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The Antidiabetic Agent Sodium Tungstate Activates Glycogen Synthesis through an Insulin Receptor-independent Pathway*

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Sodium tungstate is a powerful antidiabetic agent when administered orally. In primary cultured hepatocytes, tungstate showed insulin-like actions, which led to an increase in glycogen synthesis and accumulation. However, this compound did not significantly alter the insulin receptor activation state or dephosphorylation rate in cultured cells (CHO-R) or in primary hepatocytes, in either short or long term treatments. In contrast, at low concentrations, tungstate induced a transient strong activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) after 5–10 min of treatment, in a similar way to insulin. Moreover, this compound did not significantly delay or inhibit the dephosphorylation of ERK1/2. ERK1/2 activation triggered a cascade of downstream events, which included the phosphorylation of p90rsk and glycogen synthase-kinase 3β. Experiments with a specific inhibitor of ERK1/2 activation and kinase assays indicate that these proteins were directly involved in the stimulation of glycogen synthase and glycogen synthesis induced by tungstate without a direct involvement of protein kinase B (PKB/Akt). These results show a direct involvement of ERK1/2 in the mechanism of action of tungstate at the hepatic level.

The antidiabetic properties of sodium tungstate have been widely reported. In several animal models of type 1 and 2 diabetes, this compound reduces and, in most cases, normalizes glycemia when administered orally and does not cause hypoglycemia (1–4). In addition, several reports have also shown antidiabetic actions for other tungsten derivatives, paratungstate and pertungstate (5, 6). Tungstate treatment increases the total amount and translocation of GLUT4 in muscle (7) and restores hepatic glucose metabolism (1) in streptozotocin-induced diabetic rats. In Zucker diabetic fatty rats, this compound also markedly reduces hypertriglyceridemia (4). Tungstate administration stimulates insulin secretion (2, 8) and regenerates β-cell population in neonatally streptozotocin-treated rats (2). *In vitro*, tungstate or pertungstate treatment mimics most of the metabolic effects of insulin in primary cultured adipocytes, which include the stimulation of lipogenesis, hexose uptake, glucose oxidation, and inhibition of lipolysis (6). In contrast to other transition metal derivatives, sodium tungstate has a low toxicity profile in both short and long term treatments (for a review, see Refs. 9 and 10).

Despite considerable data on the pharmacological and metabolic effects of sodium tungstate, there is a lack of information on its molecular mechanisms of action. This compound has been described as a phosphatase inhibitor (11–16). Interestingly, Foster *et al.* (14) described the inhibitory effects of this compound on glucose-6-phosphatase *in vitro*, an action that may contribute to a decrease in gluconeogenic activity *in vivo*.

As described above, tungstate mimics most of the metabolic effects of insulin. The initial steps in insulin action involve the binding of this hormone to its receptor and the subsequent activation of its tyrosine kinase activity. This process results in the phosphorylation of several substrates, which, among other actions, ultimately trigger the activation of glycogen synthesis. Stimulation of glycogen deposition is one of the most significant consequences of insulin action in liver. Therefore, the analysis of the effects of tungstate on glycogen deposition may greatly contribute to identify its mechanism of action.

Here we present the first analysis of tungstate action on several components of the insulin transduction cascade. Tungstate exerted insulin-like actions in primary cultured rat hepatocytes as it increased glycogen deposition. The actions of this compound were not mediated by the insulin receptor (IR)[1] as its phosphorylation state remained unchanged after treatment. In contrast, there was a clear transient phosphorylation and consequent activation of ERK1/2. These protein kinases triggered the phosphorylation of glycogen synthase kinase-3β (GSK3β) and p90rsk1, and ultimately, the activation of glycogen synthase (GS). These data indicate that ERK1/2 plays a key role in tungstate-stimulated glycogen synthesis in liver and therefore contributes to the antidiabetic action of this compound.

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[1] The abbreviations used are: IR, insulin receptor; IRS-1, insulin receptor substrate-1; ERK1/2, extracellular signal-regulated kinases 1 and 2; GS, glycogen synthase; GSK3β, GS kinase-3β; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MAPKAP-K1, MAPK-activated protein kinase-1; PKB, protein kinase B; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; CHO, Chinese hamster ovary.

EXPERIMENTAL PROCEDURES

Materials—Sodium tungstate was from Carlo Erba (Milan, Italy). Tissue culture media and supplements were from Sigma and Invitrogen. Fetal calf serum (FCS) was from Biological Industries (Ashrat, Israel). ERK1/2 and phosphotyrosine (4G10) antibodies were from Upstate Biotechnology (Waltham, MA). p90rsk antibody was from Sigma, GSK3α/β and c-Myc antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-p44/42 MAPK (Thr-202/Tyr-204), phospho-GSK-3β (Ser-9), phospho-p90rsk (Ser-380), PKB/Akt, phospho-PKB/Akt (Thr-308), and phospho-PKB/Akt (Ser-473) antibodies were from Cell Signaling (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies were from Cell Signaling. PD98059 was from Sigma. Enzymes and biochemical reagents were from Sigma, unless otherwise indicated. All other chemicals were of analytical grade.

Cell Lines—CHO-R cells (17) were cultured in Ham's-F12 nutrient mixture supplemented with 10% FCS and 0.7 mg/ml Geneticin. 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS.

Hepatocyte Isolation and Culture—Collagenase perfusion was used to isolate hepatocytes from male Wistar rats (180–225 g) fasted for 24 h, as described previously (18). Cells were suspended in DMEM, supplemented with 10 mM glucose, 10% FCS, 100 nM insulin, and 100 nM dexamethasone (Sigma) and then seeded onto plastic plates of 60-mm diameter treated with 0.1% gelatin (Sigma) at a final density of 8×10^4 cells/cm^2. After cell attachment (4 h at 37 °C), the medium was replaced by DMEM without glucose, FCS, and hormones, or alternatively, by DMEM with 5 mM glucose, 10% FCS. Hepatocytes were incubated a further 14–20 h at 37 °C before starting the experiments.

Glycogen and GS Measurement—To measure glycogen content, cell monolayers were scraped into 30% KOH, and the extract was then boiled for 15 min and centrifuged at 5000 $\times$ *g* for 15 min. Glycogen was measured in the cleared supernatants, as described (19).

Glycogen synthesis was measured after incubation of primary cultured hepatocytes with 2 μCi/ml [U-^{14}C]glucose (PerkinElmer Life Sciences) for 2 h in DMEM with 10 mM glucose. On termination of the incubations, hepatocyte monolayers were washed three times with 150 mM NaCl and extracted in 0.1 M NaOH. Extracts were deproteinized with trichloroacetic acid (10%, w/v) containing glycogen carrier, and the radioactivity incorporated into glycogen was measured.

