Original Article

Bradykinin Augments Insulin-Stimulated Glucose Transport in Rat Adipocytes via Endothelial Nitric Oxide Synthase–Mediated Inhibition of Jun NH_2-Terminal Kinase

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An increase in bradykinin has been suggested to contribute to the enhanced insulin sensitivity observed in the presence of ACE inhibitors. To investigate a potential direct, nonvascular effect on an insulin target tissue, the effect of bradykinin on glucose uptake and insulin signaling was studied in primary rat adipocytes. Whereas basal glucose uptake was not altered, bradykinin augmented insulin-stimulated glucose uptake twofold, which was blocked by HOE-140, a bradykinin B_2 receptor antagonist. The bradykinin effect on glucose uptake was nitric oxide (NO) dependent, mimicked by NO donors and absent in adipocytes from endothelial NO synthase$^{-/-}$ mice. Investigation of insulin signaling revealed that bradykinin enhanced insulin receptor substrate-1 (IRS-1) Tyr phosphorylation, Akt/protein kinase B phosphorylation, and GLUT4 translocation. In contrast, insulin-stimulated extracellular signal–regulated kinase1/2 and Jun NH_2-terminal kinase (JNK) activation were decreased in the presence of bradykinin, accompanied by decreased IRS-1 Ser^{307} phosphorylation. Furthermore, bradykinin did not enhance insulin action in the presence of the JNK inhibitor, SP-600125, or in adipocytes from JNK1$^{-/-}$ mice. These data indicate that bradykinin enhances insulin sensitivity in adipocytes via an NO-dependent pathway that acts by modulating the feedback inhibition of insulin signaling at the level of IRS-1. *Diabetes* 55:2678–2687, 2006

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Received for publication 28 November 2005 and accepted in revised form 21 June 2006.

2DG, 2-deoxyglucose; ACEI, ACE inhibitor; AngII, angiotensin II; ARB, angiotensin receptor blocker; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal–regulated kinase; GFP, green fluorescent protein; iNOS, inducible nitric oxide synthase; IRS-1, insulin receptor substrate-1; JNK, Jun NH_2-terminal kinase; KKP, kallikrein-kinin pathway; LDM, low-density microsome; L-NAME, N_ω-nitro-L-arginine methyl ester hydrochloride; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PTIO, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazol-1-oxyl-3-oxide potassium salt; RAS, renin-angiotensin system; SNP, sodium nitroprusside; TNF, tumor necrosis factor.

DOI: 10.2337/db05-1538

Insulin resistance is associated with obesity and type 2 diabetes (1). Factors implicated in the pathogenesis of insulin resistance include nutrient excess, elevated levels of tumor necrosis factor (TNF)-α, resistin, decreased adiponectin, and hyperinsulinemia (rev. in 2,3). Insulin resistance has also been associated with dyslipidemia and hypertension (4). Although these associations have been coined the "metabolic syndrome" (5), the cellular and molecular mechanisms of these linkages are not completely understood.

In the context of hypertension, a number of trials have been conducted in subjects at high risk for the development of cardiovascular disease. The Heart Outcomes Prevention Evaluation (HOPE) study demonstrated that therapy with ramipril, an ACE inhibitor (ACEI), decreased cardiovascular events by 35% and, unexpectedly, significantly reduced the incidence of type 2 diabetes (6). Given the strong association between hypertension and insulin resistance, one possible explanation is that ACE inhibition improves insulin sensitivity. There have been several reports of hypoglycemia concomitant with apparent potentiation of the action of hypoglycemic agents in individuals with type 2 diabetes who received ACEIs (7). Furthermore, ACE inhibition enhanced insulin sensitivity in vivo in dogs and humans (8,9).

Blood pressure is regulated by the renin-angiotensin system (RAS) and the kallikrein-kinin pathway (KKP) (10). The main product of the RAS is angiotensin II (AngII), which is produced in a reaction catalyzed by ACE. AngII is a vasopressor and has been shown to contribute to hypertension and atherogenesis. Bradykinin is the major active peptide of the KKP, which counterregulates the RAS. Bradykinin acts by binding to G-protein–coupled receptors, the B_1 and B_2 receptor, and signals predominantly through the constitutively expressed B_2 receptor to mediate most of its actions in vascular tissue via the activation of endothelial nitric oxide (NO) synthase (eNOS) through a Ca^{2+}-dependent pathway (11,12). Inhibition of ACE, which is also called kininase II, not only blocks the production of AngII, but also elevates the circulating levels of bradykinin (13).

Both the RAS and KKP are present in the circulation as well as in many tissues (10,14). The question of whether decreased AngII or elevated bradykinin mediates the enhanced insulin sensitivity observed in the presence of ACEIs has been addressed in part by the use of AngII

