The B2 Receptor of Bradykinin Is Not Essential for the Post-Exercise Increase in Glucose Uptake by Insulin-Stimulated Mouse Skeletal Muscle

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Summary
Bradykinin can enhance skeletal muscle glucose uptake (GU), and exercise increases both bradykinin production and muscle insulin sensitivity, but bradykinin’s relationship with post-exercise insulin action is uncertain. Our primary aim was to determine if the B2 receptor of bradykinin (B2R) is essential for the post-exercise increase in GU by insulin-stimulated mouse soleus muscles. Wildtype (WT) and B2R knockout (B2RKO) mice were sedentary or performed 60 minutes of treadmill exercise. Isolated soleus muscles were incubated with [3H]-2-deoxyglucose ±insulin (60 or 100 μU/ml). GU tended to be greater for WT vs. B2RKO soleus with 60 μU/ml insulin (P=0.166) and was significantly greater for muscles with 100 μU/ml insulin (P<0.05). Both genotypes had significant exercise-induced reductions (P<0.05) in glycemia and insulinemia, and the decrements for glucose (~14 %) and insulin (~55 %) were similar between genotypes. GU tended to be greater for exercised vs. sedentary soleus with 60 μU/ml insulin (P=0.063) and was significantly greater for muscles with 100 μU/ml insulin (P<0.05). There were no significant interactions between genotype and exercise for blood glucose, plasma insulin or GU. These results indicate that the B2R is not essential for the exercise-induced decrements in blood glucose or plasma insulin or for the post-exercise increase in GU by insulin-stimulated mouse soleus muscle.

Key words
Glucose transport • Insulin sensitivity • Insulin resistance • Kinin • Physical activity

Introduction
A single exercise bout can lead to subsequently improved whole body insulin sensitivity, and skeletal muscle is the major tissue that accounts for this exercise-induced improvement in glucose disposal (Henriksen 2002, Richter et al. 1982). The increased glucose uptake by muscle is evident several hours after exercise cessation in vivo (Richter et al. 1989), and persists when rodent skeletal muscles are dissected out after exercise and studied in vitro (Cartee et al. 1989, Hamada et al. 2006).

Skeletal muscle cells express the B2 receptor of bradykinin, B2R (Duka et al. 2006, Figueroa et al. 1996, Rabito et al. 1996), which is important for bradykinin's influence on glucose uptake (Beard et al. 2006, Duka et al. 2001, Figueroa et al. 1996). We studied mice that were null for B2R (B2 receptor of bradykinin knockout, B2RKO) and normal, wildtype (WT) mice under sedentary and post-exercise conditions to test the hypothesis that the B2R is essential for the post-exercise increase in glucose uptake by insulin-stimulated skeletal muscle.

**Methods**

**Materials**

Human recombinant insulin was from Eli Lilly (Indianapolis, IN). 2-Deoxy-[3H]glucose and [14C]mannitol were from Perkin-Elmer (Boston, MA). Reagents and apparatus for SDS-PAGE, nonfat dry milk, and nitrocellulose membranes were from Bio-Rad Laboratories (Hercules, CA). A bicinchoninic acid assay kit for total protein determination and SuperSignal WestDura Extended Duration Substrate for immunodetection were from Pierce Biotechnology (Rockville, MD). Other reagents were from Sigma-Aldrich (St. Louis, MO).

**Animals**

Animal care was approved by the University of Michigan Committee on Use and Care for Animals. Male mice null for the B2 receptor of bradykinin (B2RKO; strain 002641) and wildtype (WT) control mice (strain 101045; B6129SF2/J) were from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in plastic cages and provided a standard diet (Lab Diet, PMI Nutrition International, Richmond, IN) and water ad libitum.

**Treadmill exercise protocol**

All mice (8-18 wk old) were familiarized with treadmill (Columbus Instruments, Columbus, OH) running for 10 min on 2 consecutive days (1st day at 12-22 m/min; 2nd day at 12-25 m/min). On the morning after the 2nd familiarization day, the WT and B2RKO mice were assigned to either a sedentary or exercised group. The exercise protocol consisted of 3 consecutive 20 min-periods of progressive interval exercise (5 min at 15 m/min, 10 min at 20 m/min, and 5 min at 25 m/min with 0 % slope) totaling 60 min of running (Hamada et al. 2006). Access to food was removed for all mice at the time that the protocol began. All of the exercised mice completed the 60 min protocol, after which exercised and sedentary mice were anesthetized (intraperitoneal injection of pentobarbital sodium, 50 mg/kg body wt).

