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EFFECT OF INSULIN ON DNA SYNTHESIS AND KINETIC PARAMETERS OF THYMIDINE KINASE DURING LIVER REGENERATION

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SUMMARY

The effect of insulin on cell proliferation *in vivo* has been studied in hepatectomised streptozotocin- diabetic rats. The extent of cell proliferation in sham and hepatectomized- control, diabetic and insulin treated rats were monitored by determining DNA content and [^{3}H]thymidine incorporation into DNA. The kinetic parameters of thymidine kinase a regulatory enzyme for DNA synthesis was also studied in these groups. The rate of DNA synthesis in liver of streptozotocin -diabetic rats was significantly higher 24 hrs post-hepatectomy compared to control and insulin treated diabetic groups. Kinetic studies of thymidine kinase revealed that there was no change in the Michaelis -Menten constant (K_m) whereas maximum velocity (V_{max}) was elevated in the diabetic hepatectomized groups compared to control and insulin treated hepatectomized groups. Thus our study elucidates the role of insulin in thymidine kinase activity and DNA synthesis.

INTRODUCTION

Liver regeneration provides a model system to study controlled cell proliferation *in vivo.* Normally quiescent hepatocytes constitute one of the few terminally differentiated cell types in the adult body which retains the ability to proliferate(1). DNA synthesis is initiated by partial hepatectomy (PH) in 12-16 hrs and continues until hepatic mass attains presurgery state in about 2 weeks(2,3). Previous studies on the humoral control of liver cell proliferation have suggested that various hormones may act as signals or regulators for stimulating DNA synthesis after PH(4). There have been reports implicating insulin in the regulation of hepatic regenerative processes. Intravenous infusion of insulin and glucagon into normal adult rats triggered small but significant DNA synthesis in hepatocytes(5,6). Previous evisceration including pancreatic resection largely suppressed liver DNA synthesis 24 hours after PH in untreated rats but in animals that received peripheral injections of insulin and glucagon it was not suppressed(7). Peripheral infusion of

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insulin antiserum substantially blocked hepatic DNA synthesis 24 hours after PH in rats(5). Primary cultures of rat hepatocytes could be stimulated to synthesize DNA by epidermal growth factor in combination with insulin and glucagon.Cultured hepatocytes degenerate and die in absence of insulin and glucagon.(8). The number of insulin binding sites was significantly increased and the ratio of insulin to glucagon binding was markedly increased after PH in rats(9-12). Tyrosyl phosphorylation of insulin receptor substrate-1(IRS-1) a specific target molecule for insulin β-subunit kinase was strikingly enhanced prior to major wave of DNA synthesis after PH. Phosphatidyl-inositol-3-kinase which is involved in growth pathway was seen to be associated with IRS-1 following tyrosyl phosphorylation *in vivo*(13). However plasma insulin levels were observed to decline or remain stationary after PH(14-17). Cornell (9) suggested that higher glycogen in the liver of control rats can maintain higher plasma glucose and thus higher circulating insulin concentrations than those of hepatectomized groups. Also, hepatectomized animals do not feed after surgery. Fed sham animals therefore provide inappropriate controls. Pezzino(17) concluded from his experiments that the alteration in serum insulin levels and insulin receptor binding in liver are due to surgical stress and decreased food intake.

Thymidine kinase (TK E.C 2.7.1.21) which is an enzyme of the pyrimidine salvage pathway catalyzes the phosphorylation of thymidine to thymidylate. In resting eukaryotic cells the level of thymidine triphosphate as well as other deoxyribonucleoside triphosphate is low and the activity of TK is barely detectable. Thus the production of thymidylate by these enzymes is believed to be rate limiting for DNA replication(18,19). The activities of these enzymes have been reported to increase in the proliferative phase of eukaryotic cells including regenerating liver after PH(20,21,22). In the present study, the regulatory role of insulin on the activity of TK and on DNAsynthesis during liver regeneration was studied in streptozotocin (STZ)-diabetic rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (350-400g) were divided into six groups,- control sham hepatectomy (CSH), control hepatectomy (CH), diabetic sham hepatectomy (DSH), diabetic hepatectomy (DH), diabetic insulin sham hepatectomy (DISH), and diabetic insulin hepatectomy (DIH). Each group contained 4-6 animals.

