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RESEARCH ARTICLE

Successive contractile periods activate mitochondria at the onset of contractions in intact rat cardiac trabeculae

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Wüst RC, Stienen GJ. Successive contractile periods activate mitochondria at the onset of contractions in intact rat cardiac trabeculae. *J Appl Physiol* 124: 1003–1011, 2018. First published January 11, 2018; doi:10.1152/japplphysiol.01010.2017.—The rate of oxidative phosphorylation depends on the contractile activity of the heart. Cardiac mitochondrial oxidative phosphorylation is determined by free ADP concentration, mitochondrial Ca^{2+} accumulation, mitochondrial enzyme activities, and Krebs cycle intermediates. The purpose of the present study was to examine the factors that limit oxidative phosphorylation upon rapid changes in contractile activity in cardiac muscle. We tested the hypotheses that prior contractile performance enhances the changes in NAD(P)H and FAD concentration upon an increase in contractile activity and that this mitochondrial "priming" depends on pyruvate dehydrogenase activity. Intact rat cardiac trabeculae were electrically stimulated at 0.5 Hz for at least 30 min. Thereafter, two equal bouts at elevated stimulation frequency of 1, 2, or 3 Hz were applied for 3 min with 3 min of 0.5-Hz stimulation in between. No discernible time delay was observed in the changes in NAD(P)H and FAD fluorescence upon rapid changes in contractile activity. The amplitudes of the rapid changes in fluorescence upon an increase in stimulation frequency (the on-transients) were smaller than upon a decrease in stimulation frequency (the off-transients). A first bout in glucose-containing superfusion solution resulted, during the second bout, in an increase in the amplitudes of the on-transients, but the off-transients remained the same. No such priming effect was observed after addition of 10 mM pyruvate. These results indicate that mitochondrial priming can be observed in cardiac muscle in situ and that pyruvate dehydrogenase activity is critically involved in the mitochondrial adaptation to increases in contractile performance.

NEW & NOTEWORTHY Mitochondrial respiration increases with increased cardiac contractile activity. Similar to mitochondrial "priming" in skeletal muscle, we hypothesized that cardiac mitochondrial activity is altered upon successive bouts of contractions and depends on pyruvate dehydrogenase activity. We found altered bioenergetics upon repeated contractile periods, indicative of mitochondrial priming in rat myocardium. No effect was seen when pyruvate was added to the perfusate. As such, pyruvate dehydrogenase activity is involved in the mitochondrial adaptation to increased contractile performance.

glucose; mitochondrial energetics; myocardium; pyruvate; repeated contractions

INTRODUCTION

In a healthy heart, an increase in contractile activity results in a rapid increase in the rate of oxidative phosphorylation in the mitochondria (in situ mechanical-energetic coupling). In isolated mitochondria, an increase in free ADP concentration provides a feedback signal regulating mitochondrial oxidative phosphorylation with first-order Michaelis-Menten kinetics (e.g., Ref. 10). However, the in vivo ADP concentration does not change significantly upon a rapid increase in cardiac workload (3), indicating that other factors contribute to the regulation of oxidative phosphorylation in intact cardiac muscle. Indeed, in intact cell systems, oxidative phosphorylation is governed by a higher order, allosteric mechanism (2, 27, 32, 34). The process underlying this control mechanism that has received the most attention is the accumulation of mitochondrial calcium, increasing Krebs cycle enzyme activities and/or mitochondrial complexes, effectively resulting in an increase in the rate of mitochondrial respiration (2, 14). These processes are influenced by the buffering capacity of phosphocreatine (15), substrate availability (12, 26), transporter activities (22), and the dependency of Krebs cycle enzyme activities on the concentration of intermediates and cofactors (36).

The consequence of these activity-related alterations is that cardiac mitochondrial bioenergetics are likely time dependent, similar to skeletal muscle (24, 35). In skeletal muscle, this prior exercise effect, generally referred to as "priming" (see Ref. 24), has been proposed to be the result of increased microvascular O_2 delivery (19, 23) and/or the increase in pyruvate dehydrogenase (PDH) activity upon an increase in repeated contractions (alleviating the intramuscular "inertia") (16, 17, 20, 29). More recently, studies using isolated skeletal muscle fibers suggest that the activation of the mitochondrial complexes shows a time delay at onset of contractions (35) and that oxidation of NADH is faster following prior contractions (13). We previously demonstrated that cardiac trabeculae do not show a time delay in changes in NAD(P)H and FAD fluorescence upon an increase in contractile output, indicative of an effective coupling between contractile and energetic function (34). Whether cardiac muscle shows any alterations in contractile and mitochondrial function after a prior bout of increased contractile activity is, however, unknown.

Here, we adopted a photometry-based technique to simultaneously measure contractile properties and NAD(P)H and FAD fluorescence in intact rat cardiac trabeculae (4–8, 30, 31, 34). NAD(P)H and FAD fluorescence showed mirror-like respo-

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nses upon a rapid change in stimulation frequency (34). The NAD(P)H and FAD responses upon an increase in stimulation frequency consist of an early rapid response, which coincides with the increase in contractile output (21). The rapid drop in NAD(P)H fluorescence is indicative of net NADH oxidation through mitochondrial complex I. Similarly, the rapid rise in FAD fluorescence reflects net $FADH_2$ oxidation through mitochondrial complex II.

To study time-dependent aspects of contractile and mitochondrial function, we applied two repeated bouts of increased stimulation frequency (1, 2, and 3 Hz) while the stimulation frequency was 0.5 Hz before and in between bouts. We tested the hypothesis that previous increased contractile activity enhances the changes in NAD(P)H and FAD fluorescence (priming). Since PDH activity is known to be higher at the start of the primed contractions (17) and can alleviate the metabolic inertia related to activation of the Krebs cycle enzymes, we added pyruvate to glucose-superperfused rat trabeculae to activate PDH. We hypothesized that this would alter NAD(P)H and FAD kinetics and mitigate the metabolic effects of successive contractions in cardiac muscle, as suggested from ^{31}P-NMR studies (12).

MATERIALS AND METHODS

Animals and Ethical Approval

All protocols were in accordance with the guidelines of the Animal Experimental Welfare Committee of the VU University Medical Centre (Amsterdam, The Netherlands), where all experiments were performed.

