Lipolysis and cyclic AMP response to isoproterenol in diaphragms from control and dystrophic mice

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Abstract A comparison was made of the sensitivity of lipolysis (glycerol and free fatty acid release) and of cyclic AMP production to the action of isoproterenol in diaphragms from control and dystrophic Bar Harbor mice at 7 weeks of age. An increased lipolytic response was observed in diaphragms from dystrophic mice that was more apparent in the males, and was demonstrable when cyclic AMP was used instead of isoproterenol. The increased glycerol and free fatty acid release in response to isoproterenol and cyclic AMP cannot be explained by a higher triglyceride content of diaphragms from dystrophic mice, because it was found to be similar to that of controls when it was estimated by biochemical and light microscopic techniques. The increased lipolytic response was not paralleled by changes in cyclic AMP levels, which were found to be similar in diaphragms from control and dystrophic mice, whether in the basal or the stimulated state. It was concluded that the lipolytic apparatus in muscles from dystrophic mice shows an increased sensitivity to isoproterenol that seems to be related to events more intracellular than the cAMP production step.—**Abumrad, N. A., H. M. Tepperman, and J. Tepperman.** Lipolysis and cyclic AMP response to isoproterenol in diaphragms from control and dystrophic mice. *J. Lipid Res.* 1980. **21:** 156–161.

Supplementary key words glycerol · free fatty acids

It has been suggested that the primary defect in muscular dystrophy is closely associated with an abnormally "leaky" cell membrane (1). Several studies have been carried out on membrane-associated enzymes in a search for supporting evidence for the leaky membrane theory. Reports of the activity of adenylate cyclase from dystrophic muscle were contradictory and of uncertain physiologic relevance. In homogenates of Duchenne dystrophic muscle, Canal, Frattola, and Smirne (2) showed a low basal adenyl cyclase activity while Mawatari, Takagi, and Rowland (3) found the basal enzyme levels to be higher than normal. Stimulation of the adenylate cyclase by catecholamines was decreased in both studies mentioned as well as in those of Susheela et al. (4). In cell cultures of dystrophic muscle, supranormal basal levels of the enzyme and insensitivity to epinephrine were reported (5). These findings are in contrast with those of an increased sensitivity of the contractile apparatus of the dystrophic muscle to catecholamines (6). Some evidence supports a pathogenetic role of catecholamines in certain forms of dystrophy. Increased cathecholamine levels were described in tissues of dystrophic animals (7) and experimental myopathy could be produced by inhibiting catecholamine metabolism (8). However, few attempts were made to correlate membrane changes and metabolic effects of the hormone in the dystrophic muscle. The aim of this study was to observe isoproterenol effects on cAMP generation and on triglyceride breakdown in diaphragms from control and dystrophic mice. The metabolic effect examined was lipolysis, since we showed, in an accompanying report, that it is stimulated by cAMP (9).

MATERIALS AND METHODS

Dystrophic mice (129 B_6F_1 Jdy) and their litter mate controls were obtained from Jackson Labs (Bar Harbor, ME). These mice, which are hydrids of the two strains 129 and B_6, are homozygous for dystrophy. They present the same clinical picture of the disease as each of the parent strains but show increased vigor and withstand shipping better (10). The mice were utilized at 7 weeks of age. They were killed by cervical dislocation and their diaphragms were rapidly excised and "intact" hemidiaphragms were prepared as described in more detail elsewhere (9). Glycerol (11) and FFA (12) release were measured in the incubation medium (Krebs Ringer bicarbonate buffer) unless specified otherwise. For determination of cAMP levels, tissue and medium were treated with 1 N perchloric acid; cAMP was extracted (13) and assayed according to the Gilman method (14), using cAMP binding pro-

Abbreviations: TG, triglyceride; FFA, free fatty acids; cAMP, cyclic AMP; dy, dystrophic.

