Regulation of Mitochondrial [NADH] by Cytosolic [Ca\(^{2+}\)] and Work in Trabeculae From Hypertrophic and Normal Rat Hearts

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**Abstract**—Pressure overload hypertrophy has previously been shown to reduce contractility but paradoxically to increase O\(_2\) consumption rates at a given force. Because O\(_2\) consumption rates are related to mitochondrial [NADH] ([NADH]\(_m\)), we tested the hypothesis that with hypertrophy, control of [NADH]\(_m\) may be altered. Left ventricular trabeculae were isolated from banded and control rat hearts, and fluorescence spectroscopy was used to monitor [NADH]\(_m\) and cytosolic [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_c\)). The hearts from banded rats developed hypertrophy (heart-to-body weight ratio increased from 4.1±0.1 to 4.9±0.1 mg/g) and hypertension (systolic arterial pressure increased from 117±4 to 175±5 mm Hg). Muscle workload was increased by stepwise increases in pacing frequency (up to 2 Hz). After increased work, [NADH]\(_m\) fell and then slowly recovered toward control levels. When work was decreased, [NADH]\(_m\) overshoot control values and then slowly returned. The Ca\(^{2+}\)-independent initial fall was larger for trabeculae from rats with hypertrophied hearts than from control rats (eg, 17±2% versus 11±1% when work was increased by increasing the frequency from 0.25 to 1 Hz). At 1 Hz, average [Ca\(^{2+}\)], was 280 nmol/L, and the Ca\(^{2+}\)-dependent [NADH]\(_m\) recovery was larger for trabeculae from rats with hypertrophied hearts (17±4% versus 10±2%) despite similar average [Ca\(^{2+}\)]. At steady state after Ca\(^{2+}\)-dependent recovery, there was no difference in [NADH]\(_m\) (fall of 1±2% versus 1±1%). Furthermore, the Ca\(^{2+}\)-dependent overshoot was larger for trabeculae from hypertrophied hearts than from control hearts (increase of 14±2% versus 9±2% when frequency was decreased from 1 to 0.25 Hz). We conclude that (1) there is initially a larger imbalance in NADH production versus consumption rate in hypertrophy (because NADH fell more) and (2) the Ca\(^{2+}\)-dependent recovery mechanism is enhanced in hypertrophy (because NADH recovered and overshoot more), thus compensating for the larger imbalance. (Circ Res. 1998;82:1189-1198.)

**Key Words:** muscle • force • ATP hydrolysis • oxidative phosphorylation • indo-1 fluorescence

Myocardial hypertrophy may be associated with diastolic dysfunction, slowed relaxation, and reduced ejection. Although there may be reduced expression of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, a reduced contractile relaxation rate and Ca\(^{2+}\) transport rate may be associated with altered cardiac energetics, eg, reduced metabolic rate. Surprisingly, studies of pressure-induced right ventricular hypertrophy have shown that muscle metabolism increased (ie, increased O\(_2\) consumption rate at a given force) in some studies but not in another. This result is in contrast to volume-induced hypertrophy, in which (active) energy metabolism was unaltered, as determined from O\(_2\) consumption rates or activation heat measurements.

Altered energy control may be due to alterations in mitochondrial function, myofibrillar energetics, Ca\(^{2+}\) transport processes, or altered cytosolic ADP buffering (by creatine kinase). In previous studies, we have demonstrated a dynamic control of [NADH]\(_m\) on increased workload. Specifically, we found that increased work caused an initial Ca\(^{2+}\)-independent fall of [NADH]\(_m\) that was followed by a Ca\(^{2+}\)-dependent recovery of [NADH]\(_m\) back toward control levels. This control of [NADH]\(_m\) suggests two mechanisms: the first is possibly dependent on high-energy phosphates; the second, on Ca\(^{2+}\)-dependent stimulation of NADH production. Therefore, if hypertrophy is associated with an altered energy state (eg, altered [ADP]), this may alter the first control mechanism. The second control mechanism may also be altered, because previous studies have shown that increased amounts of mitochondrial [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_m\)) are retained in pressure-hypertrophied right ventricular myocardium.

In the present study, we isolated trabeculae from pressure-induced left ventricular hypertrophied rat hearts. [Ca\(^{2+}\)] and regulation of [NADH]\(_m\) were studied in hypertrophied and control hearts. Consistent with altered energy metabolism, we found that [NADH]\(_m\) fell more in trabeculae from hypertrophied hearts than in trabeculae from control hearts. Furthermore, consistent with increased amounts of [Ca\(^{2+}\)]\(_m\), there was a larger Ca\(^{2+}\)-dependent [NADH]\(_m\) recovery in trabeculae from hypertrophied hearts.
Selected Abbreviations and Acronyms