Experimental Conditions

The isolation procedure of the cardiac trabeculae was as described previously (31, 34). In short, male Wistar rats (~300 g, $n = 10$) were anaesthetized by isoflurane inhalation. The heart was rapidly removed and retrogradely (Langendorff) perfused with a modified Krebs-Henseleit (Tyrode) solution, consisting of the following (in mM): 118 NaCl, 4.5 KCl, 0.5 $CaCl_2$, 0.33 NaH_2PO_4, 1 $MgCl_2$, 25 $NaHCO_3$, and 10 glucose, gassed with 95% O_2-5% CO_2 at pH 7.45, and supplemented with 20 mM 2,3-butanedione monoxime to inhibit crossbridge cycling. A thin trabecula (diameter: <200 μm) from the right ventricle was dissected and mounted in an experimental chamber between a force transducer and a micromanipulator. The trabecula was constantly superfused with oxygenated Tyrode solution (without 2,3-butanedione monoxime) to guarantee adequate oxygen supply. For the pyruvate superfusion ($n = 6$), the glucose containing Tyrode solution was supplemented with 10 mM sodium pyruvate. We refer to this latter solution as pyruvate and to the solution with only glucose as glucose. The optimal length of the preparation was determined while the trabecula was stimulated at 0.5 Hz, and the preparation was stretched until developed tension was maximal. On the basis of a recent study, we estimate that sarcomere length at rest was 2.1 μm (9). At optimal length, the diameters were measured in two perpendicular directions and the cross-sectional area was calculated assuming an elliptical cross section. Temperature was kept at 27°C to maintain stability of the preparation during the experiments.

Experimental Setup

A detailed description of the setup is given in Wüst et al. (34). In short, the trabecula was supramaximally stimulated and the central part of the trabecula was illuminated at 340 nm to asses NAD(P)H fluorescence, immediately followed by illumination at 480 nm for FAD fluorescence. Emitted light with a wavelength of 455 nm was guided to a photomultiplier tube (H7360–02MOD; Hamamatsu, Japan) for detection of NAD(P)H fluorescence. FAD fluorescence at 520 nm was detected with a second photomultiplier tube. To minimize movement artifacts and bleaching, fluorescence of NAD(P)H and FAD was examined for a period of 10 ms each at 1-s intervals, during the final diastolic phase of the contraction. Previously, we showed that NAD(P)H in similar trabeculae was 63 ± 10% reduced at 0.5 Hz, and FAD was 57 ± 19% oxidized at 0.5 Hz (34). In cardiac tissue, the NAD(P)H fluorescence signal primarily arises from the mitochondrial pool of NADH (11) and the contribution of NADPH is negligible (25).

Experimental Protocol

The experimental protocol was started with experiments ($n = 10$ trabeculae) in glucose by recording force and NAD(P)H and FAD fluorescence signals at a stimulation frequency of 0.5 Hz for 3 min. Thereafter, the trabecula was stimulated at a higher stimulation frequency of 1, 2, and 3 Hz for at least 3 min to study the tension-frequency relationship.

Mitochondrial priming was studied after a recovery period of at least 30 min at 0.5-Hz stimulation. This was followed by a first bout of 3 min at a stimulation frequency of 1, 2, or 3 Hz, followed by a recovery period at 0.5 Hz of 3 min. Thereafter, a second bout with the same stimulation frequency as the first bout was applied. An equilibration period at 0.5-Hz stimulation of at least 30 min was applied between successive runs, and stimulation sequences at 1, 2, and 3 Hz were selected at random. In six trabeculae, this protocol was repeated after the addition of pyruvate (10 mM) to the superfusate while the trabecula was stimulated at 0.5 Hz and when a new steady state in maximal force production was attained (>45 min). This sequence (first glucose and then pyruvate) was used because the time to reach a steady state in tension development was much larger when the measurements in pyruvate-containing solution preceded those in glucose.

Calculations

Maximal (peak) tension, i.e., peak force divided by the cross-sectional area of the preparation (T_{peak}, in mN/mm^2), was determined for each contraction during the experimental runs. The steady-state T_{peak} reached was used to construct the tension-frequency relationship. The steady-state values for the maximal rate of contraction (MRC, in s^{-1}) and relaxation (MRR, in s^{-1}) normalized for T_{peak} were determined at each stimulation frequency and experimental condition. The tension-time-integral per second (TTI/s, in mN/mm^2) was determined throughout the experimental run and is equal to the averaged developed tension. It is a good indicator of total mechanical output and the associated energy utilization.

The changes in NAD(P)H and FAD fluorescence reflect net changes in concentration. As such, a decrease in NAD(P)H fluorescence indicates that NADH utilization by complex I is larger than NADH supply by the Krebs cycle, whereas the opposite holds for the changes in FAD (through complex II). Background values for NAD(P)H and FAD fluorescence were subtracted, and all values during each experimental run were normalized to the initial value (100%). A very marginal rundown of NAD(P)H and FAD fluorescence over the experimental run of ~15 min was accounted for by correcting the slope of the decline in fluorescence.

The kinetics of the slow changes in NAD(P)H and FAD fluorescence in pyruvate- containing solutions were fitted using a single exponential equation in Prism 5 (GraphPad Software, La Jolla, CA).

Statistical Analysis

Comparisons were made using ANOVA repeated-measures (with substrate, bout, and/or stimulation frequency as within factors, followed by Bonferroni post hoc tests) or paired *t*-tests where appropriate. The level of significance was set at $P < 0.05$. Statis-

tical analyses were performed using SPSS 20.0 (IBM SPSS Statistics, Armonk, NY) or Prism 7 (GraphPad Software). All data are presented as means ± SD.

RESULTS

Contractile Properties and NAD(H) and FAD Fluorescence Changes in Pyruvate

Figure 1 shows typical examples of the effect of a prior bout of increased contractile activity on TTI/s, NAD(P)H, and FAD fluorescence during an experimental run, in which stimulation frequency was increased to 3 Hz in glucose. No time delays in the abrupt changes in NAD(P)H and FAD fluorescence were observed upon the changes in TTI/s.

