Leptin-dependent Phosphorylation of PTEN Mediates Actin Restructuring and Activation of ATP-sensitive K+ Channels

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Leptin activates multiple signaling pathways in cells, including the phosphatidylinositol 3-kinase pathway, indicating a degree of cross-talk with insulin signaling. The exact mechanisms by which leptin alters this signaling pathway and how it relates to functional outputs are unclear at present. A previous study has established that leptin inhibits the activity of the phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10), an important tumor suppressor and modifier of phosphoinositide signaling. In this study we demonstrate that leptin phosphorylates multiple sites on the C-terminal tail of PTEN, an important tumor suppressor and modifier of phosphoinositide signaling. In this study we demonstrate that leptin phosphorylates multiple sites on the C-terminal tail of PTEN in hypothalamic and pancreatic β-cells, an action not replicated by insulin. Inhibitors of the protein kinases CK2 and glycogen synthase kinase 3 (GSK3) block leptin-mediated PTEN phosphorylation. PTEN phosphorylation mutants reveal the critical role these sites play in transmission of the leptin signal to F-actin depolymerization. CK2 and GSK3 inhibitors also prevent leptin-mediated F-actin depolymerization and consequent ATP-sensitive K⁺ channel opening. GSK3 kinase activity is inhibited by insulin but not leptin in hypothalamic cells. Both hormones increase N-terminal GSK3 serine phosphorylation, but in hypothalamic cells this action of leptin is transient. Leptin, not insulin, increases GSK3 tyrosine phosphorylation in both cell types. These results demonstrate a significant role for PTEN in leptin signal transmission and identify GSK3 as a potential important signaling node contributing to divergent outputs for these hormones.

Efficient signaling by leptin and insulin is essential for the maintenance of body energy homeostasis, with disruptions in these processes strongly associated with diabetes and obesity (1, 2) and, at least for insulin, neurodegenerative disorders such as Alzheimer disease (3, 4). In recent years there has been a significant increase in understanding the intracellular signaling processes associated with the actions of insulin on a wide variety of cell types (5). However, our knowledge of leptin signaling is less advanced, with most studies indicating that leptin and insulin share many signaling intermediates in common, often leading to similar cellular outcomes (6, 7). In particular, signaling through the STAT (signal transducers and activators of transcription), mitogen-activated protein kinase, and PI3K pathways have been reported extensively in numerous cell types for both leptin and insulin (5, 8).

Nevertheless, leptin and insulin can cause differing and sometimes opposing cellular outputs, even on the same cell type. This is demonstrated in hypothalamic neurons, where electrophysiological or imaging studies show differential outcomes for leptin and insulin action (9–11). Thus, although superficially leptin may utilize the same signaling pathways as insulin, the exact nature of the leptin-induced signaling intermediates and their interplay with one another and with individual effectors is still relatively unknown. Recently, it was demonstrated that although leptin, like insulin, raises PtdIns(3,4,5)P₃ levels in hypothalamic cells, the mechanism underlying the increase by leptin differs from that of insulin. Whereas insulin increased PI3K activity, leptin had little effect on PI3K activity. Instead, leptin inhibited the lipid and protein phosphatase, PTEN, which resulted in increased PtdIns(3,4,5)P₃ levels in the presence of active PI3K (12). Previously, PI3K-dependent leptin signaling had been shown to open ATP-sensitive (Kₘₐₜₚ) channels in rat hypothalamic neurons (13) and in rat and mouse insulin-secreting cells (12, 14, 15), resulting in cell hyperpolarization and inhibition of firing. Kₘₜₚ activation by leptin is dependent on actin depolymerization in both cell types (12–15). The connection between leptin-driven PI3K activity, actin remodeling, and Kₘₜₚ opening appears not to be due simply to increased PtdIns(3,4,5)P₃ but may also require coincident inhibition of PTEN protein and lipid phosphatase activity through increased PTEN phosphorylation (12).

In this study we have focused on the mechanisms by which leptin alters PTEN phosphorylation in hypothalamic and insulin-secreting cells. The biological activity of PTEN toward anionic lipid substrates is considered to be dependent on its phosphorylation status at various residues between Ser-362 and Ser-385, located in the PTEN C-terminal tail (Fig. 1A), with phosphorylation maintaining PTEN in an inactive state (16). The major sites of phosphorylation are Ser-370 and Ser-385 (17, 18), with significant phosphorylation at a cluster of sites (Ser-380, Thr-382, and Thr-383) close to Ser-385. Mutations of
Leptin and PTEN Phosphorylation

