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HORMONAL INFLUENCE ON PHOSPHOLIPASE D AND TRIGLYCERIDE LEVELS IN THE LIVER; INTERACTION WITH PHOSPHATIDIC ACID PHOSPHOHYDROLASE ENZYME

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ABSTRACT

Several studies suggest that insulin resistance is associated with alterations in the lipid levels particularly with the triglycerides. This study was conducted to ascertain the effects of pancreatic hormone insulin and adrenergic neurotransmitter norepinephrine (NE) on the phospholipase D (PLD) enzyme activity and triglycerides levels in goat liver. Goat Liver homogenates were subjected to treatment with insulin and norepinephrine and the PLD activity was ascertained. Pretreatment with phosphatidic acid phosphohydrolase inhibitor, Propranolol potentiated the effects of insulin and NE on PLD by 16.28% and 21.32 % respectively. On the other hand PLD inhibitor 2, 3 Bisphosphoglycerate (2,3 BPG) attenuated the effects of insulin and NE on PLD activity. A Similar trend was seen with the 2,3 BPG treatment on triglyceride levels estimated in the goat liver. It can be suggested from our results that phospholipase D enzyme mediated pathway may be partially responsible for the synthesis and regulation of triglyceride levels in the goat liver.

KEYWORDS: Phospholipase D, Triglycerides, Insulin, Norepinephrine, Liver, Propranolol.

INTRODUCTION

In the mammals, liver is an important organ and serves not just as the store house of the body but also is the organ for metabolic degradation and synthesis of new molecules. Several studies suggest that the *de novo* lipogenesis in the liver is significantly increased in patients with non- alcoholic fatty liver disease (NAFLD).[1] Patients with NAFLD also have increased levels of serum triglycerides and lower levels of high-density lipoproteins. The etiology of these lipid abnormalities may in part be related to insulin resistance, which is a common

feature in patients with (NAFLD).[2] On the other hand, the peptide hormone insulin regulates glucose homeostasis by modulating the action of a multitude of metabolic enzymes in the liver, adipose tissue, and skeletal muscle. Upon insulin receptor activation and phosphorylation of the insulin-receptor substrates (IRSs), insulin signaling branches into several pathways leading to glucose uptake, lipogenesis, glycogen synthesis, glycolysis, and other anabolic processes.[3] However, glucose metabolism by activation of glycogen synthase, mitochondrial pyruvate dehydrogenase, and other regulatory enzymes through protein dephosphorylation, remain incompletely explained by these models for insulin action. Insulin resistance is associated with alterations in proteins involved in lipid metabolism including the glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD).[4] The accumulated lipids induce oxidative stress, resulting in production of cytokines and reactive oxygen species which in turn activate apoptosis thereby initiating a sequence of disease events from steatosis to nonalcoholic steatohepatitis (NASH), which progress into fibrosis and cirrhosis.[5,6,7] Degradation of membrane phosphatidylcholine (PC) by the PLD/PAP pathway is now considered to be an important route of diacylglycerol (DG) formation in stimulated cells.[8] Phosphatidic acid phosphohydrolase enzyme mediated (PAP) actions could therefore play a critical role in maintaining the balance between signaling molecules PA and DG and in the salvage of both of these agonist-sensitive molecules for general glycerolipid synthesis.[9] The adrenergic neurotransmitter, NE has been shown to stimulate the PLD activity and increase triglyceride levels in the liver.[10] NE has also been shown to stimulate the PLD activity in other tissues like the brain and blood vessels.[11,12] This study was thereby conducted to evaluate the effects of the enzyme PA phoshphohydrolase on insulin and NE mediated actions on the PLD enzyme activity and triglyceride levels in goat liver.

METHODS

A piece of about 100g goat Liver was obtained in buffer after sacrifice and preserved at -20 º C.