Av [Ca\(^{2+}\)] = time-average [Ca\(^{2+}\)],  
Av force = time-average (developed) force  
BDM = 2,3-butanedione monoxime  
[Ca\(^{2+}\)] = cytosolic [Ca\(^{2+}\)]  
[Ca\(^{2+}\)] mitochondrion = mitochondrial [Ca\(^{2+}\)]  
Hyp = hypertrophic, hypertrophied  
Iratio = background corrected indo-1 fluorescence ratio  
N385, N456 = fluorescence at 385 and 456 nm before indo-1 loading  
NADH = semicalibrated [NADH]\(_m\) from Nratio (Equation 2) or nicotineamide adenine dinucleotide (reduced form)  
[NADH]\(_m\) = mitochondrial [NADH]  
NADH MAX = maximum NADH after decreased work (Figure 3)  
NADH MIN = minimum NADH after increased work (Figure 3)  
NADH REC = recovery of NADH after prolonged work (Figure 3)  
NADH SS = NADH at steady state after prolonged work (Figure 3)  
Nratio = fluorescence ratio before indo-1 loading  
Rel Av force = Av force relative to Av force at 0.25 Hz  
R\(_{min}\), R\(_{max}\) = minimum and maximum of Iratio  
S\(_{385}\), S\(_{456}\) = fluorescence ratio (0 [Ca\(^{2+}\)] vs max [Ca\(^{2+}\)]) of indo-1 at 456 or 385 nm  
t1/2 = half-time of force relaxation  

Materials and Methods

Animal Preparation and Hemodynamic Measurements

Male Sprague-Dawley rats (Harlan Sprague Dawley, Inc, Indianapolis, Ind) weighing 150 to 200 g were anesthetized with an intramuscular injection of ketamine (60 to 90 mg/kg) and xylazine (1 to 2 mg/kg). Constriction of the suprarenal abdominal aorta was produced by using a tantalum hemoclip and modified clip applicator (set at closure equivalent to a 25-gauge needle) as described elsewhere. Sham-operated animals underwent dissection of the abdominal aorta without the placement of the hemoclip. Additional animals, which did not undergo any surgical procedure, were also used. Fifteen to 18 weeks after surgery, all animals were anesthetized for measurement of blood pressure. A 3F micrometer-tipped catheter (MNI-Gaeltec) was introduced via the right carotid artery and inserted to the level of the aortic arch for systemic pressure recording. Immediately after catheterization, a thoracic incision was made, hearts were excised and weighed, and trabeculae were dissected. All procedures and the care of the rats were in accordance with institutional guidelines, which met or exceeded those of the American Physiological Society and the American Association for Accreditation of Laboratory Animal Care.

Trabecular Preparation and Solutions

Hearts were briefly perfused before the trabeculae were removed by using a modified Krebs-Henseleit perfusion solution containing (in mmol/L except as noted) NaCl 108, KCl 6, MgCl\(_2\) 1.2, CaCl\(_2\) 2.0, NaHCO\(_3\) 24, glucose 4, sodium pyruvate 10, 20 U/L insulin, and BDM 30; the solution was equilibrated with a 95% O\(_2\)/5% CO\(_2\) gas mixture to produce a pH of 7.40. BDM was added to abolish contraction and to minimize damage to the trabeculae during dissection.

Each heart had several ellipsoidal trabeculae running along the free left ventricular wall, and a trabecula was selected on the basis of its suitability for mounting in our experimental setup. The averages of the short and long axes, respectively, were 0.14±0.02 and 0.32±0.06 mm for the control group and 0.09±0.002 and 0.14±0.02 mm for the hypertrophic group. The trabeculae were isolated from the ventricular wall while being superfused with Krebs-Henseleit buffer in a manner similar to that described previously, except that both ends of each trabecula were connected to a portion of the ventricular wall. The isolated trabecula was thereafter mounted in a muscle chamber (containing Krebs-Henseleit buffer with BDM) on a Nikon Diaphot inverted microscope. After a 10-minute rest period, the trabecula was paced at 0.5 Hz and superfused at 15 mL/min with BDM-free Krebs-Henseleit solution. Force was optimized by stretching the trabecula and was measured during isometric contractions. All measurements were performed at room temperature (~24°C to 26°C).

[NADH]\(_m\), Measurements and Calibration

[NADH]\(_m\) was assessed by using methods similar to those described previously. In brief, the trabecule were excited by light at 350 nm, and fluorescence was detected at 385 (N385) and 456 (N456) nm. The use of these tissue light isosbestic wavelengths accounts for possible changes in tissue light absorbance, eg, due to hypoxia. However, as we have demonstrated, the trabeculae were not hypoxic at high pacing rates. The N456 signal predominantly arises from NADH and motion artifacts. In contrast, the reference signal at N385 (due to autofluorescence and possibly a small component of backscattered light) is mainly sensitive to motion artifacts. We therefore used our previously developed method to eliminate motion artifacts from the NADH fluorescence signal at N456 by dividing by the reference signal, thus obtaining an Nratio:

\[
Nratio = \frac{N456}{N385}
\]

Because N385 and N456 gradually decreased during the experimental protocols and at different rates, Nratio was normalized relative to its value at a pacing frequency of 0.25 Hz (control value). The ratio was further calibrated by assuming a minimum (maximally oxidized) value of [NADH]\(_m\) corresponding to an Nratio of 0.49 (at 1 Hz). The relative [NADH]\(_m\) was thus calculated from the measured Nratio to obtain semicalibrated NADH according to the following formula:

\[
NADH = \frac{Nratio - 0.49 \times Nratio (1 Hz)}{Nratio (0.25 Hz) - 0.49 \times Nratio (1 Hz)}
\]

Note that the calibration procedure causes the changes in NADH to be up to twice as large as the changes in Nratio (eg, a 10% change in Nratio would result in a 15% to 20% change in the calibrated NADH).

