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Leptin regulates tau phosphorylation and amyloid through AMPK in neuronal cells

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ABSTRACT

Leptin, which serves as a lipid-modulating hormone to control metabolic energy availability, is decreased in Alzheimer's disease (AD) patients, and serum levels are inversely correlated to severity of dementia. We have previously described the effects of leptin in reducing amyloid β protein both *in vitro* and *in vivo*, and tau phosphorylation *in vitro*. Herein, we systematically investigated the signaling pathways activated by leptin, leading to these molecular endpoints, to better understand its mechanism of action. Inhibition of amyloid β production and tau phosphorylation in leptin-treated human and/or rat neuronal cultures were both dependent on activation of AMP-activated protein kinase (AMPK). Direct stimulation of AMPK with the cell-permeable activator, 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAR), replicated leptin's effects and conversely, Compound C, an inhibitor of AMPK, blocked leptin's action. The data implicate that AMPK is a key regulator of both AD-related pathways.

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It is widely accepted that amyloid β (Aβ) is associated with neurodegeneration in AD [1,2]. Soluble Aβ oligomers, prior to plaque buildup, exert toxic effects leading to synaptic loss, neuronal death, and dementia [3]. However, for sporadic AD, representing the overwhelming majority of AD cases, there is an astonishing absence of concrete indication of a particular cause that triggers the Aβ cascade.

On the other hand, neurofibrillary tangles (NFT), which result from hyperphosphorylation of the microtubule-interacting protein tau, and tau oligomers, termed paired helical filaments (PHF), have also been shown to be associated with microtubule destabilization and neurodegeneration [4].

Compared to Aβ plaques, the distribution of NFT within the brain correlates better with neurodegeneration and cognitive decline [5]. However, tangles are seen in other tauopathies including supranuclear palsy and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), which do not present with deposits of Aβ [6,7]. These findings suggest that tangle formation may be triggered by a number of neurodegenerative insults brought on by earlier events in these CNS disorders; yet again, only speculations exist as to what those may be.

A significant correlation between mid-life obesity and dementia later in life has been reported [8,9]. However, weight loss and a decline of leptin levels are common in AD [10]. Leptin is a peptide hormone synthesized by adipocytes. Within the central nervous system, leptin crosses the blood brain barrier to bind specific receptors in the hypothalamus to mediate food intake, body weight and energy expenditure [11]. However, accumulating evidence suggests that leptin receptors, expressed in abundance in the hippocampus [12], may be mediating other functions as well. The diverse pathways of leptin action suggest that leptin may have diverse signaling pathways.

We have found that leptin can reduce Aβ levels both *in vitro* and *in vivo* [13]. Additionally, we have shown that leptin can reduce tau phosphorylation in neuronal cultures [14]. Herein, we investigated the signaling pathways activated by leptin to mediate these effects.

Materials and methods

Reagents and antibodies. Minimum essential medium (MEM) was purchased from ATCC (Manassas, VA). Trypsin-EDTA and penicillin solution were purchased from MP Biomedicals (Solon, Ohio). Fetal bovine serum (FBS), all-trans retinoic acid (RA) and human recombinant leptin were purchased from Sigma–Aldrich (St. Louis, MO). 5-Aminoimidazole-4-carboxyamide ribonucleoside (AICAR) was purchased from Cell Signaling Technology (Danvers, MA). Compound C, 2-Hydroxy-4-(((4-methylphenyl)sulfonyloxy)acetyl)amino)-benzoic acid (S3I-201), 5-(2,2-Difluoro-benzo[1,3]dioxol-5-ylmethylene)-thiazolidine-2,4-dione (522DB13D), 1L6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-*sn*-glycerocarbonate (1L6HCI), 2-(4-Chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one (24C44F5P) and H-Trp-Glu-OH (G3335) were purchased from EMD Chemicals (Gibbstown, NJ). The final Concentrations and incubation

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times for all inhibitors or activators were based on previous reports [15–20], with additional information presented as Table 1. All kinase inhibitors were used at or in excess of previously identified inhibitory concentrations; with concentration-dependent inhibition of the target kinases confirmed by immunoblot (data not shown).

