evaluated by immunoblotting with anti–GLUT-1 antibody (data not shown).

Effect of HOE-140 treatment on tyrosine phosphorylation of insulin receptor β-subunit and IRS-1 and PI 3-kinase activity associated with anti–phosphotyrosine antibody immunoprecipitate. Without insulin injection, phosphorylation of the insulin receptor and IRS-1 was not detected in all 4 groups (Fig 7). On the other hand, insulin injection markedly increased tyrosine phosphorylation of both the insulin receptor β-subunit and IRS-1. HOE-140 treatment in sedentary rats did not change

Table 2. Body Weight of Rats, Weight and Protein Concentration of Dissected Muscle, and Specific Activity of Marker Enzyme in Homogenate and Membrane Fractions Prepared From Muscle

Parameter	S Group (n = 6)	E Group (n = 6)	E-H Group (n = 6)
Body weight (g)	208 ± 8	212 ± 9	208 ± 14
Muscle weight (g)	4.15 ± 0.35	4.05 ± 0.23	4.07 ± 0.19
Protein concentration (mg/mL)			
Homogenate	9.30 ± 1.04	8.96 ± 1.11	8.90 ± 0.98
PM	3.70 ± 1.19	3.22 ± 1.24	3.74 ± 1.50
LDM	2.95 ± 1.18	2.83 ± 0.46	2.79 ± 1.14
K*p*NPPase activity (nmol · mg^{-1} · min^{-1})			
Homogenate	6.3 ± 0.4	6.8 ± 0.7	6.6 ± 0.5
PM	116.4 ± 12.4	113.2 ± 9.6	120.2 ± 11.4
Recovery (%)	9.8 ± 0.6	8.6 ± 1.2	10.2 ± 0.8
Fold enrichment	19.4 ± 2.2	18.5 ± 3.1	20.2 ± 3.5
LDM	31.1 ± 3.2	31.2 ± 1.4	31.4 ± 1.3
Recovery (%)	2.0 ± 0.2	1.9 ± 0.4	1.9 ± 0.3
Fold enrichment	4.8 ± 0.9	4.5 ± 0.8	4.7 ± 0.7

NOTE. Values are the mean ± SEM (n = 6). Total K*p*NPPase activity of the homogenate and membrane fraction was determined by multiplying their specific activities by the respective protein recoveries. Percent recovery represents the product of total enzyme activity of the membrane fraction and total enzyme activity of the homogenate multiplied by 100.

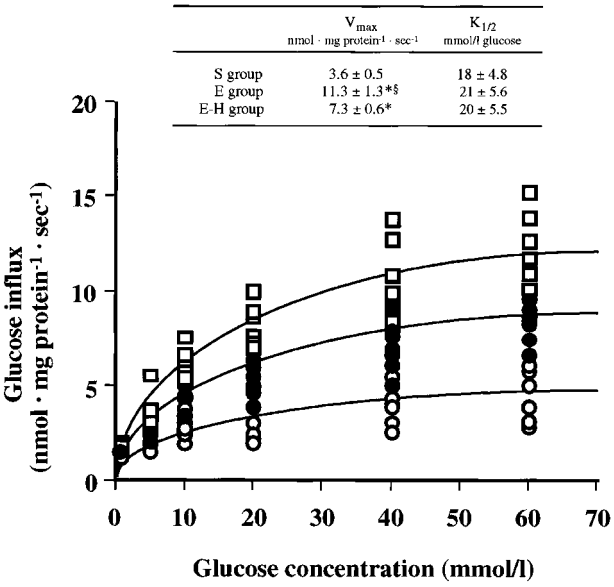

Fig 5. Carrier-mediated glucose influx versus glucose concentration for skeletal muscle plasma membranes isolated from S, E, and E-H rats. Plotted values of S (○), E(□), and E-H (●) groups are the rates of D-glucose and L-glucose uptake at each glucose concentration. The fitted line represents the nonlinear least-squares fit of the data. *Inset,* V_{max} and $K_{1/2}$ were calculated by a nonlinear least-squares fit of the data. Results are the mean ± SEM of 6 paired experiments. *$P < .05$ *v* S group. §$P < .05$ *v* E-H group.

