ETHANOL CONSUMPTION AFFECTS STRESS RESPONSE AND INSULIN BINDING IN TISSUES OF RATS

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Objective. The mechanisms of harmful effect of excessive chronic ethanol intake on cardiovascular and neuronal systems, metabolic processes and protective effects of low ethanol intake on cerebrovascular and cardiovascular disturbances are not yet known. The aim of present study was to investigate the effect of short-term and long-term ethanol consumption on food intake, plasma levels of corticosterone (CS), glucose (G) and insulin (INS) in intact rats and in animals exposed to immobilization stress (IMO) or restrain stress (RESTR). The insulin binding to specific membrane receptors in adipocytes, muscle and liver was also determined.

Methods. Adult male rats were fed liquid diet without and with ethanol (5 % per weight) for 9-12 days (short-term intake) and at the end the animals were exposed to acute IMO stress. The second group of rats was fed solid diet and without or with ethanol in drinking water (6 % per volume) for 52 days (long-term ethanol intake). A part of these animals was exposed to repeated restrain stress during 42 days. The groups of pair fed rats with the same food intake as in ethanol diet fed animals were in both experiments. The food intake, plasma glucose, insulin and corticosterone levels were determined. Plasma cell membranes were isolated from adipose, liver and skeletal muscle tissues and insulin binding to specific receptors was determined.

Results. Decreased food consumption was observed after ethanol intake. Increased plasma G and CS were noted in rats fed ethanol diet for a short time. Plasma insulin levels were not affected by ethanol intake. Exposure to IMO stress resulted in increase of plasma G levels in controls and pair fed groups. A higher increase of CS plasma levels after IMO stress was noted in rats with ethanol intake for a short-time, however, no changes of plasma CS were noted after repeated exposure to restrain stress. Plasma levels of insulin were decreased after IMO and restrain stresses, while in rats fed ethanol diet for a short-time insulin decrease was deeper as compared to controls. The binding capacity of insulin receptors in adipose tissue was elevated in rats fed ethanol diet and deep decrease of insulin binding was noted in rats exposed to stress. In liver insulin binding was elevated after short-term ethanol intake and stress exposure resulted in decrease of insulin binding in ethanol fed rats. The binding capacity of insulin receptors in skeletal muscle was not changed in rats fed ethanol diet.

Conclusions. The results showed 1. differences in short term and long term ethanol intake on basal glucose, insulin and corticosterone levels, 2. effects of ethanol intake on changes of insulin plasma levels and insulin binding in adipose and liver tissues after exposure to stress, 3. effects of short term ethanol intake on the response of plasma hormones to immobilization stress.

Key words: Ethanol intake - Stress response - Glycemia - Insulinemia - Insulin biding - Rats

Excessive and chronic alcohol intake is considered as one of risk factors for the onset and development of hypertension, arteriosclerosis, liver and brain damages with consequent neurological disturbanc-

es, diabetological complications, (KAO et al. 2001; JOHNSON et al. 2001; HASSAN et al. 2002). On contrary, several epidemiological studies have shown, that low and moderate alcohol consumption elicits favorable effects as for lower myocardial attacks and diminished complications of diabetes mellitus type II (TANASESCU et al. 2001; LEE et al. 2001; HU et al. 2001). However, the mechanism of harmful effects of long lasting excessive alcohol abuse on the functions of liver, heart, immune system, central and peripheral neural system as well as protective effects of moderate alcohol intake on cardiovascular and cerebrovascular disturbances are not yet known (KLATSKY et al. 1990; WOOD et al. 1996; KOOB 1996; KHISTI et al. 2002). Acute alcohol intake decreases lipid oxidation favoring fat deposition, however, chronic excessive alcohol abuse results in metabolic disturbances with body weight loss and diminution in of adipose tissues. Significant reduction in body mass increment in rats was found after drinking ethanol in tap water in previous experiment (STRBAK et al. 1998). It was repeatedly demonstrated that insulin has stimulatory effect on lipogenesis in adipocytes and the changes of insulin action and insulin binding could affect the accumulation of lipids in fat depots (FICKOVA and MACHO 1983; MACHO et al. 2000). High ethanol intake induces insulin resistance (ONISHI et al. 2003) and decreases insulin stimulated glucose uptake in rat adipocytes (RACHDAUI et al. 2003), however; light to moderate alcohol consumption is associated with enhancement of insulin sensitivity (FURUYA et al. 2003). The main problems of individual differences in alcohol tolerance and of multiple effects of ethanol on cellular metabolism still remain unresolved. There are not uniform data concerning ethanol intake effects on mechanism of insulin action (FURUYA et al. 2003) and on the insulin binding in target tissues.

