Ethanol inhibits insulin receptor substrate-1 tyrosine phosphorylation and insulin-stimulated neuronal thread protein gene expression

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Neuronal thread proteins (NTPs) are molecules that accumulate in the brains of patients with Alzheimer's disease, and may play a key role in both normal and neurodegenerative neuritic sprouting. In this investigation we determined whether NTP expression is up-regulated by insulin, an important neurotrophic factor that stimulates differentiation-associated neurite outgrowth, and studied the effects of ethanol, a known inhibitor of growth factor receptor tyrosine phosphorylation, on NTP expression and insulin-mediated signal transduction cascade in neuronal [primitive neuroectodermal tumour cell line 2; (PNET2)] cells. PNET2 cells were treated with 50 m-units/ml insulin in the presence or absence of 100 mM ethanol for 0.2–96 h, and cell proliferation and expression of NTP molecules were investigated by metabolic labelling, immunoprecipitation and immunohistochemical staining. Insulin stimulation resulted in an immediate increase in the levels of three (38, 18 and 15 kDa) of

five NTP species (the others were of 26 and 21 kDa), followed by a decline in expression within 120 min; however, studies performed up to 96 h of culture demonstrated up-regulation by insulin of all five NTP species. Ethanol either abolished or severely muted the short- and long-term insulin-mediated up-regulation of NTP expression, and substantially reduced insulin-mediated neuronal differentiation. The effects of ethanol on NTP gene expression were associated with impaired insulin-mediated tyrosine phosphorylation of both the insulin receptor β subunit and the insulin receptor substrate-1 (IRS-1), resulting in decreased association of phosphatidylinositol 3-kinase with IRS-1. The findings suggest that ethanol may inhibit NTP expression associated with central nervous system neuronal differentiation by uncoupling the IRS-1-mediated insulin signal transduction pathway.

INTRODUCTION

Neuronal thread proteins (NTPs) are a group of immunoreactively related, developmentally regulated, molecules expressed in the brain [1–4] and in neuronal cell lines [5]. Distinct but related thread protein molecules have been identified in cells of pancreatic, hepatic and gastrointestinal origin [6–9]. NTP gene expression is up-regulated during cell proliferation and neuritic sprouting [1–4]. In previous studies we have demonstrated accumulation of the ~ 21 kDa NTP species in brains of patients with Alzheimer's disease [2–4]. Increased NTP expression in Alzheimer's disease may reflect the widespread aberrant neuritic sprouting that correlates with the severity of dementia and characterizes an important aspect of the neurodegenerative pathology [10–12].

Preliminary studies demonstrated insulin-mediated up-regulation of NTP expression in neuronal cell lines of peripheral and central nervous system (CNS) origin [13,14]. Insulin is an important neurotrophic factor that increases DNA synthesis, cytoskeletal protein and immediate-early gene expression, and differentiation-associated neuritic growth [15–25]. In the CNS, insulin binds to neuron-specific insulin receptors [26] which then, like insulin receptors in other cell types [27–34], become auto-phosphorylated, and thereby activate a cellular signalling cascade [35,36]. Insulin receptor activation leads to phosphorylation of tyrosine, serine and threonine residues on a number of cellular proteins, including the p185 insulin receptor substrate-1 (IRS-1), mitogen-activated protein kinase 2, casein kinase II, protease-activated kinase II, S6 kinases and Raf-1 kinase [36–40]. Among

these, least is known about the role of the IRS-1 signalling pathway with respect to neuronal gene expression.

Ethanol is a potent inhibitor of DNA synthesis [41–44], neuronal development, neuronal gene expression [45–51] and insulin- [52] and cyclic AMP-mediated signal transduction [53]. Chronic ethanol neurotoxicity causes neurodegeneration in the mature CNS [54,55]. One mechanism of ethanol neurotoxicity may involve uncoupling of the insulin signal transduction pathways, as previously demonstrated in hepatocytes [52]. In addition, ethanol inhibits growth of NIH 3T3 fibroblasts and C6 glioblastoma cells following stimulation by insulin-like growth factor-1 (IGF-1), principally through a decrease of auto-phosphorylation of the IGF-1 receptor [56,57].

The present study examines changes in the levels of NTP expression in a CNS-derived primitive neuroectodermal tumour cell line (PNET2) following treatment with insulin or ethanol, and explores the effect of ethanol pretreatment on insulin modulation of NTP gene expression. The mechanism of ethanol action on NTP gene expression was investigated by analysis of the insulin-mediated signal transduction cascade [27–34].

EXPERIMENTAL

Cell lines and culture conditions

The PNET2 cell line is of human CNS origin [58] and expresses NTP molecules [5]. Preliminary studies suggested growth and differentiation responsiveness, and modulation of NTP gene expression by insulin stimulation [13,14]. PNET2 cell lines were

Abbreviations used: CNS, central nervous system; IGF, insulin-like growth factor; IR β , insulin receptor β subunit; IRS-1, insulin receptor substrate-1; NTP, neuronal thread protein; Th, thread protein monoclonal antibody; PI 3-kinase, phosphatidylinositol 3-kinase; PNET2, primitive neuroectodermal tumour cell line 2.

