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Alcohol Alters Insulin-Like Growth Factor-1-Induced Transforming Growth Factor- β 1 Synthesis in the Medial Basal Hypothalamus of the Prepubertal Female Rat

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Abstract

Objective—Insulin-like growth factor-1 (IGF-1) and transforming growth factor β 1 (TGF β 1) are produced in hypothalamic astrocytes and facilitate luteinizing hormone-releasing hormone (LHRH) secretion. IGF-1 stimulates release by acting directly on the LHRH nerve terminals and both peptides act indirectly through specific plastic changes on glial/tanycyte processes that further support LHRH secretion. Because the relationship between these growth factors in the hypothalamus is not known, we assessed the ability of IGF-1 to induce TGF β 1 synthesis and release and the actions of alcohol (ALC) on this mechanism prior to the onset of puberty.

Methods—Hypothalamic astrocytes were exposed to medium only, medium plus IGF-1 (200 ng/ml) or medium plus IGF-1 with 50 mM ALC. After 18 hours, media were collected and assayed for TGF β 1. For the *in vivo* experiment, prepubertal female rats were administered either ALC (3g/kg) or water via gastric gavage at 0730 h and at 1130h. At 0900 h, saline or IGF-1 was administered into the third ventricle. Rats were killed at 1500 hrs and the medial basal hypothalamus (MBH) was collected for assessment of TGF β 1, IGF-1 receptor (IGF-1R) and Akt.

Results—IGF-1 induced TGF β 1 release ($p < 0.01$) from hypothalamic astrocytes in culture, an action blocked by ALC. *In vivo*, IGF-1 administration caused an increase in TGF β 1 protein compared to controls ($p < 0.05$), an action blocked by ALC as well as a PI3K/Akt inhibitor. IGF-1 stimulation also increased both total ($p < 0.01$) and phosphorylated ($p < 0.05$) IGF-1R protein levels, and phosphorylated Akt levels ($p < 0.01$), which were also blocked by ALC.

Conclusions—This study shows that ALC blocks IGF-1 actions to stimulate synthesis and release of hypothalamic TGF β 1, total and phosphorylated IGF-1R and phosphorylated Akt levels further demonstrating the inhibitory actions of ALC on puberty-related events associated with hypothalamic LHRH release.

Keywords

IGF-1; TGF β 1; Alcohol; puberty; hypothalamus

INTRODUCTION

The onset of puberty relies on complex interactions within the hypothalamus that lead to increased secretion of luteinizing hormone-releasing hormone (LHRH). Any substance that can alter prepubertal LHRH release can negatively influence the pubertal process. It is well established that alcohol (ALC) diminishes the secretion of LHRH and subsequently, delays the onset of female puberty in rodents (Dees and Skelley, 1990) and primates (Dees et al., 2000; Dissen et al., 2004). The increased level of LHRH secretion needed at puberty is due to a decrease in inhibitory neurotransmission and the enhanced developmental responsiveness to excitatory neurotransmission. Additionally, in recent years, it has been recognized that the increase in prepubertal LHRH release is facilitated by the maturation and interactive participation of hypothalamic glial-neuronal communications (Ma et al., 1997; Mahesh et al., 2006; Ojeda et al., 2008; Srivastava et al., 2011).

A specific subset of glial cells, known as astrocytes, lie within the medial basal hypothalamus (MBH) and are known to facilitate prepubertal LHRH release by producing cell adhesion molecules and growth factors. Insulin-like growth factor-1 (IGF-1) and transforming growth factor β 1 (TGF β 1) are examples of glial-derived peptides that can stimulate release of the LHRH from the MBH (Hiney et al., 1991; Srivastava et al., 2014) during prepubertal development. IGF-1 acts within the median eminence (ME) to stimulate LHRH release directly from nerve terminals via prostaglandin-E₂ (PGE₂) mediation (Hiney et al., 1998), or indirectly by working with estradiol (E₂) to affect the remodeling of tanycyte end feet that surround LHRH nerve terminals (Cardona-Gomez et al., 2000, Fernandez-Galaz et al., 1997). TGF β 1 is released from the tanycytes of the ME via stimulation by specific glial products, TGF α and PGE₂, which initiates retraction of tanycyte processes to better allow for entry of LHRH into hypophyseal portal blood (Prevot et al., 2003). Since the respective contributions of IGF-1 and TGF β 1 on prepubertal LHRH secretion are important, the interrelationship between these growth factors warrants investigation. Furthermore, since ALC is capable of affecting glial-neuronal influences (Hiney et al., 2003, Srivastava et al., 2011; 2014), assessing the actions and interactions between IGF-1, TGF β 1 and ALC are important with regard to understanding the mechanism(s) by which this drug of abuse suppresses LHRH secretion and disrupts pubertal development. Hence, the present study has utilized hypothalamic astrocytes grown in culture, as well as *in vivo* experimentation to assess these issues.

