Cardiac adenosine production in rat genetic models of low and high exercise capacity

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HERITABILITY STUDIES suggest a substantial genetic component to endurance exercise capacity in humans (6, 25). Nevertheless, none of the genes that determine the difference between low and high capacity for endurance exercise has yet been identified. A long-term goal of our laboratory is to define the genetic basis for variation in exercise capacity in mammals. Divergent inbred strains, in which one strain ranks low and the other high for phenotypes of interest, are useful models for exploring the genetic basis of complex traits (14, 35). In previous work (3), we found that significant variation exists for endurance running capacity between 11 inbred strains of rats; the Copenhagen (COP) rats demonstrated the lowest running capacity with an average distance run of 298 ± 30 m, whereas the DA rats recorded the highest capacity and ran for 840 ± 64 m. In addition, intrinsic cardiac contractility (Langendorff-Neely working heart model) correlated (r = 0.86) positively with endurance running capacity among these 11 inbred strains. Isolated hearts from DA rats have on average a 50% greater cardiac output compared with hearts from COP rats.

Evolution has favored biochemical and physiological systems that utilize atmospheric oxygen (1). Oxidation reactions that use molecular oxygen as the final electron acceptor obtain a greater yield of ATP relative to substrate-level phosphorylation. Because all cells use free energy derived from the catabolism of ATP, adenosine has long been a candidate for modulating cellular response during increases in metabolic rate (4). The breakdown of ATP and the subsequent increase in AMP concentration lead to an increase in the production of adenosine and inorganic phosphate via dephosphorylation of AMP by the enzyme 5'-nucleotidase (28, 29). Much evidence exists that receptors for adenosine are involved in a feedback regulation of numerous effects to regulate oxygen delivery with metabolic demands that depend on oxidative phosphorylation. Indeed, numerous studies demonstrate that adenosine is produced during periods of decreased O2 delivery or increased metabolic activity (2, 16, 19, 20). Therefore, we tested the specific hypothesis that a differential capacity to produce adenosine in cardiac and skeletal muscle between the strains is a candidate intermediate phenotype to help explain the differences in aerobic running capacity and isolated cardiac function. Adenosine production was measured by the activity from both soluble 5'-nucleotidase and membrane-bound ecto-5'-nucleotidase.

METHODS

Animals. Rats were from Copenhagen (COP/Hsd) and DA (DA/OlaHsd) strains initially obtained from Harlan Sprague Dawley (Indianapolis, IN) and bred in house at the Medical College of Ohio. The COP strain was originated by Curtis (13) at Columbia University Institute for Cancer and bred for resistance to tumors. The DA strain was initiated by Odell at the Oak Ridge National Laboratory and completed at the Wistar Institute in 1965 (41). The strain DA was so design...
nated because it expressed the d blood group allele and had agouti coat color. The housing environments were uniform for both strains. Both strains were housed in one inbred colony room with only littersmates of the same sex sharing cages (2 rats/cage) from the time of weaning. Rats were weaned between 26 and 28 days of age. Rats were fed standard rat chow (Ralston Purina, diet 5001) and water ad libitum and placed on a 12:12-h light-dark cycle with the light cycle coinciding with daytime. All procedures were approved by the Institutional Animal Care and Use Committee before commencement of the present study.

Sample acquisition. Heart and skeletal muscle samples were taken from female and male rats at 12 wk of age. Each rat was anesthetized with pentobarbital sodium (50 mg/kg) via an intraperitoneal injection. After negative reflex tests to ensure deep anesthesia, each rat was heparinized (1,000 IU/kg) intraperitoneally, and the heart was removed from the chest. The right and left ventricles were separated and frozen in liquid nitrogen. Immediately after removal of the heart, the left gracilus muscle was isolated, resected, and frozen in liquid nitrogen. All samples were stored at −70°C until assayed.