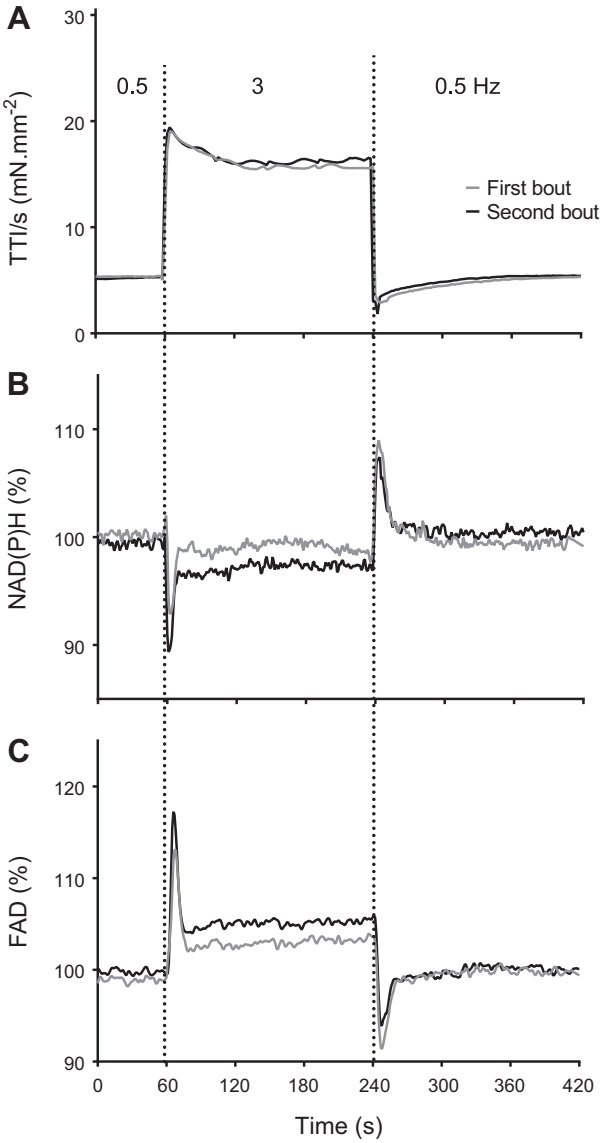

Fig. 1. Representative example of an experimental run in glucose. *A*: tension-time-integral per second (TTI/s). *B*: NAD(P)H fluorescence. *C*: FAD fluorescence in glucose. The first 3 min at increased stimulation frequency (here 3 Hz) were recorded after a period of at least 30 min of stimulation at 0.5 Hz (first bout in gray lines). This was followed by a 3-min stimulation at 0.5 Hz and a subsequent bout at a 3-Hz stimulation for 3 min (second bout in black lines). Note that the changes in NAD(P)H (*B*) are opposite to those in FAD (*C*).

Table 1. *Contractile properties of rat cardiac trabeculae in glucose- and pyruvate-containing superfusion solution in the first and second bout*

	Glucose		Pyruvate	
	First bout	Second bout	First bout	Second bout
T_{peak}, mN/mm^2				
0.5 Hz	21.4 ± 5.9	21.3 ± 6.4	35.1 ± 12.8*	33.5 ± 13.1*
1 Hz	21.5 ± 6.2	23.2 ± 4.0	33.8 ± 13.1*	35.5 ± 14.0*
2 Hz	26.7 ± 9.3#	27.7 ± 8.7#	38.9 ± 12.8*	41.4 ± 13.2*#
3 Hz	30.0 ± 9.2#	30.3 ± 8.2#	43.0 ± 11.0*#	47.5 ± 9.5*#
TTI/s				
0.5 Hz	1.6 ± 1.0	1.6 ± 0.9	3.8 ± 2.6*	3.7 ± 2.7*
1 Hz	3.0 ± 1.7#	3.0 ± 1.8#	6.8 ± 4.6*#	7.1 ± 4.7*#
2 Hz	5.7 ± 2.6#	5.9 ± 2.6#	13.7 ± 5.7*#	14.4 ± 5.8*#
3 Hz	9.9 ± 4.1#	10.8 ± 4.2#	19.9 ± 8.8,#	20.1 ± 9.2*#
MRC				
0.5 Hz	12.5 ± 0.5	12.9 ± 0.7	9.1 ± 1.1*	9.7 ± 1.3*
1 Hz	13.8 ± 0.6#	13.9 ± 0.7	10.4 ± 2.0*	10.1 ± 1.8*
2 Hz	14.9 ± 0.5#	15.2 ± 0.3#	12.1 ± 1.3*#	12.0 ± 1.2*#
3 Hz	16.7 ± 0.8#	16.7 ± 1.1#	12.9 ± 1.0*#	13.0 ± 1.1*#
MRR				
0.5 Hz	7.0 ± 2.7	8.3 ± 1.0	6.3 ± 0.8*	6.9 ± 0.6*
1 Hz	8.3 ± 1.2#	8.3 ± 1.2	6.8 ± 0.8*	6.8 ± 0.9*
2 Hz	9.3 ± 1.6#	9.5 ± 1.8#	7.9 ± 1.3*#	8.1 ± 1.2*#
3 Hz	10.7 ± 2.3#	10.7 ± 2.3#	9.3 ± 2.1*#	9.8 ± 2.1#

Values are means ± SD. T_{peak}, maximal tension (mN/mm^2); TTI/s, tension time integral per second (mN/mm^2), which is equal to average tension; MRC, maximal rate of contraction, normalized for T_{peak} (s^{-1}); MRR, maximal rate of relaxation, normalized for T_{peak} (s^{-1}). *$P < 0.05$, between substrates. #$P < 0.05$, compared with 0.5 Hz (ANOVA, followed by a Bonferroni post hoc test).

Contractile Properties

The frequency-dependent steady-state contractile properties in the second bout were not significantly different from the first bout (Table 1). These results indicate that contractile activity is not influenced by a prior bout of high stimulation and that the difference in contraction-related energy utilization between subsequent bouts, if any, would be very small.

