

# Insulin-Stimulated Glucose Uptake Does Not Require p38 Mitogen-Activated Protein Kinase in Adipose Tissue or Skeletal Muscle

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It has been proposed that p38 mitogen-activated protein kinase (MAPK) isoforms sensitive to the pyridinylimidazole compounds SB 203580 and SB 202190 may participate in the acute insulin-dependent activation of glucose transporters recruited to the plasma membrane of adipocytes and skeletal muscle. Here, we explore whether these kinases support the insulin stimulation of glucose uptake in these tissues by investigating the effects of a genetic loss in p38 $\beta$  and that of the p38 MAPK inhibitor SB 203580. Glucose uptake in adipocytes and soleus muscle was stimulated by insulin by up to fourfold irrespective of whether tissues were isolated from wild-type or p38 $\beta$ -null mice. Consistent with this finding, mice lacking p38 $\beta$  exhibited normal glucose tolerance, insulinemia, and glycemia compared with their wild-type counterparts. Insulin-stimulated glucose uptake was not inhibited by SB 203580 when adipocytes were preincubated with the drug at a cytocrit of 50%, but intriguingly, uptake was suppressed (by 35%) when the cytocrit was reduced by one-half. Despite the activation of glucose uptake at the higher cytocrit, insulin failed to induce any detectable activation of p38 MAPK, whereas p38 signaling was robustly activated by anisomycin in a SB 203580-sensitive manner. Although insulin also failed to induce any detectable activation of p38 MAPK in muscle, insulin-dependent glucose uptake was reduced by SB 203580 (~44%) in muscle of both wild-type and p38 $\beta$ -null mice. Our results indicate that p38 $\beta$  is not required for insulin-stimulated glucose uptake in adipocytes or muscle. Moreover, given that insulin fails to promote any significant activation of p38 MAPK in these tissues and the finding that sensitivity of glucose uptake, but not that of the kinase, to SB 203580 can be influenced by cytocrit, we suggest that p38 signaling is unlikely to participate in any putative activation of transporters recruited to the cell surface by insulin and that SB 203580 suppresses insulin-

stimulated glucose transport by a mechanism unrelated to its inhibitory effect on p38 MAPK. *Diabetes* 54:3161–3168, 2005

**A**n important aspect of mammalian physiology involves the homeostatic regulation of blood glucose concentration, which is primarily controlled by the action of insulin. Postprandial increases in blood glucose are shadowed by a rise in circulating insulin that helps promote the disposal of glucose into key target tissues, such as skeletal muscle and fat (1–3). Whereas it is widely accepted that skeletal muscle, by virtue of its large contribution to body mass, represents the major site of insulin-mediated glucose disposal, both tissues contribute toward the lowering of blood glucose (4). The stimulation in glucose uptake elicited by insulin in both skeletal muscle and fat is achieved principally by the increased translocation of the insulin-regulated glucose transporter GLUT4 to the plasma membranes of these tissues from specialized intracellular storage pools (3). The increase in surface GLUT4 subsequently facilitates glucose uptake from the extracellular milieu resulting in a fall in blood glucose. However, in addition to the recruitment of transporters from a subcellular pool, it has been suggested that the intrinsic activity of recruited carriers may also be subject to enhancement by insulin (3,5). Although the translocation step has been established for more than two decades and is now an accepted paradigm that explains how insulin promotes an increase in plasma membrane GLUT4 abundance, there is still considerable controversy and debate as to whether transporters undergo an activation step at the cell surface (5). The suggestion that this latter step may exist is supported by work showing that although exogenous delivery of phosphatidylinositol 3,4,5-trisphosphate (a phosphatidylinositol 3-kinase reaction product) induces GLUT4 translocation to the plasma membrane in muscle and fat cells, this alone does not elicit an increase in glucose uptake (6). There is also some suggestion that the translocation step can be distinguished from the proposed carrier activation step based on their differential sensitivity to wortmannin, a phosphatidylinositol 3-kinase inhibitor (7). The latter being much more sensitive to low wortmannin concentrations than the former (7). The idea that recruited GLUT4 molecules may undergo activation has gathered considerable favor in some camps based on data showing that although insulin recruits the transporter to the plasma membrane in muscle and fat cells, the

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3-OMG, 3-O-methyl glucose; HRP, horseradish peroxidase; KRBB, Krebs-Ringer bicarbonate buffer; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKAP-K2, MAPK-activated kinase 2; PKB, protein kinase B.

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subsequent increase in glucose uptake can be reduced significantly by the pyridinylimidazole compounds SB 203580 and SB 202190 (8–10). These compounds have been used extensively as inhibitors of p38 $\alpha$  and p38 $\beta$  mitogen-activated protein kinase (MAPK), and thus by extension, it has been suggested that one or both of these isoforms of p38 MAPK (because neither the  $\gamma$ - nor  $\delta$ -isoforms are sensitive to these inhibitors) may participate in the acute insulin-induced activation of GLUT4 transporters at the cell surface (11).

In an attempt to assess whether p38 MAPK facilitates insulin-dependent increases in glucose uptake, we have investigated the effects of insulin and SB 203580 on glucose uptake in adipocytes and skeletal muscle from wild type and mice lacking p38 $\beta$  MAPK. Our data indicate that targeted disruption of the p38 $\beta$  MAPK gene has no discernable effect on basal or insulin-stimulated glucose uptake in these tissues, suggesting that this isoform does not participate in the hormonal activation of glucose transport. We also present data showing that although SB 203580 inhibits insulin-stimulated glucose uptake in skeletal muscle and in adipocytes under certain circumstances, the observed suppression in glucose uptake is likely to stem from effects of the inhibitor that are unconnected with its inhibitory effect on p38 MAPK.

