We have compared the properties of β-adrenergic receptors in slow-twitch, oxidative skeletal muscles (soleus) from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats at three different ages. The investigation was based on the hypothesis that the increase in Na⁺ content and decrease in fatigue resistance observed previously in the soleus of SHR might be the result of a down regulation of muscle β-receptors. Activation of β-adrenergic receptors in skeletal muscle stimulates sarcolemmal sodium-potassium adenosine triphosphatase, which produces an efflux of Na⁺ and an influx of K⁺. Receptor down-regulation would be expected to reduce hormonal stimulation of Na⁺ pump activity, particularly during exercise. The results of receptor binding studies, however, and an investigation of cyclic adenosine monophosphate (cAMP) production in response to applied epinephrine indicated that there were no significant differences in receptor properties in the soleus muscles of SHR and WKY rats. Receptor number and affinity were the same in the two strains, and the rate, magnitude, and duration of the increase in cAMP in response to 10⁻⁶ M epinephrine were also similar. β-Adrenergic receptor down-regulation does not appear to be a generalized phenomenon in tissues of SHR, despite the appearance of other physiological changes in the tissue. (Hypertension 1989;14:54-60)
Hypertension has been shown to be associated with a "down-regulation" of β-receptors in cardiac muscle, leading to a reduction in the catecholamine-stimulated positive inotropic response.\(^9,11\) \(β\)-Receptor down-regulation is also purported to occur in vascular smooth muscles in hypertensive animals.\(^9\) In the present study, we tested the possibility that a progressive, age-related decrease in sarcolemmal \(β\)-receptor number, affinity, or coupling to adenyl cyclase could be responsible for the age-related decrease in soleus fatigue resistance in SHR by limiting \(Na^+\) pump activation during exercise. A newly described muscle slice preparation\(^12\) was used to determine the specific binding of the \(β\)-adrenergic antagonist \([3H]CGP12177\) to soleus muscles from SHR and normotensive WKY rats at three different ages. The slice preparation was also used to assess coupling between \(β\)-receptors and sarcolemmal adenylate cyclase in the two strains at two different ages.

Materials and Methods

Preparation

Male SHR and WKY rats were used in the experiments. The rats were purchased from Taconic Farms, Germantown, New York, or were bred and raised in our own colony. Rats from each strain were divided into the following three age groups: 1) young (6–8 weeks), 2) adult (16–18 weeks), and 3) old (24–28 weeks). These age groups were chosen to represent a time when blood pressure was increasing, the time at which peak pressure was attained, and a time at which pressure was high but stable.

Rats were housed individually in standard plastic cage and had access to food and water ad libitum. They were maintained under a 12-hour light/dark cycle at 18°C. Each rat was weighed just before muscle removal, and arterial blood pressure was recorded in the conscious animal by means of the tail-cuff occlusion technique. The rat was then anesthetized by an injection of sodium pentobarbital (35–40 mg/kg i.p.), and both soleus muscles were isolated and removed. The rat was killed by an overdose of anesthetic after muscle removal.

Muscle Slice Preparation

Soleus \(β\)-receptors were characterized with the muscle slice technique introduced by Watson-Wright and Wilkinson.\(^12\) Isolated soleus muscles were placed in ice-cold, oxygenated Dulbecco's phosphate-buffered saline (DPBS), pH 7.4, and trimmed of superficial nerves, blood vessels, and connective tissue. Each soleus was split in half longitudinally to generate more slices per muscle. The divided muscles were placed on the stage of a Mcllwain tissue chopper (Brinkmann Instrs., Inc., Westbury, New York), and 1-mm thick transverse slices were prepared. Each slice was placed in the well of a tissue culture plate (24 wells/plate) containing 0.54 ml DPBS.

\(β\)-Adrenergic Receptor Assay

A tritiated, hydrophilic \(β\)-adrenergic receptor antagonist, 4-\(3\text{-tert-}\)butylamino-2-hydroxypropoxy)-5,7-[\(3H\]benzimidazol-2-one (\([3H]\)CGP12177), obtained from New England Nuclear (Boston, Massachusetts), specific activity 49 Ci/mmol, was chosen to determine the density and affinity of skeletal muscle \(β\)-receptors in the muscle slice preparation. Since Watson-Wright and Wilkinson\(^12\) demonstrated that ligand binding is proportional to the wet weight of a muscle slice, each slice was weighed, and the wet weight of tissue in each well was standardized. In general, each well contained one or two slices providing 12±2 mg tissue.

