Stimulation of glucose uptake by chronic vanadate pretreatment in cardiomyocytes requires PI 3-kinase and p38 MAPK activation

Annie Tardif, Nathalie Julien, Jean-Louis Chiasson, and Lise Coderre

Research Center, Centre hospitalier de l’Université de Montréal (CHUM), and Department of Medicine, University of Montreal, Montreal, Canada H2W 1T7

Submitted 28 March 2002; accepted in final form 26 January 2003

Tardif, Annie, Nathalie Julien, Jean-Louis Chiasson, and Lise Coderre. Stimulation of glucose uptake by chronic vanadate pretreatment in cardiomyocytes requires PI 3-kinase and p38 MAPK activation. Am J Physiol Endocrinol Metab 284: E1055–E1064, 2003. First published December 10, 2002; 10.1152/ajpendo.00134.2002.—Vanadate, an inhibitor of tyrosine phosphatases, has insulin-mimetic properties. It has been shown that acute vanadate administration enhances glucose uptake independently of phosphatidylinositol (PI) 3-kinase and p38 MAPK. However, therapeutic vanadate use requires chronic administration, and this could potentially involve a different signaling pathway(s). Thus, we examined the mechanisms by which chronic vanadate exposure (16 h) stimulates glucose uptake in primary cultures of adult cardiomyocytes. The effect of vanadate on the activation of insulin-signaling molecules was evaluated 60 min after its withdrawal and in the absence of insulin. We therefore evaluated the persistent effect of vanadate on the insulin-signaling cascade. Our results demonstrate that preincubation with low vanadate concentrations (25–75 μM) induces a dose-dependent increase in glucose uptake. The augmentation of this process was not due to alterations in GLUT1 or GLUT4 protein levels, transcription, or de novo protein synthesis. Chronic vanadate exposure was associated with activation of the insulin receptor, insulin receptor substrate-1 (IRS-1), PKB/Akt, and p38 MAPK. Furthermore, inhibition of PI 3-kinase or p38 MAPK by wortmannin and PD-169316, respectively, significantly inhibited vanadate-mediated glucose uptake in cardiomyocytes. Thus, over time, different (albeit overlapping) signaling cascades may be activated by vanadate.

insulin signaling; protein kinase B/Akt; diabetes; vanadium; heart; phosphatidylinositol 3-kinase

Despite the ability of the heart to use multiple substrates to meet its energy requirements, this organ has one of the highest rates of glucose utilization in vivo (18, 45). Decreased glucose uptake has been observed in both type 1 and type 2 diabetic subjects (25, 29, 45), and it has been proposed that abnormal regulation of this process has a role in the development of cardiac dysfunction (13). In db/db mice, a model of type 2 diabetes characterized by a reduction in glucose utilization and myocardial dysfunction, selective overexpression of the major glucose transporter GLUT4 expressed in the heart normalized these abnormalities (4). Interestingly, vanadate administration to streptozotocin-induced diabetic rats improved both insulin action and myocardial function in these animals (49). Furthermore, it has been shown that chronic vanadate exposure augments glucose uptake and improves ischémic tolerance in the hypertrophied heart (41). Together, these data suggest that glucose is an important cardiac substrate and that vanadate, by stimulating glucose uptake, could have a beneficial effect on the diabetic heart.

Vanadium is a trace element found in most cells in concentrations ranging from 0.1 to 1 μM (31). Vanadium compounds, such as vanadate, are inhibitors of protein tyrosine phosphatases that can mimic a number of the metabolic actions of insulin. In vivo, vanadate lowers blood glucose in diabetic animal models and in humans (5, 7, 10, 31, 37, 38, 47). Oral administration of this compound also restores GLUT4 expression in skeletal muscle of streptozotocin-induced type 1 diabetes (38). Despite these studies, the mechanism underlying the activation of glucose uptake by vanadate remains unclear. Insulin binds to its receptor and stimulates its tyrosine kinase activity. It has been reported that acute exposure to vanadate either stimulates (20, 32) or has no effect (28) on the tyrosine phosphorylation and activation of the insulin receptor. The activated insulin receptor phosphorylates the insulin receptor substrates (IRSs) on tyrosine residues (3, 26, 39, 48). Phosphorylated IRS-1 then binds to the SH2 domain of p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase), and activates the p110 catalytic subunit of the enzyme. This activation step is required for the stimulatory effect of insulin on glucose uptake (27, 33, 50). In contrast, the role of PI 3-kinase in the regulation of glucose uptake by vanadate is still controversial. Studies with wortmannin have demonstrated that, although vanadate mediates PI 3-kinase activation, this step is not required for the stimulation of glucose uptake (9, 43). One of the effectors of PI 3-kinase signaling is the protein kinase B (PKB)/Akt (2). It has been demonstrated that recruitment of glucose transporter vesicles by insulin (8, 14, 17, 44) but not vanadate (43) requires PKB/Akt activation. In addition to the IRS-1/PI 3-kinase pathway,

