Cyclic Adenosine Monophosphate Accumulation and β-Adrenergic Binding in Unweighted and Denervated Rat Soleus Muscle

Christopher R. Kirby, Christopher R. Woodman, Dale Woolridge, and Marc E. Tischler

Unweighting, but not denervation, of muscle reportedly “spares” insulin receptors, increasing insulin sensitivity. Unweighting also increases β-adrenergic responses of carbohydrate metabolism. These differential characteristics were studied further by comparing cyclic adenosine monophosphate (cAMP) accumulation and β-adrenergic binding in normal and 3-day unweighted or denervated soleus muscle. Submaximal amounts of isoproterenol, a β-agonist, increased cAMP accumulation in vitro and in vivo (by intramuscular [IM] injection) to a greater degree (P < .05) in unweighted muscles. Forskolin or maximal isoproterenol had similar in vitro effects in all muscles, suggesting increased β-adrenergic sensitivity following unweighting. Increased sensitivity was confirmed by a greater receptor density (B_max) for [125I]iodo-(−)-pindolol in particulate preparations of unweighted (420 ± 10−18 mol/mg muscle) than of control or denervated muscles (285 ± 10−18 mol/mg muscle). The three dissociation constant (K_d) values were similar (20.3 to 25.8 pmol/L). Total binding capacity (11.4 fmol/muscle) did not change during 3 days of unweighting, but diminished by 30% with denervation. This result illustrates the “sparing” and loss of receptors, respectively, in these two atrophy models. In diabetic animals, IM injection of insulin diminished cAMP accumulation in the presence of theophylline in unweighted muscle (−66% ± 2%) more than in controls (−42% ± 6%, P < .001). These results show that insulin affects cAMP formation in muscle, and support a greater in vivo insulin response following unweighting atrophy. These various data support a role for lysosomal proteolysis in denervation, but not in unweighting, atrophy.

From the Departments of Biochemistry and Physiology, College of Medicine, University of Arizona, Tucson, AZ. Supported by National Aeronautics and Space Administration (NASA) Grant No. NAG2-384 (to M.E.T.), NASA Graduate Researcher Program award (to C.R.K.), National Institutes of Health Training Grants No. NS-07309 and No. HL-07249 to the Department of Physiology, and Biomedical Research Support Grant No. RR-05675 to the College of Medicine.

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bovine insulin/mL. Muscles were then transferred for 10 minutes to fresh Krebs-Ringer bicarbonate buffer containing 5 mmol/L glucose, 25 mmol/L theophylline (to inhibit phosphodiesterase), 1.5% bovine serum albumin (fatty acid-free), and 10 μL bovine insulin/mL with or without isoproterenol or forskolin (as indicated in the figures and tables). Insulin was included during incubations to duplicate conditions used in previous studies that demonstrated an increased isoproterenol response of glycogen metabolism in unweighted soleus muscle.4

Following incubation, muscles were blotted, frozen in liquid nitrogen, and homogenized in a Duall tube containing 0.5 mL acidic ethanol (1 mol/L HCl:ethanol, 1:100). Homogenates were transferred to Eppendorf tubes and centrifuged at 12,000 × g for 15 minutes. The supernatant solution was saved, and the pellet was washed with 0.5 mL ethanol:water (2:1) and centrifuged for an additional 10 minutes. Supernatants were combined and evaporated to dryness under a stream of nitrogen at 55°C. The residue was dissolved in 50 mmol/L TRIS and 4 mmol/L EDTA buffer (pH 7.5), and then frozen at −20°C until assayed. The volume of buffer (0.1 to 2.0 mL) for dissolving the residue was selected so that a 50-μL aliquot of the sample would fall within the range of maximum sensitivity (0.5 to 4.0 pmol) for the cAMP assay. cAMP was assayed using a commercial protein-binding kit (Amersham, Arlington Heights, IL). Except for bovine insulin (Calbiochem, San Diego, CA), chemicals were obtained from Sigma.

