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# Diminished Respiratory Responses of Brown Adipocytes Isolated from BIO 14.6 Dystrophic Hamsters[1] (41606)

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*Abstract.* Catecholamine-induced thermogenesis is significantly diminished in BIO 14.6 cardiomyopathic hamsters as demonstrated by a reduced increase in oxygen consumption of these hamsters in response to administered isoproterenol. This decreased responsiveness is accompanied by a reduction in the amount of brown adipose tissue, a major nonshivering thermogenic effector. The present study demonstrates that the metabolic responses of individual brown fat cells are also altered in the dystrophic hamster. That is, 1 $\mu M$ norepinephrine, the physiological mediator of nonshivering thermogenesis, evoked rates of oxygen consumption that were significantly lower in brown adipocytes isolated from the BIO 14.6 hamsters than in those from normal controls. Additionally, the dystrophic adipocytes exhibited: decreased maximal activity (per cell as well as per milligram protein) of citrate synthase; decreased cell size; and decreased amounts of protein per cell. These data indicate that the nonshivering thermogenic capacity of the intact BIO 14.6 hamsters reflects altered characteristics of the individual brown adipocytes themselves, as well as decreased amounts of the tissue.

The BIO 14.6 strain of cardiomyopathic dystrophic hamsters exhibits a reduced capacity for nonshivering thermogenesis (1). This reduction is manifested in the intact animal as a smaller increase in the rate of oxygen consumption in response to systemically administered catecholamines than that seen in normal hamsters (1). In an attempt to determine the basis for this reduced oxidative response, we have been examining several characteristics of dystrophic brown adipose tissue, the major contributor to catecholamine-induced nonshivering heat production in rodents (2). Our studies (3) confirm the report by Himms-Hagen and Gwilliam (4) that the brown adipose tissue of the BIO 14.6 hamster is considerably reduced in mass; we have also found (3) that several enzymes associated with aerobic metabolism exhibit significantly lower levels of activity per unit of tissue mass (i.e., maximal activity/gram of brown adipose tissue). This latter observation suggests that the individual brown fat cells in the BIO 14.6 dystrophic hamster may differ from their normal counterparts with respect to their thermogenic responsiveness to catecholamines.

The present study was thus undertaken to evaluate the possibility that the decreased magnitude of the catecholamine-induced oxygen consumption in the dystrophic hamsters reflects changes in the responses of individual brown fat cells as well as changes in the amount of brown adipose tissue present in the animal. To this end, we isolated brown adipocytes from normal and dystrophic hamsters and compared: (a) the effects of norepinephrine (NE) on their rates of oxygen consumption ($\dot{V}O_2$); and (b) the activities of citrate synthase, an enzyme whose maximal activity is generally taken to be correlated with flux through the TCA cycle [see reference (10)].

**Methods.** Brown adipocytes were isolated from male, dystrophic BI0 14.6 (Bio-Research Consultants, Cambridge, Mass.) and normal (Charles River Lakeview Labs, Newfield, N.J.) Syrian hamsters, aged 16–20 weeks. The animals were housed in groups of three to five at 22–24°C on a 12-hr light/dark cycle (0630–1830) and provided with Simonsen White Diet (Simonsen Labs, Inc.) and water *ad libitum*. All experiments were performed in the months of January–March; and to minimize effects of seasonal variability, experiments using dystrophic cells were alternated with those using normal cells.

On the morning of the experiment, two normal or 3 dystrophic hamsters were decap-

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itated, and the interscapular, subscapular, cervical, and axillary brown fat pads were removed and placed in a modified Krebs–Ringer–bicarbonate buffer containing 4% bovine serum albumin (BSA, Pentex Fraction V, Miles Labs., Kankakee, Ill.) at pH = 7.3 at 37°C (KRBA). The tissue was cleaned of adhering white fat and connective tissue, weighed, minced and transferred to a plastic Erlenmeyer flask (25 ml) containing crude collagenase (Worthington; 27 mg for normal tissue, 13.5 mg for dystrophic tissue) and soybean trypsin inhibitor (Sigma; 6 mg for normal tissue, 3 mg for dystrophic) in a final volume of 10 ml KRBA. This suspension was incubated at 37°C for 25 min in a slowly shaking water bath under continual gassing with 99% $O_2$–1% $CO_2$. The resulting mixture was then filtered through filter silk, the filtrate discarded, and the cells remaining in the silk gently expressed into fresh KRBA (15 ml), mixed, and allowed to rise at room temperature. The infranatant was removed, the cells were resuspended to 15 ml with fresh KRBA and the mixing, rising, and infranatant removal processes were repeated one more time—these steps taking place at room temperature. The number of adipocytes present was determined by counting aliquots of the cell suspension in a hemocytometer; and the final volume of the cell suspension was adjusted to $2.7 \times 10^6$ cells/ml KRBA and maintained at 37°C. An aliquot of these cells was rapidly frozen and stored at −85°C for subsequent enzyme analysis. The remainder were used for the respiration studies.