Indo-1 Measurement and [Ca\(^{2+}\)], Calibration

After measurement of [NADH]\(_m\), the trabecula was loaded with the free acid of indo-1 (Molecular Probes) as described elsewhere. Loading with the free acid rather than with indo-1-AM eliminates Indo-1 compartmentalization. [Ca\(^{2+}\)], was calculated according to the following formula:

\[
[Ca^{2+}] = \frac{R_{min} - \text{Iratio}}{K_i \times S_{385} \times S_{456} - \text{Iratio}}
\]

Iratio is the commonly used Indo-1 ratio after subtraction of the background fluorescence (N385 and N456) at each emission wavelength (385 and 456 nm). S\(_{385}\), S\(_{456}\) is the ratio between the 456-nm indo-1 emission intensity in the absence of [Ca\(^{2+}\)], and with saturating [Ca\(^{2+}\)]. R\(_{min}\) and R\(_{max}\) are the Iratio values determined in the absence of [Ca\(^{2+}\)], and at a saturating [Ca\(^{2+}\)], respectively. The constants S\(_{385}\), S\(_{456}\), R\(_{min}\), and R\(_{max}\) were determined in a manner similar to that previously described by Brandes et al (see the Appendix). In contrast to S\(_{385}\), R\(_{min}\), and R\(_{max}\), which are instrument dependent, the Ca\(^{2+}\) dissociation constant K\(_i\) is instrument independent. To compare our results with others, we used K\(_i\) = 256 mmol/L (Molecular Probes) because this is a commonly used value. Note that the use of an incorrect value for K\(_i\) simply scales [Ca\(^{2+}\)], linearly, and [Ca\(^{2+}\)], may therefore easily be obtained for other values of K\(_i\) by rescaling [eg, a K\(_i\) of 638 nm\(^2\)] would increase our reported values by a factor of...
The Banding Procedure Caused Hypertrophy, Hypertension, and Slowed Relaxation

Figure 1 shows the relationship between the aortic systolic pressure and the heart-to-body weight ratio for hearts from the 3 groups studied: banded (n=8), sham operated (n=6), and unoperated (n=3). Systolic pressure was proportionally related to the heart-to-body weight ratio regardless of group. There were no differences in the pressure or heart-to-body weight ratio between the sham-operated and unoperated animals. Five of the 8 banded hearts were both hypertrophied (heart-to-body weight ratio >4.6 mg/g) and hypertensive (systolic pressure >160 mm Hg). However, the other 3 hearts did not develop hypertrophy (heart-to-body weight ratio <4.2 mg/g), were only mildly hypertensive (130 mm Hg < systolic pressure <150 mm Hg), and were therefore not included in further analyses. The hearts were consequently divided into 2 groups: hypertrophied (Hyp) (consisting of the 5 hearts that were hypertensive and developed hypertrophy) and control (consisting of a total of 9 unoperated and sham-operated hearts).

To also verify that the isolated trabeculae from the hypertrophied hearts showed impaired function, the half-time of relaxation (ie, t1/2) of twitch force (before indo-1 loading) was measured. The Table shows the pooled data of systolic pressure, heart-to-body weight ratio, and t1/2. The systolic pressure and heart-to-body weight ratio were significantly larger for the Hyp than for the control group. The t1/2 values were also significantly larger for the Hyp than for the control group (except at 2 Hz). Furthermore, the t1/2 values decreased

<table>
<thead>
<tr>
<th>Systolic pressure, mm Hg</th>
<th>117±4</th>
<th>175±5</th>
<th>+50*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart/body weight, mg/g</td>
<td>4.14±0.11</td>
<td>4.87±0.06</td>
<td>+18*</td>
</tr>
<tr>
<td>t1/2, ms</td>
<td>0.25 Hz</td>
<td>171±13</td>
<td>232±22</td>
</tr>
<tr>
<td></td>
<td>0.5 Hz</td>
<td>163±12</td>
<td>226±18</td>
</tr>
<tr>
<td></td>
<td>1 Hz</td>
<td>144±13</td>
<td>196±16</td>
</tr>
<tr>
<td></td>
<td>2 Hz</td>
<td>103±9</td>
<td>128±9</td>
</tr>
</tbody>
</table>

Trabeculae force–relaxation times (t1/2) is the amount of time in ms for the maximum twitch force to decline by 50%. Values are mean±SEM. In comparing Hyp vs control, * indicates a significant increase (P<0.05).

