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Effect of 3,5,3′-triiodothyronine on cytosolic cAMP-dependent, cAMP-independent protein kinase and nuclear DNA-associated kinase activity in proliferating rat liver under different nutritional regimens

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Cytosolic cAMP-dependent, cAMP-independent protein kinase (PK) and DNA-associated nuclear kinase activity were compared in the proliferating liver of rats: (1) fed with commercially available basal laboratory diet; (2) fasted (2 days) and then fed with basal laboratory diet (2 days); (3) fasted (2 days), supplied with 30% glucose (1 day), and then realimented with basal laboratory diet; (4) fasted (2 days), supplied with 30% glucose (1 day), and then given a single dose of 3,5,3'-tri-iodothyronine (T_3) with realimentation (1 day with basal laboratory diet). The incorporation of ^{14}C-thymidine into rat liver DNA was increased in animals supplemented with glucose ($P < 0.01$) *and even more pronounced in these with (T_3) injection. Fasting significantly decreased both cytosolic cAMP-dependent and cAMP-independent PK activities in the rat liver, but not DNA-associated kinase activity, when compared with the ad libitum fed controls. Both of the cytosolic kinase activities were restored by glucose, while nuclear DNA-associated kinase activity was not altered. T_3 did not change the cytosolic kinase activities but significantly increased the DNA-associated kinase activity. The data obtained by Linewiever-Burk analysis of the cAMP-dependent protein kinase activity suggest that the lower activity in liver of fasted rats restored by glucose was not due to the induction of the enzyme. The specific binding of T_3 to rat liver nuclear receptors was characterized; the equilibrium association constant (Ka) and the maximal binding capacity (MBC) were evaluated. Fasting for 48 hr decreased MBC* ($P < 0.05$)*, glucose administration with refeeding reversed this value to control levels. The data representing Ka were of the same value in all groups tested. These observations suggest that T_3 may play a role as a modulator of liver cell proliferation, potentially via nuclear DNA-associated kinase activity. cAMP-dependent and cAMP-independent cytosolic kinase activities may be necessary for permitting DNA synthesis stimulated by nutritional manipulations, but these cytosolic kinase activities are clearly not sufficient for stimulating elevated DNA synthesis: increased DNA-associated kinase activity appears to be critical. Our results also support the idea that the mechanism of hepatocyte proliferation in rats caused by a nutritional regimen may differ from that found in the liver regenerating after partial hepatectomy.* (J. Nutr. 145–150, 1995.)

Keywords: liver regeneration; cytosolic and nuclear kinases; cAMP; thyroid hormone

Introduction

Partial hepatectomy in rats leads to the proliferation of cells in the remnant liver. The process of liver regeneration has been well characterized by the increase of DNA synthesis in

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Received March 18, 1994; accepted October 20, 1994.

Nutritional Biochemistry 6:145–150, 1995
© Elsevier Science Inc. 1995
655 Avenue of the Americas, New York, NY 10010

0955-2863/95/$10.00
SSDI 0955-2863(94)00020-M

the rat liver in vivo as well as in the isolated hepatocytes grown in primary culture.[1,2] In spite of the fact that molecular signals controlling the prereplicative phase of liver regeneration are becoming rapidly defined, the nature of the factors and systems that trigger or modulate this process requires elucidating. The cyclic 3′5′-adenosinemonophosphate (cAMP) influences positively or negatively proliferation of different types of cells.[3] During liver regeneration after partial hepatectomy (PH), alterations in the activity of second "messengers," like cAMP,[4] diacylglycerol,[5] and phosphoinositides[6] have been reported. The changes in the cAMP level in the rat liver following partial hepatectomy[4] and the stimulatory effect of cAMP on the growth of rat hepatocytes in primary culture[7] are consistent with the stimulatory role of the "messenger" in liver growth.