Address for reprint requests and other correspondence: L. Coderre, Research Center, Hôtel-Dieu-CHUM, 3850 St. Urbain, Montreal, (Quebec), Canada H2W 1T7 (E-mail: lise.coderre@umontreal.ca).
insulin also activates p38 MAPK. Recent data suggest that this enzyme modulates the intrinsic activity of GLUT4 and, thus, the stimulation of glucose uptake by insulin in skeletal muscle (36) and 3T3-L1 adipocytes (34, 40). In contrast, vanadate-mediated glucose uptake has been shown to be independent of p38 MAPK activation (43). Together, these results suggest that acute vanadate stimulation of glucose uptake occurs through the activation of a different signaling pathway than insulin.

Previous mechanistic studies on the insulin-mimetic properties of vanadate have focused most often on the acute effect of this compound. Utilization of vanadate as a therapeutic agent in animals or in humans requires the administration of low concentrations of this compound over a prolonged period of time. In addition, it has been shown that the effect of vanadate takes a few weeks to reach its maximum in humans (10, 37). Thus the mechanisms underlying vanadate-mediated activation of glucose uptake after chronic exposure could potentially differ from those observed during acute stimulation. The objective of this study was to better define the signaling pathways leading to increased glucose uptake in response to chronic vanadate exposure in adult cardiomyocytes.

**EXPERIMENTAL PROCEDURES**

**Chemicals.** All cell culture solutions, supplements, DNase I, and vanadyl sulfate were from Sigma-Aldrich Canada (Oakville, ON, Canada). Collagenase was obtained from Worthington Biochemical (Lakewood, NJ). Human insulin (Humulin R) was from Eli Lilly Canada (Toronto, ON, Canada). 2-Deoxy-[3H]glucose (2-DG) was purchased from NEN (Oakville, ON, Canada). Collagenase was obtained from Worthington Biochemical (Lakewood, NJ). Human insulin (Humulin R) was from Eli Lilly Canada (Toronto, ON, Canada). 2-Deoxy-[3H]glucose (2-DG) was purchased from NEN Research Products (Boston, MA). Polyclonal antibodies against GLUT1, the insulin receptor, IRS-1, and monoclonal anti-phosphotyrosine were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-Akt (Ser473), phospho-p38 MAPK (Thr180/Tyr182), Akt, and p38 MAPK were purchased from New England Biolabs (Beverly, MA). Anti-GLUT4 antibody was from Genzyme (Cambridge, MA). The polyvinylidene difluoride membrane was obtained from Immobilon-Millipore (Bedford, MA). The enhanced chemiluminescence detection system was from Amersham Pharmacia Biotech (Baie d’Urfée, QC, Canada). The Bradford protein assay kit was purchased from Bio-Rad (Mississauga, ON, Canada). All electrophoresis reagents were obtained from Roche Diagnostics (Laval, QC, Canada).

**Isolation of adult rat cardiomyocytes.** All experiments conformed to guidelines of the Canadian Council of Animal Care and were approved by the Animal Care Committee of the Centre hospitalier de l’Université de Montréal. Male Sprague-Dawley rats weighing 175–200 g were injected with heparin (500 U ip) 15 min before anesthesia with pentobarbital sodium (60 mg/kg ip). The heart was excised, and calcium-tolerant cardiomyocytes were isolated by the Langendorff method (retrograde perfusion), as described previously (42). During the whole procedure, the cells were maintained at 37°C. Briefly, the hearts were rinsed (4 ml/min) for 5 min in Krebs-Ringer (KR) buffer containing (in mM) 119 NaCl, 4.7 KCl, 1.25 CaCl2, 1.2 MgCl2, 1.2 KH2PO4, 11 dextrose, and 25 HEPES, pH 7.4. The heart was then perfused with a calcium-free KR solution for 5 min to stop spontaneous cardiac contractions. This was followed by perfusion with KR buffer supplemented with 0.05% collagenase, 15 mM 2-butanediol monoxime, and 0.5% fatty acid-free bovine serum albumin (BSA FAF) for 20 min, after which the ventricles were separated from the atria. Ventricles were minced in KR supplemented with 0.05% collagenase, 15 mM 2-butanediol monoxime, and 0.2 mg/ml DNase I. The resulting cell suspension was filtered through nylon mesh and centrifuged at 1,000 g for 45 s. The cells were then diluted and allowed to sediment in the washed solution twice. Freshly isolated cells were diluted in culture Medium 199 supplemented with 11 mM glucose, 0.2% BSA FAF, 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 10−7 M insulin, 0.1 M ascorbic acid, 100 IU/ml penicillin, 25 μg/ml gentamicin, and 100 μg/ml streptomycin, and were plated onto laminin-coated dishes. After 4 h, the cells were washed to remove damaged cells and debris. The cells were then incubated with media containing either 10−11 or 10−7 M insulin, 5.5 mM glucose, and various concentrations of vanadate (0–75 μM) for 16 h.