To measure GS activity, frozen cell monolayers were scraped using 100 μl of homogenization buffer, which consisted of 10 mM Tris-HCl (pH 7.0), 150 mM KF, 15 mM EDTA, 15 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Homogenization was performed at 4 °C with a Polytron homogenizer. Homogenates were centrifuged at 10,000 $\times$ *g* for 15 min at 4 °C; supernatants and sediments were recovered for determinations. Sediments were resuspended in 100 μl of the same buffer. GS activity was measured in the presence or absence of 6.6 mM glucose-6-phosphate (20). The ratio of these two activities (−glucose-6-phosphate/+glucose-6-phosphate) is an estimate of the activation state of the enzyme.

Cell Treatments and Phosphorylation Analysis—CHO-R cells were grown to confluence and then deprived of FCS for 14–16 h. Tungstate and insulin treatments were performed in serum-free medium unless otherwise indicated. Plates were flash-frozen in liquid nitrogen and processed for protein extract preparation. Cells were scraped with 400 μl/60-mm plate of cold 30 mM Tris-HCl, pH 7.4, 25 mM NaCl, 1% (v/v) Triton X-100, 0.1% SDS, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM EGTA, 20 nM okadaic acid, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin. After 10 min on ice, extracts were centrifuged 10 min at 4 °C at 13,000 $\times$ *g*. Protein concentration from the supernatants was measured using the Bio-Rad protein assay. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schüll), and immunoblotted with selected antibodies; the immunoblots were developed using an enhanced chemiluminescence detection system (Amersham Biosciences), following the manufacturer's instructions. ERK1/2 activity was measured after immunoprecipitation of cell extracts with the phospho-p44/42 MAPK antibody and assayed with recombinant ELK1 as substrate using the p44/42 MAPK assay kit (Cell Signaling).

Transfection Experiments—These were performed as described in Ref. 21. Briefly, 293T cells were plated 24 h before transfection at a density of 3×10^5 cells/60-mm plate and transfected with empty plasmid pSG5 or the derived vector containing Pyst1 cDNA. Cells were incubated in medium containing 0.5% FCS for 24 h and treated with 1 mM tungstate for 1 h. They were then treated with 100 nM epidermal growth factor for 5 and 10 min in the presence or absence of 1 mM tungstate, and ERK1/2 phosphorylation was analyzed by Western blot as described above.

Statistical Analysis—Results were analyzed for significance by analysis of variance and unpaired Student's *t* test. $p < 0.05$ was considered significant.

RESULTS

Tungstate Induces Glycogen Deposition in Rat Primary Cultured Hepatocytes—Stimulation of hepatic glycogen deposition is one of the most characteristic metabolic actions of insulin. When primary cultured hepatocytes were incubated with insulin (100 nM) or increasing concentrations of tungstate (10 μM, 100 μM, and 1 mM) in the presence of glucose, they accumulated (Fig. 1*A*) and synthesized (Fig. 1*B*) higher amounts of glycogen as compared with untreated controls. These results indicate that tungstate exerts insulin-like actions in primary cultured hepatocytes. Therefore, we analyzed whether the key components of the insulin signaling cascade were modified by this compound.

Tungstate Treatment Does Not Phosphorylate the Insulin Receptor in CHO-R Cells or Primary Cultured Hepatocytes—Our first approach was to compare the effects of insulin and tungstate on the tyrosine phosphorylation profile in Chinese hamster ovary cells that overexpress human wild type insulin receptor (CHO-R) (17). This is a suitable cellular model to study the downstream events of the insulin transduction cascade. CHO-R cells were treated with insulin or with increasing concentrations of sodium tungstate for 5, 10, 60 min, and overnight (14 h). Cell extracts were then analyzed by Western blot using antibodies that react to phospho-tyrosine residues. Insulin produced a rapid phosphorylation of the β-subunit of its receptor, and several higher molecular weight bands, one of which had a molecular weight compatible with the insulin receptor substrate-1 (IRS-1) (Fig. 2, *A–C*). In contrast, in cells treated with tungstate, there was no significant phosphorylation of the IR β-subunit at the concentrations assayed neither in short (Fig. 2*B*) nor in long term (Fig. 2*C*) treatments. Co-incubation with tungstate plus insulin did not increase the phosphorylation of the IR β-subunit above the levels observed when the cells were treated with insulin alone (not shown).

Similarly, when primary cultured hepatocytes were incubated with tungstate under conditions in which the compound stimulated glycogen deposition, no significant increase in the phosphorylation of the IR β-subunit was observed (Fig. 2*D*). In contrast, incubation with insulin, which also stimulated glycogen deposition to a similar extent, induced the phosphorylation of this receptor (Fig. 2*D*).

Since tungstate is a phosphatase inhibitor, we tested whether this compound could prevent IR dephosphorylation in CHO-R cells stimulated by insulin and thus improve downstream insulin signaling. Briefly, CHO-R cells were treated with insulin for 10 min, the hormone was removed, and the effect of tungstate on the phosphorylation state of the IR β-subunit was evaluated by Western blot analysis at a range of time points. No significant differences were detected between tungstate-treated and untreated cells (Fig. 2*E*). These results indicate that tungstate increased glycogen deposition without modifying the IR β-subunit phosphorylation.

Tungstate Treatment Activates ERK1/2 in CHO-R Cells and Primary Cultured Hepatocytes—The tyrosine phosphorylation pattern in tungstate-treated CHO-R cells showed an additional protein band of 40–44 kDa, which was also present in insulin-treated cells (Fig. 2*F*). The molecular mass of the phosphorylated band was compatible with MAPK family members.

