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PANCREATIC REGENERATION AFTER CHRONIC ETHANOL FEEDING IN RATS

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Increase of phosphatidic acid (PA) accumulation in response to caerulein (Cae) and after subtotal pancreatectomy (SP) has been previously described and phospholipase D (PLD) derived PA involvement in pancreatic regeneration was suggested. We also described decrease of Cae stimulated PA accumulation after a single dose of ethanol (both *in vitro* and *in vivo*). The present study was undertaken in order to determine the influence of chronic ethanol feeding on basal and Cae stimulated PA accumulation and other parameters of pancreatic regeneration in control and ethanol feed rats. Male rats were pair fed *ad libitum* with an isocaloric liquid diet (Lieber De Carli) with or without ethanol. *In vitro* study: pair fed rats were killed after 2 or 6 weeks of feeding, pancreata were dissected out and weighted, dispersed pancreatic acini were then prepared and loaded with ^{3}H myristic acid in order to label the phosphatidylcholine pool. Phosphatidic acid (^{3}H PA) accumulation, in the presence of propranolol, was measured after stimulation with increasing doses of Cae. *In vivo* study: PA was measured 3 days after SP or sham operation in both groups of rats, and also after 1 h of Cae infusion (0.25 μg/kg/h). Pancreatic weight, amylase, protein, RNA and DNA content were established. Results: *In vitro* study 1) Basal PLD activity expressed as PA accumulation was significantly elevated after 6 weeks of ethanol feeding in comparison to the control values (28%). 2) Cae in doses ranging from 100 pM to 5 nM was not able to stimulate PA accumulation in isolated pancreatic acini prepared from ethanol fed rats. *In vivo* study: 1) Body weight and pancreatic weight were similar in, both the ethanol fed and the control groups, after 2 and 6 weeks. 2) Ethanol feeding significantly decreased total amylase content in the pancreas, but did not change protein, RNA and DNA content. 3) in contrast to the control animals in which SP caused a 71.1% increase of PA accumulation over the sham operation, subtotal pancreatectomy was not able to stimulate PA in rats fed with ethanol. 4) Also Cae infusion did not stimulate PA accumulation in the ethanol fed animals in comparison to the controls. Since the involvement of PLD activation in the early stages of pancreatic regeneration was postulated, our results suggest that chronic ethanol feeding can influence this process by decrease of PA production, probably because of the inhibition of hydrolytic PLD activity in the presence of ethanol. This could be one of the mechanisms responsible for pancreatic injury after chronic ethanol consumption.

Key words: *ethanol feeding, regeneration, phosphatidic acid production, phospholipase D activation*

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INTRODUCTION

Ethanol abuse is the most common cause of pancreatitis, yet the mechanism by which alcohol injures the pancreas remains unknown (1—3). Three major hypotheses have been proposed in the pathogenesis of alcoholic pancreatitis: the large duct hypothesis, the small duct hypothesis (ductal plug hypothesis), and the toxic metabolite hypothesis (4, 5). Certain toxic effects of ethanol have been attributed to oxidative metabolite of alcohol, acetaldehyde. Because acetaldehyde is primarily generated in the liver and the pancreas shows only minimal oxidative ethanol metabolism, therefore acetaldehyde induced pancreatic damage is considered unlikely (6, 7). Recently it was shown that pancreatic injury may be induced by fatty acid ethyl ester, a nonoxidative metabolite of alcohol (7—9). On the other hand it is known that ethanol exerts its pharmacological effects on the lipids of the cell membrane and can cause alteration in the function of integral membrane proteins (10, 11).

It was also shown that the injury to the pancreatic tissue during acute pancreatitis or some other destructive processes is followed by a spontaneous reparative/regenerative process (12, 13), however the mechanisms and agents involved in tissue recovery are still poorly clarified (14, 15). The data concerning pancreatic regeneration after chronic ethanol feeding are few and controversial (16, 17). On the other hand phospholipase D (PLD) activation in the exocrine pancreas has been described (18). PLD in pancreatic acini can be activated by cholecystokinin (CCK) (18), growth factors (19) or oleate (unpublished). This enzyme attacks the phospholipid substrate to form a transient phosphatidyl-PLD intermediate. With water as an acceptor, phosphatidic acid is produced, however in the presence of ethanol phosphatidylethanol (PEt) is generated and this reaction is known as transphosphatidylation. The functional significance of transphosphatidylation is unknown. PA has been recognized as a mitogenic factor in some cell systems, acting by calcium mobilization. Regarding the pancreas, an increase of PA production was observed after Cae infusion or in response to growth -associated processes stimulated by pancreatic juice diversion, subtotal pancreatectomy or in the regenerative period after acute pancreatitis (20, 21). In the presence of ethanol, inhibition of PA production in the pancreas after acute alcohol ingestion was shown *in vitro* and *in vivo* (22). It was suggested, that the reduction of PA accumulation contributes to ethanol-induced impairment of pancreatic regeneration, especially since ethanol concentration achievable *in vivo* in the blood of humans provides sufficient acceptor to support transphosphatidylation.

However there was no data concerning the influence of chronic ethanol feeding on pancreatic regeneration, PLD activation and PA production.

