KOREAN JOURNAL OF ZOOLOGY
Vol. 28, No. 4, 227-236 (1985)

Changes of LDH Subunit Combinations Induced by Tetrodotoxin

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Tetrodotoxin에 의하여 유발되는 LDH 하부단위체 조합의 변화

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(Received may 10, 1985)

요 약

LDH 동위효소의 4차구조 형성기작을 규명하기 위한 일환으로 웅성 흰쥐 복강내에 여러가지 농도의 tetrodotoxin을 수차 주사한 후, 전기영동 및 densitometry에 의해 여러 조직내의 5가지 동위효소의 상대적 분포의 변화를 조사하였다.

뇌조직에서는 H_4 동위효소가 현저히 증가하였고, 골격근조직에서 M_4 동위효소는 약간 증가한 반면 M_3H 및 M_2H_2 동위효소들은 감소되었으며, 심장조직에서는 M/H 비가 급격히 감소되었으며, 간조직에서는 소량의 H_4 동위효소 형성이 나타났다. 이러한 결과들은 Donnan막 평형의 변화가 LDH 동위효소 분포의 변화를 초래할 수 있음을 시사한다.

Tetrodotoxin에 노출된 흰쥐의 여러 조직내에서 M_3H 동위효소 형성이 감소되는 사실로 미루어 고유한 세포질내 환경이 변화되면 3M+H 조합은 쉽게 이루어지지 않는 것으로 사료된다.

원형질 막의 어떤 sodium channel은 tetrodotoxin에 민감하지 않다는 사실이 재확인 되었다.

INTRODUCTION

The tissue specificity of lactate dehydrogenase (EC. 1.1.1.27; LDH) isozymes as ascertained by various electrophoreses and by immunochemical methods is convincing evidence that isozymes are a truly general aspect of the metabolic structure of cells and that there

본 연구는 1984년도 원광대학교 교내 연구비에 의하여 이루어진 것임.

might be a precise mechanism by which quaternary structure formation is brought about. In an attempt to elucidate this mechanism, numerous investigations have focused on the *in vivo* alterations in the relative percentage of the five LDH isozymes. These included hormonal treatment (Goodfriend and Kaplan, 1964; Suleiman and Vestling, 1979), cold acclimatization (Hochachka, 1965; Shaklee *et al.*, 1977; De Costa *et al.*, 1981), change in oxygen tension (Mager *et al.*, 1968; Hellung-Larsen and Andersen, 1970), poisoning by heavy metals (Secchi *et al.*, 1970) and exposure to chemical carcinogens (Spence, 1979).

One of the methods which could result in *in vivo* alteration in LDH isozyme distribution in tissues is the artificial aberration in intracellular sodium ion concentration by tetrodotoxin (TTX). It is fairly well established that TTX selectively blocks the sodium channels along the nerve axon and the muscle fiber, preventing the inward sodium current of the action potential while leaving unaffected the outward potassium current (Kao, 1966; Katz and Miledi, 1967).

It is reasonable to suppose that organisms should utilize physiological mechanisms for adapting to pharmacological stimuli when these are appropriate (Littleton, 1983). Thus, the interruption of sodium ion flux induced by *in vivo* multiple treatment of sublethal dose of TTX would be accompanied by intracellular acclimatization in terms of energy metabolism, especially of LDH isozyme systems.

In the present work with mouse LDH isozymes in which a potent neurotoxin TTX is used for the purpose of interrupting the intracellular metabolism, we have observed several characteristics of alteration in the isozyme distribution which might be a clue to the mechanism of the quaternary structure formation of the protein.

MATERIALS AND METHODS

Albino male mice weighing 35~40 g were divided in five groups of 12 each. The first was used as control. The second to fifth were subjected to multiple intraperitoneal injection of TTX with a daily dose of 1.25 μg, 2.5 μg, 5 μg and 10 μg/kg body weight, respectively. Three individuals from each group were decapitated after 7, 14 and 21 days of the *in vivo* chemical exposure.

After decapitation, five tissues of brain, skeletal muscle, heart, kidney and liver were used in the investigation. Each tissue was ground in an ice-bathed glass homogenizer in cold demineralized water followed by centrifugation in a PR-2 International Refrigerated Centrifuge at 5,500×g for 1 hr. The resulting crude extracts were stored at −20°C until further studies. Electrophoreses on cellulose acetate strip (Millipore, ESWPO25FT) were carried out by the method of Yum *et al.* (1981).

Zymograms after the electrophoreses were subjected to densitometry with an automatic computing densitometer (Gelman, ACD-18).