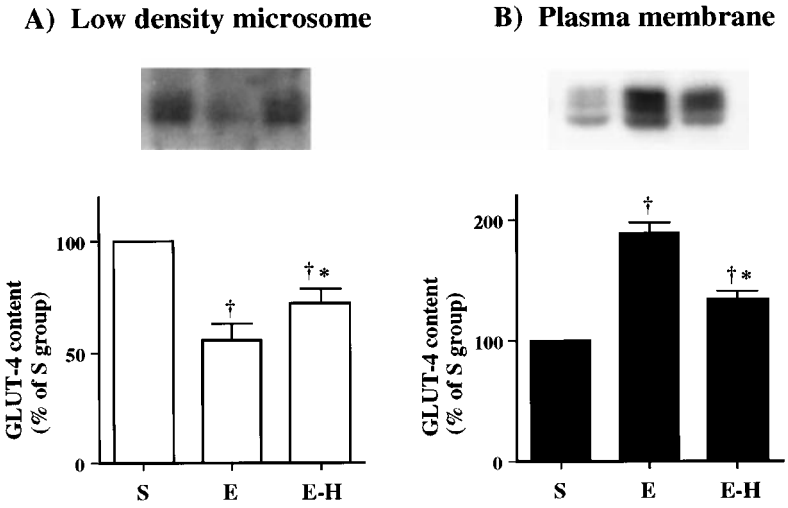

Fig 6. Effect of HOE-140 on exercise-induced GLUT-4 translocation in rat skeletal muscle. Representative results of immunoblot analysis with anti–GLUT-4 antibody in LDM (A) and PM (B) fractions and summary graph of GLUT-4 content in LDM (A) and PM (B) fractions are shown. GLUT-4 content was measured by Western blot analysis. Values are expressed as a percent of the S group. Results are the mean ± SEM (n = 6). †$P < .05$ *v* S group. *$P < .05$ *v* E group.

insulin-induced tyrosine phosphorylation of the insulin receptor β-subunit and IRS-1 (S group *v* S-H group). Exercise did not affect the insulin-induced increase of tyrosine phosphorylation of the insulin receptor β-subunit and IRS-1, while the insulin-induced tyrosine phosphorylation of the insulin receptor β-subunit and IRS-1 in the exercise group was attenuated by treatment with HOE-140 (E-H group with insulin stimulation, by 32% in insulin receptor β-subunit and by 40% in IRS-1 *v* E group with insulin stimulation). The content of insulin receptor β-subunit and IRS-1 in immunoprecipitates was comparable in all groups, as shown in the blot with each antibody (Fig 7A and B).

Similar to the results of insulin-induced phosphorylation of the insulin receptor and IRS-1, insulin-stimulated PI 3-kinase activity in immunoprecipitates with anti–phosphotyrosine antibody was not significantly different among the S, S-H, and E groups. However, PI 3-kinase activity with insulin stimulation in the E-H group was significantly attenuated by 36% compared