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maintained in Dulbecco's modified Eagle's medium supplemented with 12 % (v/v) fetal calf serum, 0.45 % (w/v) glucose and 8 mM L-glutamine (complete medium). Cells grown to 50 % confluence were subjected to overnight serum starvation to synchronize DNA synthesis. The cells were re-fed with complete medium supplemented with nothing (control), 50 m-units/ml insulin (NovolinR; Bristol-Myers Squibb, Princeton, NH, U.S.A.) or 100 mM ethanol. The levels of insulin and ethanol treatments used were derived from dose-response analyses and exploratory studies to determine the minimum exposure required to modulate the expression of insulin-responsive genes such as glyceraldehyde-3-phosphate dehydrogenase [59]. The cells were harvested at various times after re-feeding. Metabolic labelling was begun 3 h prior to cell harvesting, and NTP molecules were immunoprecipitated using monoclonal antibodies to thread proteins [1,6]. The culture medium was changed daily, and all experiments were repeated twice. DNA synthesis was assayed in triplicate cultures by measuring [3 H]thymidine incorporation into DNA [44,60].

Sources of antibodies

The Th (thread protein) monoclonal antibodies were prepared against the purified human pancreatic form of thread protein [6]. Th7, Th9 and Th10 each recognize distinct epitopes on both pancreatic and neuronal forms of the proteins [1,4]. Rabbit polyclonal antibodies to both the human IRS-1 and the β subunit of the insulin receptor (IR β) were generated with corresponding glutathione S-transferase fusion proteins that had been purified using glutathione-agarose beads and cleaved with thrombin [61,62]. Anti-[phosphatidylinositol 3-kinase (PI 3-kinase)] rabbit polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-phosphotyrosine mouse monoclonal antibody was kindly provided by Dr. Brian Drucker (Dana-Farber Cancer Center, Boston, MA, U.S.A.). Monoclonal antibodies to synaptophysin and desmin were purchased (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Polyclonal antibodies to GAP-43 were prepared from a λ gt11 fusion protein generated with a rat cDNA clone [63].

Analysis of NTP expression by immunoprecipitation and Western blot analyses

NTP expression was analysed by immunoprecipitation using lysates prepared from cells metabolically labelled with [35 S]-methionine [5]. At various times after stimulation with insulin or treatment with ethanol, the cells were harvested and then lysed in buffer containing 50 mM Tris/HCl (pH 7.5), 1 % Triton X-100, 2 mM EGTA, 10 mM EDTA, 100 mM NaF, 1 mM Na₄P₂O₇, 2 mM Na₃VO₄, 100 mg/ml PMSF, 1 mg/ml aprotinin, 1 mg/ml pepstatin A and 1 mg/ml leupeptin. The clarified supernatant fractions collected after centrifuging the samples at 14000 g for 10 min were stored at -40 °C. Protein concentrations were measured by the Lowry colorimetric assay [64], and the lysate volumes were adjusted to achieve 1 mg of total protein/ml.

NTP molecules were immunoprecipitated from 1 ml lysate samples using 10 μ g of a Th9 + Th7 + Th10 cocktail of monoclonal antibodies to human thread protein [5]. The immune complexes were captured by Protein A-Sepharose beads (Pharmacia LKB, Uppsala, Sweden) [62], solubilized in buffer containing 2 % SDS and 0.7 M β -mercaptoethanol, and then fractionated by SDS/PAGE [5]. In addition, aliquots of the lysates were simultaneously immunoprecipitated using non-relevant monoclonal antibodies to desmin as a negative control. As a positive control for protein loading, Class I antigen was immunoprecipitated

from the same lysates using the W6/32 HLA monoclonal antibody [65]. The time-dependent changes in NTP expression were assessed by volume densitometric scanning of non-saturated autoradiographs (Molecular Dynamics ImageQuant).

To determine whether prior exposure to ethanol interferes with insulin-mediated modulation of NTP expression, PNET2 cells were cultured for 5 days with complete medium plus or minus 100 mM ethanol. The 50 % confluent cultures were subjected to overnight serum starvation, followed by stimulation with 50 m-units/ml insulin, maintaining the same levels of ethanol exposure throughout. The cells were harvested 0, 10, 20, 30, 60 and 120 min (short-term studies) and 0, 12, 24 and 48 h (long-term studies) after insulin stimulation. Metabolic labelling was begun 3 h prior to cell harvesting, and NTP molecules were immunoprecipitated using the cocktail of Th monoclonal antibodies. The medium was changed daily with fresh ethanol additions, and the cells were grown in sealed flasks to minimize ethanol evaporation. All experiments were repeated twice.

Immunocytochemistry

Cells cultured in 8-well chamber slides were examined for NTP expression by immunofluorescence staining. The cells were fixed in 2 % buffered paraformaldehyde solution, rinsed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3) and permeabilized with 0.1 % saponin (Sigma) in PBS. Non-specific antibody binding was blocked with 3 % non-immune horse serum. The cells were incubated for 1 h at 37 °C with 5 μ g/ml Th9 mouse monoclonal antibody. Replicate chamber slide cultures were immunostained with antibodies to synaptophysin and GAP-43 as positive controls, and with anti-desmin as a negative control. The immunofluorescence staining reaction was revealed by the ABC method using affinity-purified biotinylated goat anti-mouse IgG (5 μ g/ml) and fluorescein or rhodamine-conjugated avidin (20 μ g/ml) (Vector Laboratories, Burlingame, CA, U.S.A.).