**Glucose uptake in primary cultures of cardiomyocytes.** On the day of the study, the cells were washed twice with 2 ml of KR buffer to remove insulin, glucose, and vanadate. The cells were then incubated for 30 min in 1 ml of fresh KR buffer containing no glucose, insulin, or vanadate. The glucose uptake assay was measured over a period of 30 min after the addition of 0.5 μCi/ml 2-DG, as described previously (42). Thus we evaluated the effect of prior vanadate exposure on this process (Fig. 1). In parallel experiments, cardiomyocytes that were not treated with vanadate were stimulated acutely with KR buffer (basal) or insulin for 30 min. The assay was terminated by three rapid washes with 1 ml of ice-cold KR buffer.
buffer. Cells were disrupted with 1 ml of 0.5 M NaOH for 60 min at 37°C, and cell-associated radioactivity was determined by scintillation counting. Glucose uptake was expressed as a percentage of basal uptake.

Preparation of cardiomyocytes for immunoprecipitation or immunoblotting. On the day of the study, the cells were washed twice with 4 ml of medium containing no insulin or vanadate. The cells were then incubated in 2 ml of medium containing no insulin or vanadate for 1 h. Therefore, the stimulatory effect of vanadate on the insulin-signaling cascade was evaluated without the compound or insulin present in the medium. In parallel experiments, cells that were not treated with vanadate were stimulated acutely with 10⁻⁷ M insulin in KR buffer or buffer alone (basal) for 5 min except for the determination of p38 MAPK phosphorylation, when the cells were incubated with insulin for 10 min. The reaction was stopped by three rapid washes with ice-cold KR buffer. Cells were lysed in buffer containing 25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS). The lysate was then centrifuged for 10 min at 12,000 g at 4°C to remove insoluble material, and the resulting supernatant was used for immunoprecipitation or immunoblotting. Protein content was evaluated by use of the Bradford assay.

Immunoprecipitation. Cell lysates were incubated with either the insulin receptor or IRS-1 antibodies at 4°C overnight. The antigen-antibody complexes were immunoprecipitated with protein A Sepharose beads for 2 h at 4°C. The immunoprecipitate was then washed three times in lysis buffer before solubilization in Laemmli buffer.

Gel electrophoresis and immunoblotting. Samples were electrophoresed on SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes for Western blotting. The membranes were blocked for 1 h in 5% BSA, fraction V, followed by incubation with the appropriate primary antibody. The membrane was subsequently incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase, and the antigen-antibody complex was detected with the enhanced chemiluminescence method. Quantitative analysis was performed using a scanning densitometer.

Statistical analysis. Statistical analysis was performed with analysis of variance (ANOVA) for multiple comparisons, followed by a Fisher post hoc test. All data are reported as means ± SE. Glucose uptake data are means of at least five independent experiments. For the immunoblot experiments, densitometric analysis was performed on at least three independent experiments. A value of P < 0.05 was considered significant.