Further experiments indicated the involvement of cAMP in the regulation of proliferative activation by triggering deoxyribonucleic acid (DNA) synthesis in hepatocytes through cytosolic cAMP-dependent protein kinase.[8] These authors reported increased cAMP-dependent protein kinase activity in the remnant rat liver cytosol following partial hepatectomy as the result of new enzyme synthesis. No further evidence has however been provided on the role of cAMP-dependent protein kinase in the regulation of proliferation of hepatocytes. Rat liver regeneration is significantly influenced also by the system of nutritional manipulation.[9]

Using this system, when rats were denied protein for 3 days and then refed amino acids, similar changes in proto-oncogene expression were observed in the liver as seen after partial hepatectomy. However, lack of *c-fos* expression was found in the liver with the above given nutritional regimen suggesting that the stimulation of hepatocyte proliferation by nutritional or metabolic changes occurs via mechanism(s) different from those expected after partial hepatectomy.[10] Fasted rats realimented with standard diet after partial hepatectomy exhibited active regeneration compared with those realimented with glucose.[11] The hormones that primarily regulate metabolism of energy substrates are also those that potently modulate liver regeneration.[12] T_3 was found to stimulate the proliferative rate in liver regeneration, yet the mode of its action is unknown.[13,14] This hormone increased the level of cAMP in white fat cells without affecting adenylate cyclase or cAMP phosphodiesterase activity.[15] In contrast, T_3 was found to activate myocardial rat adenylate cyclase.[16] Since mitogenic signals for rat liver proliferation are generated by nutrition and thyroid hormones, we decided to examine whether cAMP plays a role in the control of nutrition-hormone–induced hepatocyte cell proliferation as it does in the case of partial hepatectomy.[8] For this purpose we utilized an experimental model of nutritional manipulation and T_3 administration which induced liver cell proliferation in intact rats.[17]

Methods and materials

Male Wistar rats purchased from Velaz (Prague, Czech Republic), weighing 180 to 220 g, were randomized into four groups, 8 animals each. The animals were housed in stainless-steel cages at room temperature of 22°C and 12 hr/12 hr light/dark cycle. Rats were fed ad libitum with basal diet commercially available (Velaz Prague, Czech Republic) consisted of protein:lipid:carbohydrates = 26:13:61 cal%, originating from wheat bran 50 wt%, oats bran 17 wt%, casein 15 wt%, dry milk 13 wt%, vitamins, and biofactors 5 wt%. The fatty acid composition of the diet used expressed in wt% as follows: saturated fatty acids 43.7, monosaturated fatty acids 32.9, omega-6 polyunsaturated fatty acids 18.2, and omega-3 polyunsaturated fatty acids 2.3. The first group (A) of animals was fed commercially available (Velaz) basal laboratory diet ad libitum. The second group (B) fasted for 2 days and fed the next 2 days. The third group (C) of animals fasted for 2 days, then for 1 day the rats were supplied with 30% glucose only, and on the following day they were realimented with basal laboratory diet. The fourth group (D) underwent a similar diet regimen as the third group, however, simultaneously with realimentation they received a single dose of 20 μg/100 g B.W. of T_3 intraperitoneally. The animals of each group were killed by decapitation in the morning on the fifth day.

Incorporation of [^{14}C] thymidine into DNA

Rats were given an intraperitoneal injection of 44.4×10^5 Bq/kg of [^{14}C]thymidine 1 hr before death. They were killed by cervical dislocation and liver was removed into 5% (w/v) trichloroacetic acid, homogenized, washed three times with ethanol, washed twice at 70°C with ethanol-ether (3:1, v/v), and dried. DNA was extracted in 5% (w/v) trichloroacetic acid at 90°C for 20 min. The DNA was determined by the method described by Burton[18] and radioactivity was counted on LKB Rack Beta scintilation counter.

Isolation and extraction of nuclei

Purified rat liver nuclei from nonpooled tissue were freshly prepared by the procedure described by DeGroot and Torresani[19] all subsequent steps being carried out at 0 to 4°C. Tissue was minced and homogenized in 0.32 mol/L of sucrose, 1 mmol/L of $MgCl_2$, 0.1 mmol/L of PMSF, and 1 mmol/L of DL-dithiothreitol. The liver homogenate was centrifuged at 1,000*g*, and the crude pellet was washed with the same medium and centrifuged repeatedly at 1,000*g*. The pellet was mixed with 2.3 mol/L of sucrose containing 1 mmol/L of $MgCl_2$, 0.1 mmol/L of PMSF, and 1 mmol/L of DL-dithiothreitol and then treated by ultracentrifugation at 220,000*g* for 30 min in the swing-out SW 41 rotor in a Beckman L5-50 model ultracentrifuge.

