A study has been made of the I^{125} insulin binding and postbinding effects on excised soleus muscles from the 129 ReJ strain of dystrophic mice. Results are compared with those in sex- and weight-matched controls. The data suggest that, in the range of physiological hormone concentrations, the affinity of insulin receptors on dystrophic muscles is less than normal and that the insulin-dependent uptake of both 2-deoxyglucose (2-DG) and aminoisobutyric acid (AIB) is impaired. These findings are taken to indicate that many of the biochemical and electrophysiological abnormalities observed in murine dystrophy could arise from some genetic defect in the receptor proteins controlling uptake of raw materials.

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AN INSULIN RECEPTOR DEFECT IN MURINE MUSCULAR DYSTROPHY

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The general biological properties of dystrophic muscle have been extensively studied in genetically susceptible animals, chiefly the 129 ReJ-dy strain of mice. Biochemical, histopathological, electrophysiological, and other aspects have been thoroughly reviewed elsewhere.[18,34,44] Two very broad classes of abnormalities can be distinguished, those relating to membrane structure and function[32] and those evident in the cell contents, including the contractile proteins themselves. Perhaps the best known surface defects are "leaks" of both cations,[12,23,50] and such intrafiber contents as amino acids[3,8,47] and enzymes.[25,42] The membranes in dystrophy are not able to maintain normal electrical potentials[13,19,36] and there may be a tendency toward spontaneous firing.[20] These abnormalities have usually been taken to mean that the dystrophic muscle membrane is structurally altered, so that ions, amino acids, and proteins can simply leak down their respective concentration gradients. Little has been discovered about the cause of the observed membrane changes, and even less has been said about the sort of metabolic abnormality which might mimic such physical changes.

The salient intrafiber defects include numerous structural changes,[32,38,41] weak[3,4,11] and slowed contractions,[19,22] and relaxation.[11,19,22,35] It has also been found that there is an increased rate of protein turnover[47,49] and increased breakdown,[43] the latter increasing the size of the intracellular free amino acid pool.[47] With these changes there is profound atrophy, impairment of normal growth and repair,[6,16] and reduced ability to survive transplantation.[7,21,45,46] Energy sources are also impaired, owing to defective glucose and glycogen metabolism[14,48] and significant impairment in the muscle's ability to use acetylcarnitine, palmitate, and palmitylcarnitine[47] because of changes in the mitochondrial membrane. The dystrophic muscles may accumulate fatty acids and cholesterol, but the afflicted animals do not show elevated serum lipids, inability to produce ketone bodies, or myoglobinuria characteristic of a specific mitochondrial transport enzyme deficiency.[2]

In attempting to account for such diverse surface and intrafiber abnormalities in dystrophic muscles, some interest attaches to the hormone insulin, since disturbances in both its secretion[33] and effects[15,17] have been reported in mouse dystrophy and in the myotonic form of human dystrophy.[15,39] Further, insulin's actions begin at the surface membrane,[31] with macromolecules capable of regulating numerous intracellular energy-releasing and protein-synthesizing systems.[6,9,29] Defects in the insulin-binding-transduction system could thus lead to impairment of several metabolic cascades necessary for the muscle's function and survival.

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The present experiments are an attempt to learn if there is an abnormality of, or an availability of insulin ligands on the surface of skeletal muscles of dystrophic mice.

MATERIALS AND METHODS

All experiments were performed using excised muscles from mice which were anesthetized, then killed with ether. Male dystrophic mice of the 129 ReJ-dy strain were obtained at 4–6 weeks of age from the Jackson Laboratories (Bar Harbor, ME), along with age- and sex-matched nondystrophic littermate controls. Albino mice, weight-matched to the dystrophic mice, were also used as controls, since the healthy littermates weighed about twice as much as the clinically affected mice at the time of the experiment. The animals were kept from 1–2 weeks for special feeding and hydration prior to use. The dystrophic mice were fed a finely crushed powder made from the same standard chow blocks used for controls, the food being placed so that they had no trouble reaching it. Water was delivered by a special tube providing maximum accessibility. On this regimen even the most severely emaciated mice gained weight rapidly and appeared in good condition at the time of use. The importance of this waiting and feeding period is that it prevents the major changes in insulin binding and effects seen when animals are underfed.[5] Muscles from albino mice used as a second control behaved exactly as those from the clinically unaffected 129 ReJ littermates, and results from the two control groups were pooled.

