Central insulin signaling is critical for the prevention of insulin resistance. Hyperinsulinemia contributes to insulin resistance, but it is not yet clear whether neurons are subject to cellular insulin resistance. We used an immortalized, hypothalamic, clonal cell line, mHypoE-46, which exemplifies neuronal function and expresses the components of the insulin signaling pathway, to determine how hyperinsulinemia modifies neuronal function. Western blot analysis indicated that prolonged insulin treatment of mHypoE-46 cells attenuated insulin signaling through phospho-Akt. To understand the mechanisms involved, time-course analysis was performed. Insulin exposure for 4 and 8 h phosphorylated Akt and p70-S6 kinase (S6K1), whereas 8 and 24 h treatment decreased insulin receptor (IR) and IR substrate 1 (IRS-1) protein levels. Insulin phosphorylation of S6K1 correlated with IRS-1 serine phosphorylation and the mTOR-S6K1 pathway inhibitor rapamycin prevented IRS-1 serine phosphorylation. The proteasomal inhibitor epoxomicin and the lysosomal pathway inhibitor 3-methyladenine prevented the degradation of IRS-1 and IR by insulin, respectively, and pretreatment with rapamycin, epoxomicin, or 3-methyladenine prevented attenuation of insulin signaling by long-term insulin exposure. Thus, a sustained elevation of insulin levels diminishes neuronal insulin signaling through mTOR-S6K1-mediated IRS-1 serine phosphorylation, proteasomal degradation of IRS-1 and lysosomal degradation of the IR.

(Central insulin actions are critical for the maintenance of glucose and energy homeostasis. Nutrient homeostasis is regulated by insulin actions on hypothalamic neurons (1), where insulin receptors (IR) are highly expressed (2). Mice with a neuron-specific IR knockout (3) or IR substrate (IRS)-2 knockout (4) exhibit an obese, hyperinsulinemic, insulin-resistant phenotype. Intracerebroventricular (icv) injection of insulin causes an anorectic response in mammals (5–7) and decreases hepatic glucose output (8). Furthermore, hypothalamic overexpression of the critical insulin signaling proteins, IRS or Akt, improves insulin sensitivity and enhances the glycemic response (9).

At the cellular level, insulin signal transduction can be attenuated through multiple mechanisms, including serine phosphorylation of IRS-1, IRS-1 degradation, and IR degradation. IRS-1 has numerous phosphorylation sites, consisting of both tyrosine and serine residues (10). Tyrosine residues are required for the activation of IRS proteins, whereas serine residues may be involved in a negative feedback loop, functioning to deactivate IRS proteins. IRS-1 can be serine phosphorylated through numerous pathways, including the c-Jun N-terminal kinase (11–13), IκB kinase (IKK) (10), protein kinase C (PKC) (10, 14) and p70-S6 kinase (S6K1) (15) pathways. In separate studies, prolonged insulin stimulation results in the internalization and degradation of the IR (16–19) and the degradation of IRS-1 (20, 21). Importantly, none of these studies have been performed in the brain or in neurons, and it is un-
known whether neurons are subjected to cellular insulin resistance after prolonged exposure to insulin.

Insulin resistance is a hallmark feature of type 2 diabetes mellitus (T2DM) and obesity. There are numerous etiologies for insulin resistance, including lipotoxicity, inflammation, endoplasmic reticulum (ER) stress, oxidative stress, and hyperinsulinemia (22). Hyperinsulinemia coexists with insulin resistance in obese and metabolic syndrome phenotypes. High fasting plasma insulin predicts T2DM independent of insulin resistance (23). The data are presented as the mean ± SEM and analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Statistical significance was determined using ANOVA with Bonferroni’s multiple-comparison test.

Results

Characterization and functional analysis of mHypoE-46 neurons

The mHypoE-46 neuronal cells were immortalized and characterized as previously reported (32). The cells ex-
press key genes important for our study, such as the IR, IRS-1 and -2, NPY, and AgRP (33). mHypoE-46 neurons were treated with 10 nM insulin or vehicle over 60 min, and the relative levels of pAkt were analyzed using Western blot analysis. Insulin significantly induced phosphorylation of Akt from 5–60 min (Fig. 1A), indicating that our cells are responsive to insulin and that the classical phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway is functional in our cell model (Fig. 1B).