Rabbit anti-Akt (pSer^{473}), -Jak2 (pTyr$^{1007/1008}$), -AMPKα (pThr^{172}), -GSK-3β (pSer^{9}), -PPARγ (81B8), p38 MAPK (pThr^{180}/Tyr^{182}) (28B10) mAb, tau (pSer^{396}) mAb and tau (tau46) mAb were purchased from Cell Signaling. PHF-tau mAb (clone AT8) was purchased from Pierce Biotechnology (Rockford, IL). PHF-1 mAb was a gift from Dr. Peter Davies, Albert Einstein College of Medicine (Bronx, NY). Rabbit anti-tau (pThr^{181}) was purchased from Sigma–Aldrich. Rabbit anti-α-tubulin mAb was purchased from Affinity BioReagents (Golden, CO).

Culture and neuronal induction of cell lines. The human neuroblastoma cell line, SH-SY5Y, was purchased from ATCC. Cell culture was performed according to manufacturer's specific guidelines. Cells were propagated in MEM containing 10% FBS. Neuronal differentiation was performed as described previously [14].

Culture of rat primary neurons. Primary rat cortical neurons were purchased from BrainBits LLC (Sprinfield, IL), and cultured as per manufacturer's instruction and as described previously [14].

Protein extraction and immunoblotting. Neuronal cells were treated with leptin (100 nM; 1600 ng/ml) for 4 h or AICAR (2 mM) or specific inhibitors for 1 h, unless otherwise specified, and then harvested by scraping. Protein extraction from cell pellets and immunoblots were peformed as described previously [14]. All primary antibodies, except tau-pSer^{396}, total tau (1:500), and PHF-tau, AT8 (1:200), and secondary antibodies were used at final dilutions of 1:1000 and 1:10,000, respectively.

$A\beta_{(1-40)}$ quantification. $A\beta_{(1-40)}$ levels in cell culture media were determined using the Human β-Amyloid 1–40 Colorimetric Immunoassay kit (Invitrogen; Carlsbad, CA), according to manufacturer's specific instructions. $A\beta_{(1-40)}$ levels were calculated from a standard curve developed with OD at 450 nm using 8 serial dilutions of known concentration.

Statistical analysis. Statistical data analyses were performed with analysis of variance and Tukey–Kramer multiple comparisons test. Densitometric analyses were performed using the UN-SCAN-IT gel 6.1 software (Silk Scientific; Orem, UT). $p < 0.05$ was considered statistically significant.

Results

Upstream signaling events mediated by leptin to regulate tau phosphorylation

Tau phosphorylation at AD-related sites in human neuroblastoma SY5Y cells has been reported to increase with RA-induced differentiation (RA-SY5Y) [21]. These changes have been attributed to an increase in the absolute levels of tau during differentiation, rather than hyperphosphorylation of the protein. Due to this increased basal expression, RA-SY5Y cells represent a convenient culture system to monitor changes in human tau phosphorylation.

We previously utilized RA-SY5Y to examine the cause-effect relationship between leptin, and tau phosphorylation [14]. We reported a reduction of tau phosphorylation by leptin treatment. This effect was also achieved by AICAR treatment, a cell-permeable activator of the downstream signaling protein AMPK and conversely antagonized by an AMPK inhibitor. Herein, we expanded these studies to other signaling molecules, known to act upstream or downstream of AMPK and explored their role in regulating tau phosphorylation following leptin treatment of neuronal cells in culture.

RA-SY5Y were treated with leptin in the presence or absence of inhibitors (Table 1) to known kinases. Phosphorylation of tau at several different epitopes served as our experimental endpoint (Fig. 1). Comparisons were made relative to cells treated with leptin alone or vehicle (non-treated). Inhibitors of AMPK, Akt (protein kinase B) or p38 MAP kinase significantly ($p < 0.05$) impeded leptin's ability to reduce tau phosphorylation (panels I–III, lanes 4, 6 and 7) but notably, the PI3K inhibitor did not (panels I- III, lane 5). Cells treated with specific inhibitors in the absence of leptin did not show a significant change in tau phosphorylation (data not shown).