In the light of these information and concepts the aim of the present study was to investigate:

1. The influence of chronic ethanol feeding on basal and Cae-stimulated PA production *in vitro* in isolated rat pancreatic acini

2. The influence of chronic ethanol feeding on PA production in response to growth-stimulated processes as subtotal pancreatectomy or intravenous Cae infusion at a trophic dose (0.25 μg/kg/h)

3. The influence of chronic ethanol feeding on the other trophic parameters in the pancreas.

MATERIALS AND METHODS

Materials

Bovine serum albumine (BSA; fraction V and fatty acid free), soybean trypsin inhibitor type 2-S (SBTI), N-2-hydroxylethyl piperazine-N^1-2-ethane sulfonic acid (HEPES), standards and solvents for thin-layer chromatography for enzymes and nucleic acid determination were purchased from Sigma (ST. Louis, MO). Purified collagenase was from Worthington Biochemicals (Freehold, NJ). Silica gel TLC plates were obtained from Merck (Warsaw, Poland). ^3H myristic acid (56 Ci/mmol) was from Du Pont (Wilmington, DE). Caerulein (Takus) was from Pharmacia (Freiburg, Germany).

Animals

Male Wistar rats weighing 200—220 g (n = 126) were housed in separated cages in rooms maintained at 20° ± 2° C using a 12-hour dark cycle. Care was provided in accordance with the procedures outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals (Bethesda, MD).

Experimental design

Animals were pair fed *ad libitum* for 6 weeks with an isocaloric liquid Lieber DeCarli diet containing/or not ethanol in a doses of 10—12 g per day. Every week the animals were weighted. After 6 weeks of ethanol feeding the rats were decapitated, pancreas was dissected as quickly as possible, free of connective tissue, lymph nodes and weighted.

Surgical procedures

For caerulein infusion, rats were anesthetized with ketamine and jugular vein was cannulated with Silastic catheter. The dilutions of Cae for *in vivo* infusion were made in saline containing 0.05% BSA. Cae was infused intravenously at a dose of 0.25 μg/kg/h at rates of 1 ml/h during 1 h. For subtotal pancreatectomy the rats were anesthetized with ketamine and removal of 90% of the pancreas (including splenic, gastric, and duodenal segments) was done by gently swabbing the tissue away from its vascular and peritoneal attachments (23). Animals were decapitated 3 days after subtotal pancreatectomy.

Preparation of pancreatic acini

Acini were prepared as reported by Peikin *et al.* (24) from a 300 mg pieces of pancreatic tissue. For the *in vitro* study, acini from 5 pancreata were pooled and resuspended in 32 ml of an enriched HEPES buffered solution (in mM, 24.1 HEPES, 98 NaCl, 6 KCl, 2.1 KH_2PO_4, 0.5 $CaCl_2$, 1.2 $MgCl_2$, 5 sodium pyruvate, 5 sodium fumarate, 5 sodium glutamate, 11.4 glucose, and 0.01% (w/v)

SBTI, 0.5% (w/v) BSA fatty acid free, adjusted to pH 7.4). For the *in vivo* study, acini prepared from each animal were resuspended in 5 ml of an enriched HEPES buffered solution.

Assays

The pieces of pancreas were homogenized using a motor-driven ground-glass homogenizer either in 0.6 mol/L perchloric acid for nucleic acids determination or in ice-cold Tris-HCl buffer (pH = 8.0) for protein, α-amylase and chymotrypsin assays. Protein was determined as described by Lowry *et al.* (25), with BSA as a standard. RNA and DNA were extracted as described by Mainz *et al.* (26). DNA was determined according to Volkin and Cohn using calf thymus DNA as the standard (27). RNA was hydrolyzed overnight in 0.3 N KOH and measured by determining the absorption at 260 nm of the final 0.1 N PCA extract, as described by Munro and Fleck (28). Tissue α-amylase and chymotrypsin activities were determined according to Bernfeld and Hummel respectively (29, 30).

PA measurement

After 1 h of incubation with 5 μCi/ml of ^{3}H myristic acid, the 4 ml of acini in each flask was washed twice and resuspended in freshly oxygenated medium containing 200 μM propranolol, to favor PA accumulation. For the *in vitro* study, phosphatidylcholine (PC) hydrolysis basal and in response to different doses of Cae was measured after 20 min of incubation with Cae. For *in vivo* study acini from each pancreas were processed separately and PA was measured after 20 min of incubation with propranolol (18). At the end of each time period, 1 ml of acini was removed and quickly centrifuged at 10,000 g in a microcentrifuge for 15 s. The supernatant was removed, the pellet washed with incubation medium, then 2 ml of methanol: 10 mM glycine (5:2 v/v) was added and cells were detached mechanically with a spatula. To this methanol:glycine mixture, 1 ml of chloroform was added and mixed; 1 ml of chloroform and 1 ml of water were then added to this mix, and the phases were separated by a 5-min centrifugation at 1000 g (31). Radioactivity present in the chloroform phase was determined. The samples of the chloroform phase with standard added were dried under a stream of nitrogen and redissolved in 50 μl of chloroform. PA was separated in a solvent system containing chloroform: acetone: methanol: acetic acid: water (50:20:15:10:5, vol/vol) (18, 32). After separation and exposure to iodine vapor, the area containing PA was scraped, and radioactivity was counted. Radioactivity in PA was expressed as a percentage of total radioactivity in the chloroform phase.

