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Effect of leptin on liver alcohol dehydrogenase

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Abstract

The effect of leptin on liver alcohol dehydrogenase (ADH) was determined in male rats. Administration of one or three daily doses of leptin (1 μg/g of body weight intraperitoneally) increased ADH activity. Leptin enhanced ADH synthesis without an effect on ADH degradation. Leptin did not change ADH mRNA, indicating that the effect of leptin in enhancing ADH occurs at the post-transcriptional level. Leptin increased eukaryotic initiation factor (eIF) 2α, eIF2B activity, and the eIF4E–eIF4G complex, while it decreased the inhibitory complex of eIF4E with the eIF4E-binding protein-1 (4E-BP1). Leptin increased mammalian target of rapamycin (mTor) that phosphorylates 4E-BP1. In conclusion, leptin increases liver ADH activity and ADH protein due to an increase in synthesis which occurs at the post-transcriptional level. The effect of leptin in enhancing translational initiating factors may be of significance in the regulation not only of ADH but also of many other proteins.

Keywords: Alcohol dehydrogenase; Leptin; Eukaryotic initiation factors; Mammalian target of rapamycin

Liver alcohol dehydrogenase (ADH:alcohol:NAD oxidoreductase, EC 1.1.1.1) is the principal enzyme responsible for ethanol oxidation. Liver alcohol dehydrogenase (ADH) is affected by a variety of hormones. Stress, which stimulates the hypothalamo–hypophyseal–adrenocortical axis and the sympathetic nervous system, increases the enzyme activity [1]. GH increases liver ADH due to increased synthesis that is initiated at the level of transcription [2,3]. The effects of stress and GH were associated with increases in the rate of elimination of ethanol [1–3].

Endotoxin originating from intestinal bacteria is an important mediator of hepatocellular inflammation in the intragastric feeding rat model of alcoholic liver disease [4,5]. We recently demonstrated that lipopolysaccharide (LPS), the endotoxin component of gram-negative bacteria, increased liver ADH [6]. An increase rate of formation of acetaldehyde (AC) caused by an enhanced ADH activity may contribute to worsening of alcoholic liver injury caused by endotoxin.

Leptin is a cytokine that belongs structurally to the long-chain helical cytokine family, which includes IL-3, IL-11, and IL-12, and GH [7]. Leptin was shown to enhance liver fibrosis produced by a single dose of CCl_4 or by chronic administration of thiocetamide [8,9]. Administration of LPS to rats increases plasma leptin [10]. Since GH and LPS both increase ADH, it is quite likely that leptin has a similar effect. An increase in ADH with increased formation of AC is a potential mechanism for enhancement of fibrogenesis by leptin in alcoholic liver disease.

The purpose of this study was to determine the effects of leptin on liver ADH. This study shows that leptin enhances liver ADH and that this effect occurs at the post-transcriptional level.

Methods

Animals and materials. Male Sprague–Dawley rats were obtained from Charles River Laboratories, Wilmington, MA. All animals received humane care in compliance of the guidelines from the Animal Care and Use Committee of the Johns Hopkins University. Murine leptin was obtained from Biomol, Plymouth Meeting, PA. Sterile [4–5-^{3}H]L-leucine and [^{14}C]sodium bicarbonate ($NaH^{14}CO_3$) were obtained from ICN

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Biomedicals, Irvine, CA. [8-^{3}H]Guanosine 5′-diphosphate ([^{3}H]GDP) was obtained from Amersham Biosciences, Piscataway, NJ.

Animal treatment. Rats received intraperitoneal (i.p.) injections of leptin (1 μg/g of body weight) either once or for 3 consecutive days, while controls received isovolumetric amounts of saline. The animals were sacrificed at 6 and 24 h after one injection or 2 h after the third daily injection. Approximately 400–500 mg of the liver was homogenized in 4 vol of 0.25 M sucrose in 0.1 M Tris–HCl buffer, pH 7.4, centrifuged at 10,000*g* for 10 min, and the resulting supernatant was centrifuged at 100,000*g*. The 100,000*g* supernatant (cytosol) was used immediately for the determination of ADH activity. The remainder of the supernatant was frozen at −80 °C for determinations of LDH activity and ADH protein. One section of 1.0–1.2 g of the liver was processed for RNA isolation.