Muscle Preparation. The soleus muscle was used, which, though it is less severely affected by the disease process,[7] showed typical histological changes of murine dystrophy. Such changes included fiber splitting, fiber size variability, occasional segmental necrosis, some basophilic staining, and reduction in muscle weight, but replacement of muscle fibers by fat or connective tissue was only rarely seen. The soleus muscle also had the advantage that it could be removed en toto without fiber damage, which had been found in preliminary experiments to cause major changes in hormone binding. Another advantage of using the soleus muscle was that it had been extensively studied by earlier investigators in terms of insulin binding and effects.[30] Excision of the muscle was extremely simple and was accomplished by removing the overlying gastrocnemius muscle, transecting the remaining distal soleus tendon and gently lifting the belly until the proximal tendon could be cut. After removal from the ether-killed animals, the muscles were washed for a minimum of 1 hour in a large volume of buffered, gassed medium containing 2 m*M* pyruvate.[30] The muscles were tested in pairs, a control and a dystrophic preparation side by side in the various incubating media. Weights were recorded prior to incubation, after blotting and removing all excess fat and tendon under a dissecting magnifier. All specimens were incubated at 21°C–22°C in 2.0 ml of buffered nutrient medium of the composition described by Bretag.[4] The small incubation cups were slowly agitated in a floating shaker covered by a lid whose edges dipped below the bath water. This allowed 95% O_2 and 5% CO_2 to be delivered to the open cups at slightly above atmospheric pressure. The muscles remained fully relaxed, except those that were damaged in dissection. Results from the latter were discarded. To minimize adherence of insulin to cup walls, all media contained defatted bovine serum albumin (BSA) at a concentration of about 10 mg/ml.[10] At the end of incubation, the muscles were pinned onto a silicon-rubber holder and washed with repeated changes of ice-cold medium over a 30-minute period. Prior to dissolving, all the muscles were blotted as dry as possible to remove tracer accumulated in extracellular fluid.

Separate groups of muscles were used to record insulin binding and insulin effects. This technique avoided the significant errors introduced by removing aliquots and by adding a second tracer. For the I^{125} insulin assay, the whole muscles were counted. For the C^{14}-labeled hexose and amino acid uptake studies, each muscle was dissolved by heating to 60°C for 4 hours in 2 ml of Protosol$^{(R)}$ (New England Nuclear Corp., Boston, MA).

Radioactive Materials. I^{125}-iodinated, monocomponent porcine insulin from two sources was used. One preparation was kindly provided from the laboratories of Dr. Gerald Reaven (VA Medical Center, Palo Alto, CA), with an estimated specific activity of from 125–165 μCi/μg. The other was obtained as a standard commercial preparation from New England Nuclear Corp., with a specific activity of about 90 μCi/μg. There was no detectable difference in results with the two products, and the data were pooled. C^{14}-2 deoxyglucose (2-DG) and C^{14} aminoisobutyric acid (AIB) were also obtained from New England Nuclear Corp.

Binding Studies. To estimate the amount of "specific" insulin binding to the muscles, control and dystrophic specimens were incubated separately in

solution containing only a trace of labeled hormone (0.2–0.3 n*M*) or in solution with the same trace plus a great excess (8,000 n*M*) of "cold" insulin (monocomponent, zinc-free porcine insulin provided by Dr. R. E. Chance, Eilly Research Lab., Indianapolis, IN). The noninsulin-displaceable fraction of total radioactivity was taken as nonspecific binding and was subtracted from results in all other concentrations of hormone. To test the ability of control and dystrophic muscles to degrade insulin, aliquots of medium were counted before and after muscle incubation. The fraction of radioactivity remaining in the supernate after precipitation in 10% TCA was no more than 3% of the total with either set of muscles.