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TABLE 1. Diaphragm weight and DNA content of age-matched (7 weeks) control and dystrophic mice

Mouse Type	Body Weight	Diaphragm Weight	Ratio[a]	Diaphragm DNA		Leg DNA
	g	*mg*	*mg/g*	*Total μg*	*μg/mg tissue*[b]	*μg/mg tissue*[b]
Control	23.8 ± 1.1	60.6 ± 1.6	2.5 ± 0.4	109 ± 5.2	2.3 ± 0.3	2.4 ± 0.5
Dystrophic	16 ± 0.7	52 ± 1.4	3.5 ± 0.3	118 ± 7.0	2.8 ± 0.3	6.3 ± 0.6
n	22	22	22	12	12	12
P^c	<0.001	<0.05	<0.001	>0.05	>0.05	<0.001

[a] Ratio refers to diaphragm weight divided by body weight.
[b] DNA is expressed as μg/mg wet weight.
[c] Comparisons of the means (represented ± their standard errors) were made by the Student's *t* test ($P < 0.05$ is statistically significant).

tein kindly donated by Dr. R. Richman. Tissue DNA content was measured in the pellets obtained by centrifuging the perchloric acid extracts (15). For measurement of tissue triglycerides, diaphragms were quickly frozen in liquid nitrogen; triglycerides were extracted by chloroform–methanol 2:1 and measured as described previously (16). For light microscopy, tissue pieces were fixed in formalin; frozen sections were obtained and were stained for fat with Sudan IV.

RESULTS

Diaphragm weight and DNA content

Dystrophic mice weighed less than their age-matched controls (**Table 1**). The diaphragm weight was also lower in the diseased mice, although the diaphragm to body weight ratio was higher in the latter group. Total DNA content of the diaphragm, as well as DNA expressed per milligram were found to be similar in both groups; however μg DNA per milligram weight was almost threefold higher in the hind limbs from dystrophic mice.

Lipolytic response to isoproterenol

Isoproterenol (2 μg/ml) was more efficient in releasing glycerol and FFA from hemidiaphragms of dystrophic mice (**Fig. 1**) than from those of normal controls. Basal lipolysis was similar in both groups while the response to isoproterenol was larger in the dystrophic mice as shown by the significantly higher percentage increase over basal values.

cAMP production after isoproterenol

Isoproterenol (**Table 2**) stimulated cAMP production equally in control and dystrophic mice, the increase being approximately twofold in both groups. Glycerol values in Table 2 refer to total glycerol measured in a perchloric acid extract of the tissue and medium and they show the same pattern as was noted in Fig. 1, i.e., more lipolysis in response to isoproterenol is observed in the muscles from dystrophic animals although a similar stimulation of cAMP production is seen in both groups.

An unexpected finding in this study was the observation of a significant difference in the response to isoproterenol between male and female mice (**Table 3**). A larger isoproterenol-promoted increase in cAMP concentration was observed in female mice when compared to their male littermates. However, muscle from male dystrophic mice showed a higher isoproterenol-stimulated glycerol release than did muscle from female dystrophic mice. Cyclic AMP levels were not affected by dystrophy in male or female mice, whether in the basal or the stimulated state.

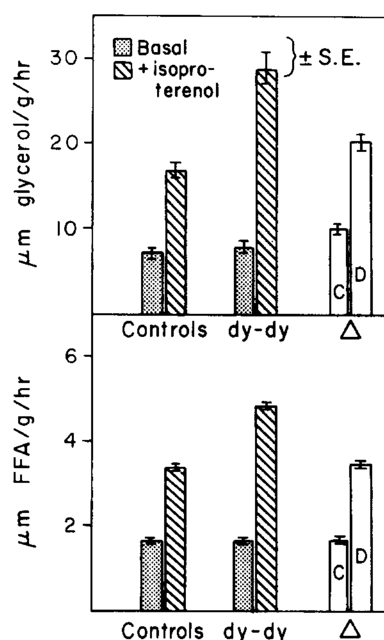

Fig. 1. Effect of isoproterenol (2 μg/ml) on glycerol and FFA release from diaphragms of control and dystrophic mice. Glycerol and FFA were measured in the medium and are expressed as μmoles per g wet weight. The percentage increases over basal were: For control mice; glycerol release 201%, FFA release 196%; for dystrophic mice; glycerol release 438%, FFA release 302%.