Materials

Tritiated thymidine (18 Ci/mmole) was purchased from BARC (India). ATP, streptozotocin, thymidine were from Sigma Chemicals Co. USA. Glucose kit (GOD-POD) was purchased from MERCK while the other chemicals were standard commercial products of analytical grade.

Induction of diabetes

Diabetes was induced by giving intrafemoral injection of a single dose of streptozotocin (60 mg/kg body weight) under anaesthesia. Control rats were injected with vehicle (50 mM sodium citrate buffer, pH4.5)(23).Insulin was administered subcutaneously to DISH and DIH groups 3 days after STZ injection. The cumulative dose varied between 6.5-12 units depending on the plasma glucose levels. At 0 hrs and 18 hrs post hepatectomy,a further 0.25 units of insulin was administered to the insulin treated groups.

Partial hepatectomy
Control and experimental animals were fasted for 12 hrs prior to surgery in order to deplete hepatic glycogen. Fasting was continued until sacrifice. Partial hepatectomy was performed according to the method of Higgins and Anderson(24). Animals were sacrificed 24 hrs post-hepatectomy by decapitation. Liver was perfused *insitu* with 27 mM citrate buffer.
Analytical Methods
Thymidine incorporation into DNA was carried out by injecting 10μCi of $[^3H]$ thymidine intraperitoneally 1hr before sacrifice. DNA was extracted in 5N trichloroacetic acid according to the method of Schneider(25) and quantified by diphenylamine procedure(26). The RNA content of liver homogenate was measured by the orcinol reaction after extraction with alkaline digestion according to the procedure of Fleck and Munro(27). Protein was determined by the method of Lowry *et al* (28).
Enzyme Assay
A 10% liver homogenate was prepared in 50 mM Tris HCl buffer pH 7.5. It was centrifuged at 36,000g for 30 min. TK was assayed by determining the conversion of $[^3H]$Thymidine to$[^3H]$Thymidine monophosphate [TMP] by the binding of the latter nucleotide to DEAE cellulose disk(29). The range of substrate concentrations used for the kinetic studies was between 50-1500μM. The 60μl reaction mixture contained 5mM [3H]thymidine (0.5μci), 10mM ATP,100mM NaF, 10mM $MgCl_2$ 0.1M Tris-HCl buffer,pH 8.0 and the liver supernatent fraction(2.5μg protein). After incubation at 37^0C for 15 min the reaction was stopped by placing the mixture in a boiling water bath for 3 min followed by immersing in an ice bath. Aliquots of 50 μl were spotted on Whatman DE 81 paper discs which were washed with 1 mM ammonium formate, water and three times with methanol. Disks were allowed to dry overnight. The dried disks were placed in counting vials and spotted with 0.3 ml of 0.2M KCl in 1M HCl. Radioactivity was measured in 10ml of liquid scintillation cocktail. Kinetic constants were determined from linear regression analysis of plots of inverse of reaction velocity versus the inverse of substrate concentration (Lineweaver Burke plot).The activity was calculated per mg protein.

RESULTS

The diabetic state of the streptozotocin injected rats was assessed on the basis of hyperglycaemia, glycosuria and body weight. Animals with plasma glucose in excess of 250mg/dl were defined as diabetic (Table 1). These animals also exhibited glycosuria and decreased body weight compared to the controls .

The hepatectomized animals were observed to have significantly higher levels of total protein compared to their respective controls($p<0.05$) [Table 2]. However the diabetic hepatectomized rats exhibited significantly lower protein levels compared with the other hepatectomized groups. The protein levels correlated well with the RNA levels which were significantly lower in the diabetic hepatectomized group compared to the other hepatectomized groups($p<0.05$) [Table-2]. Diabetic sham operated animals exhibited the lowest levels of RNA while insulin treated hepatectomized animals exhibited the highest level of RNA. The RNA : Protein ratio is a measure of the capacity for protein synthesis. Diabetic sham operated animals

TABLE-1: Glucose levels (mg dl^{-1}) after STZ injection

Experiment	Days after I.V. injection			
	0	3	7	14
Control	81.74 ± 2.08	80.03 ± 2.90	83.04 ± 1.49	77.50 ± 0.41
Diabetic	81.87 ± 1.69	490.69 ± 15.38*	384.73 ± 27.72*	339.90 ± 8.13*
Diabetic& insulin	82.88 ± 2.05	338.11 ± 10.90*	242.20 ± 39.53	170.67 ± 18.91

*Animals with blood glucose above 250mg.dl^{-1} were considered diabetic.
Values are mean ±S.E.M.of 4-6 separate determinations.

