PII S0024-3205(97)00147-1

KINETIC PARAMETERS OF THYMIDINE KINASE AND DNA SYNTHESIS DURING LIVER REGENERATION : ROLE OF THYROID HORMONES

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Summary

The role of thyroid hormones in DNA synthesis and in the activity of Thymidine kinase (TK), a key regulatory enzyme of DNA synthesis was studied in proliferating hepatocytes *in vivo*. Liver regeneration after partial hepatectomy was used as a model for controlled cell division in rats having different thyroid status - euthyroid, hypothyroid and 3,3',5'-triiodo-L-thyronine (T_3)-treated hypothyroid. Partial hepatectomy caused a significant elevation of DNA synthesis ($p<0.01$) in all the three groups compared to their sham-operated counterparts. Hypothyroid hepatectomised animals showed significantly lower ($p<0.01$) level of DNA synthesis than euthyroid hepatectomised animals. A single subcutaneous dose of T_3 to hypothyroid sham-operated animals resulted in a significant increase ($p<0.01$) of DNA synthesis in the intact liver. This was comparable to the level of DNA synthesis occurring in regenerating liver of euthyroid animals. In hypothyroid hepatectomised animals, T_3 showed an additive effect on DNA synthesis and this group exhibited maximum level of DNA synthesis ($p<0.01$). Studies of the kinetic parameters of TK show that the Michelis-Menten constant, (K_m) of TK for thymidine was altered by the thyroid status. K_m increased significantly ($p<0.01$) in untreated hypothyroid animals when compared to the euthyroid rats. T_3 treatment of hypothyroid animals reversed this effect and this group showed the lowest value for K_m ($p<0.01$). Thus our results indicate that thyroid hormones can influence DNA synthesis during liver regeneration and they may regulate the activity of enzymes such as Thymidine kinase which are important for DNA synthesis and hence cell division.

Liver regeneration after partial hepatectomy provides an excellent *in vivo* model for studying the factors involved in the transition of cells from quiescence to proliferation (1). Studies on the role of

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thyroid hormones in influencing this phenomenon have shown that T_3 can induce proliferative responses after subcutaneous administration in the intact liver (2,3). Thyroidectomy was shown to inhibit liver regeneration and this was reversed by Thyroxine (T_4) administration (4). The regenerative response of intact liver after subcutaneous T_3 administration is shown to mimic the DNA synthesis pattern induced by 40% hepatic resection (5). Thymidine kinase (E.C.2.7.1.21) is a key regulatory enzyme involved in DNA synthesis. TK activity is elevated in regenerating liver, neoplastic tissues and in developing rat liver(6,7,8). In this study the proliferative response of the liver in relation to the thyroid hormone status and the role of thyroid hormones in regulating the activity of thymidine kinase has been evaluated.

Methods

Male Wistar rats weighing 250-350g were maintained on a 12h light/12h dark cycle and were given food and water *ad libitum*. Animals were divided into 6 groups-Control (Euthyroid) Sham operated (CS), Control (Euthyroid) hepatectomized (CH), Hypothyroid Sham operated (HS), Hypothyroid hepatectomized (HH), Hypothyroid T_3 Sham operated (HT$_3$S), Hypothyroid T_3 hepatectomized (HT$_3$H). Hypothyroidism was induced by intra-peritoneal (i.p.) dose of 2-Thiouracil (10 mg/kg body weight) for 14 days (9). Working stock of 2-Thiouracil (2.5 mg/ml) was prepared by dissolving in 0.2N NaOH and making up with saline. T_3 was dissolved in 0.1N NaOH and made up with saline (1 mg/ml). T_3 was administered as a single subcutaneous dose (62.5µg/100g body wt) 4 hours before hepatectomy (2). Partial hepatectomy was done according to the procedure described by Higgins and Anderson (10). Animals were sacrificed 22 hrs after partial hepatectomy by decapitation. Liver was perfused *in situ* with 0.027M citrate buffer to flush out blood from liver lobes with a constant flow rate of 30 ml/min for 5 minutes. Tissues were stored at -70°C for various experiments.