### Insulin pretreatment attenuates insulin-mediated phosphorylation of Akt

Insulin can induce insulin resistance both in vivo and at the cellular level, leading to decreased IR signaling and reduced PI3K activity (17, 24, 30, 34). To determine the effects of hyperinsulinemia on neuronal insulin signaling, we pretreated mHypoE-46 neurons with a supraphysiological concentration of insulin (1000 nM) for 48 h. After pretreatment, the cells were washed twice, placed in fresh medium for 1 h, and then rechallenged with 10 nM insulin to determine IR responsiveness. Using Western blot analysis, we determined that insulin pretreatment attenuated insulin-mediated Akt phosphorylation at 5 min by 100 ± 5% (Fig. 1C). The same effect was seen at 15 min (data not shown).

After attaining a state of cellular insulin resistance, we determined the minimal concentration and time of insulin pretreatment required to significantly attenuate insulin signaling. The mHypoE-46 neurons were pretreated with 10–1000 nM insulin for 4–24 h before rechallenge with insulin (10 nM). A minimum of 100 nM insulin for 8 h was required to significantly block insulin signaling, as indicated (Fig. 1D), and 10–1000 nM insulin pretreatment for 8 and 24 h attenuated insulin activation of phospho (p)-Akt (Fig. 1D; 100 and 1000 nM at 8 h, 70 ± 3.8% and 70 ± 9.4%; 100 and 1000 nM at 24 h, 83 ± 9.4% and 89.5 ± 10%).

To determine the time required after removal of insulin for restoration of insulin signaling, we performed a reverse time course in which the cells were pretreated with 100 nM insulin for 24 h an then washed and placed in fresh medium. From 2–12 h after pretreatment removal, the neuronal cells were rechallenged with insulin (10 nM) to determine their responsiveness, and pAkt was analyzed using Western blot analysis. Insulin signaling through Akt was significantly decreased for up to 6 h after removal of insulin pretreatment (Fig. 2A). Interestingly, when we analyzed IRβ and IRS-1 protein levels during the reverse time course, we found that both IRβ and

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**FIG. 1.** Pretreatment with insulin inhibits insulin-induced phosphorylation of Akt upon rechallenge. A, Relative pAkt was measured using Western blot analysis and normalized to total Akt as a loading control (Ctrl). Solid circles indicate insulin treatment (10 nM), and white squares indicate vehicle. B, mHypoE-46 cells were pretreated with 25 μM LY294002, 1 μM wortmannin, or DMSO vehicle for 1 h before being treated with 10 nM insulin (black bars) or vehicle (white bars). Relative pAkt levels were normalized to total Akt. C, mHypoE-46 cells were pretreated with insulin (1 μM) or water vehicle for 48 h, as indicated in the figure. The cells were washed, allowed to recover in fresh medium for 1 h, and rechallenged with 10 nM insulin (black bars) or vehicle (white bars). Relative pAkt levels were normalized to total Akt. D, mHypoE-46 cells were pretreated with water vehicle or 0.01, 0.1, or 1 μM insulin for 4 h (circle), 8 h (triangle), and 24 h (diamond), as indicated in the figure. After washing and 1 h recovery, the cells were rechallenged with 10 nM insulin for 15 min. Relative pAkt levels were normalized to G8 and are shown as fold increase over non-pretreated control (square). All data are shown with SEM (n = 3 independent experiments). ***, P < 0.001; **, P < 0.01; *, P < 0.05.
IRS-1 were significantly decreased for up to 8 h, correlating with the decreased activation of Akt (Fig. 2, B and C).