## RESEARCH DESIGN AND METHODS

All reagent-grade chemicals, human insulin, cytochalasin B, and collagenase type II were obtained from Sigma-Aldrich (Poole, U.K.). SB 203580 was purchased from Tocris (Bristol, U.K.). Complete protein phosphatase inhibitor tablets were obtained from Boehringer-Roche Diagnostics (Basel, Switzerland). Polyclonal antibodies recognizing p38 $\alpha$ /SAPK2a and p38 $\beta$ /SAPK2b were generated in-house. The MAPK-activated kinase 2 (MAPKAP-K2) antibody has been described previously (12). Phospho-specific p38/SAPK2 Thr<sup>180</sup>/Tyr<sup>182</sup>, protein kinase B (PKB) Ser<sup>473</sup>, and horseradish peroxidase (HRP)-anti-rabbit IgG were from Cell Signaling NEB (Herts, U.K.). HRP-anti-sheep/goat IgG and the enhancer cheluminescent Supersignal were from Pierce (Rockford, IL). X-ray films were from Konica Minolta Medical Graphic Imaging Europe.

The generation of mice lacking p38 $\beta$  MAPK will be described in detail elsewhere (12a). Briefly, exons 2–7 of the murine p38 $\beta$ /SAPK2b gene were deleted in embryonic stem cells using standard targeting techniques. Deletion was confirmed by Southern blot analysis of genomic DNA. Embryonic stem cells were used to generate chimeric mice that were crossed onto C57Bl6 mice. p38 $\beta$ <sup>+/−</sup> mice from these crosses were used to generate p38 $\beta$ <sup>−/−</sup> mice, and the presence of the knockout was confirmed by PCR analysis and immunoblotting. Mice were maintained under specific pathogen-free conditions and in accordance with local and national ethical guidelines. Where indicated, wild-type and transgenic animals were fasted overnight before surgical excision of epididymal fat and soleus muscle. In some experiments, epididymal fat from overnight-fasted male Sprague-Dawley rats (~200 g; Bantin & Kingman, Hull, U.K.) was also used for glucose uptake studies.

**Analysis of blood glucose, insulin, and glucose tolerance.** For analysis of blood glucose and insulin, blood was collected by “tail-tipping” fed or overnight-fasted mice. Blood insulin was assayed using a nonradiometric ELISA kit (Crystal Chem, Chicago, IL) with mouse insulin as a standard. Blood glucose was measured using the Esprit Blood Glucose Monitoring System (Bayer). For analysis of glucose tolerance, fasted animals were injected intraperitoneally with 2 mg/g glucose, and blood glucose was subsequently assayed at the times indicated.

**Isolation of adipocytes.** Adipocytes were isolated by collagenase digestion of fat pads by the method of Rodbell (13) and maintained at 37°C in Krebs-Ringer phosphate buffer (120 mmol/l NaCl, 1.3 mmol/l CaCl<sub>2</sub>, 4.8 mmol/l KCl, 1.2 mmol/l MgSO<sub>4</sub>, 3.1 mmol/l NaH<sub>2</sub>PO<sub>4</sub>, 12.5 mmol/l Na<sub>2</sub>HPO<sub>4</sub>, and 2 mmol/l pyruvate, pH 7.4) containing 1% BSA and at 50% packed cell volume and for some experiments, where indicated, at 25% cytocrit.

**Hexose transport in isolated adipocytes and skeletal muscle.** Uptake of [<sup>3</sup>H]2-deoxyglucose was assayed as previously described (14,15). Briefly, 400- $\mu$ l aliquots of the adipocyte suspension at 50% cytocrit (or 25%, where indicated) were preincubated in the absence or presence of 100 nmol/l insulin and 10  $\mu$ mol/l cytochalasin B. Incubations were started by addition of 50  $\mu$ mol/l [<sup>3</sup>H]2-deoxyglucose (Dupont; specific activity, 26.2 Ci/mmol). Uptake

assays were terminated after 1 min by the rapid centrifugation of 300  $\mu$ l adipocyte suspension through a 100- $\mu$ l di-isooctyl phthalate oil cushion (Fluka/Sigma-Aldrich, Dorset, U.K.) for 20 s. Nonspecific cell-associated radioactivity was corrected by the inclusion in the uptake medium of [<sup>14</sup>C]inulin (specific radioactivity, 1.4 mCi/mmol).

Isolated skeletal muscle incubation was performed as described previously (16), and glucose uptake was assayed using the method described by Bruning et al. (17). Briefly, tendons from both ends of each muscle were tied with suture and mounted on an incubation apparatus. Muscles were incubated in 8 ml Krebs-Ringer bicarbonate buffer (KRBB) (117 mmol/l NaCl, 2.5 mmol/l CaCl<sub>2</sub>, 1.2 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 1.2 mmol/l MgSO<sub>4</sub>, and 24.6 mmol/l NaHCO<sub>3</sub>, pH 7.4) containing 2 mmol/l pyruvate in the presence or absence of 100 nmol/l insulin and/or 10  $\mu$ mol/l SB 203580 for 60 min at 37°C. Glucose uptake was then measured in 2 ml KRBB containing 1 mmol/l 2-deoxy-D-[1-<sup>3</sup>H]glucose (1.5  $\mu$ Ci/ml) and 7 mmol/l D-[<sup>14</sup>C]mannitol (0.45  $\mu$ Ci/ml) in the presence or absence of 100 nmol/l insulin at 30°C for 10 min. Both incubation and transport buffers were continuously gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. At the end of the uptake assay, muscles were immersed in KRBB containing 80  $\mu$ mol/l cytochalasin B on ice to terminate glucose transport, and muscles were quickly frozen in liquid nitrogen. Muscles were weighed and processed by incubating in 250  $\mu$ l 1 mol/l NaOH at 80°C for 10 min, neutralized with 250  $\mu$ l 1 mol/l HCl, and particulates were precipitated by centrifugation at 13,000g for 1 min. <sup>3</sup>H and <sup>14</sup>C radioactivity present in 350  $\mu$ l of the supernatant was measured by scintillation counting. From the <sup>14</sup>C specific radioactivity, the extracellular water volume present in the muscle was calculated. This enables the extracellular <sup>3</sup>H volume to be determined and subtracted from the total <sup>3</sup>H radioactivity to calculate 2-deoxy-D-[1-<sup>3</sup>H]glucose transported per gram of muscle per hour.