NAD(P)H and FAD Responses

Early fast responses. In glucose, the time to reach the initial minima (and maxima) in NAD(P)H and FAD responses was similar in NADH(P)H and FAD, and the values were averaged. Time to reach minima and time to reach maxima were larger for 1 Hz (9.6 ± 3.8 s) compared with 2 and 3 Hz (6.3 ± 2.0 and 6.6 ± 1.8 s, respectively; $P = 0.004$) and were also longer for the on-transient (8.3 ± 2.8 s) compared with the off-transient (5.9 ± 2.3 s; $P = 0.003$). No differences in time course were observed between the first and second bout ($P = 0.97$) nor between NAD(P)H and FAD fluorescence ($P = 0.99$). These results suggest that the time to reach the initial minima (and maxima) was reached faster at higher stimulation frequencies but was not influenced by prior contractions.

Amplitudes of the early rapid changes. The amplitudes of the early rapid changes in NAD(P)H fluorescence were linearly related to the changes in average tension (ΔTTI/s), both during the on- and off-transients (Fig. 2, *A* and *B*). In glucose, the initial decline in NAD(P)H during the second bout was larger than during the first bout ($P = 0.001$; Fig. 2*A*), and the difference increased with stimulation frequency (interaction effect: $P = 0.001$). This effect was not explained by the contractile output in the second bout, because the averaged

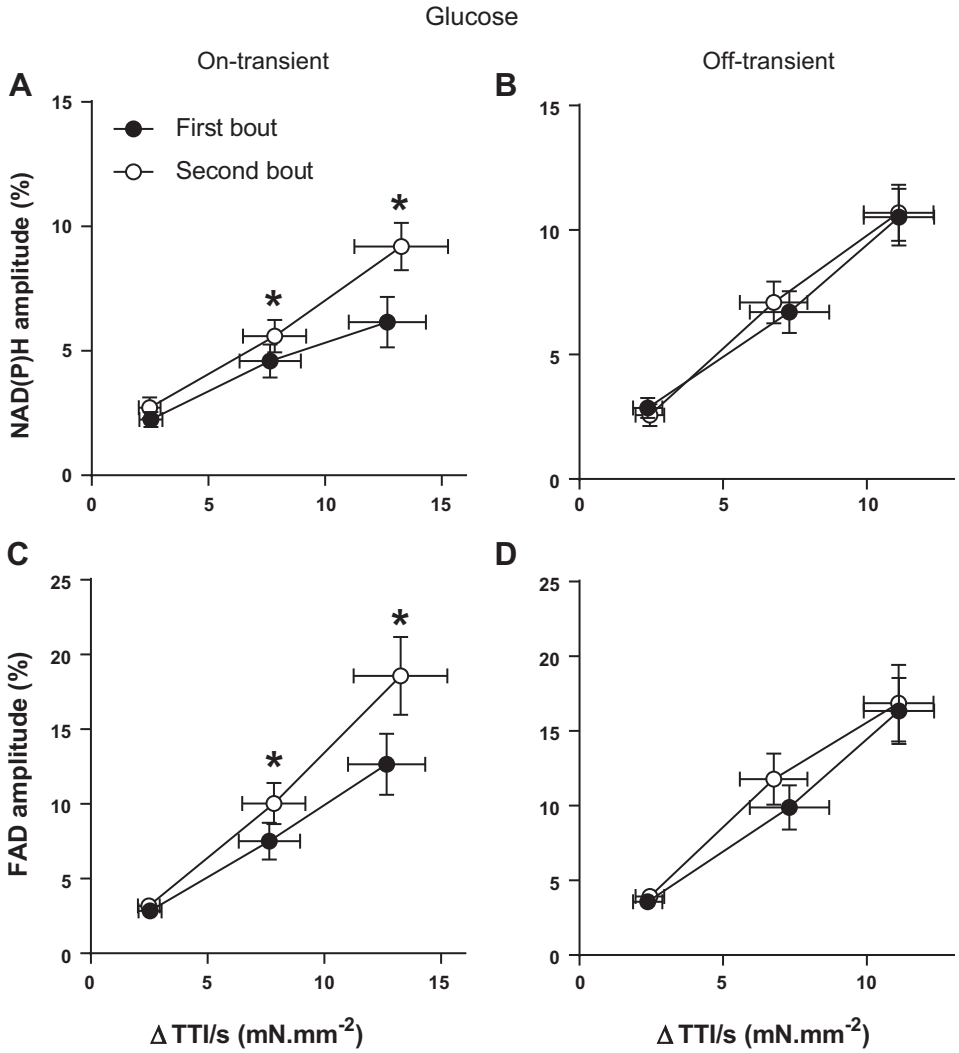

Fig. 2. Effects of a prior contractile period on the amplitude of the early changes in NAD(P)H and FAD fluorescence in glucose. NAD(P)H amplitude plotted as a function of in the change in the tension time integral per second (ΔTTI/s) for the on-transient (*A*) and off-transient (*B*) in the first and second bouts at 1, 2, and 3 Hz in glucose ($n = 10$). Corresponding FAD data for the on-transient (*C*) and off-transient (*D*). In glucose, prior contractions resulted in larger NAD(P)H and FAD amplitudes in the on-transient (*A* and *C*) but not in the off-transients (*B* and *D*). The on-off asymmetry in NAD(P)H and FAD amplitude was also reflected in differences in the slope. Results are shown as means ± SE. $*P < 0.05$, between first and second bout (ANOVA, followed by a Bonferroni post hoc test).

slope between ΔTTI/s and NAD(P)H amplitude was different between the first and second bout: 0.59 ± 0.22 vs. 0.80 ± 0.29%·mm^{-2}·mN^{-1}, respectively ($P = 0.008$; Fig. 2*A*). No differences in NAD(P)H during the off-transient were observed between the first and second bout ($P = 0.599$; Fig. 2*B*). This suggests that the effects on mitochondrial activation are fully developed during the first bout at elevated stimulation frequency and are maintained during the second bout. Similar effects were observed in the averaged slope of the relation between FAD amplitude and ΔTTI/s in glucose during the on-transient (1.17 ± 0.62 and 1.65 ± 0.76%·mm^{-2}·mN^{-1} for first vs. second bout, respectively; $P < 0.001$; Fig. 2*C*), whereas also in this case the slopes of the off-transient during the first and second bout were not different ($P = 0.894$; Fig. 2*D*).

Taken together, these results indicate that the fast NAD(P)H and FAD kinetics in glucose are modified by prior bouts at elevated stimulation frequency. Importantly, the amplitude of the second bout was larger, indicative of large NAD(P)H and $FADH_2$ utilization rates in the electron transport system, compared with NAD(P)H and $FADH_2$ production rates in the Krebs cycle.