Signal transduction pathway

The insulin-mediated signal transduction pathway was assessed by examining the levels of IRS-1 and IR β , insulin-responsive tyrosine phosphorylation of IRS-1 and IR β , and the association between PI 3-kinase and tyrosine-phosphorylated IRS-1. Cell lysate supernatants were reacted with either anti-IRS-1 rabbit polyclonal antibody or anti-IR β rabbit polyclonal antibody overnight at 4 °C and incubated with Protein A-Sepharose beads to precipitate the immune complexes. The beads were washed extensively in lysis buffer, and the immunoprecipitates were fractionated by SDS/PAGE under denaturing and reducing conditions. The proteins were transferred on to polyvinylidene difluoride nylon membrane (Millipore), and probed with the respective antibodies. Tyrosine phosphorylation of IRS-1 and IR β was detected on the same blots re-probed with mouse monoclonal anti-phosphotyrosine antibodies, and PI 3-kinase association with IRS-1 was detected by re-probing IRS-1 immunoprecipitants with rabbit polyclonal anti-(PI 3-kinase). Antibody binding was detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies and enhanced chemiluminescence (ECL) reagents (Amersham Corp., Arlington Heights, IL, U.S.A.). The time-dependent quantitative changes in protein expression or tyrosine phosphorylation were assessed by densitometric scanning of non-saturated autoradiographs (Molecular Dynamics ImageQuant).

RESULTS

Effects of insulin and ethanol treatments on DNA synthesis

Peak-level DNA synthesis occurred 12 h after re-feeding in the control and insulin-treated cultures (Figure 1). Insulin treatment had little effect on peak levels of DNA synthesis relative to the control. Ethanol exposure (100 mM) delayed peak DNA synthesis by 12 h, but had no significant effect on the total duration of DNA synthesis relative to control cultures.

Effects of insulin and ethanol treatments on the expression of different NTP species in PNET2 cells

Five different species (~38, ~26, ~21, ~18 and ~15 kDa) of NTP molecules were detected by metabolic labelling and immunoprecipitation, or by Western blot analysis under non-reducing conditions of PNET cell lysates using a cocktail of Th7+Th9+Th10 monoclonal antibodies, as described previously [5]. Antibody specificity was demonstrated by the failure to detect NTP molecules when non-relevant monoclonal antibodies to desmin were substituted for the Th monoclonal antibodies. In addition, NTP molecules could not be immunoprecipitated from lysates of non-relevant hepatocellular carcinoma cell lines (results not shown).

NTP expression was sharply modulated by treatment with insulin and by the proliferation status of the cells. Low levels of NTP were observed in overnight serum-starved and in 96 h confluent cultures, both of which contained non-proliferating PNET2 cells. Within 24 h of re-feeding with complete medium, all cultures became proliferative, and the levels of NTP expression increased, regardless of treatment (Figure 2). However, PNET2 cells treated with 50 m-units/ml insulin expressed strikingly higher levels of the various NTP species compared with control or ethanol-treated cultures. At the same time, acute exposure to 100 mM ethanol had no effect on the levels of NTP expression relative to the control.

Inhibition of insulin-mediated up-regulation of NTP expression by prior treatment with 100 mM ethanol

Within 20 min of insulin stimulation, the levels of the 38, 18 and 15 kDa NTP species had increased nearly 2-fold, and further sustained increases in the expression of these NTP molecules occurred within 30–60 min. After 120 min of insulin stimulation, NTP expression/synthesis was reduced to basal levels (Figures 3a and 3b). Expression of the 26 and 21 kDa NTP molecules was not altered during this short period of insulin stimulation. A 5-day prior exposure of PNET2 cells to 100 mM ethanol completely abolished the insulin-mediated up-regulation of the 38, 18 and 15 kDa NTP species in the short-term studies. Positive control immunoprecipitation studies using antibodies to Class I antigen demonstrated approximately equal loading of protein in both the insulin-treated and ethanol+insulin-treated cultures (Figure 3a). However, for uncertain reasons, ethanol treatment consistently resulted in dissociation of Class I molecules on SDS/PAGE, although the total amount of Class I protein was unaffected as measured by densitometric scanning.

Extended periods (12–48 h) of insulin stimulation in proliferating PNET2 cultures resulted in sustained 3–5-fold elevations in the expression of all NTP molecules (Figures 4a and 4b). Treatment with 100 mM ethanol for 5 days prior to insulin stimulation resulted in marked reductions in the insulin modulation of NTP expression. However, unlike the short-term studies, the response to insulin was nearly but not completely abolished, except with respect to the 38 kDa NTP molecule. With regard to the 26, 18 and 15 kDa NTP species, ethanol pretreatment muted

Figure 1 DNA synthesis curves for PNET2 cells cultured in medium supplemented with 12% (v/v) fetal calf serum in the presence or absence of insulin (50 m-units/ml) or 100 mM ethanol

[³H]Thymidine incorporation into DNA was measured in four replicate cultures at various intervals after re-feeding following overnight serum starvation. Data represent means ± S.D.