Ethanol itself, besides its metabolic effects is also a stressogenic factor and in combination with other stressor the effects are more probably additive than antagonistic (POHORECKY 1990; ALKANA et al. 1996). It was reported that ethanol intake in rats resulted in increase of catecholamine and corticosterone response to stress (TAYLOR et al. 1982; TIAGARAJAN et al. 1989; WEINBERG et al. 1996). Because high plasma levels of glucocorticoids decrease the sensitivity of tissues to insulin (KAHN et al. 1981; CARO and ARMATRUDA 1982) the exaggerate response to stress after ethanol intake could affect the insulin binding and insulin receptors in insulin target tissues. The aims of our experiments were to study the effects of short-term or long-term ethanol consumption in rats on plasma levels of glucose, insulin and corticosterone, on insulin binding in selected tissues in intact and in animals exposed to stress (immobilization and restrain stress).

Materials and Methods

Experiment A: Short term ethanol intake. Adult male Sprague-Dawley rats (Charles River Laboratories, Germany, initial BW 361±3 g) were fed commercially produced (Bioserve, Frenchtown, NJ, USA) liquid diet ad libitum: 1. control liquid diet (CLD), 2. ethanol liquid diet (ELD, 5 % ethanol per weight in diet, intake ad libitum, mean daily ethanol intake 0.88 g/100 g BW), 3. pair fed rats (PF1**,** offered an amount of CLD diet equal to amount consumed by ethanol group). The animals were on these diets for 9-12 days, diets were isocaloric per volume and the amount of consumed diet was registered daily. Half number of the animals from each group (16-20 animals per group) was exposed to immobilization stress (IMO, KVETNANSKY and MIKULAJ 1970) for 2 hours. The animals were sacrificed immediately after the IMO stress; blood was collected for plasma hormone level analysis. Liver, muscle (m. quadriceps) and epidydimal fat pads were removed and immediately frozen in liquid air. Plasma membranes were isolated from these tissues for determination of insulin binding capacity of specific receptors.

Experiment B: Long term ethanol intake. Adult male rats (Sprague-Dawley rats, Harlan Laboratories, USA, initial BW 258±4 g) were fed: 1. control solid diet and drinking water ad libitum (C); 2. ethanol in drinking water (6 %), solid diet ad libitum (E**,** mean daily intake of ethanol was 0.45 g/100 g BW); 3. pair fed group (PF2**)**, receiving average amount of food consumed by ethanol group on previous day, tap water ad libitum. Animals were fed these diets for 52 days, 20 animals per group. Half number of rats from each group was exposed to restrain stress (GLAVIN et al. 1994) for two hours daily (restrain in wire mesh cylinders, 6 cm in diameter), during 42 days. The animals were sacrificed immediately after

last stress exposure; blood, muscle (m. quadriceps) and epidydimal fat pads were collected for biochemical analysis. Procedure for immobilization and protocols for ethanol intake have been previously approved by the Animal Care Committee at the Institute of Experimental Endocrinology.

Plasma insulin (Linco- Ria kit, St. Charles, MO, USA) and corticosterone (Sigma Biosciences, USA) levels were determined by radioimmunoassay, glucose by enzymatic assay (Super GL, Germany). Plasma membranes were isolated from rat tissues according to SAKAMOTO et al. (1980) and binding of insulin was determined according to ZORAD et al. (1985). The binding capacity of insulin receptors was calculated from multilinear regression curves by a computer fitted program LIGAND (MUNSON and RODBARD 1980).

Results

Lower food consumption (124±2 ml/day in CLD and 62±2 ml/day in ELD) and lower body weight gain (34±3 g CLD and 5±2 g ELD) were observed after short term ethanol intake as compared to control animals fed *ad libitum*. Increased levels of glucose and slight elevation of corticosterone in plasma were noted in rats fed ethanol diet for a short time with higher daily ethanol intake (Table 1).