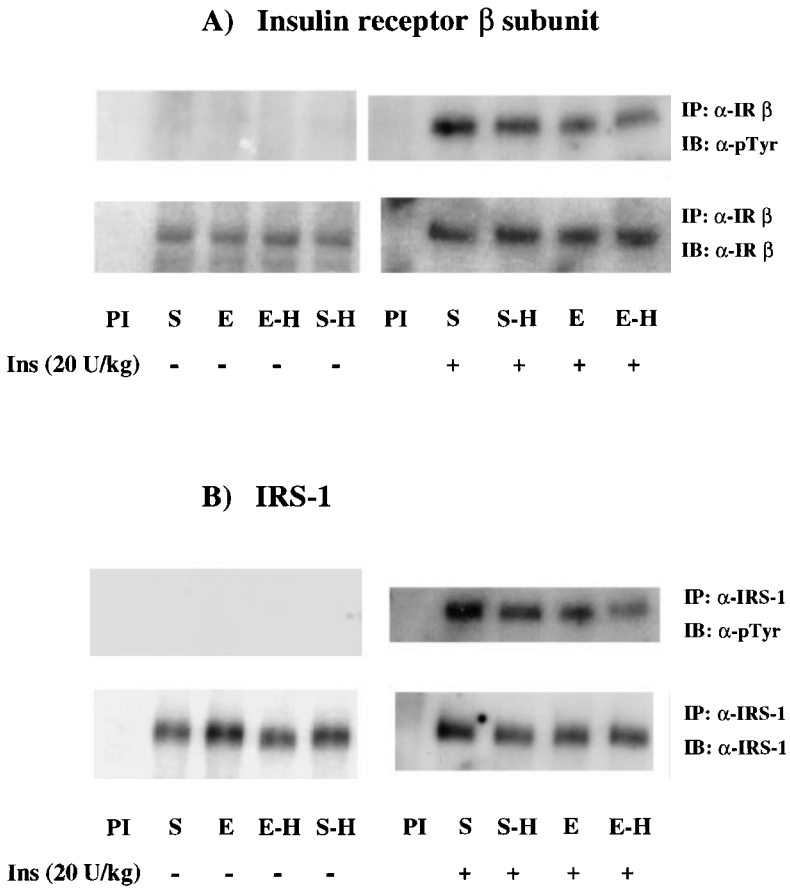

Fig 7. Effect of exercise on insulin-stimulated tyrosine phosphorylation of insulin receptor β-subunit and IRS-1 in rat skeletal muscle. Aliquots of protein (5 mg) from rat skeletal muscle were immunoprecipitated with anti–insulin receptor antibody (A) or anti–IRS-1 antibody (B) and then immunoblotted with anti–phosphotyrosine antibody (A and B, top panels) or blotted with either anti–insulin receptor β-subunit (A lower panel) or anti–IRS-1 antibody (B lower panel). Left and right panels in both A and B indicate data in the condition without (left) and with (right) insulin, respectively. The phosphorylation level and content of insulin receptor β-subunit and IRS-1 were examined. Each experiment was performed 3 times on separate occasions. A representative blot is shown. PI, preimmune IgG; Ins, insulin.

with the E group (Fig 8). There was no significant difference in PI 3-kinase activity in all groups measured in anti-p85 immunoprecipitates (data not shown).

DISCUSSION

In the present study, we first demonstrated that the plasma bradykinin concentration increased during exercise in both normal subjects and type 2 diabetic patients with relatively good glycemic control, but not in those with poor glycemic control. To confirm the results observed in human studies, we performed animal experiments. In protocol 1, we evaluated the acute effects of 1-hour swimming exercise in nondiabetic rats and rats with mild or severe STZ-induced diabetes. Our results showed that exercise increased plasma bradykinin in nondiabetic and mildly diabetic rats, but not in severely diabetic rats. These results are compatible with those of the human studies.

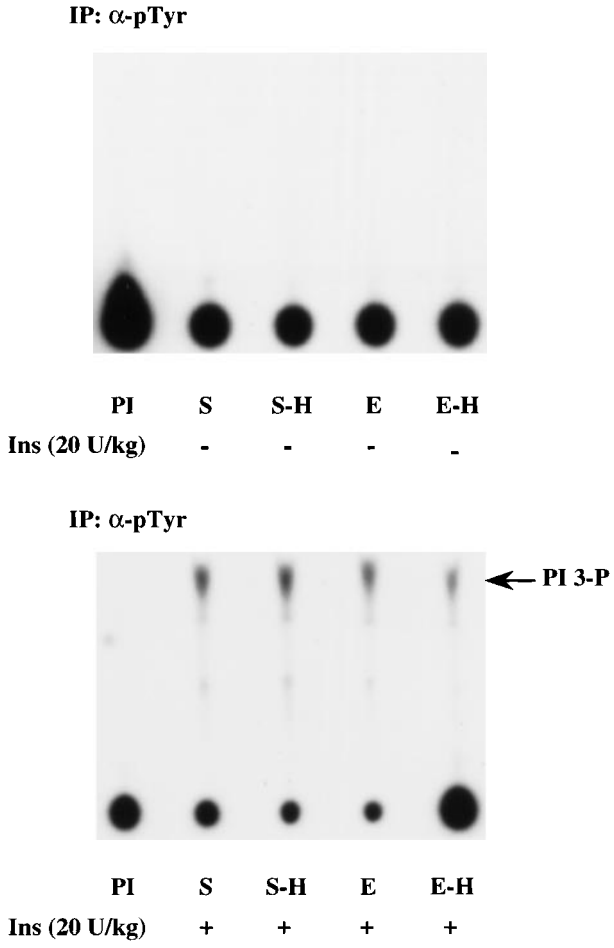

Fig 8. Effect of exercise on insulin-stimulated PI 3-kinase activity in rat skeletal muscle. Equal amounts (5 mg) of solubilized protein were immunoprecipitated with anti–phosphotyrosine antibody. PI 3-kinase assay was performed in the presence of phosphatidylinositol and [γ-^{32}P]ATP, and lipid products were separated by thin-layer chromatography. Spots that comigrated with a PI 3-phosphate (PI 3-P) standard were quantified by densitometry. Upper and lower panels indicate data in the condition without (upper) and with (lower) insulin, respectively. The experiment was performed 3 times on separate occasions. A representative blot is shown. PI, preimmune IgG; Ins, insulin.