Transport Studies. Insulin effects on hexose and amino acid transport were recorded in paired experiments, using separate muscles from those used for binding studies. Data from nondystrophic 129 strain littermate controls and normal albino mice were pooled and compared with results in clinically dystrophic animals. In tests using 2-DG, glucose in the nutrient medium was replaced by iso-osmolar sucrose. The aim of this substitution was to maximize the uptake of labeled, nonusable sugar. In the AIB experiments, glucose was left in the solution. The incubation time was 1 hour at 22°C, after which the muscles were washed with flowing, ice-cold medium for 30 minutes and then blotted as dry as possible. The muscles were then dissolved and counted in a scintillation counter. Insulin-independent (basal) uptake was recorded in all experiments by incubating separate muscles in hormone-free medium for the same period. Roughly, the same test concentrations of insulin were used for the metabolic as for the binding experiments.

RESULTS

Insulin-displaceable I^{125} insulin binding could be demonstrated in all the preparations and was taken as evidence for the presence of hormone-specific receptors on the muscles. Though the fraction specifically bound represented a very variable portion of the total, there appeared to be no significant difference in the proportions of specific and nonspecific binding on control and dystrophic muscles exposed to a large excess of unlabeled hormone. Using lower concentrations of "cold" insulin, it was possible to plot a rough dose response curve of insulin binding, and, as shown in Fig. 1, the hormone-receptor interaction appeared to saturate at about the same final level in both disease and control muscles. Also, at the lowest test

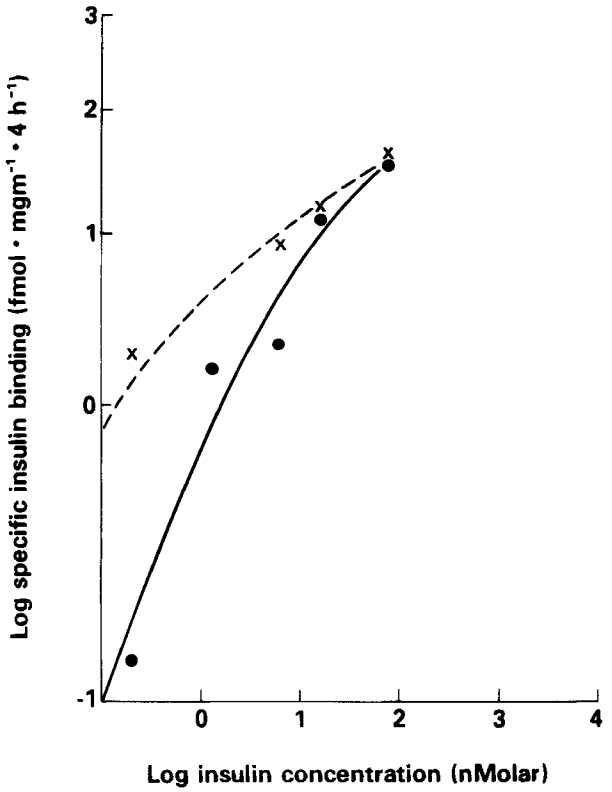

FIGURE 1. Comparison of insulin binding in control and dystrophic murine soleus muscle. Experiments at 21°C–22°C. Nonspecific binding subtracted from each point, which represents an average of eight muscles. Curves fitted by eye. Note log scales. (●) Dystrophic, (X) control values. Binding estimated with various concentrations of unlabeled porcine monocomponent insulin and I^{125} labeled insulin, 0.35 nm.

insulin concentrations, the amount specifically bound is less than control in the dystrophic preparations ($P > 0.01$, <0.05). Thus, in the range of physiological insulin concentrations the hormone affinity of the receptors on the dystrophic fibers appeared to be reduced in vitro.

Figure 2 illustrates the results with 2-DG. Unlike the case with binding studies, the labeled hexose uptake curves do not plateau at the same maxima ($P = 0.002$). Dystrophic muscles assimilated less of the sugar at all the tested insulin concentrations, and a slight difference in mean, basal (insulin-independent) uptake can also be seen ($P > 0.05$, <0.10) (see, however, Olson and Smith[40]). The binding data do not indicate a major reduction in the number of available receptor sites, and the impairment of hexose transport is therefore consistent with either a defect in the receptor binding properties or in some aspect of postbinding activation of the transporter unit. The much lower