Intramuscular Injections

Animals were injected intramuscularly (IM) as described previously14 and adapted from Gerard et al.14 Rats were tranquilized with Innovar-Vet. Both hindlimbs were shaved and the skin was swabbed with ethanol. An incision was made through the outside of the leg, and a curved blunt forceps was used to hook the soleus muscle. Then, 0.9% saline containing theophylline (62.5 mmol/L) was injected into the left muscle. The injection for the right muscle also included either isoproterenol (2.5 μmol/L) or insulin (10 μL/mL). After 20 minutes, the muscles were excised and frozen in liquid nitrogen. Muscles were homogenized and cAMP content was determined as described above.

Hormone-Binding Study

Particulate preparations were obtained from muscles frozen in liquid nitrogen as described by Ligget et al.15 Muscles were minced in ice-cold buffer (10 mmol/L TRIS, 5 mmol/L EDTA, pH 7.4) and homogenized in 20 volumes of the same buffer with a Polytron P10 (Brinkman Insts, Westbury, NY) at maximum speed for three 10-second bursts. Homogenates were filtered over nylon mesh (1 mm2) and centrifuged at 37,000 × g for 20 minutes at 4°C. The pellet was resuspended in the same buffer and washed twice using similar centrifugations. Final suspensions (~7 mg muscle wet weight/mL) were in incubation buffer (75 mmol/L TRIS, 25 mmol/L MgCl2, 5 mmol/L EDTA, pH 7.4).

For the binding assay, preparations (100 μL) were incubated for 60 minutes at 25°C with [125I]iodo(-)-pindolol (2,200 Ci/mmol; New England Nuclear, Boston, MA) in a final volume of 150 μL incubation buffer.15 The reaction was terminated by adding 10 mL ice-cold incubation buffer and vacuum filtering through a Whatman GF/C glass fiber filter (Whatman International, Maidstone, England). Filters were washed with an additional 30 mL incubation buffer, and bound radioactivity was measured in a gamma-counter. Non-specific binding was determined by linear regression of binding that occurred in the presence of 1 μmol L-propranolol. Specific binding was calculated as the difference between total and non-specific binding.

Data Analysis

Receptor densities (Bmax) and apparent dissociation constants (Kd) were estimated by multiple iterative nonlinear analysis of saturation binding data using the computer program, LIGAND (Elsevier-Biosoft, Cambridge, UK). Specific binding expressed per milligram muscle or per whole muscle was calculated using the total wet weight of tissue or total number of muscles represented by the 100-μL particulate preparation used in the saturation binding experiments. Testing for significant differences between means (P < .05) was done by a paired Student's t test or by factorial ANOVA with a post hoc Scheffe F test or Fisher exact probability test. Differences in percent effects of isoproterenol or insulin injections between groups were analyzed by the Mann-Whitney U test. All results are expressed as means ± SE for the number of muscles indicated in each table or figure.

RESULTS

Muscle and Body Masses

Weight-bearing (control) animals weighed approximately 15 g less initially than unweighted and denervated animals (Table 1), so that final soleus muscle mass would be more closely matched for incubations. Masses of muscles used for cAMP determinations were similar in weight-bearing and denervated muscles, while those from unweighted animals were slightly (6%) smaller. Similar final muscle size diminished the possibility of different diffusion distances. Unweighted animals gained less than weight-bearing or denervated animals. Since food consumption is similar in unweighted and weight-bearing animals,11 this weight-gain difference is likely due to the mild stress effects associated with tail-cast suspension.15 In both cAMP accumulation and hormone-binding experiments (not shown), the ratio of muscle to body mass, an index of muscle atrophy, was less in unweighted and denervated animals than in control animals.

cAMP Accumulation In Vitro

One potential mechanism for the greater effects of isoproterenol on glycogen metabolism in unweighted muscle could be postreceptor alterations, such as in adenylate cyclase activity. Therefore, we measured accumulation of cAMP (in the presence of theophylline) following incubation with or without forskolin, which activates adenylate cyclase independent of the β-adrenergic receptor.16 The only difference detected in basal cAMP accumulation was a lower (P < .05) amount in unweighted than in denervated muscle (Table 2). Forskolin treatment increased cAMP.

