

# 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 [<sup>3</sup>H]-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

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## 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).

The mechanisms whereby prior exercise initiates the improvement in skeletal muscle insulin sensitivity remain incompletely understood. Presumably, events that occur during exercise trigger the processes that subsequently lead to increased insulin-stimulated glucose transport. Exercise induces increased production of a circulating nonapeptide bradykinin (Blais *et al.* 1999, Boix *et al.* 2005, Langberg *et al.* 2002, Stebbins *et al.* 1990, Taguchi *et al.* 2000). Bradykinin can favor a subsequent increase in insulin-stimulated glucose uptake (Beard *et al.* 2006, Duka *et al.* 2001, Henriksen and Jacob 1995, Henriksen *et al.* 1996, Henriksen *et al.* 1998, Henriksen *et al.* 1999, Miyata *et al.* 1998). These observations raised the possibility that bradykinin may participate in the post-exercise increase in skeletal muscle insulin sensitivity.

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- $^3\text{H}$ glucose and  $^{14}\text{C}$ 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 (Rockford, IL). Anti-phospho-Akt Thr<sup>308</sup> and secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG) were from Cell Signaling Technology. 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  $\mu\text{U}/\text{ml}$  insulin was used for mice in Experiment 1; 100  $\mu\text{U}/\text{ml}$  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 % O<sub>2</sub>-5 % CO<sub>2</sub>).

After the 1st incubation step, muscles were transferred to a 2nd vial containing KHB with 0.1 % BSA, 1 mM 2-deoxyglucose (2-deoxy- $^3\text{H}$ glucose, 6 mCi/mmol), 9 mM mannitol ( $^{14}\text{C}$ 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-

**Table 1.** Body mass and tissue/body mass ratios and glucose and insulin concentrations.

	<b>WT Sedentary</b>	<b>WT Exercise</b>	<b>B2RKO Sedentary</b>	<b>B2RKO Exercise</b>
<i>Body mass, g</i>	26.4±0.6	26.3±0.7	26.8±0.5	26.4±0.6
<i>Fat pad/body mass ratio (mg/g)</i>	6.5±0.4	7.0±0.4	4.6±0.3*	4.4±0.3*
<i>Soleus/body mass ratio (mg/g)</i>	0.36±0.02	0.34±0.1	0.39±0.2*	0.38±0.2*
<i>Gastrocnemius/body mass ratio (mg/g)</i>	4.7±0.1	4.7±0.1	4.9±0.3	5.0±0.1
<i>Blood glucose, mmol·l<sup>-1</sup></i>	7.85±0.51	6.74±0.48†	8.91±0.25*	7.65±0.31†*
<i>Plasma insulin, ng·ml<sup>-1</sup></i>	0.673±0.12	0.307±0.06†	0.455±0.06*	0.205±0.04†*

Values are means ± SE, n=21-22 for body mass; n=21-22 for soleus/body mass ratio; n=8 for gastrocnemius/body mass ratio; and n=14-16 for epididymal fat pad/body mass ratio and glucose and insulin concentrations. \**P*<0.05 (WT vs. B2RKO). †*P*<0.05 (Sedentary vs. Exercise).

cold lysis buffer (0.5 ml) containing 20 mM Tris-HCl, 150 mM NaCl, 1 % NP-40, 1 mM activated Na<sub>3</sub>VO<sub>4</sub>, 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 ~1hr before being centrifuged (15,000g, 15 min). Aliquots from supernatants were quantified for [<sup>3</sup>H] and [<sup>14</sup>C] 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 Thr<sup>308</sup>. 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 Thr<sup>308</sup> 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