Results

The Banding Procedure Caused Hypertrophy, Hypertension, and Slowed Relaxation

Figure 1 shows the relationship between the aortic systolic pressure and the heart-to-body weight ratio for hearts from the 3 groups studied: banded (n=8), sham operated (n=6), and unoperated (n=3). Systolic pressure was proportionally related to the heart-to-body weight ratio regardless of group. There were no differences in the pressure or heart-to-body weight ratio between the sham-operated and unoperated animals. Five of the 8 banded hearts were both hypertrophied (heart-to-body weight ratio >4.6 mg/g) and hypertensive (systolic pressure >160 mm Hg). However, the other 3 hearts did not develop hypertrophy (heart-to-body weight ratio <4.2 mg/g), were only mildly hypertensive (130 mm Hg < systolic pressure <150 mm Hg), and were therefore not included in
Effects of Increased Frequency on Force, Av Force, and [NADH]m

Figure 2 shows a typical experiment (using a control trabecula) in which pacing frequency was increased from a control value of 0.25 Hz to 0.5, 1, or 2 Hz and then returned to 0.25 Hz. Increasing frequency did not cause large changes in the twitch amplitudes but did cause a gradual increase in the calculated Av force, as we have also shown previously. The typical undershoot and overshoot of [NADH]m when the frequency was increased and decreased, respectively, were also observed here.

In Figure 3, the changes in [NADH]m are compared for a typical control and a Hyp trabecula when the frequency was increased from the control value of 0.25 to 1 and back to 0.25 Hz. The initial fall of [NADH]m after increased work at 1 Hz may have been caused by a [Ca2+]c-independent mechanism, resulting in a minimum level of [NADH]m (NADH MIN). This minimum level depends on the stimulation of oxidative phosphorylation (synthesis of ATP), which in turn, is related to the ATP hydrolysis rate of the trabeculae by mechanical work and by other processes, such as Ca2+ transport. Figure 3 also shows that this initial fall was larger in the Hyp than in the control trabeculae. After prolonged stimulation, a Ca2+-dependent mechanism caused slow recovery of [NADH]m, resulting in a new steady-state value that is typically slightly below the control level. Note that the steady-state level depends on both the ATP synthesis, consuming NADH, and the Ca2+-dependent stimulation of NADH production, presumably via increased [Ca2+]c. In this typical example, the recovery was larger for the Hyp than for the control trabeculae, resulting in similar steady-state levels both close to the control values at 0.25 Hz. When the frequency was reduced from 1 back to 0.25 Hz, [NADH]m initially increased above control levels and then slowly returned (overshoot). This phenomenon can be explained by continued Ca2+ stimulation with increasing stimulation frequency for both the control and the Hyp group.

Effects of Increased Frequency on [Ca2+]c and Av [Ca2+]c

While the fall of NADH (to the minimum) is related to Av force, the NADH recovery is related to [Ca2+]c. It is therefore important to investigate the effects of Av [Ca2+]c, as well as Av force, on [NADH]m.

Figure 5 shows an example of the effects of increasing the frequency on force and [Ca2+]c, as well as the calculated Av force and Av [Ca2+]c, by using a protocol identical to that used for studying [NADH]m (Figure 2). As previously, Av force increased with frequency and, as expected, so did Av [Ca2+]c. Figure 6 shows pooled data of the effect of increasing frequency on Av [Ca2+]c for the control and Hyp trabeculae. At an extrapolated value of 0 Hz, Av [Ca2+]c is equal to resting (diastolic) [Ca2+]c, and was ~150 nmol/L for both control and Hyp trabeculae. Increasing the frequency caused proportional increases in Av [Ca2+]c in both groups: 141±9 nmol/L/Hz for the control and 103±10 (nmol/L)Hz for the Hyp trabeculae (no significant difference).
The relationship between Rel Av force and Av $[\text{Ca}^{2+}]_c$ was also similar for both groups (not shown), with slopes of $0.032 \pm 0.003$ (nmol/L)$^{-1}$ for Hyp and $0.024 \pm 0.001$ (nmol/L)$^{-1}$ for control (no significant difference). These slopes depend on the sensitivity of the myofilaments to $[\text{Ca}^{2+}]_c$ but may differ from the steady-state force–Ca$^{2+}$ relationship because they also depend on the time course of contraction.

$[\text{NADH}]_m$ Initially Falls to Lower Levels (Lower NADH MIN) in Hyp Muscle

Figure 7A shows pooled data of the effect of increasing the frequency on the NADH MIN for trabeculae from Hyp and control hearts. Increasing the frequency is expected to increase the ATP hydrolysis rate by the myofilaments and by energy-dependent Ca$^{2+}$ transport (eg, by sarcoplasmic reticulum Ca$^{2+}$-ATPase). Although both groups showed a larger fall of $[\text{NADH}]_m$ (decreasing NADH MIN) with frequency, this trend was more pronounced in the Hyp trabeculae. For example, at 1 Hz, Hyp fell $17 \pm 2\%$, whereas control fell only $11 \pm 1\%$. The slopes, or NADH MIN sensitivity to frequency, were significantly different: $-0.133 \pm 0.003$ Hz$^{-1}$ and $-0.189 \pm 0.012$ Hz$^{-1}$ for the control and Hyp trabeculae, respectively. The Hyp trabeculae were therefore $42\%$ more sensitive to increased pacing frequency.