DNA synthesis was studied by thymidine incorporation. [^{3}H]Thymidine (10 µCi, sp. activity, 18 Ci/mmol) was injected i.p. 1 hour before sacrifice. DNA was extracted with 5% TCA according to Schneider (11). Radioactivity was measured in Cocktail-T containing Triton X 100 in Wallac Liquid Scintillation Counter. The incorporation of thymidine in the acid soluble fraction was expressed as dpm/g wet weight. DNA was quantified by diphenylamine procedure (12). RNA was extracted by alkali lysis method (13) and was estimated by using orcinol reagent. Total soluble protein was measured according to Lowry *et al.* (14).

The activity of liver thymidine kinase was measured by determining the conversion of [^{3}H]thymidine to [^{3}H]thymidine monophosphate (TMP) and by the binding of monophosphate to DEAE cellulose disc (7). Liver was homogenized in 0.1M Tris HCl pH 7.5 containing 0.25 M sucrose and 10 mM β-mercaptoethanol to make a 10% homogenate. Supernatant collected after centrifugation at 12000 xg for 1 hr at 4°C was used as crude enzyme. A 60 µl reaction mixture contained 0.5 mM [^{3}H] thymidine , 10 mM ATP, 100 mM NaF, 10 mM MgCl$_2$ and enzyme diluted in Tris (0.1M, pH 8.0) containing 2.5 µg of protein. Reaction mixture was incubated for 15 minutes at 37°C. Reaction was arrested by plunging into boiling water bath for 3 minutes and then immersed in ice bath. 50µl of the reaction mixture was spotted on DE 81 disc and immediately immersed in and washed with 1mM Ammonium formate, water and 3 times with methanol. Discs were dried and 0.3 ml of 0.2M KCl in 1M HCl was spotted to release the bound TMP and activity

Results

The proliferative response of the liver was assessed by DNA synthesis and DNA content (Table I). Hepatectomy caused a significant increase ($p < 0.01$) in DNA synthesis in the three groups compared to the respective shams at 22 hours of regeneration. The level of DNA synthesis was lower in hypothyroid hepatectomised animals compared to their euthyroid counterparts. DNA synthesis in the intact liver of hypothyroid T_3 treated sham-operated animals was higher than the basal level ($p < 0.01$) and it was comparable to that of euthyroid hepatectomised rats. The maximum level ($p < 0.01$) of DNA synthesis was observed in T_3 treated hypothyroid hepatectomised animals. The DNA content also showed a similar pattern as DNA synthesis.

Hepatectomy resulted in significant ($p < 0.05$) elevation of RNA content in euthyroid and hypothyroid groups (Table II). T_3 administration to hypothyroid sham operated animals resulted in a significant ($p < 0.05$) increase in the RNA content compared to that of euthyroid intact liver. The maximum increase ($p < 0.01$) of RNA content was observed in the T_3 treated hepatectomised animals. Sham operated euthyroid and hypothyroid rats showed comparable protein content (Table II). T_3 treatment resulted in 30% increase of protein content in intact liver of hypothyroid animals when compared to euthyroid sham operated animals. The protein content was significantly higher ($p < 0.05$) in hepatectomised euthyroid animals when compared to the respective sham. In hypothyroid group and T_3 treated hypothyroid animals, hepatectomy did not cause any significant increase in the protein content.

Observations on the kinetic parameters of Thymidine kinase showed that the Michaelis Menten constant, (K_m) for thymidine varied with the thyroid status (Table-III). In hypothyroid untreated animals K_m increased significantly ($p < 0.01$) when compared to the euthyroid group. T_3 treatment of hypothyroid animals reversed this effect and the K_m was lower than that observed in euthyroid animals ($p < 0.01$). Within each group, hepatectomy did not cause a significant change in the K_m. Hepatectomy caused a significant increase ($p < 0.05$) in the maximal velocity (V_{max}) of thymidine kinase, at 22 hours of regeneration in euthyroid and in hypothyroid untreated animals. The V_{max} did not show any significant change due to hepatectomy in T_3 treated hypothyroid group. Moreover, the V_{max} of this group was the lowest compared to other groups ($p < 0.01$).

