Biochemical Pharmacology, Vol. 35, No. 5, pp. 865–867, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

SHORT COMMUNICATIONS

Liver regeneration in streptozotocin-diabetic rats

(*Received* 23 *May* 1985; *accepted* 22 *August* 1985)

Liver regeneration after partial hepatectomy (PH) has been investigated for many years in attempts to understand processes that control mammalian cell proliferation. It has been proposed that hepatocyte proliferation is controlled by humoral factors including hormones [1]. Among them, much attention has been devoted to insulin. Peripheral insulin-antiserum infusions virtually inhibit rat hepatic DNA synthesis at 24 hr after PH [2]. Split portacaval transposition studies in both intact and pancreatectomized animals indicate that hepatocyte proliferation critically depends on insulin [3]. It was also reported that insulin and glucagon promote synergistically hepatocyte DNA synthesis in pancreatectomized 70%-hepatectomized rats [4]. On the other hand, liver DNA synthesis is suppressed if partially hepatectomized animals receive a peripheral venous infusion of insulin [5]. Furthermore, arterial insulin levels are known to fall after PH [6]. To shed more light on the effect of insulin, we evaluated regenerative responses of streptozotocin-diabetic rats after PH. We followed hepatic DNA content and activities of key enzymes in DNA synthesis, i.e. thymidylate synthetase (TS; EC 2.1.1.45) and thymidine kinase (TK; EC 2.7.1.21). TS is responsible for *de novo* synthesis of thymidylate (dTMP) by the methylation of dUMP, and TK, which is an enzyme of the pyrimidine salvage pathway, catalyzes the phosphorylation of thymidine to dTMP. The levels of these enzymes are barely detectable in resting eukaryotic cells but increase dramatically in a proliferative phase including regenerating liver after PH [7] or acute carbon tetrachloride intoxication [8]. Thus, these enzymes are believed to be rate determining for DNA replication [9, 10].

Materials and methods

Male Wistar rats (190–210 g) were used in this study. The animals were made diabetic by a single intraperitoneal injection of streptozotocin (45 mg/kg body wt) following an overnight fast. Control rats received 0.15 M NaCl. The rats were permitted free access to food (MF, Oriental Yeast Co., Osaka, Japan) and water at all times. Four days after injection, partial (68%) hepatectomy (PH) was carried out according to the procedure of Higgins and Anderson [11].

At various times (24, 48 and 72 hr) after PH, the animals were killed under ether anesthesia. Blood was withdrawn from the abdominal aorta and the liver was perfused *in situ* with 0.9% NaCl. Plasma glucose of these blood samples was monitored utilizing commercially available kits (Glucose-Test Wako, Osaka, Japan). The excised livers were homogenized in 50 mM Tris–HCl buffer, pH 7.5, containing 0.25 M sucrose, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA (the tissue buffer ratio was 1:4). The DNA content of liver homogenate was measured by the diphenylamine reaction [12] after extraction with trichloroacetic acid according to the procedure of Schneider [13]. Protein was determined by the method of Lowry *et al.* [14], using bovine serum albumin as standard. After a 20% liver homogenate was centrifuged at 36,000 *g* for 30 min at 4°, the supernatant fraction was used as the enzyme preparation. The activities of TS and TK were determined as described previously [8] and expressed as pmoles of product formed/min/mg protein at 37°. Statistical analyses of data were by Student's *t*-test.

Results and discussion

Liver regeneration was estimated at every 24 hr after PH for 3 days. Data are shown in Table 1. The liver regeneration of control rats after PH was monitored by gross variables such as liver weight and DNA content, which increased steadily. Activity of TS elevated at 24 and 48 hr after PH reached maximal level, which was eleven times compared with non-hepatectomized (sham-operated) rats. Maximal activity of TK was attained at 24 hr after PH and the value was twelve times as much as that of the sham-operated rat.

The streptozotocin-treated group was defined as diabetic on the basis of high plasma glucose (about 450 mg/dl), as shown in Table 1. Liver weight and DNA content of the diabetic animal at 24 and 48 hr after PH showed similar levels to those of the control group. At 72 hr after PH, liver weight and DNA content in the diabetic group were higher than those in the control in spite of the lower body weight. Concerning TS and TK, no significant difference was observed between the control and the diabetic rats except that TS activity at 72 hr following PH was significantly higher for the diabetic group. Liver weight (at 24, 48 and 72 hr) and DNA content (at 72 hr) per 100 g body weight were significantly larger in the diabetic group than in the corresponding control. These observations demonstrated that the liver of the streptozotocin-diabetic rat caused similar or even enhanced DNA synthesis after PH compared with that of the control as evidenced by the enzymatic activities of TS and TK, which were rate-determining enzymes in DNA synthesis, as well as liver weight and DNA content.