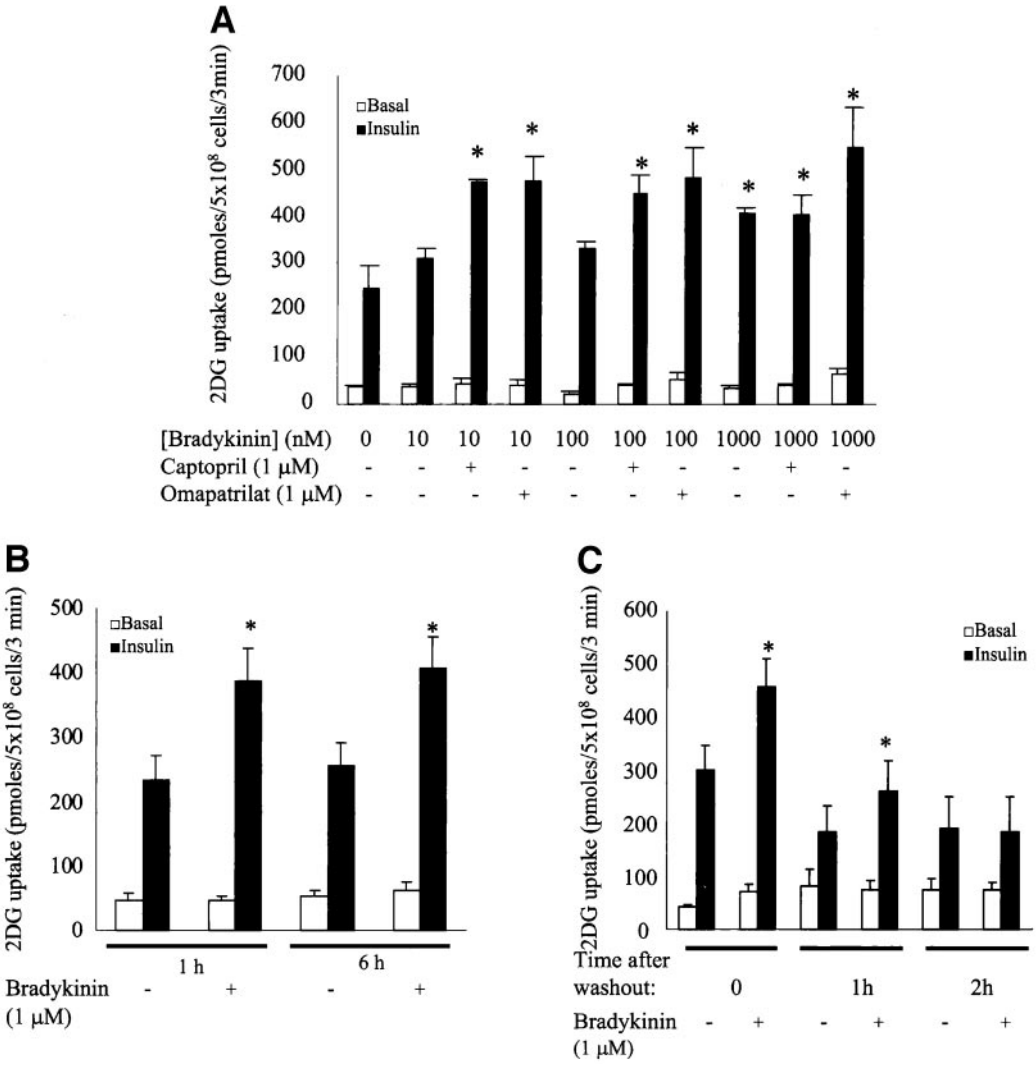

FIG. 1. Bradykinin augments insulin-stimulated glucose uptake in rat adipocytes. *A*: Isolated rat adipocytes were pretreated with captopril, omapatrilat, or vehicle for 1 h, followed by bradykinin ± insulin for 30 min, and 2DG uptake was assayed. *B*: Adipocytes were incubated with bradykinin or vehicle for 1 h or 6 h ± insulin for the final 30 min. *C*: Adipocytes were incubated ± bradykinin for 1 h and washed, and 2DG uptake ± insulin was assayed at $t = 0$, 1, and 2 h after washing. Values are means ± SE ($n = 5$). *$P < 0.02$ vs. insulin without bradykinin.

receptor blockers (ARBs). In rodent studies, ARBs were not as effective as ACEIs in increasing insulin action (15), and infusion of bradykinin improved insulin action in Zucker *fa/fa* rats (16). These data point to bradykinin as playing a major role. Although many effects of bradykinin have been attributed to actions on vascular tissue and blood flow (17), the possibility of direct effects on insulin target tissues has not been excluded and its mechanism has not been elucidated. Thus, we tested the effect of bradykinin on insulin sensitivity in isolated rat adipocytes using glucose uptake as a metabolic outcome and explored the interaction of the bradykinin and insulin signal transduction pathways.

RESEARCH DESIGN AND METHODS

Dulbecco's modified Eagle's medium was purchased from Invitrogen (Burlington, ON, Canada) and collagenase type I from Worthington (Lakewood, NJ). Bio-Rad protein assay reagent was from Bio-Rad (Hercules, CA) and LumiGlo chemiluminescent substrate kit from KPL (Gaithersburg, MD). The bradykinin assay kit was obtained from Peninsula Laboratories (San Carlos, CA). The Jun NH_2-terminal kinase (JNK) activity immunoassay kit was from Calbiochem (San Diego, CA). Anti-bradykinin B_2 receptor, anti-Akt/protein kinase B (PKB), anti-Akt2/PKBβ, anti–phospho-Akt/PKB (Thr^{308}), anti–phospho-Akt/PKB (Ser^{473}), anti-p38, anti–extracellular signal–regulated kinase (ERK)1/2, anti–phospho-ERK1/2 (Thr^{202}/Tyr^{204}), anti-JNK, anti–phospho-JNK (Thr^{183}/Tyr^{185}), and anti-pY antibodies were from Cell Signaling (Beverley, MA). Anti-neuronal NO synthase (nNOS), anti-eNOS, and anti-inducible NO synthase (iNOS) antibodies were from BD Transduction Laboratories (San Diego, CA). Anti–insulin receptor substrate-1 (IRS-1), anti–phospho-IRS-1 (Ser^{307}), and anti-p85 antibodies were from Upstate (Lake Placid, NY). Monoclonal anti-hemagglutinin antibody was from Covance (Princeton, NJ). Omapatrilat was kindly provided by Bristol-Myers Squibb (St. Laurent, QC, Canada). Rhodamine red–conjugated anti-IgG1 antibody was from Jackson Immunoresearch (West Grove, PA). Paraformaldehyde was from Fisher Scientific (Ottawa, ON, Canada). Vectashield was from Vector Laboratories (Burlingame, CA). All other chemicals were from Sigma Aldrich (Oakville, ON).

Animal protocols were approved by the Animal Care Committee of the Toronto General Hospital and carried out in accordance with the guidelines of the Canadian Council of Animal Care. Male Sprague-Dawley rats (180–220 g) were obtained from Charles River (St. Constant, QC, Canada). The following strains of male mice (7 weeks old) were obtained from The Jackson Laboratories (Bar Harbor, ME): $eNOS^{-/-}$, $nNOS^{-/-}$, $JNK1^{-/-}$, $JNK2^{-/-}$, and controls B6129SF2/J and C57BL/6J. Animals were given rodent chow and water ad libitum, exposed to a 12-h light/dark cycle, and acclimatized for 1 week before experiments.

Isolation of epididymal fat pads. Male Sprague-Dawley rats or male mice were killed, epididymal fat pads were removed, and adipocytes were isolated as described (18). After collagenase digestion, adipocytes were filtered through 20-μ mesh and washed three times with 3% BSA–Krebs-Ringer bicarbonate HEPES buffer (118 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 5 mmol/l $NaHCO_3$, 30 mmol/l HEPES, and 1 mmol/l pyruvate, pH 7.4).

2-deoxy-D-[^{3}H]-glucose uptake assay. Isolated adipocytes were incubated with various concentrations of bradykinin and/or NO inhibitors/scavengers/donors for the times indicated. Basal and insulin-stimulated (100 nmol/l for 30 min at 37°C) rates of glucose uptake were assayed as described (18).

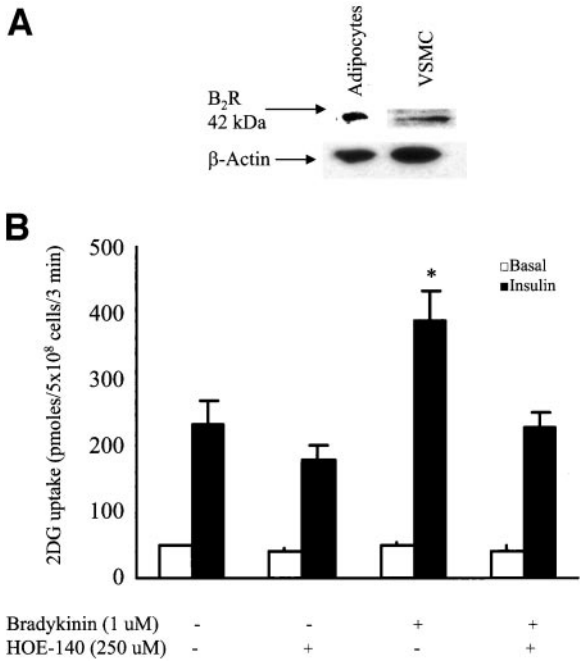

FIG. 2. Bradykinin increases insulin-stimulated glucose uptake via the B_2 receptor (B_2R). *A*: Protein (20 μg) from adipocyte and cultured vascular smooth muscle cell (VSMC) lysates was immunoblotted for B_2 receptor and actin. *B*: Adipocytes were pretreated with the B_2 receptor antagonist HOE-140 for 20 min, followed by bradykinin ± insulin. Values are means ± SE (n = 5). *P < 0.02 vs. insulin without bradykinin.