the cluster site residues have significant effects on PTEN stability and membrane and protein binding (17, 19, 20). It is thought that PTEN is constitutively phosphorylated and not linked to physiological signaling events such as stimulation of cells with insulin or growth factors (21, 22). However, we previously demonstrated that leptin stimulation of hypothalamic cells reduced PTEN phosphatase activity and increased PTEN phosphorylation at the cluster site (12). This increased phosphorylation was sensitive to inhibition of protein kinase CK2, previously implicated as the main kinase phosphorylating PTEN at these residues (17, 18). CK2 has also been reported to phosphorylate PTEN at Ser-370 and Ser-385 (Fig. 1A) using in vitro kinase assays (17, 23), resulting in a 30% depression of PTEN phosphatase activity (18). In addition, GSK3β has been reported to phosphorylate PTEN at Ser-362 and Thr-366 in human embryonic kidney 293T cells, with Thr-366 phosphorylation reducing the biological activity of PTEN.

Consequently, we have assessed whether leptin mediates phosphorylation of PTEN at Ser-370, Ser-385, and Thr-366 in a CK2- and GSK3-dependent manner and further examined the contribution of Ser-370 and Thr-366 to downstream events such as actin re-organization and KATP channel opening using hypothalamic and insulin-secreting cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Islet Isolation—The mouse hypothalamic cell line N29/4 was cultured as previously described (24). MIN6 cells (obtained with permission from Jun-ichi Miyazaki, Osaka University) were used between passages 24 and 30 and grown in Dulbecco’s modified Eagle’s medium containing 25 mM glucose and supplemented with 12.5% heat-inactivated fetal calf serum, 4 mM l-glutamine, 100 g/mL of 95% air and 5% CO2. Wild-type C57Bl/6 mice (20–25 g) were and 1% penicillin and streptomycin in a humidified atmosphere incubated at room temperature for 20 min to form the trans-

Western Blotting—N29/4 and MIN6 cells, grown in 6-well plates, were exposed to normal saline for 30 min before treatment with leptin, insulin, or saline in the presence and absence of LY294002 (Sigma), DMAT, or CT99021 for the required time (inhibitors added 20–30 min before challenge). Protein isolation, content, immunoblotting, and analysis procedures were as previously described (13). PTEN and phospho-PTEN antibodies, Ser-380/Thr-382/Thr-383 from Cell Signaling (1:5000), Ser-380/Thr-382/Thr-383 were from Cell Signaling (1:5000), and Ser-370 and Thr-366 were from Nick Leslie (1:500). phosphorylated (p)-PKB (Ser-473) and p-GSK3 (Ser-9/Ser-21) were obtained from Cell Signaling and used at 1:1000. p-GSK3 (Tyr-216/Tyr-279), PKB, and GSK3 antibodies (all polyclonal) were obtained from BD Transduction Laboratories and used at 1:2500 (p-GSK3) and 1:1000 (PKB and GSK3). Monoclonal anti β-ac-
defined as the amount that catalyzes the phosphorylation of 1 nmol of substrate in 1 h.

Electrophysiology—Mouse cultured β-cells were superfused at room temperature (22–25 °C) with normal saline. Whole-cell current and voltage clamp recordings were used to monitor membrane potential and macroscopic current, respectively, using an Axopatch 200B amplifier. Whole-cell experiments were maintained in current-clamp mode to monitor the cell resting membrane potential, with short excursions into voltage clamp mode to examine macroscopic current-voltage relations. In voltage-clamp recordings the membrane potential was held at –70 to –20 mV steps of 2 s duration, with 20 ms between pulses applied (range of voltages, –160 to –40 mV). Whole cell voltage clamp data were analyzed using pCLAMP 8.2 software (Molecular Devices). Current clamp data were recorded and stored onto digital audio tapes and replayed for analysis and illustration on a Gould TA240 chart recorder. Recording electrodes were pulled from borosilicate glass and had resistances of 5–10 megaohms when filled with pipette solution, which comprised 140 mM KCl, 5 mM MgCl₂, 3.8 mM CaCl₂, 3 mM MgATP, 10 mM EGTA, 10 mM HEPES, pH 7.2 (free [Ca²⁺] of 100 nM). Application of drugs was made via the bath perfusion system except for DMAT and the GSK3 inhibitors, which were present in the pipette solution.

Statistical Analysis—Data are presented as the means ± S.E. unless otherwise stated. All statistical analyses were performed using analysis of variance, one sample t test, Student’s paired or unpaired t tests. A probability level of 0.05 was considered significant.