TABLE-2: Total Protein ,Total RNA levels and RNA:Protein ratio

Experiment	Total Protein (mg .g^{-1} wet weight)	Total RNA (mg .g^{-1} wet weight)	RNA: Protein
CSH	61.38 ± 2.31	8.84 ± 0.05	0.14
CH	84.82 ± 4.50*†	9.92 ± 0.60	0.12
DSH	59.17 ± 6.22*	4.75 ± 0.19*	0.08
DH	69.30 ± 1.20*†	7.27 ± 0.19†	0.10
DISH	62.38 ± 3.85*	8.71 ± 0.25	0.14
DIH	80.13 ± 2.91*†	11.88 ± 1.45*†	0.15

*$p < 0.05$ compared to control sham.(CSH).
†$p < 0.05$ compared to respective sham
Values are mean ±S.D. of 4-6 separate determinations
CSH-Control sham hepatectomy,CH-Control hepatectomy,
DSH-Diabetic sham hepatectomy,DH-Diabetic hepatectomy,
DISH-Diabetic insulin sham hepatectomy,DIH-Diabetic insulin hepatectomy

showed 43% decrease in the capacity for protein synthesis compared to control sham operated and diabetic insulin treated sham operated animals[Table2].Diabetic hepatectomised animals showed a 17% decrease in the RNA: protein ratio compared to normal hepatectomised animals and a 33% decrease compared to insulin treated diabetic hepatectomised animals. The rate of DNA synthesis was determined by the incorporation of [^{3}H]thymidine into DNA. Diabetic hepatectomised animals showed the highest level of DNA synthesis compared to all other groups ($p<0.05$) (Table-3). Hepatectomy and insulin treatment caused a significant increase($p<0.05$) in DNA synthesis. Also, the DNA content of hepatectomised control,diabetic and insulin treated diabetic groups increased significantly compared to their respective control groups ($p<0.05$ and $p<0.01$;Table-3).

Results from the kinetic parameters of Thymidine kinase showed that the Michaelis Menten constant, K_m was not altered significantly between the groups after hepatectomy (Table 4). The Maximal velocity V_{max} was significantly ($p<0.05$) increased in the diabetic hepatectomised liver when compared to control and insulin-treated hepatectomised animals. The V_{max} of TK was significantly ($p<0.05$) increased in all hepatectomised groups compared to respective controls. When the sham-operated groups were compared, the diabetic insulin treated showed a significantly ($p<0.05$) higher V_{max} compared to diabetic sham which was higher than control sham (Table 4).

DISCUSSION

The requirement of insulin as a positive regulator for liver cell proliferation is fully established *in vitro*(30). Hepatocytes in culture, in chemically defined media degenerate and die in the absence of insulin(31). Suppression of hepatic DNA synthesis in partially hepatectomised rats by exogenous insulin infusions suggest that high plasma levels of insulin are inhibitory for liver regeneration(32). This correlates well with the observation that plasma insulin levels decline after partial hepatectomy(30,33).This decrease of plasma insulin levels after PH led Leffert and Koch (30) to advance a hypothesis that hypoinsulinemia and hyperglucagonmeia was a general characteristic of enhanced proliferative capacity.

The results of this study indicated that the liver of STZ-diabetic rats showed enhanced DNA synthesis 24 hours post-hepatectomy compared to control and insulin-treated diabetic groups. This was evidenced by high total DNA levels, thymidine incorporation and the V_{max} of TK of this group. Also,our observations suggest that hepatectomy and insulin administration per se can trigger DNA synthesis Insulin treatment to diabetic hepatectomised animals caused an increased DNA content.

The diabetic state which does not represent a zero level but a relative deficiency of plasma insulin was reported to promote proliferative response of the liver cell following PH in the early hours of liver regeneration (34). Younger *et al.* (35) studied the effect of administration of insulin

TABLE-3 :Total DNA levels and DNA synthesis

Experiment	Total DNA (mg.g^{-1} wet weight)	DNA synthesis (dpm g^{-1} wetweight.)
CSH	0.65 ± 0.04	319.1 ± 74.4
CH	0.93 ± 0.07*‡	458.4 ± 20.1†$
DSH	0.75 ± 0.22	367.1 ± 43.9
DH	1.31 ± 0.24*‡	566.2 ± 20.4†$
DISH	0.76 ± 0.05	508.5 ± 14.0†
DIH	0.92 ± 0.03*	515.0 ± 11.8†