Sample preparation. Tissue homogenates were prepared for measurement of soluble 5'-nucleotidase and membrane-bound ecto-5'-nucleotidase activity, as described previously (9). Each sample was thawed and weighed. All procedures were carried out at 4°C. For right and left ventricle, 20 ml of glycerol buffer was added per gram of tissue. For skeletal muscle, 10 ml of glycerol buffer was added per gram of tissue. Samples were homogenized with four 15-s bursts and poured through gauze. After the total volume was measured, the homogenate was centrifuged for 1 h at 150,000 g. After the supernatant was removed, the pellet was resuspended in an equal volume of glycerol buffer. The samples were dialyzed against 50 vol of glycerol buffer containing 0.1% activated charcoal for 4 h. After dialysis, all samples were aliquoted and stored at −70°C.

Sample analysis. 5'-Nucleotidase activity (i.e., adenosine production) in both the pellet (ecto-5'-nucleotidase) and supernatant (soluble 5'-nucleotidase) fractions was measured using a spectrophotometric assay for inorganic phosphate production (28, 29, 37). Because AMP is hydrolyzed to adenosine and inorganic phosphate in a 1:1 ratio, the measurement of the rate of production of inorganic phosphate is exactly equivalent to the rate of production of adenosine. This particular assay has been extensively validated in our laboratory (8–10) as well as others (37). The assay reaction mixture contained 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-NaOH (pH 7.3), 0–2 mM AMP, 1 mM MgCl₂, 5 μM erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), and 5 μM iodotubercidin. EHNA and iodotubercidin were present in the reaction mixture to inhibit adenosine deaminase and adenosine kinase, respectively. This particular assay has been extensively validated in our own laboratory (8–10) as well as others (37). The assay reaction mixture contained 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-NaOH (pH 7.3), 0–2 mM AMP, 1 mM MgCl₂, 5 μM erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), and 5 μM iodotubercidin. EHNA and iodotubercidin were present in the reaction mixture to inhibit adenosine deaminase and adenosine kinase, respectively. The reaction was incubated at 37°C for 11 min and terminated by the addition of 10% trichloroacetic acid at 4°C. The sample was centrifuged at 4,500 revolutions/min (rpm) for 5 min. A 1-ml sample of the supernatant was removed and placed in a fresh centrifuge tube. Acid molybdate (0.3 ml) and butyl acetate (1 ml) were added, and the mixture was vortexed for 1 min. The sample was centrifuged for 1 min at 3,000 rpm. Inorganic phosphate was measured with a Gilford Response II spectrophotometer at a wavelength of 310 nm. The rate of adenosine production was measured as nanomoles of phosphate produced per minute of incubation per milligram of protein.

Experimental design. To compare the rates of adenosine production between DA and COP rats, the 5'-nucleotidase assays were performed using a reaction mixture containing 50 mM MOPS (pH 7.3), 2 mM MgCl₂, 5 μM EHNA, 5 μM iodotubercidin, and 90 μM AMP [i.e., the approximate Michaelis-Menten constant (Km) of the membrane-bound 5'-nucleotidase]. The amount of protein in each sample was also measured using the method of Lowry et al. (30). Data are presented as means ± SE. Possible differences between the DA and COP rat strains were evaluated using Student’s t-test for independent samples. Statistical significance was defined by a P value <0.05.

Although we have extensively validated in dog heart homogenates (8, 10) that the AMP-phosphorylating activity in pellet fractions is due to ecto-5'-nucleotidase, we conducted several experiments here to confirm this in the rat. For these experiments, left ventricular samples were pooled from both DA and COP strains to serve as a control.

The effect of α,β-methyleneadenosine 5'-diphosphate (AOPCP) on 5'-nucleotidase was determined using a reaction mixture containing 50 mM MOPS (pH 7.3), 90 μM AMP, 2 mM MgCl₂, 5 μM EHNA, 5 μM iodotubercidin, and 0, 10, 150, or 300 μM AOPCP. Control reactions in which samples were incubated at 0°C for 11 min were carried out for all assays. In addition, all assays were performed over times for which the reactions were linear at 37°C. All reagents were acquired from Sigma Chemical (St. Louis, MO), except for EHNA and iodotubercidin, which were acquired from Research Biochemicals International (Natick, MA).