Western blotting. Adipocytes were solubilized by adding 200 μl of lysis buffer (50 mmol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.5 mmol/l sodium orthovanadate, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mmol/l sodium fluoride, 5 mmol/l sodium pyrophosphate, 10 mmol/l sodium β-glycerophosphate, 0.1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml okadaic acid) per milliliter of cells followed by homogenization. After centrifugation, the fat was removed, and the infranatant was stored at −70°C. Laemmli sample buffer (2×) with 0.006% bromphenol blue was added to equal aliquots of protein (Bradford assay) and boiled for 5 min. For mitogen-activated protein kinase (MAPK) analysis, cellular protein was extracted using boiling Laemmli sample buffer. For GLUT4 analysis, urea sample buffer was used (6.86 mol/l urea, 4.29% SDS, 43 mmol/l Tris-HCl, and 300 mmol/l dithiothreitol). For immunoprecipitation of Akt2/PKBβ, 750 μg of whole cell lysates were incubated with 4 μg of anti-Akt2 antibody at 4°C overnight, followed by addition of 50 μl of protein A-agarose beads for 2 h, and the samples were centrifuged and washed three times. After separation by SDS-PAGE, proteins were transferred onto nitrocellulose membranes, which were blocked with 5% milk- or BSA–Tris-buffered saline with 0.1% Tween for 1 h at room temperature. Membranes were incubated overnight with primary antibodies, washed with Tris-buffered saline with 0.1% Tween and incubated with horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature. Membranes were washed, reacted with LumiGlo chemiluminescence, and exposed to film.

Subcellular fractionation of rat adipocytes. Adipocytes were fractionated as described previously (19). Briefly, cells were washed, homogenized (250 mmol/l sucrose, 2 mmol/l EDTA, 2.5 mmol/l Tris-HCl, pH 7.4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μmol/l phenylmethylsulfonyl fluoride) and then centrifuged at 13,100*g* for 20 min at 4°C to remove the fat cake. The infranatant was centrifuged at 31,000*g* for 1 h to yield the low-density microsomes (LDMs). The pellet from the first spin was layered over a sucrose cushion and centrifuged at 75,000*g* for 1 h. The interphase was removed and spun at 39,000*g* for 20 min to yield the plasma membrane. The purity and yield of LDM and plasma membrane were similar in control and bradykinin-treated cells. The relative amounts of LDM and plasma membrane GLUT4 were determined by normalizing the intensities of the bands from the immunoblots for the protein yield of each fraction.

Immunofluorescence and confocal microscopy. Primary adipocytes were electroporated as described previously (20). Briefly, 400 μl of adipocyte suspension (lipocrit of 60%) were added to an equal volume of Dulbecco's modified Eagle's medium containing 7.5 μg of pQBI25 plasmid encoding hemagglutinin–GLUT4–green fluorescent protein (GFP) (kindly provided by S.W. Cushman, Yale University, New Haven, CT). Using the Gene Pulser XCell System (BioRad), cells were electroporated in 0.4-cm gap-width cuvettes with two pulses of 25 μF at 800 V followed by one pulse of 1,050 μF at 200 V. Eighteen hours after transfection, the adipocytes were washed, treated or not treated with bradykinin (1 μmol/l) for 1 h and/or insulin (100 nmol/l) for 30 min, and incubated with mouse monoclonal anti-hydroxylamine antibody (1:150) and donkey anti-mouse IgG antibody conjugated with rhodamine red (1:100), fixed in 4% paraformaldehyde in PBS, and mounted on glass slides using Vectashield mounting medium as described (21). Fluorescence was observed using the Leica TCS SP2 Confocal Laser Scanning Microscope and analyzed with Leica Confocal Software.

Induction of iNOS. iNOS was induced with a single injection of lipopolysaccharide (LPS) (4 mg/kg) (22). Eight hours later, total cell proteins (100 μg) were separated by SDS-PAGE and immunoblotted for iNOS (1:350).

Quantification of concentration of bradykinin. Media in which the adipocytes were incubated were collected at various times after addition of bradykinin. The bradykinin concentration was determined by an enzyme immunoassay (Peninsula).

Quantification of JNK activity. Adipocytes, treated or not treated with bradykinin and/or insulin, were lysed. JNK activity was then determined using the kit as described by the manufacturer (Calbiochem).

Statistical analysis. Values shown are means ± SE. Results were analyzed by ANOVA, and differences were deemed significant at $P < 0.05$.

RESULTS

Bradykinin enhances insulin-stimulated glucose uptake in rat adipocytes via the B_2 receptor. Whereas treatment of primary rat adipocytes for 30 min with 0–10 μmol/l bradykinin had no effect on basal 2-deoxyglucose (2DG) uptake, insulin-stimulated 2DG uptake was enhanced in a dose-dependent manner, peaking at 1 μmol/l ([pmol · 5 × 10^8 $cells^{-1}$ · 3 min^{-1}] control basal 35.4 ± 2.8, insulin 246.2 ± 47.7, bradykinin basal 33.7 ± 5.4, and bradykinin + insulin 374.8 ± 12.7; $P < 0.05$, insulin vs. bradykinin + insulin) (Fig. 1*A*). The effect of bradykinin was maintained at 1 and 6 h (Fig. 1*B*). To determine the duration and reversibility of the effect of bradykinin, the adipocytes were washed after 1 h of incubation. Insulin-stimulated 2DG uptake remained enhanced for 1 h after washout, but this effect was no longer significant at 2 h (Fig. 1*C*).

Although effective bradykinin concentrations are in the nanomolar range in vivo, bradykinin is subject to degradation by cell-associated kininases. Indeed, the concentration of bradykinin markedly decreased upon incubation with adipocytes from 1 μmol/l to 49 ± 3 nmol/l after 1 h and to 608 ± 52 pmol/l after 6 h. To examine the effect of kininase inhibition, adipocytes were pretreated with either the ACEI captopril (1 μmol/l) or the ACEI/neutral endopeptidase inhibitor omapatrilat (1 μmol/l) for 1 h before the addition of bradykinin. Both captopril and omapatrilat shifted the dose-response curve to the left so that as little as 10 nmol/l bradykinin significantly enhanced insulin-stimulated 2DG uptake. (Fig. 1*A*).

The presence of the B_2 receptor in rat adipocytes was demonstrated by immunoblotting (Fig. 2*A*). In addition, pretreatment of adipocytes with the B_2 receptor antagonist HOE-140 (250 μmol/l) blocked the enhancement by bradykinin of insulin-stimulated glucose uptake (Fig. 2*B*).

Bradykinin action on insulin-stimulated 2DG uptake involves NO and eNOS. Bradykinin leads to NO production in endothelial cells, which in turn signals its vasodilatory actions (11–13). To investigate whether bradykinin also acts via the generation of NO in adipocytes, we first immunoblotted the three isoforms of NO synthase (NOS): eNOS, nNOS, and iNOS. Under standard conditions, isolated rat adipocytes expressed primarily eNOS, with no nNOS and barely detectable levels of iNOS (Fig. 3*A*).

