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Regulation of rat liver NADP$^+$ -isocitrate dehydrogenase during aging

R.N. SINGH YADAV and S.N. SINGH
Biochemistry Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005

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Abstract. In an attempt to understand the mechanism of aging in relation to the differences in enzyme regulation, the induction and kinetic properties of NADP$^+$ -isocitrate dehydrogenase of the liver of immature (6 weeks), mature (13 weeks), adult (33 weeks) and old (85 weeks) female rats were studied. The specific activity of the cytoplasmic and mitochondrial NADP$^+$ -isocitrate dehydrogenase increased up to the adult age (33 weeks) and decreased in the old rats (85 weeks). Overiectomy decreased and estradiol administration induced activity of both the mitochondrial and eytoplasmic enzyme in the liver ol immature, mature and adult rats but had no significant effect in old rats. However, the activity of mitochondrial NADP$^+$ -isocitrate dehydrogenase decreased and eytoplasmic NADP$^+$ -isocitrate dehydrogenase increased following ovariectomy in old rats (85 weeks). Hormone-mediated induction of enzyme activity was actinomycin D sensitive. The K_m for isocitrate and NADP, K_i value for oxalomalate, heat stability and electrophoretic mobility of the purified enzyme from the cytosol fraction of the liver of immature and old rats were similar. It can he concluded that the enzyme does not change structurally with age.

Keywords. Isocitrate dehydrogenase; induction; purification; electrophoretic mobility; temperature sensitivity.

Introduction

The activities of several enzymes either decrease, or increase as a function of the age of the organism (Kanungo, 1970). A possible cause for such changes may be related to the template activity of the corresponding gene(s). We have studied two isoenzymes of NADP$^+$ -linked isoeitrate dehyrogenase (EC 1.1.1.42), from the cytoplasm and mitochondria as markers to obtain some insight into this problem.

Although alterations in the isoenzyme pattern of lactate dehydrogenase (EC 1.1.1.27) of various tissues as a function of age of the rats have been reported (Singh and Kanungo, 1968), the induction of the isoenzymes of NADP$^+$ -isocitrate dehydrogenase has not been studied. Both cytosolic and mitochondrial NADP$^+$ -isocitrate dehydrogenase differ in their electrophoretic mobility and immunological pattern (Lowenstein and Smith, 1962), suggesting that their synthesis may be governed by two separate genes. The cytoplasmic enzyme supplies NADP$^+$ for reductive biosynthesis (Lowenstein, 1961), whereas mitochondrial enzyme is involved in the production of energy (Stein *et al.,* 1967). It has been reported that sex steroids modulate this enzyme in the brain and pituitary of rats (Luine, *et al.,* 1974,1975). It was of interest to investigate the changes that occur in the isoenzymes of NADP+-isocitrate dehydrogenase of the liver during aging of the rats. These include alterations in the levels of the isoenzyme and regulation.

Part of this work was presented at the 48th Annual General Meeting of the Society of Biological Chemist, India, 1979.

Materials aad methods

Materials

Albino rats of Wistar strain (*Rattus norvegicus albinus*) were used. They were maintained at 24±2°C with 12 h light period followed by 12h dark period. The rats were fed on freshly prepared diet containing flour and vitaminised milk-powder in the ratio of 4:1 in water with added table salt. The diets were supplemented with gram (*Cicer arietinum*) on alternate days. Tap water was supplied *ad libitum.* Female rats of four age groups, representing immature (6), mature (13), adult (33) and old (85 weeks) were used in this study. All chemicals used were of the analytical grade. All the biochemicals were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