The binding assay was conducted at 30°C for 150 minutes with the muscle slices contained in 0.54 ml DPBS and the required amount of [\(3H\)]CGP12177. Measurement of total binding was performed in triplicate. Nonspecific binding was determined in duplicate by incubating slices with an excess of unlabeled isoproterenol (10⁻⁹ M) and [\(3H\)]CGP12177. Saturation-binding equilibrium assays were performed with ligand concentrations between 0.05 nM and 2.5 nM. At the end of the incubation, slices were washed twice for 10 minutes each time in 0.8 ml ice-cold DPBS to remove unbound ligand. Radioactivity was measured by transferring the muscle tissue in each well to a scintillation vial containing 5 ml Aquasol (New England Nuclear). Radioactivity was counted in a Beckman Scintillation Counter (Beckman Instrs., Inc., Irvine, California) with a [\(3H\)] counting efficiency of 43%. The specific binding of CGP12177 to muscle slices was determined as the difference between total binding and nonspecific binding. Free ligand concentrations were determined by counting 0.1-ml aliquots of the incubation medium.

The specificity of [\(3H\)]CGP12177 binding to \(β\)-receptors was tested by determining the displacement of the ligand in the presence of each of the following compounds: 1) the \(β\)-agonist isoproterenol, 2) nonselective \(β\)-antagonists (−)\(β\)-propranolol and DL-propranolol, 3) the selective \(β\)-antagonist atenolol, and 4) the \(α\)-adrenergic antagonist phentolamine. The concentrations of these compounds ranged between 10⁻⁷ and 10⁻¹⁰ M. The radioligand concentration used was 1.25 nM.

Data Analysis and Statistics for Receptor Binding Assay

Data were obtained from four to five muscles from each strain at each age (four to eight rats/strain/age group) and analyzed by means of the nonlinear regression computer program LIGAND,\(^13\) which determined \(B_{max}\), the number of receptors present, and \(K_d\), the equilibrium dissociation constant. A Hill Plot\(^14\) was also generated, and the Hill coefficient was
determined. Statistical significance was tested by analysis of variance or the unpaired t test.

Measurement of Cyclic Adenosine Monophosphate

The increase in concentration of cyclic adenosine monophosphate (cAMP) in soleus muscle slices generated in response to the introduction of exogenous epinephrine was determined to assess the efficacy of coupling of muscle β-receptors to adenylate cyclase.

Tissue Preparation for Cyclic Adenosine Monophosphate Assay

Rats 6–8 weeks and 24–28 weeks of age were anesthetized with pentobarbital sodium (35–40 mg/kg i.p.). Soleus muscles from both legs were removed and placed in cold, oxygenated (95% O₂ and 5% CO₂) Liley’s solution of the following composition (mM): NaCl 137, KCl 5, CaCl₂·2H₂O 2, MgCl₂·6H₂O 1, NaH₂PO₄ 1, NaHCO₃ 24, and glucose 11.1. The muscles were cut into 1-mm thick slices on the McIlwain tissue chopper, and the slices were allowed to equilibrate for 45 minutes in oxygenated Liley’s solution, pH 7.4, at 34°C.

Individual slices were incubated in one of several concentrations of epinephrine (10⁻⁸–10⁻⁹ M) to determine the dose–response relation. Two to four muscle slices were tested separately at each concentration. Muscle slices and epinephrine were incubated in a total buffer volume of 0.5 ml for 2 minutes. The basal level of cAMP was determined by incubating slices in the absence of the drug. After 2-minute incubation, slices were quickly frozen in 0.8 ml ethanol and HCl (60:1) solution that was cooled on dry ice. The frozen tissue was homogenized, and cAMP was extracted as indicated below.