Slow recovery toward steady-state during high workload. While T_{peak} (and TTI/s) reached a new steady state after ~20 s, NAD(P)H and FAD fluorescence did not stabilize until ~90 s (at 3 Hz) or ~160 s (at 1 Hz). Compared with the initial values before the increase in the rate of stimulation, the final values at 3 Hz in glucose for NAD(P)H tended to be lower (95 ± 6%; $P = 0.09$) and were higher for FAD (110 ± 10%; $P = 0.02$). No differences were observed in the final values between the first and second bout ($P = 0.49$). In glucose, the slow recovery phase was not very pronounced and showed a variable time course, which precluded characterization.

Contractile Properties and NAD(H) and FAD Fluorescence Changes in Pyruvate

Contractile properties. T_{peak} at 0.5 Hz was on average 53 ± 22% larger in pyruvate than in glucose ($P = 0.008$; Table 1; Fig. 3*A*). MRC and MRR were significant lower in pyruvate. These contractile differences were maintained at higher stimulation frequencies. The tension-frequency relationships were positive both in glucose and in pyruvate ($P = 0.001$; Fig. 3*B*). These contractile alterations resulted in an approximately two-fold increase in the steady-state TTI/s in pyruvate compared with glucose ($P = 0.034$; Fig. 3*C*). TTI/s increased linearly with stimulation frequency, but the absolute increase in TTI/s

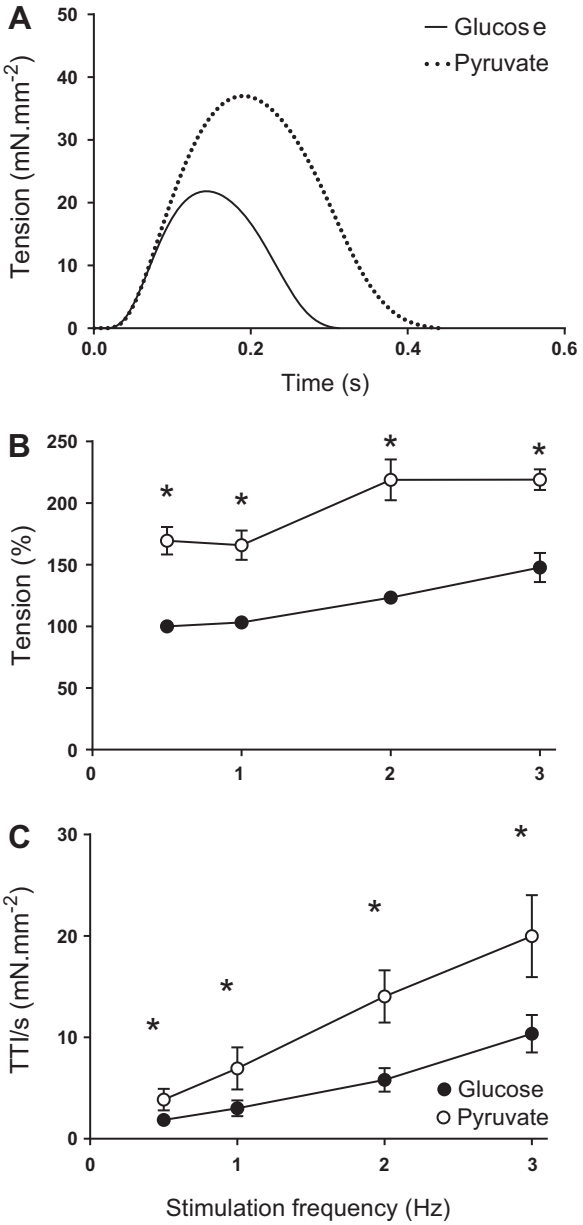

Fig. 3. Force-frequency relationship in glucose and pyruvate containing solutions. *A*: steady- state tension response in glucose and pyruvate containing solution at a stimulation frequency of 0.5 Hz. Both amplitude and duration of the tension transients in pyruvate were larger than in glucose. *B*: a positive tension-frequency relationship was observed in glucose as well as in pyruvate. *C*: the tension-time-integral per second (TTI/s) was linearly related to stimulation frequency in both glucose and pyruvate, but the slopes were different. Data are means ± SE ($n = 6$). *$P < 0.05$, between substrates (ANOVA, followed by a Bonferroni post hoc test).

was larger in pyruvate compared with glucose (interaction effect: $P = 0.002$; Fig. 3*C*). No differences in T_{peak}, TTI/s, MRC, and MRR between the first and second bout were observed in pyruvate (Table 1).

Early fast responses. Figure 4 shows typical examples of the effect of a prior bout of increased contractile activity on TTI/s, NAD(P)H, and FAD fluorescence during an experimental run in pyruvate, in which stimulation frequency was increased to 3 Hz.

As was the case in glucose, no time delays in the abrupt changes in NAD(P)H and FAD fluorescence were observed upon the changes in TTI/s. In pyruvate-containing solution, the time to reach the initial minima (and maxima) in NAD(P)H and FAD responses was not different between the first and second bout, and the data were pooled [8.9 ± 3.7 and 9.3 ± 1.3 s for NAD(P)H and FAD, respectively]. Time to reach minima and time to reach maxima were larger for 1 Hz (9.8 ± 1.8 s) compared with 2 and 3 Hz (6.3 ± 1.6 and 6.6 ± 0.9 s, respectively; $P < 0.001$) and were longer for the on-transient (10.4 ± 2.3 s) compared with the off-transient (7.4 ± 2.2 s; $P < 0.001$). The increases in average