Increases of plasma glucose levels were observed in control and pair fed rats after exposure to acute immobilization stress. The high glucose levels in ethanol fed rats were not additionally elevated after exposure to IMO stress (Table 1). The marked increase of corticosterone plasma levels was observed after IMO stress in all groups, however; the highest values of plasma corticosterone were noted in ELD fed animals (Table 1). Plasma levels of insulin were slightly decreased after exposure to IMO stress in control and pair fed rats, however, a deep decrease of plasma insulin was noted in animals fed ethanol diet with higher ethanol daily consumption (Table 1).

After long-term ethanol intake, body weight gains (115±4 g in C and 111±5 g in E) were similar in control and ethanol groups, however, decreased solid food intake (27±2 g per day in C and 20±2 g per day in E) was noted in animals with ethanol in drinking water. Plasma insulin, glucose and corticosterone levels were not affected by this lower daily ethanol intake even for a longer time (Table 2). Only a slight increase of corticosterone in plasma was noted in pair fed rats.

Table 1
Plasma levels of glucose, insulin and corticosterone in control (CLD), in pair fed animals (PF1) and in rats with short term ethanol intake (ELD)

	Intact			Immobilized		
Group	Glucose	Insulin	Corticosterone	Glucose	Insulin	Corticosterone
CLD	7.7 ± 0.4	1.04 ± 0.32	73 ± 24	9.9 ± 0.5^{+}	0.82 ± 0.13	479 ± 44^{+}
ELD	13.4 ± 1.6§	1 06 ± 0.25	154 ± 51	12.5 ± 1.0	0.27 ± 0.05$^{+\S}$	726 ± 138$^{\S+}$
PF1	7.3 ± 0.4	0.77 ± 0.10	72 ± 20	12.3 ± 1.0^{+}	0.49 ± 0.05^{+}	620 ± 130^{+}

INTACT – resting conditions, IMO – immobilization stress, corticosterone in nmol/ L, insulin in nmol/ L, glucose in mmol/ L , CLD – control liquid diet, ELD – control liquid diet with 5% of ethanol per weight for period of 9-12 days, PF 1 – animals fed CLD but in the amount of intake of ethanol group, § CLD to ELD $p < 0.05$; + Intact to IMO $p< 0.05$

Table 2
Plasma levels of glucose, insulin and corticosterone in controls (C) in pair fed (PF2) and in rats with long term ethanol intake (E) before and after exposure to stress

	Intact			Restrain stress		
Group	Glucose	Insulin	Corticosterone	Glucose	Insulin	Corticosterone
C	8.2 ± 0.2	0.36 ± 0.05	92 ± 25	8.1 ± 0.3	0.25 ± 0.03^{+}	127 ± 21
E	7.8 ± 0.2	0.36 ± 0.06	90 ± 29	7.6 ± 0.3	0.24 ± 0.03^{+}	107 ± 31
PF2	8.3 ± 0.1	0.27 ± 0.01	146 ± 26	8.3 ± 0.2	0.24 ± 0.01	105 ± 15

Corticosterone in nmol/ L, insulin in nmol/ L, glucose in mmol/ L, C - controls fed with solid diet ad libitum, E – ethanol group, fed with solid diet and ethanol in drinking water (6 % per volume), PF2 pair fed group, fed with solid diet in the amount equal to the intake of ethanol group, § C to E $p< 0.05$; + Intact to Restrain stress $p< 0.05$

The repeated exposure of animals to restrain stress (RESTR) has no effect on plasma glucose levels (Table 2). However, a moderate decrease in plasma insulin levels was noted after RESTR stress. No significant changes of plasma levels of corticosterone levels after repeated restrain stress exposure were observed (Table 2).

The binding of insulin to specific plasma membrane receptors was estimated in insulin target tissues to evaluate if the alcohol intake could affect the insulin action. Both, high affinity-low capacity (R1) binding sites and low affinity- high capacity (R2) binding sites were calculated. No changes in R1 sites were observed in fat tissue after ethanol intake or exposure to IMO stress. However, a small increase of R2 sites capacity was noted in adipocytes from animals fed ethanol diet for a short time (Fig. 1). Significant decrease of insulin binding to R2 sites was found in all groups after exposure to IMO stress (Fig. 1).