We next explored whether any of the above kinases become phosphorylated following leptin treatment (Fig. 2). Typically, phosphorylation results in refolding, and this triggers a change in kinase activity. Leptin significantly increased ($p < 0.05$) the phosphorylation of Janus kinase 2 (Jak2), AMPK, p38 and Akt (Fig. 2A–E) compared to vehicle (non-treated). In parallel, treatment with AICAR, caused similar changes to the above kinases, with the exception of Jak2 (Fig. 2B) which was not affected since AMPK is downstream of Jak2.

We lastly examined whether leptin regulates glycogen synthase kinase-3β (GSK-3β), a direct substrate for Akt and known inducer of tau hyperphosphorylation [22]. Phosphorylation of GSK-3β at Ser^{9} by Akt leads to enzymatic inactivation and both leptin or AICAR treatment (Fig. 2F) significantly ($p < 0.05$) increased the phosphorylation at that site compared to vehicle (Fig. 2G).

In addition to the neuroblastoma cells we investigated the effect of leptin and AICAR on AMPK and Akt in primary cortical neurons. In agreement with the results described above, leptin increased phosphorylation of pSer^{473} Akt by 30 ± 4% ($n = 3$) and pThr^{172}AMPK by 75 ± 9% ($n = 3$). In addition, AICAR increased phosphorylation of pSer^{473} Akt by 32 ± 3% ($n = 3$) and pThr^{172}AMPK by 71 ± 6% ($n = 3$).

In summary, the data confirmed leptin's ability to reduce phosphorylation of tau and further implicate the involvement of several principal kinases, among which are AMPK, p38 MAP kinase and Akt.

Leptin regulates Aβ release via AMPK

From our previous work, it was shown that leptin can reduce Aβ levels both *in vitro* and *in vivo* [13]. Mechanistically, this is achieved by changes in the lipid composition of membrane lipid rafts, presumably due to leptin's lipolytic activity. This results in a compromised β-secretase (BACE) activity towards amyloid precursor protein (APP) cleavage, ultimately reducing cellular production of Aβ [13]. Leptin was also capable of promoting the Lipoprotein Receptor-related Protein-1 (LRP)-directed, apoE-dependent Aβ uptake, further contributing to the lowering of extracellular levels of Aβ [13].

Based on our findings, we decided to investigate how the pathways leading to Aβ production and tau phosphorylation, both of which are modulated by leptin, are interconnected. The peroxisome proliferator-activated receptor-γ (PPARγ) is a transcription factor known to regulate BACE, a key enzyme in APP processing [23]. PPARγ levels have been shown to increase *in vivo* with leptin administration [24].

Table 1
Kinase inhibitors and their inhibitory constants.

Kinase	Inhibitor designation	Inhibition constant	Concentration used	Reference
AMPK	Compound C	K_i = 109 nM	1 μM	[15]
STAT3	S3I-201	Inhibits STAT3-dependent tumor growth at 100 μM	100 μM	[16]
PI3K	522DB13D	IC_{50} = 4.5 μM	20 μM	[17]
Akt	1L6HCI	IC_{50} = 5 μM	10 μM	[18]
p38	24C44F5P	IC_{50} = 35 nM	100 nM	[19]

Kinase inhibitors were used within the present cell culture model at or in excess of previously identified inhibitory concentrations. In addition, the PPARγ antagonist, G3335 (K_D = 8 μM), was applied at 30 μM [20]. Concentration-dependent inhibition of the target kinases were confirmed by immunoblot (data not shown).