Although the ATP hydrolysis rate by energy-dependent Ca$^{2+}$ transport may be similar in trabeculae from Hyp and control hearts, the Av force and consequent ATP hydrolysis rates by the myofilaments may differ. It is therefore also necessary to compare NADH MIN to Av force. However, because NADH MIN was calculated relative to its value at 0.25 Hz, relative changes in NADH were compared with relative changes in Av force. (Incidentally, these relative changes in Av force, versus Av force at 0.25 Hz, are independent of muscle cross-sectional area.) Figure 7B shows that for the same increase in Rel Av force, NADH MIN was still lower for the Hyp trabeculae. For example, when the frequency was increased from 0.25 to 1 Hz, Av force increased to $\approx 4 \times$ the value at 0.25 Hz for both preparations, but NADH MIN was significantly lower for the Hyp (0.827) than for control (0.894) trabeculae. Increasing Rel Av force caused decreasing NADH MIN, and this fall of $[\text{NADH}]_m$ was again larger for the Hyp trabeculae. The slopes, or NADH MIN sensitivity to Rel Av force, were significantly different: $-0.0362 \pm 0.001$ and $-0.052 \pm 0.003$ for the control and Hyp trabeculae, respectively. The Hyp trabeculae were therefore $44\%$ more sensitive to an increase in Rel Av force, similar to the $42\%$ larger sensitivity of NADH MIN to increased frequency.
Consequently, at the same Av $[\text{Ca}^{2+}]$, affects both the ATP hydrolysis rate and Av $[\text{Ca}^{2+}]$ recovery, which presumably controls $[\text{Ca}^{2+}]_{m}$, recovery was larger for Hyp than control trabeculae (ie, at identical and large values of $[\text{Ca}^{2+}]_{m}$). NADH at steady state was lower for Hyp than for the control trabeculae. Reduced Av $[\text{Ca}^{2+}]$, which is expected to reduce NADH recovery, is therefore not the cause of the slightly lower NADH at steady state of Hyp at 2 Hz. Even though recovery was larger for the Hyp trabeculae (at the same Av $[\text{Ca}^{2+}]$; see Figure 8), the recovery was apparently not large enough to overcome the larger $[\text{NADH}]_{m}$ decrease (see Figure 7A and 7B).

Figure 10 shows the NADH overshoot as a function of Av $[\text{Ca}^{2+}]$, for the Hyp and control trabeculae. The results are qualitatively similar to those for NADH recovery (Figure 8). Increasing Av $[\text{Ca}^{2+}]$, caused increased NADH MAX, and the Hyp trabeculae were significantly more sensitive to increased Av $[\text{Ca}^{2+}]$; the slope was $1.11 \pm 0.07$ (μmol/L)$^{-1}$ for Hyp and $0.74 \pm 0.04$ (μmol/L)$^{-1}$ for control trabeculae. The [NADH]$_{m}$ at steady state is expected to depend on both ATP synthesis (causing NADH consumption) and Av $[\text{Ca}^{2+}]$, which presumably controls $[\text{Ca}^{2+}]_{m}$ and thereby NADH production. Figure 9A shows the relationship between NADH at steady state and pacing frequency, which affects both the ATP hydrolysis rate and Av $[\text{Ca}^{2+}]$. As we have shown previously, the trend is for NADH at steady state to decrease slightly with increasing frequency, but for the control trabeculae this decrease was not significant. There was also no significant decrease for the Hyp trabeculae except at 2 Hz, where it was barely significant ($P=0.045$). Pairwise comparison between Hyp and control at each frequency yielded no significant differences.

Figure 9B similarly shows the relationship between NADH at steady state and Av $[\text{Ca}^{2+}]$, for the 2 groups. At similar Av $[\text{Ca}^{2+}]$ ($\approx 350$ nmol/L), NADH at steady state was lower for the Hyp than for the control trabeculae. Reduced Av $[\text{Ca}^{2+}]$, is expected to reduce NADH recovery, is therefore not the cause of the slightly lower NADH at steady state of Hyp at 2 Hz. Even though recovery was larger for the Hyp trabeculae (at the same Av $[\text{Ca}^{2+}]$; see Figure 8), the recovery was apparently not large enough to overcome the larger [NADH]$_{m}$ decrease (see Figure 7A and 7B).

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0.68±0.023 (μmol/L)⁻¹ for control trabeculae, ie, 63% more sensitive.

Discussion

The present study is the first to compare the regulation of [NADH]m by pacing frequency, Av force, and Av [Ca²⁺], in hypertrophied versus normal myocardium. Pyruvate and glucose were used as substrates to rule out alterations in energy metabolism due to changes in substrate utilization (eg, reduced fatty acid oxidation). The main and novel finding of the present study was that the [NADH]m regulation differed with hypertrophy. Immediately after an increase in pacing frequency or Av force, the fall of [NADH]m was larger, and with a sustained higher pacing rate, NADH recovery was also larger. These changes with hypertrophy may be attributed to enhanced stimulation of oxidative phosphorylation (consistent with larger [ADP]) immediately after increased work and a larger increase in [Ca²⁺]m during periods of prolonged work.