RESULTS

We used the leptin- and insulin-sensitive hypothalamic cell line N29/4 (12, 24) to examine PTEN phosphorylation at Thr-366, Ser-370, and Ser-385 (Fig. 1A) on stimulation with leptin (10 nM) or insulin (10 nM). Leptin rapidly (<5 min) increased the level of p-PTEN at all three sites (Fig. 1A), an action sustained for at least 60 min for each site (data not shown). This leptin-mediated increase in p-PTEN was unaffected by the presence of the PI3K inhibitor, LY294002 (10 μM; n = 6 for each; Fig. 1B). Previous studies indicated that the cluster site, Ser-385 and Ser-370, may be phosphorylated by protein kinase CK2 (17, 18, 23), and Thr-366 may be phosphorylated by GSK3β (23). Consequently, we examined leptin phosphorylation of Thr-366, Ser-370, and Ser-385 PTEN residues in the absence and presence of DMAT (a CK2 inhibitor (26, 27) and CT99021 (a specific GSK3 inhibitor (28, 29)). DMAT (10 μM) prevented leptin from phosphorylating Ser-370 and Ser-385 but not Thr-366 in N29/4 cells (Fig. 1, B and C). Conversely, leptin phosphorylation of Thr-366 was prevented by CT99021 (2 μM), whereas phosphorylation of Ser-370 and Thr-366 was unaffected by this inhibitor (Fig. 1, B and C). In contrast, 10 nm (Fig. 1D) or 50 nm (data not shown) insulin had no effect on p-PTEN levels in N29/4 cells at Thr-366 or Ser-370 residues, with the Ser-385 site not tested.

Leptin re-organization of the actin cytoskeleton is dependent on PI3K activity and inhibition of the lipid and protein phosphatase activity of PTEN. Overexpression of cluster site p-PTEN mutants demonstrated that phosphorylation at this site was required to limit PTEN phosphatase activity and prevent leptin-induced F-actin depolymerization (12). Accordingly, we have examined whether phosphorylation of PTEN at Ser-370 and/or Thr-366 is also required for transmission of the leptin signal to F-actin. First, we sought to determine whether phosphorylation of Thr-366 or Ser-370 altered the activity of PTEN in cells by overexpression of PTEN mutants with alanine or aspartic acid substitutions. PtdIns(3,4,5)P₃ and F-actin levels were assessed by staining with a monoclonal PtdIns(3,4,5)P₃ antibody and Alexa 488-conjugated phalloidin respectively, after transfection of PTEN mutants into N29/4 cells. In control experiments overexpression of wild-type PTEN had no effect per se but prevented leptin from decreasing F-actin (Fig. 2A). Overexpression of T366A PTEN or S370A PTEN reduced
PTEN phosphorylation at Ser-370 and Thr-366 sites led us to explore whether this action of leptin was sensitive to CK2 and/or GSK3 inhibition. The presence of the GSK3 inhibitor, CT99021 (2 μM) or the CK2 inhibitor DMAT (10 μM) alone had no effect on F-actin (data not shown). Leptin decreased the mean F-actin level, and in the presence of CT99021 this was significantly reduced (Fig. 2F). The combined presence of CT99021 and DMAT inhibited F-actin depolymerization by leptin significantly more than CT99021 alone but did not completely occlude leptin from affecting F-actin levels (Fig. 2F). Nevertheless, the presence of the GSK3 and CK2 inhibitors at concentrations that significantly reduced PTEN phosphorylation also significantly reduced the ability of leptin to depolymerize F-actin.

Phosphorylation of PTEN in β-Cells and Kmatched ATP Activation—It was previously demonstrated that Kmatched ATP channel opening and hyperpolarization of mouse pancreatic β-cells by leptin could be inhibited by the lipid phosphatase dead G129E PTEN mutant and mimicked by the dual lipid and protein phosphatase dead C124S PTEN mutant (12). This is consistent with inhibition of PTEN lipid and protein phosphatase activity for the successful transmission of the leptin signal to Kmatched ATP channels in β-cells. As leptin has not been shown to alter p-PTEN status in β-cells, we used MIN6 β-cells to explore this action of leptin. Although the effects of leptin on p-PTEN were not as robust as observed for N29/4 cells, leptin (10 nM) rapidly (∼1 min) increased phosphorylation of the cluster site, Ser-370 and Thr-366, actions sustained for at least 30 min in each case (Fig. 3, A and B). Insulin (10 nM) had no effect on PTEN phosphorylation levels at any of these sites over this time period (Fig. 3, A–C). The CK2 inhibitor DMAT (2 μM) inhibited leptin-mediated PTEN phosphorylation at Ser-370 and Thr-366, whereas the

PtdIns(3,4,5)P3 levels alone is not a predictor or indeed sufficient to initiate PTEN-dependent downstream signaling events such as F-actin depolymerization.

