Almitrine and doxapram decrease fatigue and increase subsequent recovery in isolated rat diaphragm

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McGuire, Michelle, Michael F. Carey, and John J. O’Connor. Almitrine and doxapram decrease fatigue and increase subsequent recovery in isolated rat diaphragm. J. Appl. Physiol. 83(1): 52–58, 1997.—The effects of almitrine bimesylate and doxapram HCl on isometric force produced by in vitro rat diaphragm were studied during direct muscle activation at 37°C. Doxapram and almitrine ameliorate respiratory failure clinically by indirectly increasing phrenic nerve activity. This study was carried out to investigate possible direct actions of these agents on the diaphragm before and after fatigue of the fibers. Two age groups of animals were chosen (6–14 wk (group 1) and 50–55 wk (group 2)) because it is known that increasing age decreases a muscle fiber’s resistance to fatigue. Muscle strips were isolated from both group 1 and group 2 and directly stimulated (2-ms pulse duration, 5–15 V) to produce twitch tensions of 1.3 and 2.1 N/cm², respectively. At low concentrations, doxapram (=20 µg/ml) and almitrine (=12 µg/ml) had no effect on twitch contraction or 100-Hz tetanic tension. However, 40 µg/ml doxapram and 30 µg/ml almitrine increased twitch tension by 9.0 ± 1.4 and 11.6 ± 1.9%, respectively, in animals of group 2 (n = 5). A fatigue protocol consisting of low-frequency stimulation (30-Hz trains, 250-ms duration every 2 s for 5 min) caused a reduction of twitch tension in animals of group 1 (48 ± 4% of control) and group 2 (28 ± 4% of control). At 90 min postfatigue, the twitch tension recovered to 72 ± 3 and 42 ± 2% of control values in group 1 and group 2, respectively. In the presence of doxapram (20 µg/ml), there was a significant increase in the recovery of twitch tension at 90 min in group 1 and group 2 (84.5 ± 3.2 and 80.1 ± 2.8%, respectively) compared with controls at 90 min postfatigue. In the presence of almitrine (12 µg/ml), there was a full recovery from fatigue in group 1 animals (100% of control) and a recovery to 95.6 ± 2.1% of control in group 2 animals at 90 min. These results demonstrate a significant improvement in the rapidity and magnitude of recovery from fatigue in the rat diaphragm muscle in the presence of both doxapram and, especially, almitrine. These effects may be due to changes in intracellular calcium, ADP/ATP ratios, or oxygen free radical scavenging.

almitrine bimesylate; doxapram hydrochloride; muscle fatigue; direct stimulation

RESPIRATORY MUSCLE FATIGUE is well recognized as one of the major causes of respiratory failure (5), and many pharmacological agents have been used in the treatment of this condition with variable success. Diaphragmatic contractility has been reported to be increased by aminophylline, which also increases phrenic nerve activity (2, 3, 9). The peripheral chemoreceptor stimulators almitrine and doxapram have also been used to increase ventilation in respiratory failure by indirectly increasing efferent phrenic nerve activity (19). It seems illogical that using these agents to increase phrenic nerve activity to an already fatigued diaphragm should increase diaphragmatic performance. The fact that this does happen in some cases prompted us to study the effects of almitrine and doxapram on fatigued diaphragm in vitro.

It has been recently shown that prolonged periods of muscular ischemia and deoxygenation can induce muscle injury that is free radical mediated (1, 25, 31). During repetitive contraction of muscle fibers, there is an increase in free radical concentration in the myocyte (26, 27). Almitrine has recently been shown to have oxygen free radical-scavenging ability (6, 7). It also increases calcium release from the sarcoplasmic reticulum and is used in the treatment of respiratory failure (32). At present, however, the basic mechanisms of action of almitrine are unknown. Doxapram is another compound reported to be useful in the treatment of respiratory failure (4, 23), although its direct mode of action on myocytes is not well documented.

