Arch. Gerontol. Geriatr., 3 (1984) 167–174
Elsevier

AGG 00069

Regulation of citrate synthase and phosphoenolpyruvate carboxykinase by hydrocortisone in the liver of aging rats

Ramesh Sharma * and Swaraj K. Patnaik

Department of Life Sciences, Manipur University, Canchipur, Imphal – 795003, India

(Received 2 November 1983; revised version received 2 April 1984; accepted 3 April 1984)

Summary

The activities and hormonal regulations of citrate synthase (CS) and phosphoenolpyruvate carboxykinase (PEPCK) in the liver of male rats of various ages were studied. It has been observed that the activity of CS increases gradually as a function of age of the rat. The activity of PEPCK, on the other hand is highest in the liver of adult rats. Adrenalectomy causes no significant change in the activity of CS of the liver of young, adult and old rats. However, this treatment decreases significantly the activity of PEPCK of the liver of rats of all the ages. Administration of hydrocortisone to adrenalectomized rats depresses and induces, respectively, the activity of CS and PEPCK in the liver of young and adult rats but not in the old rats. This hormone mediated effect of the enzymes decreases with increasing age of the rats. Treatment of actinomycin D prior to hydrocortisone administration tends to normalize the depressed level of CS. However, this inhibitor inhibits the PEPCK induction.

citrate synthase and phosphoenolpyruvate; aging; hydrocortisone

Introduction

Oxaloacetic acid is converted to phosphoenolpyruvate (PEP) or condenses with acetyl-CoA to form citrate in the hepatic cells. Citrate synthase (citrate oxaloacetate lyase (CoA-acetylating) EC 4.1.3.7) is the first enzyme of the Krebs cycle which catalyses the conversion of oxaloacetic acid and acetyl-CoA to citric acid, a step which is considered to be the major regulatory site for the Krebs cycle activity (Srere, 1972). Phosphoenolpyruvate carboxykinase (PEPCK; GTP: oxaloacetate carboxylase (transphosphorylating) EC 4.1.1.32) is a key enzyme of gluconeogenic pathway (Tilghaman et al., 1976). It catalyses reversible decarboxylation of

* *Address for correspondence*: Biochemistry Division, Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi – 221005, India.

oxaloacetate to PEP. The relative amount of this enzyme in the mitochondrial and cytosolic fractions of animal tissues varies greatly from one species to another (Nordlie and Lardy, 1963). In rat liver, it is predominantly cytosolic (Nordlie and Lardy, 1963). However, CS is exclusively mitochondrial (Srere, 1972).

There are only few conditions which are known to alter the total activity of CS in rat tissues. Prolonged exercises of rat caused a 2-fold increase in the activity of muscle CS (Holloszy et al., 1970). Kirsten and Kirsten (1972) have demonstrated that aldosterone injection causes a transient of 30% increase in the level of CS. Mukherjee et al. (1976) reported that the activity of hepatic CS increases by 2- to 3-fold in vitamin B_{12} deficiency. Cytosolic PEPCK responds adaptively to hormonal and dietary influences (Shrago et al., 1963; Lardy et al., 1965). Gunn et al. (1975) reported that glucocorticoids appear to stimulate the synthesis of PEPCK in hepatoma cells in vitro since the induction of this enzyme by these steroids is inhibited by actinomycin D. It has also been reported that administration of dexamethasone increases and decreases, respectively, the activity of PEPCK and CS of the liver of rat (Heitzman et al., 1972). For the present studies, CS and PEPCK were selected as the model enzymes since the former is a key regulatory enzyme of the Krebs cycle and the latter, a key enzyme of gluconeogenesis. The rate of oxidation of glucose through Krebs cycle and its synthesis through gluconeogenesis might be controlled by the activities of CS and PEPCK since these enzymes are regulated endogenously by the cellular concentration of oxaloacetate. We report here the differential regulation of CS and PEPCK by hydrocortisone in the liver of rats through different phases of their life span.

Materials and Methods

Animals

Male albino rats of Wistar strain of three different age groups (6, 30 and 90 wk) were used. They were kept at $24 \pm 2°C$ under a controlled illumination programme that provided a 12-h light period followed by a 12-h dark period. The rats were fed with a freshly prepared diet containing wheat flour and vitaminized milk powder in the ratio of 4 : 1 usually given at 6 P.M. daily. The food and water were supplied ad libitum. All the chemicals used were of analytical grade. The biochemicals were purchased from Sigma Chemical Co., U.S.A.