**Blood glucose and plasma insulin**

Blood was collected from the tail using heparinized capillary tubes, prior to anesthetization, in sedentary mice and in exercise mice immediately following the 60 min treadmill protocol. Blood glucose was determined using an Accu-Check® Aviva (Roche Diagnostics, Indianapolis, IN) hand-held blood glucose meter. Blood was transferred to microcentrifuge tubes, centrifuged, and the plasma collected was used to assay insulin with the ALPCO Diagnostics™ Insulin (Mouse) Ultrasensitive EIA kit, catalog no. 80-INSMSU-E01 (Alpco Diagnostics, Salem, NH).

**Tissue dissection and in vitro soleus incubation**

Paired soleus muscles from anesthetized mice were excised and incubated using a 2-step incubation protocol. During the 1st step, muscles were placed in vials containing 1.5 ml of Krebs-Henseleit Buffer (KHB) supplemented with 0.1 % bovine serum albumin (BSA), 2 mM sodium pyruvate, and 6 mM mannitol in the absence or presence of insulin (60 μU/ml insulin was used for mice in Experiment 1; 100 μU/ml insulin was used for mice in Experiment 2) for 60 min. During all incubation steps, vials were placed in a heated (35 °C), shaking water bath and continuously gassed from above (95 % O2-5 % CO2).

After the 1st incubation step, muscles were transferred to a 2nd vial containing KHB with 0.1 % BSA, 1 mM 2-deoxyglucose (2-deoxy-[3H]glucose, 6 mCi/mmol), 9 mM mannitol ([14C]mannitol, 0.053 mCi/mmol), and the same insulin concentration as the previous step. Muscles were incubated at 35 °C for 15 min and then rapidly blotted on ice-cold filter paper, trimmed, freeze clamped, and stored at –80 °C until processed.

Epididymal fat pads and gastrocnemius muscles were removed from some mice and weighed.

**Muscle homogenization**

Frozen muscles were weighed, transferred to prechilled glass tubes and homogenized in: 1) 0.5 ml of perchloric acid (muscles from Experiment 1) or 2) ice-
cold lysis buffer (0.5 ml) containing 20 mM Tris-HCl, 150 mM NaCl, 1 % NP-40, 1 mM activated Na3VO4, 2 mM EDTA, 2 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 μg/ml leupeptin (muscles from Experiment 2). Lysis buffer was used for the muscles analyzed for both glucose uptake and immunoblotting. Samples homogenized in perchloric acid were centrifuged (15,000g, 15 min), and samples homogenized in the lysis buffer were rotated for ~1 hr before being centrifuged (15,000g, 15 min). Aliquots from supernatants were quantified for [3H] and [14C] using a liquid scintillation counter, and glucose uptake was calculated (Cartee and Bohn 1995).

**Immunoblotting**

Portions of samples processed in lysis buffer were analyzed by immunoblotting for phospho-Akt Thr308. Total protein concentration of the supernatants used for immunoblotting was determined by the bicinchoninic acid assay. Samples were resolved on a 10 % SDS-PAGE gel and transferred to nitrocellulose in electrotransfer buffer overnight at 4 °C. Blots were incubated in blocking solution [Tris-buffered saline (TBS) with 0.1 % Tween 20 (TBST) and 5 % nonfat dry milk] for 1 hr at room temperature, washed with TBST and then incubated with anti-phospho-Akt Thr308 overnight at 4 °C. Blots were washed with TBST and incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG). Blots were washed of excess antibody with TBST and then subjected to SuperSignal enhanced chemiluminescence (Thermo Scientific, Rockford, IL). Immunoreactive protein was quantified by densitometry (Alpha Innotech, San Leandro, CA). The mean value for WT sedentary samples on each immunoblot, expressed in densitometry units relative to total protein, was adjusted to equal 1.0. Each sample value was expressed relative to the adjusted mean value for the WT sedentary control.

**Statistical analyses**

Statistical analyses used Sigma Stat version 2.0 (San Rafael, CA). Data are expressed as means ± SE. Two-way ANOVA was used to determine significant differences, and a Tukey post hoc test was used to identify the source of significant variance. A P value ≤0.05 was considered statistically significant.

**Results**

There were no statistically significant interactions (exercise x genotype) for any of the measurements that were made.

**Body and tissue masses**

Body mass was similar between genotypes, but the epididymal fat pad/body mass ratio was ~30-37 % lower (P<0.001) for B2RKO compared to WT mice (Table 1). The soleus/body mass ratio was ~8-12 % greater (P<0.05) for B2RKO vs. WT mice, but there was not a significant effect of genotype on gastrocnemius/body mass ratio.

**Blood glucose and plasma insulin**

Glycemia was 14 % greater in B2RKO vs. WT mice under both sedentary and exercised conditions (P<0.01; Table 1). Within each genotype, exercise caused a similar ~14 % decrease in blood glucose (P<0.05). Plasma insulin was ~32 % lower (P<0.05) in the B2RKO
vs. WT mice in sedentary and exercised conditions. In both genotypes, exercise caused a 55% decrease in plasma insulin (P<0.001).