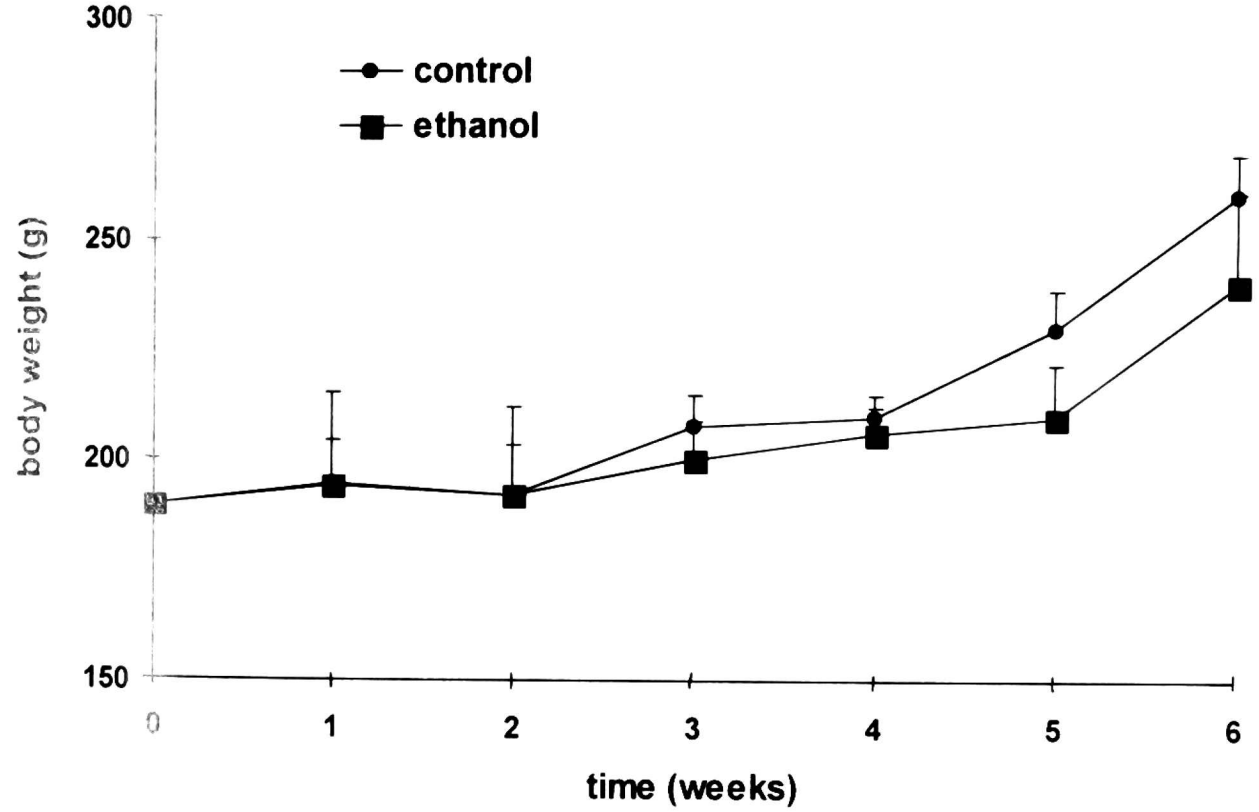
Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using Student's t test. P values < 0.05 were considered significant.

RESULTS

Body weight and pancreatic weight of control and ethanol fed animals

All animals were pair fed and weighted every week. Body weight and pancreatic weight of pair feeding animals did not differ during the observation time (*Fig. 1*).