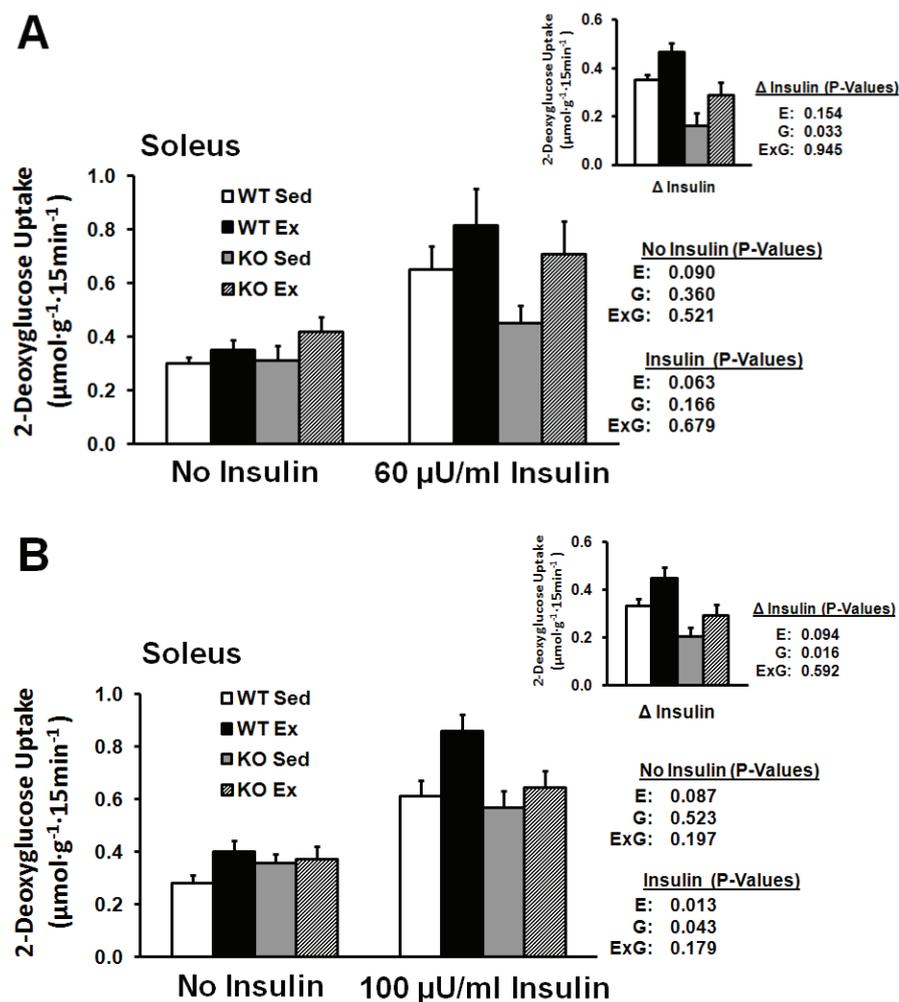
There were no statistically significant interactions (exercise x genotype) for any of the measurements that were made.

#### 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



**Fig. 1.** Glucose uptake by isolated soleus muscles (Experiment 1: without or with 60  $\mu\text{U}/\text{ml}$  insulin (**A**); Experiment 2: without or with 100  $\mu\text{U}/\text{ml}$  insulin (**B**) from WT and B2RKO mice that were sedentary (SED) or exercised (EX). Insets of (A) and (B) represent the insulin-stimulated increase in glucose uptake (delta insulin) calculated by subtracting the basal value from the insulin-stimulated value of paired muscles. Values are mean  $\pm$  SE for 9 or 15-16 muscles per group (A and B, respectively). \* $P < 0.05$ . E, main effect of exercise treatment; G, main effect of genotype; ExG, interaction between main effects.