**Long-term insulin treatment pAkt, S6K1, and IRS-1 ser1101 and decreased IR and IRS-1 protein levels**

We next determined which signaling pathways are affected by sustained insulin exposure and focused on Akt and S6K1, because S6K1 is downstream of Akt and is involved in the development of cellular insulin resistance through serine phosphorylation of IRS-1 (15). mHypoE-46 neurons were treated with insulin from 4–24 h, and protein was analyzed using Western blot analysis. At 4 and 8 h after treatment with 10–1000 nM insulin, relative pAkt levels were significantly increased (Fig. 3A; 10, 100, and 1000 nM at 4 h, 2.25 ± 0.085-, 2.79 ± 0.082-, and 3.76 ± 0.1-fold; 10, 100, and 1000 nM at 8 h, 1.82 ± 0.07-, 2.55 ± 0.05-, and 2.99 ± 0.09-fold), and relative p70-S6K levels were also significantly increased (Fig. 3B; 10, 100, and 1000 nM at 8 h, 4.51 ± 0.19-fold; 10, 100, 1000 nM at 8 h, 1.62 ± 0.14-, 2.32 ± 0.12-, and 2.95 ± 0.27-fold). By 24 h, pAkt and pS6K1 levels returned to basal.

IRβ protein expression was significantly decreased at 8 and 24 h by 100 and 1000 nM insulin (Fig. 3C; 100 and 1000 nM at 8 h, 45 ± 2.4 and 63 ± 0.7%; 100 and 1000 nM at 24 h, 71 ± 3.5 and 76 ± 4%). IRS-1 protein expression was significantly decreased at 8 h by 1000 nM insulin and at 24 h by 100 and 1000 nM insulin (Fig. 3D; 1000 nM at 8 h, 41 ± 7%; 100 and 1000 nM at 24 h, 53 ± 0.2 and 63 ± 8.2%). To determine whether the decrease of IRβ and IRS-1 by insulin was occurring only at the level of the protein, quantitative real-time RT-PCR was performed to analyze the levels of IR and IRS-1 mRNA. mHypoE-46 cells were treated with 100 nM insulin over a 48-h time course. IRβ and IRS-1 mRNA expression levels were unchanged with insulin treatment from 4–48 h (Fig. 3, E and F). This indicates that prolonged insulin treatment acts to decrease IR and IRS-1 protein levels as opposed to altering levels of IR and IRS-1 mRNA expression.

As previously mentioned, S6K1 can serine phosphorylate IRS-1, leading to inhibition of IRS-1. We analyzed whether insulin treatment leads to IRS-1 serine phosphorylation through S6K1, specifically looking at IRS-1 serine 307 and 1101 because these serine residues have been shown to be involved with IRS-1 inhibition (14, 15). Serine 307 showed no phosphorylation with insulin treatment (data not shown), whereas serine 1101 was phosphorylated by 100 nM insulin from 15 min to 4 h (Fig. 4C), indicated by a higher molecular weight band on the Western blot, due to hyperphosphorylation of IRS-1; IRS-1 contains over 180 serine residues and thus, depending on the level of hyperphosphorylation, has different electrophoretic mobility (35). Importantly, the phosphorylation of Akt and S6K1 followed a similar temporal pattern to pIRS-1 ser1101 (Fig. 4, A and B), indicating a correlation between the phosphorylation of the three proteins. To determine whether the mammalian target of rapamycin (mTOR)-S6K1 pathway was involved in the phosphorylation of IRS-1 serine phosphorylation, we pretreated the cells with 10 nM rapamycin (an mTOR-specific inhibitor) for 1 h before treatment with insulin (100 nM). Rapamycin did not affect pAkt (Fig. 5A) but did inhibit the phosphorylation of S6K1 by insulin by 100 ± 0.1% and decreased basal pS6K1 significantly below vehicle control levels (Fig. 5B). Rapamycin also inhibited the serine phosphorylation
The inhibitors epoxomicin, rapamycin, and 3-MA restored insulin signaling