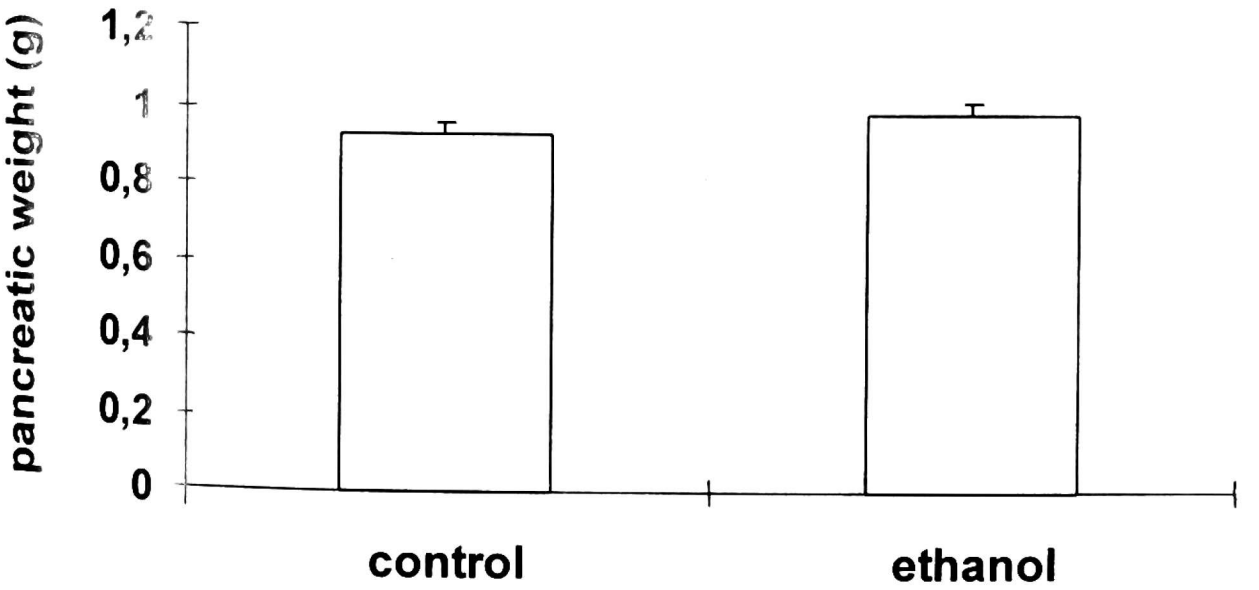


Fig. 1. Body weight and pancreatic weight of control and ethanol fed rats during 6 weeks of observation. Male Wistar rats 200—220g were used for all experiments (n = 126). Rats were pair fed with isocaloric liquid diet containing or not ethanol in approx. doses of 10—12 g/day. Animals were weighted every week (*Fig. 1A*). After 6 weeks pancreata were removed and weighted (*Fig. 1B*). Values are means ± SE of control (n = 12) and ethanol fed group (n = 12).

Protein, amylase and chymotrypsin content in the pancreas of the control and ethanol fed rats

As shown in *Fig. 2* protein and chymotrypsin contents were comparable in the control and ethanol fed animals after 6 weeks of feeding. Amylase content however was significantly lower in the ethanol group.

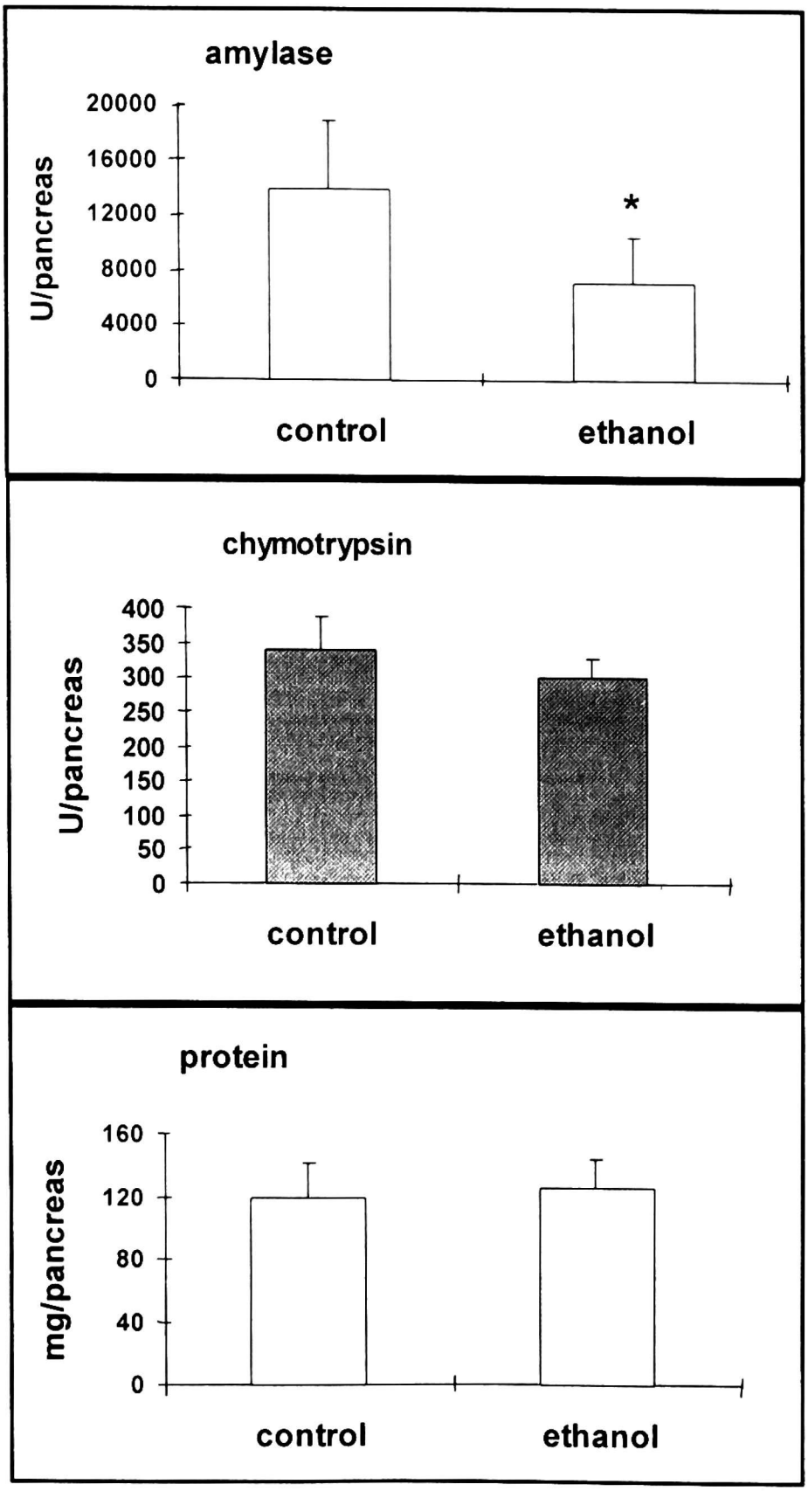


Fig. 2. Amylase, chymotrypsin, and protein content in pancreatic tissue of ethanol fed and control rats after 6 weeks of experiment. After 6 weeks of ethanol feeding amylase, chymotrypsin and protein content in pancreatic tissue were measured as described in the methods. Amylase and chymotrypsin content were expressed as units (U) per whole pancreas, protein content was expressed as mg per whole pancreas. Results are the means ± SE of controls (n = 12) and the ethanol fed group (n = 12). * significantly different from controls ($p < 0.05$)

RNA and DNA content in the pancreatic tissue of the control and ethanol fed animals

Also RNA and DNA contents did not significantly differ in the ethanol fed animals when compared to the controls (*Fig. 3*).