Assay of isoenzymes

The rats were killed by cervical dislocation, the liver was excised, chilled in ice-cold 0.9% NaCl. A 10% homogenate was prepared in 0.25 M sucrose containing 0.1 mM EDTA and centrifuged at 700 *g* for 10 min in a high-speed IEC (PR-6 model) refrigerated centrifuge. The supernatant was again centrifuged at 14,000 *g* for 30 min. The supernatant obtained was used for the assay of cytoplasmic $NADP^+$ -isocitrate dehydrogenase. The pellets were suspended in 0.25 M sucrose containing 0.1 mM EDTA. To solubilise the enzyme, an equal volume of phosphate buffer (0.1 M, pH 7.4) containing 10% glycerol and 0.5% Triton x-100 was added to t he mitochondrial suspension which was then homogenised and centrifuged. This was used for the assay of mitochondrial enzyme by monitoring the NADPH produced. Both the isoenzymes were assayed in an Unicam SP-500 spectrophotometer (Watanabe *et al.,* 1974). One unit of enzyme is defined as that amount which forms 1 μ mol NADPH/min at 25°C. The protein content of the fractions was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The data were statistically analysed for students 't' test according to Garret (1966).

Effect of ovariectomy and estradiol on isocitrate dehydrogenase isoenzymes

The rats of all the ages were divided into four groups, each group comprised of 4-5 rats. Rats in group I were injected with 1.0 ml of 0.9% NaCl intraperitoneally. Rats in groups II, III and IV were bilaterally overiectomised and maintained for 21 days. On the 22nd day, rats in group II received 1.0 ml 0.9% NaCl injection and served as ovariectomised controls. Rats in group III were administered estradiol (20 μg/100. g body weight in 1.0 ml of 0.9% NaCl) intraperitoneally. Rats in group IV were injected actinomycin D (10 μg/100 g body weight in 1.0 ml of 0.9% NaCl) 1 h prior to the hormone administration. All the injections were given intraperitoneally at 2.00 p.m. and the rats were killed at 6.00 p.m. on the same day.

Purification of $NADP^+$ -isocitrate dehydrogenase

The $NADP^+$ -isocitrate dehydrogenase was purified from the cytosol fraction of the liver of immature (6 weeks) and old (85 weeks) rats by the procedure described by Islam (1972) with certain modifications. All the operations were carried out at 2°C unless otherwise indicated. The livers from 5-6 rats were pooled for each age group.

Results

Levels of isoenzyme

The levels of both cytoplasmic NADP$^+$ -isocitrate dehydrogenase and mitochondrial NADP$^+$ -isocitrate dehydrogenase increased during active growth phase and adulthood (table 1). Thereafter, the activity decreased significantly in old rats of 85 weeks.

Effect of ovariectomy and estradiol on isoenzymes

Table 1 shows that the levels of both the isoenzymes of the liver of rats in the different age groups decrease is significantly following ovariectomy, except that the cytoplasmic enzyme of old rats (85 weeks) increased significantly. Estradiol administration induced both the isoenzymes in all the age groups. However, the mitochondrial enzyme of old rats (85 weeks) did not show any significant change on estradiol treatment. The degree of response was greater in 6 and 13 weeks as compared to that of 33- and 85-weeks old rats. Actinomycin D injection to ovariectomised and estradiol treated rats restored the enzyme level to that in the normal ovariectomised control rats (table 1).

Studies with the purified enzyme

Table 2 gives the protocol for the purification of NADP-isocitrate dehydrogenase from the liver cytosol of immature (6) and old (85 weeks) normal female rates, respectively for the enzyme from 6 and 85 week-old rats. Figure 1 gives the elution pattern of enzymes from livers of rats of two ages from DEAE-cellulose columns. The enzyme from both the ages were eluted at the same concentration of KCl. The apparent K_m values for isocitrate and NADP$^+$ are the same for the enzyme from both, 6 and 85 week old rats (table 3). The K_i value for oxalomalate were 8.5 μM and 9.0 μ M for the immature and the old rats respectively (table 3). The activity of purified enzyme from the two age groups of rats at different temperatures (figure 2) were similar.

The enzyme from both immature and old rats had identical mobility when subjected to Polyacrylamide gel electrophoresis at 4°C in 0.01 M citrate phosphate buffer pH 1.0, containing 10% glycerol and 0.1 mM EDTA. A constant current of 4 mA/gel was applied for 3 h. One of the gels was stained for protein with 0.25% coomassie brilliant blue R (prepared in 10% trichloroacetic acid) after fixing it with 12.5% trichloroacetic acid for 1 h. For specific staining of NADP$^+$ -isocitrate dehydrogenase activity, the gels were incubated in the staining mixture for 30 min at 37°C. The Staining mixture consisted of 0.1 M Tris/HCl pH 7.4, 8.0 mM isocitrate, 4.0 mM $MgCl_2$, 0.26 mM NADP, 0.25 mM nitroblue tetrazolium and 0.18 mM phenazine methosulphate according to the method of Henderson (1965).

