Influence of Plasma Hormone Levels on Various Stimulant-Induced Hepatic DNA Synthesis in Carbon Tetrachloride-Intoxicated Rats

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Abstract—The effects of insulin, glucagon, isoproterenol and carbachol on the regeneration of injured liver were investigated in rats treated with carbon tetrachloride (CCl₄). These agents effectively potentiated hepatic DNA synthesis in rats both at 48 and at 72 hr after CCl₄ intoxication. The maximal stimulatory effects of the agents on the synthesis coincided in time with the peak of elevation in basal DNA synthesis following the intoxication. Plasma levels of insulin and triiodothyronine were decreased before the elevation of basal DNA synthesis in CCl₄-treated rats. The possible relationship of these changes in plasma hormones to the potentiated effects of the agents on DNA synthesis was examined in rats treated with streptozotocin (STZ) or methylthiouracil (MTU). The agents caused no potentiation in STZ-treated rats. On the other hand, in MTU-treated rats, isoproterenol and carbachol significantly stimulated DNA synthesis, but this was not the case with insulin and glucagon. These results suggest that the pancreatic hormonal, ,3-adrenergic and cholinergic stimulations play positive roles in regulating liver regeneration after CCl₄ intoxication. Furthermore, the hypothyroid state developed in CCl₄-treated rats may provide favorable conditions for the stimulation of DNA synthesis by isoproterenol and carbachol. It is unlikely, however, that insulin deficiency contributes to potentiations in the regenerative responses of the injured liver.

Liver regeneration after partial hepatectomy has been the subject of many studies in relation to the regulatory mechanisms of cell proliferation. Many factors have been suggested to be involved in the regulation of liver regeneration (1). It has been shown that the pancreatic hormones, insulin and glucagon, play positive roles in liver regeneration (2, 3). The pancreatic hormones also stimulate DNA synthesis in intact liver (4) and in cultured liver cells (5, 6). In addition, the role of catecholamines in liver regeneration has been reported in experiments with adrenergic agents. Isoproterenol, a ,3-adrenergic agonist, increases DNA synthesis in cultured liver cells prepared from partially hepatectomized rats (7), and propranolol, an antagonist, injected 8 hr after partial hepatectomy delays the initiation of DNA synthesis (8). On the other hand, little work has been done to examine the regeneration of intoxicated liver with a hepatotoxin such as carbon tetrachloride (CCl₄) or D-galactosamine (9). It is not yet clear whether or not the pancreatic hormones and catecholamines play positive roles in regulating the regeneration of injured liver. The process of liver regeneration after intoxication with carbon tetrachloride is reportedly distinct from that of regeneration after partial hepatectomy (10).

The purpose of the present study is to examine the effects of the pancreatic hormones, catecholamine and cholinergic agonist on the regeneration of CCl₄-intoxicated rat liver.

On the other hand, some investigators have reported that "specific" alterations in blood hormone levels, i.e., low insulin and iodothyronines levels and a high glucagon level,
occur before the initiation of hepatic DNA synthesis in partially hepatectomized rats (11) and in CCl₄-treated rats (12). These "specific" endocrine changes are assumed to be related to the liver proliferative state (13). However, it remains obscure whether or not any one or more of these endocrine changes are causally related to the initiation of hepatic DNA synthesis.

In this study, the author also examined a possible relationship of alterations in blood hormone levels to the effects of the pancreatic hormones, catecholamine and cholinergic agonist on the regeneration of the injured liver.

Materials and Methods

Animals: Male Wistar rats, fed standard laboratory chow ad lib., were used for the experiments.

Carbon tetrachloride (1 ml/kg, b.w.) in 50% olive oil solution was administered to rats, weighing 260–320 g, by a stomach tube. Normal control rats received olive oil alone.

Streptozotocin (STZ) treatment was performed as described by Steiner (14). After an overnight fast, rats, weighing 280–310 g, were given a single intravenous injection of streptozotocin (65 mg/kg) freshly dissolved in 0.02 M-citrate buffer, pH 4.5. Control rats were given the buffer alone. At three days after the treatment, the body weight was 250±4 g in STZ-treated rats and 294±4 g in control rats, and the blood glucose level in non-fasting STZ-treated rats reached over 250 mg per 100 ml. Rats were used for the experiments at four days after STZ treatment.

Methylthiouracil (6-methyl-2-thiouracil) treatment was performed as described by Okajima and Ui (15). A 0.3% solution of methylthiouracil (MTU) was given to rats as drinking water for 30 days. After the treatment, body weights were 368±5 g for control rats and 274±4 g for the rats treated with MTU, and goiters were observed in MTU-treated rats. Rats were used for the experiments after the 30-day treatment with MTU.