RESULTS

Chronic exposure to vanadate stimulates glucose uptake in cardiomyocytes. Acute activation of glucose transport by millimolar concentrations of vanadate has been observed in many tissues, including adipocytes, cardiomyocytes, and skeletal muscle (19, 20, 31, 41, 43). However, chronic vanadate exposure could stimulate glucose uptake at different concentrations from the one observed for acute exposure. In addition, after chronic administration of vanadate in vivo, improved blood glucose is maintained for ≥2 wk, suggesting that the effect of this compound endures for some time (37). To address these possibilities, cardiomyocytes were incubated with various concentrations of vanadate and either 10⁻¹¹ or 10⁻⁷ M insulin for 16 h. On the day of the study, the cells were washed and incubated in KR buffer containing no insulin or vanadate, and glucose uptake was examined (see Fig. 1). As shown in Fig. 2, activation of glucose uptake by chronic vanadate pretreatment is dose dependent and is not affected by the concentration of insulin present during the pretreatment period (10⁻¹¹ vs. 10⁻⁷ M). Our results demonstrate that the concentrations of vanadate required to stimulate glucose uptake are ≥20 times lower than what has been previously observed for acute stimulation of this process (11, 19, 20, 31, 41, 43). At 50 and 75 μM, vanadate induced a 4.1- and 6.3-fold stimulation in glucose uptake, respectively (P < 0.01). In comparison, acute insulin stimulation of cardiomyocytes that were not exposed to vanadate augmented this process 2.4-fold. At concentrations of ≥100 μM, we noted that vanadate started to become toxic for the cardiomyocytes, and a subset of cells lost their characteristic rod-shaped form (data not shown). Thus, in subsequent experiments, cardiomyocytes were incubated with media containing 10⁻⁷ M insulin.

Vanadate stimulation of glucose uptake is independent of transcription and de novo protein synthesis in cardiomyocytes. Because the cells were treated with vanadate for 16 h, the possibility existed that the increased glucose uptake was due to elevated levels of either GLUT1 or GLUT4 proteins. As shown in Fig. 3, chronic exposure to 50 μM vanadate did not modulate GLUT1 or GLUT4 protein levels in cardiomyocytes. Alternatively, vanadate could regulate glucose uptake by altering the transcription or translation of signaling molecules in cardiomyocytes. To address this possibility, we evaluated the effect of actinomycin D, an inhibitor of transcription, on vanadate-mediated glucose uptake. Cardiomyocytes were incubated with 5 μg/ml actinomycin D with or without vanadate for 16 h before determination of glucose uptake. Our results showed that inhibition of transcription had no effect on the
stimulation of glucose uptake by vanadate in cardiomyocytes (Fig. 4). To assess the role of newly synthesized proteins, cardiomyocytes were cultured with 100 μM cycloheximide, a protein synthesis inhibitor, with or without vanadate for 16 h, and glucose uptake was evaluated. Cycloheximide had no effect on the activation of glucose uptake by vanadate (Fig. 4).

**Vanadate increases tyrosine phosphorylation of the insulin receptor and IRS-1.** To identify possible mechanisms involved in the regulation of glucose uptake in cardiomyocytes, we examined the effect of chronic vanadate pretreatment on the activation of several insulin-signaling molecules. Cardiomyocytes were cultured with various concentrations of vanadate for 16 h. On the day of the study, the cells were washed and incubated in the absence of vanadate for 60 min before determination of the protein levels and tyrosine phosphorylation states of the insulin receptor and IRS-1. Chronic vanadate exposure did not alter the concentration of either the insulin receptor or IRS-1 (data not shown). However, this treatment induced a dose-dependent increase in tyrosine phosphorylation of both proteins in cardiomyocytes. As shown in Fig. 5, pretreatment with 75 μM vanadate induced a 5- and 15.5-fold increase in the phosphorylation of the insulin receptor and IRS-1, respectively (P < 0.05). In comparison, acute insulin stimulation in cells that were not exposed to vanadate induced a 4.2- and 13-fold in-

Fig. 3. Total GLUT4 and GLUT1 immunoreactive protein in control and vanadate-treated cardiomyocytes. Cells were pretreated with 50 μM vanadate in medium containing 10^{-7} M insulin for 16 h. Vanadate and insulin were removed 60 min before cells were harvested, and GLUT1 and GLUT4 protein levels were determined by Western blot. A: representative immunoblot of GLUT1 and GLUT4 protein levels; B: densitometric scanning signal from GLUT1 and GLUT4 proteins (arbitrary units).

Fig. 4. Effect of cycloheximide and actinomycin D on vanadate-mediated glucose uptake in cardiomyocytes. Cells were incubated with medium containing 10^{-7} M insulin and 50 μM vanadate for 16 h. Medium was replaced with KR buffer containing no vanadate or insulin, and glucose uptake was determined (see Fig. 1). *P < 0.05, basal vs. vanadate-pretreated cardiomyocytes.

Fig. 5. Insulin receptor and IRS-1 tyrosine phosphorylation in response to vanadate in cardiomyocytes. Cells were incubated with medium containing 10^{-7} M insulin and the indicated vanadate concentration for 16 h. Medium was changed, and vanadate and insulin were removed 60 min before evaluation of the insulin receptor and IRS-1 tyrosine (Tyr) phosphorylation. In parallel experiments, cells cultured without vanadate were stimulated for 5 min with insulin. A, top: representative immunoblot of Tyr-phosphorylated insulin receptor; bottom: densitometric scanning signal from Tyr-phosphorylated IRS-1 (arbitrary units). B, top: representative immunoblot of Tyr-phosphorylated IRS-1; bottom: densitometric scanning signal from Tyr-phosphorylated IRS-1 (arbitrary units). †P < 0.05, basal vs. insulin-stimulated cardiomyocytes; *P < 0.05, basal vs. vanadate-pretreated cells.