To determine the mechanisms used by insulin to attenuate insulin signal transduction, three specific inhibitors were used: epoxomicin, rapamycin, and 3-MA. Epoxomicin inhibits the catalytic subunit of the 20S proteasome, preventing proteasomal protein degradation; rapamycin inhibits mTOR, preventing activation of p70-S6K; and 3-MA inhibits autophagy and lysosomal sequestration, the initial steps required for lysosomal protein degradation. mHypoE-46 neurons were pretreated with the inhibitors 50 μM epoxomicin, 10 nM rapamycin, or 10 mM 3-MA or DMSO vehicle for 1 h, followed by insulin (100 nM) or water vehicle treatment for 8 h, after which the phosphorylation of Akt by insulin was analyzed. Consistent with previous results, 100 nM insulin pretreatment in DMSO vehicle control attenuated insulin-mediated Akt phosphorylation. Interestingly, pretreatment with epoxomicin, rapamycin, or 3-MA significantly increased the induction of pAkt by insulin rechallenge after prolonged insulin exposure by 1.98 ± 0.36- and 2.27 ± 0.52-fold, respectively, partially restoring insulin signaling (Fig. 6A). This indicates that the three pathways, proteasomal protein degradation, mTOR-S6K1, and lysosomal protein degradation, may be involved with the development of cellular insulin resistance and that prevention of only one of these mechanisms is not sufficient to fully restore insulin signaling. When p70-S6K was analyzed with inhibitor treatments, rapamycin blocked phosphorylation of p70-S6K in control and insulin-pretreated samples, whereas 3-MA restored phosphorylation of p70-S6K in insulin-pretreated samples (Fig. 6B). Epoxomicin restored IRS-1 levels in the presence of 100 nM insulin to 84 ± 8.6% of control (Fig. 6C), whereas 3-MA restored IRS-1 levels to 87 ± 9.3% of control (Fig. 6D). Therefore, our data indicate that in the mHypoE-46, hypothalamic cell model, insulin increases the degradation of IRβ through the lysosomal pathway and degrades IRS-1 through the proteasomal pathway.
Discussion

Until recently, the brain was not considered a major contributor to overall glucose homeostasis. Through elegant transgenic experiments, which produced neuron-specific knockouts or overexpression of insulin signaling components, the hypothalamus is now recognized as a critical regulator of insulin action (9). However, exactly how hypothalamic neurons sense insulin and the mechanisms through which neurons develop insulin resistance are not fully understood. We have been able to approach this issue through which neurons develop insulin resistance are not.

mTOR-S6K1 pathway (15). We found that sustained insulin treatment induced Akt, S6K1, and IRS-1 ser1101 phosphorylation, whereas rapamycin inhibited phosphorylation of S6K1 and, subsequently, IRS-1 serine phosphorylation. Furthermore, the mTOR-S6K1 pathway attenuates insulin signal transduction in the mHypoE-46 neuronal cell model. These results agree with the study by Tremblay et al. (15) performed in myocytes, indicating that in neurons, insulin may also act through the mTOR-S6K1 pathway to inhibit insulin signaling and that this likely involves IRS-1 ser1101 phosphorylation. Of note, IRS-1 ser307 did not appear to be phosphorylated by insulin in our cells (data not shown).

FIG. 4. Insulin induces phosphorylation of IRS-1 ser1101 in a temporally similar pattern to Akt and S6K1 phosphorylation. mHypoE-46 cells were treated with 100 nM insulin (solid circles) or vehicle (white squares) and protein was isolated from 5–240 min. Protein was analyzed using Western blot analysis and p-specific antibodies against Akt (A), S6K1 (B), and IRS-1 ser1101 (C), and all blots were probed with Gb as a loading control (Ctrl). Data are shown with SEM (n = 4 independent experiments). ***, P < 0.001; **, P < 0.01.

In vivo and in vitro (17, 24, 30, 34), and it is thus suggested that hyperinsulinemia may cause or exacerbate insulin resistance (22, 23). We found that hyperinsulinemia attenuates the phosphorylation of Akt, in agreement with previous studies performed in myocytes and adipocytes, which indicate that treatment with 5–100 nM insulin decreases cellular insulin actions (26, 27, 34). Interestingly, the mHypoE-46 neuronal cells required a minimum of 100 nM insulin to significantly affect insulin signaling, whereas studies in peripheral cells indicate that as low as 5 nM insulin can diminish insulin signaling (27). This may be indicative of neuronal cells having a greater tolerance to the effects of hyperinsulinemia than peripheral cells.