Liver nuclei were then washed once in ice-cold SMCT buffer (0.32 mol/L of sucrose, 10 mmol/L of Tris-HCl, pH 7.4, 0.1 mmol/L of PMSF, and 1 mmol/L of DL-dithiothreitol) in the presence of 0.25% Triton X-100 and once in the absence of Triton X-100. This method for isolation of liver nuclei gives a highly purified nuclear fraction as proven by fluorescence microscopy[20] and a protein/DNA ratio of 3.01 ± 0.26 and RNA/DNA ratio of 0.081 ± 0.011.[18]

The nuclear nonhistone proteins, containing the T_3 receptor fraction, were obtained directly by extracting purified liver nuclei in NaMPTD buffer (0.3 mol/L of NaCl, 1 mmol/L of Mg Cl_2, 10 mmol/L of Tris-HCl, 25 mmol/L NaH_2PO_4, pH 8.0, and 1 mmol/L of DL-dithiothreitol) at 0°C, for 1 hr, and separated from the fraction of disrupted nuclei by ultracentrifugation at 135,000*g* (Ti 50.3 rotor, a Beckman model L5-50 ultracentrifuge).

Binding of ^{125}I-T_3 to nuclear receptors

The assays on the specific binding of ^{125}I-T_3 (specific activity 44.4 TBq/g, commercially available from the Institute of Isotopes, Hungarian Academy of Sciences, Budapest, Hungary) were performed at 22°C in 0.5 ml of NaMPTD buffer (pH 8.0).

Samples containing approximately 200 μg/ml of nuclear proteins were incubated for 2 hr with both 7.4×10^{-11} mol/L of ^{125}I-T_3 and increasing concentrations (from 1.5×10^{-10} to 3.0×10^{-9} mol/L) of T_3. Nonspecific binding of the labeled ligand was

distinguished by 3.0×10^{-7} mol/L of T_3 per sample. After incubation protein–bound T_3 was separated from unbound T_3 by adding 0.5 ml of Dowex-1X8 (Cl^-, 200 × 400 mesh) anion exchange resin of a 80 mg/ml of suspension in NaMTD buffer (pH 8.0) at 0 to 4°C[19] to each test tube. After short vortexing, the suspension was placed on an ice bath for 10 min, then vortexed and the supernatant was collected after a 2 min centrifugation at 1,000*g*. Then 0.5 ml of the supernatant was decanted and its radioactivity was quantified in a gamma spectrometer (model 4000, Beckman, Fullerton, CA USA).

Determinations of DNA-associated nuclear kinase activity

The incubation medium, a total of 250 μL volume for each assay, contained: 100 μL of isolated DNA binding protein solution prepared from liver nuclei[21] containing 100 μg of proteins, 1×10^{-6} mol/L of $MgCl_2$ 5 μCi of γ-^{32}P-ATP–specific activity 220 TBq g_{-1} Buckinghamshire UK, 1×10^{-2} mol/L of Tris-HCl (pH 7.5). The incubation of samples was carried out at 37°C for 60 min. The proteins were precipitated with 1 ml of 33% trichloroacetic acid. The precipitates were collected on Millipore HAWP 0.45 U filters and washed with 10 ml of 25% trichloroacetic acid. The filters were transferred into 5 ml of Unisolve (Koch Light Lab.) and the radioactivities measured in a LKB Rackbeta 1217 spectrometer.

Determination of cytosolic cAMP-dependent and cAMP-independent protein kinase activities

Liver tissue cytosol was prepared as described previously.[8] The samples (20 μg of protein in sample) were assayed for cAMP-dependent protein kinase activity in 250 μL of a reaction mixture containing 2×10^{-3} mol/L of Tris-HCl, pH 7.5, 5×10^{-5} mol/L of 2-mercaptoethanol, 5×10^{-3} mol/L of $MgCl_2$, 10×10^{-3} mol/L of isobuthylmethylxantine, 1×10^{-6} mol/L of cAMP, 100 μg of substrate type II-AS histones (Sigma, Deisenhoten, Deutschland) and 20×10^{-6} mol/L of [γ-^{32}P]ATP-specific activity 220 TBq/g. The incubation was carried out at 25°C for 5 min and the reaction was stopped by adding 1 ml of 25% trichloroacetic acid. The samples were then filtered on a 0.22 μm pore Millipore filter and washed with 2 ml of 5% trichloroacetic acid. The radioactivity associated with the filters was measured as mentioned previously. All data are presented as mean + SEM and the Duncan test was used to evaluate significance.[22]

Results

In order to re-examine whether the above described regimen leads to liver cell proliferation in the rat, the method of [^{14}C]thymidine incorporation into DNA[13] was used. As shown in *Table 1*, specific activity of DNA in the rat liver was comparable between fed (group A) and 2 days fasted (group B) animals. However, when the rats fasted for 2 days, then receiving glucose for 1 day and were refeeding with basal laboratory diet for another day (group C), the rat liver–specific activity of DNA increased significantly, compared with group A and B ($P < 0.01$). In group D, a single dose of T_3 administered at the beginning of realimentation caused a further significant rise of specific activity of DNA in the rat liver.