Table 1. Muscle and Body Masses of Animals Used for cAMP Determinations

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Body Mass (g)</th>
<th>Soleus Mass (mg)</th>
<th>Soleus-Body (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>61.7 ± 1.0</td>
<td>76.2 ± 1.1</td>
<td>0.383 ± 0.009</td>
</tr>
<tr>
<td>Unweighted</td>
<td>75.1 ± 1.1*</td>
<td>86.2 ± 1.1†</td>
<td>28.1 ± 0.4*†</td>
</tr>
<tr>
<td>Denervated</td>
<td>76.2 ± 1.0</td>
<td>91.6 ± 1.0</td>
<td>28.6 ± 0.4</td>
</tr>
</tbody>
</table>

NOTE. Values are means ± SE for 42 to 47 animals.

*P < .05 unweighted versus denervated vs weight-bearing by ANOVA.

†P < .05 unweighted versus denervated by ANOVA.
accumulation in a dose-dependent manner in all conditions. A maximal effect was achieved at 0.5 mmol/L. Accumulation of cAMP in normal muscle did not differ from that in unweighted or denervated muscles at all forskolin concentrations tested. However, at the higher concentrations of forskolin, denervated muscle accumulated more cAMP than did unweighted muscle. These results suggest that the site of enhanced isoproterenol response in the unweighted soleus is likely proximal to the adenylate cyclase catalytic subunit in the β-adrenergic receptor-effector cascade.

Isoproterenol stimulated cAMP accumulation in a dose-dependent fashion in all three muscles (Fig 1). In accordance with an enhanced isoproterenol response of glycogen metabolism, cAMP accumulation was markedly greater in unweighted than in weight-bearing or denervated muscles. Weight-bearing and denervated muscles showed similar responses. These differences in cAMP accumulation could not be attributed to variable muscle integrity, as cAMP in the medium was below the detectable (0.2 pmol) limit under all conditions. These data suggest an enhanced isoproterenol sensitivity of cAMP accumulation in unweighted relative to weight-bearing muscles.

cAMP Accumulation In Vivo

To evaluate the β-adrenergic response of cAMP accumulation in vivo, soleus muscles were injected with 0.9% saline containing theophylline with or without isoproterenol (Table 3). In the absence of isoproterenol, cAMP accumulation was lower (−46%) in unweighted than in weight-bearing muscles. Injection of isoproterenol elevated muscle cAMP content in both conditions. In agreement with in vitro results, both the absolute and percent increases of cAMP accumulation were greater in unweighted compared to weight-bearing muscles.

A proportionately smaller solution volume had been injected into the unweighted muscles than into the weight-bearing muscles. Even so, it was possible that the differential responses, in part, were a result of a difference in isoproterenol concentration in the muscle. To estimate the approximate agonist amount present, we used the percent in vivo responses from Table 3 and the in vitro dose curve in Fig 1 to predict the approximate average isoproterenol concentration in these muscles. For both control and unweighted muscles, a concentration of 0.24 μmol/L was estimated. Thus, the difference reported in Table 3 was characteristic of the muscles, and was not a consequence of some large difference in agonist concentration.

In vivo basal cAMP accumulation was significantly less in unweighted muscle (Table 3). Since the insulin sensitivity of unweighted soleus is increased and insulin reportedly reduces cAMP levels in some tissues, we tested in vivo whether insulin effects could account for the basal difference in cAMP accumulation. Thus, unweighted or weight-bearing muscles of diabetic animals were injected with theophylline with or without insulin (Table 4). Diabetic animals were used to abolish the potential influence of circulating insulin on muscle cAMP levels. Elimination of insulin abolished the difference in cAMP accumulation detected in the absence of isoproterenol (Table 3). Injection of insulin significantly reduced the accumulation of cAMP in both conditions, but produced an even greater

<p>| Table 2. Effect of Forskolin on cAMP Accumulation in Vitro |</p>
<table>
<thead>
<tr>
<th>Forskolin (mmol/L)</th>
<th>Normal</th>
<th>Unweighted</th>
<th>Denervated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.6 ± 0.6</td>
<td>7.2 ± 0.5*</td>
<td>9.9 ± 0.6</td>
</tr>
<tr>
<td>0.1</td>
<td>81.4 ± 7.01</td>
<td>81.6 ± 5.3</td>
<td>73.0 ± 6.4</td>
</tr>
<tr>
<td>0.3</td>
<td>137.0 ± 12.5</td>
<td>138.3 ± 6.6</td>
<td>136.7 ± 11.6</td>
</tr>
<tr>
<td>0.5</td>
<td>169.4 ± 16.2</td>
<td>173.8 ± 7.4</td>
<td>192.9 ± 16.9</td>
</tr>
<tr>
<td>1.0</td>
<td>178.9 ± 1.81</td>
<td>142.8 ± 7.0</td>
<td>215.2 ± 34.0</td>
</tr>
</tbody>
</table>