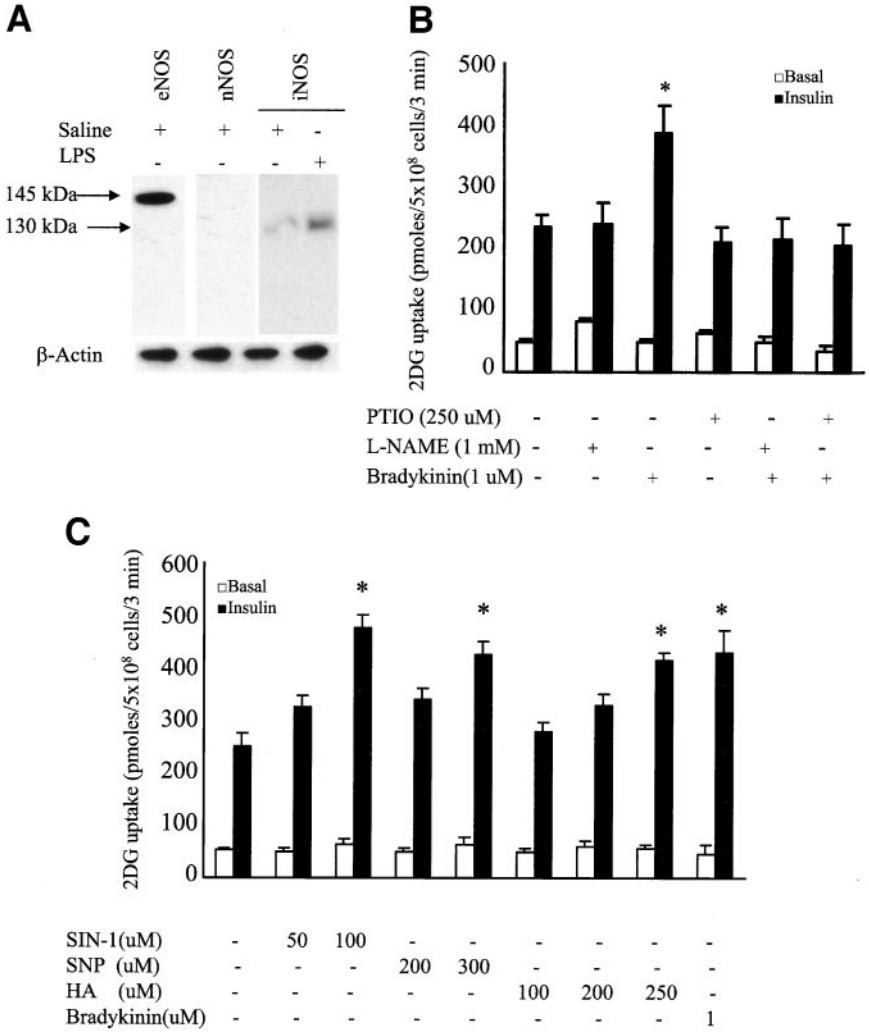

FIG. 3. NO is required for bradykinin action. *A*: Adipocyte lysates from saline-injected rats were immunoblotted for eNOS, nNOS, and iNOS and from LPS-injected rats for iNOS. Representative immunoblot ($n = 3$). *B*: Adipocytes were preincubated with L-NAME, PTIO, or vehicle for 1 h and then with bradykinin ± insulin ($n = 6$). *C*: Adipocytes were incubated (30 min) with 3-morpholinosydnonimine (SIN-1), SNP, and hydroxylamine (HA) ± insulin. Values are means ± SE ($n = 5$). *$P < 0.05$ vs. insulin without bradykinin.

However, iNOS could be induced by LPS administration after 8 h (Fig. 3*A*). To probe the role of NO, adipocytes were incubated with and without bradykinin in the presence and absence of either the NOS inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME) (1 mmol/l) or the NO scavenger 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazol-1-oxyl-3-oxide potassium salt (PTIO) (250 μmol/l), followed by assessment of basal and insulin-stimulated 2DG uptake. Both L-NAME and PTIO blocked the enhancement by bradykinin of insulin-stimulated 2DG uptake (Fig. 3*B*). To test whether NO generation was sufficient to mimic the effect of bradykinin, adipocytes were incubated for 30 min with and without insulin in the absence and presence of one of three NO donors: sodium nitroprusside (SNP), 3-morpholino-sydnonimine, and hydroxylamine. These significantly enhanced insulin-stimulated 2DG uptake comparable to the effect of bradykinin (Fig. 3*C*). Insulin-stimulated 2DG uptake remained elevated after NO donors for up to 6 h, similar to bradykinin (data not shown). The above data indicate that NO is the mediator of the enhanced insulin-stimulated 2DG uptake by bradykinin.

The eNOS isoform is required for bradykinin action in adipocytes. The requirement for NO synthesis and the detection of eNOS led us to test the effect of bradykinin in adipocytes lacking eNOS or nNOS. Bradykinin enhanced insulin-stimulated 2DG uptake in adipocytes from $nNOS^{-/-}$ mice and control mice to a similar extent (Fig. 4*A*). However, although adipocytes from $eNOS^{-/-}$ mice had a slightly increased basal and decreased insulin-stimulated 2DG uptake compared with controls, bradykinin did not alter either basal or insulin-stimulated glucose uptake (Fig. 4*B*). Because the defect in the action of bradykinin in $eNOS^{-/-}$ mice was presumed to be due to an inability to generate NO, it was predicted that the adipocytes would respond to an NO donor. Indeed, treatment of $eNOS^{-/-}$ adipocytes with hydroxylamine (500 μmol/l) augmented insulin-stimulated glucose transport (Fig. 4*B*). Thus, the presence of eNOS is critical for bradykinin to enhance insulin-stimulated glucose uptake.

Bradykinin augments insulin-stimulated GLUT4 translocation. To explore the mechanism by which bradykinin increases insulin-stimulated glucose uptake, GLUT4 translocation was determined. Whereas bradykinin alone minimally increased plasma membrane GLUT4 (NS), it significantly augmented the effect of insulin to induce translocation (GLUT4 content in the plasma membrane as a percentage of total GLUT4 from densitometric analysis: control basal 21.5 ± 4.9, control + insulin 55.6 ± 7.2, bradykinin basal 27.8 ± 8.0, and bradykinin + insulin 83.5 ± 3.9%; $P < 0.05$ vs. control + insulin) (Fig. 5*B*).