Table 1 Incorporation of ^{14}C-thymidine into rat liver DNA

Group	dpm × 10^3/mg DNA
A	0.756 ± 0.082
B	0.632 ± 0.046
C	2.421 ± 0.152, $P < 0.01$ vs. A, B
D	4.657 ± 0.181, $P < 0.05$ vs. C

The characteristics of the T_3-specific binding to T_3 receptors were determined in rat liver nuclear proteins as described in the methods and materials section. From the data plotted and expressed according to Scatchard (*Table 2*) it is evident that the Ka values are similar in all the groups studied. *Table 2* shows also the data representing the MBC which was found significantly lower ($P < 0.05$) after 2 days of fasting (group B) compared with fed rats (group A). Glucose administration for 1 day with 1 day refeeding (group C) reversed the MBC to the control level and it remained unchanged after T_3 administration (group D). cAMP-dependent and cAMP-independent protein kinase activity in cytosol is presented in *Figure 1*. A significant decrease ($P < 0.01$) in cytosolic cAMP-dependent and cAMP-independent PK activities in liver of fasted rats (group B) was recorded in comparison with groups A, C, and D. No changes in these activities were found in groups C and D, as compared with fed rats (group A). Both of the cytosolic kinase activities in fasted rats were restored by glucose ($P < 0.05$) (groups C and D). T_3 did not increase cytosolic cAMP-independent or cAMP-dependent kinase activities when compared with the ad libitum fed or glucose treated groups.

We further investigated whether the increase in cytosolic cyclic AMP-dependent protein kinase activity was due to the synthesis of new molecules of the enzyme. We carried out kinetic studies of the cAMP-dependent PK activity as a function of in vitro added cAMP in cytosol fractions from rat liver of group A and D. *Figure 2* shows that the apparent Michaelis constant (K_m) for cAMP determined in the samples from fed rats (0.45 μM) is similar to that found in the group D (0.41 μM) in which the proliferation of rat liver cells was found.

The activity of DNA-associated kinase activity is shown in Figure 3. When 100 μg of DNA binding proteins was incubated in the presence of (γ-^{32}P) ATP, an incorporation of ^{32}P into TCA precipitable material was found. The time course of the incorporation of ^{32}P showed markedly lower values in the fed (A) and fasted group (B), in the groups fasted and subsequently treated with glucose (C), when

Table 2 Comparison of binding characteristics of relative association constants (Ka) and maximal binding capacity (MBC) in hepatic nuclear T_3 receptors in rats kept on different nutritional regimens. Results are expressed as mean ± SEM. Significantly different value from the groups A, C, and D

Group	Ka (L/mol × 10^9)	MBC (pmol/mg)
A	2.93 ± 0.43	0.39 ± 0.075
B	1.72 ± 0.52	0.16 ± 0.092*
C	1.85 ± 0.49	0.48 ± 0.101
D	2.33 ± 0.22	0.37 ± 0.082

*$P < 0.05$.

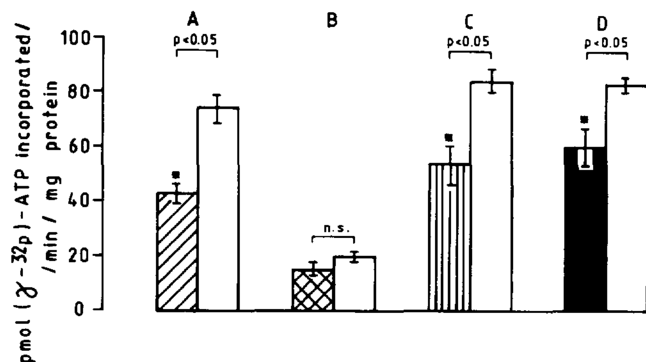

Figure 1 Cytosolic cAMP-dependent and cAMP-independent protein kinase activity during liver regeneration. The first marked bars represent values in the absence and open bars after the addition of cAMP added into incubation medium. Results are expressed as mean ± SEM. $^*P < 0.01$, group B in comparison to values of groups A, C, and D (the first bars).