Table 1. Effects of ovariectomy, estradiol and actinomycin D on the activity levels of NADP-isocitrate dehydrogenase isoenzymes in the liver of female rats of various ages.

Treatments	Specific activity of $NADP^+$-isocitrate dehydrogenase isoenzymes							
	Mitochondrial enzyme				Cytoplasmic enzyme			
	Age in weeks							
	6	13	33	85	6	13	33	85
Normal	129.03±1.41(5)	163.02±2.86(6)	130.85±3.48(5)	112.48±0.86(5)	297.58±3.85(5)	335.97±6.55(6)	317.50±3.54(5)	253.10±1.98(5)
Overiectomised	74.02±8.56(5) $P<0.01$	101.18±0.75(6) $P<0.001$	86.68±0.43(5) $P<0.001$	102.16±1.47(4) $P<0.01$	243.10±2.90 $P<0.001$	264.10±4.01(6) $P<0.001$	264.72±1.28(5) $P<0.001$	279.03±6.78(4) $P<0.01$
Ovariectomised + estradiol	131.98±2.87(5) $P<0.01$	152.68±2.76(5) $P<0.001$	112.59±2.36(5) $P<001$	104.31±1.90(5) P : NS	317.99±0.77(5) $P<0.001$	353.18±1.96(5) $P<0.001$	301.55±5.62(5) $P<0.01$	297.13±2.93(5) $P<0.05$
Ovariectomised+actinomycin D+ estradiol	88.10±7.00(4) $P<0.01$	84.10±1.27(5) $P<0.001$	103.29±2.06(4) $P<0.02$	96.30±1.10(5) $P<0.01$	203.32±3.47(4) $P<0.001$ 243.10±2.90(5)	265.74±4.17(5) $P<0.001$	243.72±6.68(4) $P<0.01$	237.61±4.36(5) $P<0.001$

Table 2. Summary of partial purification of cytoplasmic $NADP^+$-isocitrate dehydrogenase from liver of immature (6 weeks) and old (85 weeks) normal female rats

Age	Fractions	Volume (ml)	Total enzyme activity (units)	Total protein (mg)	Specific activity (units mg protein)	Fold purification	Yield (%)
6 weeks	Supernatant (40,000 *g*)	95	228.95	939.12	0.24	—	100
	First $(NH_4)_2SO_4$ fraction (50-70%)	30	179.70	214.10	0.57	2.33	78.48
	Second $(NH_4)_2SO_4$ fraction (40-60%)	20	154.20	167.92	0.91	3.76	67.35
	DEAE-Cellulose chromatography fraction	20	76.28	17.89	4.26	17.49	33.31
	Third $(NH_4)_2SO_4$ fraction (51-69%)	5	42.35	6.26	6.76	27.75	18.49
85 weeks	Supernatant (40,000 *g*)	90	234.16	985.45	0.23	—	100
	First $(NH_4)_2SO_4$ fraction (50-70%)	32	189.45	348.94	0.54	2.28	80.89
	Second $(NH_4)_2SO_4$ fraction (40-60%)	18	150.63	184.45	0.81	3.43	64.31
	DEAE-cellulose chromatography fraction	25	69.45	18.14	3.82	16.11	29.65
	Third $(NH_4)_2SO_4$ fraction (51-60%)	6	38.02	6.56	5.79	24.39	16.23