Effect of hormone

Pilot experiments were undertaken to find the time and dose dependence of these enzymes towards hydrocortisone in rats of various ages. Maximum response of the enzymes was obtained 3 days after the hormone administration at a dose of 5.0 mg/100 g body wt. The rats of each age group were divided into four sets, each having 4–5 rats. The set I rats served as the norm. The rats of sets II, III and IV were bilaterally adrenalectomized (A/d). These rats were given 0.9% NaCl ad libitum instead of water for 10 days. On the 11th day, the set II rats were administered 1.0 ml of 0.9% NaCl intraperitoneally (i.p.) and these rats served as

control for the induction studies. The rats belonging to sets III and IV were given an i.p. dose of hydrocortisone (5.0 mg/100 g body wt., suspended in 1.0 ml of 0.9% NaCl) at the fixed time of day for 3 days. The set IV rats were also given actinomycin D (10.0 μg/100 g body wt., suspended in 0.9% NaCl), 1 h prior to hydrocortisone administration for 3 days. All the rats were killed 3 h after the final hormone injection.

Tissue preparation

The rats were killed by cervical dislocation at a fixed time of day (i.e. 18.00 h) in order to avoid fluctuations in enzyme levels due to circadian rhythm. Their livers were removed, washed in normal saline and blotted dry on a filter paper. A 10% (w/v) homogenate of the tissue was prepared in 0.25 M sucrose at 2 ± 1°C using a Potter–Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was filtered through a double layered cheese cloth and centrifuged for 15 min at $700 \times g$ at 0°C to sediment nuclei. The resulting supernatant was centrifuged further for 30 min at $14{,}000 \times g$ at 0°C to sediment mitochondria. The clear supernatant was used for spectrophotometric assay of PEPCK (Ballard and Hanson, 1967). The enzyme activity was routinely measured at 25°C by the reduction of NADH (0.15 mM) in 1.0 mM imidazole/HCl buffer (final pH 6.7), containing 50 mM $NaHCO_3$, 1.25 mM IDP, 2.0 mM $MnCl_2$, 2.0 mM GSH, 20 U of malate dehydrogenase, 0.05 ml of the suitably diluted supernatant and 1.5 mM PEP. One unit of the enzyme was defined as that amount which is required to oxidize 1.0 μmol of NADH/min at 25°C.

The crude mitochondrial pellet was washed once with the homogenizing solution and centrifuged. The mitochondrial pellets were suspended in 5.0 mM potassium phosphate buffer, pH 7.5 containing 0.25 M sucrose. This suspension was used for the assay of CS within 3 h of solubilization. The activity of CS was determined by measuring the initial rate of the reaction at 412 nm (Srere et al., 1963). The reaction mixture (final volume 3.0 ml) contained 100 mM Tris–HCl buffer (pH 8.1), 0.1 mM acetyl-CoA, 0.1 mM 5,5′-dithiobis (2-nitrobenzoate), 0.5 mM oxaloacetate and the suitably diluted enzyme (0.05 ml). The reaction was carried out at 25°C and was initiated by the addition of oxaloacetic acid. One unit of this enzyme is the amount that catalyses the liberation of 1 μmol of CoA-SH/min under the standard conditions of the assay. The activities of both the enzymes are expressed as U/mg protein. Protein content (mg/g wet weight) of the supernatant and the mitochondrial fractions were determined (Lowry et al., 1951). All the data were statistically analysed (Garrett, 1966). The level of significance (P) between two sets of data was calculated according to Student's t-test. P values, which were 5% or lower for two sets of data were taken as significant.

Results and Discussion

Our data indicate that the activity (U/mg protein) of citrate synthase shows a gradual increase in the liver as a function of age of the rat (Table I). The slight increase in the activity of this key Krebs' cycle enzyme in the liver of old rat may