FIGURE 2. Effect of PTEN phosphorylation mutants on PtdIns(3,4,5)P3 and F-actin levels in N29/4 cells. Wild-type PTEN and T366A PTEN (A) and S370A PTEN (B) were overexpressed in N29/4 cells (upper panels, Cy3 secondary antibody on cells treated with monoclonal PTEN antibody) and F-actin visualized with Alexa 488-phalloidin (lower panels) with and without stimulation by 10 nM leptin. Note that untransfected cells in the secondary antibody on cells treated with monoclonal PTEN antibody) and F-actin visualized with Alexa 488-phalloidin (lower panels) with and without stimulation by 10 nM leptin. Data are shown as a ratio of control, untreated cells. C, PtdIns(3,4,5)P3 levels in N29/4 cells transfected with T366D PTEN or S370D PTEN were investigated using a PtdIns(3,4,5)P3, (a ratio of control) in N29/4 cells after treatment with these PTEN phosphorylation mutants. D and E, F-actin levels were visualized (lower panels) in N29/4 cells transfected (upper panels) with T366D PTEN (D) or S370D PTEN (E) in the absence (left) and presence (right) of 10 μM LY294002 (LY, D) or 10 nM leptin (E). The bar graphs show mean levels of F-actin after treatment of N29/4 cells overexpressing T366D PTEN (D) or S370D PTEN (E). Data are shown as a ratio of control, untreated cells. F, cellular F-actin levels in the absence or presence of 10 nM leptin and in cells pretreated with 2 μM CT99021 alone (CT) or in combination with 10 μM DMAT (D) before leptin challenge. Note that F-actin is visualized in this series of experiments with rhodamine-conjugated phalloidin. The bar graph denotes mean levels of F-actin-treated cells. Data are shown as the ratio of control, untreated cells. Values represent the mean ± S.E. for 3–4 experiments per group. *p < 0.05 compared with the appropriate labeled control for each treatment. Scale bars, 10 μM. PIP3, PtdIns(3,4,5)P3.
GSK3 inhibitor CT99021 (10 μM) inhibited phosphorylation at Thr-366 only (Fig. 3D).

Consequently, we predicted that the F-actin reduction and opening of K\textsubscript{ATP} channels by leptin (12, 13) would be susceptible to inhibition of CK2 and GSK3. Therefore, we examined the ability of leptin to hyperpolarize the membrane potential and activate K\textsubscript{ATP} currents in whole-cell current and voltage clamp recordings, respectively, from mouse primary cultured pancreatic β-cells in the absence and presence of DMAT and various GSK3 inhibitors. In control experiments β-cells were dialyzed with an electrode solution containing 3 mM ATP, which resulted in a mean membrane potential of −41.8 ± 1.5 mV (n = 22) under current clamp conditions. Application of leptin (10 nM) resulted in a slowly developing hyperpolarization (Fig. 4A) to a mean value of −69.4 ± 1.6 mV (n = 22, p < 0.05), which was reversibly inhibited (to −34.5 ± 1.9 mV; n = 17, p < 0.05) by the sulfonylurea tolbutamide (100 μM). Voltage clamp analysis (Fig. 4A) showed that leptin increased the mean cell slope conductance from 0.73 ± 0.11 to 1.62 ± 0.22 nanosiemens (n = 19; p < 0.05), an action reduced by tolbutamide to 0.42 ± 0.04 nanosiemens (n = 14; p < 0.05), indicating that

Leptin and PTEN Phosphorylation

![Image](https://www.jbc.org/content/284/14/9337/F3.large.jpg)

**FIGURE 3.** Leptin increases PTEN phosphorylation in MIN6 pancreatic β-cells. A, phosphorylated PTEN levels in MIN6 cells using the phospho-specific PTEN antibodies indicated (cluster site, Ser-370 and Thr-366), in response to 10 nM leptin or 10 nM insulin for the indicated times (minutes). B, the bar graph shows mean normalized levels of phosphorylated PTEN at the cluster phosphorylation site (380/2/3) for untreated cells and for cells stimulated with leptin (filled bars; n = 6) and insulin (open bars; n = 8) up to 30 min (*, p < 0.05, versus control (C) or insulin). C, bar graph shows the mean normalized levels of phosphorylated Thr-366 PTEN after stimulation with 10 nM insulin for 5 and 30 min. D, bar graphs showing mean normalized levels of phosphorylated Thr-366 PTEN and Ser-370 PTEN under non-stimulated conditions (C) and after stimulation with 10 nM leptin in the absence (L) and presence of DMAT (L+D) and CT99021 (L+CT; n = 7 each). *, p < 0.05.