Discussion

The pattern of DNA synthesis in regenerating rat liver has been well defined. DNA synthesis which is negligible in the normal liver exhibits an abrupt rise at about 14-18 hrs and reaches a peak at 22-26 hrs of partial hepatectomy (15). A significant increase in [^{3}H] thymidine incorporation to DNA was observed at 22 hrs of regeneration. DNA content was also significantly higher in hepatectomized animals supporting our observations on DNA synthesis. In hepatectomized hypothyroid animals the DNA synthesis was higher than sham operated hypothyroid animals but was lower than their respective euthyroid group. Thyroidectomy has been shown by Canzanelli *et al.* to reduce DNA synthesis (4). The decreased DNA synthesis in animals having depleted thyroid hormone levels by 2-Thiouracil treatment agrees with their observations. Obata *et al.* (16) have reported that the rate of regeneration is not affected by the thyroid status when rate of restoration of liver weight was used as an index for regeneration. In our studies we have used [^{3}H]thymidine incorporation as the index to assess regeneration.

In our experiments T_3 was injected to hypothyroid animals 4 hours prior to hepatectomy to

TABLE-I

DNA Synthesis And DNA Content In The Liver
Of rats of Various Experimental Groups.

EXPERIMENT	DNA SYNTHESIS (dpm/g wet wt.)	DNA CONTENT (mg/g wet wt.)
CS	366 ± 03	0.491 ± 0.09
CH	$567 \pm 32^{*f}$	$0.751 \pm 0.10^{*f}$
HS	362 ± 28	0.466 ± 0.01
HH	$469 \pm 35^{*f}$	$0.576 \pm 0.04^{*}$
HT$_3$S	$493 \pm 25^{*}$	$0.570 \pm 0.03^{*}$
HT$_3$H	$702 \pm 38^{*f}$	$0.789 \pm 0.04^{*f}$

$*$p<0.01 compared to Control Sham
fp<0.01 compared to respective sham.
Values are Mean ± S.E.M. of 4-6 separate determinations.
CS-Control Sham Hepatectomy, CH-Control Hepatectomy,
HS- Hypothyroid Sham Hepatectomy,HH-Hypothyroid Hepatectomy,
HT$_3$S-Hypothyroid T$_3$ treated Sham Hepatectomy,
HT$_3$H-Hypothyroid T$_3$ treated Hepatectomy.

Several reports have suggested that T$_3$ has a proliferative effect in intact animal (2,3). Francavilla et al (5) have shown that a single subcutaneous dose of T$_3$ can mimic a 40% hepatic resection in timing and in magnitude. The growth associated genes which are expressed during liver regeneration are also expressed due to T$_3$ administration (5). Our results also suggest a proliferative effect of T$_3$.

We have observed an increase of DNA synthesis and an increase in DNA content in T$_3$ treated sham operated hypothyroid animals which were comparable with hepatectomized

TABLE-II

RNA And Protein Content In The Liver Of Rats Of Various Experimental Groups.

EXPERIMENT	RNA CONTENT (mg/g wet weight)	PROTEIN CONTENT (mg/g wet weight)
CS	2.6 ± 0.57	88 ± 28
CH	$3.5 \pm 0.11^{*}f$	$149 \pm 26^{*}$
HS	2.9 ± 0.23	95 ± 10
HH	$3.4 \pm 0.17^{*}f$	100 ± 06
HT$_3$S	$3.5 \pm 0.28^{*}$	$126 \pm 18^{*}$
HT$_3$H	$4.8 \pm 0.25 \dagger f$	123 ± 14

$^{*}p < 0.05$ when compared to Control Sham.

f $p < 0.05$ when compared to respective sham.

$\dagger p < 0.01$ compared to Control Sham

Values are Mean$\pm$ S.E.M. of 4-6 separate determinations.

