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Ethanol inhibits leptin-induced STAT3 activation in Huh7 cells

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Abstract Leptin, an adipocyte-derived hormone, regulates food intake and energy expenditure in the hypothalamus via its receptor, member of the class I cytokine receptor family. Leptin resistance has been observed in rodents and in humans. However, the mechanisms could not be explained in most cases of human obesity, except for rare cases with mutations in the leptin receptor. Recent reports demonstrated that ethanol inhibited the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway activated by some members of the class I cytokine receptor family. In this study, we examined the effects of ethanol on the leptin-induced JAK/STAT signaling pathway using human hepatoma cell lines transiently expressing long form of the leptin receptor. A 30 min pretreatment with ethanol dose-dependently inhibited the leptin-induced STAT3 phosphorylation. Furthermore, to determine the time course of ethanol inhibitory effects, the cells were incubated in 10 mM ethanol for various times. Partial inhibition of leptin-induced STAT3 activation was seen after 1 min of treatment with ethanol and completely inhibited after 30 min pretreatment. SB 202190, a p38 mitogen-activated protein kinase (MAPK) inhibitor, partly prevented this inhibition by ethanol of leptin-induced STAT3 activation. These findings suggest that ethanol time- and dose-dependently inhibits the leptin action, in part via p38 MAPK.

Key words: Leptin receptor; Ethanol; p38 mitogen-activated protein kinase; JAK/STAT pathway

1. Introduction

Leptin, the product of the obese gene [1], is an adipocyte-derived hormone that plays a central role in the control of food intake and energy homeostasis [2–4]. Leptin has also been shown to have effects on peripheral cell types such as pancreatic β cells [5], endothelial cells [6], hematopoietic cells [7] and hepatocytes [8]. Leptin acts via receptors that are homologous in sequence to class I cytokine receptors. These include receptors for interleukin-6 (IL-6), leukocyte inhibitory factor (LIF), and granulocyte colony stimulating factor (G-CSF) [9,10]. The leptin receptor (OBR) gene encodes at least five alternatively spliced forms [10]. The spliced isoform OBRb, referred to as the long form, is expressed at a high level in the hypothalamus and at a low level in peripheral tissues [10,11]. OBRb has a long cytoplasmic region containing several motifs for signal transduction through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway [12]. OBRb couples ligand binding to the induction of tyrosine phosphorylation through its association with JAK2. Receptor dimerization leads to JAK2 association, tyrosine phosphorylation and activation of JAK2. The activated JAK2 phosphorylates a distal tyrosine (Y1138) on the receptor, as well as STAT proteins. Although leptin appears to activate STAT1, STAT3, STAT5 and STAT6, leptin only activates STAT3 in the rodent hypothalamus [13]. The db/db mice and Zucker rats (fa/fa), lacking functional leptin receptors, have distinctive resistance to leptin [12,14]. Although Clement et al. [15] identified mutations of the human OBR gene, they are extremely rare [16]. In obese humans, elevated serum leptin concentrations suggest reduced sensitivity to leptin [17]. Several studies provided the mechanism for leptin resistance [18–20]. However, the molecular basis for leptin resistance in most cases of obesity in both humans and rodents is still unknown. Several orexigenic molecules have been reported and some of them antagonize leptin action in vivo [2,21,22]. Little is known about the direct interaction between these candidates as leptin, and leptin receptor-mediated signals. Alcohol, when consumed as an aperitif, generally increases appetite. There have been several studies concerning the effects of ethanol intake on plasma leptin levels in rodents and humans [23,24]. However, the effects of ethanol on leptin receptor signals remain unknown. Recently, Chen et al. [25] reported that ethanol inhibits IL-6-induced STAT3 activation in rat hepatocytes. In a more recent report, DeVito et al. [26] demonstrated that prolactin (PRL)-induced JAK2 activation was blocked by ethanol in rat astrocytes. The molecular mechanisms for these effects of ethanol have not been established. Several in vitro studies have demonstrated that ethanol stimulates the activity of important kinases, including protein kinase C (PKC) [27], p44/42 mitogen-activated protein kinase (MAPK) [28] and p38 MAPK [29]. In this study, we examined the effects of ethanol and p38 MAPK on the leptin-induced JAK/STAT pathway in cultured human hepatoma cells.