* p<0.01 compared to CSH. ‡p<0.01 compared to respective sham
† p<0.05 compared to CSH. $p<0.05 compared to respective sham.
Values are mean ±S.D. of 4-6 separate determinations
CSH-Control sham hepatectomy,CH-Control hepatectomy,
DSH-Diabetic sham hepatectomy,DH-Diabetic hepatectomy,
DISH-Diabetic insulin sham hepatectomy,DIH-Diabetic insulin hepatectomy

to severely diabetic rats and found that this led to a marked proliferation of cells. Their results validate the growth promoting action of insulin.

The low levels of insulin in the diabetic conditions are sufficient to promote proliferative responses of the liver cell after partial hepatectomy as observed in our experiments. Probably, the low levels of insulin sensitises the insulin receptor for its ligand resulting in active hepatic extraction of insulin, thereby promoting DNA synthesis.

Insulin consumption is accelerated in the remnant liver after PH (9-12). Caruna *et al.*(11) proposed that increased uptake of insulin after PH may reflect increased binding of these hormones to hepatocyte receptor by which hepatic proliferation is induced. Demouzon *et al.*(36) concluded from their experiments that long term culture with high glucose concentrations increases the amount of insulin receptors and their tyrosine kinase activity. The insulin receptor sensitization as a result of elevated glucose and depleted insulin in the diabetic state may result in increased binding of insulin leading to enhanced proliferation. This receptor sensitization in diabetic state explains our observation of triggered DNA synthesis in diabetic and insulin treated

TABLE-4: Kinetic parameters of Thymidine Kinase

Experiment	Vmax(x10^6) (pmoles $mg^{-1}min^{-1}$)	Km (x10^4) (μM)
CSH	17.30 ± 0.68	3.60 ± 0.13
CH	28.27 ± 4.34*†	3.96 ± 0.50
DSH	20.92 ± 3.86*	3.54 ± 0.26
DH	34.68 ± 1.20*†	3.46 ± 0.38
DISH	25.05 ± 1.30*	3.00 ± 0.43
DIH	29.26 ± 2.57*†	3.71 ± 0.92

*$p<0.05$ compared to control sham.
†$p<0.05$ compared to respective sham.
Values are mean ±S.D. of 4-6 separate determinations.
CSH-Control sham hepatectomy,CH-Control hepatectomy,
DSH-Diabetic sham hepatectomy,DH-Diabetic hepatectomy,
DISH-Diabetic insulin sham hepatectomy,DIH-Diabetic insulin hepatectomy,
V_{max} - Maximal velocity,K_m - Michaelis Menten constant.

shams even without PH. Francavilla *et al.*(37) observed that following partial hepatectomy insulin binding sites are increased after 24 and 48 hrs.

The diabetic rats, as observed from our experiments, maintained low levels of protein, RNA and RNA: Protein ratio, in accordance with what was reported previously (38,39). This, however, did not seem to interfere with the regenerative capacity of the liver. Regardless of the fact that DH group showed reduced capacity for protein synthesis and total protein levels. it exhibited enhanced DNA synthesis and V_{max} for TK. This may be as a result of increased catalytic efficiency of the enzyme due to more efficient functioning leading to higher turnover rates per given catalytic site (40). This function is probably under the control of insulin.

Thus, from our study the activity of TK correlated closely with the rate of DNA synthesis and hence cell proliferation. These results suggest that insulin is a factor in the control system involved in liver regeneration. It might exert its effect by regulating the activity of thymidine kinase ,a key enzyme for DNA synthesis.

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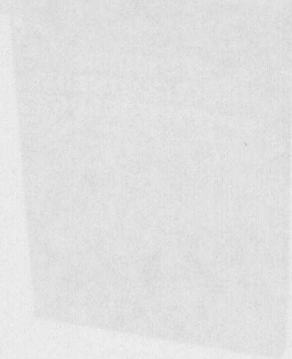

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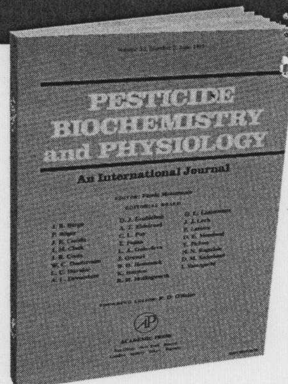

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