The requirement of insulin as a positive regulator for liver cell proliferation is fully established by *in vitro* [15] and *in vivo* [2–4] studies. Suppression of hepatic DNA synthesis in partially hepatectomized rats by exogenous insulin infusion [5] suggests that high plasma insulin level is inhibitory for liver regeneration. This correlates with the observation [6] that plasma insulin level declines after PH in adult rats. Leffert and Koch [16] advanced a hypothesis that the particular endocrine pattern such as hypoinsulinaemia and hyperglucagonaemia was a general characteristic of enhanced proliferative capacity and explained the benefit of hypoinsulinaemia according to the physicochemical properties of interaction between insulin and its receptor. Our experiment indicates that the diabetic condition, i.e. relative deficiency (not zero level) of plasma insulin does not prevent but rather promotes proliferative responses of the liver cell following PH at least in the early stage of regeneration. The mechanism of the phenomenon remains to be explored.

The present results are not consistent with the report [17] that incorporation of [^{3}H]thymidine into liver DNA in alloxan-diabetic rats was only 63% of the normal at 28 hr following PH. However, temporal incorporation of radioactive thymidine is suggested not to reflect quantitatively the amount of DNA synthesis [18, 19]. Besides, the exogenous [^{3}H]thymidine is metabolized by the salvage pathway (via TK), and therefore the *de novo* pathway (via TS) cannot be evaluated. Recently we reported that the integration of the activities of TS and TK correlated closely with the amount of DNA synthesis in liver regeneration

Table 1. Effects of streptozotocin-induced diabetes on liver regeneration after PH

Treatment	Time after PH (hr)	Body weight (g)	Liver weight (g)	Liver weight (g/100 g body wt)	Liver DNA (mg)	Liver DNA (mg/100 g body wt)	Enzymatic activities (pmoles/min/mg protein) TS	Enzymatic activities (pmoles/min/mg protein) TK	Plasma glucose (mg/100 ml)
Control	24	195.7 ± 3.9	3.48 ± 0.08	1.78 ± 0.04	7.23 ± 0.27	3.70 ± 0.13	64.78 ± 5.07	201.51 ± 24.24	123.22 ± 2.78
	48	196.4 ± 1.9	4.67 ± 0.18	2.45 ± 0.04	10.47 ± 0.29	5.32 ± 0.17	75.58 ± 5.82	163.03 ± 14.29	137.7 ± 2.09
	72	201.8 ± 2.1	5.99 ± 0.28	2.96 ± 0.08	14.21 ± 0.83	6.96 ± 0.31	56.42 ± 4.70	111.21 ± 5.97	140.25 ± 7.80
Streptozotocin	24	188.0 ± 2.8	3.73 ± 0.11	1.99 ± 0.07*	6.63 ± 0.18	3.53 ± 0.08	60.00 ± 6.60	180.60 ± 16.18	451.4 ± 27.10*
	48	187.0 ± 6.12	5.06 ± 0.21	2.70 ± 0.08*	10.44 ± 0.42	5.73 ± 0.18	95.70 ± 10.30	137.90 ± 14.62	496.0 ± 15.06*
	72	183.0 ± 3.0†	6.99 ± 0.21*	3.82 ± 0.07*	17.10 ± 0.78*	9.33 ± 0.30*	78.45 ± 9.06†	104.61 ± 15.50	435.4 ± 26.16*

Streptozotocin (45 mg/kg body wt) was injected 4 days before PH. At 24, 48 and 72 hr after PH, liver weight, DNA content, activities of TS and TK, and plasma glucose level were determined as described in Materials and Methods. Values are expressed as mean ± S.E. of five to eighteen animals.
*† Symbols indicate significant differences from the corresponding controls
* $0.01 > P$; and † $0.05 > P > 0.01$.

[20], and thus both TS and TK must be followed to quantitate DNA synthesis.