The time course of the epinephrine-induced generation of cAMP was also determined. The dose–response experiments indicated that 10⁻⁸ M epinephrine produced a maximal increase in muscle cAMP concentration. Therefore, this concentration of epinephrine was used in the time-course experiments. Slices were incubated with epinephrine for the following time intervals (minutes): 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0. Three to six measurements were obtained at each time point. At the end of the incubation period, slices were transferred rapidly to test tubes containing 1 ml cold ethanol and HCl solution and homogenized in a Polytron homogenizer (Brinkmann Instrs., Inc.) at setting 7 for 35–40 seconds. The homogenate was centrifuged at 3,000g for 20 minutes (temperature 2–4°C). The supernatant containing cAMP was decanted to a test tube and evaporated to dryness by heating at
Results

Receptor Binding of CGP12177

The binding characteristics of CGP12177 in soleus muscle slices were similar in both strains and at all three ages studied. Binding was saturable (Figure 1). Nonspecific binding was 22–30% at ligand concentrations between 0.1 and 2.7 nM. The ligand was bound to a single homogeneous population of binding sites as indicated by the linearity of the Scatchard plot (Figure 1, insert). Hill plots gave a Hill coefficient between 0.97 and 1.02, which suggests neither negative nor positive cooperative interaction between β-receptors in hypertensive skeletal muscle.

Competitive displacement experiments were conducted to determine the specificity of [3H]CGP12177 binding to β-receptors. Results are shown in Figure 2. Propranolol and alprenolol, nonselective β-antagonists, and isoproterenol, a β-agonist, competed for binding with [3H]CGP12177 and gave an IC50 of 4.9 × 10^-8 M, 4.9 × 10^-8 M, and 2.5 × 10^-8 M, respectively. The specificity of binding to β-receptors was tested with atenolol, a selective β1-antagonist, and phentolamine, an α-adrenergic antagonist. [3H]CGP12177 binding was not influenced to any appreciable extent by the presence of either compound; thus, a preference for β1-receptor sites was indicated.

Comparison of β-Receptor Density and Affinity in Soleus Muscles of SHR and WKY Rats

Table 1 summarizes the β-receptor binding results for muscles of SHR and WKY rats at the three ages studied. No statistical differences were found between either the density of receptors or the affinity of β-receptor binding in the two strains at any age or between ages in the same strain.

Comparison of Cyclic Adenosine Monophosphate Concentration in Soleus Muscles

Activation of adenylate cyclase is closely associated with hormone binding to β-receptors.16,17 It is possible that aberrant coupling between receptor and enzyme in skeletal muscles from hypertensive animals retards the production of cAMP and the expression of the hormonal effect. This possibility was examined by determining the increase in cAMP concentration, which followed treatment of muscle slices with epinephrine. The results of these experiments are shown in Figures 3 and 4. A concentration of 10^-6 M epinephrine produced the greatest increase in muscle cAMP in both rat strains at the two ages studied (6–8 weeks and 24–28 weeks), as indicated by the dose–response curves (Figures 3 and 4, upper panels). Incubation in 10^-6 M epinephrine also generated an increase in muscle cAMP with the same temporal pattern in both strains (Figures 3 and 4, lower panels). Muscles from 24–28-week-old SHR had a significantly greater (p<0.001) basal concentration of cAMP than did those from WKY rats, but the rate and relative magnitude of the increase in the presence of 10^-8 M
FIGURE 3. Upper panel, dose-response curves of effect of epinephrine on soleus muscle slices from 6-8-week-old SHR (●) and 6-8-week-old WKY rats (▲). Slices were incubated in indicated concentration of epinephrine for 2 minutes and then frozen and assayed for cyclic adenosine monophosphate (cAMP). Values are mean±SEM of muscle slices from two to four rats of each strain. Lower panel, time course of increase in cAMP in muscle slices from 6-8-week-old SHR (●) and 6-8-week-old WKY rats (▲) in presence of $10^{-6}$ M epinephrine. Values are mean±SEM of slices from two to four rats of each strain. Basal concentration of cAMP is indicated by values at 0-minute time point.