RESULTS AND DISCUSSION

Changes in the relative percentages of five isozymes

Sodium channel has been found to contain two kinds of binding sites for neurotoxins. One binding site is for the alkaloids such as batrachotoxin and veratridine (Amy and Kirshner, 1982) and scorpion venom (Barhanin *et al.*, 1982) which cause a partial opening of the sodium channel. A second characteristic of the sodium channel is the monovalent cation-translocating site which can be blocked by TTX (Catterall, 1975).

Table 1 summarizes the conspicuous increase of the H_4 isozymes in mouse brain, after exposure to TTX, to such an extent that nearly all experimental brain tissues have twice as much H_4 isozyme as control brain. Such an increase of H_4 isozyme could be also found in amphibian brain tissues. De Costa *et al.* (1983), in their demonstration of a seasonal variation in energy metabolism, reported that there is a continuous decrease in M-type LDH isozyme from autumn to summer in brain tissues from a naturally occurring population of *Discoglossus pictus pictus.*

Aside from interruption of afferent and efferent nerve impulses to the muscle, TTX has direct blocking action on skeletal muscle fibers (Nakajima *et at.*, 1962). After the exposure to TTX, the M_4 isozyme of mouse skeletal muscle tissues was slightly increased while the M_3H and M_2H_2 isozymes were decreased (Table 2).

Ogura (1958) found that, after a single subcutaneous injection of crystalline TTX, detectable amounts of TTX are present in the rat's kidney, heart, liver, lung, intestine,

Table 1. Effects of *in vivo* multiple treatment of TTX on the relative percentage of five LDH isozymes in mouse brain tissue.

Daily dose of TTX (μg/kg)	Days	Relative percentage					M/H
		M_4	M_3H	M_2H_2	MH_3	H_4	
Control		14.0	27.4	31.4	17.5	9.7	1.20
1.25	7	15.5	22.1	21.7	18.9	21.8	0.91
	14	18.8	24.6	22.8	17.9	15.9	1.13
	21	13.3	24.2	23.9	18.3	20.3	0.92
2.50	7	14.9	24.0	23.5	19.0	18.6	0.98
	14	13.7	24.2	26.7	17.8	17.6	0.99
	21	11.5	22.8	26.1	20.4	19.2	0.88
5.00	7	18.0	27.6	23.6	15.2	15.6	1.19
	14	16.4	23.0	25.5	18.7	16.4	1.04
	21	16.5	25.0	23.3	18.1	17.1	1.06
10.00	7	13.6	25.1	24.8	19.0	17.5	0.98
	14	13.5	25.4	23.6	18.1	19.4	0.96
	21	13.6	26.7	22.7	18.0	19.0	0.98

brain and blood. The peak concentration in these tissues was reached in 20 minutes. By exposure of mouse to TTX, the percentages of M_4 and M_3H isozymes in heart were reduced while those of the other three isozymes showed an increase and, thus, the M/H ratio was strikingly reduced (Table 3). This reduction was contrary to our expectation

Table 2. Effects of *in vivo* multiple treatment of TTX on the relative percentage of five LDH isozymes in mouse skeletal muscle tissue.

Daily dose of TTX (μg/kg)	Days	Relative percentage			
		M_4	M_3H	M_2H_2	MH_3+H_4
Control		76.5	11.5	6.6	5.4
1.25	7	84.1	9.3	3.7	2.9
	14	85.7	6.1	4.5	3.7
	21	89.0	5.2	3.5	2.3
2.50	7	85.1	5.4	4.9	4.6
	14	90.9	4.2	3.1	1.8
	21	92.3	4.0	2.5	1.2
5.00	7	85.1	5.8	3.8	5.3
	14	88.2	5.6	4.7	1.5
	21	91.2	4.5	2.6	1.7
10.00	7	79.0	7.2	6.4	7.4
	14	87.5	5.2	3.3	4.0
	21	85.3	6.0	4.1	4.6

Table 3. Effects of *in vivo* multiple treatment of TTX on the relative percentage of five LDH isozymes in mouse heart tisssue.