**Preparation of whole-cell and tissue lysates.** In some experiments, adipocytes from epididymal fat pads, heart, and soleus muscle were lysed after experimental manipulation (see figure legends) in an appropriate volume of lysis buffer (50 mmol/l Tris, pH 7.4, 0.27 mol/l sucrose, 1 mmol/l sodium orthovanadate, pH 10, 1 mmol/l EDTA, 1 mmol/l EGTA, 10 mmol/l sodium  $\beta$ -glycerophosphate, 50 mmol/l NaF, 5 mmol/l sodium pyrophosphate, 1% [wt/vol] Triton X-100, 0.1% [vol/vol] 2-mercaptoethanol, 0.1  $\mu$ mol/l microcystin-LR, and protease inhibitors). Whole-cell and muscle lysates were centrifuged (15,000g, 4°C for 10 min) and stored at −80°C until required.

**Determination of protein concentration.** Protein concentrations were determined by the method of Bradford (18) using BSA as standard.

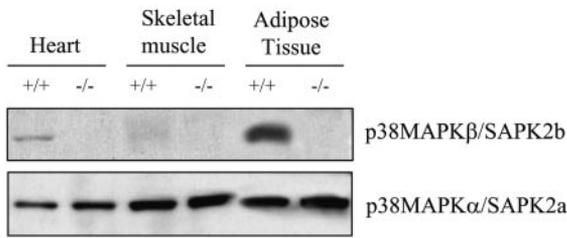
**Immunoblotting.** Cell and tissue lysates were subjected to SDS-PAGE on 10% polyacrylamide gels as described by Laemmli (19). Proteins were transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ), blocked for 2 h at room temperature in 5% (wt/vol) skimmed milk/Tris-buffered saline (50 mmol/l Tris-HCl, pH 7.6, and 150 mmol/l NaCl) supplemented with 0.1% (vol/vol) Tween 20, and probed with antibodies against p38 $\alpha$ /SAPK2a (1  $\mu$ g/ml), p38 $\beta$ /SAPK2b (1  $\mu$ g/ml), phospho-p38/SAPK2 Thr<sup>180</sup>/Tyr<sup>182</sup> (1:1,000), or phospho-PKB Ser<sup>473</sup> (1:1,000). Nitrocellulose membranes were washed three times in Tris-buffered saline/0.1% (vol/vol) Tween 20 for 5 min before incubation with either HRP-anti-sheep IgG (1:10,000) or HRP-anti-rabbit (1:1,000). Protein signals were visualized using enhanced chemiluminescence by exposure to Konica autoradiographic film.

**Analysis of MAPKAP-K2 activity.** MAPKAP-K2 was immunoprecipitated from 50 to 100  $\mu$ g adipocyte homogenate using MAPKAP-K2 antibody coupled to protein G sepharose for 1 h at 4°C. Immunoprecipitates were washed in 0.5 mol/l NaCl, 50 mmol/l Tris-HCl, pH 7.5, 0.27 mol/l sucrose, 1 mmol/l sodium orthovanadate, pH 10, 1 mmol/l EDTA, 1 mmol/l EGTA, 10 mmol/l sodium  $\beta$ -glycerophosphate, 50 mmol/l NaF, 5 mmol/l sodium pyrophosphate, and 1% (wt/vol) Triton X-100; twice in 50 mmol/l Tris, pH 7.5, 1 mmol/l EGTA, and 0.5 mol/l NaCl; and twice in 50 mmol/l Tris-HCl, pH 7.5, and 1 mmol/l EGTA. The assay was initiated by the addition of 35  $\mu$ l of a mix of 50 mmol/l Tris-HCl, pH 7.5, 100  $\mu$ mol/l EGTA, 2.5  $\mu$ mol/l protein kinase inhibitor, 0.1% (vol/vol) 2-mercaptoethanol, 30  $\mu$ mol/l substrate peptide KKLNRTLVA, 100  $\mu$ mol/l [ $\gamma$ -<sup>32</sup>P]ATP, and 10 mmol/l MgCl<sub>2</sub> and incubated for 10 min at 30°C. Reactions were stopped by pipetting onto P81 paper and washing in 75 mmol/l orthophosphoric acid. Incorporation of <sup>32</sup>P radioactivity was measured by Cerenkov counting using a Wallac 1409 liquid scintillation counter.

**Statistical analyses.** For multiple comparisons, statistical analysis was performed using one-way ANOVA followed by a Newman-Keuls post hoc test. Data analysis was performed using GraphPad Prism software and considered statistically significant at  $P < 0.05$ .

## RESULTS

**p38 $\beta$  MAPK expression in heart, skeletal muscle, and adipose tissue.** Isoform-specific antibodies against p38 $\alpha$  and p38 $\beta$  MAPK were used to probe heart, skeletal muscle, and adipose tissue extracts from wild-type and p38 $\beta$