**AJP-Endocrinol Metab • VOL 284 • JUNE 2003 • www.ajpendo.org**
crease in the phosphorylation of the insulin receptor and IRS-1, respectively \((P < 0.05)\).

Vanadate-stimulated glucose uptake requires PI 3-kinase activation. Previous studies have demonstrated that activation of PI 3-kinase is required for insulin-stimulated glucose uptake \((27, 33, 50)\). However, the role of this enzyme in the regulation of vanadate-mediated glucose uptake is still unclear \((9, 43)\). We therefore determined whether this enzyme participates in the stimulation of glucose uptake by vanadate in cardiomyocytes. Cells were preincubated with 50 \(\mu\)M vanadate for 16 h and then incubated for 30 min with 500 nM wortmannin, an inhibitor of PI 3-kinase, before the determination of glucose uptake. As shown in Fig. 6, inhibition of PI 3-kinase suppressed the activation of glucose uptake by either vanadate pretreatment or acute insulin stimulation in cardiomyocytes. Similar results were obtained with 10 \(\mu\)M LY-294002, another inhibitor of PI 3-kinase \((\text{data not shown})\).

Vanadate increases PKB/Akt phosphorylation in cardiomyocytes. We also evaluated the effect of chronic vanadate exposure on PKB/Akt content and activation in cardiomyocytes. Prolonged exposure to vanadate did not alter the concentration of PKB/Akt \((\text{data not shown})\). This treatment, however, increased PKB/Akt phosphorylation levels in a dose-dependent manner \((\text{Fig. 7A})\). At 75 \(\mu\)M, vanadate augmented PKB/Akt phosphorylation 3.2-fold \((P < 0.05)\). In comparison, acute insulin stimulation induced a 4.1-fold increase in the phosphorylation of this enzyme \((P < 0.001)\).

Vanadate is a more powerful activator of p38 MAPK than insulin in cardiomyocytes. Recent studies have suggested that p38 MAPK is a key signaling intermediate in the regulation of glucose transport by insulin \((34, 36, 40)\). We therefore determined whether vanadate regulates this enzyme in cardiomyocytes. As shown in Fig. 7B, vanadate is a more powerful activator of p38 MAPK than insulin. In cardiomyocytes, acute insulin stimulation induced a 2- to 3-fold increase in p38 MAPK activity, whereas prolonged exposure to 75 \(\mu\)M vanadate provoked a 17-fold increase in enzyme phosphorylation \((P < 0.05)\).

PD-169316 inhibits glucose uptake and p38 MAPK activation in response to vanadate pretreatment in cardiomyocytes. To determine whether p38 MAPK activation had a role in vanadate-enhanced glucose uptake, cardiomyocytes were incubated with 2 or 5 \(\mu\)M of the p38 MAPK inhibitor, PD-169316, for 30 min. As shown in Fig. 8A, both concentrations of inhibitor abolished acute insulin-stimulated glucose uptake \((P < 0.05)\). PD-169316 also decreased vanadate-mediated activa-

![Fig. 6. Effect of wortmannin on vanadate-mediated glucose uptake in cardiomyocytes. Cells were incubated with medium containing 10^{-7} M insulin and 50 \(\mu\)M vanadate for 16 h. Medium was replaced with KR buffer without vanadate or insulin. Cardiomyocytes were then treated with 500 nM wortmannin or vehicle (Me2SO) for 30 min before determination of glucose uptake. In parallel experiments, cells cultured without vanadate were stimulated with insulin for 30 min. \(\dagger\) \(P < 0.005\), basal vs. insulin-stimulated cardiomyocytes; \(\ddagger\) \(P < 0.001\), basal vs. vanadate-pretreated cells; \(\dagger\dagger\) \(P < 0.001\), insulin-stimulated cells vs. wortmannin-treated cells; \(\ast\ast\) \(P < 0.001\), vanadate-pretreated cells vs. wortmannin-treated cells.](E1059)