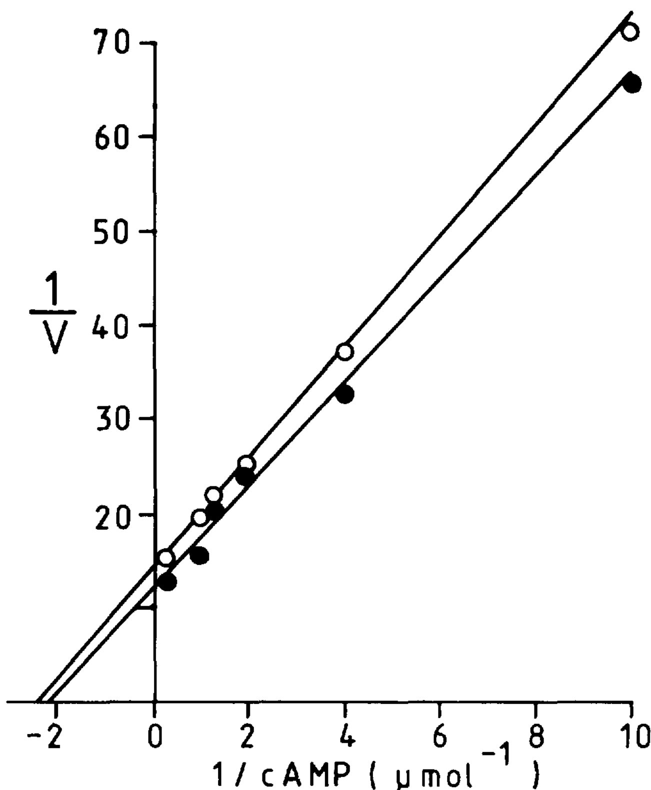

Figure 2 Linewiever–Burk analysis of the cytosolic cAMP-dependent protein kinase activity as a function of cAMP in control (group A) and fasted + glucose + T_3 (group D) rats. Each point represents the value of one of three similar experiments.

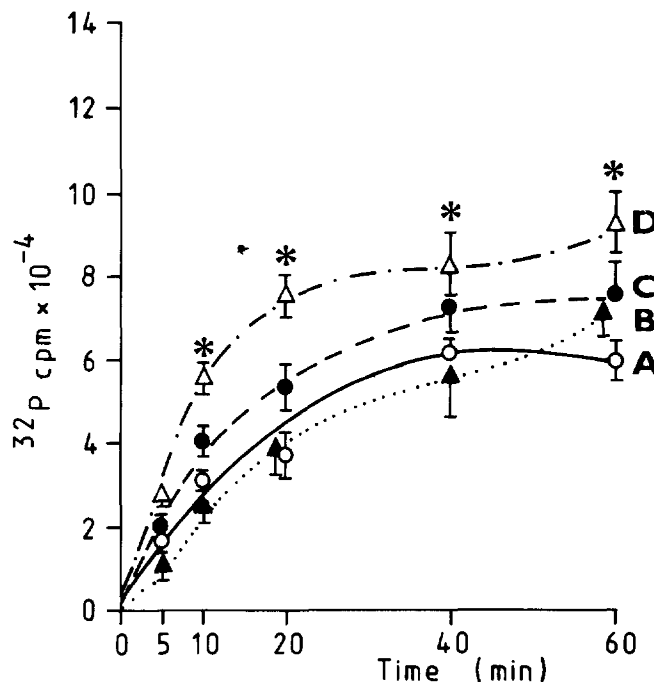

Figure 3 Time course of the DNA-associated protein kinase activity of four different experimental groups, intact controls (A), fasted (B), fasted + glucose (C), fasted + glucose + T_3 (D). Each point represents the value of 6 rats as the mean ± SEM. $^*P < 0.01$, group D compared with groups A, B, and C.

compared to the glucose + T_3 group (D). The results show that DNA-associated kinase activity was not altered by glucose (group C); moreover, the activity of the enzyme increased significantly ($P < 0.01$) with the T_3 treatment (group D).