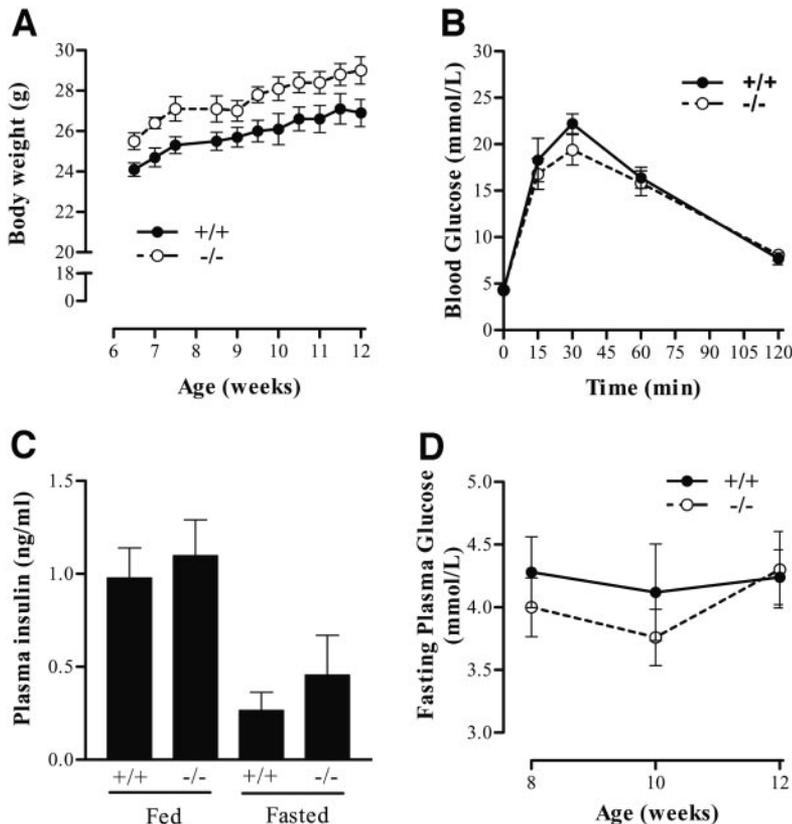


**FIG. 1.** p38 $\alpha$  and p38 $\beta$  MAPK abundance in heart, skeletal muscle, and adipose tissue of wild-type (+/+) and p38 $\beta$ -null (-/-) mice. Tissue lysates (100  $\mu$ g protein) were resolved by SDS-PAGE and immunoblotted with isoform-specific antibodies against p38 $\alpha$  and p38 $\beta$  MAPK. The blot is representative of three separate experiments.

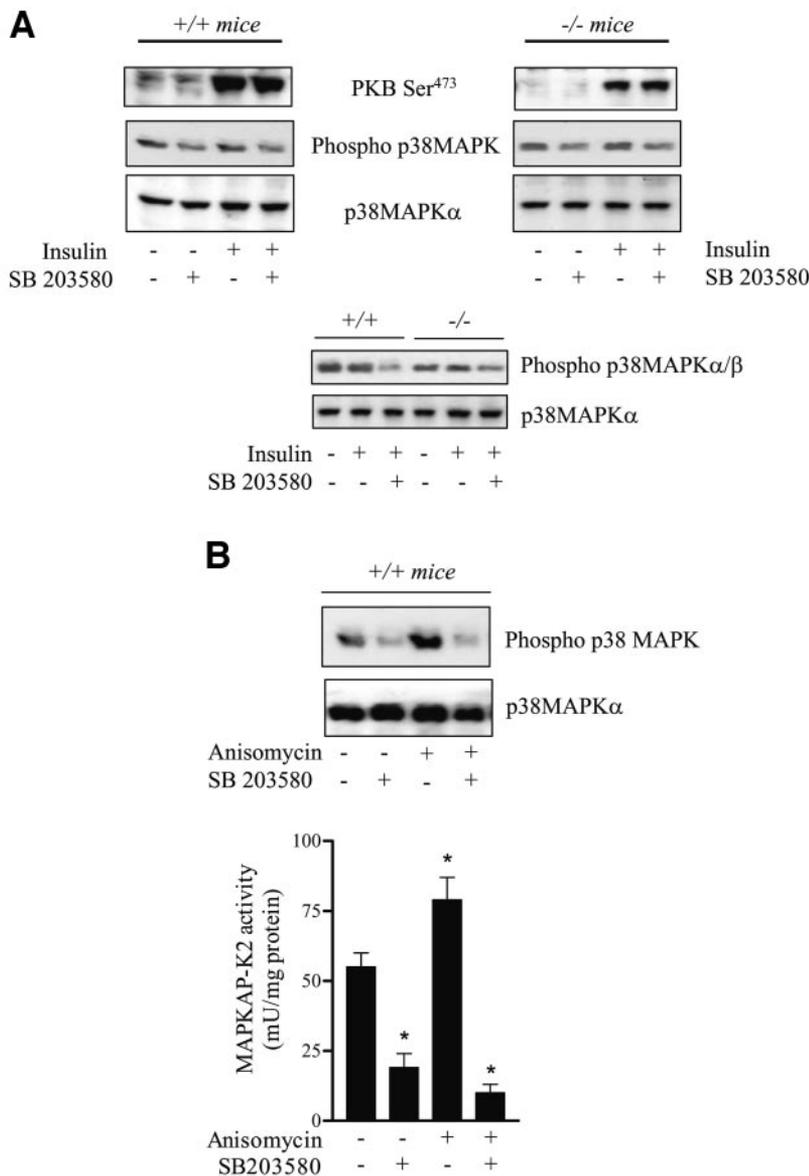
MAPK-null mice. Figure 1 shows representative immunoblots of the three tissues examined and reveals that, in wild-type animals, adipose tissue possesses the greatest abundance p38 $\beta$  MAPK, whereas, by comparison, skeletal muscle contains the least. As expected the  $\beta$ -isoform was absent from all three tissues when these were harvested from the p38 $\beta$  MAPK-null mice. The lower blot in Fig. 1 shows the relative abundance of p38 $\alpha$  MAPK. Skeletal muscle and adipose tissue possess near equivalent amounts of this protein kinase on a per milligram protein basis. Neither skeletal muscle nor adipose tissue shows any compensated changes in the expression p38 $\alpha$  MAPK as a result of a loss in the  $\beta$ -isoform.

**Body weight, glucose tolerance, plasma insulin, and glucose.** p38 $\beta$  MAPK-null mice are both viable and fertile. Body weights of mice lacking p38 $\beta$  MAPK were monitored over a 6-week period and were found to be marginally heavier (on average by  $\sim 2$  g) than their wild-type counterparts (Fig. 2A). It remains unknown at present whether this small weight difference reflects a true global or tissue-specific increase in mass because it failed to achieve