Figure 2 Modulation of NTP protein expression by cell proliferation and insulin stimulation

PNET2 cells were subjected to overnight serum starvation, and NTP expressed was assayed 0, 24 and 96 h after re-feeding with complete medium containing no supplements (control; C), 100 mM ethanol (Et) or 50 m-units/ml insulin (I). NTP molecules immunoprecipitated from metabolically labelled cell lysates using Th monoclonal antibodies were fractionated by SDS/PAGE (top). Equivalent protein content within the cell lysates was demonstrated by the similar levels of HLA Class I antigen immunoprecipitated from the same lysates (bottom).

(by 15–20-fold) and abbreviated (by approx. 5-fold) the insulin-mediated up-regulation of these proteins compared with the findings in cultures treated with insulin alone (Figures 4a and 4b). SDS/PAGE analysis also demonstrated that all NTP species in the insulin-stimulated control cultures, except for the 38 kDa NTP, were present as doublets or triplets, whereas in the ethanol-treated/insulin-stimulated cultures the NTP species were expressed as single bands (Figure 4a). Western blot analysis using ¹²⁵I-labelled anti-phosphotyrosine antibodies demonstrated tyrosine phosphorylation of these immunoprecipitated NTP molecules (results not shown), suggesting that the doublet

Figure 3 Short-term study of NTP modulation by insulin, and the effects of 5 days of prior treatment with 100 mM ethanol

(a) Autoradiographs demonstrating the insulin-mediated up-regulated expression of the 38, 18 and 15 kDa NTP species, and the virtual absence of such expression in insulin-stimulated cultures exposed to 100 mM ethanol. Doublet bands represent phosphorylated molecules demonstrated by binding of anti-phosphotyrosine antibody (results not shown). Lane M contains molecular-mass standards. Class I antigen immunoprecipitated from the same lysates, demonstrating similar loading in all lanes. Ethanol treatment causes dissociation of Class I molecule, but no change in the abundance of the total protein. (b) Volume densitometric analysis of the autoradiographs in (a) demonstrating 4–5-fold insulin-mediated up-regulation of three distinct NTP species and inhibition of the insulin effect by prior treatment with 100 mM ethanol.

Figure 4 Long-term study of NTP modulation by insulin, and the effects of 5 days of prior treatment with 100 mM ethanol

(a) Autoradiographs demonstrating the insulin-mediated up-regulated expression of all NTP species, and the markedly low-level expression in insulin-stimulated cells exposed to 100 mM ethanol. Class I antigen immunoprecipitated from the same lysates, demonstrating similar loading in all lanes. Ethanol treatment causes dissociation of Class I molecule, but no change in the abundance of the total protein. (b) Volume densitometric analysis of the autoradiographs in (a) demonstrating 3–5-fold insulin-mediated up-regulation of all NTP species and a markedly muted insulin effect in cells previously and continuously treated with 100 mM ethanol.

Figure 5 Demonstration of NTP expression in PNET2 cells by immunofluorescence

Cells were cultured for 24 h (**a–c**) or 96 h (**d–f**) in the presence of 12% (v/v) fetal calf serum alone (**a, d**), or containing 50 m-units/ml insulin (**b, e**) or 50 m-units/ml insulin + 100 mM ethanol (**c, f**). Insulin alone resulted in increased NTP immunoreactivity in the cell bodies and neurites (small processes) (compare **b** with **a**). Ethanol treatment abolished insulin-mediated up-regulation of NTP expression (compare **c** with **b** and **f** with **e**). In contrast, control (**g**) and ethanol-treated (**h**) 24 h (and 96 h; results not shown) cultures had similar levels of synaptophysin gene expression. Panel (**i**) shows a 24 h control culture stained with primary antibody omitted (negative control).

or triplet NTP bands in insulin-stimulated cultures represent different phosphorylated states of the NTP molecules.

Effect of insulin stimulation and ethanol treatment on immunocytochemical expression of NTPs

Immunofluorescence staining with the Th9 monoclonal antibody revealed uniform to granular cytoplasmic NTP immunoreactivity (Figure 5). Within 24 h of insulin stimulation, PNET2 cells exhibited neurite extension as previously described for other neuronal cell lines and primary fetal brain cultures [15–25,39]. Immunoreactive NTP expression was at a high level and was distributed throughout the perikarya and neurites (Figure 5a). At the same time, synaptophysin (Figure 5g) and GAP-43 (results not shown) expression were also high-level, with similar cellular distributions. After 48 h of insulin stimulation, the NTP immunoreactivity detected by the Th9 monoclonal antibody was reduced compared with 24 h cultures, and was coarsely granular and asymmetrically distributed within the cells (Figure 5b). After 96 h, the insulin-stimulated cultures exhibited relatively low levels of NTP immunoreactivity within both the perikarya and neuritic processes (Figure 5c).

Ethanol treatment prior to insulin stimulation resulted in no

distinct morphological abnormality other than an apparent reduction or absence of neurites, as reported by others [46]. There were no differences in cell attachment or viability relative to cultures not subjected to ethanol treatment. After 24 h of insulin stimulation, the levels of immunoreactive NTP expression were strikingly lower than in the non-ethanol-exposed cultures (Figure 5d). In addition, synaptophysin immunoreactivity (Figure 5h) as well as GAP-43 immunoreactivity (results not shown) were reduced relative to insulin-treated control cultures. At 48 h and beyond, the insulin-mediated up-regulation of immunoreactive NTP expression was virtually abolished by prior ethanol treatment (Figures 5e and 5f), similar to the findings in the metabolic labelling studies.