METHODS

Animals and surgery

Eighteen-day pregnant female rats of the Sprague-Dawley line were purchased from Charles River (Boston, MA) and allowed to deliver pups normally in the Texas A&M University lab animal facility. For the *in vivo* study, female pups were weaned at twenty-one days of age and housed four per cage under controlled conditions of light (lights on, 0600h; lights off, 1800h) and temperature (23 C), with ad libitum access to food (Harland Teklad Diet, Madison, WI) and water. All procedures performed were approved by the University Animal Care and Use Committee and in accordance with the NAS-NRC Guidelines for the

Care and Use of Laboratory Animals. Surgical anesthesia was an intraperitoneal injection of 2.5% Tribromoethanol (0.5ml/60g body weight).

Cell Culture Study

Hypothalamic astrocytes were obtained from the hypothalami of 1–2 day old rats. Briefly, tissues were triturated in dissociation medium [Modified Eagles Medium (MEM), Life Technologies Corp., Carlsbad, CA] containing 2× penicillin-streptomycin (Pen-Strep, Life Technologies Corp.) and Dispase (1.5 units per ml) using Sigmacote treated 10 ml glass pipettes. Dissociated cells were removed after 10 minutes of gentle stirring and placed in MEM containing 1× Pen-Strep and 10% fetal bovine serum to stop the digestion process. Fresh dissociation medium was added to remaining tissue and the procedure repeated for a total of 4 extractions. DNaseI (8,000 Units/ml; Sigma-Aldrich, Saint Louis, MO) was added after the first extraction. Astrocytes were plated at a density of 1×10^6 cells. Culture medium was changed after 24 hrs and then twice per week. On day 7 of culture, cells were shaken (200 rpm) for 18 hrs under atmosphere of 5% CO₂-95% O₂ at 37 C to remove oligodendrocytes and neurons. Following shaking, the astrocytes were recovered by trypsin-EDTA and re-plated in 6 well plates at 400,000 cells/well. Once astrocytes reached 90% confluence, wells were washed with PBS and replaced with serum-free Dulbecco's modified Eagles medium (DMEM, Life Technologies Corp.) without phenol red and supplemented with transferrin (100 µg/ml, Sigma-Aldrich) and putrescine (100 µM, Sigma-Aldrich) [astrocyte defined medium, ADM]. After 12 hours, media was removed, and ADM containing either 25mM, or 50mM or 70mM doses of ALC was added to each respective 6 well plate of astrocytes. ALC levels were maintained by adding 2.5 µl of 25% ethanol every 3 hrs. After 18 hrs, media was collected and measured for TGFβ1 by enzyme-linked immunosorbent assay (ELISA). Once the minimal effective ALC dose was determined, the experiment was repeated as above, except after 12 hrs in serum-free media, the astrocytes were challenged with the following: ADM only; ADM with IGF-1 (200 ng/ml) and ADM with IGF-1 plus ALC (50 mM). After 18 hrs, media was collected and TGFβ1 was measured ELISA.

Animal Study

Twenty-four day old female rats were implanted with third ventricular cannulae as described previously (Dees et al., 2005). After 4 days of recovery, the animals were divided into three groups. At 0730 hrs, groups 1 and 2 were administered water and group three received ALC (3g/kg; 1.5 ml 25% ALC/100 g rat) by gastric gavage. This dose of ALC was chosen because a single intragastric injection yields a moderate blood ALC concentration that is capable of consistently suppressing LH release in immature female rats (Hiney et al., 2003). The animals were left undisturbed for 90 min to allow time for ALC absorption. At 0900 hours, groups 2 and 3 received a third ventricular injection of IGF-1 (Prospec-Tany TechnoGene Ltd., Ness Ziona, Israel) at a dose of 200 ng/3µl saline which we have used previously (Hiney et al., 1996; 2009). Group 1 (control) was injected with an equal volume of saline. The injections were delivered into the third ventricle over a 1 min period of time. A 2g/kg dose of ALC or water was administered gastrically at 1130 hrs (4 hrs after the initial dose) to maintain moderately elevated blood levels of ALC over the course of the day (Hiney et al., 2003). All animals were killed by decapitation at 1500 hrs, 6 hrs post IGF-1 or