Yamada *et al.* [21] reported that liver mitochondria was impaired in alloxan-diabetic rats and argued that this lesion caused a decrease in the ability of liver regeneration based on high mortality of the diabetic animal on PH. Among their variables concerning mitochondrial activity, RCR (respiratory control ratio) and Phos/cyt. *a* (moles of ATP formed per second divided by the moles of cytochrome aa_3) decreased most and the levels were about 40% of the normal values in the severest diabetic group, whose survival rate was zero after PH [21]. However, they did not provide evidence showing depressed proliferation of the hepatic cell. On the other hand, Van den Bogert *et al.* [22] described that a reserve capacity of mitochondria for ATP formation was very large and liver regeneration was not blocked when mitochondrial function was diminished to as low as 20% of the normal by intoxication with oxytetracycline. The present results also show that the liver regeneration was not prevented by diabetic condition even if mitochondrial oxidative phosphorylation was partially inhibited. Therefore, low survival rate of diabetic animals following PH reported by Yamada *et al.* [21] may be ascribed to other metabolic defects encountered in diabetes.

In summary, the liver of the streptozotocin-diabetic rat caused similar or even enhanced regenerative responses following partial hepatectomy compared with that of the control on the basis of activities of thymidylate synthetase and thymidine kinase (which are rate-determining enzymes in DNA synthesis) as well as gross variables such as liver weight and DNA content.

*Department of Food Science and Nutrition
Nara Women's University
Nara 630, Japan; and
‡Department of Public Health
Faculty of Medicine
Kyoto University
Kyoto 606, Japan

RIEKO NAKATA*
IKUYO TSUKAMOTO*†
MASAMITSU MIYOSHI*
SHOSUKE KOJO‡

REFERENCES

1. H. L. Leffert, K. S. Koch, T. Moran and B. Rubalcava, *Gastroenterology* **76**, 1470 (1979).
2. N. L. Bucher, U. Patel and S. Cohen, in *Hepatotrophic Factors*, CIBA Foundation Symposium No. 55 (Eds. R. Porter and J. Whelan), p. 95. Elsevier, Excerpta Medica, North-Holland, Amsterdam (1978).
3. T. E. Starzl, K. A. Porter, J. A. Francavilla, J. Benichou and C. W. Putnam, in *Hepatotrophic Factors*, CIBA Foundation Symposium, No. 55 (Eds. R. Porter and J. Whelan), p. 111. Elsevier, Excerpta Medica, North-Holland, Amsterdam (1978).
4. N. L. R. Bucher and M. N. Swaffield, *Proc. natn. Acad. Sci. U.S.A.* **72**, 1157 (1975).
5. J. Simek, V. Chmelar, J. Melka, J. Pazderka and Z. Charvat, *Nature, Lond.* **213**, 910 (1967).
6. H. Leffert, N. M. Alexander, G. Faloona, B. Rubalcava and R. Unger, *Proc. natn. Acad. Sci. U.S.A.* **72**, 4033 (1975).
7. R. Labow, G. F. Maley and F. Maley, *Cancer Res.* **29**, 366 (1969).
8. R. Nakata, I. Tsukamoto, M. Miyoshi and S. Kojo, *Biochem. Pharmac.* **34**, 586 (1985).
9. R. L. Blakely, *The Biochemistry of Folic Acid and Related Pteridine*, p. 231. American Elsevier, New York (1969).
10. A. Kornberg, *DNA Replication*, p. 39. Freeman, San Francisco (1980).
11. G. M. Higgins and R. M. Anderson, *Archs Path.* **12**, 186 (1931).

† To whom correspondence should be addressed.

12. K. Burton, *Biochem. J.* **62**, 315 (1956).
13. W. C. Schneider, *Meth. Enzym.* **3**, 680 (1957).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. H. L. Leffert, *J. Cell Biol.* **62**, 792 (1974).
16. H. L. Leffert and K. S. Koch, in *Hepatotrophic Factors*, CIBA Foundation Symposium No 55 (Eds. R. Porter and J. Whelan), p. 61. Elsevier, Excerpta Medica, North-Holland, Amsterdam (1978).
17. L. R. Younger, J. King and D. F. Steiner, *Cancer Res.* **26**, 1408 (1966).
18. J. D. Simnett and J. M. Fisher, *J. cell. Physiol.* **81**, 171 (1973).
19. T. Youdale and J. P. MacManus, *J. cell. Physiol.* **86**, 495 (1975).
20. R. Nakata, I. Tsukamoto, M. Nanme, S. Makino, M. Miyoshi and S. Kojo, *Eur. J. Pharmac.*, **114**, 355 (1985).
21. T. Yamada, M. Yamamoto, K. Ozawa and I. Honjo, *Ann. Surg.* **185**, 35 (1977).
22. C. Van den Bogert, M. Lont and A. M. Kroon, *Biochim. biophys. Acta* **722**, 393 (1983).

