Biochemical Pharmacology, Vol. 32, No. 20, pp. 3059–3063, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

INFLUENCE OF CHRONIC ETHANOL TREATMENT ON ALPHA$_1$-ADRENERGIC AND VASOPRESSIN RECEPTOR-STIMULATED PHOSPHATIDYLINOSITOL SYNTHESIS IN ISOLATED RAT HEPATOCYTES

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(*Received* 4 *November* 1982; *accepted* 11 *March* 1983)

Abstract—Adult male rats were given nutritionally balanced high fat (34% corn oil) diets containing either ethanol or isocalorically substituted sucrose for 4–5 weeks. Phosphatidylinositol–phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine contents of whole hepatocytes were not altered in ethanol-treated rats. However, vasopressin and alpha$_1$-adrenergic stimulation of [^{32}P]-incorporation into phosphatidylinositol of isolated hepatocytes from ethanol-treated rats was increased substantially compared to that of controls. Yet, chronic ethanol treatment had no effect on hepatic alpha$_1$ receptor density or affinity as measured by [^{3}H]prazosin specific binding. These results suggest that the supersensitive phosphatidylinositol response to this alpha$_1$ agonist observed in hepatocytes from ethanol-treated rats occurred distal to cell surface receptors and may be similar for vasopressin.

Stimulation of phosphatidylinositol (PhI) degradation with its compensatory resynthesis is coupled to the activation of a wide variety of cell surface receptors in several different tissues and is thought by some investigators to be a mechanism whereby cell-surface calcium permeability and intracellular calcium mobilization are regulated [1–4]. This phenomenon, commonly known as the "PhI effect," is generally quantitated by monitoring receptor stimulated incorporation of [^{32}P] into PhI. In some tissues, receptor activation also stimulates the turnover of phosphatidic acid as well as polyphosphoinositides. [^{32}P]-Incorporation into other phospholipids such as phosphatidylcholine and phosphatidylethanolamine is not altered by receptor activation [5, 6]. In isolated rat hepatocytes, the PhI effect can be elicited by such glycogenolytic hormones as vasopressin, angiotensin and epinephrine [3, 5]. It has been postulated that activation of glycogen phosphorylase by these hormones is due to a rise in intracellular free [Ca^{2+}] consequent to PhI degradation [3]. However, this claim has been refuted recently. Others have shown that the kinetics of the PhI effect in hepatocytes appear to be too slow to play a role in either Ca^{2+} mobilization or its resulting metabolic effects, i.e. activation of glycogen phosphorylase activity [7]. In addition, these authors have demonstrated that the PhI effect in hepatocytes is calcium dependent. Thus, the physiological relevance of the PhI effect remains controversial.

The nature of the biochemical coupling of cell-surface receptors to the activation of enzymes responsible for accelerated PhI degradation and resynthesis is also not well understood. Even less is known about possible dietary influences on the hepatic PhI effect. Since it is well known that chronic ethanol intake leads to considerable alterations in hepatic lipid composition [8: review] as well as function [9, 10], it was of interest to study the PhI effect in rat hepatocyte preparations whose lipid composition was altered as a result of chronic consumption of a high-fat containing ethanol liquid diet. We have reported previously that this diet results in increases in hepatic phospholipid fatty acid saturation as well as dramatic increases in cholesterol [11].

METHODS

Male Sprague–Dawley rats weighing 150–200 g were housed individually and pair-fed for 4–5 weeks nutritionally balanced liquid diets of which 34% of the total caloric content was derived from corn oil [12]. The amount of ethanol added was 36% of the total caloric intake. In control animals, sucrose was substituted isocalorically for ethanol. Animals consumed ethanol at approximately 12 g per kg per day.

The isolation of parenchymal cells has been described previously [13]. Freshly isolated hepatocytes were resuspended to a final concentration of 45 mg wet wt tissue/ml in 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing: NaCl, 144 mM; KCl, 5 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2.5 mM; and glucose, 5 mM. Representative aliquots (200 μl) of this cell suspension were added to vials containing 50 μl [^{32}P] (5–10 μCi/vial) in the above buffer plus drugs when indicated. All assays were run in triplicate. Cells were incubated with shaking at 37° for 30 min. The reaction was stopped with the addition of 5 ml $CHCl_3$–CH_3OH (2:1) containing 0.02% butylated hydroxytoluene (BHT). The lipid extract was first partitioned with 1 ml of 10 mM NaH_2PO_4 dissolved