Estimation of Phospholipase D enzyme activity

A modified technique was used to determine the PLD activity in the liver homogenate.[13] For each experiment, 3 grams of goat liver was homogenized in 12 ml of HBSS. 1.0 ml of the homogenate was added in different test tubes which contained either NE (10µg) or insulin (20 U) or Propranolol (100µM) or 2,3 BPG (10µg) pretreatment in combination added with insulin and NE. These were incubated at 37°C for about 25 minutes each. The solution was

centrifuged at room temperature at 8000 rpm for 10 mins. The supernatant was collected and added with 0.9 ml of cold substrate solutions, which contains 0.14 M choline chloride (in Tris-HCl buffer), 0.48mM of 4-Aminoantipyrine (in H_2O), 2.1 mM of phenol (in H_2O), 4.92 U/ml of Peroxidase from horse radish. The control readings were taken without adding the drug to the homogenate and compared with the test samples. The incubation of the mixture was conducted at 37°C for 1 hr and finally the optical density measured at 500 nm absorbance. The PLD activity was calculated and depicted as the unit/ml activity using the different concentrations of the PLD enzyme standards.

$$\text{Units per ml activity} = \frac{\text{OD (Sample)} - \text{OD (Blank)} \times Vt}{12 \times 0.5 \times 1 \times Vs}$$

Vt - is total volume of the cuvette

Vs - is sample volume.

12 - is millimolar extinction coefficient of quinoneimine dye under the assay conditions (cm^2/micromole)

0.5 - is a factor based on the fact that one mole of $H2O_2$ produces half a mole of quinoneimine dye

1 - is light path length (cm)

Determination of Liver triglyceride levels: The Liver triglyceride content determination was done as per a modified technique described by Zhenyuan Song *et al.*[14] For this purpose, 3 grams of liver was homogenized in about 12 ml of HBSS solution. Drugs and phospholipid modulators were added to the incubation mixture and the reaction was continued for about 30 minutes. The reactions were stopped using 0.6 ml of trichloroacetic acid (TCA). 2.0 ml of chloroform: methanol (2:1) was added to the reaction mixture and the reaction was spun at 10000 rpm for 10 minutes. After centrifugation the supernatant was separated and added with 0.5 ml of Phosphate buffered saline and 1% Triton X. Finally the 4-(pyridyl-2-azo) resorcinol dye was added to the mixture and after 15 minutes the optical density was measured at 540 nM using a UV-Vis spectrophotometer (LAB India, UK). The triglyceride levels were determined using the standard triglyceride values.

RESULTS

Figure 1 and 2 depict the results of PLD activity obtained with the agonists alone i.e Insulin, Norepinephrine and the lipid altering agents i.e Propranolol and 2,3 BPG. Alone *per se* the insulin and NE were able to increase the PLD activity by 12.06 and 19.51 % respectively. Pretreatment with phosphatidic acid phosphohydrolase inhibitor, Propranolol potentiated the effects of insulin and NE on PLD by 16.28% and 21.32 % respectively. On the other hand PLD inhibitor 2,3 Bisphosphoglycerate (2,3 BPG) attenuated the effects of insulin and NE on PLD activity by -8.66 % and 2.45 % respectively . A Similar trend was seen with the 2,3 BPG treatment on triglyceride levels estimated in the goat liver as clearly shown in the Table 1.

Legend for Figure 1: Estimation of phospholipase D activity in liver homogenate. The liver homogenates were treated with vehicle as control group, Norepinephrine: NE, Insulin, Propranolol, 2,3 BPG alone. The values are represented as % change from control values of 627 U/ml of n=5 experiments.

Legend for Figure 2: Estimation of phospholipase D activity in liver homogenate. The liver homogenates were treated with vehicle as control group, NE, Insulin, after pretreatment with Propranolol, or 2,3 BPG. The values are represented as % change from control values of 627 U/ml of n=5 experiments.

Legend for Table 1: Estimation of Triglycerides in liver homogenate. The liver homogenate were treated with vehicle as control group, Norepinephrine: NE, Insulin and alone or in combination with Propranolol or 2,3 BPG. The triglyceride values are represented as % change from control i.e 26.71mg/g liver tissue.