Biochemical Pharmacology, Vol. 35, No. 5, pp. 867–869, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

The inhibition of rat adipocyte ecto-5′-nucleotidase by xanthines is not related to lipolysis

(*Received* 10 *June* 1985; *accepted* 23 *September* 1985)

The effects of alkylxanthines on fat cell metabolism especially the stimulation of lipolysis are regarded as the result of the combination of adenosine receptor antagonism and cAMP-phosphodiesterase inhibition. Since alkylxanthines have been reported to inhibit 5′-nucleotidase in several tissues [1, 2] and Newby *et al.* [3] showed that the enzyme of adipocytes is predominantly a cell surface enzyme we investigated if alkylxanthines inhibited the cleavage of exogenously added 5′-AMP and if there was any relation to the effect of the agents on lipolysis.

Methods

Isolation and incubations of rat adipocytes were performed in a Krebs–Ringer medium buffered with 4-(2-hydroxyethyl)-piperazine ethanesulfonic acid (10 mM; pH 7.4). the epididymal fat pads of male, fed Wistar rats (140–180 g) were digested with collagenase according to Rodbell [4]. Cytocrit determination was accomplished by the addition of aliquots of cells to hematocrit capillaries and subsequent centrifugation [5].

Ecto-5′-nucleotidase of the fat cells [3] was tested after preincubation with the agents under investigation. The velocity of the hydrolysis of exogenously added [2-^{3}H]-5′-AMP (20,000–30,000 cpm) and unlabelled 5′-AMP at the concentrations of 5, 50 and 200 μM was determined in duplicate with 500 μl aliquots of the treated cells. After 2, 3 or 10 min respectively the reaction was stopped and the remaining nucleotide precipitated by a $ZnSO_4$ (0.3 M)/$Ba(OH)_2$ (0.3 N) procedure [3]. After a minute centrifugation (10,000 *g*) the amount of label remaining in 500 μl of the clear supernatant was determined. Blank values were obtained by the simultaneous addition of $ZnSO_4$ and cells to the substrate. After subtraction of these blank values the velocity of 5′-AMP hydrolysis was expressed as nmol substrate hydrolysed per μl lipid per min. The selective inhibitor of 5′-nucleotidase α,β-methylene ADP was added to the substrate at a concentration of 50 μM. Lipolysis of the same pretreated cells was evaluated by the determination of medium glycerol content according to [7].

Results and discussion

Theophylline dose-dependently inhibited the hydrolysis of 5′-AMP (200 μM) by rat adipocytes (Fig. 1). The maximal inhibition of 40% was achieved by 5 mM theophylline. PGE_2 (1 μg/ml) failed to significantly alter the enzyme inhibition by the methylxanthine. In contrast, this concentration of the prostaglandin completely antagonized the lipolysis stimulated by theophylline (Fig. 1).

Time course experiments of 5′-AMP (200 μM) hydrolysis at a cytocrit of 1% were linear for at least 30 min in the absence and the presence of theophylline (1.7 mM). The methylxanthine without delay caused a 30% inhibition of the hydrolysis of the nucleotide at all tested time points.

The kinetics of the hydrolysing activity are shown in Fig. 2 in the form of a Hanes plot (S/v vs S) for one representative

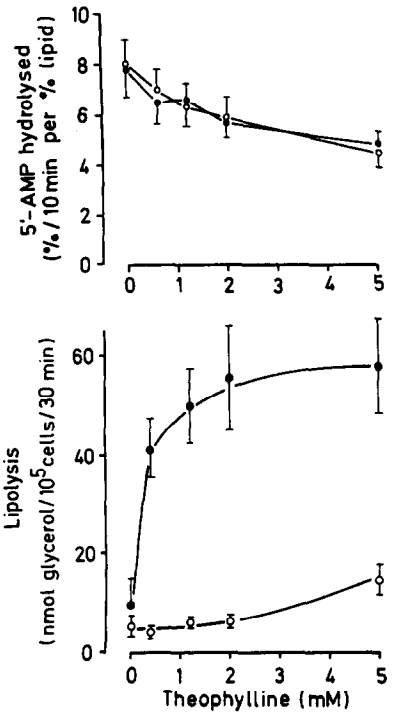

Fig. 1. Effects of theophylline on 5′-nucleotidase (upper panel) and on lipolysis (lower panel) in isolated adipocytes in the absence (●) or presence (○) of prostaglandin E_2 (1 μg/ml). Data on inhibition of 5′-AMP (200 μM) hydrolysis are the means ± standard error of 4 experiments. Mean cell cytocrit was 1.1% in nucleotidase experiments. Data on lipolysis are the means ± standard error of 3 experiments. The mean cell concentration was 2.04×10^5 cells/ml.