saline. Trunk blood was collected at that time for subsequent assessment of blood ALC concentrations by an enzymatic method using a diagnostic kit purchase from Sekisui Diagnostics P.E.I. Inc. (Prince Edwards Island, Canada). Brains were removed, placement of the third ventricular cannulae verified, and the animals were confirmed to be in the juvenile stage of pubertal development (Dees and Skelley, 1990). A block of tissue containing the MBH was dissected as described previously (Hiney et al., 2009). All tissues were stored frozen until assayed for their TGF β 1, total and phosphorylated (p)-IGF-1 receptor (IGF-1R), and total and phosphorylated (p)-Akt protein expressions by Western blot analysis. In a separate experiment without ALC, IGF-1 was administered as above either in the presence or absence of an phosphatidylinositol 3 (PI3) kinase/Akt inhibitor (LY294002; 10 mM/4 μ l in 3V) to determine if the IGF-1 effect on TGF β 1 was mediated by Akt.

Immunoblotting

Brain tissues were homogenized in 1% Igepal CA-630, 20 mM Tris-Cl, pH (8.0), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 10 mM sodium pyruvate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.25% protease inhibitor cocktail (Sigma-Aldrich) at 4 C. The homogenates were incubated on ice for 30 min and centrifuged at 12,000Xg for 15 min. The concentration of total protein in the resulting supernatant was determined by the RC DC protein assay (Bio-Rad Laboratories) using bovine serum albumin as standard. Immunoblot analysis was performed by solubilizing the proteins (100 μ g) in a sample buffer containing 25 mM Tris-Cl, pH 6.8, 1% SDS, 5% β -mercaptoethanol, 1mM EDTA, 4% glycerol and 0.01% bromophenol blue and electrophoresed through 8% SDS-PAGE for IGF-1R and 12% for TGF β 1 and Akt under reducing conditions. The separated proteins were electrophoretically transblotted onto polyvinylidene difluoride membranes. Following transfer, membranes were blocked with 5% nonfat dried milk/0.1% Tween 20 in PBS (pH 7.4) for 3 hr and subsequently incubated at 4 C overnight with rabbit anti-total or anti-p-IGF-1R (1:400; Abcam Inc, Cambridge, MA), rabbit anti-TGF β 1 (1 μ g/ml; Sigma Aldrich, Saint Louis, MO), rabbit anti-total or anti-p-Akt (1:1000; Cell Signaling Tech. MA). After the incubation, membranes were washed in PBS/0.1% Tween-20 and then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1: 30,000; Santa Cruz Biotech., CA) for 2 hour at room temperature. Following washing, the specific signals were detected with the enhanced chemiluminescence (Western Lightning Plus-ECL, PerkinElmer, Shelton, CT) and quantified with NIH Image J software version 1.43 (National Institutes of Health, MD). Subsequently, membranes were stripped using Re-Blot Plus kit (EMD Millipore, Temecula, CA) and reprobed with mouse monoclonal antibody to β -actin and goat anti-mouse secondary antibody, to normalize for the amount of sample loading. Following washing, the detection and quantitation of β -actin was conducted as described above.

TGF β 1 Analysis

TGF β 1 released into the media was determined by an ELISA kit purchased from Promega (Madison, WI). The sensitivity of this assay was 15 pg/ml.

Statistical Analysis

Differences between groups were analyzed by ANOVA with post hoc testing using the Student-Newman-Keuls multiple comparison test. These statistical tests were conducted with INSTAT software (GraphPad Software, San Diego, CA). Probability values less than 0.05 were considered significantly different.

RESULTS

In vitro effect of ALC on TGF β 1 secretion from primary hypothalamic astrocytes

An ALC dose response was conducted on primary hypothalamic astrocytes to determine the minimal effective dose of ALC needed to inhibit TGF β 1 release into the medium (Figure 1A). Astrocytes exposed to ADM plus 25 mM ALC showed a slight but non-significant decrease in the amount of TGF β 1 secretion in the medium. However, ADM with 50 mM or 70 mM dose of ALC inhibited the release of TGF β 1 from astrocytes ($p < 0.01$). Thus, the 50 mM dose was used in the following experiment. Figure 1B shows the basal release of TGF β 1 (ADM only) and that the addition of IGF-1 to the media markedly stimulated the astrocytes to release the peptide ($p < 0.01$). Exposure of the hypothalamic astrocytes to 50 mM ALC blocked the IGF-1 induced release of TGF β 1.