To confirm these findings, adipocytes were transfected

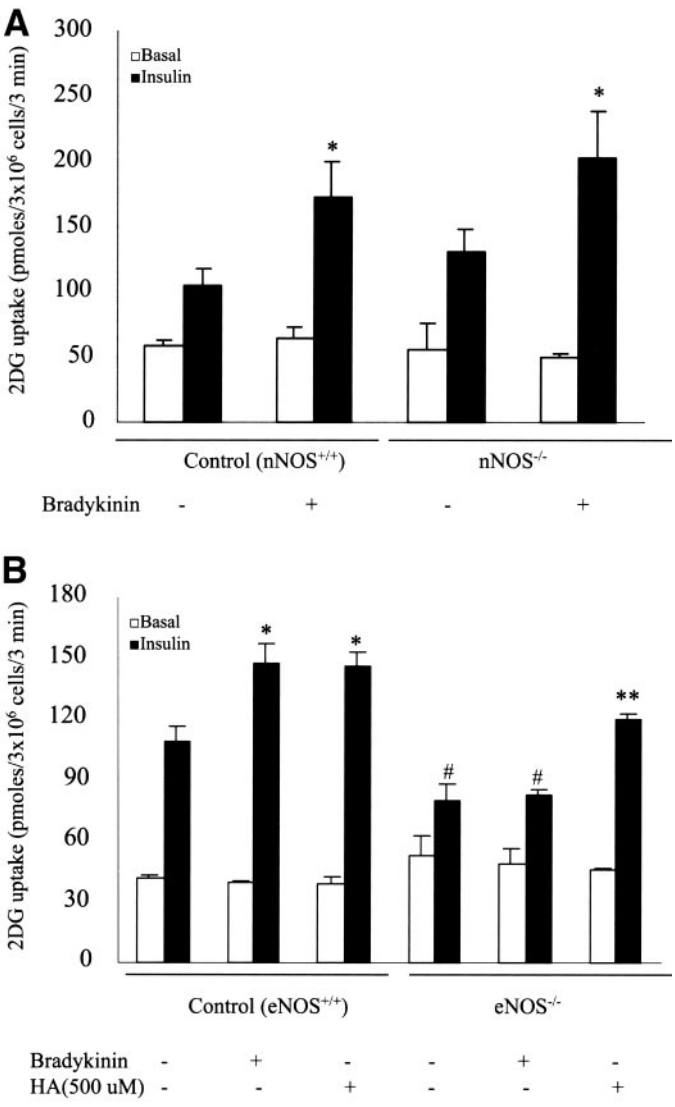

FIG. 4. Requirement of eNOS for bradykinin action on insulin-stimulated glucose uptake. Adipocytes were isolated from control (nNOS$^{+/+}$) and nNOS$^{-/-}$ control (eNOS$^{+/+}$) mice (*A*) and eNOS$^{-/-}$ mice (*B*) and treated with vehicle, bradykinin, or hydroxylamine (HA) (30 min) ± insulin. Values are means ± SE (n = 4). *A*: *P < 0.05 vs. control insulin or nNOS$^{-/-}$ insulin without bradykinin. *B*: *P < 0.05 vs. control insulin without bradykinin or HA; **P < 0.01 vs. eNOS$^{-/-}$ insulin with or without bradykinin; #P < 0.04 vs. basal eNOS$^{-/-}$.

with a hemagglutinin epitope–tagged GLUT4-GFP–expressing cDNA. The hemagglutinin tag is inserted in the exofacial loop of GLUT4. Thus, in nonpermeabilized cells, anti-hemagglutinin will bind only to GLUT4 that is inserted into the plasma membrane (20,21). Imaging confirmed that bradykinin-enhanced GLUT4 translocation (mean fluorescence intensity representing cell surface hemagglutinin-GLUT4-GFP [arbitrary units]: control basal 1.0 ± 0, control + insulin 1.99 ± 0.13, bradykinin basal 1.17 ± 0.015, and bradykinin + insulin 2.57 ± 0.08; P < 0.04 vs. control + insulin (n = 3) (Fig. 5*C*).

Bradykinin augments insulin signaling in rat adipocytes. Recruitment of GLUT4 to the plasma membrane by insulin is mediated by a signaling pathway that involves Tyr phosphorylation of IRS-1 and activation of phosphatidylinositol 3-kinase (PI3K) and Akt/PKB (23). Insulin stimulation led to an increase in IRS-1 pY and p85 association (Fig. 6*A*). Treatment with bradykinin alone did not alter basal levels but significantly enhanced these effects of insulin (Fig. 6*A*). Similarly, although bradykinin alone had no effect on Akt/PKB phosphorylation on Thr^{308} and Ser^{473}, insulin-stimulated phosphorylation was significantly augmented (Fig. 6*B*). Of the three isoforms of Akt/PKB, Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ (24), Akt2/PKBβ is involved in insulin-stimulated GLUT4 translocation to the plasma membrane (25,26). Thus, Akt2/PKBβ was immunoprecipitated from whole cell lysates, separated by SDS-PAGE, and immunoblotted for total Akt2/PKBβ and Ser^{473}. Bradykinin significantly enhanced insulin-stimulated Akt2/PKBβ pSer^{473} phosphorylation (Fig. 6*C*).

Bradykinin decreases insulin-stimulated phosphorylation of ERK1/2 (p42/p44 MAPK) and JNK. Apart from the PI3K-Akt/PKB signaling pathway, insulin also leads to the activation of the MAPK pathway (23,27). Thus, we examined the effect of bradykinin ± insulin on the phosphorylation levels of p38 MAPK, ERK1/2 (p42/p44 MAPK), and JNK using phospho-specific antibodies that recognize their activated forms. Insulin did not stimulate the phosphorylation of p38 MAPK in adipocytes, consistent with previous reports (28) (data not shown), whereas the phosphorylation of ERK1/2 and p54 JNK was increased. It was noted that expression of p46 JNK appeared very weak to undetectable in adipocyte lysates. Unexpectedly, in contrast to the findings with Akt/PKB, bradykinin decreased the insulin-stimulated phosphorylation of both ERK1/2 and p54 JNK (Fig. 7*A*). The functional consequence of decreased phosphorylation was confirmed by the finding of decreased kinase activity in JNK immunoprecipitates (phospho-c-Jun corrected for total JNK [arbitrary units]: control basal 1.0, control + insulin 1.69 ± 0.01, bradykinin basal 0.81 ± 0.06, and bradykinin + insulin 0.96 ± 0.08; P = 0.014, control + insulin vs. bradykinin + insulin) (Fig. 7*B*).

These somewhat surprising results suggested a potential mechanism by which bradykinin could increase insulin signaling to glucose transport. It has been reported that IRS-1 may be a substrate of JNK (27) and ERK1/2 (29). In particular, phosphorylation of Ser^{307} of IRS-1 by JNK has been found to be associated with decreased insulin-stimulated IRS-1 Tyr phosphorylation and insulin resistance (27,30). Exposure to bradykinin significantly decreased IRS-1 Ser^{307} phosphorylation in response to insulin, accompanied by an increase in IRS-1 pY (Fig. 7*C*). Phosphorylation of Ser^{616} of IRS-1 by ERK1/2 also reduces insulin signaling (29). Bradykinin decreased insulin-stimulated phosphorylation of IRS-1 Ser^{616} (Fig. 7*B*). These data suggested that inhibition of JNK and/or ERK activity and subsequent IRS-1 Ser^{307} and Ser^{616} phosphorylation, respectively, could mediate the enhancement of insulin signaling by bradykinin.

To test whether inhibition of either JNK or ERK could replicate the effect of bradykinin to enhance glucose uptake, adipocytes were preincubated with either the MEK inhibitor PD-98059 or the JNK inhibitor SP-600125. Inhibition of JNK but not of MEK increased insulin-stimulated 2DG uptake comparable to bradykinin. Coincubation of the JNK inhibitor with bradykinin did not result in any further increase in insulin-stimulated 2DG uptake (Fig. 7*C*).

The JNK1 isoform appears to be responsible for the phosphorylation of IRS-1 Ser^{307} and induction of insulin resistance. Thus, JNK1$^{-/-}$ but not JNK2$^{-/-}$ mice are insulin sensitive and resistant to high-fat diet–induced