ADH activity. ADH activity was determined in the liver cytosol at 37 °C by the method of Crow et al. [11]. The reaction mixture was 1.0 ml and consisted of 0.5 M Tris–HCl, pH 7.2, 18 mM ethanol, 2.8 mM NAD^+, and 0.01 ml of the cytosol. Blank reactions were run without ethanol. One unit of enzyme activity is defined as the formation of 1 μmol of NADH per min. ADH activity was expressed per mg of protein. Cell protein was determined by the method of Lowry et al. [12]. LDH activity was determined by the method of Plagemann et al. [13].

Immunoreactive protein of ADH. Immunoreactive ADH protein was assayed by quantitative enzyme linked immunosorbent assay (ELISA) as described previously [14]. The antisera to the enzyme were produced in rabbits by subcutaneous injections of purified ADH in Freund's adjuvant. The antisera are purified with protein A–Sepharose CL-4B affinity chromatography prior to use in the assay.

Determination of messenger RNA (mRNA) by quantitative real time PCR. Total cellular RNA was isolated using the guanidine isothiocyanate procedure of Chomcynski and Sacchi [15] as described previously [3]. The concentration of the isolated RNA was determined from the optical density at 260 nm and its purity from the 260/280 ratio. The isolated RNA was initially stored at −80 °C. The cDNA template was synthesized with the Superscript III first-strand synthesis system (InVitrogen, Carlsbad, CA) using 5 μg of total RNA. ADH mRNA was determined by the TaqMan gene expression assay of Applied Biosystems, Foster City, CA. Glyceraldehyde 3 phosphate dehydrogenase (GADPH) mRNA was used as a reference control. The gene-specific oligonucleotide primers for rat ADH (#Rn01522111) and for rodent GADPH (#4308313) were obtained from Applied Biosystems. The reaction conditions were 50 °C for 2 min, 95° for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To quantify ADH gene expression, the $\Delta\Delta C_t$ method was used to calculate relative fold changes normalized against GADPH gene expression.

ADH synthesis and degradation. To determine the effect of leptin on ADH synthesis, 16 male Sprague–Dawley rats were fasted overnight following which 8 were injected with leptin, 1 μg/g body weight i.p., while 8 were injected with isovolumetric amounts of saline. Six hours later [4–5-^{3}H]L-leucine (100 μC/μmol per 100 g body weight in a 0.1 ml vol) was injected i.p. The animals were sacrificed 1 h after the isotope injection. The livers were removed and homogenized in 4 vol of 0.25 M sucrose in 0.1 M Tris–HCl buffer, pH 7.4, containing a proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 0.1 mM dithiothreitol (DDT), and 1 mM phenylmethanesulfonyl fluoride (PMSF). The homogenate was centrifuged and the liver cytosol was prepared as described above. A portion of the cytosol was used for the assay of ADH activity and the remainder was frozen at −80 °C for determination of the incorporation of radioactive leucine into ADH.

For the determination of ADH turnover, 40 rats received a single i.p. injection of 1.0 mCi of $NaH^{14}CO_3$ (55 mCi/mmol) per 100 g of body weight as described previously [16]. Starting immediately after the injection of the isotope, leptin (1 μg/g body weight) was given i.p. each day to one-half the rats, while the other one-half were given isovolumetric amounts of saline. Eight animals, 4 injected with leptin and 4 controls, were sacrificed on days 1, 2, 3, 4, and 5 after the administration of the isotope. The livers were processed to isolate the liver cytosol as described above.