statistical significance with the low number of animals (five in each group) that were followed over the 6-week period. If p38 $\beta$  MAPK contributes to the hormonal activation of glucose transport, then it is conceivable that its absence may result in impaired glucose homeostasis. However, Fig. 2B shows that compared with wild-type mice, animals lacking p38 $\beta$  do not exhibit any major deviation in glucose tolerance. Analysis of blood insulin from fed and fasted animals also fails to unveil any significant differences between the control and knockout mice (Fig. 2C), and similarly, values for fasting glycemia were comparable between the two animal groups (Fig. 2D). **Effects of insulin, anisomycin, and SB 203580 on p38 MAPK signaling in murine adipocytes.** Because p38 $\beta$  MAPK-null mice still express the SB 203580-sensitive p38 $\alpha$ -isoform, it was necessary to examine the effects of insulin and test the efficacy of SB 203580 on p38 MAPK phosphorylation/signaling. Figure 3A shows that adipocytes isolated from epididymal fat pads of wild-type or p38 $\beta$  MAPK-null mice exhibit basal p38 MAPK phosphorylation, possibly reflecting that this stress-signaling kinase may have become activated as a result of the adipocyte isolation procedure. This basal phosphorylation was reduced slightly in cells that had been preincubated with the p38 MAPK inhibitor, SB 203580. Regardless of whether this compound was present or absent we were unable to detect any significant insulin-induced increase in p38 MAPK phosphorylation. Under these circumstances phosphorylation of PKB on its regulatory hydrophobic serine residue was elevated as expected in adipocytes from both wild-type and p38 $\beta$  MAPK-null mice (Fig. 3A). To assess the effects of a loss in p38 $\beta$  expression on the relative intensity of the phospho-p38 signal observed in adipocytes from wild-type and knockout mice, lysates were run side by side and immunoblotted using the phospho-specific antibody.



**FIG. 2.** Analysis of body weight, glucose tolerance, and blood insulinemia and glycemia in wild-type and p38 $\beta$  MAPK-null mice. **A:** Body weight of five age-matched wild-type (+/+) and p38 $\beta$  MAPK-null (-/-) mice was monitored over a 6-week period as shown. **B:** Glucose tolerance in wild-type and p38 $\beta$  MAPK-null mice (five animals in each group) was performed after an intraperitoneal injection of glucose, and blood glucose was assayed as described in RESEARCH DESIGN AND METHODS. **C:** Serum insulin was assayed from fed and overnight-fasted wild-type and p38 $\beta$ -null mice (five animals per group) using an enzyme-linked immunosorbent assay-based assay as described in RESEARCH DESIGN AND METHODS. **D:** Blood glucose from fasted mice at three different ages (as indicated) was assayed using the Esprit Blood Glucose Monitoring System. All values represent means  $\pm$  SEM from five separate animals.

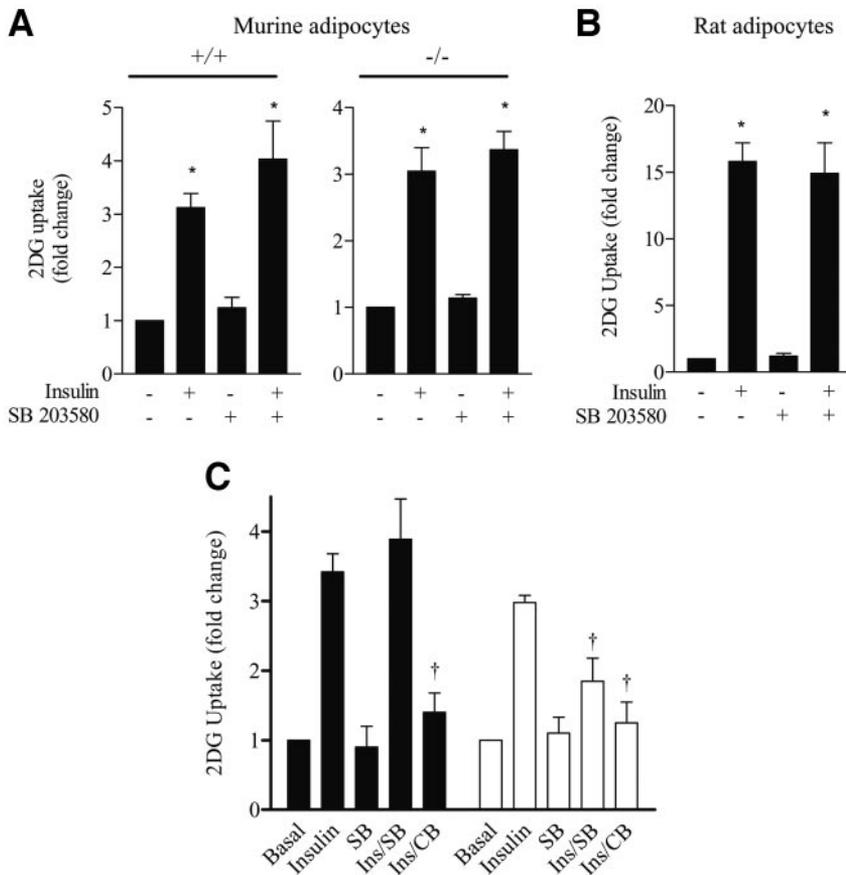


**FIG. 3.** Effects of insulin and anisomycin on p38 MAPK and MAPKAP-K2 activation in primary mouse adipocytes. **A:** Isolated adipocytes from wild-type (+/+) and p38β MAPK-null (-/-) mice were incubated in the absence or presence of SB 203580 (10 μmol/l, 60 min) and/or 100 nmol/l insulin (10 min). Adipocytes were lysed after the incubation with drug and/or hormone and lysates (50 μg protein) immunoblotted with antibodies against phospho-PKB Ser<sup>473</sup>, phospho-p38 MAPK (T<sup>180</sup>/Y<sup>182</sup>), or native p38α MAPK. **B:** Adipocytes isolated from wild-type mice were also subjected to incubation with anisomycin (10 μg/ml, 60 min) in the absence or presence of SB 203580 (10 μmol/l, 60 min). Cells were lysed and either immunoblotted with phospho-p38 MAPK (T<sup>180</sup>/Y<sup>182</sup>) and native p38α MAPK or assayed for MAPKAP-K2 activity as described in RESEARCH DESIGN AND METHODS. The immunoblots are representative of three separate experiments, and the values shown in the bar graph are means ± SEM from three separate experiments. \*Significant changes from the untreated control (*P* < 0.05).