![Image](https://www.jbc.org/content/284/14/9337/F4.large.jpg)

**FIGURE 4.** Activation of K\textsubscript{ATP} channels in mouse pancreatic β-cells by leptin is occluded by the presence of CK2 or GSK3 inhibitors. Representative whole-cell current clamp recordings from a mouse cultured β-cell dialyzed with an electrode solution containing 3 mM ATP. In this and subsequent panels the trace begins ~2 min after obtaining the recording configuration. Leptin (10 nM), tolbutamide (Tolb, 100 μM), or diazoxide (Dzx, 200 μM) were applied for the time indicated on each trace (A–E). Cont, control. The action of leptin on β-cell membrane potential was determined in the absence (A) and presence of 10 μM DMAT (B), 2 μM CT99021 (C), 10 μM kenpaullone (D), and 2 μM AR-A014418 (E) added to the electrode solution and, hence, cell interior. The bar graphs to the right of each trace denote the mean slope conductance, determined from voltage clamp current-voltage relations in the absence and presence of leptin. In control cells (A) the hyperpolarization and increased slope conductance induced by leptin are inhibited by the presence of tolbutamide. For drug-treated cells leptin has no effect on mean cell conductance, although in each case application of 200 μM diazoxide hyperpolarizes the cells, which is associated with a significant increase in slope conductance. *, p < 0.05, compared with control.
the increase in current responsible for the augmented slope conductance and β-cell hyperpolarization by leptin is due to $K_{ATP}$ channel opening (14, 15, 30). The presence of DMAT (10 mM) in the electrode solution (and, therefore, cell interior) resulted in a mean resting membrane potential of $-44.8 \pm 3.4$ mV ($p > 0.05$ compared with control). Subsequent addition of leptin caused a small, insignificant ($p > 0.05$) hyperpolarization to $-52.4 \pm 8.4$ mV ($n = 5$; Fig. 4B), which was significantly different ($p < 0.05$) from the control leptin response. Voltage clamp analysis showed that DMAT also prevented leptin from increasing the β-cell mean slope conductance (Fig. 4B). The $K_{ATP}$ channels were operational in the presence of DMAT as β-cells were readily responsive (Fig. 4B) to the direct-acting $K_{ATP}$ activator, diazoxide (200 μM), which caused hyperpolarization to $-75.2 \pm 1.7$ mV ($p < 0.05$) and increased slope conductance from $0.82 \pm 0.07$ to $3.1 \pm 0.8$ nanosiemens ($n = 5$; $p < 0.05$). CT99021 (2 μM), in the electrode solution, resulted in a mean β-cell membrane potential of $-41.5 \pm 3.3$ mV. Application of leptin (Fig. 4C) caused hyperpolarization to $-51.5 \pm 4.1$ mV, which although less than the control leptin response, was significantly different ($n = 5$, $p < 0.05$) from the mean resting potential before leptin challenge. However, voltage clamp analysis showed that in the presence of CT99021 leptin did not significantly increase the β-cell slope conductance (Fig. 4C). As a small, but significant hyperpolarization of β-cells was elicited by leptin in the presence of CT99021, we decided to confirm that GSK3 inhibition does occlude leptin-mediated β-cell hyperpolarization and increase in $K_{ATP}$ current. Consequently, we examined the effects of the GSK3 inhibitors, AR-A0144-18 (28, 31) and kenpaullone (29) on leptin-induced opening of $K_{ATP}$. The presence of AR-A0144-18 (2 μM) or kenpaullone (10 μM) in the electrode solution did not alter ($p < 0.05$) β-cell membrane potential ($-40.4 \pm 1.6$ mV and $-35.2 \pm 2.1$ mV, respectively) compared with control recordings. Leptin (10 nM) caused a small hyperpolarization of the β-cells for both AR-A014418 (to $-51.8 \pm 6.4$; $n = 5$) and kenpaullone (to $-45.5 \pm 4.6$ mV; $n = 6$), which was not significant ($p > 0.05$) for either inhibitor (Fig. 4, D and E). Voltage clamp analysis confirmed that the presence of either GSK3 inhibitor prevented an increase in slope conductance by leptin (Fig. 4, D and E). Diazoxide (200 μM), in contrast, reversibly hyperpolarized β-cells in the presence of each GSK3 inhibitor (Fig. 4, C–E), CT99021, to $-73.0 \pm 2.2$ mV ($n = 5$; $p < 0.05$), AR-A014418 to $-72.4 \pm 4.1$ mV ($n = 5$, $p < 0.05$), and kenpaullone, to $-72.3 \pm 2.4$ mV ($n = 6$, $p < 0.05$) and significantly increased the mean slope conductance to values comparable for control β-cells ($p < 0.05$).