make this tissue more aerobic during this phase of its life span since the amount of this enzyme in the cell is directly correlated with the ability of the cell to utilize oxygen (Srere, 1969). The findings are consistent with the earlier findings on lactate dehydrogenase and malate dehydrogenase (Singh and Kanungo, 1968; Sharma and Patnaik, 1982a). The activity of PEPCK of the liver, on the other hand, is significantly higher in the adult rat as compared to those of the young and old rats (Table I). It is of great interest to mention here that the observed changes in the level of PEPCK show similar trends with those of the isoenzymes of alanine aminotransferase of the liver as a function of age of the rat (Patnaik and Kanungo, 1974). Although cytosolic alanine aminotransferase participates in active gluconeogenesis its activity decreases in old age. A decrease in the activity of two other gluconeogenic enzymes (i.e. glucose 6-phosphatase and fructose 1, 6-diphosphatase) in the liver of aging rat has been reported earlier (Singhal, 1967). The higher level of PEPCK in adult rat may be due to the higher metabolic status of this animal at this phase of its life span. A decrease in its activity in old age may also be correlated with a decrease in the rate of synthesis of corticoids as a function of age (Serio et al., 1969) since this enzyme is regulated by corticosteroids (Shrago et al., 1963). Our studies also show that the protein content (mg/g wet weight) of the cytosolic and mitochondrial fractions of the liver remains unchanged in rats of all the ages (Table IV).

Adrenalectomy causes no significant effect on the activity of CS of the liver of rats of all the ages (Table II). However, this treatment decreases the activity of liver PEPCK in rats of all ages (Table III). This shows that the activity of hepatic CS is not very dependent on the adrenal gland of the animal. The percent decrease in the activity of hepatic PEPCK following adrenalectomy is highest in the adult rat which is correlated with a higher endogenous level of this enzyme at this phase of its life span. Administration of hydrocortisone to adrenalectomized rats causes a depression in the activity of CS of the liver of young and adult rats (Table II). In contrast to the observations on CS, it has been observed that hydrocortisone increases significantly

TABLE I

Activity of citrate synthase (CS) and phosphoenolpyruvate carboxykinase (PEPCK) of the liver of normal male rats of different ages.

Age (wk)	Enzyme activity (U/mg protein)$\times 10^3$			
	CS		PEPCK	
	Mean SD	P	Mean SD	P
6	25.40 ± 0.70		30.00 ± 2.40	
		< 0.01		< 0.001
30	29.60 ± 0.83		47.60 ± 1.20	
	(+16%)		(+58%)	
		< 0.02		< 0.001
90	33.50 ± 1.10		28.20 ± 1.30	
	(+13%)		(−41%)	

The data were collected from 4 to 5 rats of each age group. Standard deviation (SD) and the levels of significance ($P < 0.05$) are given. +, Increase; −, decrease.

TABLE II

Effects of adrenalectomy (A/d), hydrocortisone (HC) and actinomycin D (A) on the activity of citrate synthase of the liver of male rats of various ages.

Treatments	Enzyme activity (U/mg protein)$\times 10^3$					
	6 wk		30 wk		90 wk	
	Mean SD	P	Mean SD	P	Mean SD	P
Normal	25.40 ± 0.70		29.60 ± 0.83		33.50 ± 1.10	
		NS		NS		NS
A/d	26.60 ± 1.70		30.50 ± 1.50		39.20 ± 1.20	
	(NE)		(NE)		(NE)	NS
		< 0.01		< 0.01		
A/d + HC	18.00 ± 1.50		23.00 ± 0.90		39.70 ± 1.10	
	(−33%)		(−25%)		(NE)	
		< 0.01		< 0.01		NS
A/d + A + HC	26.10 ± 2.10		27.10 ± 0.98		38.50 ± 1.50	
	(+45%)		(17%)		(NE)	

The data were collected from 4 to 5 rats of each age group. Standard deviation (SD) and the levels of significance ($P < 0.05$) are given. +, Increase; −, decrease; NE, no effect; NS, not significant.

the activity of liver PEPCK in young and adult rats (Table III). Administration of actinomycin D prior to the hormone treatment tends to normalize the depressed level of CS. However, this inhibitor prevents the induction of PEPCK by hydrocortisone. Thus, the above findings provide a firm support to the general hypothesis (Krebs, 1966) that oxaloacetic acid, an intermediate of gluconeogenesis, is diverted from the Krebs cycle to gluconeogenesis due to the higher level of the enzymes which convert it to PEP, thereby diminishing the rate of this cycle.

Our present observations as well as the earlier findings of Heitzman et al. (1972) confirm the above-mentioned hypothesis that increase in the rate of gluconeogenesis

TABLE III

Effects of adrenalectomy (A/d), hydrocortisone (HC) and actinomycin D (A) on the activity of phosphoenolpyruvate carboxykinase of the liver of male rats of various ages.