The KRBA utilized for isolating brown adipocytes contained 4% bovine serum albumin, 136 m*M* NaCl, 4.7 m*M* KCl, 2.5 m*M* $CaCl_2$, 1.18 m*M* $KH_2PO_4$, 1.18 m*M* $MgSO_4$, and 6.65 m*M* $NaHCO_3$. For the $\dot{V}O_2$ measurements, the KRBA placed in the flasks contained two additional components: 400 U/ml penicillin and 400 μg/ml streptomycin (penicillin/streptomycin solution from Grand Island Biol. Co., Grand Island, N.Y.). A third Krebs–Ringer–bicarbonate buffer (KRB) without BSA, penicillin, or streptomycin but having the same ionic composition as KRBA was used to solubilize the ascorbate (see below). KRBA solutions were prepared fresh daily, filtered (0.45 μm Millipore filter, Millipore Corp., Bedford, Mass.), and adjusted to pH 7.3 by gassing with 1% $CO_2$ and 99% $O_2$ (with a Pyrex sintered glass disc filter) for 30 min. KRB was prepared fresh weekly and filtered daily prior to use. Norepinephrine (NE; ℓ-arterenol bitartrate, Sigma) was solubilized in the antioxidant, ascorbate (0.10 mg/ml in KRB), and the pH was adjusted to 7.4 with 3 *M* Tris (Trizma base, Sigma). A diethanolamine–$CO_2$ buffer containing 13.17 m*M* thiourea, 2.0 *M* $KHCO_3$, 0.88 *M* HCl, and 4.17 *M* diethanolamine was equilibrated with 1% $CO_2$ and 99% $O_2$ at 37°C immediately prior to use.

Rates of oxygen consumption of the isolated brown adipocytes in the presence and absence of NE were determined with a Gilson differential respirometer (submarine model). A constant 1% $CO_2$ environment was maintained during the experiment by placing the diethanolamine–$CO_2$ buffer into the center well of each flask along with filter paper to increase the surface area (5). KRBA and adipocytes were pipetted into the main chamber of each flask so that the final cell concentration was $2 \times 10^5$ cells/ml. These additions were made at 37°C. At time ($t$) = 0, ascorbate (control) or NE (experimental) was added to the main chamber of each flask. The flasks were then removed from the 37°C water bath, attached to manometers, and immersed in the Gilson water bath (~37°C) at 15 sec intervals. The flasks were closed to the atmosphere 10 min after placement in the Gilson bath. Manometric readings were taken at 5-min intervals from $t$ = 15 to $t$ = 90 min. Equilibration of the system took approximately 30 min, after which time oxygen consumption rates were stable.

Citrate synthase (E.C. No. 4.1.3.7) was measured using the method of Bass *et al.* (6) and Srere (7). The reaction medium (pH = 7.5) totaled 1 ml and contained: 100 m*M* Tris–HCl, 2.5 m*M* EDTA, 0.1 m*M* 5,5′-dithiobis-(2-nitrobenzoic acid), 0.2 m*M* acetyl CoA, and 0.5 m*M* oxalacetate. Activity was measured at 25°C as the change in absorbance at 412 nm on a Varian 635 spectrophotometer. The thawed cell suspension ($2.7 \times 10^6$ cells/ml) was diluted 1:20 with a 0.1 *M* phosphate buffer, pH = 7.5, and kept on ice. This diluted suspension was then sonicated (Sonifier Cell Disruptor, Model 185D, Heat Systems–Ultrasonics, Inc., Plainview, N.Y.)

twice for 10-sec intervals with 30-sec pauses in-between. The suspension and sonicator were kept on ice in minimize temperature increases. Immediately prior to enzymatic analysis, a fresh dilution of the homogenate (1:50) was made. Duplicates were run on all samples. Use of different enyzme concentrations indicated that substrates were not limiting. Protein was assayed by the method of Lowry *et al.* (8) using bovine serum albumin as the standard.

For all data, statistical significance ($P \leq 0.05$) between means was determined using either the Student's $t$ test (for comparison of two means) or an analysis of variance and Newman–Keuls test (for comparing more than two means).