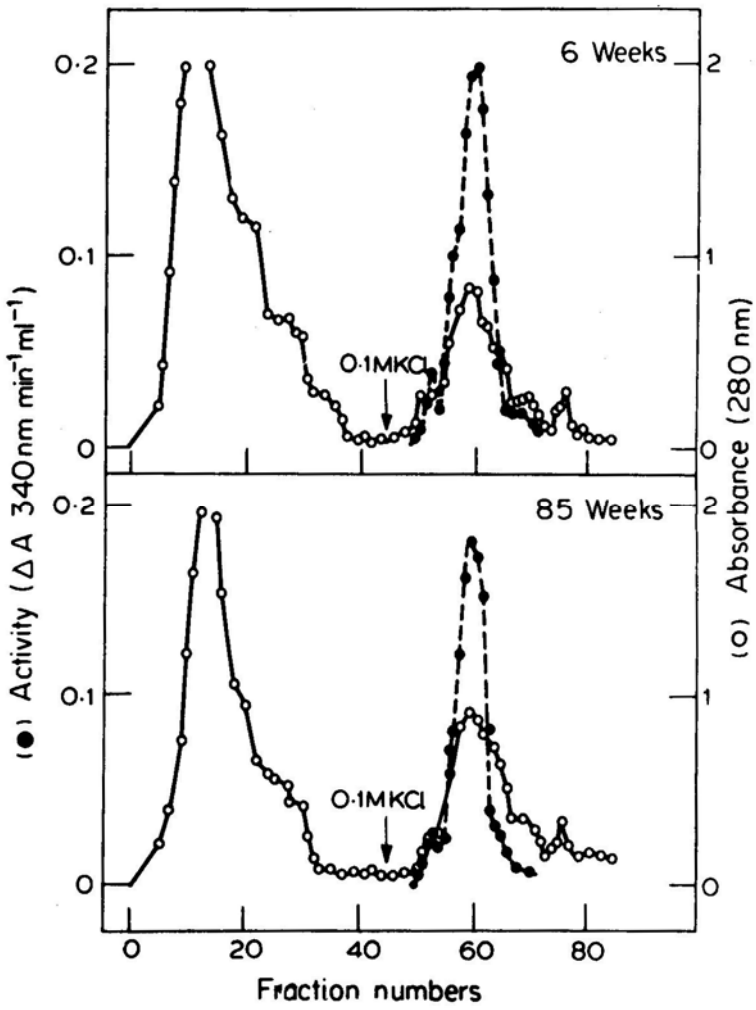

Figure 1. Elution pattern of NADP$^+$ -isocitrate dehydrogenase of liver cytosol of immature (6 weeks) and old (85 weeks) normal female rats on DEAE-Cellulose column.
(●- - - ●) enzyme activity, (O- - -O) protein absorbancy at 280 nm.

Table 3. K_m and K_i of cytoplasmic-NADP$^+$ -isocitrate dehydrogenase of the liver of immature (6-weeks) and old (85-weeks) normal female rats

	6 weeks	85 weeks
K_m for isocitrate	6.00×10^{-6} M	6.40×10^{-6} M
K_m for NADP	2.70×10^{-5} M	2.85×10^{-5} M
K_i for oxalomalate	8.50×10^{-5} M	9.00×10^{-5} M

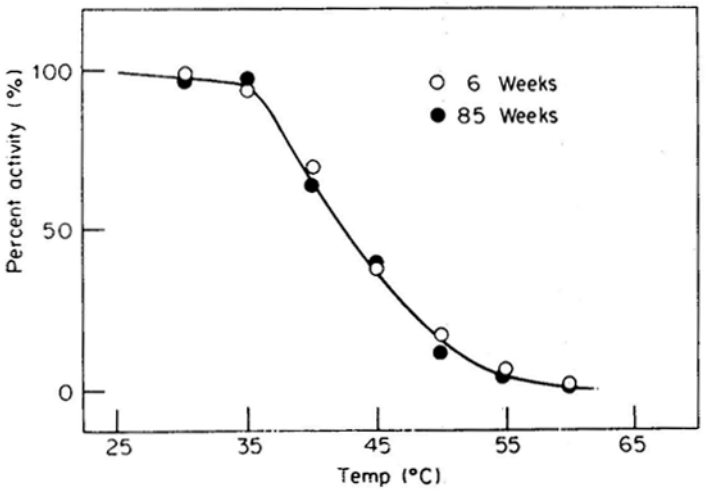

Figure 2 Effect of temperature on the activity of cytoplasmic $NADP_+$ -isocitrate dehyrogenase of liver of immature (6 weeks) and old (85 weeks) normal female rats.