2. Materials and methods

2.1. Materials

Recombinant mouse leptin was obtained from R&D Systems (Minneapolis, MN, USA). The JAK2 expression vector was a generous gift

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Abbreviations: JAK, Janus kinase; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; OBRb, long form leptin receptor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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of Dr. Rikiro Fukunaga (Osaka University, Osaka, Japan). All reagents for cell culture and transfection were obtained from Life Technologies (Gaithersburg, MD, USA). Anti-JAK2, anti-STAT3 and anti-phospho-leptin receptor (Y1138) antibodies were from Upstate Biotechnology (Lake Placid, NY, USA). Anti-phosphospecific STAT3 antibody was from New England Biolabs (Beverly, MA, USA). Anti-phosphospecific JAK2 antibody was from Bio Source (Camarillo, CA, USA). Anti-leptin receptor antibody was a generous gift of Dr. Radek C. Skoda (Basel University, Basel, Switzerland). Nitrocellulose membranes were from Scleicher and Schuell (Keene, NH, USA). The enhanced chemiluminescence (ECL) detection system, anti-rabbit IgG-conjugated horseradish peroxidase was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). SB 202190, a p38 MAPK inhibitor, was purchased from Calbiochem (La Jolla, CA, USA).

2.2. Cell culture and transient transfection

Huh7 cells were grown in RPMI supplemented with 5% fetal calf serum, 50 U/ml penicillin G and 50 μg/ml streptomycin under 5% CO_2. 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 10% fetal calf serum, 50 U/ml penicillin G and 50 μg/ml streptomycin under 5% CO_2. The cDNA encoding mouse leptin receptor long form was generated as described previously [30]. For Western blotting experiments, cells were grown in six-well plates and transfected using 5 μl of LipofectAMINE (Life Technologies, Rockville, MD, USA) and 2 μg of DNA per well. 48–72 h after transfection, including 15 h of serum deprivation, the cells were incubated for 15 min with 1 nM leptin as previously described [31]. Cells were then washed three times with ice-cold phosphate-buffered saline (PBS) and dissolved in lysis buffer (1% Nonidet P-40, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 20 mM Tris).

2.3. Immunoprecipitation and immunoblotting

The cell lysates were centrifuged at 20 000 × *g* at 4°C for 20 min. The supernatants were incubated with anti-OBR antibody and protein A agarose beads on a rotating wheel at 4°C overnight. Immune complexes were collected by centrifugation and washed three times in lysis buffer before the samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/0.1% Tween 20 (TBST) at room temperature for 2 h and then incubated with antibodies in 5% milk overnight at 4°C. After three washes, for 5 min each in TBST at room temperature, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (1:1000) in 1% milk for 1.5 h at room temperature and again washed three times with TBST. The targeted proteins were detected using enhanced chemiluminescence (ECL) following the instructions of the manufacturer. The membranes were stripped in Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