To measure the incorporation of the isotopes into ADH protein, 400 μL of cytosol was precipitated with antibody to ADH. The antigen–antibody complex was allowed to form for 2 h at 25 °C. The antigen–antibody complexes were precipitated from the incubation mixture with protein A bearing *Staphylococcus aureus* cells (Pansorbin, Calbiochem, San Diego, CA). The precipitate was centrifuged at 12,000*g* for 3 min and the pellet was resuspended in 0.05 M Tris–HCl buffer, pH 7.4, containing 300 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1% Na deoxycholate, and 0.025 M sucrose [17]. The pellet was precipitated and washed 3 times in the same buffer, and then resuspended in 0.125 M Tris–HCl buffer, pH 6.2, containing 6 M urea, 3% sodium dodecyl sulfate, and 5% mercaptoethanol and heated for 5 min at 95 °C. After cooling, the suspension was centrifuged and the supernatant was subjected to SDS–polyacrylamide electrophoresis [16]. The gels were fixed in a solution of 28% trichloroacetic acid and stained with Coomassie brilliant blue R-250. The protein bands corresponding to the 40,000 molecular weight subunit of ADH were identified from the electrophoresis of the purified enzyme [17]. The gels were sliced, incubated into Protosol, and counted in Ecolite (ICN, Biochemicals, Aurora, OH). The radioactivity incorporated into ADH was corrected for background radioactivity in adjacent slices not containing proteins. In the case of the ADH turnover study with $NaH^{14}CO_3$, the fractional rates of degradation (K_d) and synthesis (K_s) were obtained from the slopes of the regression lines of the decreases in total and specific radioactivity, respectively [18]. The absolute rate of synthesis (V) was obtained by multiplying K_s by the total liver ADH activity.

Western blot analysis of eukaryotic initiation factors (eIFs). Liver was homogenized in 7 vol of buffer composed of 20 mM Tris–HCl, pH 7.4, 250 mM sucrose, 100 mM KCl, 1 mM DDT, 50 mM NaF, 1 mM PMSF, 1 mM benzamidine, and 0.5 mM vanadate. The samples were centrifuged to 10,000*g* for 10 min and the determinations were done with the resulting supernatant as described by Lang et al. [19]. The samples were mixed with 2× Laemmli SDS buffer boiled, centrifuged, subjected to electrophoresis at 60 mA in a 12.5% polyacrylamide gel, and transferred to nitrocellulose membranes. The membranes were washed and subsequently blocked with (5% wt/vol) non-fat milk in 25 mM Tris–HCl, pH 7.4, and the membranes were incubated with goat polyclonal antibodies to either eIF2α, phosphorylated eIF2α or eIFB2ε (Cell signaling Technology, Beverly, MA) at 4 °C overnight. After repeated washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution, Amersham Life Science) at room temperature for 1 h. The membranes were washed and visualized by enhanced chemiluminescence reaction (ECL-plus, Amersham Biosciences).

Phosphorylation of mTOR. The phosphorylation of mTor was determined in the 10,000*g* liver supernatants as described by Kimball et al. [20]. The Western blots were carried out with rabbit polyclonal mTor and phospho-mTOr (Ser 2448) antibodies (Cell Signaling Technology).

Quantitation of eIF4E–eIF4G and eIF4E–4E-BP1complexes. The associations of eIF4E with eIF4G or 4E-BP1 were determined as described by Kimball et al. [20]. Briefly, eIF4E was immunoprecipitated from the 10,000*g* liver supernatant with rabbit polyclonal eIF4E antibody (Santa Cruz), the precipitate resolved by SDS–PAGE, and transferred to nitrocellulose membranes. One set of membranes was re-probed with rabbit polyclonal anti-eIF4G antibody, and another set of membranes re-probed with rabbit 4E-BP1 (both from Santa Cruz). The membranes were visualized by the enhanced chemiluminescence reaction. The blots were later inactivated and re-probed with anti-eIF4E antibody. The values obtained with the anti-eIF4G and anti-4E-BP1 antibodies were normalized to eIF4E present in the samples.

Determination of eIF2B activity. Liver eIF2B activity was determined in 10,000*g* liver supernatants from the rate of exchange of [^{3}H]GDP in the eIF2 [^{3}H]GDP complex for non-radioactively labeled GDP as described by Kimball et al. [21,22]. A binary complex of eIF2 and [^{3}H]GDP was formed by incubation for 10 min at 30 °C in a mixture containing 50 mM MOPS, pH 7.4, 100 mM KCl, 1 mM DDT, 200 μg/ml bovine albumin, 1.3 μM [^{3}H]GDP (10.7 Ci/mmol), and recombinant human liver eIF2α (Cell Sciences, Canton, MA). Two millimolar magnesium acetate was added and the binary complex was placed on ice. A 35 μL aliquot of the liver supernatant was mixed with 140 μL of a reaction buffer containing 50 mM Mops, pH 7.4, 209 μM GDP, 2 mM magnesium acetate, 100 mM