Analysis of the effects of ethanol on the insulin-mediated signal transduction pathway during NTP expression

PNET2 cells were analysed to determine the possible mechanisms of the ethanol-mediated inhibition of insulin action on NTP gene expression. Insulin-stimulated PNET2 cells, in the presence or absence of prior treatment with 100 mM ethanol, expressed similar basal levels of IR β (Figure 6). In the insulin-stimulated control cultures, the levels of IR β changed only slightly over time

Figure 6 Analysis of the insulin-mediated signal transduction pathway in PNET2 cells by immunoprecipitation and Western blot analysis

IR β and IRS-1 levels were similar in insulin-stimulated cells and in cells that were treated with 100 mM ethanol prior to insulin stimulation. However, insulin-stimulated tyrosine-phosphorylated (PY) IR β and IRS-1 were markedly decreased by pretreatment with ethanol. Consequently, the association of PI 3-kinase (PI3K) with tyrosine-phosphorylated IRS-1 was also significantly reduced in the ethanol-treated PNET2 cultures.

(60 min), whereas in the ethanol-treated cultures, IR β increased nearly 2-fold within 10 min of insulin stimulation. However, basal levels of tyrosine-phosphorylated IR β were significantly higher in the insulin-stimulated control cultures than in the ethanol-pretreated insulin-stimulated cultures. More important, insulin-mediated tyrosine phosphorylation of IR β occurred rapidly (within 10 min) in the control cultures, whereas a markedly muted and delayed response (30 min) was observed in the ethanol-pretreated cultures.

Basal levels of IRS-1 protein were similar in the insulin-stimulated control and ethanol-pretreated cultures. In addition, the levels of IRS-1 protein did not change in response to insulin stimulation in either group. Basal levels of tyrosine-phosphorylated IRS-1 were also similarly low in both control and ethanol-pretreated cultures. Within 10 min of insulin stimulation, the tyrosine phosphorylation of IRS-1 began to increase, with high levels achieved at 60 min in the control cultures. In contrast, treatment with 100 mM ethanol abolished the insulin-mediated increase in tyrosine phosphorylation of IRS-1 in PNET2 cells. Correspondingly, the association between PI 3-kinase and tyrosine-phosphorylated IRS-1 was markedly reduced in the ethanol-treated cultures. Therefore pretreatment and sustained exposure to 100 mM ethanol did not result in decreases

in the levels of the IRS-1 or IR β proteins themselves, but was associated with significant decreases in the insulin-mediated tyrosine phosphorylation of IRS-1 and IR β and in the subsequent binding of PI 3-kinase to tyrosine-phosphorylated IRS-1.

DISCUSSION

The present study demonstrates that NTP expression in PNET2 cells is exceptionally insulin-responsive, and that the insulin-mediated effects can be abrogated by prior and sustained exposure to 100 mM ethanol. The inhibitory action of ethanol was associated with the uncoupling of upstream tyrosine phosphorylation events in the insulin signal transduction cascade. PNET2 cells were derived from a human CNS primary primitive neuroectodermal tumour [58], with a neuronal phenotype demonstrated by immunoreactive expression of GAP-43, synaptophysin and neurofilament [3]. Insulin treatment of PNET2 cells resulted in prominent neurite extension and increased immunoreactive expression of synaptophysin and GAP-43, i.e. neuronal differentiation. Insulin stimulation has been demonstrated to enhance either DNA synthesis or differentiation of neuronal and glial cell lines, as well as cultured fetal brain cells [15–25,36,66]. IGF-1 and -2 are highly related members of the insulin growth factor family, and IGF molecules bind to specific receptors with high affinity to stimulate mitogenesis or differentiation of neuronal cells [23,26,67–69]. IGF-1 is abundantly present in fetal calf serum [68], and PNET2 cells probably do express IGF receptors, given the ubiquitous and abundant presence of these receptors in CNS neurons [23,26,67–71]. Since IGF-1 receptor kinases also tyrosine-phosphorylate IRS-1 [69], a synergistic effect between insulin and IGF-1 on NTP expression is possible. Previous studies have demonstrated that insulin stimulation in the absence of serum was sufficient to modulate gene expression and neuritic growth of neuronal cell lines and primary fetal neuron cultures [16,25,71]. The present studies were not conducted using serum-free medium because complete requirements for maintenance and growth of PNET2 cells under these conditions have not been determined. Abundant expression of IR β in the PNET2 cells is consistent with a direct action of insulin in modulating NTP expression and neuronal differentiation.

Ethanol at 100 mM is within the range measured in the blood of actively drinking alcoholics [72]. This level of ethanol exposure can interfere with DNA synthesis [41–44], gene expression [45–57] and cellular signalling [52,53]. In PNET2 cells, treatment with 100 mM ethanol had no significant overall effect on DNA synthesis (although attainment of peak levels was delayed), cell viability or cell surface attachment. However, ethanol treatment profoundly inhibited the insulin-mediated up-regulation of NTP expression, together with the insulin-stimulated neuritic growth and synaptophysin expression. Ethanol neurotoxicity leading to impaired neurite formation [46], inhibition of neuronal differentiation [73–76] and altered gene expression [45,47,77] has been described both *in vivo* and *in vitro*. Furthermore, in a previous study we demonstrated transient ethanol-mediated down-regulation of p36 (annexin II), another insulin-responsive gene in PNET2 cells [77]. This finding suggests that one of the major adverse effects of ethanol on neuronal cells is through the inhibition of insulin-mediated gene up-regulation.