To check whether this protein corresponded to a MAPK, we performed a series of Western blots in extracts from CHO-R cells and primary cultured hepatocytes using antibodies that recognize ERK1/2 only when phosphorylated at conserved thre-

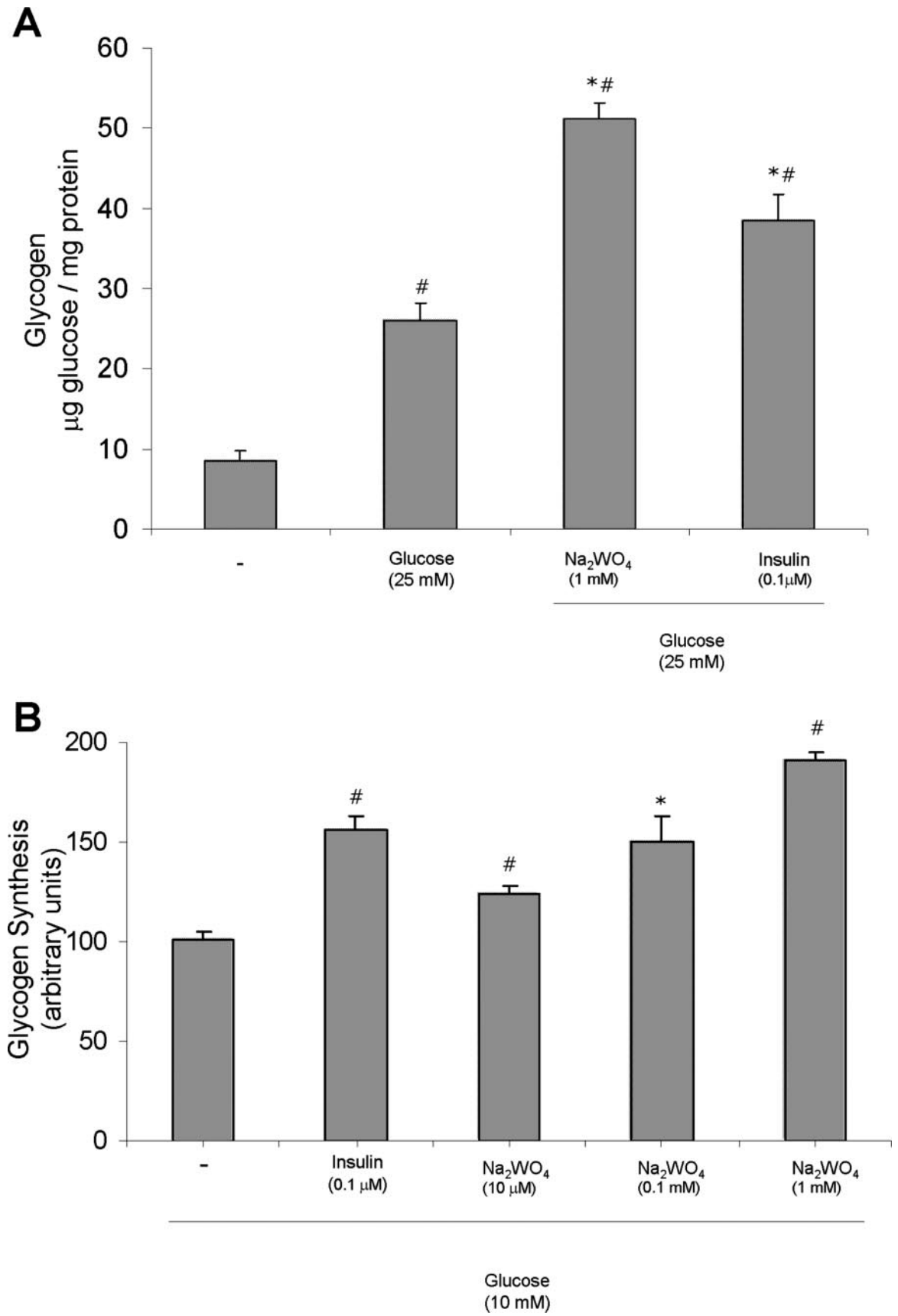

FIG. 1. **Glycogen accumulation and synthesis in primary cultured hepatocytes treated with tungstate.** Hepatocytes were preincubated in the absence of glucose for 20 h. *A*, glycogen accumulation measured after 2 h of incubation in the various experimental conditions. Glycogen content is expressed as μg of glucose/mg of protein. #, $p < 0.001$ as compared with treatment without glucose (−). *, $p < 0.001$ as compared with 25 mM glucose. *B*, glycogen synthesis measured as the incorporation of [U-^{14}C]glucose after 2 h of incubation in the various experimental conditions. #, $p < 0.001$ as compared with 10 mM glucose control (−). *, $p < 0.05$ as compared with 10 mM glucose control (−). Values are expressed in arbitrary units. Data represent the mean ± S.E. for three independent experiments.