In vivo effect of ALC on IGF-1 induced TGF β 1 protein synthesis

Since IGF-1 stimulated astrocytes to secrete TGF β 1 *in vitro*, we assessed the ability of the peptide to stimulate TGF β 1 protein synthesis in prepubertal female rats *in vivo*. The blood ALC concentrations for these acute *in vivo* experiments were measured and the BACs showed a mean ($\pm$ SEM) of 154 ± 10.2 mg/dL at the time the tissues were collected. Figure 2A and B demonstrates that the central administration of IGF-1 stimulated an increase ($p < 0.05$) in TGF β 1 at 6 hours post injection when compared to control animals injected with saline. Additionally, the acute exposure to ALC blocked the IGF-1-induced synthesis of TGF β 1. To determine if Akt signaling is directly involved with IGF-1 induced stimulation of TGF β 1, we utilized LY294002, a highly selective inhibitor of PI3 kinase which blocks Akt phosphorylation. Figure 2C and D demonstrate that LY294002 blocked IGF-1-induced TGF β 1 protein synthesis thus indicating PI3 kinase/Akt signaling is involved.

In order to determine the mechanism of ALC's action on IGF-1 signaling in this brain region, we evaluated the content of IGF-1R and its phosphorylation and whether phosphorylation of Akt was affected by ALC in these same tissues. Figure 3 demonstrates that IGF-1 stimulated the synthesis of total IGF-1R protein ($p < 0.01$; Fig. 3A and B) and its phosphorylation ($p < 0.05$; Fig. 3C and D), respectively, and that this action was inhibited by ALC. Subsequently, we assessed total and p-Akt, a transduction signal that is activated by IGF-1. Figure 4A shows a representative gel showing the expression of p-Akt and total Akt in the MBH. Total Akt levels are unchanged by IGF-1 and ALC and therefore, the ratio between total Akt and p-Akt was calculated to determine the level of p-Akt. Figure 4B shows that p-Akt protein expression in the MBH was increased ($p < 0.01$) in the animals 6 h after central administration of IGF-1, compared to the basal levels expressed by the control animals that received saline. Conversely, the ALC-treated animals showed suppressed levels of p-Akt in the MBH.

DISCUSSION

Hypothalamic astrocytes synthesize and secrete various growth factors which are involved in bi-directional glial-neuronal communications contributing to LHRH release. Because of the well-known effects of IGF-1 on astrocyte function and cell to cell communication in the hypothalamus, and because IGF-1Rs have been localized on glial cells in the median eminence (Bohannon et al., 1986; Lesniak et al., 1988) we first assessed whether IGF-1 could stimulate TGF β 1, another growth factor that like IGF-1, is also produced and secreted by hypothalamic astrocytes (Buchanan et al., 2000; Galbiati et al., 1996; Melcangi et al., 1995). Our results are the first to show that IGF-1 can stimulate astrocytes in culture to release TGF β 1. This is the first indication of a potential role for IGF-1 in the control of TGF β 1. We also observed that ALC blocked both basal secretion and IGF-1 stimulated TGF β 1 secretion. Because astrocytes secrete IGF-1, our results suggest that their response to the available peptide, whether endogenous/basal or supplemented was suppressed by ALC.

Our *in vivo* studies further assessed the relationships between IGF-1 and ALC with regard to the control of hypothalamic TGF β 1. In this regard, we showed that the central administration of IGF-1 induced TGF β 1 protein synthesis, that this IGF-1 action utilized the Akt transduction pathway, and that the stimulated TGF β 1 was blocked by ALC. To begin determining mechanism of these actions, we assessed the upstream effects on the IGF-1R, and on the IGF-1 activation of Akt (Cardona-Gomez, 2002). Our results indicated that IGF-1 induced an increase in the content and phosphorylation of IGF-1R protein, and that this was associated with the concomitant increase in p-Akt protein. Importantly, ALC blocked both of these actions of IGF-1 within the MBH. It seems likely, at least in part, that the suppression in p-Akt was due to the upstream action of ALC to inhibit IGF-1R synthesis; however, we cannot rule out a possible action of ALC to directly alter Akt signaling as well. This ALC effect has been observed in other areas of the prepubertal female brain, in that acute ALC exposure inhibits IGF-1-induced KiSS-1 gene expression by altering p-Akt signaling in the anteroventral periventricular nucleus, which is located in the rostral area of the hypothalamus (Hiney et al., 2010). Additionally, following chronic ALC exposure to late juvenile female rats, the level of p-Akt was suppressed in the MBH when compared to basal levels in the control animals (Srivastava et al., 2009).