RESULTS

5'-Nucleotidase activity in the membrane pellet fraction of the left and right ventricles accounted for the vast majority (90 and 80%, respectively) of the total tissue adenosine production. The left ventricular pellet fraction was evaluated for comparison to previously published characteristics of purified ecto-5'-nucleotidase. In the presence of increasing concentrations of AMP, the enzyme followed Michaelis-Menten kinetics, with a Km of 89 μM (Fig. 1). As shown in Fig. 2, measurable activity of ecto-5'-nucleotidase was not dependent on MgCl₂. The activity was, however, substan-
Fig. 2. Effect of MgCl₂ on ecto-5'-nucleotidase activity. Reaction mixture contained 50 mM MOPS (pH 7.3), 90 μM AMP, 0–10 mM MgCl₂, 5 μM EHNA, and 5 μM iodotubercidin. Enzyme activity was not dependent on MgCl₂ presence for activity. MgCl₂, however, substantially increased the activity of ecto-5'-nucleotidase activity. Samples were pooled from both DA and COP strains to serve as a control.

Comparison of cardiac ecto-5'-nucleotidase activity between DA and COP rats in the membrane pellet fraction is shown in Fig. 3. In the left ventricle, ecto-5'-nucleotidase activity was found to be 22.4% higher in the DA than in the COP strain (10.93 ± 0.51 vs. 8.91 ± 0.71 nmol·min⁻¹·mg protein⁻¹, P = 0.018). In the right ventricle, ecto-5'-nucleotidase activity was 46.1% higher in the DA than in the COP rats (10.91 ± 0.76 vs. 7.48 ± 0.44 nmol·min⁻¹·mg protein⁻¹, P = 0.0008). There were no significant differences in soluble 5'-nucleotidase activity between the DA and COP strains in either the left (1.16 ± 0.13 vs. 1.42 ± 0.15 nmol·min⁻¹·mg protein⁻¹, P = 0.11) or right (2.27 ± 0.21 vs. 2.38 ± 0.16 nmol·min⁻¹·mg protein⁻¹, P = 0.35) ventricles.

As shown in Fig. 4, assays in skeletal muscle revealed no significant difference in membrane-bound ecto-5'-nucleotidase activity between the DA and COP strains (1.66 ± 0.16 vs. 1.84 ± 0.21 nmol·min⁻¹·mg protein⁻¹, P = 0.50). There was also no difference in the soluble 5'-nucleotidase between the DA and COP strains (1.31 ± 0.04 vs. 1.37 ± 0.11 nmol·min⁻¹·mg protein⁻¹, P = 0.33). As shown in Table 1, all groups, with one exception, showed no significant difference between DA and COP in protein content. Left ventric-
Table 1. Comparison of protein content between DA and COP rat strains in cardiac and skeletal muscle tissue

<table>
<thead>
<tr>
<th>Group</th>
<th>DA, mg protein/g tissue</th>
<th>COP, mg protein/g tissue</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left ventricle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>112.2 ± 4.0</td>
<td>115.2 ± 1.2</td>
<td>0.24</td>
</tr>
<tr>
<td>Supernatant</td>
<td>94.9 ± 1.1</td>
<td>90.0 ± 0.96</td>
<td>0.001*</td>
</tr>
<tr>
<td>Right ventricle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>100.4 ± 2.2</td>
<td>101.5 ± 2.2</td>
<td>0.36</td>
</tr>
<tr>
<td>Supernatant</td>
<td>60.1 ± 0.4</td>
<td>58.0 ± 1.4</td>
<td>0.09</td>
</tr>
<tr>
<td>Skeletal muscle</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>79.9 ± 7.0</td>
<td>82.2 ± 8.9</td>
<td>0.38</td>
</tr>
<tr>
<td>Supernatant</td>
<td>28.6 ± 1.2</td>
<td>28.5 ± 1.1</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Values are means ± SE. Protein content was measured according to Lowry et al. (30). *Protein content was 5.1% greater in the left ventricle supernatant of the DA rats compared with the Copenhagen (COP) rats. Protein content for none of the other samples differed between the DA and COP rats.