Discussion

After partial hepatectomy, cellular signals such as growth factors, neurotransmitters, and hormones control the proliferation of hepatocytes in the regenerating rat liver. The action of these agents results from their binding to specific receptors, thereby they activate a signaling cascade within the cell. In the regenerating liver, the mode of action of these substances in the regulation of cell proliferation is a subject of controversy. After partial hepatectomy, the number of receptors for the epidermal growth factor (EGF) was decreased in residual hepatocytes of the rat.[23,24] In the regenerating mouse liver, however, the EGF binding to its receptors as well as the EGF receptor mRNA levels were increased after partial hepatectomy.[25]

Transient increases of cAMP level, which are probably necessary for DNA synthesis, were reported in the early stages of liver regeneration.[26] Cytosolic cAMP-dependent protein kinase, as the major cellular receptor for cAMP, was found to be increased in the rat liver after partial hepatectomy as a result of its new synthesis.[8] In rats kept for several days on a diet consisting of only 20% glucose or on a diet free of protein (and consequently high in carbohydrate), a single protein or amino acid meal induced a substantial rise in DNA synthesis and in mitosis. However, rats were first subjected to a fast and then given an identical meal, no mitogenic response was obtained.[27] Considering these findings, we carried out experiments on fasted rats receiving glucose (group C) or glucose + T_3 (group D) and a standard diet. In both groups DNA synthesis was accelerated compared with the fed (A) and fasted (B-without glucose) groups. Cytosolic cAMP-independent or cAMP-dependent protein kinase activities in rats fasted for 2 days (B) was decreased and restored by glucose, while T_3 had no effect on the cytosolic kinases. (*Figure 1*). The mechanism

of the increase of PK activity in fasted rats with glucose treatment and realimentation (group C) to level of fed rats (A) is unknown. An enhancement of the cytosolic cAMP-dependent PK activity was found in all groups tested, with the exception of the groups of fasted rats (B), suggesting that catalytic units of PK were rather constant in these groups. In contrast, disproportion between regulatory and catalytic units was reported in regenerating rat liver after partial hepatectomy.[28]

With regard to the findings of PKC and PKA levels, investigated by the method of immunoblotting, in the liver of hypothyroid rats compared with euthyroid animals,[29] the effect of thyroid hormone on other types of protein kinase in the regenerating rat liver cannot be excluded. The increase of the cytosolic cAMP-dependent PK activity found in the ad libitum fed group (A) or fasted and glucose treated rats (group D), as calculated from the Linewiever and Burk plot, was not presumably due to new enzyme synthesis. This result, which is not in agreement with the other data found in the regenerating rat liver after partial hepatectomy,[8] supports the notion that the mitogenic signals activating liver growth by a shift in nutrients most likely may differ somewhat from those generated by partial hepatectomy.[9] Increased DNA-associated protein kinase activity in the proliferating rat liver found in the presence of thyroid hormone may indicate the importance of this enzyme in the process of DNA replication.[21]

The observed decreases in the MBC values of T_3 receptors in fasted rats (group B) are in agreement with the results reported by several investigators, although the Ka values in each group remained essentially unaltered.[30–32] Administration of 30% glucose (group C) and 30% glucose plus T_3 (D) led in both groups to an increase of the number of nuclear T_3 receptors when compared with control levels (group A). The mechanism by which this alteration may occur is unclear. Fasting, especially in rats, is a situation in which many metabolic alterations may occur (e.g., increased ketogenesis, acidosis, weight loss, and relative dehydration). It has also been reported that administration of glucagon to rats reduced the number of nuclear T_3 receptors in the liver.[33]

Since starvation changes glucagon and corticosterone plasma concentration[34] and these hormone levels are known to affect nuclear T_3 receptor concentration,[35,36] these altered hormone levels may be responsible for affecting nuclear T_3 receptor capacity. Fasting is a great stimulus for the release of glucagon and its suppression, an energy-dependent process.[37,38] Thus, recovery of the lower nuclear T_3 receptor after fasting observed in our experiments could presumably be a result of glucagon decrease due to glucose administration and rat refeeding. Nuclear protein concentration in the rat liver was also reported to be decreased by starvation.[39] In other experiments the changes of nuclear T_3 receptor in the liver were parallel to those of nuclear protein concentrations. These data provide further insight into the regulation of the concentration of nuclear T_3 receptor.

In summary, our findings suggest that one possible pathway by which tri-iodothyronine as a comitogen regulates proliferation of hepatocytes might be via the activation of the DNA-associated protein kinase activity. From the results of our experiments on cytosolic cAMP-dependent protein kinase activity we can conclude that signals for proliferation of rat liver cells, generated by partial hepatectomy, differ from those evoked by nutrient/metabolic imbalance.

Acknowledgments

The authors thank Mrs. H. Farkasova for her technical assistance. This research was supported by a grant from the Slovak Grant Science Agency, Grant 2/999426.

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