**Results and Discussion.** The amount of brown fat obtained from the dystrophic hamsters used in this study was significantly less than that seen in the normal hamsters, a finding consistent with previous observations (3, 4). However, the cell yield from the dystrophics was more than 6 times greater than that from the normals ($20.4 \pm 3.9 \times 10^6$ cells/gm, dystrophics; $3.07 \pm 0.51 \times 10^6$ cells/gm, normals). This greater cell yield can be explained, at least in part, by the fact that the dystrophic cells were considerably smaller in size than were the normal cells. Thus, each gram of dystrophic brown fat undoubtedly contained more adipocytes than did each gram of normal tissue. Moreover, the smaller size of the dystrophic cells probably rendered them less susceptible to breakage during the isolation procedure than were the larger, more fat-filled adipocytes from the normal hamsters.

Approximately 30 min was required for the respiratory apparatus to stabilize after the flasks were attached. From this time, until termination of the experiment ($t$ = 90 min), the amount of oxygen consumed by the isolated adipocytes increased linearly with time (Fig. 1). This linearity was manifested by the cells isolated from the normal as well as dystrophic hamsters and by cells respiring in the presence or absence of NE (Fig. 1). The temporal constancy of the oxygen consumption rates indicates that: (a) the cells remained viable throughout the perod in which respiration was measured; and (b) the antibiotics present in the flask prevented significant bacterial growth.

As summarized in Table I, resting (i.e., in the absence of NE) values of $\dot{V}O_2$ were significantly higher in the normal brown adipocytes than in the dystrophic cells. Although NE increased respiration of both dystrophic and normal brown adipocytes, the values of $\dot{V}O_2$ observed in the presence of 0.1 or 1 $\mu M$ NE were greater in normal than in dystrophic cells. Even when comparing NE-induced $\dot{V}O_2$ (i.e., $\dot{V}O_2$ in the presence of NE minus $\dot{V}O_2$ in the absence of NE), at 1 $\mu M$ NE, normal cells exhibited a significantly greater increase (~47%) in $\dot{V}O_2$ than did the dystrophic cells. Such differences in NE-induced respiration were not observed at 0.1 $\mu M$ NE (Table 1). These data demonstrate that on a per-cell-basis, the respiratory response of normal cells to 1 $\mu M$ NE [a concentration that elicits maximal rates of oxygen consumption in these normal cells (5)] is considerably greater than that of dystrophic cells.

The lower response of dystrophic cells could reflect a number of factors. One possibility is that these dystrophic cells have a decreased number of $\beta$-adrenergic receptors mediating the thermogenic response to NE. This, however, does not appear to be the case (9). An alternate explanation for the lower $\dot{V}O_2$ values is that the dystrophic cells have a decreased capacity to respire as a result of a decreased complement of oxidative "machinery." In support of this possibility is our observation that the brown adipocytes from the normal hamsters appeared to be considerably larger than those from the dystrophic hamsters when viewed microscopically during counting of the isolated cells. That this size difference could be accompanied by differences in the amount of oxidative enzymes rather than simply differences in the amount of stored fat is indicated by the significantly lower amount of protein present per cell (Table II). In view of this decrease (about 55%), a quantitative measure of oxidative capacity was desired. To this end, citrate synthase activities of the isolated cells were measured. Citrate synthase operates in the TCA cycle, and its maximal activity is generally considered to be proportional to the maximal flux through the cycle and, at least in some systems, to the oxidative potential of the cells [see (10) for discussion and references].

Citrate synthase activity per cell was indeed significantly less in the isolated dystrophic cells