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TABLE 2. cAMP and glycerol production after isoproterenol in control and dystrophic mice

Mouse Type	−Isoproterenol	+Isoproterenol	% Change	−Isoproterenol	+Isoproterenol	% Change
		pmol cAMP/mg[a]			*μmol glycerol/g/hr*	
Normal	5 ± 0.4	9.7 ± 0.5	+91 ± 6	5.9 ± 0.5	11 ± 0.6	+92 ± 5
Dystrophic	4.4 ± 0.2	8.4 ± 0.4	+90 ± 7	4.4 ± 0.3	12.7 ± 0.8	+207 ± 10
P[b]	>0.05	>0.05	>0.05	>0.05	>0.05	<0.025

[a] cAMP and glycerol were determined in the perchloric acid extracts of tissue and medium. cAMP is expressed as pmol/mg wet weight/hr. The cAMP values per μg DNA were: 1.6 ± 0.1, 2.6 ± 0.2 for normal mice minus and plus isoproterenol, and 1.6 ± 0.1, 2.8 ± 0.3 for dystrophic mice (−) and (+) the drug.
[b] Each value is the mean of six observations ± SEM.

Effect of 15mM cAMP on lipolysis

This experiment was designed to reveal whether the increased lipolytic response to isoproterenol in muscle from dystrophic mice was related to events beyond cAMP generation. It is apparent in **Fig. 2** that there is more lipolysis in response to 15mM cAMP in diaphragms from dystrophic mice. Basal lipolysis was again found to be similar in the muscles from both groups.

Triglyceride content of hemidiaphragms

At the age the animals were used, we could not measure an increased triglyceride content in hemidiaphragms from dystrophic mice. Levels similar to those observed in normals were found (20.7 mg/g ± 2 versus 17.2 mg/g ± 3 in normal and dystrophic mice respectively). Light microscopic examination confirmed these results, since no significantly increased fat accumulation was observed in diaphragm muscles from dystrophic mice. However, areas of fat deposits and necrosis were noted in sections from hind limb muscles (**Fig. 3**).

DISCUSSION

Diaphragm, weight, and DNA content

It is apparent from Table 1 that there is a small decrease in absolute diaphragm weight in dystrophic animals but that the process seems slower than wasting in other muscles of the animal, which leads to an apparent diaphragm enlargement (increased diaphragm to body weight ratio). Total diaphragm DNA content and DNA per mg weight were not changed by dystrophy, despite a small increase in the latter which could be due to the loss in weight of the diaphragm. The increase is much more pronounced in hind limb-DNA per mg weight, where the muscle mass loss is obviously marked. These results seem to support the view (17) that the reported increase in mouse leg-DNA per unit weight is a result of the muscle weight decrease caused by dystrophy, while total DNA per leg remains constant. However, an actual enlargement of the diaphragm muscle together with increases in total DNA content were described in the dystrophic hamster at 90 and 110 days of age (18). Although our data suggest that, at the age the mice were studied (7 weeks), a slow weight loss occurs in the diaphragm muscle, a careful study is necessary to evaluate whether diaphragm hypertrophy occurs in these mice during the progression of the disease. We did observe a trend toward an increased total diaphragm-DNA in dystrophic mice. However, the scatter of the data prevented differences between dystrophic and normal mice from reaching statistical significance.

Lipolysis response to isoproterenol

The finding of similar basal glycerol and FFA release in normal and dystrophic mice suggests that triglyceride mobilization is not disturbed by dystrophy. On the other hand, sensitivity to isoproterenol is increased (Fig. 1). The increased response is not related to a larger cAMP production (Table 2) because

TABLE 3. Effect of isoproterenol on cAMP and glycerol production by diaphragm tissue of male and female control and dystrophic mice

Mouse Type	−Isoproterenol	+Isoproterenol	% Change	−Isoproterenol	+Isoproterenol	% Change
		pmol cAMP/mg[a]			*μmol glycerol/g/hr*	
Male-controls	4.8 ± 0.3[b]	7.7 ± 0.4	62 ± 6	7.6 ± 0.6	11.9 ± 0.7	60 ± 4
Male-dy	4.8 ± 0.4	8.1 ± 0.4	74 ± 7	6.1 ± 0.6	12.8 ± 0.6	120 ± 8
Female-controls	3.7 ± 0.2	7.5 ± 0.4	120 ± 11	8.2 ± 0.5	12.0 ± 0.6	51 ± 5
Female-dy	4.1 ± 0.3	8.1 ± 0.2	105 ± 12	7.3 ± 0.5	12.3 ± 0.5	70 ± 5

[a] cAMP and glycerol were determined in perchloric acid extracts of tissue and medium.
[b] Each value is the mean of six observations ± the standard error.