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in 0.1 M HCl. The subsequent lower phase was then washed an additional six times with $CHCl_3$–CH_3OH–H_2O (3:48:47, by vol.) to remove trace amounts of unincorporated [^{32}P]. Phosphatidylethanolamine (PhE), phosphatidylinositol–phosphatidylserine (PhI–PhS), and phosphatidylcholine (PhC) were isolated on silica gel H plates with $CHCl_3$–CH_3OH–CH_3COOH–H_2O (25:15:4:2, by vol.) [14] which contained 0.02% BHT. Individual lipid spots were visualized with iodine vapors, scraped directly into vials and counted by liquid scintillation spectroscopy. In pilot studies employing two-dimensional TLC to separate PhS from PhI, it was found that receptor stimulation of [^{32}P]-incorporation into PhS was negligible compared to that incorporated into PhI. These results were similar to those observed in pineal gland [15]. Thus, receptor-stimulated ^{32}P-labeling in the PhI–PhS spot was considered to be due to [^{32}P]-incorporation into PhI only. In other experiments, individual phospholipids from hepatocytes were isolated as above and quantitated by measuring phospholipid phosphorus [16].

The alpha$_1$-adrenergic receptor population of hepatocytes was assessed by Scatchard analysis of specific binding of [^{3}H]prazosin. Aliquots (100 μl) of tissue homogenate (40 mg/ml) were incubated with four concentrations of [^{3}H]prazosin (17.7 Ci/mmole) in a final volume of 2 ml containing 50 mM sodium potassium phosphate buffer, pH 7.4. Incubations were carried out for 30 min at 25°. Membrane bound [^{3}H]prazosin was trapped at the end of the incubation period by rapid vacuum filtration of the incubation mixture through Whatman glass fiber filters (GF/B). The filters were rinsed with three aliquots (5 ml) of 50 mM sodium potassium phosphate buffer, and trapped radioactivity was measured subsequently by liquid scintillation spectrophotometry with 45% efficiency. Binding in the presence of 10 μM phenotolamine was defined as nonspecific. Protein was measured by the method of Lowry *et al.* [17] using bovine serum albumin as the standard.

RESULTS

Chronic consumption of ethanol in a high fat liquid diet had no significant effect on either endogenous levels of the PhI–PhS mixture or that of the major phospholipids, PhC and PhE (Table 1). These results are consistent with recent reports in which rat hepatic phospholipid content was not altered after chronic treatment with ethanol containing high fat liquid diets compared to pair-fed control [11, 18].

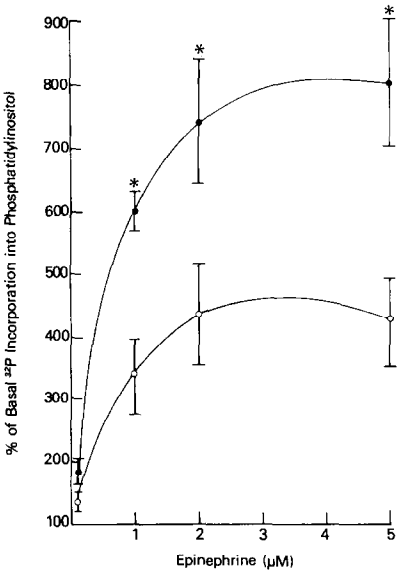

Fig. 1. Dose–response curves for (−)-epinephrine stimulation of [^{32}P]-incorporation into PhI in hepatocytes from control (○) and ethanol-treated (●) rats. Data are expressed as the means ± S.E.M. from four to five experiments, each done in triplicate. An asterisk (*) indicates $P < 0.05$.

Basal incorporation of [^{32}P] into PhI was not affected by chronic ethanol treatment. Mean values ± S.D. for control and ethanol-treated animals were 1362 ± 288 and 1627 ± 115 cpm/μg phospholipid P respectively. These counts were based on 1×10^7 cpm [^{32}P] added per incubation tube. In control hepatocytes from rats fed a high liquid diet, the stimulation of [^{32}P] incorporation into PhI by epinephrine was dose dependent and reached a maximum of over 400% of basal values in the presence of 2 μM epinephrine (Fig. 1). Maximum stimulation of 500% occurred with vasopressin at a concentration of 23 nM (Table 2). The magnitude of stimulation by each of these hormones was similar to that reported for hepatocytes of rats fed regular low fat laboratory chow [4, 5]. Compared with controls, the ability of epinephrine (Fig. 1) and vaso-

Table 1. Hepatic phospholipid levels after chronic ethanol treatment*

	Phospholipid phosphorus (μg/mg wet wt tissue)	
	Controls	Ethanol
Phosphatidylinositol–phosphatidylserine	0.195 ± 0.023	0.217 ± 0.053
Phosphatidylethanolamine	0.355 ± 0.026	0.375 ± 0.064
Phosphatidylcholine	0.595 ± 0.026	0.617 ± 0.060

* Rats were maintained on a high fat liquid diet with or without ethanol for 4–5 weeks. Individual hepatic phospholipids were isolated and quantitated as described in Methods. Results are expressed as the mean ± S.D. for two separate experiments in which phospholipid content was assayed in duplicate.