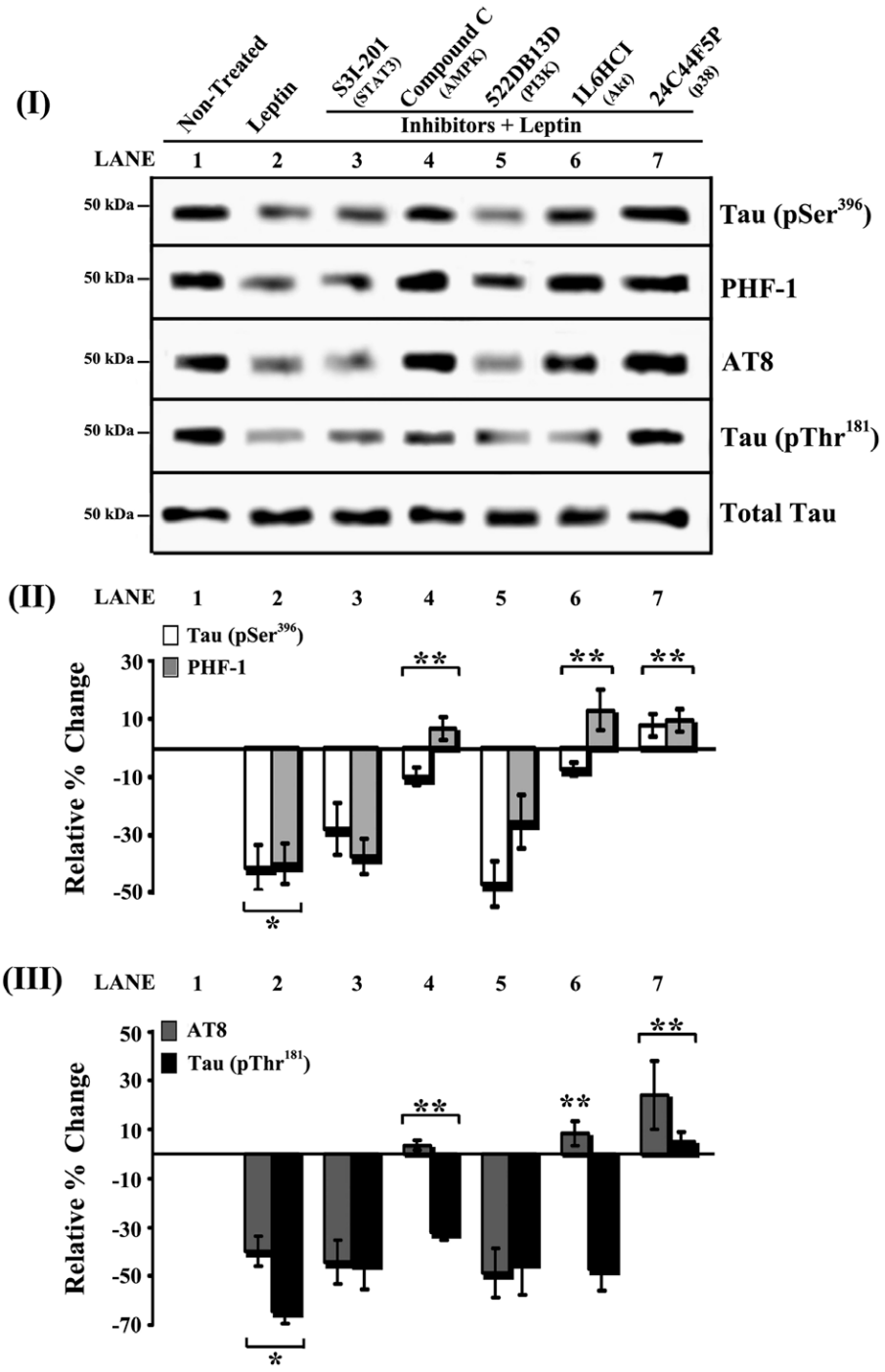

Fig. 1. Leptin-mediated signaling pathways regulating tau phosphorylation. RA-SY5Y were incubated with inhibitors to known downstream effectors of leptin signaling (STAT3, AMPK, PI3K, Akt, p38) in the presence of leptin (100 nM; 1600 ng/ml—lanes 3–7), or non-treated (vehicle—lane 1). Cells treated with leptin alone served as positive control (lane 2). Whole-cell lysates were prepared and analyzed by immunoblot (Panel I) with phosphorylated tau-specific antibodies (pSer^{396}, PHF-1, AT8 or pSer^{181}). Membranes were stripped and re-probed with total tau antibody for normalization. Representative blots are shown, $n = 3$. Normalized tau bands were analyzed by densitometry and results (panels II and III) are presented as the mean ± SD percent change, relative to non-treated samples, which were arbitrarily assigned a value of 0. * vs. non-treated (vehicle—lane 1). ** vs. leptin alone (lane 2).