CS-Control Sham Hepatectomy, CH-Control Hepatectomy,

HS- Hypothyroid Sham Hepatectomy, HH-Hypothyroid Hepatectomy,

HT$_3$S-Hypothyroid T$_3$ treated Sham Hepatectomy

HT$_3$H-Hypothyroid T$_3$ treated Hepatectomy.

reports (18,19) suggest that there is an increased RNA synthesis early after partial hepatectomy in rat liver. The activities of RNA polymerase I, II and III are elevated at 18 hrs of regeneration (20,21,22). Ljungquist et al. (23) have observed increased rRNA synthesis during mouse liver regeneration. We have observed an increase in RNA content in all hepatectomized groups - euthyroid, hypothyroid and T$_3$ treated hypothyroids. When compared with respective sham operated control, an increase in RNA content was observed in T$_3$ treated sham operated hypothyroid group. A further increase was observed in hepatectomized T$_3$ treated group. All these

TABLE-III

Kinetic Parameters Of Thymidine Kinase In The Liver Of Rats Of Various Experimental Groups.

EXPERIMENT	$K_m (\times 10^4)$ μM	$V_{max} (\times 10^7)$ (pmoles/mg/min)
CS	3.27 ± 0.28	3.07 ± 0.04
CH	3.30 ± 0.14	3.98 ± 0.27*
HS	5.75 ± 0.60†	3.03 ± 0.44
HH	5.80 ± 0.90†	4.10 ± 0.80*
HT$_3$S	1.80 ± 0.50†	1.68 ± 0.20†
HT$_3$H	2.20 ± 0.50†	1.50 ± 0.13†

* $p < 0.05$ when compared to Control Sham.
† $p < 0.01$ compared to Control Sham.
Values are Mean± S.E.M. of 4-6 separate determinations.
CS-Control Sham Hepatectomy, CH-Control Hepatectomy,
HS- Hypothyroid Sham Hepatectomy, HH-Hypothyroid Hepatectomy,
HT$_3$S-Hypothyroid T$_3$ treated Sham Hepatectomy
HT$_3$H-Hypothyroid T$_3$ treated Hepatectomy.

    Brown *et al.* (24) have observed that T$_3$ is capable of stimulating the rate of protein synthesis in skeletal muscle. Peavy *et al.* (25) have observed that thyroidectomy resulted in a 20% decrease in the synthesis of nonsecretory proteins. T$_3$ has a role in the post-translational modification of the secretory proteins. We have observed an increase in protein content in hepatectomised euthyroid animals. But the response to hepatectomy was not seen in hypothyroid as well as T$_3$ treated hypothyroid group. There was an increase in protein content in T$_3$ treated group compared to the basal level.

significant elevation of V_{max} at 22 hours of regeneration. The increase in V_{max} implies an increased catalytic efficiency of the enzyme during the DNA synthetic phase of liver regeneration. The alteration in V_{max} in euthyroid hepatectomised group correlates with the observed increase in DNA synthesis. An increase in V_{max} was also observed due to hepatectomy in hypothyroid animals and this may account for the increased DNA synthesis in these animals compared to respective sham.

The serum thyroid hormone levels are reported to decline by 24 hours post hepatectomy (27). In hypothyroid condition, serum T_3 levels are depressed (28). In these two groups, hepatectomy caused an increase in V_{max} implying that the V_{max} of TK may be negatively regulated by the levels of thyroid hormone. The K_m of TK did not show any change due to hepatectomy in any of the groups. However, the K_m of the enzyme varied with the thyroid status of the animal. Hypothyroidism resulted in a significant increase in the K_m of TK. This signifies a decreased affinity of the enzyme for thymidine. The DNA synthesis in hypothyroid animals was lower than that of euthyroid animals, though the V_{max} did not vary between these groups. The decreased affinity of the enzyme could account for this observation. The V_{max} of T_3 treated hypothyroid group was the lowest compared to other groups. T_3 treatment was done 4 hours prior to hepatectomy. The serum T_3 levels peak 24 hours after T_3 administration (5). The enzyme kinetics were measured when circulating T_3 levels were high. The affinity of the enzyme increased significantly with T_3 treatment. The presence of T_3 may negatively regulate the V_{max} of the enzyme and positively regulate the affinity of the enzyme for thymidine. The increase in affinity of the enzyme for thymidine may account for the high level of DNA synthesis observed in the T_3 treated animals. Thymidine kinase is an allosteric enzyme. T_3 might affect the affinity of the enzyme by bringing about conformational changes by directly or indirectly acting as an allosteric regulator of thymidine kinase.

Acknowledgement

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