Animal Model

We have produced hypertrophy (and hypertension) by using an animal model that resulted in a high survival rate (in our case, none of the banded animals died before the heart was removed). Impaired cardiac function was evident from the slowing of force relaxation rates in trabeculae that were isolated from the hypertrophied hearts. This is in agreement with previous studies in which this model caused decreased rates of relaxation and Ca²⁺ decline. It is also notable that this model shows a significant decrease in sarcoplasmic reticulum Ca²⁺-ATPase mRNA protein expression and thapsigargin-sensitive Ca²⁺-transporting activity in homogenates. However, previous experiments from this laboratory have shown that isolated left ventricular myocytes were hypertrophied, but that Ca²⁺ current, sarcoplasmic reticulum Ca²⁺ load, unloaded shortening relaxation times, and Ca²⁺ rates of decline were unaltered.

Measurements of Av Force and Av [Ca²⁺]c

Figures 4 and 6 demonstrated that, as expected, the Av force and Av [Ca²⁺]c increased with pacing frequency. Partially because the Hyp trabeculae had a slower relaxation rate (see Table), the Av force was larger for the Hyp trabeculae. However, the relative increase in Av force (Rel Av force) with frequency was identical in the Hyp and control trabeculae, with a normalized slope of 0.92 (Figure 4B). The deviation of the slope from unity may be explained by the faster relaxation rates with increasing frequency (see Table).

Increased Pacing Frequency Causes a Larger Initial Fall of [NADH]m in Hyp Muscle

Figure 2 shows that as the pacing frequency and the consequent ATP hydrolysis rate increased, there was a rapid initial fall of [NADH]m within ≈10 seconds. This is in agreement with previous studies, although the fall toward a steady state has, in some cases, been much slower (≈10 minutes). These in vivo results are consistent with results in isolated mitochondria, in which increased [ADP] causes decreased [NADH]m. A transient increase in [ADP] during the NADH MIN may result from an increased rate of ATP consumption, while the rate of ATP synthesis (predominantly through oxidative phosphorylation) increases less.

Figure 7 shows that an increased pacing frequency and consequent increased Rel Av force caused proportionally lower minimum values of [NADH]m as we have also shown previously. Furthermore, the NADH MIN was lower for the Hyp than for the control trabeculae. The rates of NADH consumption (eg, due to ATP synthesis) or production (due to substrate oxidation) were not measured in this study. We therefore cannot unequivocally determine whether the lower NADH MIN with Hyp might have been caused by a reduced rate of NADH production, increased rate of NADH consumption, or altered time constants for changes in flux rates.

Altered Relative Flux Rates

As work and ATP consumption rate increase, a lower NADH MIN would be expected for a larger imbalance in the NADH production versus consumption rates. There are several reasons why the NADH production versus consumption rate, at NADH MIN, may be lower in the Hyp trabeculae. First, the rate of NADH production could be lower in the Hyp trabeculae, so that for the same increase in NADH consumption rate, a larger initial decrease in [NADH]m would result. A lower rate of NADH production could result from either lower initial [Ca²⁺]m (unlikely, since NADH MIN is largely Ca²⁺ independent), decreased [ADP]/[ATP], or some other control factor.

Second, there may be a higher rate of NADH consumption in the Hyp trabeculae owing to increased stimulation of oxidative phosphorylation. This would be consistent with previous findings, which have demonstrated increased O₂ consumption rates (at a given force) with hypertrophy. Enhanced stimulation with hypertrophy might result from increased [ADP] caused by higher rates of ATP consumption or higher rates of ATP hydrolysis per unit of work (although this seems unlikely, owing to shifts in myosin isofroms). Alternatively, it has been shown that creatine kinase activity is reduced in hypertrophied hearts. Thus, while ATP consumption rates may not differ with hypertrophy, the reduced ADP buffering during increased work may lead to a larger transient increase of [ADP]. This could cause a transiently enhanced stimulation of oxidative phosphorylation (during the NADH MIN), consistent with our results.

It is also possible that the non–ATP-producing NADH consumption rate is higher in the Hyp trabeculae. This could happen if mitochondrial Ca²⁺ cycling consumes protons without generating ATP. Indeed, a larger, state 4 respiration rate (non–ATP producing) was found in mitochondria from hypertrophied hearts, although an increase in state 3 respiration rate has also been reported. An increased rate of NADH consumption is also consistent with the increased respiratory rate observed in mitochondria isolated from non-failing hypertrophied rabbit hearts.

In addition to these mechanisms, there is a decrease in mitochondrial versus myofilament volume in this type of hypertrophic rat model (eg, this ratio is 16% to 37% higher in control than in aortic banded rats). Thus, a given increment in myofilament ATP consumption rate may be expected to produce a more pronounced NADH decline in the rela-
tively smaller mitochondrial pool. This mismatch may indeed have broad-reaching energetic effects in the hypertrophied heart.