We therefore explored the roles of PPARγ and the leptin-AMPK pathway in regulating Aβ release. RA-SY5Y were treated with leptin, AICAR or vehicle for 6 h in the presence or absence of an antagonist or inhibitor to PPARγ or AMPK, respectively, and soluble $A\beta_{(1-40)}$ levels within the culture media were determined by ELISA (Fig. 3A). A significant ($p < 0.05$) decrease in soluble Aβ was observed in cells treated with leptin (lane 2) or AICAR (lane 5). These effects were negated by co-treatment with AMPK inhibitor (lanes 3 and 6) or PPARγ (lanes 4 and 7) antagonist. No change was observed in cells treated with inhibitor or antagonist alone (lanes 8 and 9).

These results present a novel finding in which Aβ production is modulated by AMPK, an energy regulator that we have also linked to phosphorylation of tau.

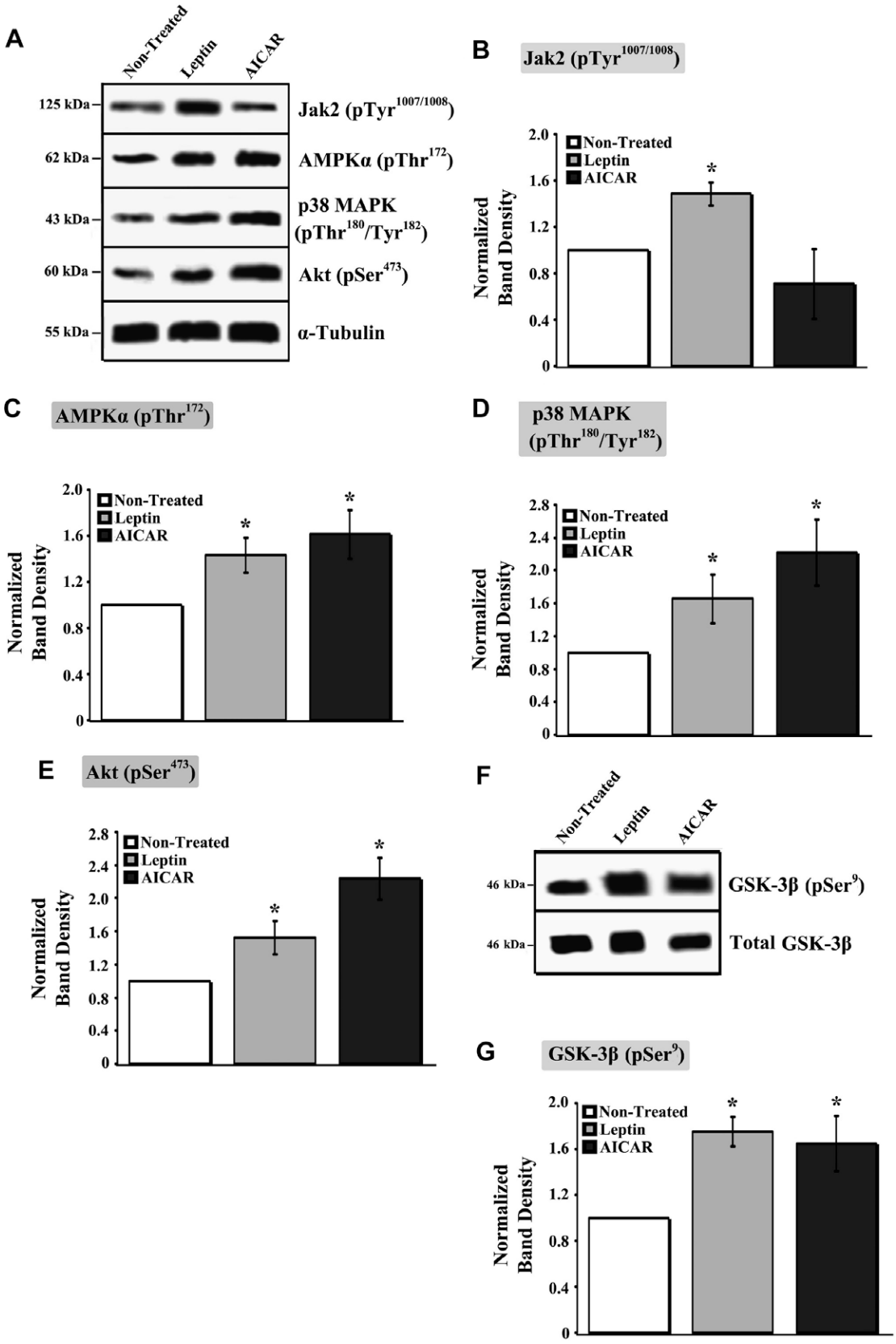

Fig. 2. Phosphorylation of downstream signaling proteins activated by leptin and AICAR. (A). RA-SY5Y were treated with leptin (100 nM; 1600 ng/ml), AICAR (2 mM) or non-treated (vehicle), and signaling proteins (Jak2, AMPK, p38, Akt) implicated in regulating tau phosphorylation were examined by immunoblot using phosphorylation-specific antibodies. Membranes were stripped and re-probed with α-tubulin antibody for normalization. Representative blots are shown, $n = 3$. Normalized bands were analyzed by densitometry and results (B–E) are presented as the mean ± SD. Phosphorylation of (F) GSK-3β ($pSer^9$) in the above cells was measured by immunoblot. Membranes were stripped and re-probed with total GSK-3β for normalization. Bands were analyzed by densitometry and results (G) are presented as the mean ± SD. * vs. non-treated.