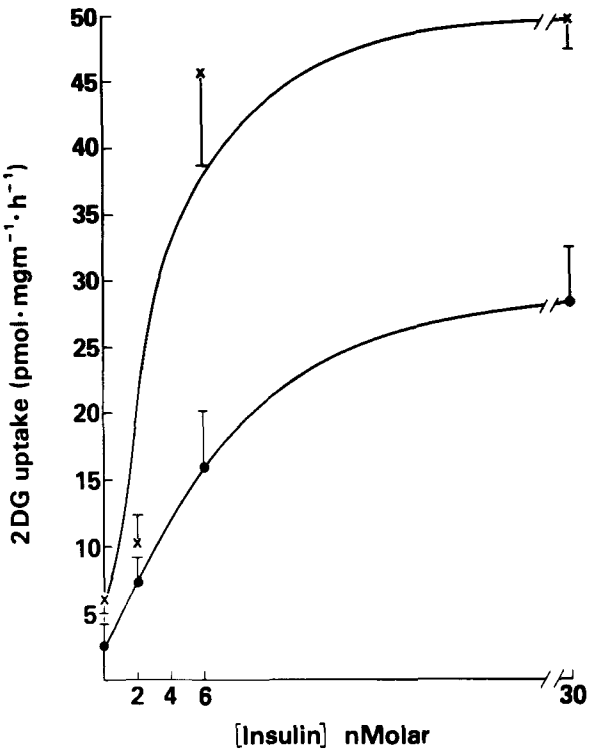

FIGURE 2. Insulin stimulation of carbohydrate (nonutilizable) uptake in control and dystrophic murine soleus muscles in vitro. Tissues incubated for 1 hour in C^{14}-labeled deoxyglucose at insulin concentrations shown. Basal and all subsequent values obtained after preliminary 1-hour wash in nutrient medium (see text) with 2 m*M* pyruvate, temperature 21°C–22°C. Each point and bar represent mean ± SD of 8–12 muscles. The curves were fitted by eye. (●) Dystrophic, (X) control values. Tissues blotted thoroughly before counting to remove tracer remaining in extracellular fluid.

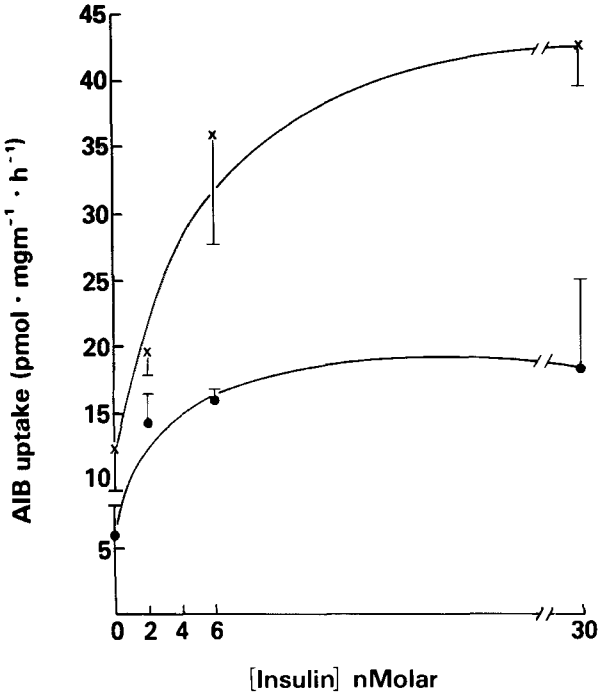

FIGURE 3. Transport of C^{14}-labeled aminoisobutyric acid into control and dystrophic soleus muscles in vitro at various insulin concentrations. Conditions as in Fig. 2. Each point and bar obtained from 12 muscles.

levels of tracer uptake than reported elsewhere[30] are presumed to be the result of tissue-blotting the muscle until it is nearly dry before counting. This technique is designed to remove the large amounts of radioactivity contained in the extracellular fluid.

Similar findings are seen in Fig. 3, which illustrates the results of AIB uptake studies. Again, the maximum response is blunted in the dystrophic fibers ($P > 0.01$, <0.05), and the noninsulin-dependent inward movement of tracer is even more depressed in the diseased specimens than with 2-DG ($P < 0.05$, >0.10). Removal of much of the extracellular fluid after incubation in this case could introduce a serious error in the estimation of AIB uptake and release rates, but the point of interest remains that the final amount of radioactivity associated with each milligram of tissue is less in the dystrophic fibers. Thus, while the sampling technique used could totally obscure very rapid uptake and release of amino acids, the net result can be visualized as depriving the dystrophic muscles of protein precursors. The problem is less important with the 2-DG studies, since the sugar, once phosphorylated, probably does not diffuse back to the extracellular fluids as can amino acids freely dispersed in the cytosol.