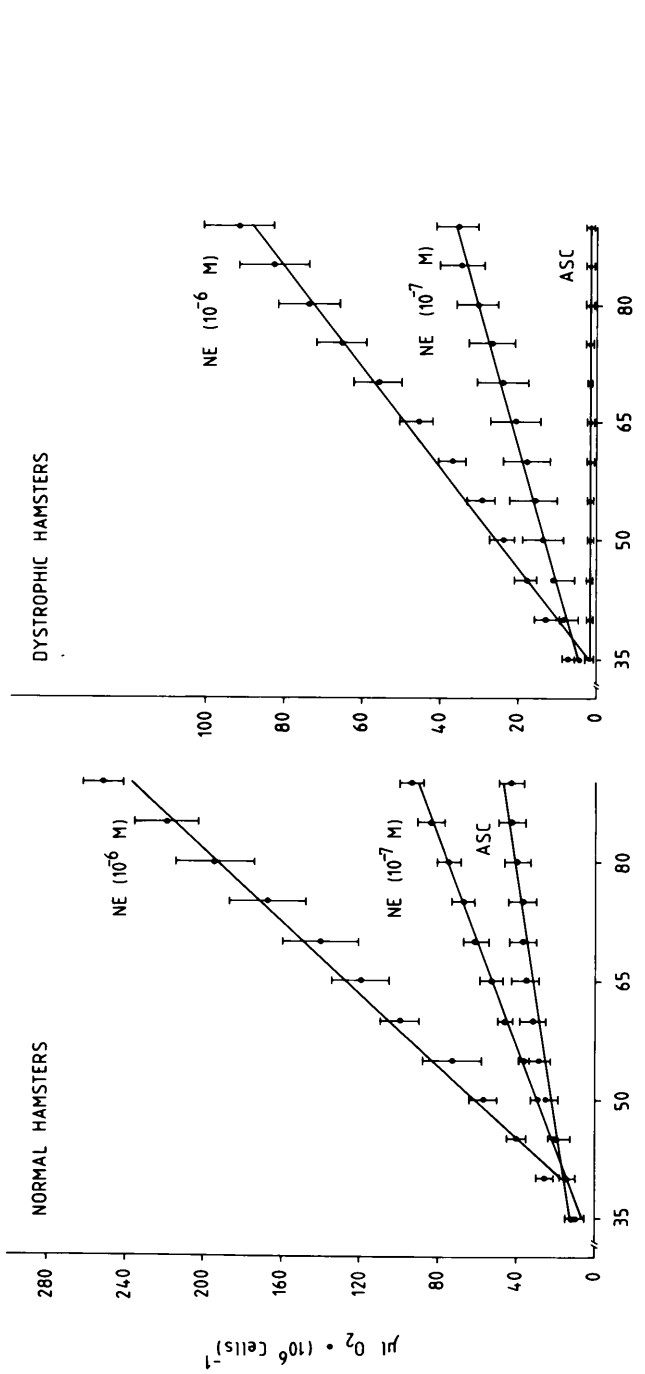


FIG. 1. Oxygen consumption of brown adipocytes isolated from normal and dystrophic BIO 14.6 hamsters. The "Normal Hamsters" graph illustrates the temporal response of one preparation of brown adipocytes, isolated from tissue pooled from two normal animals; the "Dystrophic Hamsters" graph represents the response of one preparation isolated from tissue pooled from three dystrophic hamsters. Note the different ordinate scales for the two plots. The average of three flasks ±1 standard error are presented. In some cases, the error was small enough to be contained within the symbol designating the average value. Each line represents the best fit for the data as determined by linear regression. These graphs indicate that the measured respiratory responses with norepinephrine (NE) as well as without NE (i.e., ascorbate, ASC, controls) are linear over the 90-min reaction period. Because approximately 30 min after the flasks were attached to the respirometer was required for stabilization of the apparatus, the time scale in the graphs was begun at 35 min.

TABLE I. EFFECT OF NOREPINEPHRINE (NE) ON THE RATES OF OXYGEN CONSUMPTION ($\dot{V}O_2$) OF BROWN ADIPOCYTES ISOLATED FROM NORMAL AND DYSTROPHIC HAMSTERS

| | Absolute $\mu$l $O_2/10^6$ cells · hr | | | NE-induced $\mu$l $O_2/10^6$ cells · hr | |
|---|---|---|---|---|---|
| | 0 | 1 $\mu M$ | 0.1 $\mu M$ | 1 $\mu M$ | 0.1 $\mu M$ |
| Normal | 43.12 ± 6.73 ($n$ = 12) | 184.95 ± 12.94 ($n$ = 12) | 90.0 ± 6.23 ($n$ = 11) | 142.20 ± 16.56 ($n$ = 12) | 40.77 ± 7.97 ($n$ = 11) |
| Dystrophic | 11.18 ± 3.90 ($n$ = 9) | 107.86 ± 17.66 ($n$ = 9) | 46.55 ± 4.77 ($n$ = 9) | 96.69 ± 17.55 ($n$ = 9) | 33.72 ± 2.09 ($n$ = 9) |
| $P$ ($H_0$:normal = dystrophic) | 0.05 | 0.05 | 0.05 | 0.05 | NSD |

*Note.* Values of $\dot{V}O_2$ represent $\bar{X}$ ± SE for respiration measured during the 60- to 90-min period after addition of NE (or vehicle). NE-induced respiration = $\dot{V}O_2$ in the presence of NE minus $\dot{V}O_2$ in the absence of NE. The numbers in ( ) represent the number of cell preparations examined.