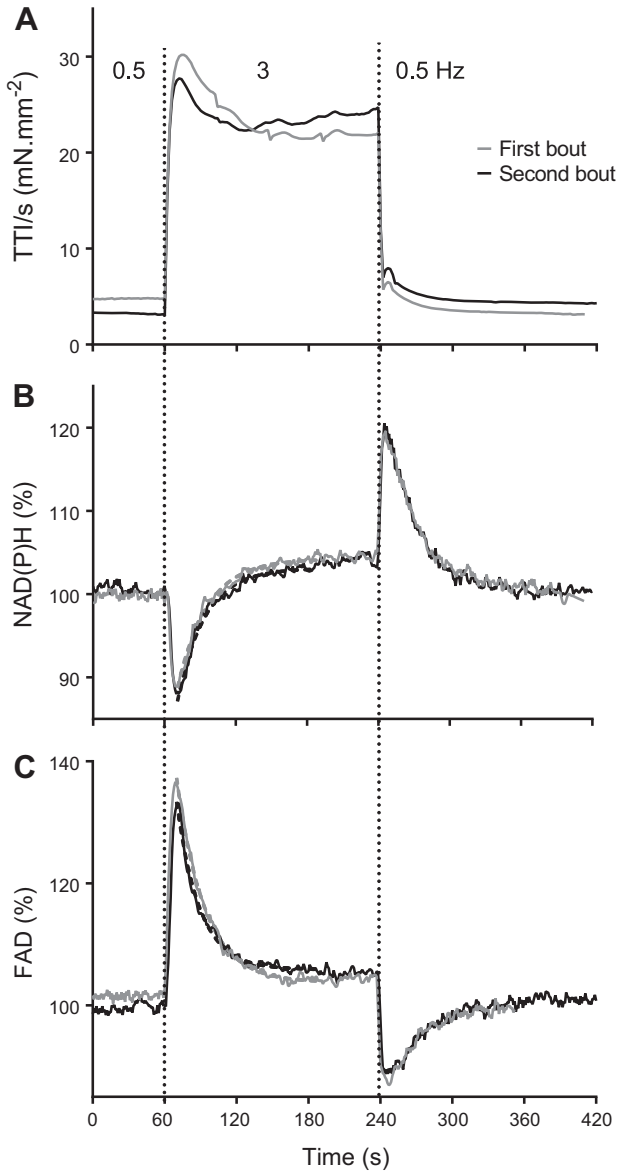

Fig. 4. Representative example of an experimental run in pyruvate. *A*: tension-time-integral per second (TTI/s). *B*: NAD(P)H fluorescence. *C*: FAD fluorescence in pyruvate. The first 3 min at increased stimulation frequency (here 3 Hz) were recorded after a period of at least 30-min stimulation at 0.5 Hz (first bout in gray lines). This was followed by a 3-min stimulation at 0.5 Hz and a subsequent bout at 3-Hz stimulation for 3 min (second bout in black lines). The dotted lines represent the fitted exponential curves. Note that the changes in NAD(P)H (*B*) are opposite to those in FAD (*C*).

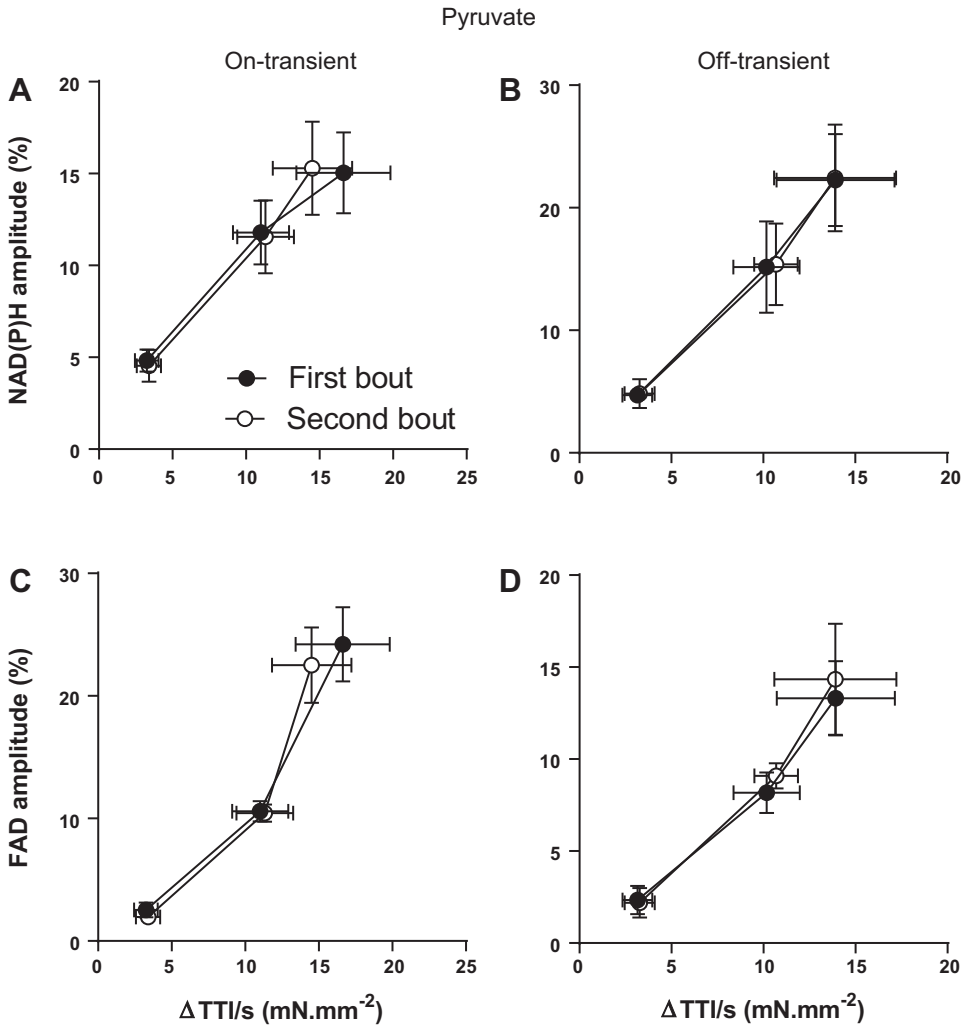

Fig. 5. Effects of a prior contractile period on the amplitude of the early changes in NAD(P)H and FAD fluorescence in pyruvate. NAD(P)H amplitude plotted as a function of the change in the tension time integral per second (ΔTTI/s) for the on-transient (*A*) and off-transient (*B*) in the first and second bouts at 1, 2, and 3 Hz ($n = 6$). Corresponding FAD data for the on-transient (*C*) and off-transient (*D*). The on-off asymmetry in NAD(P)H and FAD amplitude is reflected in a difference in slope (note the differences in scale). Results are shown as means ± SE.