Insulin resistance develops from the inactivation of signaling molecules downstream of the IR. One of the key steps in this process is the serine phosphorylation of IRS-1. Of the numerous serine residues on IRS-1, a few have recently come to light as being important for IRS-1 inhibition. IRS-1 ser307 phosphorylation is induced by many factors, including insulin, and contributes to insulin resistance (14). IRS-1 ser1101 is phosphorylated by insulin, and this can occur through the activation of the mTOR-S6K1 pathway (15). We found that sustained insulin treatment induced Akt, S6K1, and IRS-1 ser1101 phosphorylation, whereas rapamycin inhibited phosphorylation of S6K1 and, subsequently, IRS-1 serine phosphorylation. Furthermore, the mTOR-S6K1 pathway attenuates insulin signal transduction in the mHypoE-46 neuronal cell model. These results agree with the study by Tremblay et al. (15) performed in myocytes, indicating that in neurons, insulin may also act through the mTOR-S6K1 pathway to inhibit insulin signaling and that this likely involves IRS-1 ser1101 phosphorylation. Of note, IRS-1 ser307 did not appear to be phosphorylated by insulin in our cells (data not shown).

Normally, IRS-1 serine phosphorylation may be part of a negative feedback pathway involved in maintaining timely insulin signaling, but in disease states, when insulin levels are continuously elevated, IRS-1 serine phosphorylation may act to provoke cellular insulin resistance.

In addition to the attenuation of insulin signaling, IRS-1 serine phosphorylation has been shown to target the protein for proteasomal degradation. It was first observed by Haruta et al. (20) that insulin causes phosphorylation of IRS-1 through the mTOR-S6K1 pathway and targets it for degradation. Recently, Xu et al. (36) found that this occurs via phosphorylation of multiple serine residues through truncated IRS-1 constructs that the key serines reside between 1 and 574 amino acids, with a key residue being serine 527. We have found that the mTOR-S6K1 pathway is involved with attenuated insulin signaling, al-
though not due to degraded IRS-1. This does not rule out the involvement of IRS-1 serine phosphorylation targeting for proteolysis in our model, because there are other pathways through which insulin can cause IRS-1 serine phosphorylation, such as PKC. IRS-1 was degraded through the ubiquitin-proteasomal pathway, because treatment with the 26S proteasome-specific inhibitor epoxomicin prevented degradation, in agreement with previous studies. Sun et al. (21) first showed that chronic insulin induces IRS-1 degradation through the proteasomal pathway, because the 26S proteasome inhibitor lactacystin prevented insulin-induced degradation. The mechanism through which IRS-1 degradation occurs was later analyzed by Xu et al. (36) who found that a specific E3 ligase complex is involved with IRS-1 degradation. The CUL7 E3 ligase complex specifically bound serine-phosphorylated IRS-1, and the overexpression of CUL7 and another member of the complex, Fbw8, increased the decay of IRS-1. Importantly, they found that the CUL7 knockout mouse has 5-fold increased IRS-1 levels and increased activation of the Akt and MAPK pathways by insulin. By preventing the degradation of IRS-1 by prolonged insulin exposure, using epoxomicin, we restored insulin signaling through the Akt pathway. Additionally, we found that decreased IRS-1 protein levels correlated with decreased insulin signal transduction. Taken together with the previous studies, this suggests that IRS-1 degradation plays a crucial role in the development of cellular insulin resistance.

As early as 1974, it was shown that prolonged insulin exposure decreases the concentration of the IR, both in vivo and in vitro (16). Numerous studies then demonstrated that hyperinsulinemia down-regulates the IR, lead-
The inhibitors epoxomicin, rapamycin, and 3-MA partially restore insulin (Ins) activation of the Akt pathway. mHypoE-46 cells were pretreated with vehicle or 100 nM insulin and DMSO vehicle, 50 μM epoxomicin (Epox), 10 μM rapamycin (Rap), or 10 μM 3-MA for 8 h. The cells were washed and allowed to recover for 1 h before rechallenge with 10 nM insulin (black bars) or vehicle (white bars). Western blot analysis was used to measure pAkt (A), pS6K1 (B), IR (C), or IRS-1 protein (D); all blots were normalized to Gα as a loading control (Ctrl). Data are shown with s/n (n = 4–7 independent experiments). ***, P < 0.001; **, P < 0.01; *, P < 0.05.