DISCUSSION

It is clear that the Bar Harbor strain 129 dystrophic mouse is not an ideal model for the study of the myopathology itself. The animals show abundant evidence of motor root dysmyelination, slowed conduction of impulses, reduction in the number of surviving axons, and changes in axoplasmic flow.[26–28] It is also well recognized that the type 1 muscles (soleus) used for these studies are less severely affected than are the fast-twitch glycolytic type 2 fibers, and the possibility thus remains that these latter muscles, which bear the brunt of the dystrophic process in rodents,[37] may have a number of important biochemical and other changes having nothing to do with the findings reported here for the soleus muscle. On the other hand, the relatively minor pathological alterations in the soleus favor use of this muscle in studies of the present kind, in which gross error would be

introduced by replacing muscle tissue with fat or connective tissue. Nevertheless, as with all the various abnormalities reported in the mouse model, the findings in these experiments could just as well be the result of dystrophy as the cause of it.

An important feature of surface insulin binding sites is that they regulate both the sources of energy for functions of the contractile apparatus and the inward transport of materials from which structural elements are made and replaced. To the extent that muscular dystrophy follows the inadequacy of such a supply system it can be compared to metabolic myopathies. The essential difference would be that, in dystrophy, the fundamental block in energy flow begins in the transduction-transport mechanisms at the surface, rather than within the sarcoplasmic enzyme systems or intracellular organelles. Acting over time, even a minor abnormality in the properties of membrane receptor-ligand complexes could affect many subsequent metabolic steps and thus give rise to disturbances that appear to be intracellular in origin. Goldberg et al.[17] have already demonstrated that insulin is less effective in inhibiting protein catabolism in dystrophic muscle, and Ballard et al.[1] have shown that the hormone's ability to stimulate protein synthesis is also reduced in the disease. Reduced protein synthesis and possibly greater catabolism to support energy demands both could contribute to the dystrophic muscle's failure to grow, abortive efforts to repair itself, and inability to survive transplantation normally. Some of these deficits could also stem from similar abnormalities in amount or properties of receptors for other "trophic" substances.

Chronic reduction of intrafiber energy sources could also lead to biochemical and electrophysiological changes which are usually considered secondary to membrane permeability defects in dystrophy. Depolarization, along with loss of intrafiber K^+ and gain of Na^+ and Ca^{++} are often taken as evidence of membrane "leaks," but inadequate fuel for both surface and mitochondrial ion "pumps" could give rise to exactly the same findings. In the case of energy supplied from glucose, Horsfall[24] has suggested that competitive binding to insulin receptor sites by insulin-like molecules in dystrophy could prevent hormone-dependent sugar uptake, leading to the observed metabolic changes. The present findings are entirely consonant with this idea but do not distinguish between such competitive blocking and a primary defect in synthesis or placement of receptor in the developing membrane.

The outward movement of amino acids, which is seen even in carriers having no clinical disease, raises the question whether a normally high bidirectional flux of amino acids is simply converted to net efflux by reduction of active reaccumulation.

The finding of diminished insulin binding and effects on muscle does not mean the dystrophic animal would be diabetic. In fact, insulinopenic, diabetic mice have been found to show increased insulin binding and enhanced insulin responses, quite unlike what has been found here.[40] Further, dystrophic mice have been found to have normal or slightly slowed insulin levels,[33] so that the defective hormone binding and action observed here cannot be attributed to receptor "down-regulation," as is seen in insulin-resistant diabetics.[10] Since it has been observed that insulin is important in fetal growth and metabolism, it is of interest that insulin binding to monocytes of newborn humans is greater than that in adults.[48] Cells having a disturbance in the insulin receptor-transducer mechanism would thus be particularly vulnerable in early life. On this view, the entire spectrum of biochemical and structural abnormalities in muscular dystrophy could result from inadequate supplies of raw materials at a time when the cellular structure is being laid down.

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