Another pathway that IGF-1 uses to affect TGF β 1 is through activation of the Oct 2 POU–TGF α -PGE₂ pathway from adjacent glial cells. Oct 2 POU homeodomain genes are expressed in hypothalamic glia and increase during pubertal development (Ojeda et al., 1999). This gene is an upstream modulator of the TGF α , another glial growth factor involved in LHRH release (Ojeda et al., 1990). We have shown that Oct 2 genes are a link between IGF-1 and TGF α (Dees et al., 2005). Specifically, the central administration of IGF-1 stimulated Oct 2c in the MBH and furthermore, this action was blocked by ALC. In the MBH, TGF α acts via the erbB1 receptor to stimulate glial derived PGE₂ release (Ma et al., 1997; Ojeda et al., 1990; 2008). Once released, PGE₂ not only can induce LHRH release directly from its neuron terminals in the median eminence (Hiney and Dees, 1991; Ojeda et al., 1979), but it can also act on specialized glial cells called tanycytes that line the third ventricle of the hypothalamus (Prevot et al., 2003). With regard to the latter, PGE₂ stimulates the release of TGF β 1, which then causes retraction of tanycyte processes within

the median eminence to better allow for entry of LHRH into the hypophyseal portal blood. Thus, an ALC-induced suppression of TGF β 1 synthesis as shown in the present study, either alone or coupled with the ALC inhibition of IGF-1-induced PGE₂ release (Hiney et al., 1998) would markedly affect tanycyte functions related to prepubertal LHRH secretion.

In addition to the IGF-1-TGF β 1 interactions discussed above between astrocytes, tanycytes and LHRH nerve terminals within the median eminence, we suggest that these two peptides are involved in glial to neuronal communications within the arcuate nucleus (ARN) of the MBH as well. The present results have clearly demonstrated that IGF-1 induces the synthesis and release of glial TGF β 1, and that both actions are suppressed by ALC. We recently showed that TGF β 1 can stimulate LHRH release from the MBH of prepubertal female rats and that this action was blocked by ALC (Srivastava et al., 2014). It was suggested that the TGF β 1 action to induce LHRH release is indirect since the LHRH neurons are not localized within the AN of the rat (Kozłowski and Dees, 1984) as they are in the primate, including humans (Barry, 1976; Silverman et al., 1982), and because TGF β 1 receptors are not localized on LHRH nerve terminals in the ME (Bouret et al., 2004; Prevot et al., 2000). Thus, in this brain region, the TGF β 1 must first be stimulating another neurotransmitter synthesized by neurons within the ARN, which, in turn stimulates release of the LHRH peptide from the nerve terminals in the ME; hence, mediating the TGF β 1 effect. While the phenotype of this neuron has yet to be identified, it is well known that several neurotransmitters/peptides synthesized by neurons in the rat ARN can stimulate LHRH release directly from the nerve terminals (Hiney and Dees, 1991; Navarro et al., 2004; Ojeda et al., 1990; Sarkar et al., 1981; Urbanski and Ojeda, 1987). Regardless of the mechanism by which TGF β 1 stimulates LHRH release, we have demonstrated an upstream action of IGF-1 to regulate TGF β 1. Collectively, these results further demonstrate an IGF-1-TGF β 1 interaction contributing to early signaling processes controlling prepubertal LHRH secretion and that ALC clearly interferes with these communications.

In conclusion, our data demonstrates that IGF-1 can stimulate the synthesis and release of TGF β 1 and suggests that this interaction between glial products is part of a series of events resulting in facilitation of prepubertal LHRH secretion. These data indicate that IGF-1 plays a dual role in this regard. In addition to its ability to stimulate LHRH secretion directly from nerve terminals in the ME (Hiney et al., 1998), IGF-1 can also induce glial TGF β 1 release which causes retraction of the tanycyte end feet from the portal vasculature allowing entry of the LHRH peptide. Furthermore, the fact that ALC blocks the IGF-1 induced release of TGF β 1 from hypothalamic astrocytes and from MBH tissue, via actions to reduce IGF-1R synthesis further demonstrates the inhibitory actions of ALC on puberty-related events associated with hypothalamic LHRH release.