Appropriately diluted solutions of purified enzyme of immature and old rats were incubated for 15 min at a fixed temperature in a thermostatically controlled waterbath. The samples were then cooled quickly at 25°C and the enzyme activity was assayed.

Discussion

The functional capacity of an organism deteriorates with increasing age (Shock, 1960). The decline in the functional or metabolic capacity of the organism during aging may be due to alterations in the activity and inducibility of certain important rate-limiting enzymes of various metabolic pathways (Finch *et al.,* 1969; Chainy and Kanungo, 1978). Such alterations may be attributed to the differential template activity of the corresponding gene(s) (Kanungo, 1970).

In the present study, the high levels of both the isoenzymes of $NADP^+$ -isocitrate dehyrogenase during active growth phase and adulthood may be correlated with the high degree of physiological activities of animal during this phase of life. The step in the Krebs cycle, catalysed by isocitrate dehydrogenase occupies a central position in the intermediary metabolism and links a variety of synthetic and energy yielding pathways (Srere and Bhaduri, 1962). Furthermore, the metabolic step controlled by this enzyme could be rate limiting (Kadenbach *et al.,* 1964). Therefore, the high level of the isoenzymes in the young animal and decrease in the activity in the old animal, observed in this study, supports the above hypothesis.

Pitotand Yatvin (1973) reported that the removal of the organ secreting a hormone from an animal causes a change in the levels of many enzymes in different tissues (Santtiand Villee, 1971; Singhal, 1967; Gandhi and Kanungo, 1974; Giri and Singh, 1978a, b; Chainy and Kanungo, 1978). Our investigation indicates that the level of both the isoenzymes of $NADP^+$ -isocitrate dehydrogenase *in vivo* is regulated, at least in part by sex steroids throughout the life span of the rat. It is noteworthy that the degree of response to ovariectomy or estradiol treatment is maximum at maturity and thereafter decreases in old rats. This finding confirms that estradiol not only maintains the secondary sexual characters for the attainment of puberty but also increases the overall physiological activities of the animal during this phase of life.

The decrease in the response in old age may be due to the loss of ovarian function. Furthermore the possible decrease of estradiol specific receptors in the liver of old rats may in turn decrease the responsiveness of its gene (Kanungo *et al.,* 1975).

The induction of an enzyme by a hormone may be due to, (i) stimulation of transcription or translation of the mRNA specific for that enzyme; or (ii) a decrease in the rate of degradation of the enzyme; or (iii) stimulation of the rate of synthesis of a modifier molecule which interacts with the enzyme and enhances its catalytic efficiency without any change in its net synthesis or degradation (Cox *et al.,* 1971). Our observations that the induction of the enzyme was inhibited by actinomycin D show that the effect may be due to stimulation of transcription of specific *m*RNA. The conclusion was in agreement with the finding of DeAsua *et al.,* (1968) that the induction of pyruvate kinase by estradiol in the uterus of ovariectomised rats was inhibited by actinomycin D.

Studies on the purified enzyme from the cytoplasmic fraction of the liver of 6- and 85-week old rats showed that the structure and net charge of the enzyme was independent of the age of the animal. In addition the K_m and K_i values for substrates and inhibitor were similar.

The observations that the kinetic properties of alanine amino transferase (Patnaik and Kanungo, 1976) and the kinetic and immunological properties of superoxide dismutase (Reiss and Gershon, 1976) are not altered with the increasing age of the animals provide firm support to the view that enzymes synthesised in old age are structurally similar to those of young.

From these studies, it can be conlduded that the alteration in the levels and regulation of cytoplasmic and mitochondrial $NADP^+$ -isocitrate dehydrogenase of the liver observed at different phases of life span may be due to the differential template activity of the corresponding gene(s). Thus, the changes at molecular level, brought about by various endogenous factors like hormones may lead to the process of aging in an animal.

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