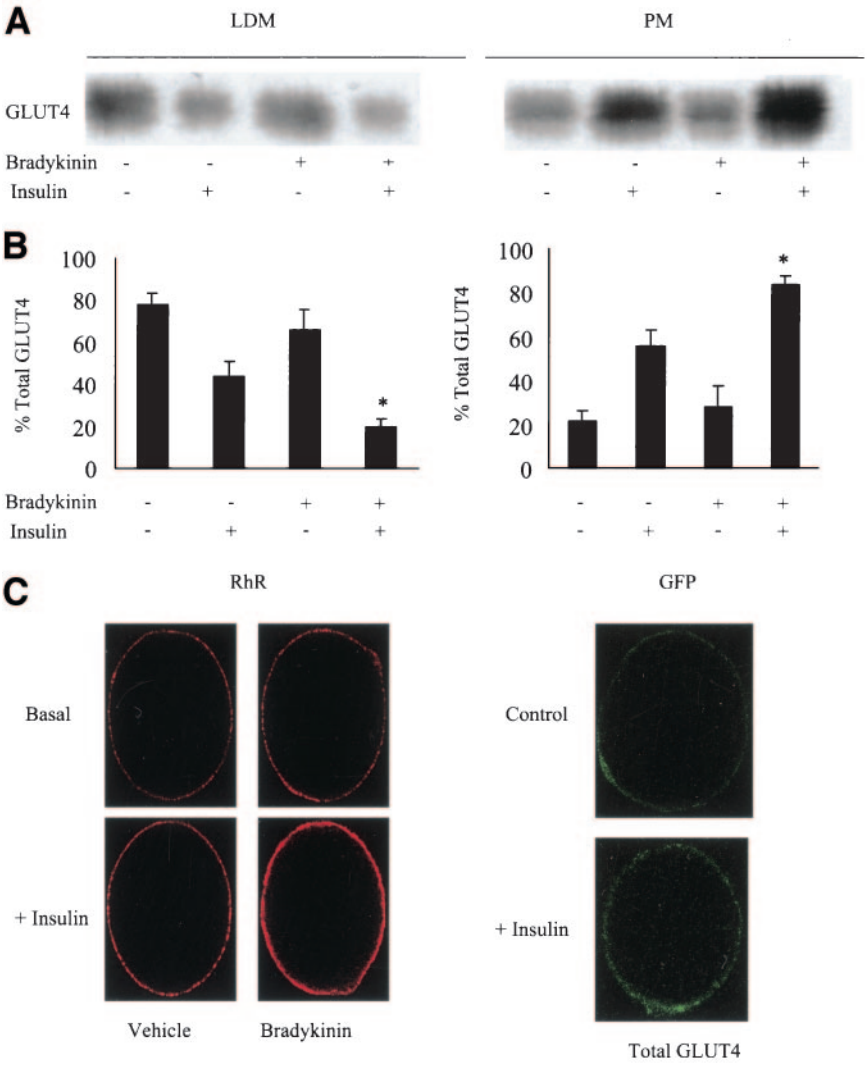

FIG. 5. Bradykinin enhances insulin-stimulated GLUT4 translocation. Adipocytes were treated with bradykinin for 1 h ± insulin (final 40 min), and LDM and plasma membrane fractions were isolated. *A*: Representative immunoblot. *B*: The total amount of GLUT4 (LDM + plasma membrane) was set at 100%. Values are means ± SE (n = 5) and represent the percentage of total GLUT4 in each fraction. *P < 0.02 vs. insulin without bradykinin. *C*: Adipocytes were transfected with a hemagglutinin-GLUT4-GFP expression vector by electroporation. After overnight incubation, cells were treated with and without bradykinin ± insulin. A representative set of cells is shown. RhR, rhodamine red signal (see text for details).

insulin resistance (31,32). Adipocytes from JNK1$^{-/-}$ mice showed increased insulin-stimulated glucose uptake, and this was not enhanced by bradykinin. In contrast, JNK2$^{-/-}$ adipocytes displayed sensitivity to insulin and bradykinin similar to that of controls (Fig. 7*D*). Taken together, these data suggest that bradykinin enhances insulin sensitivity by inhibition of JNK activation.

Bradykinin signals via NO to decrease insulin-stimulated JNK phosphorylation. To confirm the role of NO in bradykinin-mediated inhibition of JNK, adipocytes were treated with either bradykinin or the NO donor, hydroxylamine, in the presence and absence of insulin. Both bradykinin and hydroxylamine significantly reduced insulin-stimulated JNK phosphorylation (Fig. 8*A*). In addition, compared with controls, the basal level of JNK phosphorylation was significantly greater in adipocytes from eNOS$^{-/-}$ mice, and did not decrease upon bradykinin treatment (Fig. 8*B*). These data indicate that signaling via NO is involved in bradykinin-mediated inhibition of JNK phosphorylation and activation.

DISCUSSION

Bradykinin has been implicated as a vasoactive factor associated with increased insulin sensitivity (9,15,16). Both ACEIs and bradykinin improved insulin action in skeletal muscle of Zucker fatty rats in vivo (16,33) and insulin-stimulated 2DG uptake ex vivo and in vitro (34–36). In the present study, addition of bradykinin directly to isolated adipocytes increased insulin-stimulated 2DG uptake in a dose- and time-dependent manner. This effect of bradykinin was mediated through its B_2 receptor, in agreement with one previous study (35), and is consistent with the observation that mice lacking the B_2 receptor display insulin resistance (37). In another study, HOE-140, the B_2 receptor antagonist, also blunted the ability of ACEIs to improve insulin sensitivity (36). Together, these findings strongly suggest that the major mechanism by which ACEI improve insulin action is via increased levels of bradykinin rather than decreased production of AngII.

Our results demonstrate that NO is required for bradykinin to enhance insulin action in adipocytes. A number of studies investigating the putative role of NO in insulin action have focused on the vascular system. Treatment of rats with the NOS inhibitor L-N^G-monomethyl-L-arginine resulted in hypertension and insulin resistance (17). Furthermore, mice with a targeted deletion of eNOS (eNOS$^{-/-}$) also manifest hypertension and insulin resistance (38). Thus, it has been postulated that NO improves insulin action in muscle in vivo as a result of increased blood flow (17,39). In the present study, we examined the

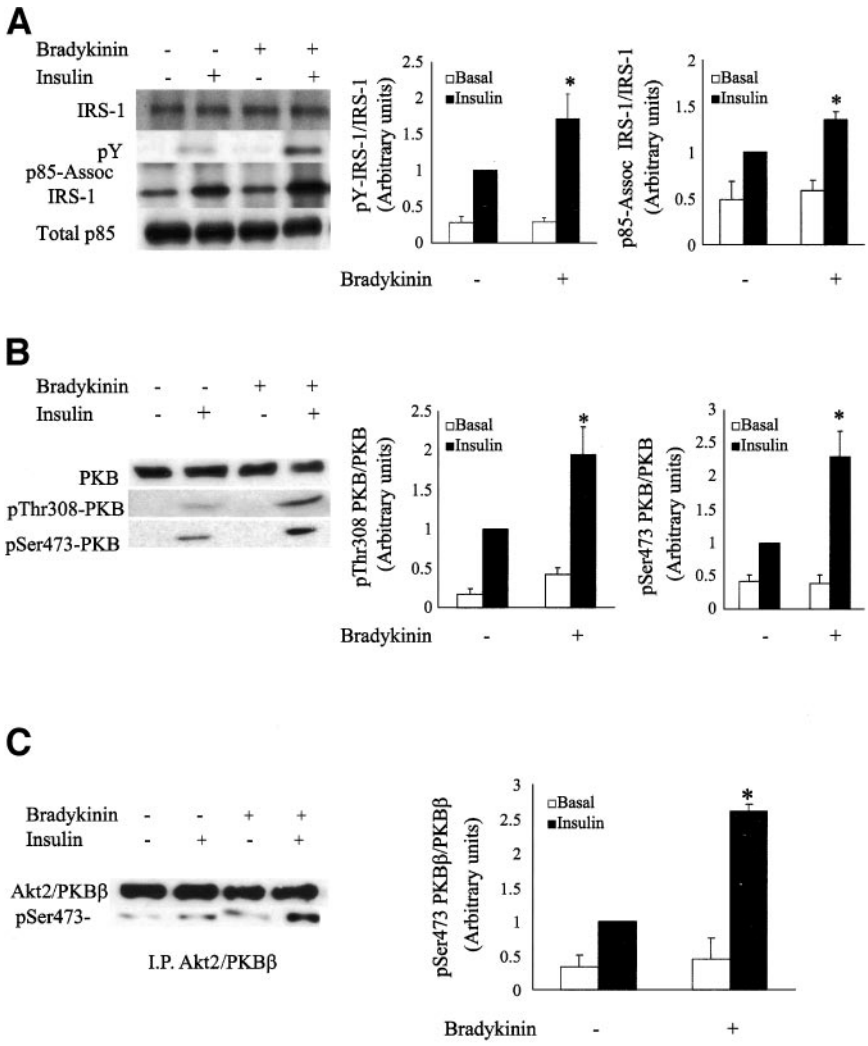

FIG. 6. Bradykinin augments insulin-stimulated IRS-1 and Akt/PKB phosphorylation. Adipocytes were treated as in the legend to Fig. 5. *A* and *B*: Representative immunoblots and means ± SE (n = 4–5). *C*: Akt2/PKBβ was immunoprecipitated from whole cell lysates. Representative immunoblots of Akt2/PKBβ and pSer^{473}-Akt2/PKBβ. (n = 3). The intensities of the bands from phospho-specific immunoblots were corrected for those of total kinases. Values are means ± SE. *P < 0.03 vs. insulin without bradykinin.