**Muscle glucose uptake**

There was not an effect of genotype on glucose uptake without insulin in either experiment (Fig. 1A and 1B) or when the data from both experiments were pooled (data not shown). Glucose uptake with insulin was lower for B2RKO vs. WT mice with 100 µU/ml insulin (P<0.05), and a non-significant trend (P=0.166) for lower values in the B2RKO mice was also evident with 60 µU/ml insulin. For the pooled data from the insulin-treated soleus, glucose uptake was significantly lower (P<0.05) in the B2RKO compared to WT mice. Paired muscles were used for glucose uptake measurements, with one muscle from each mouse incubated without insulin and the contralateral muscle incubated with insulin. We calculated the insulin-stimulated increase in glucose uptake (delta insulin) by subtracting the basal value from the insulin-stimulated value. Delta insulin was significantly lower for B2RKO vs. WT mice with 60 µU/ml (P<0.05), 100 µU/ml (P<0.05) and when the data from both experiments were pooled (P<0.001).

Glucose uptake without insulin tended to be slightly higher for exercised vs. sedentary mice in both Experiment 1 (P=0.090; Fig. 1A) and Experiment 2 (P=0.087; Fig. 1B). The pooled values for glucose uptake without insulin from both experiments were significantly greater for the exercised vs. sedentary (P<0.05). In the insulin-stimulated soleus, glucose uptake tended to be greater for the exercised vs. sedentary mice in Experiment 1 (P=0.063 with 60 µU/ml; Fig. 1A) and was significantly greater in Experiment 2 (P<0.05 with 100 µU/ml; Fig. 1B). When data from both experiments were pooled, glucose uptake of insulin-stimulated muscles was significantly greater (P<0.005) for exercised vs. sedentary mice. Delta insulin tended to be greater for exercised vs. sedentary mice with 60 µU/ml (P=0.150) or 100 µU/ml (P=0.094), and the delta insulin values were significantly greater after exercise when the data from both experiments were pooled (P<0.05).
Akt Thr\(^{308}\) phosphorylation

Soleus Akt Thr\(^{308}\) phosphorylation was not significantly altered by exercise or genotype, with or without insulin, or for delta insulin (Fig. 2).

### Discussion

Improved whole body insulin sensitivity is a hallmark-effect of prior exercise. A post-exercise elevation in insulin-stimulated glucose uptake by skeletal muscle has been found in humans (Richter et al. 1989, Wojtaszewski et al. 2000), rats (Cartee and Holloszy 1990, Cartee et al. 1989, Richter et al. 1982), and mice (Bonen and Tan 1989, Bonen et al. 1984, Hamada et al. 2006). The primary aim of this study was to determine if the B2R is essential for the post-exercise increase in glucose uptake in insulin-stimulated skeletal muscle. Prior exercise resulted in an increased glucose uptake by muscles incubated with 100 µU/ml insulin and a trend (P=0.063) for increased glucose uptake by muscles incubated with 60 µU/ml insulin. The lack of a significant interaction between genotype and exercise on glucose uptake by muscles incubated with either insulin concentration indicates that the B2R is not essential for the post-exercise increase in glucose uptake. This study focused on glucose uptake, which is a rate-controlling process for skeletal muscle glucose metabolism. However, the current results do not eliminate the possibility that the B2R may influence other aspects of muscle metabolism, including glycogen synthesis, glucose oxidation, lipid synthesis and fatty acid esterification that we did not assess.

Earlier research demonstrated that circulating bradykinin can be increased by exercise or muscle contraction (Blais et al. 1999, Boix et al. 2005, Langberg et al. 2002, Stebbins et al. 1990, Taguchi et al. 2000). Furthermore, there is substantial evidence that bradykinin can elevate glucose uptake in both intact animals (Damas et al. 1999, Duka et al. 2001, Kohlman et al. 1995, Uehara et al. 1994) and isolated cells or tissues (Beard et al. 2006, Damas et al. 2004, Henrikson et al. 1999, Isami et al. 1996, Kudoh et al. 2000, Miyata et al. 1998). It seemed possible that bradykinin could be involved in the post-contracture increase in insulin sensitivity, but because bradykinin is very rapidly degraded by kininases, experiments using exogenous bradykinin should be interpreted cautiously. Accordingly, in the current study, we used in vivo exercise by mice lacking the B2R as a novel approach to assess bradykinin’s potential importance for the persistent increase in insulin-stimulated glucose uptake after in vivo exercise. The current results for in vivo exercise extend the earlier findings using exogenous bradykinin or B2 receptor inhibitors with ex vivo muscle contractions. These results using several different approaches do not support the idea...
that exercise or contraction lead to subsequent elevation in insulin sensitivity as the result of a B2R-dependent mechanism.