Phosphorylation and Kinase Activity of GSK3—These results taken together with those of Ning et al. (12) indicate that leptin inhibits PTEN phosphatase activity via phosphorylation at multiple sites on the C terminus of PTEN, which results in increased PtdIns(3,4,5)P$_3$ levels, actin restructuring, and $K_{ATP}$ activation. These events appear to be mediated through CK2- and GSK3-dependent mechanisms. However, the dependence on PTEN Thr-366 phosphorylation on GSK3 activity and consequent influence on downstream signaling events such as $K_{ATP}$ channel regulation gives rise to a quandary. Leptin stimulation of rat arcuate increases PKB phosphorylation with subsequent GSK3 phosphorylation at Ser-9/Ser-21 (13), actions also reported for leptin on mouse skeletal muscle cells (32, 33), mouse cortical neurons (34), and MCF-7 breast cancer cells (35). These latter actions of leptin replicate those of insulin, which increases PI3K activity and raises PtdIns(3,4,5)P$_3$ levels with subsequent increased PKB and GSK3 phosphorylation (5). Importantly, the phosphorylation of GSK3 at these serine residues by PKB is associated with reduced kinase activity (36, 37).

This outcome is inconsistent with the requirement for active GSK3 causing phosphorylation of PTEN Thr-366 allowing subsequent downstream signaling. Indeed, leptin (10 nM) increases the phosphorylation of PKB (Ser-473) and GSK3 (Ser-9/Ser-21) in N29/4 hypothalamic cells (Fig. 5A), although this is not well sustained in the hypothalamic cells, as previously reported for rat arcuate (13) and mouse muscle (32, 33). Insulin (10 nM) induced a sustained rise in PKB (Ser-473) and GSK3 (Ser-9/Ser-21) phosphorylation in N29/4 cells (Fig. 5B). Similar results were obtained for MIN6 β-cells, although in this case both leptin (10 nM) and insulin (10 nM) produced sustained increases (Fig. 5, C and D) in the levels of phosphorylated PKB (Ser-473) and GSK3 (Ser-9/Ser-21). These results are ostensibly inconsistent with the view that leptin signaling to actin and $K_{ATP}$ channels requires GSK3-dependent phosphorylation and inhibition of PTEN activity. However, GSK3 also has a tyrosine residue in the activation loop of the enzyme (Tyr-216 in GSK3β and Tyr-279 in GSK3α), the phosphorylation of which is important for GSK3 activity (38, 39). Most studies conclude that this site is not physiologically regulated by growth factors or cytokines (40). Insulin (10 nM) did not affect GSK3 Tyr-216/Tyr-279 phosphorylation levels in either N29/4 cells (Fig. 6A) or MIN6 β-cells (Fig. 6C). In contrast, leptin (10 nM) rapidly increased GSK3 phosphorylation at Tyr-216/Tyr-279 in N29/4 (Fig. 6B), and MIN6 (Fig. 6D) cells, which was sustained for at least 1 h. Thus, in contrast to insulin, which increases phosphorylation of GSK3 serine sites only (consistent with kinase inhibition), leptin increases the phosphorylation level of both serine and tyrosine sites on GSK3. To define the outcome of this dual phosphorylation of GSK3, we examined the effects of leptin (10 nM) and insulin (50 nM) on GSK3 activity in N29/4 cells. Stimulation of N29/4 cells by insulin for 30 min results in a substantial inhibition of GSK3 activity, as expected from increased PKB activity causing GSK3 Ser-9/Ser-21 phosphorylation. In contrast, stimulation of N29/4 cells with leptin (10 nM) for 30 min, a time when PTEN phosphorylation is increased, PtdIns(3,4,5)P$_3$ levels are high, F-actin is depolymerized, and β-cells are hyperpolarized, results in maintained GSK3 activity (Fig. 6E). This outcome is consistent with the finding that, although both hormones increase GSK3 Ser-9/Ser-21 phosphorylation levels, leptin, but not insulin, also increases GSK3 Tyr-216/Tyr-279 phosphorylation.