Table 2. Effect of chronic ethanol treatment on vasopressin stimulation of [^{32}P]-incorporation into phosphatidylinositol of isolated rat hepatocytes*

Vasopressin (nM)	% of basal incorporation	
	Controls	Ethanol-treated
23	513 ± 97 (4)	1088 ± 72† (4)
100	510 ± 77 (3)	1115 ± 153† (3)

* Rats were maintained on high fat liquid diets as described in Methods. Results are expressed as the mean ± S.E. from the number of individual experiments indicated in parentheses.

† Indicates $P < 0.001$ when compared to corresponding control values.

Table 3. Specific binding of [^{3}H]prazosin to membrane fragments of isolated rat hepatocytes*

	K_d (nM)	B_{max} (fmoles/mg protein)
Controls		
Experiment 1	78	73
Experiment 2	67	71
Ethanol		
Experiment 1	53	64
Experiment 2	37	69

* Male rats (150–170 g) were given a high fat liquid diet containing ethanol (36% of total caloric intake) for 4–5 weeks. Ethanolic calories were replaced by sucrose in control diets. [^{3}H]Prazosin concentrations (in triplicate) were 0.125, 0.25, 0.50 and 1.00 nM. Results were determined using nonlinear least squares fit of the data. One control and one ethanol-treated animal were used for each experiment.

pressin (Table 2) to stimulate [^{32}P] incorporation into PhI was increased markedly in hepatocytes from ethanol-treated rats. Receptor-stimulated [^{32}P]-incorporation into PhI was not associated with an increase in [^{32}P]-incorporation into phosphatidylcholine in either controls or ethanol-treated rats in the present study. The control data are in agreement with previous reports [5, 6]. To determine if the supersensitive response to epinephrine in ethanol-treated rats was due to an alteration in alpha$_1$-adrenergic receptor affinity or density, saturation studies of specific [^{3}H]prazosin binding to hepatocyte membrane fragments were performed. From subsequent Scatchard analyses, no change in either maximum binding (B_{max}) or the dissociation constant (K_d) as a result of chronic ethanol feeding was observed (Table 3). Thus, the supersensitive response to epinephrine observed in Fig. 1 appears to be due to an alteration which is distal to the cell-surface receptor.

DISCUSSION

We as well as others have reported substantial increases in hepatic cholesterol levels in rats chronically fed ethanol high fat diets [11, 19]. In addition, the degree of fatty acyl saturation of various phospholipids is also increased under these conditions [11]. However, neither total phospholipid content [11] nor the individual phospholipid content measured in the present study was altered as a result of chronic ethanol consumption. This is in agreement with a recent report in which hepatic mitochondrial phospholipid content was unaltered in rats offered an ethanol high fat diet [18]. However, others have reported increases in total hepatic and mitochondrial phospholipid content after chronic administration of ethanol high fat diets [12, 20]. The reasons for this discrepancy are unclear.

In rat hepatocytes, activation of cell-surface receptors by alpha-adrenergic agonists, vasopressin and angiotensin II enhances PhI degradation and concomitant PhI resynthesis as measured by [^{32}P]-incorporation [2, 4, 21]. In rats chronically treated with an ethanol high fat diet, labeling of PhI in response to either epinephrine or vasopressin was approximately twice that observed in control animals. Interestingly, PhI degradation has been shown recently to occur within the plasma membrane [21, 22] even though phosphatidylinositol phosphodiesterase, the enzyme which catalyzes this reaction, is found mainly in the cytosol [23]. In addition, PhI resynthesis, the event measured in the present investigation, is though to occur within the endoplasmic reticulum where CDP-diacylglycerol:inositol phosphatidyltransferase is located [1]. To our knowledge, the influence of the immediate lipid environment on CDP-diacylglycerol:inositol phosphatidyltransferase activity has not yet been documented. However, it is known that phosphatidylinositol phosphodiesterase activity can be modulated by a number of different lipids [24–26] and is Ca^{2+} dependent [27]. Since chronic ethanol consumption increases hepatic Ca^{2+} binding [28] and, in addition, has multiple effects on hepatic lipid metabolism [8, 29], one could speculate that the supersensitive PhI response to hormones observed in the present study is a reflection of activation by an altered biophysical environment of one or more enzymes involved in receptor-stimulated PhI metabolism. Alternatively, an increase in Ca^{2+} binding seen after chronic ethanol treatment may be sufficient to increase the sensitivity of phosphatidylinositol phosphodiesterase to activation by hormones. The substantially decreased glycogen content previously observed in livers of ethanol-treated animals [30, 31] is probably not a result of the supersensitive PhI effect observed in the present study since others have reported that hormonal activation of glycogen phosphorylase and phospholipid turnover are most likely two independent phenomena [7]. Thus, while the physiological relevance of the PhI effect is still to be determined, we have demonstrated that this system is substantially altered after chronic ethanol treatment and may influence hepatic function.

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