3. Results and discussion

Recent reports demonstrated that ethanol inhibited the JAK/STAT pathway activated by some members of the class I cytokine receptor family [25,26]. In the present study, we examined the effects of ethanol on the leptin-induced JAK/STAT signaling pathway using human hepatoma cell lines. We tested for the presence of endogenous functional leptin receptors. The untransfected Huh7 were stimulated with 1 nM leptin for 5–30 min at room temperature and cell lysates were subsequently analyzed by Western blotting. Neither anti-phospho-STAT3 (Tyr705) nor anti-phosphotyrosine antibodies detected any specific bands in leptin-stimulated untransfected cells (data not shown). We transiently transfected OBRb cDNA to Huh7 cells. The Huh7 cells expressing OBRb were stimulated with 1–100 nM leptin at room temperature for 5–30 min. The cell lysates were immunoblotted with anti-phospho-STAT3. The phosphorylated endogenous STAT3 bands were detected after 5 min of leptin stimulation and peaked at 15 min (data not shown). To determine the dose response of the inhibitory effects of ethanol on leptin-induced STAT3 phosphorylation, the cells expressing OBRb were preincubated with 0–100 mM ethanol for 30 min at 37°C, followed by leptin stimulation for 15 min at room temperature. After a 30 min pretreatment with ethanol, 50% inhibition was observed at 0.1 mM and complete inhibition at 100 mM ethanol, indicating a dose-dependent inhibition of the leptin-induced STAT3 phosphorylation (Fig. 1A). Furthermore to determine the time course of ethanol inhibitory effects on leptin-induced STAT3 phosphorylation, the cells were incubated in 100 mM ethanol for various times. Partial inhibition of STAT3 activation was seen after a 1 min pretreatment with ethanol, and completely inhibited after 30 min pretreatment (Fig. 1B). To test whether ethanol decreased levels of STAT3 protein in Huh7 cells, we stripped and reprobed both membranes with anti-STAT3 antibodies. Ethanol did not affect the amounts of STAT3 protein (Fig. 1B). These findings suggest that ethanol time-dependently attenuates leptin-induced STAT3 phosphorylation in Huh7. Leptin binding to OBRb results in the tyrosine phosphorylation of tyrosine residues on the intracellular domain of OBRb. We investigated the effects of ethanol on the tyrosine phosphorylation of OBRb. 293 cells transiently expressing OBRb were pretreated with or without 100 mM ethanol for 30 min, followed by leptin stimulation for 10 min at room temperature. The clarified cell lysates were subjected to immunoprecipitation and immunoblotting. In 293 cells expressing OBRb, ethanol inhibited the tyrosine phosphorylation of OBRb as well as the leptin-induced STAT3 phosphorylation (Fig. 2A, B). Chen et al. [25] have shown that ethanol rapidly and dose-dependently

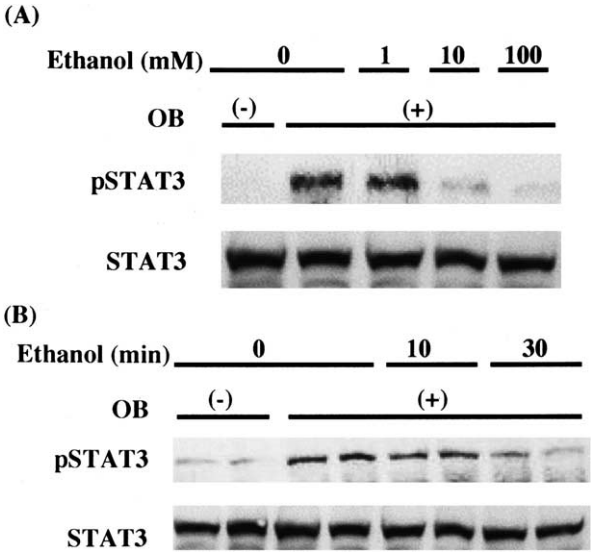

Fig. 1. Ethanol inhibits leptin-induced STAT3 activation in a dose- and time-dependent manner. A: Huh7 cells expressing OBRb were incubated with various concentrations of ethanol for 30 min at 37°C, and subsequently stimulated with 1 nM of leptin for 15 min. Cell extracts were separated by 8% SDS–PAGE and analyzed by immunoblotting with an anti-phosphospecific STAT3 antibody, then with an anti-STAT3 antibody. B: Huh7 cells expressing OBRb were incubated with 100 mM of ethanol for various times at 37°C, and subsequently stimulated with 1 nM of leptin for 15 min.