DISCUSSION

One approach for defining genetic candidates for variation in exercise capacity is to evaluate known biological and physiological phenotypes intermediate to the trait of interest (14, 35). Because of the complexity of aerobic endurance capacity in intact animals, we utilized a model developed by Joyner (24) to guide the choice of our measures of intermediate phenotypes between the two contrasting DA and COP strains. This model of maximal endurance running capacity is the product of three physiological variables: 1) the maximal rate at which oxygen and nutrient substrates can be utilized to produce energy in the form of ATP (O2 max, ml·min⁻¹·kg⁻¹), 2) the percentage of O2 max at the threshold for lactate release (%O2 max at LT), and 3) the efficiency of running (RE, km·min⁻¹·O2⁻¹). On the basis of this model, we formulated two working hypotheses. First, we hypothesized that allelic variation for each of these three complex intermediate phenotypes will largely account for the magnitude of the difference in performance. Second, we hypothesized that the VO2 max component of the Joyner model is of the greatest fractional importance. Knowledge that originated from the seminal work of Hill and Lupton (21) supported the contention that the ability of the heart to deliver oxygen is a major factor that can limit maximal aerobic capacity, especially in aerobically fit animals as described by Wagner (40) and Rowell et al. (36). Thus the capacity of the heart to maintain cardiac output in response to changes in metabolism became a logical intermediate phenotype to evaluate.

The findings of the present study demonstrate that ecto-5’-nucleotidase activity in both the left and right ventricles is greater in the DA than in the COP rat strain. This result is consistent with the hypothesis that the DA rats have a higher capacity relative to the COP rats for the cardiac formation of adenosine. In contrast, our data do not support the hypothesis that differences in skeletal muscle adenosine production contribute to the differences in running capacity between these two strains, as there was no detectable difference between DA and COP rats in the 5’-nucleotidase activity in gracilis muscle. We also found in comparing cardiac adenosine production in DA vs. COP rats that the increase in right ventricular ecto-5’-nucleotidase activity in DA rats was twice as great as the increase in left ventricular ecto-5’-nucleotidase activity. The exact significance of this difference is not known, although the greater differential effect in right ventricular adenosine production is consistent with the work of Blanck et al. (5), who found a 96% increase in right ventricular 5’-nucleotidase activity and a 48% increase in left ventricular 5’-nucleotidase activity in porcine pig hearts exposed to 15 min of global ischemia (5).

Strain differences for cardiac adenosine formation may contribute to the contrasting exercise capacities via one or more of adenosine’s various biochemical and physiological effects on the heart. The most obvious of these, in relation to exercise, is the vasodilator effect of adenosine that apparently operates in a feedback loop to match the delivery of oxygen with utilization. During sustained exercise, cardiac output increases to meet the increased utilization of oxygen by skeletal muscle. The simultaneous increases in heart rate and cardiac contraction produce increased catabolism of ATP and thus an increased concentration of AMP. Via the enzyme 5’-nucleotidase, the accumulated AMP is dephosphorylated to adenosine (22). Through its vasodilator effects on the coronary circulation, adenosine increases oxygen delivery to the heart, which allows for increased ATP synthesis.

The general hypothesis that adenosine is a functional intermediate that matches oxygen delivery with cardiac metabolism during exercise has been tested by a number of studies. McKenzie et al. (31) demonstrated a positive correlation between myocardial adenosine, coronary sinus adenosine, and coronary vascular conductance during exercise in dogs. Laxson et al. (27) provided evidence that adenosine contributes significantly to coronary vasodilation during exercise-induced cardiac ischemia in dogs. They showed that combined intracoronary administration of adenosine deaminase and the adenosine receptor antagonist 8-phenyltheophylline allowed less coronary vasodilation in a stenosed left anterior descending coronary artery during treadmill exercise. It has also been shown that 5’-nucleotidase activity is higher in cardiac tissue of exercise-trained compared with sedentary subjects (34). Other reports, however, show that adenosine levels may not be of sufficient concentration to serve as the primary substance for local metabolic control of coronary blood flow during exercise hyperemia (39).