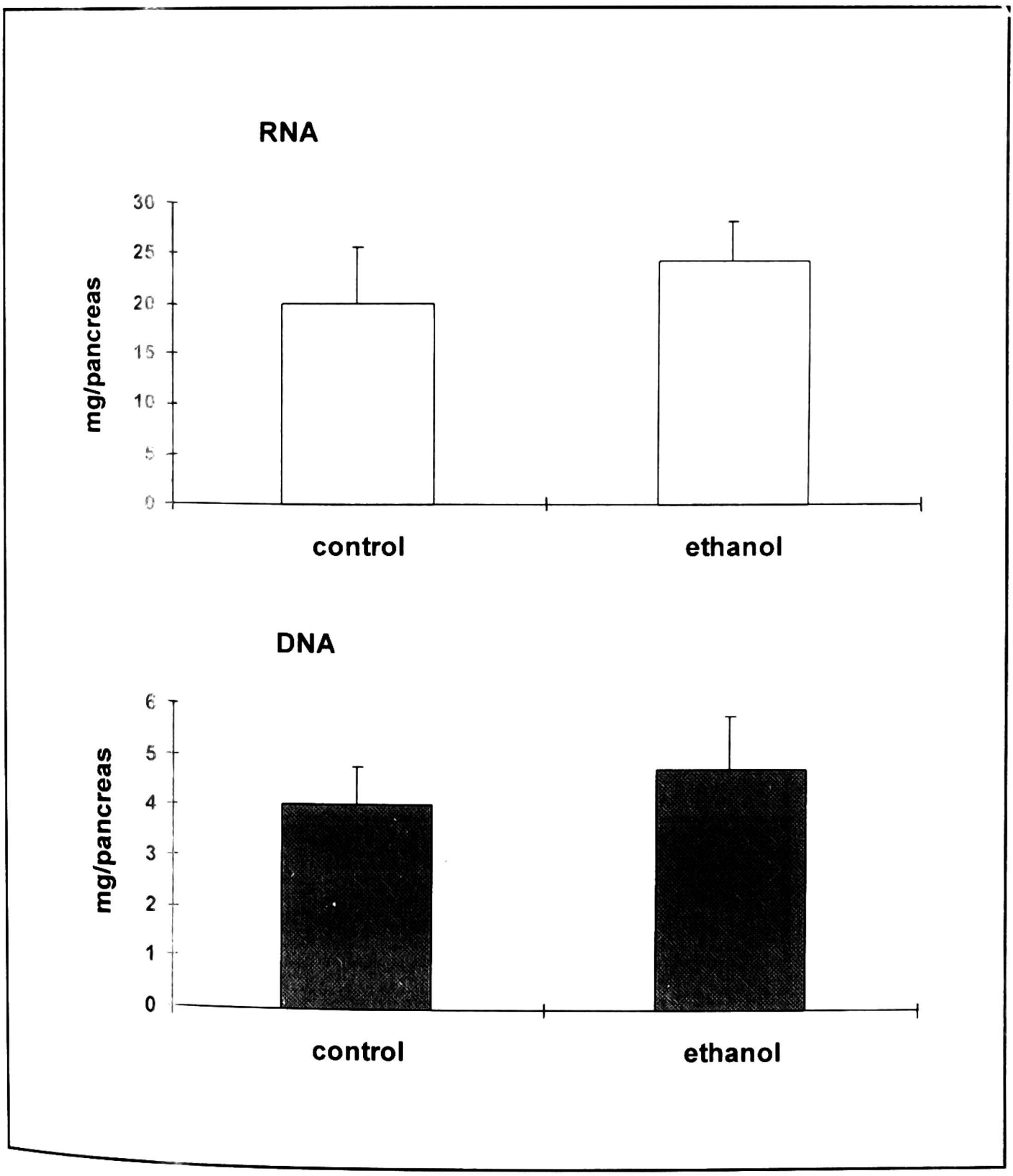


Fig. 3. RNA and DNA content in the pancreas of the control and ethanol fed animals. After 6 weeks of ethanol feeding RNA and DNA content in pancreatic tissue were measured as described in the methods. DNA and RNA contents were expressed as mg per whole pancreas. Results are the means ± SE of controls (n = 12) and the ethanol fed group (n = 12).