tension (ΔTTI/s) were larger in pyruvate than in glucose, but the NAD(P)H amplitude increased in proportion with ΔTTI/s (Fig. 5). The averaged data showed an excellent correlation between NAD(P)H amplitude and ΔTTI/s during the on-transient ($R^2 = 0.887$; $P < 0.001$; slope: 0.77 ± 0.11%·mm^{-2}·mN^{-1}) and off-transient ($R^2 = 0.864$; $P < 0.001$; slope: 1.37 ± 0.20%·mm^2·mN^{-1}), albeit with marked and highly significant differences ($P < 0.001$) in steepness. The slopes of the relation between FAD amplitude and ΔTTI/s in glucose and pyruvate were similar during the on-transient ($P = 0.403$; slope: 1.00 ± 0.0.38%·mm^2·mN^{-1}), but during the off-transient the slope in pyruvate was less than half of the slope in glucose ($P = 0.001$; 1.84 ± 0.69 vs. 0.59 ± 0.33%·mm^{-2}·mN^{-1} for glucose and pyruvate, respectively). The differences in NAD(P)H amplitudes between glucose and pyruvate at the same stimulation frequency can therefore be attributed to quantitative differences in contractile output.

Importantly, the difference in NAD(P)H and FAD amplitude between the first and second bout was absent in pyruvate ($P = 0.834$; Fig. 5). Similarly, the slopes of the relation between NAD(P)H and FAD amplitudes and ΔTTI/s were not altered ($P = 0.200$ and 0.623 for NAD(P)H and FAD, respectively). The FAD amplitudes in the second bout even tended to become smaller than in the first bout ($P = 0.065$). Taken together, these results indicate that the fast NAD(P)H and FAD kinetics are modified by prior bouts at elevated stimulation frequency in glucose but not in pyruvate.

Slow recovery toward steady-state during high workload. In pyruvate, recovery of NAD(P)H and FAD fluorescence took place in a monoexponential fashion (Fig. 6). The rate constants governing the slow recovery in NAD(P)H and FAD fluorescence in pyruvate were not different between the first and second bout of contractions during the on-transient ($P = 0.267$ and 0.924, respectively) or the off-transient ($P = 0.399$ and 0.274, respectively). This indicates that prior activity does not significantly modulate the slow adjustment in NAD(P)H and FAD kinetics in pyruvate, and therefore, the rate constants obtained during the first and second bouts were pooled. The (pooled) rate constants of the slow recovery during the on-transient (k_{on}) increased significantly with stimulation frequency [$P = 0.019$ and 0.018 for NAD(P)H and FAD, respectively; Fig. 6] but not during the off-transient (k_{off}; $P = 0.182$ and 0.274, respectively). The value of k_{on} at 3 Hz was significantly higher than k_{off} ($P < 0.05$; Fig. 6). These data indicate a more rapid adjustment of NAD(P)H and $FADH_2$ production to utilization at high stimulation frequencies in pyruvate.

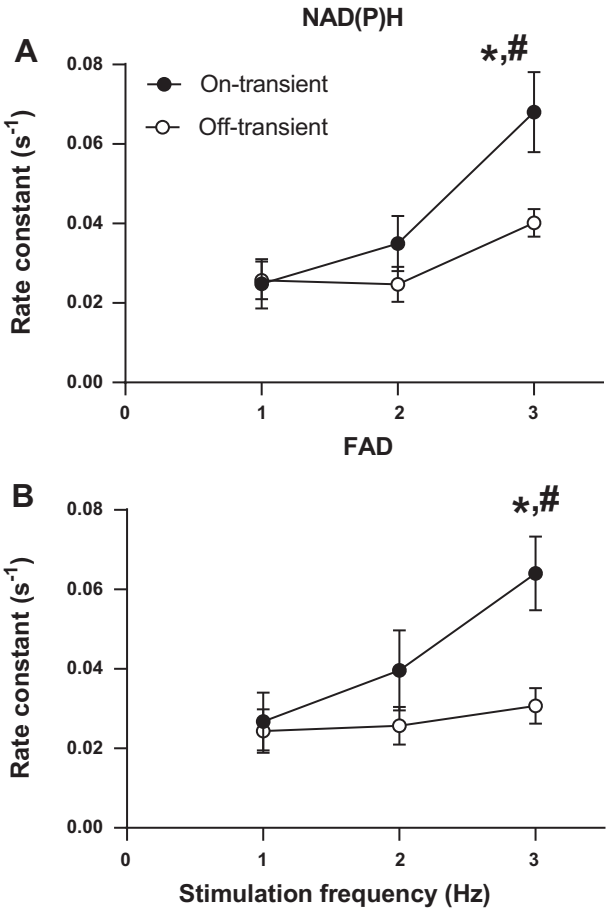

Fig. 6. Rate constants of slow recovery phases at different stimulation frequencies. *A*: rate of NAD(P)H recovery during the on- and off-transients in pyruvate. *B*: rate of FAD recovery during the on- and off-transients. Values of the first and second bout were averaged, because no differences were observed. Results are shown as means ± SE ($n = 6$). *$P < 0.05$, compared with 1 Hz. #$P < 0.05$, compared with off-transient (ANOVA, followed by a Bonferroni post hoc test ($P < 0.05$).

The final values reached at 3 Hz were larger than initial values for NAD(P)H (106.1 ± 3.7%; $P = 0.021$) but not for FAD (98.7 ± 7.2%; $P = 0.480$).

DISCUSSION

In this study, NAD(P)H and FAD kinetics were measured simultaneously, on a second-to-second basis in intact cardiac trabeculae, to study the processes underlying the early fast as well as the later slow responses to increased contractile activity. We observed that *1*) a first bout in glucose-containing superfusion solution resulted, during the second bout, in an increase in the amplitudes of the on-transients, but the off-transients remained the same; and *2*) this priming effect of the on-transient was absent after addition of pyruvate (10 mM) to the superfusate.