The bottom panel of Fig. 3A shows a marginal reduction (~20%) in the phospho-p38 signal in adipocyte lysates from p38β-null mice compared with those from wild-type mice. As before, the blot also shows that phosphorylation of p38 MAPK does not change in response to insulin compared with that seen in unstimulated adipocytes from either genotype, but it was reduced in response to cell pretreatment with SB 203580. The efficacy of SB 203580 was further investigated by monitoring the effects of the inhibitor and anisomycin [a stress-inducing agent (20)] on p38 MAPK signaling. Figure 3B shows that, unlike insulin, exposing adipocytes from wild-type mice led to enhanced phosphorylation of the kinase over and above that seen in unstimulated cells, which was not observed if adipocytes were pretreated with SB 203580. The ability of the pyridinylimidazole compound to inhibit basal as well as agonist-induced phosphorylation of p38 MAPK is completely consistent with the idea that the inhibitor can bind to both active and inactive forms of the kinase, reducing not only its activation, but also its ability to competitively bind ATP (21). One of the physiological targets of p38 MAPK is MAPKAP-K2, and in line with the finding that SB 203580 reduces both basal and agonist-mediated activation of p38

MAPK, we observed that activation of the downstream kinase was affected similarly (Fig. 3B, bottom panel).

**Adipocyte and skeletal muscle glucose uptake.** To establish whether there is a requirement for p38 MAPK to support the acute insulin-dependent increase in glucose uptake, we assayed basal and insulin-stimulated glucose transport in isolated adipocytes and soleus muscle. Figure 4A shows that irrespective of whether adipocytes were isolated from wild type or from mice lacking p38β, insulin induced a three- to fourfold increase in glucose uptake. That in absolute terms the basal glucose uptake was not statistically different between the two animal groups in any one experiment is noteworthy; but given that variation was observed between experiments, the results shown are presented as a fold change (values for basal and insulin-stimulated glucose uptake from one single experiment are given in the Fig. 4 legend). It is plausible that the β-isoform of p38 MAPK may not contribute to the regulation of glucose uptake in response to insulin or, if it does, that the α-isoform may compensate for its loss in the knockout mice. To test this possibility, hexose uptake was assayed after pretreatment of fat cells (at 50% cytocrit) with SB 203580. Despite the ability of SB 203580 to suppress basal



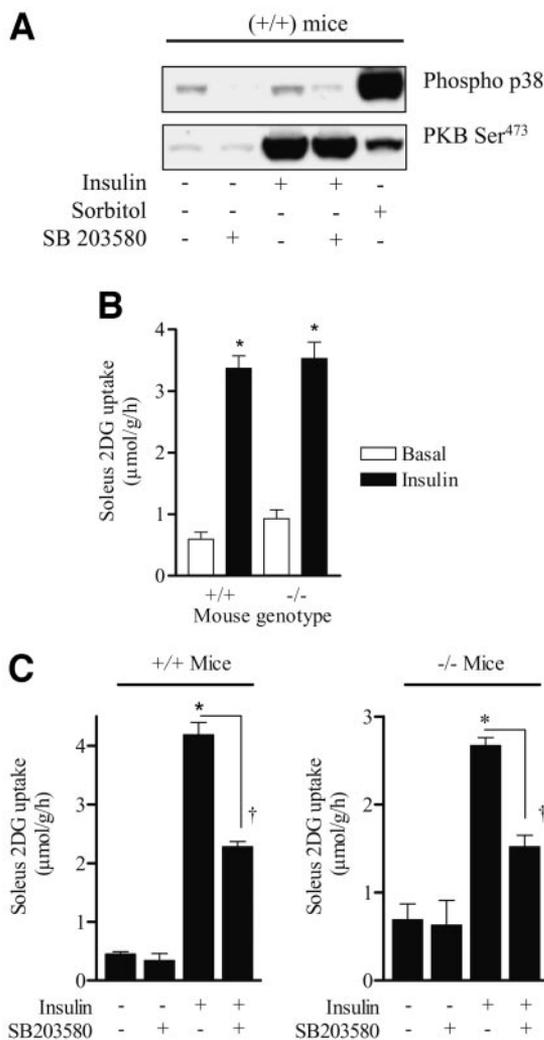
**FIG. 4.** Effects of insulin and SB 203580 on glucose uptake in adipocytes and soleus muscle from wild-type (+/+) and p38 $\beta$  MAPK-null (-/-) mice and adipocytes from rats. Adipocytes from mice (A) and rats (B) were incubated in the absence and presence of SB 203580 (10  $\mu$ mol/l, 60 min) and insulin (100 nmol/l, 30 min) before assaying 2-deoxyglucose (2DG) uptake as described in RESEARCH DESIGN AND METHODS. The mice data represent means  $\pm$  SEM from six separate experiments conducted in triplicate, whereas the rat data are means  $\pm$  SEM from three separate experiments. \*Significant change from the untreated control ( $P < 0.05$ ), whereas daggers ( $\dagger$ ) signify a statistically significant change with respect to insulin value ( $P < 0.05$ ). C: Murine adipocytes maintained at 50% (■) or 25% (□) cytocrit were used to test the effects of insulin, SB 203580, and cytochalasin B (CB; 10  $\mu$ mol/l, 60 min) on 2-deoxyglucose uptake as in A. Data for basal and insulin-stimulated glucose uptake from a single mouse adipocyte experiment conducted in triplicate (means  $\pm$  SEM) were as follows. +/+; adipocytes: basal,  $13.5 \pm 2.3$  pmol  $\cdot$  min $^{-1} \cdot$  mg $^{-1}$  protein; insulin,  $34.3 \pm 4.4$  pmol  $\cdot$  min $^{-1} \cdot$  mg $^{-1}$  protein. -/-; adipocytes: basal,  $14.1 \pm 1.5$  pmol  $\cdot$  min $^{-1} \cdot$  mg $^{-1}$  protein; insulin,  $32.2 \pm 2.9$  pmol  $\cdot$  min $^{-1} \cdot$  mg $^{-1}$  protein.

and agonist-dependent stimulation of the p38 pathway at this cytocrit (Fig. 3), the inhibitor did not exert any inhibitory effect on basal or insulin-stimulated sugar uptake (Fig. 4A). Similar experiments were performed using rat adipocytes, which we found to exhibit a much greater hormonal stimulation of glucose uptake, but again, pretreatment of rat adipocytes with SB 203580 did not lead to any detectable change in hexose uptake (Fig. 4B).