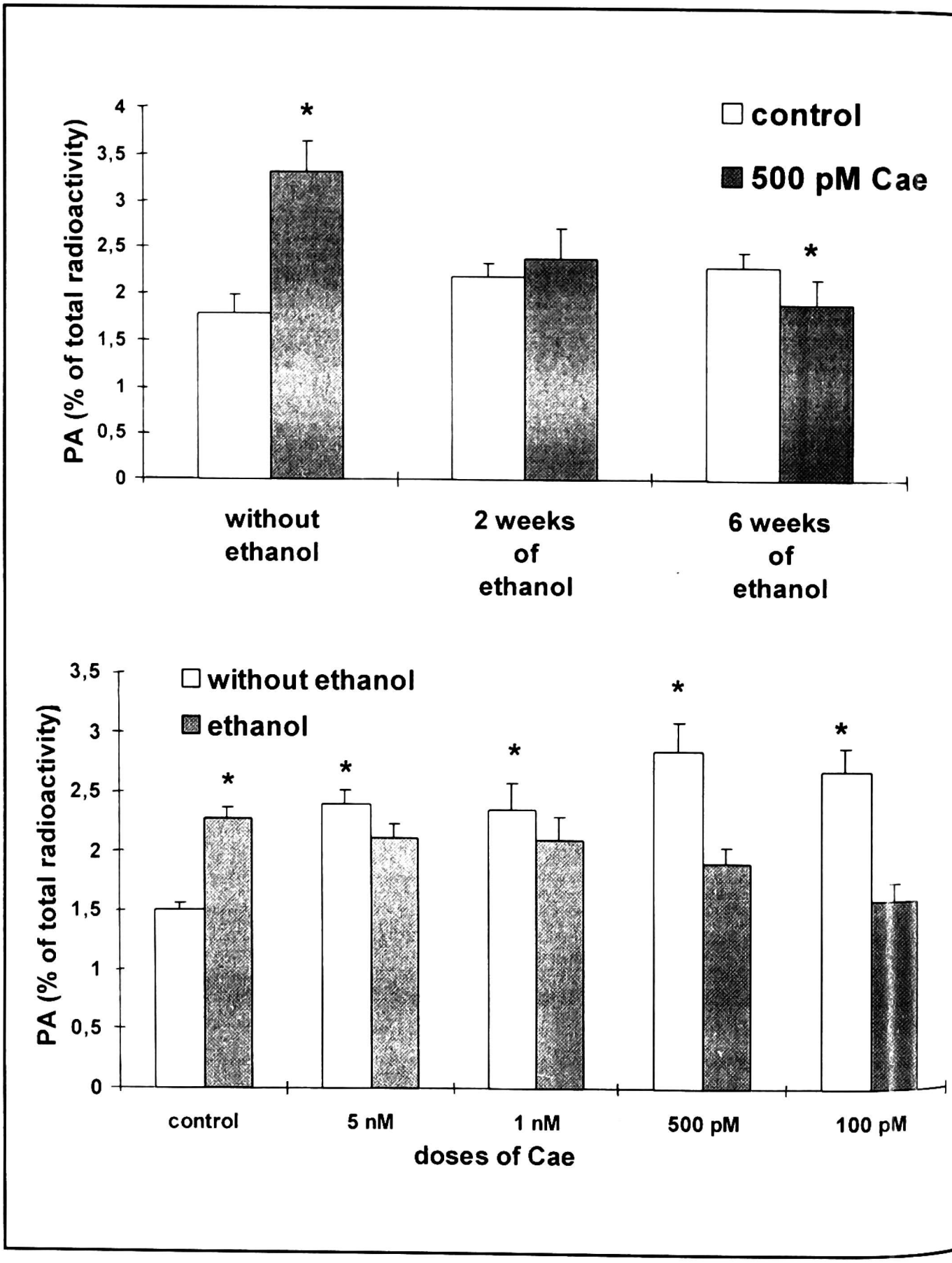


Fig. 4. The influence of Cae on PA accumulation in isolated rat pancreatic acini of control and ethanol fed animals. After 2 and 6 weeks of ethanol feeding pancreatic acini were prepared and loaded with ^{3}H myristic acid as described in the methods. Acini were then stimulated with 500 pM Cae (Fig. 4 A) and PA accumulation in the presence of 200 μM of propranolol was measured. In Fig. 4 B different doses of Cae were used to stimulate pancreatic acini, and PA accumulation was then determined. Results are the means ± SE of 3 separate experiments made in duplicate.
* significantly different from controls without ethanol ($p < 0.05$)

PA production after Cae stimulation of isolated pancreatic acini from ethanol treated and control rats after 2 and 6 weeks of ethanol feeding

Our next approach was to determine PA production in pancreatic acini stimulated with Cae in the control and ethanol feed animals. Our previous study was shown the inhibition of Cae stimulated PA accumulation *in vitro* in the presence of 0.5 up to 2% of ethanol in the medium and we suggested that ethanol can serve as a competitive inhibitor of hydrolytic activity of phospholipase D in that experimental model. We have also shown a significant inhibition of Cae stimulated PA accumulation after a single dose of ethanol (22). As shown in Fig 4A, 500 pM of Cae did not stimulate PA accumulation after 20 minutes in animals treated 2 or 6 weeks with ethanol in comparison to controls. Surprisingly, however, basal PA accumulation after 6 weeks of ethanol feeding was significantly higher than in the control animals. Since the inhibition of Cae stimulated PA accumulation in acini from the ethanol treated animals could be dependent on the dose of Cae, in the next step we checked the dose response to Cae. As is shown in *Fig. 4B* different doses of Cae were not able to significantly increase the PA accumulation in ethanol treated animals, but the basal level of PA accumulation in the ethanol treated animals was significantly higher than in the controls.

PA accumulation in pancreatic acini after Cae infusion and after subtotal pancreatectomy

In the next step we have determined the PA accumulation after 1 h of Cae infusion (0.25 μg/kg/h) in the control and ethanol fed rats. The dose of Cae was chosen according to the previous observations, that its administration for 1 h was associated with significant increase of PA accumulation (116%) and for 48 h was associated with DNA increase, thus confirming the trophic effect of Cae (22). We have also previously observed that a single dose of ethanol (40% ethanol; 5 g/kg p.o.) significantly diminished PA level (22). As shown in *Fig. 5A* in the ethanol treated animals the trophic dose of Cae did not significantly increase PA accumulation. However, as we pointed out earlier, basal PA accumulation was significantly higher in the ethanol treated animals than in the controls.