**FIG. 6.** The inhibitors epoxomicin, rapamycin, and 3-MA partially restore insulin (Ins) activation of the Akt pathway. mHypoE-46 cells were pretreated with vehicle or 100 nM insulin and DMSO vehicle, 50 μM epoxomicin (Epox), 10 μM rapamycin (Rap), or 10 μM 3-MA for 8 h. The cells were washed and allowed to recover for 1 h before rechallenge with 10 nM insulin (black bars) or vehicle (white bars). Western blot analysis was used to measure pAkt (A), pS6K1 (B), IR (C), or IRS-1 protein (D); all blots were normalized to Gα as a loading control (Ctrl). Data are shown with s/n (n = 4–7 independent experiments). ***, P < 0.001; **, P < 0.01; *, P < 0.05.

Insulin. This indicates that IR degradation also plays a key role in the development of cellular insulin resistance.

Although there are no current studies that have studied the effects of sustained insulin exposure upon neurons, there have been some recent studies that have demonstrated the induction of hypothalamic insulin resistance by nutrients. Ono et al. (40) showed that mice fed a HFD for 1 d had decreased tyrosine-phosphorylated IRS-1 and attenuated suppression of hepatic glucose production (HGP) by insulin; interestingly, IRS-2 was not affected by a HFD. They went on to show that overexpression of p70-S6K induced IRS-1 serine 1101 phosphorylation, which corresponds well with the data presented in our study; attenuated the induction of pAkt by insulin; and blunted the suppression of hepatic glucose production (HGP) by insulin. p70-S6K overexpression also led to insulin resistance and increased IRS-1 serine phosphorylation under hyperinsulinemic-euglycemic clamp conditions. Additionally, expression of dominant-negative S6K1 restored the ability of insulin to suppress HGP on a HFD and restored induction of pAkt and IRS-1 tyrosine phosphorylation by insulin. Zhang et al. (41) found that HFD induces neuronal insulin resistance and that this was mediated by the IKKβ-nuclear factor-κ-light-chain-enhancer of B cells (NFκB) pathway. Interestingly, they found that AgRP neurons-specific IKKβ knockout in mice prevented the negative effects of a HFD. Posey et al. (42) demonstrated that icv infusion of palmitic acid, a prevalent free fatty acid, decreased hypothalamic insulin signaling, which was mediated through the IKKβ-NFκB pathway. Ozcan et al. (43, 44) have found that the obese models, either diet-induced or genetic (ob/ob and db/db), have increased levels of ER stress markers and that icv administration of chemical chaperones that can prevent ER stress, 4-phenylbutyric acid or tauroursodeoxycholic acid (TUDCA), normalize the obese, diabetic phenotype in mice as well as normalizing insulin sensitivity. These studies further indicate that central insulin resistance plays an important role in the development of obesity and T2DM, again stating the need for further investigation of the mechanisms involved in the pathogenesis of neuronal insulin resistance.
tates in attenuated insulin signaling in neuronal cells. This occurs through three specific mechanisms: the inactivation of IRS-1 by serine phosphorylation, the proteasomal degradation of IRS-1, and the lysosomal degradation of the IR. The inhibition of any one of these mechanisms is sufficient to improve insulin signaling in neuronal cells. Thus, hyperinsulinemia strongly affects neuronal insulin signaling and, because central insulin actions are critical to the regulation of energy and glucose homeostasis, the central effects of insulin resistance requires more attention. The mechanisms illustrated in this study have been similarly demonstrated in studies using peripheral cells; hence, we have determined that there are possible parallel effects of insulin upon neuronal and peripheral cells. The proposition that there are similar mechanisms involved in the development of peripheral and neuronal insulin resistance is important when considering treatments for the pathophysiological states involving insulin resistance, such as obesity and T2DM; treatments that target peripheral insulin resistance may also improve central insulin resistance.

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Address all correspondence and requests for reprints to: Denise D. Belsham, Ph.D., Department of Physiology, University of Toronto, Medical Sciences Building 3247A, 1 Kings College Circle, Toronto, Ontario, Canada M5S 1A8. E-mail: d.belsham@utoronto.ca.

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