Determination of DNA synthesis: Rats were injected intraperitoneally with 5 μCi (methyl-³H) thymidine per 100 g body weight and killed 1 hr later. A portion of the liver (1 g) was homogenized in 19 volumes of cold distilled water. DNA was extracted from the homogenate as described by Munro and Fleck (16), and the amount of radioactivity incorporated into DNA was determined by counting in a Beckman LS 9000 scintillation spectrometer. DNA was measured by the method of Martin et al. (17). ³H-Thymidine incorporated into liver DNA was expressed as cpm/mg DNA. The agents or saline (as control) were injected into rats 24 hr before the estimation of DNA synthesis.

Determination of plasma insulin, glucagon and triiodothyronine: Immunoreactive insulin, glucagon and triiodothyronine were determined as described below. Whole blood (0.1 ml) withdrawn from the tail vein was mixed with 0.1 ml of saline, pH 7.4, containing 50 U of Trasylol® and 0.12 mg of EDTA. After centrifugation, the supernatant (0.1 ml) was analyzed for hormones by radioimmunoassay using commercial assay kits (Commissariat à l'Energie Atomique, France, and Dainabot Radioisotope Lab., Tokyo).

Results are given as the mean±S.E. Student's t-test was used for calculating statistical significance.

Materials: The sources of materials used in this work were as follows: (methyl-³H)thymidine (25 Ci/mmol) from the Amersham Corp.; insulin, glucagon, L-isoproterenol-D-bitartrate, carbachol, and streptozotocin from Sigma Chemical Co.; methylthiouracil and triiodothyronine from Tokyo Kasei Kogyo Co., Ltd.; and Trasylol® from Bayer AG. All other reagents were of analytical grade.

Results

Effects of the pancreatic hormones, isoproterenol and carbachol, on hepatic DNA synthesis in CCl₄-treated rats: The results are shown in Fig. 1. The agents were administered to rats at 24 or 48 hr after CCl₄ intoxication, and then, 24 hr later, DNA synthesis was determined. Basal DNA synthesis (without the agents) in normal rats was 240±72 cpm/mg DNA. The level of the basal synthesis was significantly elevated at 48 and 72 hr following CCl₄ treatment, and the peak of basal DNA synthesis (a 2.5-fold increase of that in normal rats receiving saline) was found at 48 hr. These results indicate the
regeneration of the liver after CCl₄ intoxication (18). In normal rats, DNA synthesis was not stimulated by insulin (50 mU/kg, s.c.), whereas it was increased by glucagon (500 μg/kg, i.p.) compared with that in saline-injected animals (Fig. 1). Isoproterenol, (250 μg/kg, i.p.), a β-adrenergic agonist, and carbachol (250 μg/kg, i.p.), a cholinergic agonist, had no effects on DNA synthesis in normal rats. On the other hand, 48 hr after CCl₄ administration in rats, both insulin and glucagon strikingly stimulated hepatic DNA synthesis, to 4.8-fold and 4.0-fold that of the basal synthesis, respectively. These stimulatory effects of the pancreatic hormones were still observed at 72 hr. Furthermore, isoproterenol and carbachol also produced significant increases of DNA synthesis in CCl₄-treated rats. The maximal stimulation of DNA synthesis by each agent was observed in rats at 48 hr after the intoxication and was coincident in time with the peak of elevation in basal DNA synthesis.

Alterations in plasma insulin levels in response to glucagon, isoproterenol and carbachol: Glucagon, isoproterenol and carbachol are known to stimulate the secretion of insulin from the pancreas (19). Furthermore, insulin stimulated hepatic DNA synthesis in CCl₄-treated rats (Fig. 1). Therefore, it might be possible that the enhancements in the increase of plasma insulin levels contribute to the stimulatory effects of glucagon, isoproterenol and carbachol on hepatic DNA synthesis in CCl₄-treated rats.

**Fig. 1.** Effects of the pancreatic hormones, isoproterenol and carbachol, on hepatic DNA synthesis in CCl₄-treated rats. Insulin (50 mU/kg, s.c.), glucagon (500 μg/kg, i.p.), isoproterenol (250 μg/kg, i.p.) or carbachol (250 μg/kg, i.p.) was injected into rats 24 hr before the estimation of DNA synthesis. The results are given as the mean±S.E. The number of observations is 4—7. **P<0.05, ***P<0.01, compared with the saline-injected control in normal rats. *P<0.05, **P<0.01, ***P<0.001, compared with the corresponding saline-injected control.