The inability of SB 203580 to suppress insulin-stimulated glucose uptake in adipocytes (Fig. 4A and B) is in stark contrast to that recently reported by Ribé et al. (22). One possible explanation that may account for the discrepancy is that our uptake studies were performed at a higher packed cell volume (cytocrit) than those reported by Ribé et al., and this may have adversely affected the ability of the drug to exert its full effect on adipocyte glucose transport. Because loss of p38 $\beta$  had no effect on basal or insulin-stimulated glucose uptake, we reassessed the effects of SB 203580 using adipocytes from wild-type mice but at one-half the cytocrit (i.e., 25%). Insulin caused a near threefold increase in glucose uptake at the lower cytocrit, which was comparable with that observed at the higher cytocrit (Fig. 4C). Although the data have been normalized to the untreated control at each cytocrit, it should be stressed the basal glucose uptake was  $\sim$ 50% lower in absolute terms when using one-half the normal packed cell volume (basal glucose uptake at 50% cytocrit,  $113 \pm 15$  fmol/min per 1,000 cells; basal glucose uptake at 25% cytocrit,  $55 \pm 14$  fmol/min per 1,000 cells; values are means  $\pm$  SEM from three experiments). As before, pretreatment of adipocytes with SB 203580 had no detectable impact on basal glucose uptake, whereas insulin-stimulated glucose uptake was reduced significantly by  $\sim$ 35% at

the lower, but not at the higher, cytocrit (Fig. 4C). That pretreatment of adipocytes with 10  $\mu$ mol/l cytochalasin B induced a substantial decrease in insulin-stimulated transport irrespective of what cytocrit was used is also worth noting (Fig. 4C). Similar studies were performed using rat adipocytes in which pretreatment with 10  $\mu$ mol/l SB 203580 caused a more modest reduction (28%) in insulin-stimulated, but not basal, glucose transport at the lower cytocrit (data not shown).

To test whether p38 $\beta$  participates in the control of insulin-stimulated glucose transport in skeletal muscle, we assessed the effects of insulin and SB 203580 on p38 phosphorylation and glucose uptake in isolated soleus muscle from mice. As with adipocytes, acute insulin treatment did not significantly alter the phosphorylation of p38 MAPK, whereas subjecting muscles to osmotic stress by incubation with sorbitol (used as a positive control) led to a robust induction in p38 MAPK phosphorylation (Fig. 5A). The basal p38 phosphorylation observed in the absence and presence of insulin was reduced noticeably by SB 203580 treatment. The inability of insulin to induce any phosphorylation of p38 MAPK was in sharp contrast to its effect on PKB phosphorylation in the same muscle lysates, which was enhanced considerably and which, as anticipated, was not affected by SB 203580 treatment (Fig. 5A). We subsequently assessed the effects of insulin and SB 203580 on glucose uptake in muscles of wild-type and p38 $\beta$ -null mice. Figure 5B shows that neither basal nor insulin-stimulated glucose uptake was affected by the loss of p38 $\beta$  MAPK, and although SB 203580 does not affect basal glucose uptake, the compound caused a significant reduction in insulin-stimulated glucose uptake in muscle



**FIG. 5.** Effects of insulin and SB 203580 on p38 MAPK phosphorylation and glucose uptake in isolated soleus muscle from wild-type (+/+) and p38 $\beta$  MAPK-null (-/-) mice. **A:** Isolated soleus muscles from wild-type mice were incubated in the absence or presence of insulin (100 nmol/l, 60 min) and/or SB 203580 (10  $\mu$ mol/l, 60 min) and/or sorbitol (0.5 mol/l, 60 min), after which muscle lysates were prepared and resolved (40  $\mu$ g protein) by SDS-PAGE and immunoblotted with phospho-p38 MAPK (T<sup>180</sup>/Y<sup>182</sup>) or phospho-PKB Ser<sup>473</sup> (equal loading of protein on gels was confirmed by subsequent Ponceau S staining of the transfer membrane). 2-deoxyglucose (2DG) uptake was assayed in soleus muscle from wild-type and p38 $\beta$  MAPK-null mice in the absence and presence of insulin (100 nmol/l, 60 min) (**B**) or after incubation in the absence or presence of insulin (100 nmol/l, 60 min) and/or SB 203580 (10  $\mu$ mol/l, 60 min) (**C**) from wild-type and p38 $\beta$  MAPK-null mice. The data represent means  $\pm$  SEM for up to five animals in each case. \*Significant change from the untreated control ( $P < 0.05$ ), whereas the dagger ( $\dagger$ ) represents a significant change between the two bars indicated ( $P < 0.05$ ).

of both wild-type and knockout mice by 48 and 44%, respectively (Fig. 5C).

## DISCUSSION

A key principle underpinning the idea that p38 MAPK may activate glucose transporters recruited to the surface membranes of skeletal muscle and adipose tissue by insulin is that the hormone also induces an acute activation of p38 MAPK signaling (9,23). In brown adipocytes, it has been reported that only the  $\beta$ -isoform of p38 MAPK is activated, suggesting that the  $\beta$ - and not the  $\alpha$ -isoform may signal to and activate recruited GLUT4 transporters (24). However, the ability of insulin to promote activation/

phosphorylation of p38 MAPK is not a universal finding (22,25), and even where the hormone has been shown to stimulate p38 MAPK the involvement of this signaling cascade in the acute activation of glucose transport has been questioned (26,27). Fujishiro et al. (27) have demonstrated, for example, that suppressing p38 MAPK activation by overexpression of a dominant-negative p38 MAPK did not diminish insulin-induced glucose uptake by 3T3-L1 adipocytes. There is also evidence showing that acute activation of the p38 pathway by reactive oxygen species antagonizes insulin-stimulated glucose transport in a SB 203580-sensitive manner in cultured L6 myotubes (25). In line with this observation, evidence also exists showing that sustained activation of the p38 pathway (achieved by expressing constitutively active forms of the upstream kinases, MKK3/6, or by overexpression of the drug-insensitive  $\gamma$ -isoform of p38 MAPK) reduces GLUT4 expression and insulin-stimulated glucose transport in fat cells and adult mouse muscle (27,28), whereas sustained pharmacological inhibition of p38 signaling has an insulin-sensitizing effect in 3T3-L1 adipocytes (29). Although it is accepted that distinct mechanisms may be involved in the desensitization of these tissues to insulin after acute or chronic activation of the p38 pathway, the findings run counter to the suggestion that this pathway may participate in the activation of glucose transporters recruited to the cell surface by insulin.