Treatment	Enzyme activity (U/mg protein)$\times 10^3$					
	6 wk		30 wk		90 wk	
	Mean SD	P	Mean SD	P	Mean SD	P
Normal	30.30 ± 2.40		47.60 ± 1.20		26.20 ± 1.50	
		< 0.01		< 0.001		< 0.01
A/d	19.80 ± 1.30		25.00 ± 1.70		18.10 ± 1.20	
	(−34%)	< 0.001	(−48%)	< 0.001	(−31%)	NS
A/d + HC	47.10 ± 1.20		40.60 ± 1.10		20.70 ± 1.20	
	(+137%)	< 0.001	(+62%)	< 0.001	(NE)	NS
A/d + A + HC	17.60 ± 3.10		21.40 ± 1.60		23.50 ± 0.50	
	(−63%)		(−48%)		(NE)	

The abbreviations are the same as in Table II.

TABLE IV

Protein content (mg/g wet weight) of the liver of normal male rats of different ages.

Age (wk)	Cytosolic fraction		Mitochondrial fraction	
	Mean SD	*P*	Mean SD	*P*
6	75.86 ± 1.74		45.27 ± 1.45	
		NS		NS
30	79.46 ± 2.15		43.82 ± 1.72	
		NS		NS
90	76.10 ± 1.52		47.10 ± 1.29	

The data were collected from 4 to 5 rats of each age group. Standard deviation (SD) and the levels of significance (*P*) are given. NS, not significant.

following glucocorticoid administration is related to an increase and decrease, respectively, in the activity of cytoplasmic PEPCK and CS. Further, the present observations are better clarified by the findings that actinomycin D treatment tends to normalize the depressed level of CS in the liver of young and adult rats (Table II), possibly by preventing the induction of PEPCK by hydrocortisone (Table III). Thus, the present findings indicate clearly that oxaloacetic acid is diverted more towards gluconeogenesis following the administration of hydrocortisone which is used ultimately for the synthesis of glucose. It is noteworthy to mention here that the degree of induction and repression, respectively in the activity of PEPCK and CS following hydrocortisone administration, decreases as a function of age of the rat. The impairment of induction of PEPCK in old rats may be due to a gradual loss in the level of hydrocortisone receptors (Singer et al., 1973; Roth and Adelman, 1974, 1975). A decrease in the degree of induction of hepatic cytoplasmic MDH, aspartate aminotransferase and two other gluconeogenic enzymes (i.e. glucose 6-phosphatase and fructose 1,6-diphosphatase) with advancing age of the rat has been reported earlier (Singhal, 1967; Sharma and Patnaik, 1982a, b). The induction of PEPCK by hydrocortisone is actinomycin D sensitive. Thus, it appears that the induction of this enzyme by hydrocortisone occurs at the transcriptional level and is due to an increase in the synthesis of mRNA for this enzyme (Iynedjian and Hanson, 1977).

From the above-mentioned findings, it may be concluded that the level and inducibility of CS and PEPCK are age-specific and undergo specific changes at different phases of the life span of the rat. Such alterations in the levels of these enzymes may be due to the regulatory changes in the corresponding genes which are brought about by factors such as hormones according to a specific programme (Kanungo, 1980). Such changes may be responsible for the aging of an organism.

Acknowledgements

The authors are indebted to Professor M.S. Kanungo of the Department of Zoology, Banaras Hindu University for helpful advice and use of facilities during the

tenure of this project. This research was supported by grants from the University Grants Commission, New Delhi, Nuffield foundation London and PL-480 U.S.A.

References

Ballard, F.J. and Hanson, R.W. (1967): PEPCK and pyruvate carboxylase in developing rat liver. Biochem. J., 104, 866–871.

Garrett, H.E. (1966): Statistics in psychology and education. David McKay Co. Inc., New York.

Gunn, J.M., Tilghman, S.M., Hanson, R.W., Reshef, L. and Ballard, F.J. (1975) Effect of CAMP, dexamethasone and insulin on PEP carboxykinase synthesis in Reuber H-35 hepatoma cells. Biochemistry, 14, 2350–2357.

Heitzman, R.J., Herriman, I.D. and Mallinson, C.B. (1972): Some effects of glucocorticoids on the subcellular distribution of the activities of citrate synthase and PEPCK in livers of rats and cows. FEBS Lett., 20, 19–21.