**Fig. 2.** Alterations in plasma insulin levels in response to glucagon, isoproterenol and carbachol in CCl₄-treated rats. Glucagon (A), isoproterenol (B) or carbachol (C), using the same dose as in Fig. 1, was subcutaneously injected at time 0. Glucose (D) was intravenously injected with a 50% solution, 1.5 ml/kg, at time 0. The results are given as the mean±S.E. The number of observations is 4 or 5. *P<0.05, compared with the normal controls. ○, normal control; ●, 24 hr after CCl₄ treatment; △, 48 hr after CCl₄ treatment.
treated rats. To examine this, alterations in plasma insulin levels in response to these agents were determined. As shown in Fig. 2, increases of plasma insulin by these stimulants were significantly lower in rats both at 24 and at 48 hr after CCl₄ administration than in normal rats. Furthermore, the plasma insulin responses to the glucose load were also attenuated in CCl₄-intoxicated rats, though these were statistically insignificant. It is unlikely, therefore, that the stimulations of hepatic DNA synthesis by glucagon, isoproterenol and carbachol are due to the enhancements in the increase of plasma insulin levels.

Since CCl₄ results in hepatocyte damage and decrease of hepatic blood flow in rats (Hatta, unpublished) and cats (20), the attenuation of stimulant-induced elevation in plasma insulin levels in CCl₄-treated rats may result from decreases of the insulin secretion from the pancreas rather than increases of the insulin uptake by the liver.

**Effects of deficient state of insulin or iodothyronines on the stimulation of hepatic DNA synthesis:** Plasma levels of insulin, triiodothyronine and glucagon were determined in rats after CCl₄ intoxication. As shown in Fig. 3, plasma levels of insulin and triiodothyronine were decreased, and the glucagon level was increased at 24 and 48 hr after the intoxication, in agreement with a previous report (12). Since decreases in plasma levels of insulin and triiodothyronine were observed before the elevation of DNA synthesis in CCl₄-treated rats (Figs. 1 and 3), it was studied if lowered levels of plasma hormones contribute to the stimulation of DNA synthesis by the agents, using STZ- or MTU-treated rats.

At four days after STZ treatment, plasma insulin level was significantly lower in STZ-treated rats (14.8±2.5 μU/ml) than in control rats (33.2±4.1 μU/ml). Basal DNA synthesis was significantly lower in STZ-treated rats than in the controls (Fig. 4). Insulin, at the same dose used for the CCl₄-treated rats, produced no stimulation of DNA synthesis in STZ-treated rats, while glucagon increased hepatic DNA synthesis to 1.8-fold that of the basal synthesis. However, the extent of this stimulation was almost the same as that induced by glucagon in normal rats. The effect of glucagon on DNA synthesis was not potentiated in STZ-treated rats. Furthermore, isoproterenol and carbachol failed to stimulate DNA synthesis in STZ-treated rats.

After the 30-day treatment with MTU, plasma triiodothyronine level was significantly lower in MTU-treated rats (0.36±0.03 ng/ml) than in control rats (0.55±0.04 ng/ml). As in STZ-treated rats, basal DNA

![Fig. 3. Alterations in plasma levels of insulin, triiodothyronine and glucagon following CCl₄ intoxication.](image)

Blood was withdrawn from the tail vein. Plasma hormone levels were determined by radioimmunoassay. The results are given as the mean±S.E. The number of observations is 6 for insulin (A), 4 for triiodothyronine (B) and 5 for glucagon (C). *P<0.05, **P<0.01, ***P<0.001, compared with the normal controls.
synthesis in MTU-treated rats was significantly lower than in control rats (Fig. 5). This decrease in the basal synthesis was reversed to the control level by the administration of triiodothyronine (1 mg/kg, s.c.) 24 hr before the determination of DNA synthesis. DNA synthesis in control rats, MTU-treated rats, and MTU-treated rats administered triiodothyronine was 1896±202, 1353±133, and 2394±175 cpm/mg DNA, respectively. Neither insulin nor glucagon caused any stimulation of DNA synthesis in MTU-treated rats (Fig. 5). In contrast, isoproterenol and carbachol significantly potentiated DNA synthesis in MTU-treated rats to 1.5-fold and 1.4-fold that of the basal synthesis, respectively.