![Fig. 7. PKB/Akt and p38 MAPK phosphorylation in response to vanadate pretreatment in cardiomyocytes. Cells were incubated with medium containing 10^{-7} M insulin and the indicated vanadate concentration for 16 h. Medium was changed, and vanadate and insulin were removed 60 min before determination of PKB/Akt and p38 MAPK phosphorylation. In parallel experiments, cells cultured without vanadate were stimulated for either 5 or 10 min with insulin, and PKB/Akt and p38 MAPK phosphorylation levels were examined. A, top: representative immunoblot of phospho-PKB/Akt; bottom: densitometric scanning signal from phospho-PKB/Akt (arbitrary units). B, top: representative immunoblot of phospho-p38 MAPK; bottom: densitometric scanning signal from phospho-p38 MAPK (arbitrary units). \(\dagger\) \(P < 0.001\), basal vs. insulin-treated cells; * \(P < 0.05\), basal vs. vanadate-treated cells.](E1059)
tion of glucose uptake by 35 and 65% at 2 and 5 μM, respectively (P < 0.02). Furthermore, as shown in Fig. 
8, B and C, 2 μM PD-169316 inhibited vanadate-induced p38 MAPK phosphorylation by 70%. Incubation 
with 5 μM PD-169316 did not provoke a further decrease in enzyme phosphorylation in vanadate-pre-
treated cells.

**Wortmannin inhibits PKB/Akt but not p38 MAPK phosphorylation in response to vanadate pretreatment in cardiomyocytes.** It has been shown that wortmannin can inhibit p38 MAPK activation in L6 muscle cells (35). Potentially, inhibition of vanadate-stimulated glucose uptake by wortmannin could be mediated via a decrease in p38 MAPK activation. To address this question, we examined the effect of wortmannin on p38 MAPK phosphorylation in response to acute insulin 
stimulation or vanadate pretreatment in cardiomyocytes. As a control, we also evaluated the effect of 
wortmannin on PKB/Akt phosphorylation, a down-
stream target of PI 3-kinase. As shown in Fig. 9A, 
wortmannin completely suppressed PKB/Akt activa-
tion in response to vanadate pretreatment in cardio-
myocytes. However, wortmannin did not alter van-
adate-induced p38 MAPK activation in cardiomyocytes (Fig. 9B).

**DISCUSSION**

Our results demonstrate that prior exposure to low 
concentrations of vanadate for 16 h stimulates glucose 
uptake by 35 and 65% at 2 and 5 μM, respectively (P < 0.02). Furthermore, as shown in Fig. 
8, B and C, 2 μM PD-169316 inhibited vanadate-induced p38 MAPK phosphorylation by 70%. Incubation 
with 5 μM PD-169316 did not provoke a further decrease in enzyme phosphorylation in vanadate-pre-
treated cells.

**Wortmannin inhibits PKB/Akt but not p38 MAPK phosphorylation in response to vanadate pretreatment in cardiomyocytes.** It has been shown that wortmannin can inhibit p38 MAPK activation in L6 muscle cells (35). Potentially, inhibition of vanadate-stimulated glucose uptake by wortmannin could be mediated via a decrease in p38 MAPK activation. To address this question, we examined the effect of wortmannin on p38 MAPK phosphorylation in response to acute insulin 
stimulation or vanadate pretreatment in cardiomyocytes. As a control, we also evaluated the effect of 
wortmannin on PKB/Akt phosphorylation, a down-
stream target of PI 3-kinase. As shown in Fig. 9A, 
wortmannin completely suppressed PKB/Akt activa-
tion in response to vanadate pretreatment in cardio-
myocytes. However, wortmannin did not alter van-
adate-induced p38 MAPK activation in cardiomyocytes (Fig. 9B).

**DISCUSSION**

Our results demonstrate that prior exposure to low 
concentrations of vanadate for 16 h stimulates glucose 
uptake by 35 and 65% at 2 and 5 μM, respectively (P < 0.02). Furthermore, as shown in Fig.
uptake in cardiomyocytes. Activation of this process does not involve any changes in GLUT1 or GLUT4 protein levels, nor does it require transcription or de novo protein synthesis. In cardiomyocytes, vanadate-enhanced glucose uptake depends on the activation of PI 3-kinase and p38 MAPK. This is the first demonstration that stimulation of this process by vanadate is dependent on the activation of p38 MAPK for full activity.