Although adenosine may be of some importance, it is reasonable to assume that it is only one factor in a hierarchy of regulators (11, 12, 23, 43) and that its role is state dependent. Ishibashi and colleagues (23) pro-
vide data that adenosine, nitric oxide (NO), and ATP-sensitive K+ (KATP) channels are all important coronary vasodilatory mechanisms in sustained exercise. Recent work suggests that KATP channels are the major regulators of coronary vasodilation during exercise (11, 12). However, during periods of ischemia or other situations that may result in the blockade or malfunction of the KATP channels, adenosine provides an alternative mechanism for exercise-induced coronary vasodilation (11, 12). It has also been proposed that the most important function of adenosine may not be vasodilation, but instead its role as an inhibitor of neutrophilic superoxide anion production (38). Superoxide anion has been shown to substantially attenuate the half-life of NO (18). Therefore, by inhibiting superoxide anion production, adenosine may indirectly contribute to coronary artery vasodilation by increasing NO half-life (38).

Another characterized effect of adenosine is its ability to influence myocardial contractility. The enhanced inotropic response after activation of A2a receptors has been demonstrated in both isolated rat cardiac myocytes (42) and isolated perfused rat hearts (32). We recently tested other intermediate phenotypes of cardiac function in the COP and DA strains by measuring indexes of contractile capacity in isolated papillary muscles and dispersed left ventricular cells (7). Our data demonstrate that DA papillary muscles were significantly greater than those from the COP strain for both maximal developed tension and rate of change in developed tension during contraction and relaxation. On average, myocytes from the DA rats showed a 50% greater fractional shortening than COP myocyte cells. Both the amplitude of Ca2+ transients and Na+-K+ ATPase activity in individual dispersed myocytes were higher in DA compared with COP. The contribution of adenosine to each of these intermediate phenotypes in COP and DA strains warrants future investigation.

Although a number of previous studies found an increased production of adenosine in exercising skeletal muscle (22), its role in matching blood flow to metabolism is less certain. Ballard et al. (2) found that the concentration of adenosine appearing in venous blood from the gracilis increases during contraction. Goonewardene and Karim (17) estimated that adenosine produced ~40% of the steady-state exercise hyperemia in an isolated gracilis by following vascular responses before and during administration of adenosine deaminase. In contrast, Koch and collaborators (26) found that pharmacological blockade of adenosine receptor sites did not influence the magnitude of hindlimb blood flow in conscious dogs during treadmill exercise. Although this observation does not preclude a role for adenosine in the absence of adenosine blockade, it does demonstrate that adenosine is not essential for the normal hyperemia of exercise in skeletal muscle. The current study found no difference in either soluble 5’-nucleotidase activity or membrane-bound 5’-nucleotidase activity in skeletal muscle between the COP and DA rats under basal conditions. It is possible, nevertheless, that there is differential expression between these strains for 5’-nucleotidase activity that is induced as a function of exercise.

In conclusion, data presented here demonstrate that the DA rat strain possesses a significantly higher membrane-bound ecto-5’-nucleotidase activity than the COP rat strain in the both left and right ventricles. This result is consistent with the hypothesis that the DA rats have a higher capacity relative to the COP rats for the cardiac formation of adenosine. Thus adenosine formation becomes a candidate intermediate phenotype for genomic exploration (14) that may explain part of the difference in running capacity and cardiac performance between the COP and DA strains of inbred rats as first described by Barbato et al. (3) in 1998. Identification of the genes responsible for these trait differences between low and high aerobic capacity would form a broader base for understanding the origins of such complex physiology and lead to new paths for the prevention and treatment of system-related diseases.

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