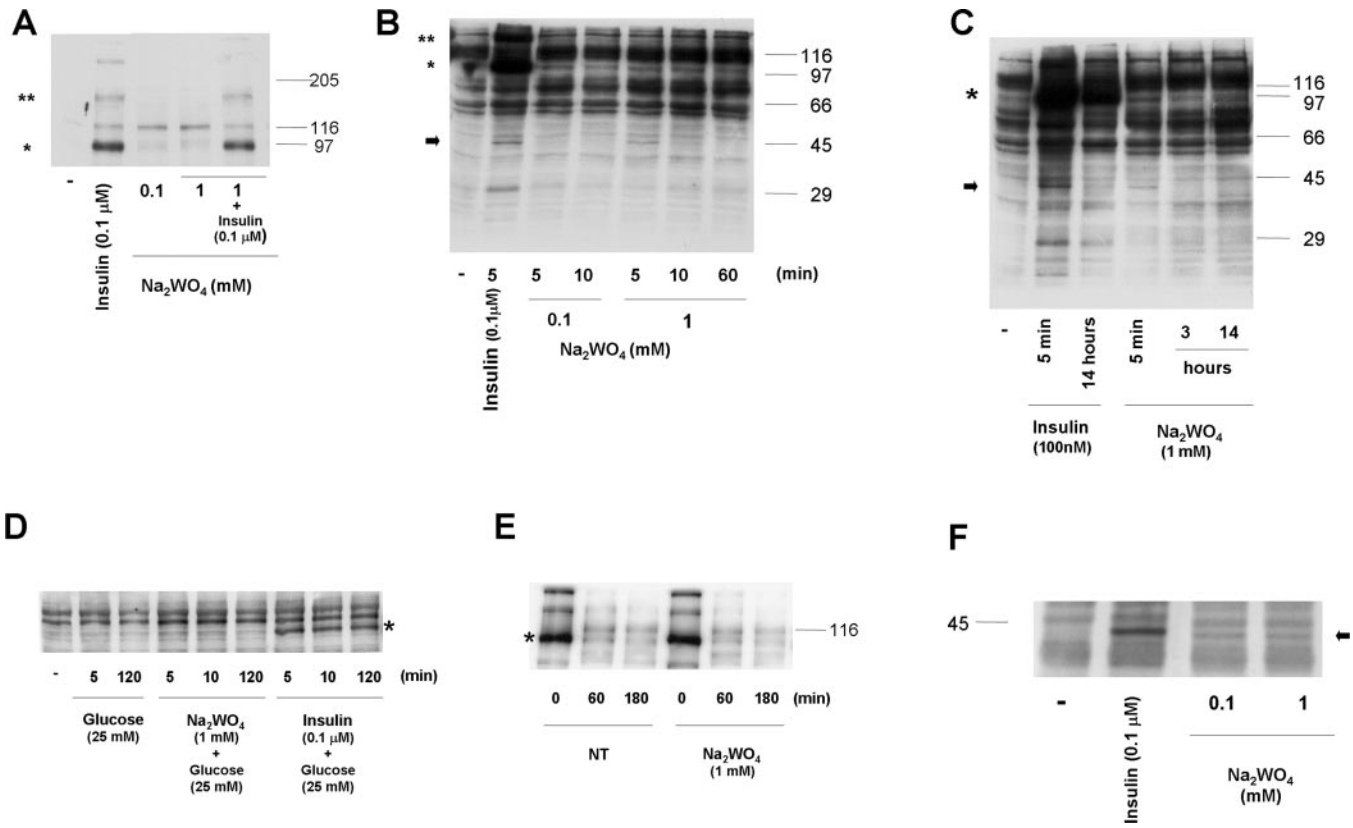

FIG. 2. **Phospho-tyrosine pattern and insulin receptor dephosphorylation in CHO-R cells and primary cultured hepatocytes.** *A–C*, CHO-R cells were treated with insulin (0.1 μM) or tungstate (0.1 and 1 mM) from 5 min to 14 h. Cells were lysed, and total protein was subjected to 8% (*A*) or 10% (*B* and *C*) SDS-PAGE and immunoblotted with 4G10 anti-phosphotyrosine antibody. *A*, 10-min treatments; *B*, short term treatments; *C*, long term treatments. As shown in *D*, hepatocytes were treated with medium with 25 mM glucose in the presence of 1 mM tungstate or 0.1 μM insulin for the times indicated. Cells were lysed, and total protein was subjected to 10% SDS-PAGE and immunoblotted with the 4G10 antibody. *E*, CHO-R cells were pretreated with 1 mM tungstate or vehicle for 20 h in FCS-free medium. Then, cells were treated with 0.1 μM insulin for 10 min, the hormone was removed, and the phosphorylation state of IR β-subunit in the presence or absence of tungstate was evaluated at 60 and 180 min by Western blot with the 4G10 antibody. *NT*, vehicle-treated cells. *F*, magnification of a 44-kDa tyrosine phosphorylated band at 10 min in CHO-R treated cells. *, the position of the IRβ-subunit. **, the position of the IRS1/2. *Arrows* indicate the molecular mass of ERK1/2. Molecular masses are expressed in kDa.

onine 202 and tyrosine 204 (numbering corresponding to human MAPK (Erk1)). Transient phosphorylation of ERK1 and 2 was observed when CHO-R cells were treated either with insulin or with tungstate; it peaked at 5–10 min, decreasing thereafter (Fig. 3*A*). Furthermore, analysis with antibodies against total ERK1/2 showed that these treatments did not significantly modify the total levels of these two kinases (Fig. 3*A*). We also observed an increase in the phosphorylation of ERK1/2 in primary cultured hepatocytes incubated with 1 mM tungstate, which peaked at about 10 min, decreasing thereafter (Fig. 3*B*). There was no significant increase in the total amount of ERK1/2 (Fig. 3*B*). No synergistic effect was observed when CHO-R cells or primary cultured hepatocytes were simultaneously incubated with tungstate and insulin (not shown).

ERK1/2 phosphorylation at consensus residues triggers the activation of these kinases (22, 23). To confirm that these phosphorylated kinases were catalytically active, we assayed ERK1/2 activity in immunoprecipitates from CHO-R cells treated either with tungstate or with insulin. Recombinant transcription factor ELK-1 was used as substrate, and the phosphorylation was monitored by Western blot with antibodies that recognize this protein when phosphorylated by ERK1/2 at serine 383. Tungstate and insulin treatment increased ERK1/2 activity (Fig. 3*C*), a finding consistent with the phosphorylation data shown in Fig. 3, *A* and *B*.

As a phosphatase inhibitor, the effect of tungstate on ERK1/2 phosphorylation may be related to the inhibition of a MAPK-phosphatase. Pyst1 (MKP-3, rVH6) is the main MAPK phosphatase involved in the inactivation of ERK1/2 (24–26). The putative inhibition of Pyst1 by tungstate would potentially lead to an increased phosphorylation of its substrates, *i.e.* ERK1/2.

A first set of experiments was performed to compare the time-dependent dephosphorylation of ERK1/2 in tungstate-treated *versus* untreated cells. Briefly, CHO-R cells were treated with insulin for 10 min, the hormone was then removed, and the effect of tungstate on the phosphorylation state of ERK1/2 was evaluated by Western blot analysis at a range of time points. No significant differences were detected between treated and untreated cells (Fig. 3*D*). To further study this point, we performed a second set of experiments in which Pyst1 was overexpressed in 293T cells. Cells were treated with 100 nM epidermal growth factor in the presence or absence of 1 mM of tungstate, and the ERK1/2 phosphorylation state was then visualized by Western blot. Pyst1 overexpression enhanced the dephosphorylation of ERK1/2. This effect was not significantly blocked or delayed by preincubation with 1 mM tungstate (Fig. 3*D*). These results indicate that no MAPK phosphatase is involved in tungstate-dependent ERK1/2 activation.

Tungstate-induced Glycogen Deposition in Rat Primary Cultured Hepatocytes Is Dependent on ERK1/2 Activation—We tested whether tungstate-induced ERK1/2 phosphorylation was involved in the stimulation of glycogen deposition triggered by this compound. For this purpose, we used PD98059, a specific inhibitor of ERK1/2 phosphorylation. Incubation of CHO-R cells and hepatocytes with this inhibitor completely