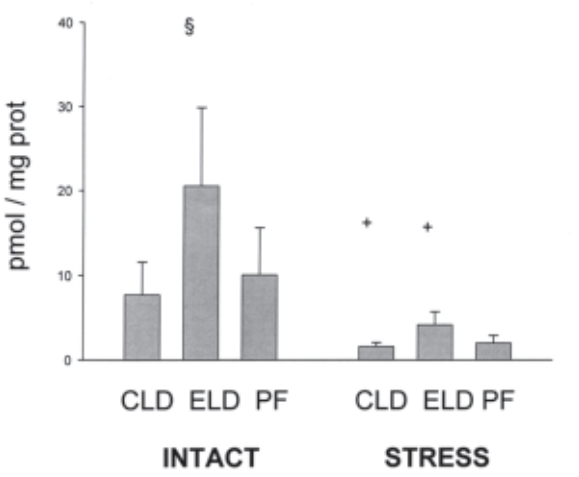

Fig 1 Binding of insulin to low-affinity high capacity binding sites of membranes from adipose tissue of intact rats (INTACT) and animals exposed to immobilization stress (STRESS). CLD – control liquid diet, ELD – liquid diet with 5% of ethanol per weight, for the period of 9-12 days, PF – animals fed CLD in the amount consumed by ethanol group. § CLD : ELD $p < 0.05$, + Intact to Stress $p < 0.05$.

Short-term ethanol intake resulted in increase of insulin binding to R1 and R2 sites in rat liver. Exposure to stress resulted in decrease of insulin binding to R2 sites in rats with ethanol intake, but no significant changes in R1 and R2 binding sites were noted in control and pair fed groups (Fig.2).

The bindings of insulin to R1 sites in adipose tissues were elevated in rats fed ethanol diet for a long time and in pair fed group (Fig. 3). A significant diminution of R1 sites was noted after exposure to repeated restrain stress in ethanol fed and pair fed rats. No significant changes of insulin binding to R2 sites of adipocyte membranes were found in ethanol and pair fed rats (pmol/mg prot C- 1.76±0.08, E- 1.76±0.60, PF2- 1.40±0.71). No changes of insulin binding to R2 sites of adipocytes were observed after exposure to repeated stress.

Insulin binding to specific receptors (R1 and R2 sites) was not changed in skeletal muscles after long-term ethanol intake or exposure to repeated restrain stress (data not presented).

Discussion

These results showed that there are differences in the effects of short-term or long-term ethanol consumption (in various doses of daily intake) on plasma glucose levels. The acute or short-term ethanol intake seems to result in elevation of glycemia, probably due to decrease in glucose uptake (Pennington et al. 1995) and in the reduction of insulin mediated glucose utilization (Avogaro et al. 1987; Onishi et al. 2003). The results of plasma glucose determinations after chronic ethanol intake in this experiment are in agreement with our previous observations (Strbak et al. 1998) demonstrating, that four-week ethanol consumption did not affected plasma glucose levels. It was noted that chronic ethanol feeding decreases insulin stimulated glucose uptake in adipocytes (Wilkes et al. 1996; Rachdaoui et al. 2003), depressed a rate of local glucose utilization in discrete brain regions (Smith et al. 2001), and diminished the incorporation of glucose into hepatocytes (VanHorn et al. 2001). In the view of these results we have expected the increase of plasma glucose levels in rats after chronic ethanol consumption. On other hand several human and animal studies suggest, that light to moderate chronic ethanol consumption is associated with enhancement of insulin sensitivity (Tanasescu et al. 2001; Furuya et al. 2003). Low ethanol consumption for four weeks resulted in rats in the same blood glucose profile during the intravenous glucose tolerance tests and showed an elevation of plasma glucose disappearance rate during the intravenous insulin tolerance test (Furuya et al. 2003). Because the different levels of daily ethanol intake could have various metabolic implications, further studies are necessary to ex-