**DISCUSSION**

Leptin phosphorylation of PTEN at the cluster site is CK2-dependent and leads to inhibition of PTEN phosphatase activity with a consequent increase in PtdIns(3,4,5)P$_3$ levels in N29/4 cells (12). Here, we show that leptin increases PTEN phosphorylation at three further sites, Thr-366, Ser-370, and Ser-385 in N29/4 cells. We also show that leptin increases p-PTEN at the
cluster site, Thr-366 and Ser-370, in MIN6 pancreatic β-cells. As demonstrated for the cluster site (12), phosphorylation of PTEN at Thr-366 and Ser-370 is important for transfer of the leptin signal downstream to cause F-actin depolymerization. Leptin-mediated reduction in F-actin levels was previously shown to be dependent on basal PI3K activity and inhibition of both lipid and protein phosphatase activity of PTEN (12). Correspondingly, overexpression of the PTEN phosphorylation site mutants, S370A PTEN and T366A PTEN, in N29/4 cells occluded leptin-driven reduction in F-actin. The Thr-366 and Ser-370 alanine and aspartic acid PTEN mutants, when overexpressed in N29/4 cells, alter PtdIns(3,4,5)P3 levels exactly as reported for the cluster site mutants. This outcome does not agree with in vitro assays, which show no alteration in enzyme activity associated with these mutants (41), unlike the corresponding mutations of the cluster site residues (12). Thus, S370D and T366D both raised PtdIns(3,4,5)P3 levels,
Leptin and PTEN Phosphorylation

indicative of reduced lipid phosphatase activity per se and dominant negative behavior with respect to native PTEN activity. Interestingly, these mutants produced different outcomes on F-actin levels and leptin signal transduction. Overexpression of T366D PTEN per se mimicked the effects of leptin by increasing PtdIns(3,4,5)P$_3$ and inducing F-actin depolymerization in a PI3K-dependent manner. This replicates the effect of overexpression of C124S PTEN and D3PTEN (cluster site mutant) in these cells (12) and is consistent with phosphorylation of the Thr-366 site reducing PTEN lipid and protein phosphatase activity. In contrast, overexpression of S370D PTEN was unable to elicit F-actin depolymerization per se even in the face of increased cellular PtdIns(3,4,5)P$_3$ levels and did not occlude leptin-mediated F-actin depolymerization. Consequently, mimicking phosphorylation by aspartate substitution at Ser-370 does not exactly replicate the effect of aspartate substitution at Thr-366 or the cluster phosphorylation site (12), with all three mutant forms of PTEN increasing PtdIns(3,4,5)P$_3$ levels but only the latter two reducing F-actin levels. The S370D PTEN mutant also does not duplicate the action of the lipid phosphatase dead, protein phosphatase active, mutant G129E PTEN. Overexpression of G129E PTEN in N29/4 cells increases PtdIns(3,4,5)P$_3$ levels with no reduction in F-actin and prevents leptin-mediated F-actin depolymerization (12), whereas the presence of S370D PTEN allows leptin depolymerization of actin. Our conclusion from these data is that S370D is lipid phosphatase dead but protein phosphatase active and that leptin inhibits the protein phosphatase activity by phosphorylation at the Thr-366 and cluster sites, whereas the protein phosphatase activity of G129E PTEN cannot be overcome by phosphorylation at these sites. Thus, phosphorylation of PTEN at Ser-370 is adequate to inhibit PTEN lipid phosphatase activity and increase PtdIns(3,4,5)P$_3$ in N29/4 cells but is not sufficient per se for efficacious transmission of the leptin signal to F-actin.

A number of phosphorylation sites have been identified within the PTEN C-terminal tail, with the protein kinase, CK2, purported to phosphorylate multiple residues, including the cluster site, Ser-385 and Ser-370, but not Thr-366 (17–19, 23, 41). PTEN protein stability and activity are considered to be dependent on phosphorylation status of the C-terminal tail (17, 18), with phosphorylation argued to reduce association of PTEN with the plasma membrane (20, 42, 43). It is presently unclear what effect leptin-induced phosphorylation will have on PTEN protein stability, as it has been reported that T366A and S370A are more stable than wild-type PTEN and GSK3 inhibition increases the stability of wild-type PTEN (41), whereas alanine mutation of the cluster site results in reduced protein stability (17, 19). The inhibition of leptin-induced phosphorylation of the cluster site (12), Ser-370 and Ser-385, in N29/4 cells and of the cluster site and Ser-370 in MIN6 cells by DMAT supports the view that CK2 is the kinase responsible for phosphorylation at these sites by this hormone. Inhibition of PI3K had no effect on leptin-mediated phosphorylation at Ser-370, Ser-385, or Thr-366 in N29/4 cells, indicating no evidence for PI3K-dependent feedback phosphorylation (44) consistent with leptin-induced phosphorylation of the cluster site. For GSK3 to phosphorylate its substrate, the substrate has to be primed by phosphorylation of a site four or five residues C-terminal of the target Ser/Thr of GSK3 (45, 46). Thus, phosphorylation of Ser-370 by CK2 is considered a priming event for subsequent GSK3 phosphorylation of Thr-366 (23), and inhibition of GSK3 blocks phosphorylation of PTEN on Thr-366 (41). Occulsion of leptin-driven increased p-PTEN on Thr-366 by the GSK3 inhibitor CT99021 in N29/4 and MIN6 cells agrees with these findings. Consequently, we expected DMAT to reduce or inhibit leptin-mediated phosphorylation of Thr-366 in these cells. This was observed for MIN6 pancreatic β-cells but not for the hypothalamic cells, where DMAT prevented leptin-induced phosphorylation of Ser-370 but not Thr-366. Thus, either an additional unidentified priming kinase is responsible or there is sufficient phosphorylation of Ser-370 remaining in DMAT-treated N29/4 cells to allow GSK3 priming. Interestingly, an in vitro study of PTEN phosphorylation has shown that phosphorylation of Ser-370 by CK2 is less efficient than that of the cluster site and that GSK3 is capable of phosphorylating PTEN at Thr-366 without priming, although at low efficiency (41). Thus, we predicted that inhibition of CK2 and GSK3 would prevent or reduce transmission of the leptin signal via PTEN to actin filament restructuring and $K_{ATP}$ channel opening. Our results show that the GSK3 inhibitor CT99021 per se or in combination with DMAT significantly reduces leptin-mediated F-actin depolymerization. Furthermore, leptin-driven hyperpolarization of pancreatic β-cells by $K_{ATP}$ activation was prevented by the presence of DMAT or any one of three structurally dissimilar GSK3 inhibitors. This is consistent with the view that leptin-mediated phosphorylation of PTEN and subsequent inhibition of its phosphatase activity is a necessary step for signal transmission to $K_{ATP}$ channels.