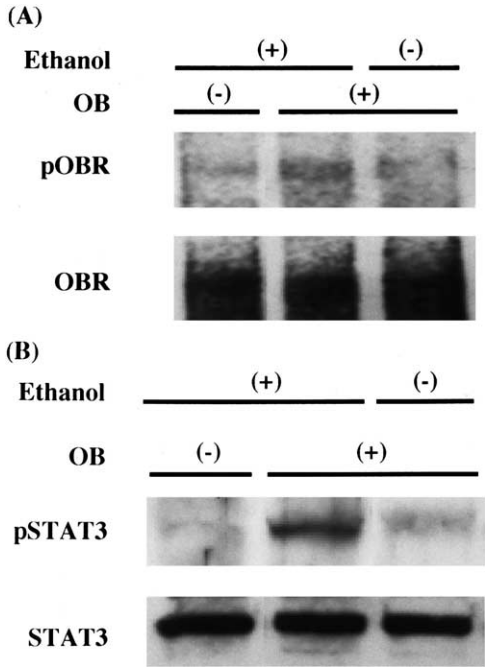

Fig. 2. Ethanol inhibits leptin-induced OBRb phosphorylation. A: 293 cells expressing OBRb were incubated with 100 mM ethanol for 30 min at 37°C, and subsequently stimulated with 1 nM of leptin for 10 min. Cell extracts were immunoprecipitated with anti-OBR antibody and immunoblotted with anti-phospho-OBR antibody. B: 293 cells expressing OBRb were incubated with 100 mM ethanol for 30 min at 37°C, and subsequently stimulated with 1 nM of leptin for 15 min. Cell extracts were immunoblotted with anti-phospho-STAT3 or STAT3 antibody.

inhibited IL-6-induced STAT3 activation in isolated rat hepatocytes. The inhibitory effects of ethanol on STAT3 phosphorylation were not due to inhibition of JAK2 activation. In this study, IL-6-induced STAT3 activation was completely inhibited at 100 mM ethanol and only slightly at 10 mM ethanol. We showed that leptin-induced STAT3 activation was completely inhibited by ethanol at lower concentration (10 mM). This concentration in blood is within the physiological relevant range, and is associated with impaired mental capabilities and coordination in humans. Agapejev et al. [32] measured the concentration of ethanol in samples of blood and CSF from patients under pathological conditions. In their study, the concentration of ethanol in blood was similar to that in CSF. These results suggest that ethanol affects the leptin effects in peripheral tissue, as well as the central nervous system. It has been reported that ethanol inhibited the prolactin-induced activation of JAK2, and subsequently, inhibited STAT3 phosphorylation in cultured astrocytes [26]. Recently, a family of cytokine-inducible proteins has been identified that appear to act as negative regulators for cytokine receptor signaling [33]. Bjorbaek et al. reported that leptin induced the expression of SOCS-3 mRNA in the hypothalamus [19] and the expression of SOCS-3 inhibited leptin-induced JAK2 activation in CHO cells [34]. To investigate whether ethanol inhibits leptin-induced JAK2 phosphorylation, Huh7 treated with 100 mM ethanol were immunoblotted with anti-phoshospecific JAK2 ($Y^{1007/1008}$). Ethanol did not affect leptin-induced tyrosine phosphorylation of JAK2 in Huh7 cells (Fig. 3). No inhibitory effects of ethanol on JAK2 phosphorylation have been seen in 293 cells expressing OBRb (data not shown). Phosphorylated tyrosine at position 1007 is critical for JAK2 activity [35]. The results suggest ethanol directly attenuates leptin-induced STAT3 and OBRb phosphorylation without inhibition of JAK2. We then examined whether the leptin-induced STAT3 phosphorylation could recover after the removal of ethanol from the medium. The cells were treated with 100 mM of ethanol for 30 min to inhibit the leptin-induced STAT3 phosphorylation, then washed three times with PBS and cultured for various times in serum-free RPMI 1640. The leptin-induced STAT3 phosphorylation gradually returned to the same level as ethanol-untreated cells, 12 h after the removal of the ethanol from the medium (Fig. 4). The mechanism of the inhibitory effects of ethanol on leptin-induced STAT3 phosphorylation was then investigated. Since these effects occurred so rapidly we speculated that the mechanism does not require de novo protein synthesis. Ethanol has been shown to modulate the activity of several protein kinases, including PKC [27], JNK [36] and MAPK [37]. It has been reported that PKC inhibitor prevents ethanol inhibition of IL-6-induced STAT3 activation in rat hepatocytes [25]. To determine whether the inhibitory effects of ethanol on leptin-induced STAT3 phosphorylation are