**Altered Time Constants**

If NADH starts to recover during the NADH fall, then the minimum NADH value would depend on the time constant for increasing the NADH consumption rate (fall) versus the time constant for increasing the NADH production rate (recovery). A lower minimum in Hyp trabeculae may therefore be reached before any significant NADH recovery has occurred (versus control, wherein some recovery might already have occurred). Because the NADH MIN in the absence of recovery is not known, it is not possible to accurately determine the time constants to test this hypothesis. However, because recovery was larger in Hyp than in control trabeculae (see Figure 8), it seems unlikely that recovery would start later in Hyp trabeculae and would thereby explain the lower NADH MIN.

**[NADH] m Recovers More at Fixed Av [Ca 2+] c in Hyp Muscle**

We previously hypothesized that the recovery of [NADH] m during prolonged work was mediated by increased [Ca 2+] m, which in turn was a result of increased [Ca 2+] c.11 In our previous study [Ca 2+] c was not measured, but when work was increased without increasing [Ca 2+] c (by increasing sarcomere length), there was no recovery, only an initial decrease after the increased workload. In the present study, the purpose of the [Ca 2+] c measurements was to compare the [NADH] m recovery of the Hyp and control trabeculae at the same Av [Ca 2+] c. Figure 8 shows that for a given Av [Ca 2+] c, the [NADH] m recovery was larger in the Hyp than in the control trabeculae. Furthermore, there was also a strong correlation between Av [Ca 2+] c and the amount of recovery, consistent with our earlier work that demonstrated recovery only when [Ca 2+] c was expected to increase. The enhanced NADH recovery, at a given Av [Ca 2+] c, for Hyp versus control trabeculae would be expected by an increased NADH production rate due to activation of mitochondrial dehydrogenases, either by a larger [Ca 2+] m or by increased sensitivity to [Ca 2+] m. Increased [Ca 2+] m in turn, may be a result of an increased rate of uptake of cytosolic Ca 2+ by the mitochondrial uniporter or by a reduced Ca 2+ removal by the mitochondrial Na/Ca 2+ exchanger.35 The relation between increased [Ca 2+] c and [Ca 2+] m has been demonstrated in intact myocytes36,37 and is consistent with the data in Figure 8. Increased Ca 2+ retention has also been observed in mitochondria isolated from hypertrophied versus control hearts.32 The removal of mitochondrial Ca 2+ is believed to be energy dependent and may therefore depend on the energy state (eg, electrochemical gradient) of the mitochondria.36

**Steady-State [NADH] m Is Similar in Hyp and Normal Muscle**

The [NADH] m at steady state depends on both the mechanism responsible for the initial fall (possibly ADP dependent) and the Ca 2+ dependent recovery mechanism. Figure 9 shows that NADH at steady state fell only slightly, suggesting that the combined effects of the two mechanisms resulted in a return of [NADH] m toward control in both the control and Hyp trabeculae. However, the barely significant decrease below control levels for Hyp at 2 Hz (or at a resulting Av [Ca 2+] c of ≈350 nmol/L; Figure 9) suggests that the combined regulation may be less effective with hypertrophy and high workloads.

**Maximum [NADH] m Is Larger in Hyp Muscle**

If increased recovery is due to increased [Ca 2+] m and the removal of [Ca 2+] m after a reduction of Av [Ca 2+] c, is not instantaneous, then it is expected that the stimulation of NADH production will remain elevated and result in an [NADH] m overshoot. Figure 10 indeed shows that this is the case, and it is consistent with the notion that a larger [Ca 2+] m is retained in the Hyp than in the control trabeculae after increased work.32

**Appendix: Determination of R min and R max**

As we have previously shown,22 R min and R max may be calculated without saturating or depleting the muscle [Ca 2+] c, according to the following formulae:

\[
R_{\text{max}} = \frac{1 - S_{385}}{1 - S_{456}} \times \frac{S_{456}}{S_{385}}
\]

\[
R_{\text{min}} = R_{\text{max}} \times \frac{S_{385}}{S_{456}}
\]