The last step of our study was to evaluate the influence of chronic ethanol feeding on PA accumulation in pancreatic acini after subtotal pancreatectomy. The subtotal pancreatectomy is a model of pancreas regeneration and a significant increase of PA accumlation 3 days after surgery was observed (20).

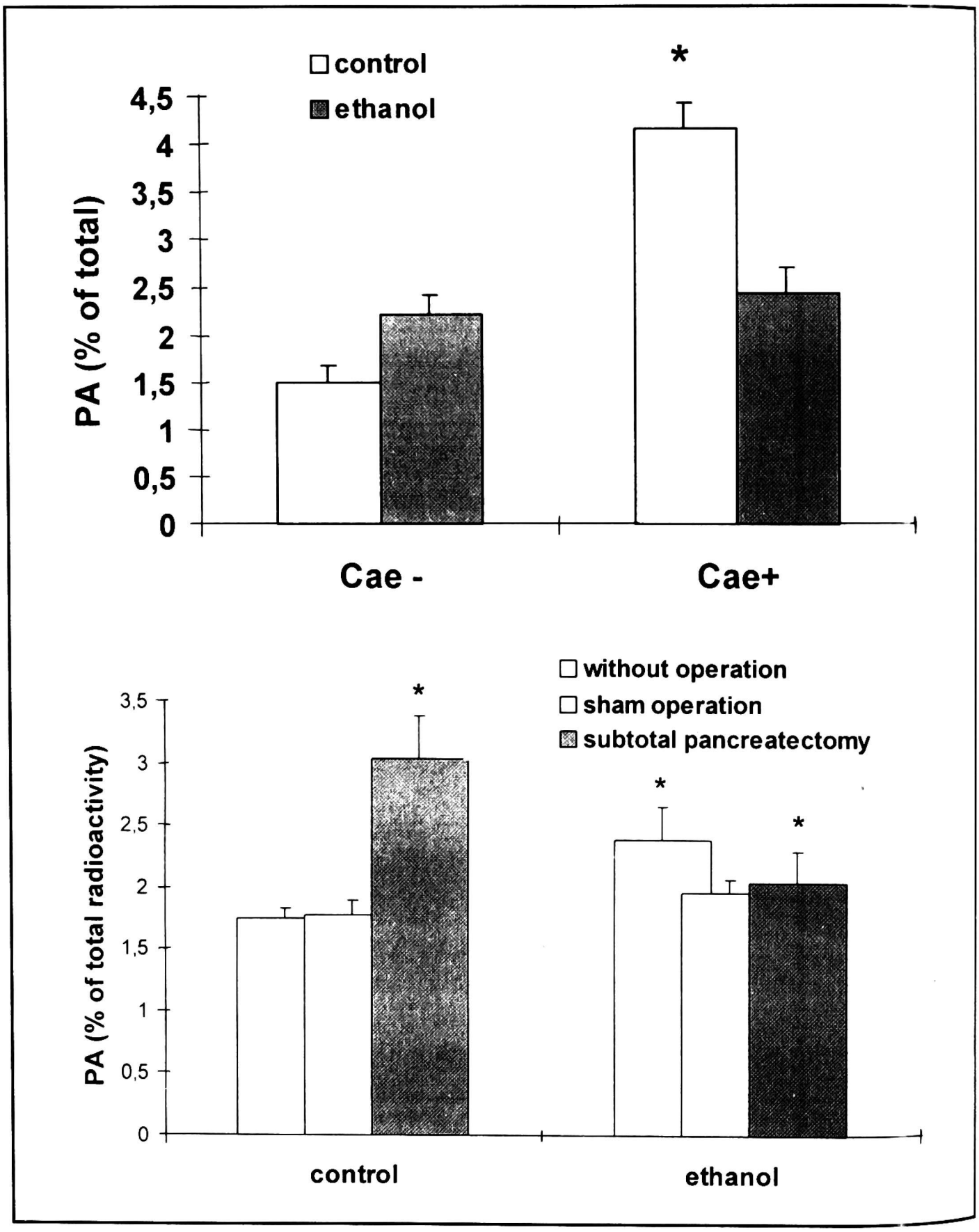


Fig. 5. The influence of Cae infusion (A) and subtotal pancreatectomy (B) on PA accumulation in control and ethanol fed animals. After 6 weeks of respective feeding Cae in a dose 0.25 μg/kg/h or saline were infused during 1 h in control and ethanol fed animals to stimulate PA accumulation. After preparation of pancreatic acini PA accumulation was measured in the presence of 200 μM of propranolol. (*Fig. 5A*). A group of animals was operated — an subtotal pancreatectomy or sham operation was performed respectively in the control and ethanol fed animals. 3 days after pancreatectomy the animals were decapitated, pancreatic acini were prepared and PA was measured as described in the methods section. (*Fig. 5B*) Results are the means ± SE (n = 6)
* significantly different from controls

In the present study, as shown in *Fig. 5B*, the PA accumulation 3 days after subtotal pancreatectomy in the ethanol treated group was significantly lower than in the control animals and did not differ from the ethanol treated animals without operation or which were sham operated.