We have observed a parallel activation of the insulin-signaling cascade and glucose uptake in response to chronic vanadate pretreatment in cardiomyocytes. The concentrations of vanadate used in this study are compatible with what has been observed in plasma after chronic treatment with vanadate (50–90 μM) in animals and in humans (1, 47). This is in contrast to the acute action of this compound, when millimolar concentrations of vanadate are required to produce an effect in vitro (11, 19, 20, 31, 41, 43). This difference in effective concentrations (acute vs. chronic) might be explained by the accumulation of vanadate in an intracellular compartment. In vivo, the maximum effect of vanadate is reached only after a few weeks of treatment (10, 31). In addition, the improvement in glyce mia is maintained after vanadate withdrawal (37). Concordant with these studies, our results show that glucose uptake and insulin-signaling molecules are still activated 60 min after the termination of vanadate pretreatment in cardiomyocytes. These results suggest that the effect of vanadate endures for some time after its withdrawal.

The requirement for lower vanadate concentrations could be due to the generation of pervanadate in the cell. It has been shown that chronic vanadate exposure increases the production of hydrogen peroxide, which can then transform the vanadate into pervanadate (15, 31). Pervanadate is a very powerful tyrosine phosphatase inhibitor and is more potent than vanadate in stimulating glucose uptake (16, 19, 37, 43, 46). The generation of pervanadate could be one of the reasons why the stimulation of glucose uptake occurs at such low concentrations under chronic treatment. However, the generation of hydrogen peroxide could also have a deleterious effect on these cells. We have observed that chronic exposure to elevated concentrations of vanadate (≥100 μM) is associated with increased cell death (data not shown). Recent studies have shown that a vanadate-mediated increase in hydrogen peroxide triggers the activation of p53 and apoptosis in epidermal cells (15). A similar mechanism could also operate in cardiomyocytes and explain the toxicity associated with elevated concentrations of vanadate that we observed.

Enhanced glucose uptake in response to chronic vanadate pretreatment does not require active transcription, de novo protein synthesis, or an increase in GLUT1 and GLUT4 protein content. This is in contrast to what has been observed in vivo, where vanadate restores GLUT4 expression in skeletal muscle (38) and adipocytes (7, 23) of streptozotocin-induced diabetic rats. However, vanadate does not increase GLUT4 protein concentrations in adipocytes from control animals (7), skeletal muscle of fa/fa rats (6), or hypertrophied failing heart (30). The stimulation of glucose uptake observed in vanadate-pretreated cardiomyocytes could result from an augmentation of the transporter present at the plasma membrane. It has been shown that insulin-mediated GLUT4 recruitment to the cell surface requires PI 3-kinase activation (27, 33, 50). Because inhibition of this enzyme suppresses vanadate-enhanced glucose uptake, stimulation of glucose uptake by vanadate could occur through recruitment of this transporter to the plasma membrane. We are currently investigating this possibility.

Chronic exposure to vanadate resulted in the activation of insulin-signaling molecules, including the insulin receptor, IRS-1, PKB/Akt, and p38 MAPK. Activation of this cascade by vanadate also induced a dose-dependent increase in glucose uptake in cardiomyocytes. The effect of vanadate on glucose uptake was independent of the concentration of insulin found in the preincubation media (10–11 or 10–7 M insulin). However, because the cardiomyocytes were cultured with insulin, they may have displayed some degree of insulin resistance. As reported by Lu et al. (20), insulin-resistant adipocytes have enhanced sensitivity to vanadate. This may be one of the reasons why we see stimulation of glucose uptake at low vanadate concentrations.

In our study, the augmentation in PKB/Akt and p38 MAPK phosphorylation after acute insulin stimulation or chronic exposure to vanadate was similar to the effect induced by either stimulus alone (data not shown). These results suggest that the signaling pathway activated by chronic vanadate pretreatment or acute insulin stimulation probably converges in the cascade leading to enhanced glucose uptake. Potentially, vanadate, by inhibiting protein tyrosine phosphatases, may preserve the phosphorylation status of insulin-signaling molecules induced by insulin during the preincubation period.

As mentioned above, inhibition of PI 3-kinase completely blocked the stimulation of glucose uptake after chronic vanadate pretreatment. This is in contrast to previous studies, in which the acute stimulatory effect of vanadate on glucose uptake in L6 muscle cells (43), skeletal muscle (9), and H9c2 cardiomyocytes (43) was shown to be independent of PI 3-kinase activation. Thus our results suggest that different signaling pathways could be activated in response to acute vs. chronic vanadate stimulation.