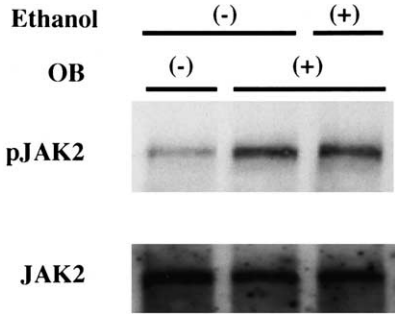

Fig. 3. Ethanol does not inhibit leptin-induced JAK2 phosphorylation. Huh7 cells were co-transfected with OBRb cDNA (1.5 μg) and JAK2 cDNA (0.5 μg). Cells expressing OBRb and JAK2 were incubated with 100 mM of ethanol for 30 min at 37°C, and subsequently stimulated with 1 nM of leptin for 5 min. Cell extracts were separated by 8% SDS–PAGE and analyzed by immunoblotting with an anti-phosphospecific JAK2 antibody.

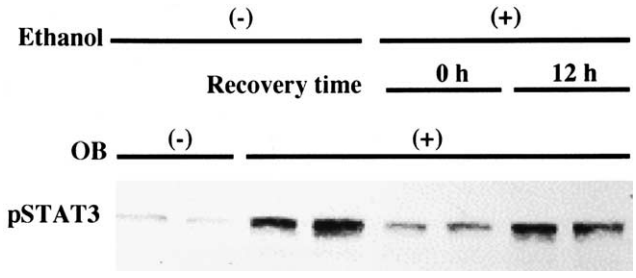

Fig. 4. Inhibitory effects of ethanol are reversible. Cells were incubated with 100 mM of ethanol for 30 min to inhibit the leptin-induced STAT3 phosphorylation and cultured for various times in serum-free RPMI 1640. The leptin-induced STAT3 phosphorylation returned to the same level as ethanol-untreated cells 12 h after the removal of the ethanol from the medium.

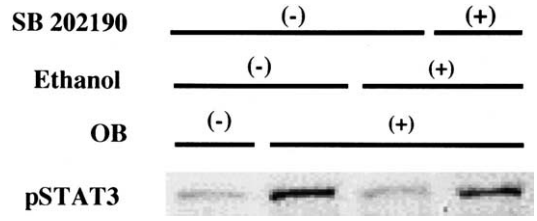

Fig. 5. SB 202190 partly blocks the ethanol inhibition of leptin-induced STAT3 activation. Cells were incubated with 1 μM of SB 202190 for 45 min prior to treatment with ethanol. Cells were stimulated with 1 nM of leptin for 15 min. Cell extracts were separated by 8% SDS–PAGE and analyzed by immunoblotting with an anti-phosphospecific STAT3 antibody.

mediated through these kinases, we investigated the inhibitory effects of ethanol using the specific inhibitors of PKC and MAPK. Rottlerin, an inhibitor of PKC, and PD 98059, an inhibitor of MEK, had no effects on the ethanol inhibition of leptin-induced STAT3 activation (data not shown). In contrast, when 1 μM of SB 202190, a p38 MAPK inhibitor, was added to the cells prior to treatment with ethanol, SB 202190 partially blocked the ethanol inhibition of leptin-induced STAT3 activation (Fig. 5). SB 202190 by itself had no effects on the STAT3 phosphorylation. These findings suggest that p38 MAPK is involved in the ethanol inhibition of leptin-induced STAT3 activation. p38 MAPK is known to be associated with inflammatory cytokines and environmental stresses. A recent study has identified p38 MAPK activated by ethanol in perfused rat liver cells [29]. In this study, p38 MAPK was rapidly activated in response to isoosmotic cell swelling induced by ethanol. In the central nervous system, the activation of p38 MAPK has been demonstrated, and is considered to be a part of the neuronal stress response [38]. Thus ethanol may inhibit leptin action via p38 MAPK in neuronal cells expressing OBRb. To test this hypothesis, further studies will be required in vivo. In summary, we examined the effects of ethanol on the leptin-induced JAK/STAT signaling pathway in Huh7 cells transiently expressing OBRb. The leptin-induced STAT3 phosphorylation was dose- and time-dependently inhibited by pretreatment with ethanol. Ethanol has no effect on the amount of STAT3 protein or leptin-induced JAK2 phosphorylation. p38 MAPK may play a role in these inhibitory effects of ethanol on leptin-induced STAT3 phosphorylation.

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