DISCUSSION

In the present study we have shown the disturbances in some intracellular events after chronic ethanol feeding. We have demonstrated a significant increase in basal phosphatidic acid accumulation in comparison to the control group and the disability of Cae to increase this level. Furthermore we have shown the lack of PA accumulation in the pancreas after growth associated processes as Cae infusion and subtotal pancreatectomy in the ethanol fed groups in comparison to the controls. There were no changes in other trophic parameters such as DNA, RNA, protein and chymotrypsin content after 6 weeks of chronic ethanol feeding.

Acute and chronic ethanol abuse is the major factor for pancreatic injury, but the mechanism of its effect is still unknown. This is due in part to the absence of an appropriate animal model of ethanol-induced pancreatitis. Most studies have focused on the analysis of chronic ethanol administration on various cellular events. It is known that ethanol can affect intracellular calcium homeostasis (33), modify protein synthesis (34), cause premature zymogen activation or the alteration in membrane lipids (10, 11, 35) and can selectively impair endocytosis in rat pancreas (36). Since phospholipase D activity in pancreatic acini was demonstrated, formation of phosphatidylethanol in pancreas in the presence of ethanol is conceivable. We have shown primarily inhibition of the PA production in pancreatic acini in the presence of ethanol in the medium or after acute ethanol ingestion, and we suggested that ethanol could act as an inhibitor of PA production, but the physiological consequences of transphosphatidylation in the pancreas are still unknown. Since ethanol induces the lack of PA production (probably PLD-derived pool), and its replacement by PEt, it may be possible to use transphospatidylation as a way to „selectively" ablate increments in PA from pancreatic membranes and cause membrane injury. In some cell types PA acts as a mitogenic agent, involved in phagocytosis, respiratory burst, proliferation etc. (37, 38). On the other hand Cae, the most potent trophic factor for pancreas, significantly stimulate PA accumulation in pancreatic acini. The increase of PA was also observed in growth associated processes as subtotal pancreatectomy or Cae infusion (20, 22). Therefore it is intriguing to speculate that reduction of PA accumulation contributes to ethanol-induced impairment of pancreatic regeneration, especially since ethanol concentration achievable *in vivo* in the blood of humans

(0.1—0.6%) provides sufficient acceptor to support transphosphatidylation (0.3—2%). This study was performed to confirm this hypothesis and to demonstrate the influence of chronic ethanol treatment on PA accumulation and other parameters of pancreatic regeneration. Our experiments with acute ethanol ingestion let us to suspect that chronic ethanol feeding by itself can impair basal pancreatic regeneration, probably by inhibiting PA production *via* PLD. Surprisingly, under basal conditions we did not observe any influence of chronic ethanol feeding during 6 weeks on the parameters of regeneration. At the same time PA accumulation was significantly higher than in the control group, what suggest the activation of an early phase of the regenerative process, however we did not observe the significant changes in other trophic parameters. One can speculate that after chronic ethanol feeding, an overexpression of PLD activity could be observed, similarly to the overexpression of this enzyme activity in the lymphocytes of alcoholics (39). Another possibility might be that basal PA level of the control and ethanol fed animals is not dependent on PLD activation, but is derived from other sources. The capability of the pancreas to regenerate is observed usually after injuring processes as acute pancreatitis or subtotal pancreatectomy or pancreas growth could be stimulated by Cae infusion in trophic doses. It is known that ethanol-induced injury of the pancreas evokes a regenerative/reparative response which is characterized by a series of morphological and biochemical adaptative responses in subcellular organnelles and an increase in protein and DNA replication. An inadequate or excessive regenerative response is of key importance in perpetuating tissue injury in the alcoholic (16). Under experimental conditions we have used, it can be suspected that repeated cellular impairment caused by chronic ethanol administration could be a source of pancreatic injury and thus could stimulate an early phase of pancreas regeneration. The relatively short time of chronic ethanol feeding could explain the lack of significant changes in other trophic parameters.

However *in vitro* in isolated pancreatic acini from ethanol fed animals, we were not able to show the increase of PA accumulation under basal conditions after different doses of Cae, the one of the most potent factor which stimulates this accumulation in the control group. The lack of increase of PA accumulation was also shown *in vivo* after the infusion of trophic doses of Cae and after subtotal pancreatectomy in ethanol fed animals. From our study with acute ethanol ingestion we conclude that ethanol can cause the inhibition of hydrolytic PLD activity, thus in the presence of ethanol, PEt is produced instead of PA. It is known that ethanol impairs the pancreas regeneration after partial pancreatectomy, some authors explain this effect by the inhibition of CCK release after ethanol (17). Our data do not support this explanation since we did not observe PA increase after exogenous Cae administration in the ethanol fed group. According to our present study the impairment of

pancreatic regeneration observed after subtotal pancreatectomy could be dependent on impairment of PA production in ethanol feed animals.

From the present study we can conclude that chronic ethanol feeding can increase basal PA production in pancreatic acinar cells, however we can observe the lack of PA accumulation after growth related processes such as Cae infusion or subtotal pancreatectomy. Since the involvement of PLD-derived PA in the early stages of pancreatic regeneration was postulated, our results can suggest that chronic ethanol feeding can affect this process, probably by the inhibition of hydrolytic PLD activity and production of PEt instead of PA. This could be one of the mechanisms responsible for pancreatic injury after chronic ethanol consumption.

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