The rat diaphragm is particularly resistant to fatigue because of its mixed composition of fiber types, namely, 27% fast twitch, glycolytic; 39% slow twitch, oxidative; and 34% fast twitch, oxidative, glycolytic (see Ref. 30). This mixed fiber type ratio is reasonably similar to that found in humans, with shortening velocities and adeninetriphosphatase activity similar to those of myosin (30). However, with repeated stimulation, the diaphragm becomes fatigued (fast-twitch glycolytic fibers have the lowest resistance to fatigue), and its ability to develop tension is reduced (29).

It has previously been demonstrated that many biochemical and morphological changes occur in skeletal muscle with aging (13). Increasing age also decreases the maximum tension developed by the diaphragm, and the older diaphragm has less resistance to fatigue (21, 33). In this study, a low-frequency protocol was used to give a reproducible fatigue and recovery profile in diaphragm muscle strips from young (6–14 wk) and more mature animals (50–55 wk; Ref. 24). The effects of almitrine and doxapram on this profile were then investigated.

METHODS

Tissue preparation and solutions. Male Wistar rats were allocated to two groups according to weight. Group 1 animals weighed 114.3 ± 24.9 g (range 50–150 g; age 6–14 wk; n = 32), and group 2 animals weighed 378.7 ± 51.2 g (range 300–450 g; age 50–55 wk; n = 28). Animals were anesthetized with chloroform and decapitated. The diaphragm and adjacent rib sections were removed in <3 min and placed in a dissection tray filled with modified Krebs-Ringer bicarbonate (KRB) solution of the following composition (in mM): 113 NaCl, 5 KCl, 1.4 CaCl₂, 0.9 MgSO₄, 1.2 NaH₂PO₄, 25 NaHCO₃, and 11.5 glucose. This solution
was aerated continuously with 95% O2-5% CO2. The diaphragm was divided along its central tendon, with one hemidiaphragm acting as control and the other used for drug experiments. A central rectangular muscle strip (4 mm wide) was dissected from each hemidiaphragm. The muscle bundle weight and optimal length (L0) for group 1 were 16.2 ± 3.4 mg and 13.1 ± 1.5 mm and for group 2 were 28.2 ± 3.8 mg and 20.1 ± 2.7 mm, respectively. These values were significantly different between groups (P < 0.01; Student's unpaired t-test; n = 20). The average cross-sectional area (CSA) of muscle bundles for group 1 and group 2 was 1.17 ± 0.19 and 1.8 ± 0.2 mm², respectively, when a muscle density of 1.056 g/cm³ was calculated for both groups.

This muscle strip was suspended vertically in a 100-ml water-jacketed organ bath filled with KRB solution at 37°C and continuously aerated as described above. The costal diaphragm was divided along its central tendon, with one half of the diaphragm included in the bath and continuously aerated with 95% O2-5% CO2. The diameter of doxapram and almitrine was measured when a muscle density of 1.056 g/cm³ was measured.

Baseline single-twitch tension (2-ms duration at supramaximal voltage; 5–15 V, every 5 min) and tetanic tensions (400-ms trains of 2-ms-duration impulses at supramaximal voltage delivered at 100 Hz; every 15 min) were measured in the two groups of rats. Twitch contraction time (times to peak contraction (CT)) and half relaxation time (RT1/2) were measured for a single twitch. Twitch tension was expressed as force per unit CSA (N/cm²).

Statistical comparisons in Fig. 2 were analyzed by unpaired Student’s t-test. Comparisons in Figs. 3 and 4 were done with one-way analysis of variance (ANOVA). Fisher's positively least significant difference test was used for post hoc comparisons. Results were considered significant at the level of P < 0.05. All measurements are expressed as means ± SE.

RESULTS

Single-twitch and tetanic tensions. Group 1 animals developed a mean twitch tension of 1.3 ± 0.1 N/cm² (n = 32), whereas the mature animals, group 2, developed a mean tension of 2.1 ± 0.2 N/cm² (n = 28). Table 1 shows the mean CT and RT1/2 for both groups as well as the ratio of twitch to 100-Hz tetanus (Po). Po carried out the fatigue protocol to that of the single-twitch tension immediately before the protocol. To assess the degree of recovery, single-twitch tension was measured every 5 min for 90 min after the fatigue protocol and compared with the single-twitch tension immediately before the protocol.