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basal and isoproterenol-stimulated levels of the nucleotide were similar in both groups of animals. Expressing the cAMP values per μg DNA did not modify the results obtained. Our results disagree with those of Susheela (19) who reported low adenyl cyclase activity in skeletal muscle of dystrophic mice. However in the same species, Canal et al. (2) found increased activity of both adenyl cyclase and phosphodiesterase, which fits better with our data since the net effect on tissue cAMP levels could be nil.

The increased lipolytic activity we observed in response to isoproterenol in diaphragms from dystrophic animals cannot be explained by the possibility that membranes of muscle cells from dystrophic mice are abnormally permeable and, thus, allow more leakage of glycerol and FFA into the medium, because the glycerol production measured in the perchloric acid extracts of the tissue and medium (Table 2) followed a pattern similar to the glycerol release (Fig. 1). This is also supported by the fact that basal lipolysis was similar in muscles from control and dystrophic mice. Our observation cannot be accounted for by a higher fat content in diaphragms of dystrophic mice since *1*) light microscopic examination of diaphragm pieces stained for fat with Sudan IV did not show more fat in the muscles from dystrophic animals, and *2*) triglyceride content was found to be similar in the diaphragms from both groups of animals at the age they were studied (7 weeks): 20 mg/g ± 3 versus 17 mg/g ± for normal and dystrophic mice, respectively.

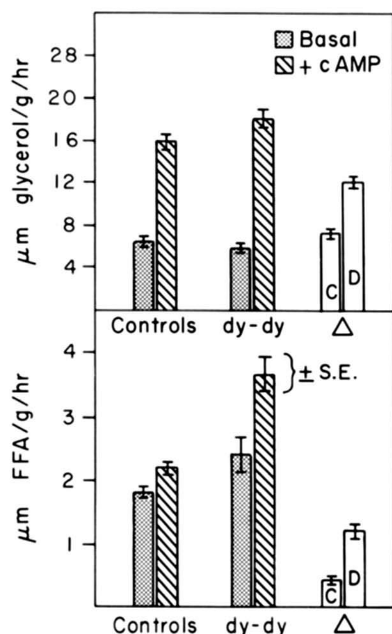

Fig. 2. Effect of cAMP (15 mM) on glycerol and FFA release from diaphragms of control and dystrophic mice. The percentage increases over basal were 89% ± (control), 193% ± 9 (dystrophic) and for glycerol: for FFA: 20% ± 3 (control), 62% ± 14 (dystrophic). All values are means of six observations and are represented ± their standard errors.

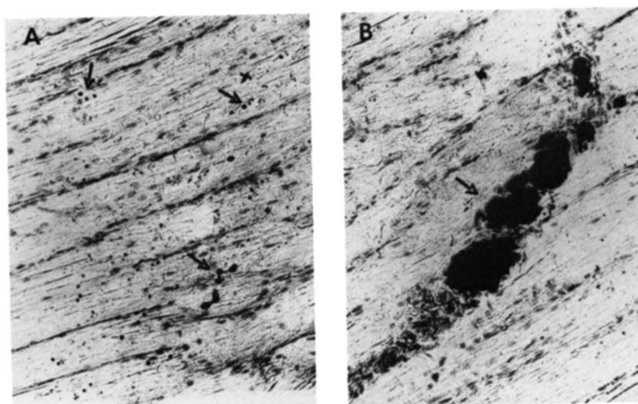

Fig. 3. Sections of the hind limb of a normal (Panel A) or dystrophic mouse (Panel B). Areas of extensive fat accumulation are observed in the muscle from the dystrophic animal. Fat droplets (arrows) were stained with Sudan IV. Magnification: ×165.

The finding of a difference in the response to isoproterenol between male and female mice suggests that sex hormones could affect catecholamine actions in muscle, at least in this particular strain of mice. With respect to dystrophy, cAMP generation was not affected by the disease in males or females. The larger stimulation of glycerol release by isoproterenol in dystrophic mice was more apparent in the males, suggesting that they are more affected by the disease. The dissociation between cAMP and glycerol production when the effect of sex in relation to dystrophy was studied adds support to the interpretation that the increased sensitivity to isoproterenol observed in the muscles from dystrophic mice does not involve a modified response of the cAMP producing system in the muscle cell membrane.