Successive Contractile Periods

Mitochondrial activity was markedly altered by a prior bout of elevated stimulation frequency but interestingly only in glucose-perfused trabeculae. In glucose, the early rapid increases in NAD(P)H and FAD fluorescence upon the second (primed) bout were considerably larger than those during the first (unprimed) period of increased stimulation. The rapid changes and the subsequent slow recovery in NADH and FAD originate, as discussed previously (see Fig. 7 in Ref. 34), from a dysbalance of the velocity of NADH/$FADH_2$ supply and demand. These differences could either be explained by an increase in the rate of NADH and $FADH_2$ oxidation, i.e., complex I and II activity, or by a blunted rise in the rate of NAD^+ and FAD reduction in the Krebs cycle. The latter possibility, however, seems unlikely because Krebs cycle enzyme activity is expected to be higher, rather than lower in the primed condition (12, 17). Rather, mitochondrial complexes (not only complex I and II, but also others) are activated to a net larger extent upon an increase in stimulation frequency and subsequent mitochondrial calcium concentration (14, 33) and remained activated during the off-transient and subsequent bouts. Indeed, in skeletal muscle fibers, mitochondrial enzyme deactivation at the off-transient is suggested to be slower than the rapid mitochondrial activation at the onset of contractions (13, 35). Here, mitochondrial enzyme activity remained elevated for at least 3 min.

There was no delay in the change in NAD(P)H and FAD fluorescence upon a change in contractile activity, and NAD(P)H and FAD fluorescence slowly recovered toward baseline values throughout the stimulation period. This was different compared with changes in NAD(P)H fluorescence in skeletal muscle (13). In a second, successive, bout of contractions in skeletal muscle, a faster NADH utilization rate and a delayed and less pronounced decrease in mitochondrial membrane potential upon an increase in stimulation frequency (13) suggest that in primed skeletal muscle mitochondria the redox potential [i.e., NAD(P)H/NAD(P)] and the proton motive force (13) are more in equilibrium. Here, we also observed larger NADH and $FADH_2$ utilization rates at the start of the primed bout, implying that mitochondrial complex activity was higher in the second compared with the first bout. Whether this would also result in alterations in mitochondrial membrane potential, faster oxygen consumption rates (at complex IV), and/or ATP phosphorylation rates (at complex V) is not known.

The observation that mitochondrial priming was only seen in glucose and not in pyruvate can be attributed to a higher PDH activity in pyruvate (26, 36). It is well established that the maximal PDH capacity is not fully exploited at high cardiac output in glucose (26). This is in contrast to the situation in pyruvate where PDH activity was higher at low and high cardiac output (26). Surprisingly, however, the NAD(P)H and FAD amplitudes were larger, rather than smaller in pyruvate, despite the higher production rates in the Krebs cycle. These results suggest that pyruvate is able to relieve the metabolic inertia (16), such that complex I and II activity adapt more rapidly at the start of the bout. Higher PDH activity also directly increases mitochondrial complex I activity through deactivation of PDH kinase (28).

Another possibly contributing factor could be a shift in the relative contributions of the oxidative and nonoxidative metabolism at the first and the second train of contractions by changes in phosphocreatine kinetics. However, the free ADP concentration does not change over a range of rate-pressure products in cardiac muscle (3). Indeed, a recent overview of the cardiac ^{31}P-MRS literature (1) found no changes in phospho-

creatine/ATP with increased heart rate. Therefore, we expect that this contribution, if present, would be small.

Substrate Dependency: Glucose vs. Pyruvate

In agreement with previous studies (18, 36), we observed a larger amplitude and duration of the contractile responses in pyruvate compared with glucose, which resulted in an approximately twofold larger TTI/s at the same stimulation frequency. This increase in contractile output is most likely attributable to cytosolic factors, such as changes in calcium handling, intracellular pH, inorganic phosphate concentration, and ΔG_{ATP} (36). The alterations in Ca^{2+} handling result in an increase in Ca^{2+} loading of the sarcoplasmic reticulum, which causes an increased Ca^{2+} release during contraction, while a decline in inorganic phosphate concentration and an increase in cytosolic pH both result in an increased Ca^{2+} sensitivity of the myofilaments (18). In concert, these factor contribute to an increase and prolongation of force production. These changes might be of therapeutic relevance (18), but the linear relationship between NAD(P)H and FAD amplitude and ΔTTI/s was preserved. Our study thus illustrates that the higher contractile output in pyruvate is accompanied by a larger energy demand, which requires an adequate mitochondrial adaptation (31). Further studies are warranted to assess potential energetic benefits of pyruvate.

Previous studies in cardiac trabeculae focused on NAD(P)H kinetics using pyruvate-perfusion only (5–8). Despite that pyruvate increased Krebs cycle enzyme activities (and speeded up NADH and $FADH_2$ utilization upon an increase in contractile activity), on-off asymmetry also occurred in pyruvate-containing medium. This asymmetry is consistent with a parallel activation of the NADH-producing and -utilizing pathways, both in glucose and in pyruvate, when energy requirements are increased. An increase in Krebs cycle enzyme and mitochondrial activities throughout the bout of increased ATP utilization likely underlies the on-off-asymmetry in NAD(P)H and FAD kinetics in glucose and pyruvate. Fast uptake and slow restitution of mitochondrial calcium upon changes in stimulation frequency (33) and its effect on NADH- and $FADH_2$-utilizing pathways could be responsible for this phenomenon.

It can be noted that the final values of the slow recovery phase at 3 Hz were larger than initial values for NAD(P)H, but not for FAD. We attribute this latter finding to the relatively large variability in FAD fluorescence in the recordings. The rapid drop in FAD concentration upon a decrease in stimulation frequency (the off-transient, Fig. 5*D*) in pyruvate was smaller than expected from the measurements in glucose. This suggests that succinate dehydrogenase (mitochondrial complex II) kinetics are altered upon the addition of pyruvate.

Conclusion

Our experiments show that a prior bout of elevated contractile activity influences mitochondrial bioenergetics and therefore provides evidence for priming of mitochondrial activity in cardiac muscle tissue. Our results also indicate that PDH activity is critically involved in the process of mitochondrial adaptation to increases in contractile performance in cardiac tissue.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.C.W. and G.J.M.S. conceived and designed research; R.C.W. and G.J.M.S. performed experiments; R.C.W. and G.J.M.S. analyzed data; R.C.W. and G.J.M.S. interpreted results of experiments; R.C.W. and G.J.M.S. prepared figures; R.C.W. and G.J.M.S. drafted manuscript; R.C.W. and G.J.M.S. edited and revised manuscript; R.C.W. and G.J.M.S. approved final version of manuscript.

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