NOTE. Muscles were incubated as described in the Methods with 10 μU insulin/mL and the forskolin concentration indicated. cAMP accumulation was also measured as described in the Methods. Values are means ± SE for 4 to 10 muscles.
*P < .05 unweighted versus denervated by ANOVA.
†P < .05 forskolin versus no forskolin by ANOVA.

<p>| Table 3. Effect of Isoproterenol on cAMP Accumulation In Vivo |</p>
<table>
<thead>
<tr>
<th>Muscle</th>
<th>Amount of cAMP (pmol/muscle)</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight-bearing</td>
<td>10.0 ± 1.7</td>
<td>40.9 ± 3.1*</td>
</tr>
<tr>
<td>Unweighted</td>
<td>5.4 ± 0.5†</td>
<td>53.7 ± 4.1†</td>
</tr>
</tbody>
</table>

NOTE. Contralateral soleus muscles in eight weight-bearing or hindlimb-suspended animals were injected with theophylline with or without isoproterenol as described in the Methods. Weight-bearing muscles were injected with 4.0 μL/100 g body weight, but unweighted muscles were injected with only 3.2 μL/100 g body weight to account for muscle size differences owing to atrophy caused by unweighting. These volumes are based on average soleus muscle sizes of 40 and 32 mg/100 g body weight for weight-bearing and suspended animals, respectively. After 20 minutes, the muscles were excised and immediately processed for analysis of cAMP accumulation as described in the Methods. Results are means ± SE.
*P < .001 isoproterenol versus no isoproterenol by ANOVA or paired t test.
†P < .05 unweighted versus weight-bearing by ANOVA.
‡P < .001 unweighted versus weight-bearing by Mann-Whitney.
Table 4. Effect of Insulin on cAMP Accumulation In Vivo

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Amount of cAMP (pmol/muscle)</th>
<th>Decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Insulin</td>
<td>With Insulin</td>
</tr>
<tr>
<td>Weight-bearing</td>
<td>9.4 ± 0.8</td>
<td>5.3 ± 0.4*</td>
</tr>
<tr>
<td>Unweighted</td>
<td>11.3 ± 1.2</td>
<td>3.8 ± 0.4*</td>
</tr>
</tbody>
</table>

NOTE. Muscles from diabetic rats were injected with theophylline with or without insulin, as in Table 3. Weight-bearing muscles were injected with 3.7 μL/100 g body weight, and unweighted muscles were injected with 2.7 μL/100 g body weight. Volumes are based on average soleus muscle sizes of 37 and 27 mg/100 g body weight for weight-bearing and suspended diabetic animals, respectively. Results are means ± SE.

*P < .01 insulin versus no insulin by ANOVA or paired t test.

Effect in the unweighted muscle, thereby restoring a significant difference, as between muscles of nondiabetic animals (see Tables 3 and 4).

β-Adrenergic Binding

To distinguish between altered hormone sensitivity and responsiveness, we measured the binding capacity of the membrane receptor. [125I]iodo-(-)-pindolol saturation binding was measured with particulate preparations from weight-bearing, unweighted, and denervated muscles. Binding appeared saturable and could be inhibited by 1 μmol L-propranolol. Specific binding occurred in “zone A” (ie, <10% of total ligand bound) and represented between 70% to 90% of total maximal binding (Fig 2). Scatchard analysis of binding data demonstrated similar receptor affinity (Kd) for weight-bearing (20.7 ± 1.9 pmol/L), unweighted (25.8 ± 3.3 pmol/L), and denervated (20.3 ± 2.1 pmol/L) muscles (Fig 3). These values agree with the Kd (19.5 pmol/L) in [125I]iodo-(-)-pindolol binding in human skeletal muscle. Maximal binding capacity per mg muscle was markedly greater in the unweighted soleus compared with the weight-bearing and denervated muscles that yielded similar results (Fig 3). When expressed relative to the whole muscle, maximal binding capacity was lower in denervated (7.8 ± 1.1 fmol/muscle) than in weight-bearing (11.2 ± 0.8 fmol/muscle) or unweighted (11.5 ± 1.2 fmol/muscle) muscles. Similar general findings were obtained for binding of [3H]dihydroalprenolol to membrane preparations from these muscles. These results suggest that the increase in β-receptor number per mg muscle in unweighted soleus must be mostly a consequence of muscle atrophy and not of an increase in the total receptor population. The reduction in total β-receptors of denervated muscle suggests that the loss of this membrane protein parallels decreases in structural proteins.