Daily dose of TTX (μg/kg)	Days	Relative percentage					M/H
		M_4	M_3H	M_2H_2	MH_3	H_4	
Control		20.1	19.5	27.0	20.6	12.8	1.14
1.25	7	11.8	14.5	39.8	22.8	11.1	0.93
	14	4.3	4.3	32.3	39.2	19.9	0.50
	21	2.5	4.7	30.7	38.3	23.8	0.45
2.50	7	8.5	11.9	34.9	32.8	11.9	0.76
	14	15.1	17.2	32.5	24.3	10.9	1.01
	21	7.5	16.5	37.8	27.6	10.6	0.84
5.00	7	3.2	4.2	37.2	40.8	14.6	0.54
	14	2.8	5.0	33.1	35.5	23.6	0.47
	21	4.8	6.1	33.1	36.9	19.1	0.54
10.00	7	3.0	5.5	36.3	37.3	17.9	0.53
	14	8.2	11.0	26.9	32.2	21.7	0.61
	21	1.7	5.6	32.8	38.1	21.8	0.47

Table 4. Effects of *in vivo* multiple treatment of TTX on the relative percentage of five LDH isozymes in mouse kidney tissue.

Daily dose of TTX (μg/kg)	Days	Relative percentage					M/H
		M_4	M_3H	M_2H_2	MH_3	H_4	
Control		11.0	24.0	25.2	23.5	16.3	0.90
1.25	7	14.8	19.2	27.1	23.6	15.3	0.95
	14	17.3	14.4	22.9	22.9	22.5	0.83
	21	7.3	9.0	27.9	32.4	23.4	0.56
2.50	7	9.7	11.6	24.6	29.7	24.4	0.62
	14	11.3	14.3	32.2	30.1	12.1	0.84
	21	19.2	17.2	23.0	23.9	16.7	0.98
5.00	7	13.5	11.8	25.0	29.0	20.7	0.73
	14	15.4	13.7	24.1	25.0	21.8	0.78
	21	12.8	14.4	23.3	26.8	22.7	0.72
10.00	7	8.8	13.3	28.4	29.6	19.9	0.68
	14	11.1	14.8	27.1	27.8	19.2	0.75
	21	10.6	14.0	24.4	28.2	22.8	0.68

that there would be a slight change in the ratio on the basis of the fact that cardiac muscle cells have shown to be fairly resistant to TTX (Hagiwara and Nakajima, 1966). By recording the maximum rate of rise in the spike, a measure of the inward sodium current, they showed that in frog ventricular fibers, TTX (150 nM) reduced it to 17 percent of the rate of untreated preparations, even though the amplitude of the overshoot was reduced by only a few millivolts. This concentration is equivalent to a dose of about 20 μg/kg in a whole animal if TTX is assumed to be equally distributed throughout body fluid.

Blocking the sodium channels in kidney tissue by TTX can significantly alter the isozyme distribution. The kidney M_3H isozyme was reduced obviously and the M/H ratio showed a decrease as in the heart tissue (Table 4).

The liver tissue showed an increase in H_4 isozyme by TTX exposure (Table 5). This increase is also met in liver of anuran amphibian during summer (De Costa *et al.*, 1983). The liver tissue differs from the other four tissues examined in that H_4 isozyme is formed by treatment of TTX although the synthetic rate of the tetramer is extremely low. Such a sudden appearance of the homotetramers could be encountered in various pathologic conditions. A significant rise in M_4 LDH isozyme, for example, were found in human atherosclerotic fibrous plaques while their putative cells of origin in an aortic media and intima have not M_4 LDH isoymes (Gown and Benditt, 1982).

Significance of alterations in isozyme distribution by TTX

The precise reason for this alteration in LDH isozymes by TTX is not clearly understood, but it may be resulted from a new intracellular ionic environment established by compulsory change in Donnan equilibrium. This suggestion also stems from our other

Table 5. Effects of *in vivo* multiple treatment of TTX on the relative percentage of five LDH isozymes in mouse liver tissue.

Daily dose of TTX (μg/kg)	Days	Relative percentage			
		M_4+M_3H	M_2H_2	MH_3	H_4
Control		84.1	12.6	3.3	—
1.25	7	79.3	14.3	3.3	3.1
	14	88.1	4.8	2.2	4.9
	21	88.0	5.2	4.0	2.8
2.50	7	85.8	7.2	3.4	3.6
	14	92.0	4.9	1.8	1.3
	21	93.1	3.6	2.2	1.1
5.00	7	84.7	7.4	4.4	3.5
	14	89.6	7.7	2.7	—
	21	90.2	5.1	2.9	1.8
10.00	7	84.6	8.0	4.6	2.8
	14	84.2	10.1	3.5	2.2
	21	83.7	10.0	2.7	3.6

finding that, when mouse is exposed to a wide concentration range of ouabain which inhibits the $(Na^+ + K^+)$-ATPase of plasma membrane, the H_4 isozyme in brain tissue shows an abrupt increase (Yum and Kim, unpublished).