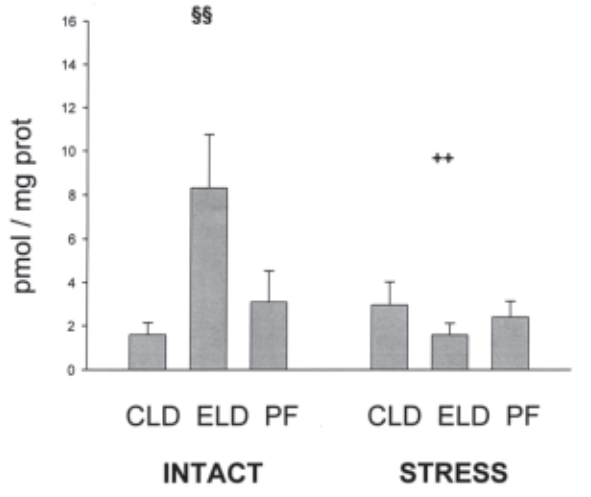

Fig 2 Binding of insulin to low-afinity high capacity binding sites in liver membranes of intact rats and animals exposed to immobilization stress. INTACT- non stressed rats, STRESS – rats exposed to stress for 2.5 hours. CLD control liquid diet, ELD – liquid diet with 5% of ethanol for period of 9-12 days, PF – pair fed group, rats fed CLD in the amount of food intake of ethanol fed animals. §§ CLD:ELD $p < 0.01$, ++ Intact to Stress $p < 0.01$

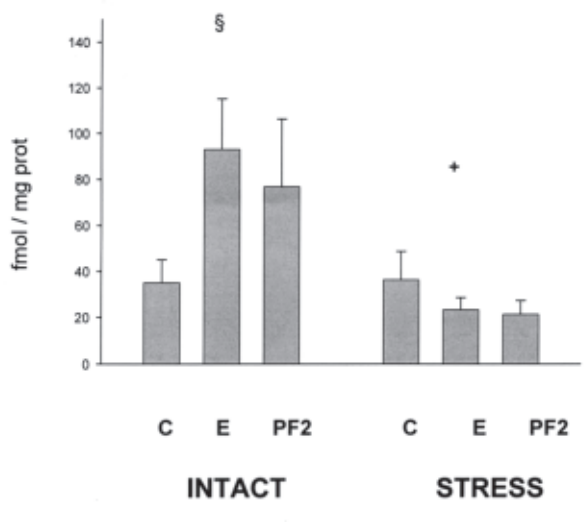

Fig 3 Binding of insulin to high-affinity low capacity binding site in adipose tissue of intact rats (INTACT) and animals exposed to restrain stress 2 hours daily for period of 42 days (STRESS). C – control solid diet, E – fed by control solid diet but ethanol 6% per volume in drinking water, PF2 – to animals offered control solid diet but in the amount of intake of ethanol group. § C:E $p< 0.05$, + Intact : Stress $p < 0.05$

plain the causes of unchanged plasma glucose levels after chronic ethanol intake in contrast to the effect of short-term ethanol intake.

Plasma levels of insulin were not affected by ethanol intake. Slight, however not significant, decrease of insulinemia was noted in pair fed rats. This observation is in agreement with the results of our previous experiment demonstrating that four-week ethanol consumption resulted in unchanged insulin plasma levels but significant reduction in insulin values was noted in pair fed rats (STRBAK et al. 1998; BENICKY et al. 2000). Unchanged insulin plasma levels in ethanol fed rats were found in spite of the fact that an ethanol induced beta-cell dysfunction was suggested in chronic alcoholics (PATTO et al. 1993). Because an inhibition of insulin uptake by liver was observed in the presence of ethanol (FAWCETT at al. 1993) it is probable that in spite of ethanol induced beta- cell dysfunction, the normal insulin plasma levels could result due to lower uptake of insulin during the transport through liver.

Ethanol intake affected the binding of insulin to specific receptors in adipocytes and hepatocytes. The increased binding of insulin to cell membranes of adipocytes after the ethanol intake was not followed by augmentation of lipids in fat depots (MACHO et al. 2000; CORNIER et al. 2000) in spite of the fact that it was repeatedly demonstrated that insulin had stimulatory effect on lipogenesis in adipocytes. (MACHO et al. 2000). These results are in agreement with previous observations in rats (STRBAK et al 1998) and in human subjects (SHELMET et al. 1998) that after chronic ethanol intake the body mass and the contents of lipids in fat depots are lowered. It was observed that increase insulin binding after ethanol intake did not change the glucose transporter content GLUT 4 in isolated adipocytes (FICKOVA et al. 1998). The observation of the function of insulin receptors in cell culture showed a decrease of phosphorylation of insulin receptor tyrosine kinase after the addition of ethanol to cultivation medium (SEILER et al. 2000), suggesting that the stimulation of lipogenesis could be lowered due to changes of insulin action at post receptor levels by the action of ethanol. Also the phosphorylation of purified insulin receptor tyrosine kinase activity was inhibited by ethanol (SEILER et al. 2000). Further it was reported that ethanol impairs insulin receptor substrate –1 mediated signal transduction in hepatocytes and neuronal cells (SASAKI et al. 1994; XU et al. 1995) affecting the stimulatory action of insulin on metabolism in these tissues. Therefore the increased binding of insulin is probably not followed

by elevated insulin stimulatory action in tissues of ethanol diet fed rats.