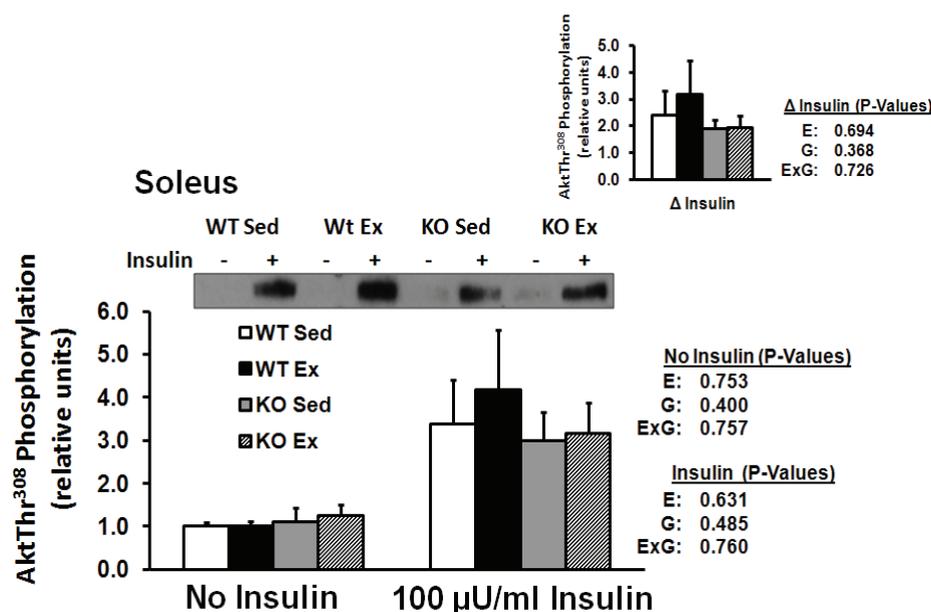
vs. WT mice in sedentary and exercised conditions. In both genotypes, exercise caused a 55 % decrease in plasma insulin ( $P < 0.001$ ).

#### 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  $\mu\text{U}/\text{ml}$  insulin ( $P < 0.05$ ), and a non-significant trend ( $P = 0.166$ ) for lower values in the B2RKO mice was also evident with 60  $\mu\text{U}/\text{ml}$  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  $\mu\text{U}/\text{ml}$  ( $P < 0.05$ ), 100  $\mu\text{U}/\text{ml}$  ( $P < 0.05$ ) 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  $\mu\text{U}/\text{ml}$ ; Fig. 1A) and was significantly greater in Experiment 2 ( $P < 0.05$  with 100  $\mu\text{U}/\text{ml}$ ; 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  $\mu\text{U}/\text{ml}$  ( $P = 0.150$ ) or 100  $\mu\text{U}/\text{ml}$  ( $P = 0.094$ ), and the delta insulin values were significantly greater after exercise when the data from both experiments were pooled ( $P < 0.05$ ).



**Fig. 2.** Akt threonine<sup>308</sup> phosphorylation in isolated soleus from WT and B2RKO mice that were SED or EX. The insets represents the insulin-stimulated increase Akt threonine<sup>308</sup> (delta insulin) calculated by subtracting the basal value from the insulin-stimulated value of paired muscles. Values are mean  $\pm$ SE for 8 muscles per group.

### Akt Thr<sup>308</sup> phosphorylation

Soleus Akt Thr<sup>308</sup> 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  $\mu$ U/ml insulin and a trend ( $P=0.063$ ) for increased glucose uptake by muscles incubated with 60  $\mu$ U/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, Henriksen *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-exercise increase in insulin-stimulated glucose transport. However, previous studies indicated that electrically stimulated contractions by isolated rat skeletal muscle in the presence of exogenous bradykinin did not cause a subsequent increase in glucose transport of insulin-stimulated muscles (Dumke *et al.* 2002). Furthermore, HOE-140, a B2R inhibitor, did not reduce the ability of prior contraction in serum to increase insulin-dependent glucose transport (Dumke *et al.* 2002). These results did not support a role for bradykinin in the post-contraction 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  $\mu$ U/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 Thr<sup>308</sup> phosphorylation was similar between B2RKO and WT mice implicating an Akt-independent mechanism for the insulin resistance found in the B2RKO muscles. Muscle Akt Thr<sup>308</sup> 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 Thr<sup>308</sup> 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.

### Acknowledgements

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