Lipolytic response to cAMP

The increased lipolytic response to cAMP observed with dystrophic mice, (Fig. 2) together with the cAMP generation data (Table 2), suggest that the lipolytic machinery is altered by dystrophy at a locus more intracellular than cAMP production. Our results contradict the report by Janaki and Susheela (20) of decreased basal triglyceride lipase levels in Duchenne dystrophic muscle. They fit better with the findings of Jato-Rodriguez, Hudson, and Strickland (21) of increased lipase activity in muscles from dystrophic mice. However, there are two main difficulties in interpreting our data within the scope of the last study mentioned. *1*) We did not observe a consistently higher than control basal lipolysis in dystrophic mouse muscle. *2*) The triglyceride lipases described by Jato-Rodriguez et al. (21) in muscle homogenates (a short

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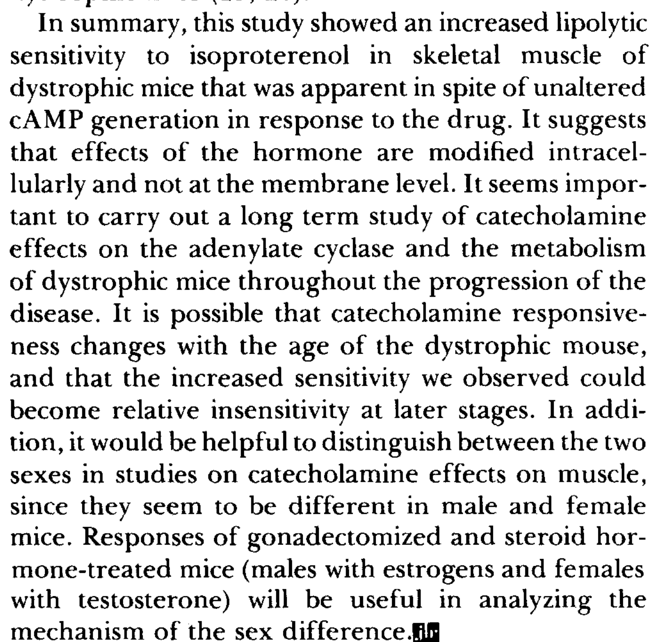
chain and a long chain TG Lipase) were insensitive to epinephrine activation in both normal and dystrophic muscle. If we assume that the effect of isoproterenol and cAMP on endogenous TG breakdown in our diaphragm preparation is carried out through the activation of a triglyceride lipase, it implies that the latter enzyme is hormone-sensitive. The inability of epinephrine to activate the lipases in the study by Jato-Rodriguez et al. (21) could be due either to the fact that it is sometimes difficult to show hormone-sensitive lipolysis in tissue homogenates (22, 23) or to the use of an inappropriate substrate for the enzyme (tributyrin and tripalmitin). In fact, recent evidence in adipose tissue homogenates indicates that the effect of epinephrine on adipose tissue triglyceride lipase can best be shown using an endogenous substrate (23, 24). On this basis, a second mechanism of lipolysis activation by epinephrine, independent of cAMP, was suggested (23). It would involve "substrate activation" of endogenous triglycerides, making them more susceptible to the action of the lipase. Calcium was hypothesized as a possible mediator of the substrate activation (22). It is tempting to postulate that the second mechanism of lipolysis activation by epinephrine is altered in dystrophic mice, especially since we showed, in an accompanying report, that calcium seems to be involved in muscle lipolysis (9) and, also, in view of the many reports of an altered capability to take up calcium by sarcoplasmic reticulum from dystrophic mice (25, 26).

In summary, this study showed an increased lipolytic sensitivity to isoproterenol in skeletal muscle of dystrophic mice that was apparent in spite of unaltered cAMP generation in response to the drug. It suggests that effects of the hormone are modified intracellularly and not at the membrane level. It seems important to carry out a long term study of catecholamine effects on the adenylate cyclase and the metabolism of dystrophic mice throughout the progression of the disease. It is possible that catecholamine responsiveness changes with the age of the dystrophic mouse, and that the increased sensitivity we observed could become relative insensitivity at later stages. In addition, it would be helpful to distinguish between the two sexes in studies on catecholamine effects on muscle, since they seem to be different in male and female mice. Responses of gonadectomized and steroid hormone-treated mice (males with estrogens and females with testosterone) will be useful in analyzing the mechanism of the sex difference.



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