Insulin produces wide-ranging effects on cellular metabolism, growth and differentiation. Upon insulin binding to the extracellular domain of the insulin receptor, there is rapid activation of the intrinsic tyrosine kinase of IR β [78]. The early molecular event linking the insulin receptor tyrosine kinase to its ultimate biological effect is the rapid tyrosine phosphorylation of IRS-1 [79] and p62, a ras-activating protein [80]. IRS-1 is a cytoplasmic

protein of 160–190 kDa, and is the major cellular substrate for insulin, IGF-1 [81] and interleukin-4 receptors [82]. IRS-1 contains 20 tyrosine phosphorylation consensus sequences, of which six appear in Tyr-Met-Xaa-Met motifs [83]. These tyrosine-phosphorylated domains will bind to SH₂-containing proteins such as the p85 subunit of PI 3-kinase, thereby up-regulating the kinase activity [84]. Members of the ras family, which is comprised of low-molecular-mass GTP-binding proteins, have now been shown to play an important role in mediating insulin action within the cell [30–34]. Thus insulin stimulation of cells enhances ras activity by increasing the amount of ras-GTP [33,34,85–87]. Activation of ras by this mechanism then results in the activation of several serine/threonine kinases in a cascade of events that reflects the many actions of insulin within the cell [85–89], leading to DNA synthesis, mitosis and transcription of regulatory genes such as *c-fos* [90]. Therefore the failure of ethanol-treated PNET2 cells to modulate the expression of highly insulin-responsive NTP gene(s) suggests that ethanol imposes major restrictions on events involved in cellular signalling.

The mechanism of ethanol-mediated impairment of the action of insulin on NTP gene expression was to abrogate or diminish tyrosine phosphorylation of the IR β and subsequent tyrosine phosphorylation of IRS-1. The same mechanism of ethanol toxicity was demonstrated previously in primary hepatocyte cultures [52], NIH 3T3 cells and C6 glioma cells [56,57]. Furthermore, ethanol treatment of cultured PNET2 cells not only impaired insulin-mediated tyrosine phosphorylation of IR β , but it also reduced the association of IRS-1 with PI 3-kinase, a signal transduction molecule thought to be important in transmitting the insulin signal to the nucleus for activation of genes associated with growth and differentiation [52].

The present study examined upstream steps involved in the insulin signal transduction cascade to determine the mechanism of ethanol interference with the insulin-modulated expression of NTP in PNET2 cells. These studies demonstrated that the ethanol-mediated down-regulation of NTP was associated with inhibition of the tyrosine phosphorylation of both IR β and IRS-1. The association between PI 3-kinase and tyrosine-phosphorylated IRS-1 was thereby reduced. On the other hand, expression of the IRS-1 and IR β proteins themselves was not diminished by ethanol treatment. These findings suggest that the inhibition by ethanol of the insulin-induced up-regulation of NTP gene expression and attendant neuronal differentiation may be mediated through the insulin intracellular signal transduction cascade.