Previous studies have shown that the venous bradykinin concentration increases after exercise in humans. Wicklmayr et al[10] and Rett et al[11] found that plasma kinin concentrations in the forearm increased after isometric work in nondiabetic subjects. They postulated that such an increase of kinins in contracting muscles was due to the activation of prekallikrein-kallikrein conversion, a decrease of plasma kininase activity by a decrease in pH, and an increase and a decrease of the venous concentration of kinins and kininogen, respectively.[27] In another study, they also demonstrated that exercise increased plasma bradykinin liberation from contracting skeletal muscle in healthy subjects and type 1 diabetic patients, but not in type 2 diabetic patients.[28] However, in the present study, we found that plasma bradykinin increased during exercise in type 2 diabetic patients and diabetic rats with relatively good glycemic control, but not in those with poor glycemic control. The differences in the results are probably due to differences in glycemic control, because other parameters were not different between the 2 groups in humans and rats, and we also considered that there was no significant difference in the intensity of exercise among the 3 groups in the human experiment. However, since we have not measured the absolute workload (watts) for each group in human studies, we cannot rule out the possibility that the blunted bradykinin response observed in poorly controlled type 2 diabetic patients could be related to differences in absolute workload. Recently, Kennedy et al[29] have reported that exercise-induced GLUT-4 translocation is normal in skeletal muscle from patients with type 2 diabetes. Since their subjects with type 2 diabetes were in a relatively better-controlled glycemic state (HbA_{1c}, 8.8% ± 0.7%), more obese (BMI, 27.2 kg/m^2), and older (49 ± 6 years) than the poorly controlled diabetic patients in our study (HbA_{1c}, 11.6% ± 2.6%; BMI, 21.7 ± 6.6 kg/m^2; age, 32 ± 6 years), it might be hard to compare these 2 results directly.

An explanation for the relationship between changes in plasma bradykinin during exercise and the condition of glycemic control in diabetes is not readily apparent. Other investigators have reported that STZ-induced diabetic rats develop a rapid decrease in kallikrein protein and mRNA levels in the kidney, the site of abundant kallikrein synthesis (the enzyme that cleaves kinin from substrate kininogen). Replacement of insulin in these rats returned the mRNA level toward normal.[30] Based on these findings and the results of the present studies in humans and rats, it is possible that kinins and kallikrein levels might decrease in various tissues and organs, including skeletal muscle, in the diabetic state. Further analysis of the kallikrein mRNA level in tissues of diabetic rats is necessary.

We observed a significant decrease in plasma glucose following exercise and an elevation in plasma bradykinin in the rat study. On the other hand, in the human study, it was shown that plasma glucose levels were not significantly different following exercise; however, plasma bradykinin levels were significantly elevated. The difference in the glycemic response to exercise between human and animal experiments could be a result of a difference in the intensity of exercise. In human studies, we loaded each individual with 20 minutes of aerobic exercise which seemed equivalent to the intensity of 50% to 80% maximal oxygen consumption, since this exercise was reported to be suitable for diabetic patients.[31] On the other hand, in

animal studies, the rats were loaded with the constant intensity of exercise consisting of a 60-minute bout of swimming without consideration of the aerobic state.

We have previously reported that increased plasma bradykinin was associated with increased glucose uptake in vivo and in vitro.[8,13,14] We extended these findings and examined in the present study the effect of elevated plasma bradykinin levels during exercise on glucose transport in rat skeletal muscle. Most previous reports have shown that exercise increases glucose transport in skeletal muscles through an increase in the V_{max} without an appreciable change in the K_m as demonstrated in saturation kinetics studies.[32,33] Such an increase in the V_{max} reflects an increase in the number of functional glucose transporter proteins present in the PM and/or an increase in the rate of glucose transport by each carrier protein.[1] In the present study, to estimate the effect of an exercise-induced increase in bradykinin on glucose transport, we studied glucose transport activity in the PM fraction prepared from muscles and confirmed that exercise increased the V_{max} of glucose transport in PM. Interestingly, HOE-140 resulted in a 35% decrease in the exercise-induced increase in the V_{max} without changing the $K_{1/2}$ value. Since HOE-140 is a specific inhibitor of bradykinin, these results suggested that bradykinin is partly involved in the increased glucose transport activity in muscle. The dose of HOE-140 used in the present experiments seemed sufficient to inhibit the effects of endogenous kinins, because we found that 150 $\mu g \cdot kg^{-1} \cdot d^{-1}$ ($<$50% of the dose of HOE-140 used in our study) produced approximately a 50% inhibition of the depressor effect of 100 ng exogenous bradykinin (data not shown).