In contrast, insulin does not cause F-actin depolymerization in N29/4 cells nor does it hyperpolarize pancreatic β-cells by opening $K_{ATP}$ channels, and this differential output may be mediated via PTEN (12). The results presented here indicate a key role for PTEN Thr-366 phosphorylation via GSK3 activity for successful transmission of the leptin signal. For this to occur, GSK3 activity has to be maintained or increased by leptin. However, leptin, like insulin, increases PKB phosphorylation with the resultant phosphorylation of GSK3 at Ser-9/Ser-21 consistent with inhibition of GSK3 activity. Indeed, both insulin and leptin increased the phosphorylation of GSK3 at Ser-9/Ser-21 in N29/4 and MIN6 cells. In contrast, leptin, but not insulin, caused a rapid and sustained increase in GSK3 phosphorylation at Tyr-216/Tyr-279 in both cell types. Phosphorylation of GSK3 at this site is considered to be activating and necessary for functional activity (38). Indeed, direct GSK3 kinase assay showed that insulin stimulation of N29/4 cells substantially reduced GSK3 activity, whereas leptin had no effect. Taken together, the semiquantitative Western blot data show that insulin significantly alters N-terminal serine phosphorylation of GSK3, which would reduce its activity toward primed substrates. However, phosphorylation of PTEN at Thr-366 in response to leptin does not appear to require priming (phosphorylation of this site is unaffected by DMAT), and this may explain why insulin does not reduce Thr-366 phosphorylation. In contrast, GSK3 in leptin-treated cells increased phosphate in Tyr-216/Tyr-279 compared with control or insulin-treated cells. This is a possible explanation why leptin and not insulin...
induces Thr-366 phosphorylation. However, there are other ways to regulate GSK3, including protein-protein interaction (e.g. Wnt signaling) and Thr-380 phosphorylation (e.g. cytokine signaling) that also require further investigation. Identification of the kinase (or phosphatase) responsible for the increase in tyrosine phosphorylation of GSK3 and development of a quantitative multiple reaction monitoring liquid chromatography-mass spectrometric approach for each of the phosphorylation sites on GSK3 would be required to elucidate the exact mechanism of control by leptin. These subtle signaling differences downstream of insulin and leptin receptors are likely to contribute to the ability of each to perform their distinct physiological actions through modulation of the same signaling pathways/molecules.

In conclusion, this study has demonstrated that leptin increases the phosphorylation of PTEN at multiple residues in a CK2- and GSK3-dependent manner. This action of leptin is required for successful transmission of the leptin signal to cause cytoskeletal remodeling, the opening of $K_{\text{ATP}}$ channels, and subsequent hyperpolarization of membrane potential. Interestingly, complete loss of PTEN activity in mouse diets (48). Dysregulation of PTEN (lipid and protein phosphatase) activity has also been implicated in certain cancers (49) and neurodegenerative disease (e.g. Parkinson disease (47)). Thus, modulation of PTEN function plays multiple and important roles in numerous physiological and pathological phenomena.

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Leptin and PTEN Phosphorylation

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