DISCUSSION

Receptor and Postreceptor Stimulation of cAMP Accumulation

Hormone effects can be characterized by altered sensitivity or responsiveness representing receptor or postreceptor modifications, respectively. Investigation herein of receptor and postreceptor stimulation of the β-adrenergic receptor-effector cascade supported our previous studies that suggested greater β-adrenergic sensitivity following unweighting. Whether via incubation (Fig 1) or IM injection (Table 3), submaximal amounts of isoproterenol increased cAMP accumulation more so in unweighted muscle. Similar responses to maximal amounts of isoproterenol increased cAMP accumulation more so in unweighted muscle. In accordance with enhanced sensitivity, this concept is further supported by the comparable postreceptor stimulation by forskolin of cAMP accumulation in unweighted and weight-bearing muscles (Table 2). Thus, increased responsiveness of adenylate cyclase cannot account for enhanced β-adrenergic effects in unweighted muscle.

These results cannot exclude enhanced G-protein–complex coupling between the receptor and adenylate
accumulation is altered 3 days after denervating the soleus receptor- nor postreceptor-mediated stimulation of cAMP. Results from the current study clearly indicate that neither vated muscle insulin resistance of carbohydrate metabolism in dener-

enol effects in unweighted or dcnervated muscles. The

amounts of insulin to assess insulin antagonism of isoproter-

enous responses between weight-bearing and un-

control and suspended animals. Therefore, differences in

responses in hormone responses between weight-bearing and un-

weighted soleus muscles are not likely due to a systemic

alteration.

A possible role of systemic effects in altered hormone responses of unweighted versus weight-bearing muscles has been evaluated by examining insulin and isoproterenol responses of the extensor digitorum longus, a hindlimb muscle unaffected by hindlimb unweighting. Insulin stimulation of glucose transport1,3 and isoproterenol stimulation of lactate production4 were similar in this muscle from control and suspended animals. Therefore, differences in hormone responses between weight-bearing and un-

weighted soleus muscles are not likely due to a systemic

alteration.

In our earlier study,4 incubations contained physiological amounts of insulin to assess insulin antagonism of isoproter-

cyclase. For instance, decreased isoproterenol response of cardiac muscle from adrenalectomized animals occurs with-

out altered β-receptor density or affinity.24 Instead, the lack of glucocorticoids may alter β-adrenergic responses at a postreceptor site. As dexamethasone treatment reverses reductions in the G_{βγ}-protein subunit mRNA of adipocytes from adrenalectomized rats and increases this mRNA in normal animals, glucocorticoids may modulate β-adrener-

gic receptor-effector coupling.25 The several-fold increases of plasma glucocorticoids17 and soleus glucocorticoid recep-

tors28 following unweighting could possibly alter the β-ad-

renergic receptor-effector cascade. Further studies are

needed to evaluate this possibility.

The inability of previous investigators to detect insulin effects on muscle cAMP metabolism could be due to the

absence of phosphodiesterase inhibitors in those studies.35,36 Furthermore, the use of muscles from diabetic

animals may allow detection of small differences in cAMP accumulation due to insulin. While the physiologic significa-

cence of these responses remains to be determined, small

changes in skeletal muscle cAMP levels by insulin could possibly result in large changes in cellular metabolism

through amplification.