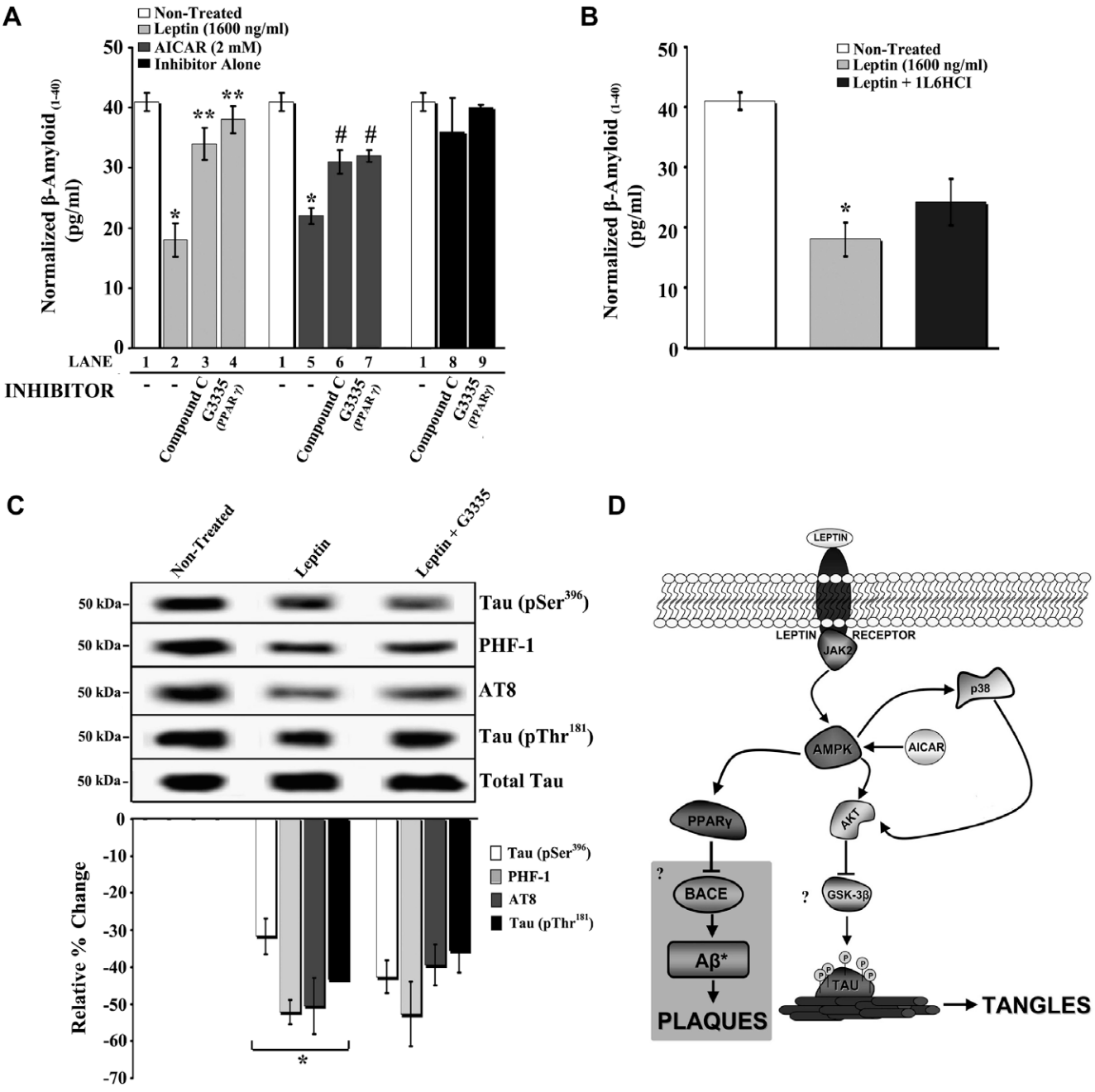

Fig. 3. Leptin and AICAR regulate Aβ production via overlapping signaling pathways. (A) RA-SY5Y were treated for 6 h with leptin (100 nM; 1600 ng/ml—lane 2), AICAR (2 mM—lane 5) and/or inhibitors to each of the following signaling proteins—AMPK (lanes 3 and 6) or PPARγ (lanes 4 and 7). Non-treated (vehicle—lane 1) cells or cells treated with inhibitor alone (lanes 8–9) served as control. Culture media was collected and assayed for $A\beta_{(1-40)}$ by ELISA. Results were normalized to total protein in cell lysates and are presented as the mean $A\beta_{(1-40)}$ concentration (pg/ml) ± SD. (B) Cells from *A* were treated with leptin, alone or in the presence of Akt inhibitor (1L6HCl). Culture media was collected and assayed for $A\beta_{(1-40)}$ by ELISA. Results are presented as in *A*. (C) Cells from *A* were treated with leptin alone or in the presence of PPARγ antagonist (G3335). Whole-cell lysates were prepared and analyzed by immunoblot with phosphorylated tau-specific antibodies as described in the legend of Fig. 1. (D) Cartoon depicting the hypothesized signaling pathways activated by leptin and AICAR in RA-SY5Y. * vs. non-treated (vehicle—lane 1). ** vs. leptin alone (lane 2). # vs. AICAR alone (lane 5).

Tau and Aβ pathways do not overlap downstream of AMPK

Finally, it was investigated whether leptin-mediated tau and Aβ pathways differentiate beyond AMPK. Since AICAR increased phosphorylation of Akt (Fig. 2E) and was unable to reduce Aβ levels in the presence of a PPARγ antagonist (Fig. 3A), we hypothesized that these signaling proteins were downstream of AMPK along their respective pathways.