Here, too, we show that irrespective of whether adipocytes were isolated from wild-type or p38 $\beta$  MAPK-null mice we could not detect any insulin-stimulated increases in p38 MAPK phosphorylation over and above that observed in unstimulated cells. This observation is in line with recent data obtained using rat adipocytes (22) and implies that p38 MAPK is either not involved in the acute hormonal stimulation of adipocyte glucose transport or that activation of surface transporters is achieved by a mechanism that does not require an attendant increase in kinase phosphorylation/activation. The finding that basal and insulin-stimulated glucose uptake is not altered in muscle or fat of mice lacking p38 $\beta$  MAPK provides compelling evidence that this isoform is not involved in the activation step of newly inserted transporters at the plasma membrane; a proposition consistent with the fact that these mice do not exhibit any major phenotypic changes with respect to whole-body glucose handling and insulin sensitivity. It is also worth drawing attention to the finding that p38 $\beta$  was virtually undetectable in skeletal muscle (Fig. 1), thereby strengthening the argument that it is unlikely to be involved in regulation of glucose transport in this tissue. The extremely low level of p38 $\beta$  in muscle would imply that p38 $\alpha$  is the de facto drug-sensitive isoform in this tissue. However, unlike p38 $\beta$ , it is difficult to apply a transgenic approach that directly tests whether p38 $\alpha$  is required to support the insulin-dependent increase in glucose transport in adipose tissue and muscle because the mouse knockout of this isoform is embryonic lethal (30).

Curiously, although SB 203580 reduced insulin-stimulated, but not basal, glucose uptake in soleus muscle, we did not initially observe this inhibition in our adipocyte studies; a finding that was in stark contrast to recent work from Ribe et al. (22), who reported that SB 203580 suppressed insulin-stimulated glucose uptake in rat adipocytes. One possible explanation for this discrepancy is that the studies reported by Ribé et al. used a lower packed cell volume (cytocrit) compared with those reported here.

This appears to be important because reducing the cytotrit by one-half enabled us to observe the inhibition in insulin-stimulated glucose transport by SB 203580. Precisely why sensitivity of the stimulated transport activity toward SB 203580 is influenced by cytotrit is unclear at present. However, it is plausible that in the presence of an elevated fat mass that would be associated with the higher cytotrit, there might be greater partitioning of the organic inhibitor into the intracellular lipid. If so, this may have the effect of reducing the free effective concentration of the inhibitor and its ability to suppress p38 signaling and glucose transport. Although we cannot exclude such partitioning effects, it seems unlikely that it would have a significant impact on the efficacy of the inhibitor given that we observe a substantial reduction in p38 signaling in response to anisomycin at the higher cytotrit (Fig. 3).

The finding that activation of p38 MAPK by anisomycin on the one hand and stimulation of glucose transport by insulin on the other exhibit different sensitivities to SB 203580 when using a higher packed cell volume argues against a role for p38 MAPK in the activation of glucose transport by insulin. If this assertion is correct, SB 203580 must inhibit insulin-stimulated glucose uptake by some other mechanism unrelated to its action on p38 MAPK. Ribé et al. (22) have recently addressed this issue by investigating the effects of SB 203580 on 3-O-methyl glucose (3-OMG) uptake in rat adipocytes under zero-trans and equilibrium exchange conditions. These investigators have shown that in the absence of 3-OMG (zero-trans), adipocytes loaded with SB 203580 display reduced insulin-stimulated glucose transport as a result of noncompetitive inhibition. However, if adipocytes are loaded with inhibitor in the presence of 3-OMG, which unlike 2-deoxyglucose is not phosphorylated and is able to engage with GLUT transporters on the endo-facial surface, the observed inhibition in transport becomes competitive, suggesting that inhibitor and substrate may compete for GLUT binding on the internal membrane face (22). If this mechanism underlies the suppressive action of the inhibitor on glucose transport, then one might have expected SB 203580 to also inhibit basal glucose uptake, which we (Fig. 4) and others do not observe (8,9). It is possible that the lack of any inhibition in basal glucose uptake reflects differences in the ability of the inhibitor to target GLUT1 vis a vis GLUT4 transporters, because the former would be the predominant carrier in the plasma membrane of unstimulated adipocytes and skeletal muscle. This suggestion is in line with very recent work from Antonescu et al. (31), who have performed cell surface photolabeling experiments and report that SB 203580 only affects labeling of membrane transporters after insulin stimulation of cells. Antonescu et al. have hitherto been leading exponents of the idea that p38 MAPK mediates a crucial signaling step that activates newly recruited glucose transporters. However, while this report was under review, Antonescu et al. revised their stance on this issue based on data from studies in which the cellular activity and expression of p38 MAPK isoforms have been manipulated. Their very recent work shows that expression of a dominant-negative p38 MAPK or a drug-resistant form of p38 MAPK in L6 muscle cells is unable to prevent the inhibition in glucose uptake elicited by SB 203580 (31). Moreover, they also find that reducing expression of both p38 $\alpha$  and p38 $\beta$  MAPK using small interfering RNA fails to confer any protection against the inhibitory effects of SB 203580 upon insulin-stimulated glucose uptake (31). In light of these

findings, they propose that the inhibition exerted by SB 203580 on insulin-stimulated glucose transport probably reflects increased susceptibility of recruited GLUT4 transporters to the drug itself, a view that is broadly compatible with the recent findings from Ribe et al. (22).

In summary, based on the collective strength of the available evidence presented here and in the recent literature, it is highly unlikely that signaling via SB 203580-sensitive isoforms of p38 MAPK contributes to activation of recruited transporters in the surface membranes of adipose tissue and skeletal muscle. There is, nevertheless, considerable evidence that indirectly supports the existence of an activation step (5), and thus defining the molecular players in this process remains a challenge for future work not only for providing a better conceptual understanding of how insulin stimulates glucose uptake, but also for identifying novel therapeutic targets whose manipulation may be of benefit in the management of impaired glycemic control normally associated with insulin resistance and diabetes.

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