In accordance with the concept that altered hormone sensitivity is a receptor-mediated phenomenon,23 β-adrener-

gic binding capacity increased during unweighting atro-

phy (Fig 3). Just as increased effects of insulin paralleled greater insulin binding capacity in unweighted soleus,1,29 enhanced isoproterenol effects in unweighted muscle can be attributed to increased β-adrenergic-receptor concentration. Mechanisms for increased β-receptor density may include: (1) up-regulation due to reduced circulating cate-

cholamines37; (2) changes in plasma glucocorticoids, which induce β-receptor expression37; or (3) sparing of membrane

receptors during unweighting atrophy.1 Catecholamine-

induced up-regulation of the β-receptor seems unlikely, as plasma catecholamines increase during the first several
days of suspension.31 As we did not detect an increase in the total receptor population, it is not likely that increased plasma glucocorticoids17 induced β-receptor expression during unweighting. However, these data cannot exclude a

role for glucocorticoids in maintaining β-receptors during unweighting atrophy. Just as for insulin receptors,1 there

are a similar number of β-receptors per whole unweighted

or weight-bearing muscle. Thus, the increase in receptor
density per mg muscle must result from preferential loss of structural proteins rather than from up-regulation. It is

noteworthy that the percent increase in β-adrenergic recep-
tors (46%) (Fig 3) and insulin receptors (50%)1 agree.

Isoproterenol responses of cAMP accumulation (Fig 1) and [125I]iodo-(-)-pindolol binding capacity per milligram

muscle (Fig 3) were similar in denervated and innervated soleus muscles. The reduced total binding capacity (fmol/
muscle) following denervation suggests that receptor and nonreceptor proteins are lost proportionately, thus preserv-

ing a receptor density comparable to innervated muscle.

This constancy of β-adrenergic binding capacity was also
evident in a mixed hindlimb muscle membrane preparation

In situ insulin treatment diminished skeletal muscle cAMP levels in previous studies.35,36 In this investigation, an effect of intracellular insulin mediators on phosphodiesterase activity was unlikely, as theophylline was always present. However, these results are consistent with insulin antagon-

ism of isoproterenol-stimulated lactate formation in muscle.4,37 While these findings support a response of cAMP metabolism to insulin in muscle, they cannot exclude possible insulin effects at sites other than adenylate cyclase. For example, insulin inhibition of cAMP-dependent protein kinase activity36 could also explain the diminished formation of lactate.

β-Adrenergic Binding Capacity in Atrophic Soleus

In previous studies reported increased effects of insulin on carbohydrate and protein metabolism in unweighted mus-

cle.1,7,25 Additionally, the greater insulin sensitivity of un-

weighted muscle leads to a lower accumulation of cAMP (Table 4). To our knowledge, this is the first investigation to demonstrate a reduced accumulation of cAMP in skeletal muscle following insulin treatment.

Several investigations have demonstrated an increased production of intracellular insulin mediators following insulin treatment of skeletal muscle.30-32 These mediators increase the activity of low-Km phosphodiesterase and decrease adenylate cyclase activity in adipocyte and hepatocyte membranes.32,34 However, neither in vitro, in vivo, nor
following 5 days of denervation.\textsuperscript{28} This similar β-adrenergic binding capacity in innervated and denervated muscles parallels their similar insulin binding capacity.\textsuperscript{4} These results support the concept that, even though both unweighted and denervated muscles undergo atrophy, certain hormone responses and receptor binding capacities differ distinctly in these models of reduced use.

Mechanisms of Proteolysis in Unweighted and Denervated Soleus

A principal goal of studies from our laboratory has been to evaluate the possibility of different mechanisms of proteolysis in unweighted and denervated soleus muscles.\textsuperscript{11} Recent evidence suggests that membrane receptors may be degraded primarily through lysosomal proteolysis.\textsuperscript{8,10} Thus, sparing of insulin receptors in unweighted,\textsuperscript{1} but not in denervated,\textsuperscript{4} muscle supports the idea that lysosomal proteolysis plays a greater role in denervation than in unweighting atrophy. Accordingly, IM injection of chloroquine, a lysosomotropic agent, diminished atrophy and in vivo proteolysis of the denervated, but not of the unweighted, soleus muscle.\textsuperscript{11} The finding here of increased β-adrenergic receptor density with unweighting, but not with denervation, atrophy also supports this hypothesis.

REFERENCES