role of NO independent of the vasculature. In isolated rat adipocytes, three different NO donors replicated the effect of bradykinin to enhance the action of insulin. In addition, inhibition of NOS or scavenging NO blocked the ability of bradykinin to augment insulin-stimulated 2DG uptake. In contrast to these data, one previous study reported that in 3T3-L1 adipocytes, a NO donor stimulated glucose uptake independent of insulin and its downstream effectors, IRS-1 and Akt/PKB (40). The concentration of SNP used in that study was >150-fold that used here (50 mmol/l vs. 300–400 μmol/l). Either the cell culture model or the NO concentration or both may have contributed to the discrepancies observed.

The importance of eNOS as a putative mediator of bradykinin action in adipocytes was demonstrated by the finding that bradykinin failed to increase insulin-stimulated 2DG uptake in adipocytes from eNOS$^{-/-}$ mice in contrast to wild-type and nNOS$^{-/-}$ mice. However, the NO donor hydroxylamine significantly enhanced insulin-stimulated glucose uptake in these adipocytes. Thus, NO production by eNOS is critical for bradykinin to enhance insulin sensitivity in adipocytes.

As opposed to eNOS, iNOS can produce large amounts of NO over long periods of time, which may be detrimental (41). Chronic exposure to high concentrations of NO or induction of iNOS by cytokine treatment of cultured skeletal muscle cells has been associated with insulin resistance (42), and iNOS$^{-/-}$ mice are protected from insulin resistance induced by a high-fat diet (43). In agreement with these reports, we found that incubation of isolated adipocytes with very high concentrations of NO donors (greater than those in Fig. 3*C*) resulted in decreased insulin-stimulated 2DG uptake (data not shown).

Investigation of the mechanism of cross-talk between bradykinin and the insulin signaling pathway revealed that the enhancement of insulin-stimulated glucose transport by bradykinin was associated with increases of *1*) GLUT4 translocation from LDMs to plasma membrane, *2*) IRS-1 pY, *3*) IRS-1 association with the p85 regulatory subunit of PI3K, and *4*) phosphorylation of Thr^{308} and Ser^{473} of Akt/PKB. These findings extend previous reports of increased Tyr phosphorylation of IRS-1 and GLUT4 translocation by insulin in the presence of bradykinin (35,44).

As part of the investigation of insulin signaling in the presence of bradykinin, we examined the MAPK family of enzymes. Bradykinin itself can activate ERK1/2 in mesangial cells (45), SW-480 cells (46), and HEK 293 cells (47). In contrast to these reports and our findings of enhanced IRS-1 and Akt/PKB phosphorylation, bradykinin caused a reduction in insulin-stimulated phosphorylation of both ERK1/2 and JNK. The effect of bradykinin on the MAPK family of enzymes in adipocytes had not been previously

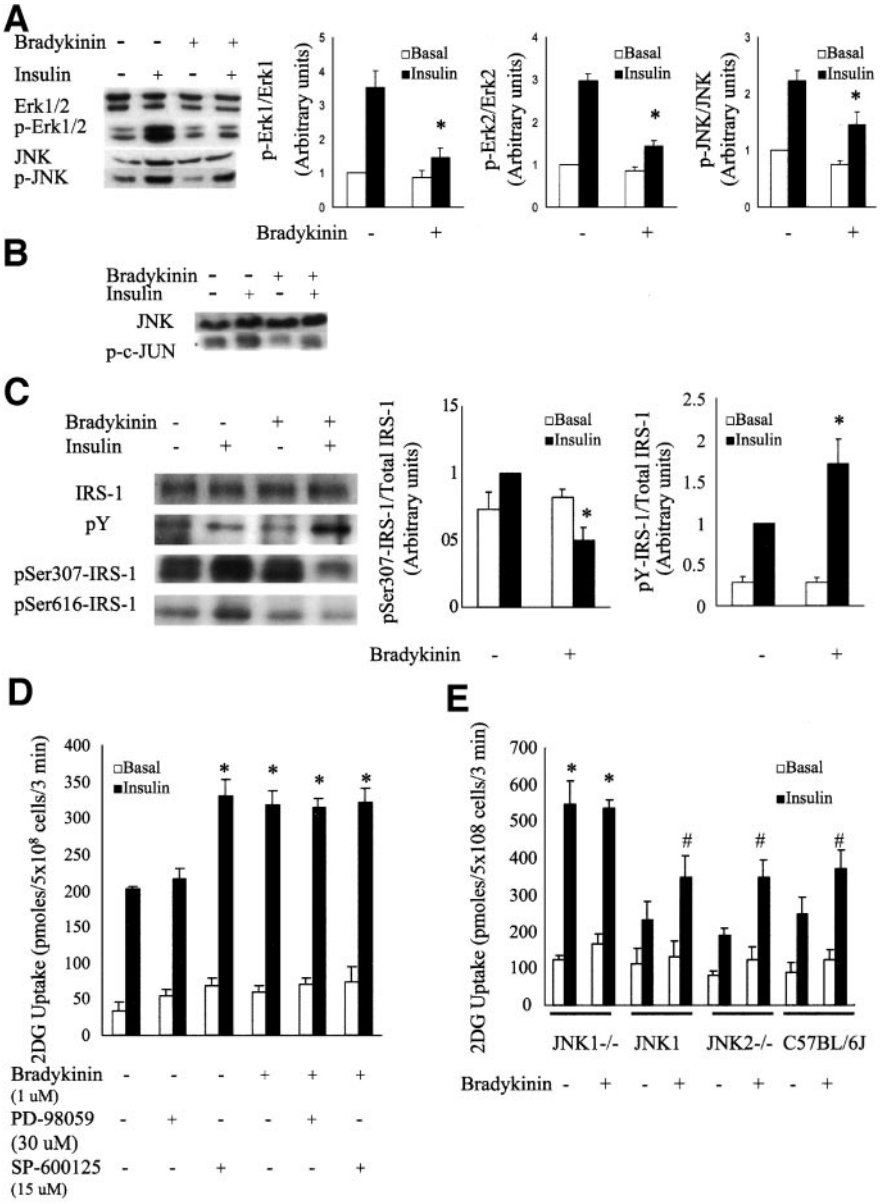

FIG. 7. Bradykinin decreases ERK1/2 and JNK phosphorylation. Adipocytes were treated as in the legend to Fig. 5. *A*: Representative immunoblots and means ± SE ($n = 5$). *B*: Immunoprecipitated JNK activity was assayed, and total JNK and phospho–c-Jun were immunoblotted ($n = 5$) (see text for quantification). *C*: Representative immunoblots and means ± SE ($n = 3$), except pSer^{616}-IRS-1 ($n = 1$). *A* and *C*: The intensities of the phospho-specific antibody bands were corrected for total kinases. *$P < 0.04$ vs. insulin without bradykinin. *D*: Adipocytes were pretreated with either SP-600125, PD-98059, or vehicle, followed by bradykinin ± insulin. 2DG uptake was assayed. Values are means ± SE ($n = 4$). *$P < 0.03$ vs. insulin without bradykinin. *E*: Adipocytes from JNK1$^{-/-}$, control (JNK1$^{+/+}$), JNK2$^{-/-}$, and C57BL/6J mice were treated with bradykinin ± insulin. Values are mean ± SE ($n = 4$). *$P < 0.04$ vs. control insulin.