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Figure 1.

Effects of ALC on hypothalamic astrocytes *in vitro*. A) TGFβ1 levels in conditioned medium of hypothalamic astrocytes following 18 hrs of exposure to different doses of ALC. Note that ALC inhibited release of TGFβ1 into the medium containing either 50mM or 70 mM dose of ALC. ** $p < 0.01$. B) TGFβ1 levels in conditioned medium of hypothalamic astrocytes following 18 hrs of exposure to astrocyte-defined medium (ADM), IGF-1 (100 ng/ml), and IGF-1 + ALC (50mM). IGF-1 increased the amount of TGFβ1 released from the astrocytes (solid bar) over basal secretion (open bar) and ALC blocked the IGF-1 induced release of TGFβ1. N=6 wells/group. ** $p < 0.01$ vs. ADM only and IGF-1+ALC.

Figure 2.

Effects of IGF-1, acute ALC exposure, and an Akt inhibitor, LY294002, on TGFβ1 protein in the MBH of prepubertal female rats. A) Representative Western blot of TGFβ1 and β-actin proteins from saline (lanes 1–3), IGF-1 (lanes 4–6), and IGF-1 + ALC (lanes 7–9) treated animals. B) Densitometric quantification of all bands assessing the TGFβ1 protein. These data were normalized to the internal control β-actin protein. IGF-1 (open bar) induced an increase in TGFβ1 over saline-treated (solid bar) animals. Note that exposure to ALC blocked the IGF-1 induced expression of TGFβ1 protein (hatched bar). C) Representative Western blot of TGFβ1 and β-actin proteins from saline (lanes 1–3), IGF-1 (lanes 4–6), and IGF-1 + LY294002 (lanes 7–9) treated animals. D) Densitometric quantification of all bands assessing the TGFβ1 protein. These data were normalized to the internal control β-actin protein. IGF-1 (open bar) induced an increase in TGFβ1 over saline-treated (solid bar) animals. Note that central administration of 10mM of LY294002 one hour prior and then again 3 and 5 hrs post IGF-1 administration, blocked the IGF-1 induced increase in TGFβ1 (hatched bar). Each bar represents the mean ± SEM of the TGFβ1/β-actin ratio. The number of animals represented by each bar is 6. *p<0.05.

Figure 3.

Effects of IGF-1 and acute ALC exposure on total and phosphorylated (p)-IGF-1R protein in the MBH of prepubertal female rats. A) Representative Western blot of IGF-1R and β -actin proteins from saline (lanes 1–3), IGF-1 (lanes 4–6), and IGF-1 + ALC (lanes 7–9) treated animals. B) Densitometric quantification of all bands assessing the IGF-1R protein. These data were normalized to the internal control β -actin protein. IGF-1 (open bar) induced an increase in IGF-1R over saline-treated (solid bar) animals. Note that ALC blocked the IGF-1 induced expression of IGF-1R protein in the MBH (hatched bar). C) Representative Western blot of p-IGF-1R and β -actin proteins from saline (lanes 1–3), IGF-1 (lanes 4–6), and IGF-1 + ALC (lanes 7–9) treated animals. C) Densitometric quantification of all bands assessing the p-IGF-1R protein. These data were normalized to the internal control β -actin protein. IGF-1 (open bar) induced an increase in p-IGF-1R over saline-treated (solid bar) animals. Note that ALC blocked the IGF-1 induced expression of p-IGF-1R protein in the MBH.

(hatched bar). Each bar represents the mean $\pm$ SEM of the IGF-1R/ β -actin ratio. The number of animals represented by each bar is 6. **p<0.01; *p<0.05.

Figure 4. Effects of IGF-1 and acute ALC exposure on total and phosphorylated (p)-Akt protein in the MBH of prepubertal female rats. A) Representative Western blot of total Akt and p-Akt proteins from saline (lanes 1–3), IGF-1 (lanes 4–6), and IGF-1 + ALC (lanes 7–9) treated animals. B) Desitometric quantification of all bands assessing p-Akt protein normalized to total Akt protein. Note that IGF-1 (open bar) induced an increase in p- Akt over saline-treated (solid bar) animals and ALC blocked the IGF-1 induced expression of p-Akt protein in the MBH (hatched bar). Each bar represents the mean $\pm$ SEM of the p-Akt/total Akt ratio. The number of animals represented by each bar is 6. ** $p < 0.01$.