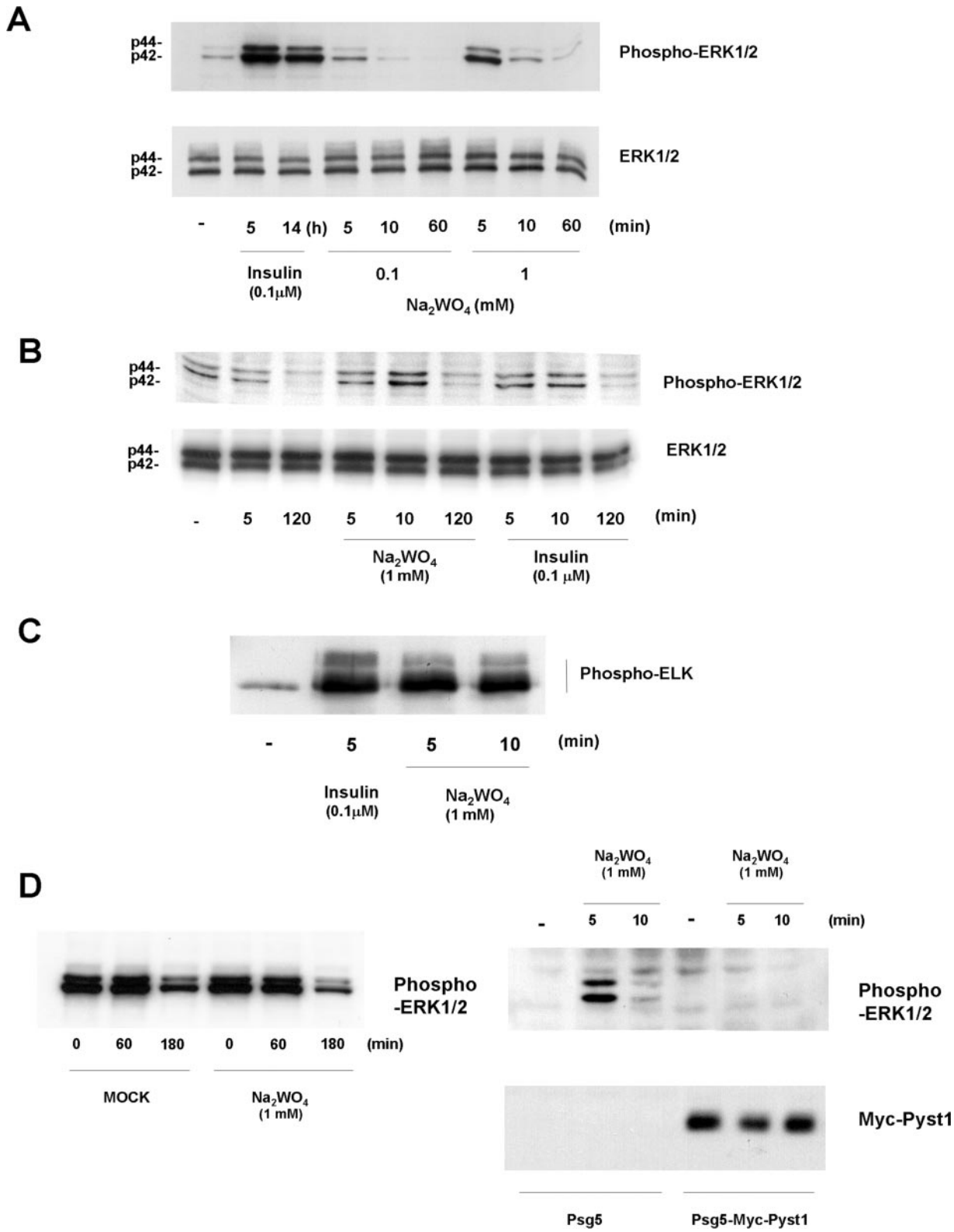

FIG. 3. **ERK1/2 phosphorylation and activity in CHO-R cells and primary cultured hepatocytes treated with tungstate.** *A*, CHO-R cells were treated with insulin (0.1 μM) or tungstate (0.1 and 1 mM) for the times indicated. Cells were lysed, and total protein was subjected to 10% SDS-PAGE and immunoblotted with phospho-p44/42 MAP kinase and ERK1/2 antibodies. *B*, hepatocytes were treated with medium with 25 mM glucose plus 1 mM tungstate or 0.1 μM insulin for the times indicated. Cells were lysed, and total protein was subjected to 10% SDS-PAGE and immunoblotted with phospho-p44/42 MAPK and ERK1/2 antibodies. *C*, CHO-R cells were treated with 0.1 μM insulin and 0.1 and 1 mM tungstate for the times indicated. ERK activity was measured after immunoprecipitation of phosphorylated ERK1/2, incubation with ELK1, and detection with a phospho-specific ELK1 antibody. *D*, *left panel*, CHO-R cells were pretreated with 1 mM tungstate or vehicle for 20 h in medium without FCS. Then, cells were treated with 0.1 μM insulin for 10 min, the hormone was removed, and the phosphorylation state of ERK1/2 in the presence or absence of tungstate was evaluated at 60 and 180 min by Western blot with the phospho-p44/42 MAPK antibody. *Right panel*, 293T cells transfected with empty pSG5 plasmid or pSG5 encoding Pyst1 were treated with 100 nM epidermal growth factor in the presence of 1 mM tungstate for the times indicated. ERK1/2 phosphorylation was monitored by immunoblot with the phospho-p44/42 MAPK antibody, as described above. *MOCK*, mock-treated.