The tissue specificity has been interpreted collectively in two ways. The differential activation of genes responsible for two subunits (Markert and Ursprung, 1962; Dawson *et al.*, 1964) would permit the tissue have its own characteristic isozyme distribution. This hypothesis to explain the spatiotemporal specificity of LDH isozymes is strongly supported by the observations that H_4 isozyme is found exclusively in primary spermatocyte of mammals and birds (Goldberg, 1971) and predominantly in the liver of gadoid and cyprinid fishes (Shaklee *et al.*, 1973) and in the eye of several groups of teleosts (Whitt, 1970b). The changes in the M/H ratio found in our results reveal that new intracellular ionic environments could influence the action of genes, especially of M gene in heart tissue.

On the other hand, differences in the degradation rates of five tetramers (Fritz *et al.*, 1969; Fritz *et al.*, 1973) or the selective formation of specific tetramers (Markert and Faulhaber, 1965; Whitt, 1970a) might account for the tissue-specific distribution of isozymes. This hypothesis supports several findings that there is no assembly of the M_3H isozyme in reptiles (Markert, 1968; Park *et al.*, 1976) and that three heterotetramers are frequently absent in fishes (Yum *et al.*, 1981).

Assumption that the subunit combination of the 3M+H is likely to be more fragile when the surrounding environment of the tetramer is deviated from its accustomed one could be derived from the early observations that M_3H isozyme of *Alosa pseudoharengus* (Clupeidae) LDHs are strongly inactivated under more basic pH condition (Shaklee, 1975)

and that M_3H isozyme of *Ophicephalus argus* (Ophicephalidae) skeletal muscle tissue lose its quaternary structure dramatically after relatively mild heat inactivation (Yum *et al.*, 1981). However, the tendency towards a reduction of M_3H isozyme in kidney, heart and skeletal muscle tissues by TTX appears to reflect that the subunit combination to form the M_3H isozyme is to be unfavorable in those intracellular environment since the half-life of mouse LDH isozymes, except that of skeletal muscle, is reported to be 4.3 to 8.2 days (Nadal-Ginard, 1978).

It should be noted that, in brain tissue of this result, while the relative percentage of M_4 isozyme remains almost unchanged, that of H_4 isozyme increases abruptly, suggesting that the new intracellular ionic environment would favor the formation of the H_4 homotetramer.

In their *in vitro* hybridization studies of porcine LDH isozyme after acid dissociation, Hermann *et al.* (1982) reported that the reassociation of subunits is dependent on the total monomer concentration and that high temperature (40°C) could enhance the formation of two homotetramers while those isozymes are not found at low temperature (0~5°C). This results mean that the differential activation of genes is not necessarily changed with the tissue-specific formation of quaternary structures.

TTX-insensitive sodium channels

In squid giant axon, it is possible to create a situation in which the initial response to depolarization is an outward movement of sodium rather than the usual inward movement by combining perfusion and voltage clamp techniques. This outward sodium movement is also blocked by TTX (Moore *et al.*, 1966). Thus, it is quite reasonable to suppose that the injected TTX would block both the influx and efflux of sodium ion. Moreover, our daily dose of 10 μg/kg for 21 days would be sufficient amount to show a pharmacological effects (Kao and Fuhrman, 1963). Indeed, in the preliminary tests, all mice received 1 μg TTX intraperitoneally died within 2 hrs. Hence it is quite evident from our results that the effect of TTX concentration on the isozyme distribution is not dependent on the agent concentration, indicating that there might occur TTX-insensitive sodium channels in plasma membrane. Although no definitive evidence is available to support the concept, efforts to find such channels have been partly successful (Patrick and Stallcup, 1979; Amy and Kirshner, 1982).

SUMMARY

In an attempt to make a scrutiny into a mechanism for the formation of quaternary structure of LDH isozymes, male mice were intraperitoneally exposed to a wide range of tetrodotoxin concentration and the changes in relative percentage of the five isozymes were monitored by electrophoresis and subsequent densitometry.

The observations that a conspicuous increase of the H_4 isozyme was demonstrated in

nearly all brain tissues, that the M_4 isozyme of skeletal muscle tissue was slightly increased while the M_3H and M_2H_2 isozymes were decreased, that the M/H ratio was strikingly reduced in heart tissue and that assembly of H_4 isozyme was revealed in liver tissue although its rate was extremely low suggest that new intracellular ionic environment established by compulsory change in Donnan equilibrium could alter the LDH isozyme distribution.

The reduction of assembly of M_3H isozyme found in mouse tissues exposed to tetrodotoxin also seems to suggest that the subunit combination of $3M+H$ is very unfavorable in an intracellular environment deviated from its accustomed one.

It was reaffirmed that there might occur TTX-insensitive sodium channels in plasma membrane.

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