Discussion

There have been very few reports on the regenerative response of injured liver (9, 21), and the stimuli that induce the regeneration of injured liver are not well known. The results presented here show that insulin and glucagon effectively stimulate hepatic DNA synthesis in rats after CCl₄ intoxication (Fig. 1). Furthermore, isoproterenol, a β-adrenergic agonist, also enhanced DNA synthesis in CCl₄-treated rats (Fig. 1). The maximal stimulatory effects of these agents were observed in rats at 48 hr after CCl₄ administration, coincidentally in time with the peak of elevation in basal DNA synthesis which represents the liver regeneration after the intoxication (18). It is likely, therefore, that catecholamine, which may act through β-adrenergic stimulation (8), and the pancreatic hormones play positive roles in regulating the liver regeneration after CCl₄ intoxication. Involvement of the pancreatic hormones and catecholamines in hepatocyte proliferation have been suggested in the regenerating liver after partial hepatectomy (2, 3, 8) and in cultured liver cells (5–7).

No reports have been made regarding the relationship between cholinergic stimulation and liver growth. In this study, carbachol, a cholinergic agonist, significantly potentiated DNA synthesis in CCl₄-treated rats and the marked stimulating effect of carbachol coincided in time with the elevation of basal DNA synthesis (Fig. 1). These results may suggest that cholinergic stimulation is also involved in the regulation of the liver regener-
The mechanisms of the potentiation in the regenerative responses in the injured liver are not clear. The relationship of "specific" alterations in plasma hormone levels, i.e., decreases of insulin and iodothyronines and an increase of glucagon, to the induction of DNA synthesis was assumed by Leffert and Koch (13) in partially hepatectomized rats. Among these hormone changes, a deficient state of insulin or iodothyronines is known to enhance the β-adrenergic action of catecholamines in liver (24, 25). Therefore, it may be possible that insulin or thyroid hormone deficiency developed in rats after CC14 intoxication (Fig. 3 and Ref. 12) is involved in the potentiated effects of the agents on DNA synthesis observed in CC14-treated rats.

However, the pancreatic hormones, isoproterenol and carbachol, had little or no effect on DNA synthesis in STZ-treated rats (Fig. 4). In addition, basal DNA synthesis was significantly lower in STZ-treated rats than in the controls. This result is compatible with the earlier observation that anti-insulin serum inhibits hepatic DNA synthesis in rats after partial hepatectomy (26) and supports the view that insulin plays an important role in hepatocyte proliferation. Thus, it seems unlikely that insulin deficiency contributes to poten­tiations in the regenerative responses of CC14-treated rats to the agents.

Basal DNA synthesis in MTU-treated rats was also lower than in the controls (Fig. 5). The administration of triiodothyronine to MTU-treated rats reversed the decrease in the basal synthesis of MTU-treated rats to the control levels. These results may indicate that thyroid hormones also regulate the proliferation of liver cells. The role of iodothyronines in liver cell proliferation has been demonstrated (27, 28). Insulin and glucagon were without effect on DNA synthesis in MTU-treated rats. In contrast, isoproterenol and carbachol produced a significant stimulation of DNA synthesis in MTU-treated rats. Therefore, the hypothyroid state developed in CC14-treated rats may provide favorable conditions for the stimulations of DNA synthesis by isoproterenol and carbachol, but not for the stimulations by the pancreatic hormones.

Thus, the potentiated effects of insulin and glucagon on DNA synthesis observed in CC14-treated rats could not be related to reduction in plasma levels of either insulin or iodothyronines alone. If hormonal changes relate to the stimulatory effects of the pancreatic hormones, a combination, rather than any one of these "specific" changes in plasma hormones, might be responsible for the induction of hepatic DNA synthesis (13).

Since the deficient state of insulin or iodothyronines caused reduction of hepatic DNA synthesis (Figs. 4 and 5), one would anticipate a decrease rather than an increase in basal DNA synthesis after CC14 intoxication. However, basal DNA synthesis was significantly increased in rats at 48 and 72 hr after the intoxication (Fig. 1). Hepatic responsiveness in DNA synthesis to the pancreatic hormonal, β-adrenergic and cholinergic stimulations were potentiated in CC14-treated rats (Fig. 1). It appears, therefore, that the elevation in basal DNA synthesis in CC14-treated rats results from the enhancement of DNA synthesis endogenously induced by these stimulations, which produce an increase greater than the decrease caused by deficiencies of insulin and iodothyronines.

The mechanisms by which the hepatic regenerative response was potentiated in CC14-intoxicated rats remain to be clarified. Nevertheless, the present study suggests that the pancreatic hormonal, β-adrenergic and cholinergic stimulations participate in the liver regeneration after CC14 intoxication.

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