The gain adjuster (bH) is defined as the slope of indo-1 fluorescence emission intensity at 385 nm (I385) versus that at 456 nm (I456) during pacing. S 385 and S 456 are obtained from an appropriate reference solution by calculating the ratio between indo-1 intensity in the absence of Ca 2+ and intensity with saturating [Ca 2+] c at 385 and 456 nm, respectively.22 Here we have improved on the determination of the gain adjuster by exploring 3 new methods of reducing motion artifacts: (1) BDM (10 to 20 mmol/L) was added to abolish contraction and therefore motion artifacts, while retaining (reduced amplitude) Ca 2+ transients. (2) Indo-1 isoabsorbic wavelength was determined in the presence of BDM by tilting an interference filter until no Ca 2+ transients were observed.22 Fluorescence was then measured (in the absence of BDM) at this isoabsorbic indo-1 wavelength (429 nm [I429]) in addition to I385 and I456. Motion artifacts were subsequently reduced by calculating motion-corrected ratios: L 385 = I385/I429 and L 456 = I456/I429. (3) In some trabeculae, addition of BDM did not completely abolish contraction, and a combination of BDM application and the motion-correcting ratio calculation minimized any residual motion artifacts. In either protocol, the gain adjuster was obtained from the relationship between the motion-reduction signals at 385 and 456 nm. The 3 methods produced identical results: bH1 = 0.373 ± 0.015, bH2 = 0.388 ± 0.013, and bH3 = 0.383 ± 0.017, and the third method was therefore used to also obtain reliable values from trabeculae in which BDM incompletely abolished contraction. S 385 and S 456 depend on the reference solution and, to a small degree, on instrumentation (mainly on filter bandwidth and excitation wavelength). In contrast to our original study in which protein solutions were used, we used heart homogenates in this study. Hearts were first briefly perfused with HEPES buffer containing 40 mmol/L HEPES (adjusted to pH 7.2) and 140 mmol/L KCl. Caffeine (10 mmol/L) and EGTA (1 mmol/L) were added to reduce [Ca 2+] m in the hearts. The caffeine and EGTA were then washed out with HEPES buffer only. The left ventricle was subsequently cut into pieces and immersed in 0.5 mL HEPES buffer with leupeptin (10 µg/mL) and aprotonin (10 µg/mL) (added to limit protease activity). The tissue was homogenized for 1 to 2 minutes at 4°C in a Polytron homogenizer (Brinkman Instruments). The homogenate was cen-
were simultaneously detected at ground intensity from the indo-1–free samples. Emission intensities $K_{50}$ as a common parameter by using a nonlinear algorithm (Origin, L). For each portion, solutions were diluted to obtain a range of concentrations from 0 to 10 mg/mL protein. Glass capillary tubes were dark brown owing to dissolved $(2 \text{ mmol/L})$, and (3) added indo-1 (5 mmol/L), and (456 nm). Figure 11 shows the relationships between fluorescence intensities were measured from the muscle chamber in solutions and the ends sealed with Future Glue, and data and assuming a hyperbolic binding function: ...

\begin{align}
S_l &= S_{l, \text{blank}} \left( \frac{l_{385, \text{blank}}} {l_{385}} \right) \\
S_{l, \text{max}} &= \frac{l_{385, \text{max}}}{l_{385}} \\
S_{l, \text{min}} &= \frac{l_{385, \text{min}}}{l_{385}} \\
S_l &= S_{l, \text{blank}} + S_{l, \text{AMP}} \left[ \text{protein} \right] + K_0
\end{align}

where $l_{385}$ is the indo-1 intensities from the Ca$^{2+}$- and EGTA-containing solutions, respectively, and $l_{385, \text{blank}}$ is the background intensity from the indo-1–free samples. Emission intensities were simultaneously detected at $\lambda=385, 429$ (indo-1 isosbestic wavelength), and 456 nm. Figure 11 shows the relationships between $S_l$ and protein concentration. As we have also shown previously, $S_l$ increases with increasing protein concentration. At high protein concentrations, the solutions were dark brown owing to dissolved myoglobin, and this significantly reduced the indo-1 fluorescence intensities (relative to the intensities from the blank). $S_l$ at concentrations $>10 \text{ mg/mL}$ were therefore extrapolated by fitting the data and assuming a hyperbolic binding function:

\begin{align}
S_l &= S_{l, \text{blank}} \left( \frac{l_{385, \text{blank}}} {l_{385}} \right) \\
S_{l, \text{max}} &= \frac{l_{385, \text{max}}}{l_{385}} \\
S_{l, \text{min}} &= \frac{l_{385, \text{min}}}{l_{385}} \\
S_l &= S_{l, \text{blank}} + S_{l, \text{AMP}} \left[ \text{protein} \right] + K_0
\end{align}

It was assumed that $K_0$ was independent of wavelength but not $S_{l, \text{blank}}$ and $S_{l, \text{AMP}}$. The 3 data sets were therefore fitted simultaneously with $K_0$ as a common parameter by using a nonlinear algorithm (Origin, MicroCal Software Inc). We thus obtained $K_0=12.3 \text{ mg/mL}$, $S_{385, \text{AMP}}=0.12$, $S_{429, \text{AMP}}=0.28$, $S_{456, \text{AMP}}=1.30$, $S_{385, \text{AMP}}=0.81$, and $S_{456, \text{AMP}}=3.22$. It is unlikely that the interactions between indo-1 and proteins in the homogenate exactly mimic the intracellular interactions, and we therefore used our previously employed ad hoc method of selecting a suitable protein concentration. Because the in vivo indo-1 isosbestic wavelength was 429 nm (ie, $S_{429}=1.00$ in vivo), it would also be required that $S_{429}=1.00$ in a protein solution of the appropriate concentration. At an (extrapolated) concentration of $\approx 15.9 \text{ mg/mL}$ (see Figure 11), $S_{429}=1.00$ (as required), $S_{385}=0.11$, and $S_{456}=2.61$, similar to our previous values.\(^{22}\)

Acknowledgments

This study was supported by National Heart, Lung, and Blood Institute grants HL-57562 to Dr Brandes and HL-52478 to Dr Bers and grants from the Deutsche Forschungsgemeinschaft and the Boehringer Ingelheim Fonds to Dr Maier. We thank Drs Ming Qi, Kenneth S. Ginsburg, and Allen Samuel for assisting with the surgery and hemodynamic measurements.

References


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_Circ Res._ 1998;82:1189-1198
doi: 10.1161/01.RES.82.11.1189

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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