To this end, RA-SY5Y were treated with leptin in the presence or absence of Akt inhibitor (Fig. 3B) or PPARγ antagonist (Fig. 3C). Inhibition of Akt was unable to significantly ($p > 0.05$) reverse the leptin-induced reduction of soluble Aβ release (Fig. 3B, dark gray bar). Likewise, inhibition of PPARγ did not significantly ($p > 0.05$) reverse the leptin-induced reduction of tau phosphorylation at all examined sites (Fig. 3C, right set of bars). This strongly implicates that following activation of AMPK by leptin, a separate pathway

involving PPARγ and BACE exist to regulate Aβ while another pathway involving Akt and GSK-3β regulates tau phosphorylation.

Discussion

We have shown that Leptin reduces Aβ levels both *in vitro* and *in vivo* [13], and inhibits tau phosphorylation in neuronal cultures [14]. Leptin is known to activate AMPK [25–27] and our present findings suggest that AMPK mediates Leptin's effect on tau phosphorylation (Fig. 1) and Aβ release (Fig. 3). Leptin signaling pathways may reflect differential AMPK responses that are tissue and neuron specific [28]. For example, it has been shown that Leptin activates AMPK in skeletal muscle [27] but inhibits AMPK in the hypothalamus [26]. Nonetheless, Leptin may also act independently of AMPK, through PI3K as shown in hippocampal neurons [12].

The underlying link between AMPK, tau phosphorylation and AD pathobiology has not been previously explored in detail (see Fig. 3D for a working model) and it is unknown as to how leptin modulates AMPK activity in the hippocampus. Leptin has been reported to activate AMPK via a Jak2-dependent, signal transducer and activator of transcription 3 (STAT3)-independent pathway in hepatic cells [29]. Inhibition of tau phosphorylation through inactivation of GSK-3β by Akt has been well-described [30]. AICAR has been shown to transiently activate Akt in hippocampal neurons [31], in agreement with our findings in RA-SY5Y (Fig. 2E). However, higher doses of AICAR and longer incubation periods may trigger negative feedback loops or be deleterious to AMPK and Akt. In addition, other signaling pathways may be involved, independent of AMPK.

Surprisingly, inhibition of p38 MAPK, a known inducer of tau phosphorylation, abrogated leptin's reducing effect on tau phosphorylation (Fig. 1) but inhibiting PI3K did not. Several reports have demonstrated that p38 can activate Akt [32] and inactivate GSK-3β [33]. Additionally, AMPK activates p38 in the ischemic heart [34], and this finding was supported in RA-SY5Y through stimulation with AICAR (Fig. 2D). Therefore, leptin may also modulate tau phosphorylation through AMPK via an alternative pathway involving p38.

We have previously shown that leptin reduces Aβ production [13] (Fig. 3A). Inhibition of AMPK or PPARγ reversed this effect, suggesting their role as mediators of the leptin/Aβ pathway. PPARγ levels have been shown to increase *in vivo* with leptin administration [24]. The link between PPARγ and Aβ has been well described [23]; however, the role of AMPK has not. It is unclear whether AMPK directly activates PPARγ through phosphorylation, or through an indirect mechanism. However, ample evidence suggests that both AMPK and PPARγ pathways affect lipid metabolism [35,36]. Thus our finding that leptin-induced Aβ reduction depends on PPARγ (Fig. 3A, lane 4) is consistent with a role for leptin and AMPK in modulating membrane lipids in neurons as we previously reported [12].

A large study found mid-life central obesity, as the greatest metabolic risk factor for developing dementia later in life [8,9], yet, interestingly, high serum leptin levels in the elderly had significantly less likelihood of cognitive decline [37]. This is in agreement with cross-sectional studies showing that people with dementia have a lower body mass index (BMI) than those without dementia, potentially due to a greater rate of BMI decline during the years immediately preceding dementia [10]. These findings suggest that a potential leptin therapy for AD could in fact be a replacement therapy. Presently, we have demonstrated that leptin regulates two major AD pathways via distinct AMPK-dependent mechanisms in neuronal cells. Leptin, and potentially AMPK activators, may provide a novel therapeutic approach to AD treatment.

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