FIGURE 4. Upper panel, dose-response curves for response of muscle slices from 24-28-week-old SHR (●) and 24-28-week-old WKY rats to epinephrine (▲). Values are mean±SEM of measurements from four to five muscles from each strain. Lower panel, time course of increase in cyclic adenosine monophosphate (cAMP) in muscle slices from 24-28-week-old SHR (●) and WKY rats (▲) in response to incubation with $10^{-6}$ M epinephrine. Values are mean±SEM of measurements from five to six muscles from each strain. The basal concentration of cAMP in each strain is indicated by values at 0-minute time point.

epinephrine was similar in the two strains. Nonetheless, the greater initial concentration insured that cAMP concentration was higher in the soleus of 24-28-week-old SHR at every time point during the 5-minute incubation with epinephrine. There also appeared to be a tendency of cAMP concentration to decrease sooner in the normotensive muscles.

Discussion

$\beta$-Receptor number, affinity, and coupling to adenylate cyclase were similar in soleus muscles of SHR and WKY rats at each of the three ages studied. Thus, unlike previous observations in cardiac muscle of SHR,9,10 a down-regulation of $\beta$-receptors did not occur in hypertensive slow-twitch skeletal muscle. The one significant difference between the two strains was an increased basal concentration of cAMP in muscles from 24-28-week-old SHR. This finding suggests that there may be some differences in metabolic regulation in hypertensive skeletal muscle, but the increase in cAMP does not appear to be a consequence of alterations in $\beta$-receptor number or hormonal responsiveness.

These observations are dependent on the reliability of the receptor binding assay. The skeletal muscle slice technique, developed by Watson-Wright and Wilkinson,12,13 together with the hydro-
philic β-ligand CGP12177 provides a convenient and accurate method for assessing receptor properties in skeletal muscle. The ligand has been shown to be suitable for the assessment of β-receptors in intact cells and in skeletal muscle slices. Moreover, its hydrophilic nature reduces the probability of its binding to internalized receptors, which removes one potential source of error in receptor-binding assays. The muscle slice technique also offers benefits in terms of ease of measurement and low levels of nonspecific binding (typically less than 30% of total binding at high ligand concentrations), and we have now shown that it is suitable for assessing hormonal activation of adenylate cyclase.

Our initial hypothesis that β-receptor down-regulation, coupled with an increase in Na+ permeability, is responsible for the depression of soleus contractile performance and fatigue resistance in SHR was not confirmed. Receptors did not decline with age, and catecholamine-initiated cAMP production was similar to that in muscles of WKY rats. The apparent stability of β-receptors in the soleus muscle of SHR, despite increasing age and severity of hypertension, contrasts with the age-dependent decrease in density reported in cardiac muscle of SHR. Down-regulation of cardiac β-receptors in SHR, however, has not been noted in every case. The association of changes in basal and hormonally stimulated levels of cAMP with hypertension is also unclear. Levels have been reported to be decreased and increased in vascular smooth muscle, decreased or unchanged in heart, and increased in blood platelets. An involvement of cAMP in the etiology of primary hypertension would appear to be tissue-specific and limited.

Our failure to show any differences in β-receptor affinity, density, or coupling to adenylate cyclase in soleus muscles of SHR at three different ages, however, does not preclude the possibility that genetic hypertension affects the sarcolemmal Na+ pump directly and differentially with age. The Na+ content of slow-twitch soleus muscles is increased by 6 weeks of age in SHR, in contrast to the normal Na+ content found in hypertensive fast-twitch gastrocnemius muscles. With increasing age, soleus Na+ content in SHR remains nearly twice that of age-matched WKY rats whereas intracellular K+ content declines in SHR and is significantly less than that in WKY rats by 28 weeks. Resting membrane potentials are significantly more hyperpolarized in muscles of 6-week-old SHR compared with WKY rats but significantly more depolarized at 24–28 weeks. These observations suggest that skeletal muscle membranes of SHR, like those of vascular smooth muscles, have an increased permeability to Na+ from an early age and that this may initiate an early compensatory increase in Na+,K+-ATPase activity that cannot be sustained in older animals. Our current efforts toward understanding the etiology of membrane-related ionic differences in skeletal muscles from SHR and WKY rats are now directed toward uncovering possible abnormalities in Na+,K+-ATPase structure or function.

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