(Table II). This was true whether the results were expressed per $10^6$ intact cells, per milligram of protein, or per $10^6$ "total" cells ("total" cells being those that were intact plus those with slightly damaged plasma membranes—as determined microscopically). Thus, the brown adipocytes isolated from the BIO 14.6 dystrophic hamsters appear to have a diminished capacity for TCA cycle activity, a reduction that can only be partially explained by their smaller size (since citrate synthase activity per milligram protein is significantly less in the dystrophic cells).

Interestingly, even though the dystrophic adipocytes exhibited a reduced oxidative response when exposed to NE (i.e., microliters $O_2/10^6$ cells), their NE-induced rates of oxygen consumption expressed per milligram protein or per unit of citrate synthase activity were actually higher than were those of the normal cells. That is, NE-induced respiration was not diminished to the same degree as was maximal citrate synthase activity or protein content in the dystrophic cells. This differential effect on respiration and citrate synthase activity was unexpected in light of the correlation between activity of this enzyme and oxidative capacity in other systems [see reference (10)]. The significance of this differential effect is not yet clear.

In summary, the findings of the present study are consistent with the view that the reduced nonshivering thermogenic response of the intact BIO 14.6 hamster to administered catecholamne (1) reflects changes in the individual brown adipocytes as well as a reduction in the total amount of brown fat tissue present. Among the differences noted in the individual brown adipocytes were decreases in cell size, decreases in oxidative en-

TABLE II. CITRATE SYNTHASE ACTIVITY (MICROMOLES SUBSTRATE CONVERTED) OF ISOLATED BROWN FAT CELLS FROM NORMAL AND DYSTROPHIC HAMSTERS

| | $\mu$mol/min · $10^6$ cells | | $\mu$mol/min · mg prot. |
|---|---|---|---|
| | Intact cells | Total cells | |
| Normal | 20.32 ± 3.42 ($n$ = 6) | 13.57 ± 2.39 (n = 5) | 1.362 ± 0.069 ($n$ = 5) |
| Dystrophic | 4.84 ± 0.48 ($n$ = 6) | 3.21 ± 0.38 ($n$ = 6) | 0.710 ± 0.030 ($n$ = 6) |
| $P$ ($H_0$:normal = dystrophic) | 0.01 | 0.01 | 0.001 |

*Note.* Values of enzyme activity represent $\bar{X}$ ± SE. Total cells include those that were intact plus those with slightly damaged cell membranes when viewed with the light microscope. Protein content averaged ($\bar{X}$ ± SE) 10.0 ± 1.86 mg/$10^6$ total cells (from normal hamsters) and 4.52 ± 0.45 mg/$10^6$ total cells (from dystrophic hamsters).

zyme content, and an attenuated thermogenic response to a maximum concentration of NE.

1. Horwitz BA, Hanes GE. Isoproterenol-induced calorigenesis of dystrophic and normal hamsters. Proc Soc Exp Biol Med **147**:392–395, 1974.
2. Foster DO, Frydman ML. Nonshivering thermogenesis in the rat. II. Measurements of blood flow with microspheres point to brown adipose tissue as the dominant site of the calorigenesis induced by norepinephrine, Canad J Physiol Pharmacol **56**:110–122, 1978.
3. Wickler SJ, Horwitz BA, Horowitz JM. Metabolic organization of muscle and brown fat in normal and dystrophic hamsters. Fed Proc **40**:518, 1981.
4. Himms-Hagen J, Gwilliam C. Abnormal brown adipose tissue in hamsters with muscular dystrophy. Amer J Physiol **239**:C18–C22, 1980.
5. O'Donnell ME, Horwitz BA. Effects of butyrate on ouabain-sensitive respiration of hamster brown adipocytes. Amer J Physiol **242**:C46–C51, 1982.
6. Bass AD, Brdiczka D, Eyer P, Hofer S, Pette D. Metabolic differentiation of distinct muscle types at the level of enzymatic organization. Eur J Biochem **10**:198–206, 1969.
7. Srere PA. Citrate synthase. In: Lowenstein JM, ed. Methods in Enzymology. New York, Academic Press, Vol 13:p3, 1969.
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
9. Cornett LE, Horwitz BA, Roberts JM. $\beta$-adrenergic receptors in brown fat from dystrophic and normal hamsters. Fed Proc **40**:254, 1981.
10. Wickler SJ. Seasonal changes in enzymes of aerobic heat production in the white-footed mouse. Amer J Physiol **240**:R289–R294, 1981.

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