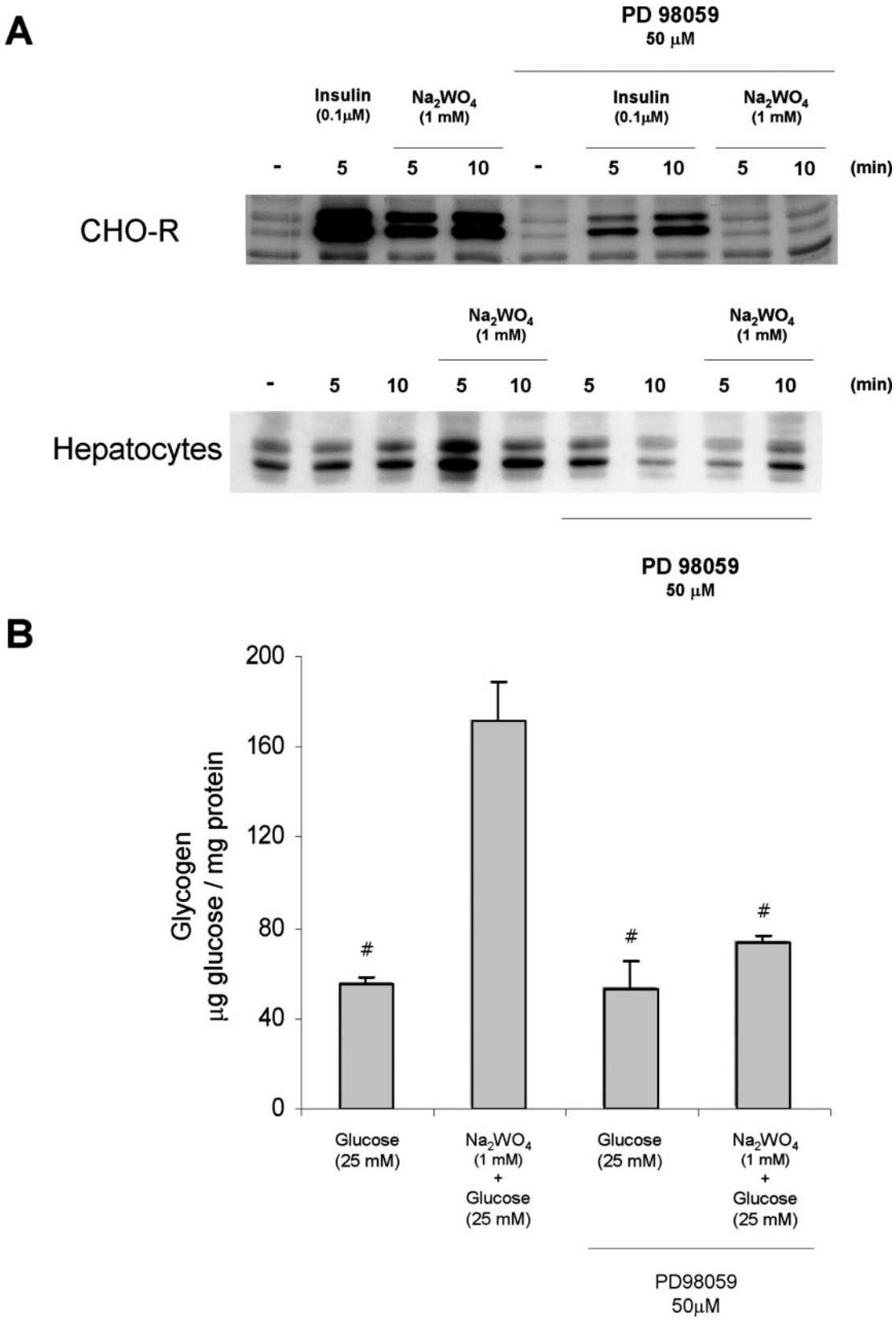

FIG. 4. **Analysis of tungstate-induced ERK1/2 phosphorylation and glycogen accumulation after treatment with PD98059.** *A*, *upper panel*, CHO-R cells were pretreated with or without PD98059 (50 μM) for 30 min. They were then incubated in the presence of the inhibitor plus 1 mM tungstate or with 0.1 μM insulin for the times indicated. ERK1/2 phosphorylation was monitored by Western blot with the phospho-p44/42 MAPK antibody. *Lower panel*, primary cultured hepatocytes were pretreated with or without PD98059 (50 μM) for 30 min. They were then incubated in the presence of the inhibitor plus 1 mM tungstate or with 0.1 μM insulin for the times indicated. ERK1/2 phosphorylation was monitored by Western blot with the phospho-p44/42 MAPK antibody. They were then assayed in the various experimental conditions in the presence of 25 mM glucose. ERK1/2 phosphorylation was monitored by Western blot with the phospho-p44/42 MAPK antibody. *B*, hepatocytes were preincubated in the absence of glucose for 20 h. Glycogen was measured after 2 h of incubation in the various experimental conditions. PD98059-treated plates were preincubated for 30 min and during the experiment with the inhibitor. Glycogen content is expressed as μg of glucose/mg of protein. #, $p < 0.001$ as compared with 25 mM glucose + 1 mM tungstate.