Acute exercise is known to increase GLUT-4 translocation without changing the total content of GLUT-4 protein in the muscle, while long-term exercise increases the amount of GLUT-4 protein.[34] It is also known that an increase in the number of glucose transporters in skeletal muscle induced by acute exercise is almost due to the translocation of GLUT-4 from the intracellular pool to the plasma membrane.[1,3,35,36] To study the effect of an exercise-induced increase in bradykinin on GLUT-4 translocation, we measured the GLUT-4 content in PM and LDM purified by fractionation with ultracentrifuge. Our results showed that HOE-140 treatment prior to exercise inhibited exercise-induced GLUT-4 translocation. However, the inhibitory effect was partial, reducing translocation by only 52% in PM. This could be explained by increases in other glucose transporters. Accordingly, we analyzed the content of GLUT-1, another isoform of glucose transporter expressed in muscle tissue.[6,37] However, the GLUT-1 content in PM and LDM after exercise was not changed. Therefore, exercise-induced GLUT-4 translocation may be regulated not only by bradykinin but also by other factors such as 5′ AMP–activated protein kinase,[38-40] adenosine,[41,42] and nitric oxide.[43,44] In the E-H group, plasma glucose was elevated following exercise despite the significant increase in glucose transport and GLUT-4 translocation compared with the S group. At first, we expected that blood glucose levels after exercise in the E-H group may not change or may slightly decrease compared with the E group, since the effect of bradykinin in the E-H group would be abolished. According to the data for glucose transport and GLUT-4 translocation, it is unlikely that the increase in blood glucose in the E-H group is due to decreased glucose uptake in skeletal muscle. Therefore, increased glucose production in the liver may be responsible for this phenomenon. Although an exact explanation is not readily apparent, it is possible that the effect of HOE-140 during exercise is different in peripheral tissues including muscle versus the liver. Further analysis to examine glucose utilization in the liver will be necessary to clarify this point.

To evaluate the effect of bradykinin on insulin sensitivity in postexercise skeletal muscle, we next examined the effect of increased bradykinin on insulin-stimulated phosphorylation of the insulin receptor and IRS-1 and PI 3-kinase activity in rat muscle in the postexercise period. Our results showed that in rats pretreated with HOE-140 prior to exercise, insulin-stimulated tyrosine phosphorylation of insulin receptor β-subunit and IRS-1 and PI 3-kinase activity were significantly lower than those in exercising but untreated rats. Moreover, the decrease in insulin-stimulated phosphorylation of IRS-1 in the E-H group paralleled the decrease of insulin-stimulated insulin receptor phosphorylation and of insulin-stimulated PI 3-kinase activation. However, HOE-140 itself did not affect the early step of insulin signaling, since tyrosine phosphorylation of the insulin receptor and IRS-1 and PI 3-kinase activity were not significantly different between the S group and S-H group. In an earlier study, we suggested that bradykinin could inhibit the dephosphorylation of insulin receptor β-subunit.[13] More recently, we also demonstrated that bradykinin enhanced insulin signaling in 32D cells transfected with B_2 receptor, insulin receptor, and IRS-1.[45] In the current study, we could not confirm the hypothesis that the increment of bradykinin during exercise directly potentiates insulin-induced tyrosine phosphorylation of the insulin receptor in skeletal muscle following exercise. The present result indicates that exercise does not increase insulin receptor tyrosine kinase activity, which is almost identical to previous data from other investigators in rat[46-48] and human[49] skeletal muscle. However, we also found that the inhibition of bradykinin signal using bradykinin antagonist resulted in a decrease in insulin receptor phosphorylation following exercise. Therefore, it is plausible that bradykinin participates in the enhancement of insulin receptor tyrosine phosphorylation in skeletal muscle after contraction.

Most previous studies indicated that exercise and insulin use distinct signaling pathways that lead to the activation of glucose transport in skeletal muscle.[1,2,50] The present study extends these findings by indicating that bradykinin might stimulate glucose transport through several signaling pathways, including an insulin-independent increase in GLUT-4 translocation in PM and an enhancement of the insulin signaling pathway through potentiation of insulin receptor tyrosine phosphorylation.

In conclusion, our human and animal experiments demonstrate that bradykinin generated in the contracting muscle during exercise is at least partly involved in modulating exercise-induced glucose uptake through enhancement of GLUT-4 translocation, resulting in lower blood glucose levels during exercise. Such an increase in plasma bradykinin was influenced by the status of glycemic control in type 2 diabetic patients.

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