Because ethanol could be stressogenic factor (CICERO 1981; GUAZA et al. 1983; RIVIER et al., 1989) and in combination with other stressors the effects are more probably aditive than antagonistic (POHORECKY 1990; ALKANA et al. 1996), the plasma corticosterone levels were determined in rats after short-term and long-term ethanol intake. An elevation of basal plasma corticosterone values was noted in short-term ethanol fed rats. No changes of plasma corticosterone levels were observed after long-term ethanol consumption. This is in agreement with the results of previous experiment demonstrating that four-week ethanol intake did not changed the basal plasma corticosterone levels (STRBAK et al. 1998). Also the repeated exposure to restrain stress was not followed by changes in corticosterone plasma values suggesting the adaptation of hypothalamo-pituitary adrenocortical axis of rats to this kind of stress.

Very interesting are results of the studies of late effects of prenatal exposure of fetal rats to ethanol, which demonstrate that there are increased responses to various stressogenic stimuli in adult rats being prenatally exposed to ethanol (WEINBERG et al. 1996; TAYLOR et al. 1982; HANNIGAN et al. 1985). Mechanisms of this prenatal exposure to ethanol on stress response and behavior of animals is not explained. Administration of a single dose of ethanol to adult rats (by intragastric cannula implanted one week prior to application of ethanol) induced a rapid increase of ACTH and prolonged elevation of corticosterone. Also the levels of epinephrine and norepinephrine were elevated more than sixty minutes after the ethanol administration (TIAGARAJAN et al. 1989). The results of our experiments showed that short-term ethanol consumption affected the response of plasma corticosterone, insulin and insulin binding to stress exposure. The elevations of plasma corticosterone after immobilization were exaggerated in ethanol diet fed animals. The decrease of plasma insulin was deeper in ethanol group exposed to immobilization. These results suggested a hypersensitivity hypothalamo-pituitary-adrenal system and endocrine pancreas of rats to stress after short-term ethanol intake.

It has been repeatedly demonstrated that high plasma levels of glucocorticoids result in lower response of tissue metabolism to insulin (OLEFSKY et al. 1975; DE PIRRO et al. 1980; KHAN et al. 1981; CARO and ARMATRUDA 1982). This could be related to the effect of glucocorticoids on insulin binding (OLEFSKY et al. 1975; DE-PIRRO et al. 1980; FANTUS et al. 1981). We have found in previous experiments that glucocorticoids participate in the regulation of insulin receptors in adipocytes of rats exposed to hypokinetic stress (MACHO and FICKOVA 1992; MACHO et al. 1999). In present experiments the decrease of insulin binding capacity (R2 receptors) in membrane from adipose and liver tissues was observed immediately after exposure to immobilization stress and this is probably related to the elevation of plasma corticosterone levels. The changes in insulin binding in adipose and liver tissues of rats exposed to immobilization stress can not be explained by down regulation of insulin receptors by plasma insulin levels (LANE 1981), because no elevation, but a decrease of plasma insulin levels was observed in short-term ethanol diet fed rats after exposure to acute stress.

In conclusion, short term ethanol intake affected plasma G and CS levels and the response of plasma CS and INS to IMO stress. Ethanol intake increased the insulin binding capacity of adipose and liver tissues, however, the insulin receptors in skeletal muscle were not changed by chronic ethanol consumption. Chronic ethanol intake did not affect plasma glucose, insulin and corticosterone levels. Repeated restrain stress in intact and chronic ethanol fed rats did not change the plasma glucose and corticosterone levels suggesting the adaptation to this stress. However, the insulin plasma levels and insulin binding in adipose tissue were lower in ethanol fed rats after exposure to stress.

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