reported. As seen in Fig. 7*A*, phosphorylation of ERK1/2 and JNK was detectable in unstimulated cells, probably due to the release of a variety of inflammatory mediators, including TNF-α and interleukin-1α and interleukin-6, by the adipocyte isolation procedure (48). These basal levels of phosphorylation were reduced but not entirely abolished by washing and preincubating the cells in 3% BSA–Krebs-Ringer bicarbonate HEPES buffer, pH 7.4, for 4–5 h at 37°C before experiments.

There is evidence that JNK activation and subsequent phosphorylation of Ser^{307} of IRS-1 results in decreased Tyr phosphorylation of IRS-1 and impaired insulin signaling (27,30). These events may occur in response to cytokines such as TNF-α (30), to free fatty acids (49), and to insulin itself (30). In the present study, we observed that insulin-stimulated phosphorylation of Ser^{307} and Ser^{616}, a site of ERK phosphorylation, of IRS-1 was decreased by bradykinin. Whereas inhibition of MEK had no effect on glucose uptake, inhibition of JNK potentiated insulin-stimulated 2DG uptake to an extent similar to bradykinin. To confirm these findings, we examined glucose uptake in adipocytes isolated from JNK1$^{-/-}$ mice, which have enhanced insulin sensitivity associated with reduced IRS-1 Ser^{307} phosphorylation (31). These adipocytes exhibited enhanced insulin sensitivity and, in contrast to JNK2$^{-/-}$ and control strains, there was no potentiation of insulin-stimulated 2DG uptake by bradykinin. Inhibitor of IκB-kinase β is an inflammatory cytokine-activated kinase that also has been implicated in insulin resistance by promoting IRS-1 Ser phosphorylation (50,51). However, we did not detect any change in inhibitor of IκB-kinase β phosphorylation (phospho-specific antibodies from cell signaling) upon treatment with insulin and/or bradykinin under the conditions of these experiments (not shown).

The link between bradykinin-mediated NO signaling to enhance glucose uptake and to inhibit JNK was confirmed by the observation that the NO donor hydroxylamine also blunted insulin-stimulated JNK phosphorylation. Furthermore, adipocytes from eNOS$^{-/-}$ mice displayed an elevated basal level of phosphorylated JNK, which was not reduced by bradykinin. Together, these data indicate that bradykinin potentiates insulin-stimulated glucose uptake

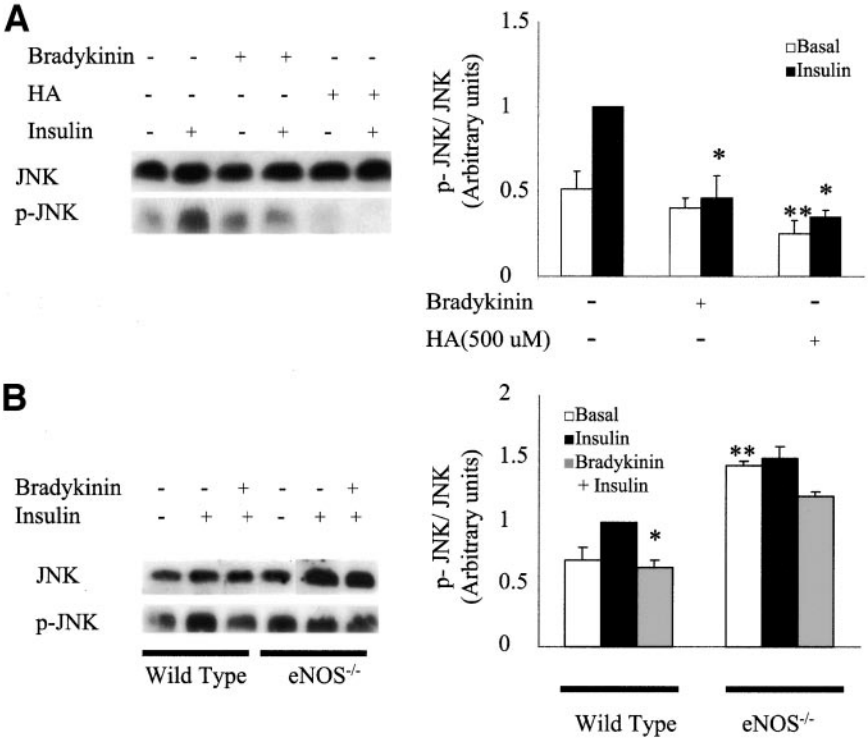

FIG. 8. NO decreases insulin-stimulated JNK phosphorylation. *A*: Adipocytes were treated with bradykinin or hydroxylamine (HA) (500 mmol/l) ± insulin. Representative immunoblots of JNK and phospho-JNK. ($n = 3$). *$P < 0.05$ vs. insulin without bradykinin; **$P < 0.03$ vs. basal without bradykinin. *B*: Adipocytes from control ($eNOS^{+/+}$) and $eNOS^{-/-}$ mice were treated as in the legend to Fig. 7. Representative immunoblots of JNK and phospho-JNK. Intensities of phosphorylated bands were corrected for total kinases. Values are means ± SE ($n = 3$). *$P < 0.04$ vs. insulin without bradykinin; **$P < 0.04$ vs. basal control ($eNOS^{+/+}$).

via the production of NO and subsequent inhibition of JNK activation. Although the mechanism by which NO inhibits JNK activity is not clear, some of its actions are exerted by *S*-nitrosylation of proteins. Endogenous NO has been reported to suppress JNK through such a thiol-redox mechanism (52), suggesting one possible mechanism for bradykinin action.

In summary, this study demonstrates that bradykinin significantly enhances insulin-stimulated glucose transport in adipocytes via a signaling pathway that involves eNOS and the generation of NO independent of blood flow. Investigation of the mechanism of cross-talk revealed that bradykinin decreases insulin stimulation of JNK, which is associated with reduced IRS-1 Ser^{307} phosphorylation and increased insulin stimulation of IRS-1 Tyr phosphorylation, p85 association, Akt/PKB phosphorylation, and GLUT4 translocation. These data present a novel mechanism that may account for the sensitization to insulin observed in the presence of ACEIs and bradykinin signaling. Elucidating the role of this regulatory pathway in normal physiology, in insulin-resistant states, and in the presence of currently used pharmacological agents such as ACEIs and ARBs will be of interest.

ACKNOWLEDGMENTS

This work was funded by a grant from the Canadian Diabetes Association (to I.G.F.) and by Grant FRN 38009 from the Canadian Institutes of Health Research (to I.G.F.). K.M.B. was supported by a Canadian Diabetes Association Doctoral Scholarship and a Banting and Best Diabetes Centre–Novo Nordisk Studentship.

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