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REFERENCES

- Ozturk, M., de la Monte, S. M., Gross, J. and Wands, J. R. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 419–423
- de la Monte, S. M., Ozturk, M. and Wands, J. R. (1990) *J. Clin. Invest.* **86**, 1004–1013
- de la Monte, S. M. and Wands, J. R. (1992) *J. Neurol. Sci.* **113**, 152–164
- de la Monte, S. M., Volicer, L., Hauser, S. L. and Wands, J. R. (1992) *Ann. Neurol.* **32**, 733–742
- Xu, Y.-Y., Wands, J. R. and de la Monte, S. M. (1993) *Cancer Res.* **53**, 3823–3829
- Gross, J., Carlson, R. I., Brauer, A. W., Margolies, M. N., Warshaw, A. L. and Wands, J. R. (1985) *J. Clin. Invest.* **76**, 2115–2126
- Terazono, K., Yamamoto, H., Takasawa, S., Shiga, K., Yonemura, Y., Tochino, Y. and Okamoto, H. (1988) *J. Biol. Chem.* **263**, 2111–2114
- Watanabe, T., Yonekura, H., Terazono, K., Yamamoto, H. and Okamoto, H. (1990) *J. Biol. Chem.* **265**, 7432–7439
- Lasserre, C., Christa, L., Simon, M.-T., Vernier, P. and Brechot, C. (1992) *Cancer Res.* **52**, 5089–5095
- McKee, A. C., Kosik, K. S. and Kowall, N. W. (1991) *Ann. Neurol.* **30**, 156–165
- Onorato, M., Mulvihill, P., Connolly, J., Galloway, P., Whitehouse, P. and Perry, G. (1989) *Prog. Clin. Biol. Res.* **317**, 781–789
- Scheibel, A. B. and Tomiyasu, U. (1978) *Exp. Neurol.* **60**, 1–8
- Xu, Y.-Y., Wands, J. R. and de la Monte, S. M. (1993) *J. Neuropathol. Exp. Neurol.* **52**, 291 (abstract)
- de la Monte, S. M. and Wands, J. R. (1993) *J. Neuropathol. Exp. Neurol.* **52**, 265 (abstract)
- Recio-Pinto, E., Rechler, M. M. and Ishii, D. N. (1986) *J. Neurosci.* **6**, 1211–1219
- Lenoir, D. and Honegger, P. (1983) *Brain Res.* **283**, 205–213
- Heidenreich, K. A. and Toledo, S. P. (1989) *Endocrinology (Baltimore)* **125**, 1451–1457
- Adamo, M., Raizada, M. K. and LeRoith, D. (1989) *Mol. Neurobiol.* **3**, 71–100
- Heidenreich, K. A., deVellis, G. and Gilmore, P. R. (1988) *J. Neurochem.* **51**, 878–887
- Recio-Pinto, E., Lang, F. F. and Ishii, D. N. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2562–2566
- DiCicco-Bloom, E. and Black, I. B. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4066–4070
- Burgess, S. K., Jacobs, S., Cuatrecasas, P. and Sahyoun, N. (1987) *J. Biol. Chem.* **262**, 1618–1622
- Mill, J. F., Chao, M. V. and Ishii, D. N. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7126–7130
- Pahlman, S., Meyerson, G., Lindgren, E., Schalling, M. and Johansson, I. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9994–9998
- Recio-Pinto, E. and Ishii, D. N. (1984) *Brain Res.* **302**, 323–334
- Gammeltoft, S., Ballotti, R., Nielsen, F. C., Kowalski, A. and Van Obberghen, E. (1988) *Horm. Metab. Res.* **20**, 436–442
- Yonezawa, K., Ando, A., Kaburagi, Y. et al. (1994) *J. Biol. Chem.* **269**, 4634–4640
- Baltensperger, K., Kozma, L. M., Cherniack, A. D., Klarlund, J. K., Chawla, A., Banerjee, U. and Czech, M. P. (1993) *Science* **260**, 1950–1952
- Tobe, K., Matuoka, K., Tamemoto, H. et al. (1993) *J. Biol. Chem.* **268**, 11167–11171
- Yamauchi, K., Holt, K. and Pessin, J. E. (1993) *J. Biol. Chem.* **268**, 14597–14600
- Li, N., Batzer, A., Daly, R. et al. (1993) *Nature (London)* **363**, 85–88
- Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A. and Schlessinger, J. (1991) *Cell* **65**, 83–90
- Korn, L. J., Siebel, C. W., McCormick, F. and Roth, R. A. (1987) *Science* **236**, 840–843
- McCormick, F. (1993) *Nature (London)* **363**, 15–16
- Adamo, M. L., Shemer, J., Roberts, C. T., Jr. and LeRoith, D. (1993) *Ann. N.Y. Acad. Sci.* **692**, 113–125
- Heidenreich, K. A. (1993) *Ann. N.Y. Acad. Sci.* **692**, 72–88
- Girault, J. A., Chama, B., Bertuzzi, G., Tixier, H., Wang, J. K. T., Pang, D. T. and Greengard, P. (1992) *J. Neurochem.* **2**, 518–528
- Rosen, O. M., Rubin, C. S., Cobb, M. H. and Smith, C. J. (1981) *J. Biol. Chem.* **256**, 3630–3633
- Heidenreich, K. A. and Toledo, S. P. (1989) *Endocrinology (Baltimore)* **125**, 1458–1463
- Denton, R. M. (1990) *Nature (London)* **348**, 286–287
- Wands, J. R., Carter, E. A., Bucher, N. L. R. and Isselbacher, K. J. (1979) *Gastroenterology* **77**, 528–532
- Duguay, L., Coutu, D., Hetu, C. and Joly, J. G. (1982) *Gut* **23**, 8–13
- Diehl, A. M., Thorgeirsson, S. S. and Steer, C. J. (1990) *Gastroenterology* **99**, 1105–1112
- Carter, E. A. and Wands, J. R. (1985) *Biochem. Biophys. Res. Commun.* **128**, 767–774
- Brodie, C. and Vernadakis, A. (1992) *Dev. Brain Res.* **65**, 253–257
- Dow, K. E. and Riopelle, R. J. (1985) *Science* **228**, 591–593
- Miles, M. F., Diaz, J. E. and DeGuzman, V. (1992) *Biochim. Biophys. Acta* **1138**, 268–274
- Clarren, S. K. and Smith, D. W. (1978) *N. Engl. J. Med.* **298**, 1063–1067
- Jones, K. L. and Smith, D. W. (1973) *Lancet* **ii**, 999–1001
- Clarren, S. K., Alvord, E. C., Jr., Sumi, S. M., Streissguth, A. P. and Smith, D. W. (1978) *J. Pediatr.* **92**, 64–67
- Peiffer, J., Majewski, F., Fischback, J., Bierich, J. R. and Volk, B. (1979) *J. Neurol. Sci.* **41**, 125–137
- Sasaki, Y. and Wands, J. R. (1994) *Biochem. Biophys. Res. Commun.* **199**, 403–409
- Diehl, A. M., Yang, S. Q., Cote, P. and Wand, G. S. (1992) *Hepatology* **16**, 1212–1219
- de la Monte, S. M. (1988) *Arch. Neurol.* **45**, 990–992
- Victor, M., Adams, R. D. and Mancall, E. L. (1959) *Arch. Neurol.* **71**, 579–588
- Resnicoff, M., Sells, C., Ambrose, D., Baserga, R. and Rubin, R. (1993) *J. Biol. Chem.* **268**, 21777–21782
- Resnicoff, M., Rubin, M., Baserga, R. and Rubin, R. (1994) *Lab. Invest.* **71**, 657–662