Our results show that vanadate is a very powerful activator of p38 MAPK and that inhibition of this enzyme reduces vanadate-mediated glucose uptake. This suggests that this enzyme participates in the activation of glucose uptake by vanadate. To gain a better understanding of the mechanisms involved in vanadate-enhanced glucose uptake and the role played by p38 MAPK, we examined the effect of wortmannin and PD-169316 on the activation of this enzyme in cardiomyocytes. Incubation with PD-169316 inhibits vanadate-mediated p38 MAPK phosphorylation in cardiomyocytes. Although the effect of PD-169316 on the
phosphorylation levels of P38 MAPK was maximal at 2 μM, a further decrease in glucose uptake was observed at 5 μM. This could not be explained by a reduction in PKB/Akt activation, because PD-169316 has no effect on vanadate-mediated PKB/Akt phosphorylation in cardiomyocytes (data not shown). It is possible that 5 μM PD-169316 inhibited the enzyme without altering its phosphorylation levels. Alternatively, other enzymes involved in glucose uptake may also be inhibited by PD-169316. Recent studies have demonstrated that high concentrations of wortmannin inhibit p38 MAPK activation in 3T3-L1 adipocytes (35). We therefore explored the possibility that part of the effect of wortmannin on glucose uptake may be mediated by a reduction in p38 MAPK activity. Our results showed that, whereas wortmannin completely suppressed PKB/Akt phosphorylation in response to either acute insulin stimulation or prior vanadate pretreatment, it did not alter the phosphorylation of p38 MAPK. Thus, in cardiomyocytes, inhibition of vanadate-enhanced glucose uptake by wortmannin is not due to its effect on p38 MAPK activation.

In cardiomyocytes, PD-169316 decreases vanadate-stimulated glucose uptake by 45%. This is comparable to what has been observed after p38 MAPK inhibition with SB-203580 in 3T3-L1, L6 muscle cells, and isolated soleus muscle (36, 40). Furthermore, inhibition of the enzyme with a dominant negative mutant of p38 MAPK also diminished the insulin-stimulated glucose uptake by 40% (34). This reduction in glucose uptake in response to acute insulin stimulation occurs in the absence of any alteration in GLUT4 protein levels present at the plasma membrane (34, 36, 40). Together, these results suggest that activation of p38 MAPK regulates plasma membrane GLUT4 intrinsic activity and that activation of the enzyme is required for maximal insulin stimulation of glucose uptake.

In cardiomyocytes, inhibition of p38 MAPK significantly diminishes the activation of glucose uptake after chronic vanadate treatment. This is in contrast to the studies of Tsiani et al. (43), who reported that p38 MAPK is not involved in acute vanadate action or pervanadate-mediated glucose transport. One possible explanation for these differences is that we are looking at the chronic effect of this compound as opposed to its acute action. Activation of the glucose transport process by chronic vanadate exposure occurs at much lower concentrations than what has been observed under acute stimulation, and this may reflect the activation of a different signaling cascade. As mentioned previously, it has been shown that, under prolonged exposure, vanadate generates hydrogen peroxide (15, 31). Recent studies have demonstrated that hydrogen peroxide plays an important role in the activation of the insulin receptor/IRS-1/PI 3-kinase signaling cascade and the stimulation of glucose uptake by insulin (21, 22). Furthermore, it has been shown that it potentiates the effect of vanadate on the stimulation of glucose uptake (12, 23, 24). Potentially, the production of hydrogen peroxide by vanadate may contribute to the augmentation of glucose uptake in response to this compound. Alternatively, divergence of the signaling pathways could be due to differential generation of pervanadate. It has been shown that, in contrast to vanadate, the inhibition of tyrosine phosphatases by pervanadate is irreversible (16). Pervanadate also appears to be more potent then vanadate in stimulating various enzymes involved in insulin signaling, such as the insulin receptor tyrosine kinase (31, 37) and PKB/Akt (43, 46). Potentially, the stimulation of glucose uptake after chronic exposure to vanadate could be due to the production of hydrogen peroxide and/or pervanadate by the cardiomyocytes.

In summary, the present study showed that low concentrations of vanadate enhance glucose uptake by activating the insulin-signaling pathway in a dose-dependent fashion in adult rat cardiomyocytes. Furthermore, stimulation of this process requires the activation of both PI 3-kinase and p38 MAPK for full activation. This is the first demonstration that activation of p38 MAPK participates in vanadate-enhanced glucose uptake. These results suggest that the regulation of glucose uptake under chronic and acute vanadate action may involve divergent, albeit overlapping, signaling pathways in these cells.

We thank James C. Engert for helpful discussions. The Canadian Diabetes Association and Association Diabète-Québec supported this work.

REFERENCES

E1063


42. Takacs A, Julien N, Baudier A, Thibault G, Srivastava AK, Chiasson JL, and Coderre L. Chronic exposure to β-hydroxybutyrate impairs insulin action in primary cultures of adult