Table 1: Triglyceride levels in goat liver

Nos.	Pretreatment	Treatment	Triglyceride levels (mg/g)	% change from control n=6
1	Vehicle	Vehicle	26.71	---
2	-	Insulin	27.56	3.18
3	-	NE	38.15	42.83
4	Propranolol	Insulin	23.47	-12.13
5	Propranolol	NE	33.53	25.53
6	2,3 -BPG	Insulin	21.78	- 18.45
7	2,3- BPG	NE	29.34	9.85

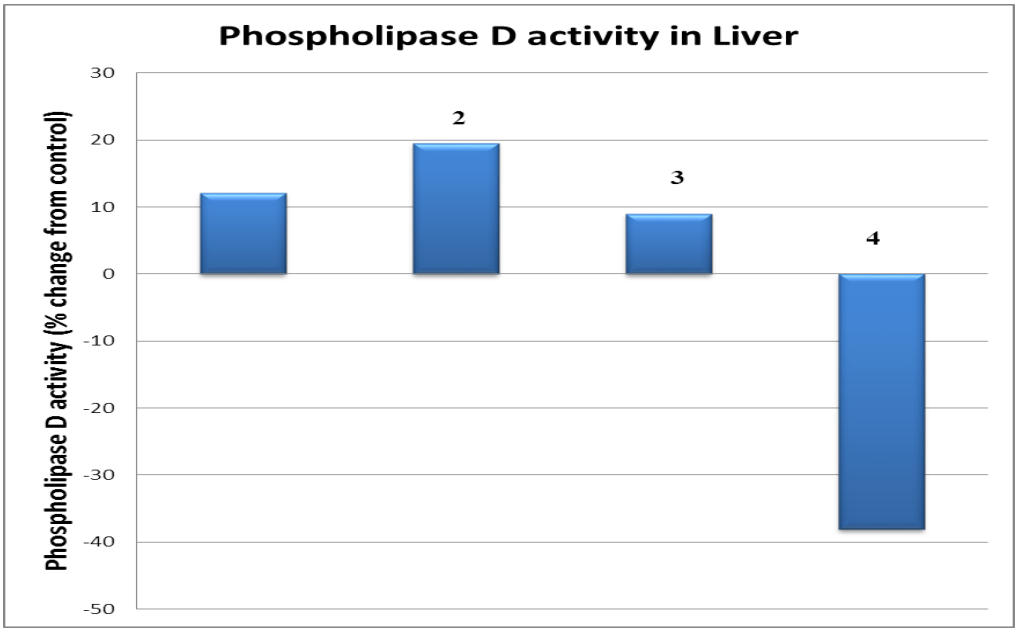

Fig.1 1) Insulin (20U) 2) NE (10µg) 3) Propranolol (100µM) 4) 2,3 BPG (10µg)

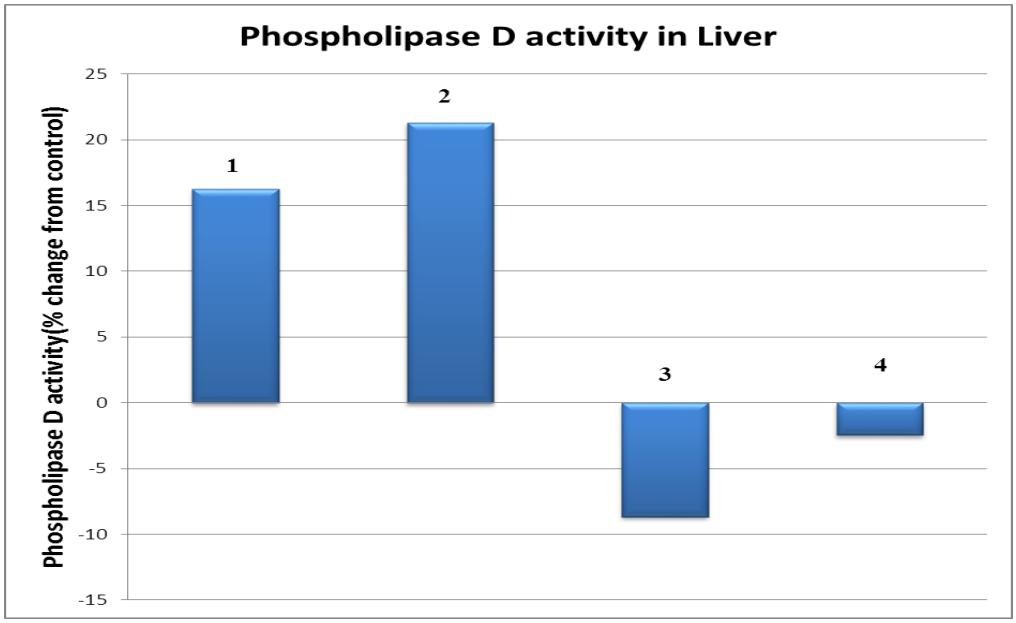

Fig.2 1) Propanolol + Insulin 2) Propanolol + NE 3) 2,3 BPG + Insulin 4) 2,3 BPG + NE