- 58 The, I., Murthy, A. E., Hannigan, G. E., Jacoby, L. B., Menon, A. G., Gusella, J. F. and Bernards, A. (1993) *Nature Genet.* **3**, 62–66
- 59 Alexander, M. C., Lomanto, M., Nasrin, N. and Ramaika, C. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5092–5096
- 60 Carter, E. A. and Wands, J. R. (1988) *Alcohol. Clin. Exp. Res.* **12**, 555–562
- 61 Nishiyama, M. and Wands, J. R. (1992) *Biochem. Biophys. Res. Commun.* **183**, 280–285
- 62 Sasaki, Y., Zhang, X. F., Nishiyama, M., Avruch, J. and Wands, J. R. (1993) *J. Biol. Chem.* **268**, 3805–3808
- 63 Karns, K. R., Ng, S.-C., Freeman, J. A. and Fishman, M. C. (1987) *Science* **236**, 597–600
- 64 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 65 Kao, K. J., Cook, D. J. and Scornik, J. C. (1986) *Blood* **68**, 627–632
- 66 Ishii, D. N., Recio-Pinto, E., Spinelli, W., Mill, J. F. and Sonnenfeld, K. H. (1985) *Int. J. Neurosci.* **26**, 109–127
- 67 Masters, B. A. and Raizada, M. K. (1993) *Ann. N.Y. Acad. Sci.* **692**, 89–101
- 68 Shemer, J., Raizada, M. K., Masters, B. A., Ota, A. and LeRoith, D. (1987) *J. Biol. Chem.* **262**, 7693–7699
- 69 Orlowski, C. C., Chernaused, S. D. and Akeson, R. (1989) *J. Cell. Physiol.* **139**, 469–476
- 70 Gluckman, P. D. (1989) *Acta Paediatr. Scand.* **349** (Suppl.), 21–25
- 71 Aizenman, Y. and deVellis, J. (1987) *Brain Res.* **406**, 32–42
- 72 Urso, T., Gavalier, J. S. and Van Thiel, D. H. (1981) *Life Sci.* **28**, 1053–1056
- 73 Dow, K. E. and Riopelle, R. J. (1990) *Neuroreport* **1**, 111–114
- 74 Bonthius, D. J. and West, J. R. (1991) *Neurotoxicol. Teratol.* **13**, 611–619
- 75 Borges, S. and Lewis, P. D. (1983) *Neuropathol. Appl. Neurobiol.* **9**, 53–60
- 76 Gressens, P., Lammens, M., Picard, J. J. and Evrard, P. (1992) *Alcohol Alcohol.* **27**, 219–226
- 77 de la Monte, S. M., Bhavani, K., Xu, Y.-Y., Puisieux, A. and Wands, J. R. (1995) *J. Neurol. Sci.* **128**, 122–133
- 78 Ullrich, A., Bell, J. R., Chen, E. Y. et al. (1985) *Nature (London)* **313**, 756–761
- 79 White, M. F., Maron, R. and Kahn, C. R. (1985) *Nature (London)* **318**, 183–186
- 80 Sung, C. K., Sanchez-Margalet, V. and Goldfine, I. D. (1994) *J. Biol. Chem.* **269**, 12503–12507
- 81 Myers, M. G., Jr. and White, M. F. (1993) *Diabetes* **42**, 643–650
- 82 Wang, L.-M., Myers, M. G., Jr., Sun, X. J., Aaronson, S. A., White, M. and Pierce, J. H. (1993) *Science* **261**, 1591–1594
- 83 Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J. and White, M. F. (1991) *Nature (London)* **352**, 73–77
- 84 Myers, M. G., Jr., Backer, J. M., Sun, X. J., Shoelson, S., Hu, P., Schlessinger, J., Yoakin, M., Schaffhausen, B. and White, M. F. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10350–10354
- 85 Porras, A., Nebreda, A. R., Benito, M. and Santos, E. (1992) *J. Biol. Chem.* **267**, 21124–21135
- 86 Kozma, L., Baltensperger, K., Klarlund, J., Porras, A., Santos, E. and Czech, M. P. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4460–4464
- 87 Medema, R. H., de Laat, W. L., Martin, G. A., McCormick, F. and Bos, J. L. (1992) *Mol. Cell. Biol.* **12**, 3425–3430
- 88 Wood, K. W., Sarnecki, C., Roberts, T. M. and Blenis, J. (1992) *Cell* **68**, 1041–1050
- 89 Haystead, C. M., Gregory, P., Shirazi, A., Fadden, P., Mosse, C., Dent, P. and Haystead, T. A. (1994) *J. Biol. Chem.* **269**, 12804–12808
- 90 Messina, J. L., Standaert, M. L., Ishizuka, T., Weinstock, R. S. and Farese, R. V. (1992) *J. Biol. Chem.* **267**, 9223–9228