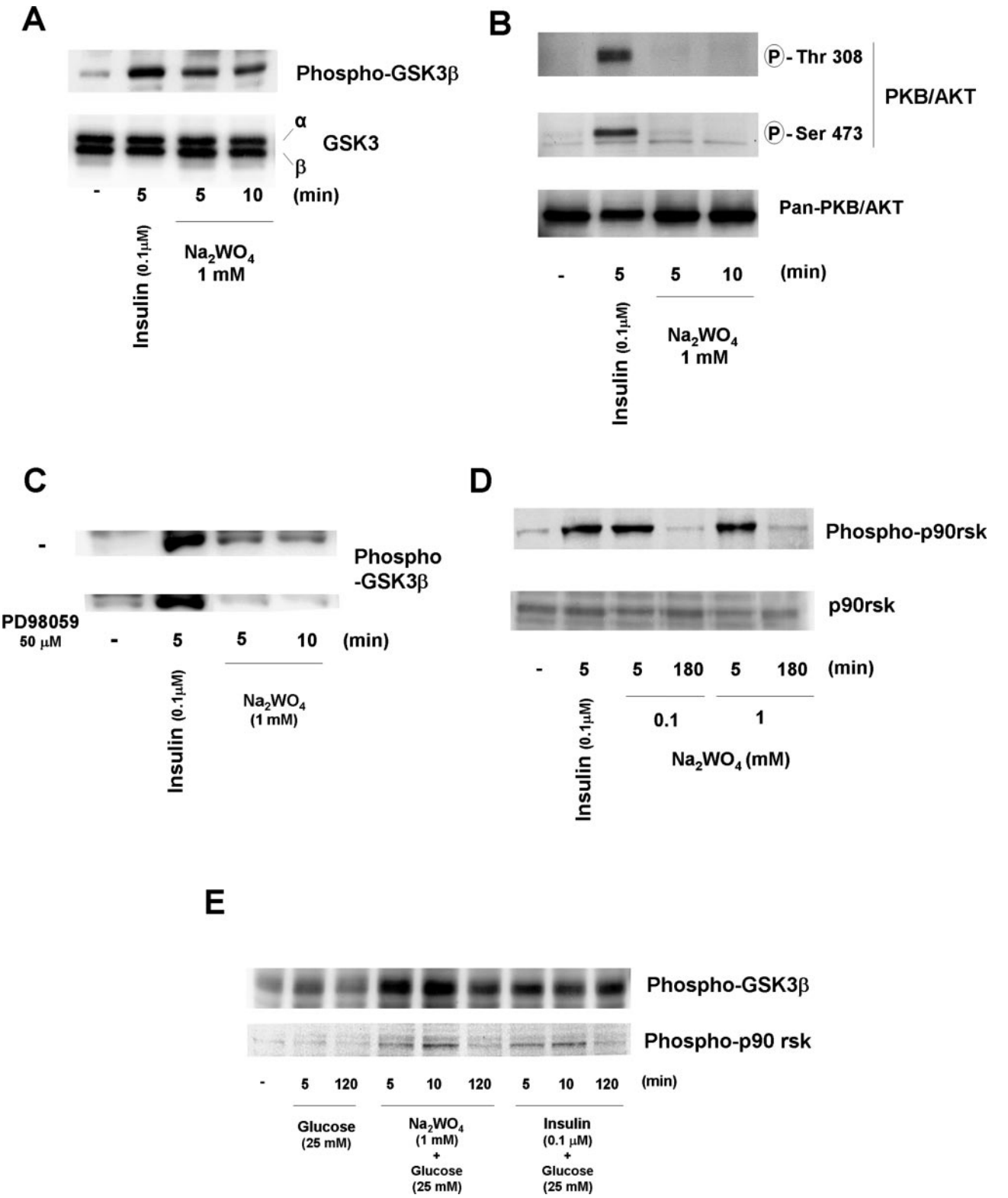

FIG. 5. **Analysis of tungstate-induced GSK3*β*, PKB/Akt, and p90rsk phosphorylation in CHO-R cells and primary cultured hepatocytes.** *A*, *B*, and *D*, CHO-R cells were treated with insulin (0.1 μM) or tungstate (0.1 and 1 mM) for the times indicated. Cells were lysed, and total protein was subjected to 10% SDS-PAGE and immunoblotted with the phospho-GSK3*β* (*A*), GSK3*α*/*β* (*A*), phospho-PKB/Akt (Thr-308, Ser-473) (*B*), PKB/Akt (*B*), phospho-p90rsk (*D*), and p90rsk (*D*) antibodies. As shown in *C*, CHO-R cells were either mock- or pretreated for 30 min with PD98059 (50 μM). They were then incubated in the presence of the inhibitor plus 1 mM tungstate or with 0.1 μM insulin for the times indicated. GSK3*β* phosphorylation was monitored by Western blot with the phospho-GSK3*α*/*β* antibody. *E*, hepatocytes were treated with medium with 25 mM glucose and 1 mM tungstate or 0.1 μM insulin for the times indicated. Cells were lysed, and total protein was subjected to 10% SDS-PAGE and immunoblotted with phospho-p90rsk and phospho-GSK3*α*/*β* antibodies.

blocked the tungstate-induced phosphorylation of ERK1/2 (Fig. 4*A*). Under these conditions, PD98059 also prevented tungstate-induced glycogen deposition in cultured hepatocytes, indicating that this process is dependent on active ERK1/2 (Fig. 4*B*).

Tungstate Treatment Leads to the Activation of p90rsk and Activation of Glycogen Synthesis in a PKB/Akt Phosphoryla-

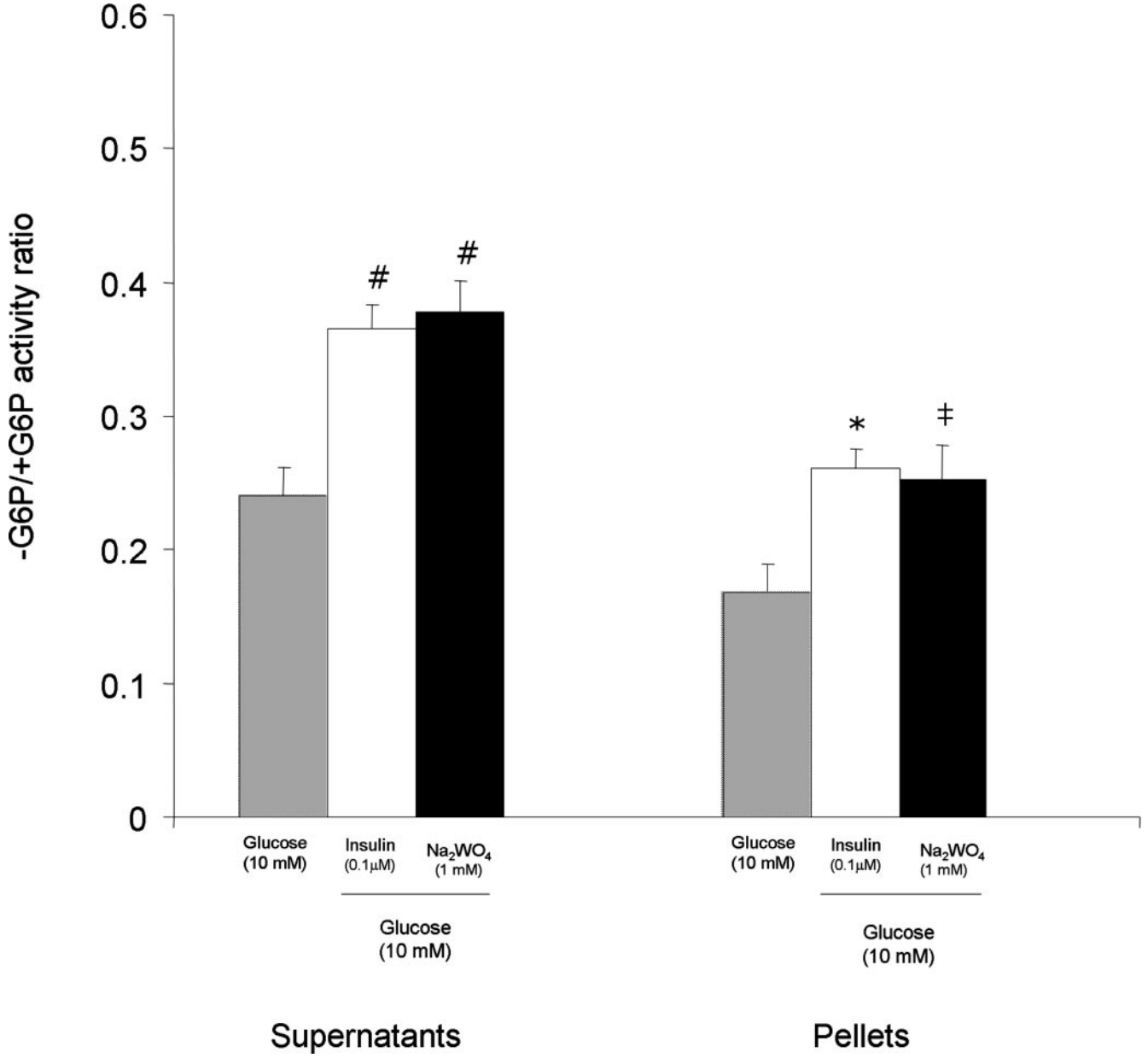

FIG. 6. **Analysis of GS activity in primary cultured hepatocytes treated with tungstate.** Hepatocytes were preincubated in the absence of glucose for 20 h. GS was measured in the soluble and particulate fractions after 2 h of incubation in the various experimental conditions. Active GS was measured in the absence of glucose-6-phosphate, as described under "Experimental Procedures." Supernatants: #, $p < 0.01$, as compared with 10 mM glucose. Pellets: *, $p < 0.01$; ‡, $p < 0.05$, as compared with 10 mM glucose. Data represent the mean ± S.E. for three independent experiments. *G6P*, glucose-6-phosphate.

tion-independent Way—The stimulation of glycogen synthesis by insulin is triggered by the phosphorylation of GSK3β at serine 9 and its concomitant inactivation. Therefore, we used specific antibodies to analyze whether tungstate treatment stimulates the phosphorylation of GSK3β in CHO-R and primary hepatocytes. Tungstate, like insulin, stimulated the phosphorylation of GSK3β at consensus serine 9 in both cell types, whereas no significant variations were observed in the total amount of this kinase (Fig. 5, *A* and *E*).