DISCUSSION

Two distinct mammalian PLD genes, *PLD1* and *PLD2*, have been cloned in the previous three decades.[15,16] The product of one of these genes, PLD1, is regulated by the small GTP-binding proteins and protein kinase C and[17] Phosphatidylcholine is the major phospholipid comprising about 50-55% of the total phospholipid content of the cell membrane and its breakdown mediated signal transduction plays critical roles in health and disease.[18] Plasma membrane preparations reportedly contain a PLD activity that is significantly stimulated by

adenosyl ribosylation factor (ARF) and Rho, but the relationship of this PLD activity to PLD1 or PLD2 has not been clearly delineated. There is still relatively little information on the mechanism of activation of Rho and ARF proteins by extracellular signals.[19,20] A few recent reports have suggested that ARF proteins may be activated by cell surface receptors and.[21], and several ARF guanine-nucleotide exchange factors have been described, some of which appear to be recruited to the cell membrane *via* the pleckstrin homology domains.[22] It has been suggested that ARF interacts with the insulin receptor, either directly or via an unknown adaptor protein, thus suggesting a mechanism by which the insulin receptor, and perhaps other related receptor tyrosine kinases, may regulate PLD activity. In our study conducted on the goat liver the results suggest a important role for the enzyme phosphatidic acid phosphohydrolase in regulating the phospholipase D and triglyceride levels. As depicted in our results shown in Fig.1 and Fig.2 and Table 1 it is evident that Propranolol (100µM) was able to reasonably increase the PLD activity of both insulin and NE in the liver homogenate but it also attenuated the triglyceride levels in the liver homogenate. It appears that propranolol with its property to block the PA phosphohydrolase enzyme was able to increase the accumulation of PA and thereby also prevent partially the conversion to 1,2 DAG. Propranolol is an established inhibitor of PA phosphohydrolase enzyme. There are two main types of the PA phosphohydrolase enzymes i.e PAP1 and PAP2. The liver has both of these enzymes. Unlike PAP1, which is highly specific for the Mg^{2+} salts of PA and LPA, PAP2 degrades a number of lipid phosphates, including S1P, ceramide- 1-phosphate (C1P), as well as PA and LPA, does not require specific ions, and is heat and sulphhydryl reagent resistant.[23,24] It is well known the 1,2 DAG is eventually converted to triacylglycerol by the DAG transferase enzyme. This contention was supported by the data obtained using a PLD inhibitor 2,3 BPG and it was able to reduce the triglyceride levels thus enhancing our view that PLD was contributing in the triglyceride synthesis in the liver. Insulin was able to increase the PLD activity and it caused a reasonable increase in the triglyceride synthesis as well.[25] Intrahepatic triglycerides (IHTG) may be an important variable in hepatic insulin resistance and excess triglycerides may be associated with generation of lipid-derived signaling molecules that inhibit insulin action.[26] The two genetic and environmental rodent models have been utilised to delineate many aspects of the NAFLD. Considering only a small proportion of individuals get afflicted with NAFLD due to genetic reasons the use of dietary models to induce changes in liver TG could be considered a more relevant approach.[27] When comparing between species consideration needs to be made for the habitual diet, which varies greatly. For example, the mice on a chow diet have a low-fat intake (4% fat by weight)

whilst humans typically consume approximately 35% total energy (TE) as fat therefore the contribution of DNL fatty acids may be of greater important for TG production[28] and steatosis development in animals than that observed in humans. In our experiments NE when added to the liver homogenate caused an increase in PLD activity and triglyceride levels. These results are in accordance with previous studies suggesting the importance of this sympathetic neurotransmitter in regulating lipid metabolism the liver.[29] Thus in conclusion it can be stated that the importance of insulin in disease states such as hepatic steatosis, insulin resistance and hyperinsulinemia as related to hypertension in both the alchohol consumers and possibly in obese people is dependent partially on PLD mediated pathway and triglyceride formation.[30]

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