The ~14% decline in blood glucose in both genotypes compares to an ~12-29% decrease in glycemia previously reported for normal mice after 60 min of treadmill exercise (Fritsche et al. 2010, Hoene et al. 2009, Howlett et al. 2002, Wojtaszewski et al. 1999). Treadmill exercise caused an ~32% decline in plasma insulin concentration in both genotypes, which compares to published results indicating an ~33-52% reduction in circulating insulin after 60 min of treadmill exercise by normal mice (Fritsche et al. 2010, Hamada et al. 2006, Higaki et al. 1999, Hoene et al. 2009). The absence of significant exercise x genotype interactions in the current study suggests that the expression of the B2R was not essential for exercise effects on either glycemia or insulinemia.

Glucose uptake by isolated muscles in the absence of insulin was not different for B2RKO compared to WT mice. However, for muscles with 100 µU/ml insulin there was a genotype-associated reduction in glucose uptake for B2RKO vs. WT mice. These results are reminiscent of the previously reported results of a study using the euglycemic-hyperinsulinemic-clamp in which Duka et al. (2001) reported a lower glucose infusion rate for B2RKO compared to WT mice. Duka et al. (2001) reported a non-significant trend for a 13% increase in fasting glycemia for B2RKO vs. WT mice which corresponds to the 14% increase in blood glucose that we found in B2RKO mice. We found a lower value for plasma insulin concentration in the B2RKO vs. WT mice, whereas Duka et al. (2001) did not find a significant difference between the genotypes for fasting insulin. Rather they found a non-significant trend for higher fasting insulin for B2RKO mice. The mice in the earlier study had been treated for 3 days prior to the clamp with captopril, an ACE inhibitor which also inhibits kininase II (the enzyme which degrades bradykinin). Furthermore, the mice in the current study were only fasted ~1 hr prior to sampling plasma for the insulin assay. Duka et al. (2001) did not explicitly describe the duration of the fast, but they cited the method used in an earlier euglycemic-hyperinsulinemic clamp study in which the animals underwent an overnight fast. The age and sex of the mice in the earlier study were not described. It is uncertain if these or other differences in experimental design account for the different effect of B2RKO on circulating insulin concentration. The lower plasma insulin concentration for B2RKO vs. WT mice suggests that there may have been decreased insulin secretion in the B2RKO animals.

The current study provides the first insulin signaling data for B2RKO mice. Muscle Akt Thr308 phosphorylation was similar between B2RKO and WT mice implicating an Akt-independent mechanism for the insulin resistance found in the B2RKO muscles. Muscle Akt Thr308 phosphorylation was also not different for exercised compared to sedentary groups, consistent with earlier data after exercise by normal mice (Hamada et al. 2006) and providing evidence that the increased insulin sensitivity after exercise, regardless of B2R expression, does not require enhanced Akt Thr308 phosphorylation.

The current results are consistent with previous studies (Cervenka et al. 1999, Schanstra et al. 2003) in which body mass was not different between B2RKO and WT mice. Schanstra et al. (2003) also found no difference between B2RKO and WT mice for food intake. A more recent study reported that B2RKO vs. WT mice had greater energy intake and energy expenditure concomitant with a ~25% reduction in total body fat content (per g body mass) determined by carcass analysis (de Picoli Souza et al. 2010). The results for lower body fat are similar to the reduction in epididymal fat pad/body mass ratio for the B2RKO compared to WT mice in the current study. They also found the gastrocnemius mass (per g body mass) of the B2RKO mice to be ~19% greater than control mice. Although gastrocnemius mass was not significantly different between genotypes in the current study, the soleus/body mass ratio was greater for B2RKO versus WT mice. The metabolic phenotype of B2RKO mice occurs despite moderate decrements in body fat and increments in skeletal muscle mass which are typically expected to favor improved insulin sensitivity.

In conclusion, mice lacking the B2R compared to WT controls had a small reduction in glucose uptake by insulin-stimulated soleus muscles concomitant with an undiminished insulin-stimulated increase in Akt phosphorylation. Earlier in vivo results from B2RKO mice undergoing a hyperinsulinemic clamp demonstrated insulin resistance, but they had not directly evaluated skeletal muscle or assessed insulin signaling. The current results demonstrate that insulin resistance of skeletal muscles lacking the B2R persists ex vivo and suggest this defect is secondary to an Akt-independent mechanism. The absence of the B2R also did not alter the effects of exercise on circulating glucose or insulin concentrations in vivo indicating that the B2R is not a major modulator
of the important effects of acute exercise on either glycemia or insulinemia. Finally, the lack of a significant interaction between exercise and genotype for glucose uptake by insulin-stimulated skeletal muscle after exercise demonstrates that the B2R is not essential for the increased glucose uptake in insulin-stimulated soleus muscle after exercise by mice.

Conflict of Interest
There is no conflict of interest.

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References