GSK3β is mainly phosphorylated by activated PKB/Akt after insulin treatment (for a review, see Refs. 27 and 28). We analyzed whether tungstate stimulated the phosphorylation of PKB/Akt at consensus threonine 308 and serine 473 using antibodies specific for these phosphorylation sites. Our results show that, in contrast to insulin, this compound did not significantly modify the phosphorylation state of this kinase in CHO-R cells (Fig. 5*B*) or in primary cultured hepatocytes (not shown). Since PKB/Akt was not apparently involved in the phosphorylation of GSK3β, we next checked whether ERK1/2 may play a role in this event. As shown in Fig. 5*C*, pretreatment of CHO-R cells with PD98059 abrogated tungstate-induced GSK3β phosphorylation. Similar results were obtained in primary cultured hepatocytes (not shown). p90rsk (MAPKAP-K1), one of the main substrates activated by phosphorylated ERK1/2, may be involved in the phosphorylation and inactivation of GSK3 (29–31). Tungstate treatment strongly stimulated the phosphorylation of p90rsk at consensus serine 380, both in CHO-R cells and in primary cultured hepatocytes, whereas no significant changes were observed in the total amount of this protein (Fig. 5, *D* and *E*). Therefore, the phosphorylation of p90rsk may be the link between ERK activation and GSK3β phosphorylation.

GSK3β inactivation may lead to the activation of GS, the key enzyme of glycogen synthesis. Incubation of primary cultured

hepatocytes with tungstate increased the GS activation state (expressed as −glucose-6-phosphate/+glucose-6-phosphate activity ratio) in both soluble and particulate fractions (Fig. 6). Moreover, tungstate-induced GS activation was greatly reduced when the cells had been incubated previously with PD98059, indicating that an active ERK1/2 kinase is required for the activation of this enzyme (not shown).

DISCUSSION

Although ample information is available on the pharmacological effects of tungstate as an antidiabetic agent, data on its molecular targets are scarce. This study is the first to focus on the identification of the molecular mechanism of action of this compound. Our work hypothesis was that tungstate directly or indirectly affects one or several components of the insulin signaling cascade and that this leads to insulin-like actions. Our results support this hypothesis as incubation of primary cultured hepatocytes with tungstate increased glycogen deposition, one of the most characteristic insulin-like effects. The increase in glycogen deposition was observed at a wide range of tungstate concentrations. It must be highlighted that tungstate did not exert any toxic effect on the primary cultured hepatocytes or the cell lines used.[2] Furthermore, the concentrations that are active *in vitro* are similar to the plasma levels measured in tungstate-treated animals (32).[3]

We considered the possibility that this compound may alter the phosphorylation of the IR β-subunit, thus mimicking the action of insulin. Tungstate did not significantly alter the phosphorylation state of the IR β-subunit, nor did it block or delay its dephosphorylation. Therefore, this compound bypassed the IR in its mechanism of action. In contrast, we observed a clear and transient activation of ERK1/2 when CHO-R cells or primary cultured hepatocytes were incubated with the compound. As a potential phosphatase inhibitor, tungstate might have kept ERK1/2 in an active state through inhibiting a MAPK phosphatase. However, this does not seem to be the case since dephosphorylation of ERK1/2 was not significantly delayed by tungstate. Furthermore, we have gained evidence that indicates that Pyst1/MKP3, the main phosphatase that acts on ERK1/2 (24–26), is not involved in tungstate-induced activation of these kinases.

The accepted mechanism by which insulin activates GS and therefore glycogen deposition is through the inactivation of GSK3β induced by phosphorylation at serine 9 (33–35). Tungstate treatment indeed promoted GSK3β phosphorylation at this consensus residue, which explains the observed activation of GS induced by this compound. Moreover, tungstate-induced GS activation and glycogen deposition were dependent on the activation of ERK1/2 since they were blocked by inhibition of the phosphorylation of these enzymes, indicating a connection between the phosphorylation of these kinases and glycogen synthesis in tungstate-treated cells. We propose that p90rsk links these two events as this protein is a substrate of ERK1/2 and is strongly activated in cells treated with tungstate and since it has been reported that it participates in the inactivation of GSK3β (29–31, 36–39).

Nevertheless, the role of p90rsk in the activation of GS is controversial. Some results show that in skeletal muscle and adipocytes, p90rsk is not significantly involved in the regulation of glycogen synthesis (40, 41). In fact, results from mice that lacked the RSK2 isoform showed that this protein is not required for activation of muscle GS by insulin but may indirectly modulate muscle GS activity and/or glycogen content (42). However, although muscle and adipose tissue mainly express the RSK2 isoform of p90rsk, a different isozyme, RSK1, is expressed in liver (43). Very little is known about RSK1 and its role in liver glycogen metabolism. In our experiments, RSK1 was stimulated by tungstate treatment in primary cultured hepatocytes, and on the basis of these results, we propose that RSK1 contributes to tungstate-induced GS activation and glycogen deposition in liver.

Surprisingly, all the phosphorylation events described were independent of the activation of PKB/Akt, the main contributor to GSK3 phosphorylation in the insulin signaling pathway. This is a remarkable result since it has been reported that the insulin- or IGF1-induced phosphorylation of GSK3 is prevented by inhibition of PKB/Akt but not of p70S6K or ERK1/2 (44).

Furthermore, tungstate-stimulated ERK1/2 activation is not correlated with an increase in the proliferation rate of CHO-R or other cell lines.[2] Taken together, these results indicate that under tungstate stimulation, the metabolic effects of the activation of these MAPKs are prevalent over the mitogenic ones.

The question remains as to what activates ERK1/2 in this experimental situation. Our data indicate that these protein kinases are not directly activated by tungstate as they are sensitive to PD98059, a MEK1/2 inhibitor. We propose that tungstate acts on one or several of the components of the ras-raf-MEK-ERK pathway.

In conclusion, we have identified the first set of molecular targets through which sodium tungstate may exert its antidiabetic action. Moreover, this study provides novel data that point to ERK1/2 as a new target for the design of antidiabetic agents.

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The Antidiabetic Agent Sodium Tungstate Activates Glycogen Synthesis through an Insulin Receptor-independent Pathway

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