Holloszy, J.O., Oscai., L.B., Don, I.J. and Mole, P.A. (1970): Mitochondrial citric acid cycle and related enzymes: adaptive response to exercise. Biochem. Biophys. Res. Commun., 40, 1368–1373.

Iynedjian, P.B. and Hanson, R.W. (1977): Increase in level of functional mRNA coding for PEPCK (GTP) during induction by cyclic adenosine 3′,5′-monophosphate. J. Biol. Chem., 252, 655–662.

Kanungo, M.S. (1980): Biochemistry of ageing. Academic Press, London.

Kirsten, R. and Kirsten, E. (1972): Redox state of pyridine nucleotides in renal response to aldosterone. Am. J. Physiol., 223, 229–235.

Krebs, H.A. (1966): In: Advances in Enzyme Regulation, Vol. 4, pp. 339–353. Editor: G. Weber, Pergamon Press, Oxford.

Lardy, H.A., Paetkan, V. and Walter, P. (1965): Paths of carbon in gluconeogenesis and lipogenesis: the role of mitochondria in supplying precursors of phosphoenolpyruvate. Proc. Natl. Acad. Sci. U.S.A., 53, 1410–1415

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951): Protein measurement with Folin phenol reagent. J. Biol. Chem., 193, 265–275.

Mukherjee, A., Srere, P.A. and Frenkel, E.P. (1976): Studies on the mechanism by which hepatic citrate synthase activity increases in vitamin B12 deprivation. J. Biol. Chem., 251, 2155–2160.

Nordlie, R.C. and Lardy, H.A. (1963): Mammalian liver PEPCK activities. J. Biol. Chem., 238, 2259–2263.

Patnaik, S.K. and Kanungo, M.S. (1974): Different patterns of induction of the two isoenzymes of alanine aminotransferase of liver of rat as a function of age. Biochem. Biophys. Res. Commun., 56, 845–850.

Roth, G.S. and Adelman, R.C. (1974): Age-dependent regulation of mammalian DNA synthesis and cell division in vivo by glucocorticoids. Exp. Gerontol., 9, 27–31.

Roth, G.S. and Adelman, R.C. (1975): Age-related changes in hormone binding by target cells and tissues, possible role in altered adaptive responsiveness. Exp. Gerontol., 10, 1–11.

Serio, M., Piolanty, P., Capelli, G., Magistris, L., Rieci, F., Anzalone, M. and Giusti, G. (1969): The miscible pool and turnover rate of cortisol in the aged, and variations in relation to time of day. Exp. Gerontol., 4, 95–101.

Sharma, R. and Patnaik, S.K. (1982a): Differential regulation of malate dehydrogenase isoenzymes by hydrocortisone in the liver and brain of rats of various ages. Dev. Growth Differ., 24, 501–505.

Sharma, R. and Patnaik, S.K. (1982b): Properties of liver cytoplasmic aspartate aminotransferase of rats of various ages. Biochem. International. 5, 561–566.

Shrago, E., Lardy, H.A., Nordlie, R.C. and Foster, D.O. (1963): Metabolic and hormonal control of PEPCK and malic enzyme in rat liver. J. Biol. Chem., 238, 3188–3192.

Singer, S., Ito, H. and Litwack, G. (1973): ^{3}H-Cortisol binding by young and old human liver cytosol protein in vitro. Int. J. Biochem., 4, 569–573.

Singh, S.N. and Kanungo, M.S. (1968): Alterations in lactate dehydrogenase of the brain, heart, skeletal muscle and liver of rats. J. Biol. Chem., 243, 4526–4529.

Singhal, R.C. (1967): Effect of age on the induction of glucose 6-phosphatase and fructose 1, 6-diphosphatase in rat liver. J. Gerontol., 22, 77–88.

Srere, P.A. (1969): Some complexities of metabolic regulation. Biochem. Med., 3, 61–72.

Srere, P.A. (1972): In: Current topics in cellular regulation, Vol. 5, pp. 229–283. Editors: B.L. Horecker and E.R. Stadtman. Academic Press, New York.

Srere, P.A., Brazil, H. and Gonen, L. (1963): The citrate condensing enzyme of pigeon breast muscle and moth flight muscle. Acta. Chem. Scand., 17, 129–134.

Tilghman, S.M., Hanson, R.W. and Ballard, F.J. (1976): In: Gluconeogenesis: Its Regulation in Mammalian Species, pp. 47–91. Editors: R.W. Hanson and M.A. Mehlman. Wiley, New York.