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Table 1. Contractile properties of rat diaphragm muscle fibers in the absence and presence of doxapram and almitrine

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Twitch Tension, N/cm²</th>
<th>Time to Peak Contraction, ms</th>
<th>Half Relaxation Time, ms</th>
<th>100-Hz Tension, N/cm²</th>
<th>Twitch-to-100-Hz Tension Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong>&lt;br&gt;Control&lt;br&gt;Control&lt;br&gt;Doxapram&lt;br&gt;(10 µg/ml) (20 µg/ml)&lt;br&gt;Almitrine&lt;br&gt;(12 µg/ml) (30 µg/ml)&lt;br&gt;<strong>Group 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>1.3 ± 0.1</td>
<td>17.7 ± 1.3</td>
<td>36.2 ± 1.9</td>
<td>8.7 ± 0.9</td>
<td>0.15 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>(4)</td>
<td>1.5 ± 0.2</td>
<td>17.8 ± 1.2</td>
<td>37.6 ± 2.3</td>
<td>8.3 ± 1.2</td>
<td>0.18 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>1.1 ± 0.2</td>
<td>18.0 ± 0.6</td>
<td>38.6 ± 1.7</td>
<td>8.3 ± 0.8</td>
<td>0.13 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>(4)</td>
<td>1.5 ± 0.1</td>
<td>15.8 ± 1.3</td>
<td>35.6 ± 0.6</td>
<td>9.4 ± 2.2</td>
<td>0.16 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>1.7 ± 0.2*</td>
<td>14.5 ± 1.7</td>
<td>35.6 ± 1.6</td>
<td>10.25 ± 1.1*</td>
<td>0.17 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>(28)</td>
<td>2.1 ± 0.2</td>
<td>28.4 ± 1.2</td>
<td>54.3 ± 1.5</td>
<td>21.7 ± 0.1</td>
<td>0.1 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>(4)</td>
<td>2.1 ± 0.4</td>
<td>27.4 ± 1.3</td>
<td>55.2 ± 2.3</td>
<td>20.7 ± 2.7</td>
<td>0.1 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>1.8 ± 0.1</td>
<td>32.1 ± 4.3</td>
<td>55.0 ± 0.5</td>
<td>21.6 ± 3.6</td>
<td>0.08 ± 0.01</td>
<td></td>
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<tr>
<td>(3)</td>
<td>2.6 ± 0.3†</td>
<td>31.4 ± 2.6</td>
<td>53.7 ± 2.8</td>
<td>23.7 ± 1.4*</td>
<td>0.11 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>2.0 ± 0.1</td>
<td>25.2 ± 0.9</td>
<td>55.8 ± 1.1</td>
<td>21.9 ± 4.1</td>
<td>0.09 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>(4)</td>
<td>2.5 ± 0.2†</td>
<td>30.2 ± 3.1</td>
<td>54.1 ± 1.7</td>
<td>25.3 ± 2.0†</td>
<td>0.1 ± 0.04</td>
<td></td>
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</table>

Values are means ± SE; n, no. of observations. *P < 0.05; †P < 0.01 compared with controls (Student's t-test).
Fig. 1. Low-frequency fatigue protocol in young and mature animals. A: characteristic twitch from an animal weighing 105 g (group 1) followed by submaximal low-frequency fatigue protocol (30-Hz trains, 250-ms duration every 2 s for 5 min) showing tetanic forces developed during repetitive contraction of muscle strip. Twitch immediately after run has decreased to 49% of control value. Arrow indicates inset to illustrate individual 30-Hz trains used in fatigue protocol. Note different time bases. B: characteristic twitch and low-frequency fatigue protocol in an animal weighing 389 g (group 2). Note treppe during fatigue run. Postfatigue twitch can be seen to have decreased to 27% of control value immediately after fatigue protocol.

The present data show that almitrine and doxapram, at concentrations that either had no effect on twitch tension or did have a significant enhancing effect on twitch tension, were able to significantly improve recovery from fatigue in young and mature animals. In the case of almitrine there was a complete and rapid recovery from fatigue. The results of this study also confirm previous work in the baboon (21) and hamster (33), which showed that the respiratory muscles of younger animals have a higher resistance to fatigue than do those of older animals. This may be due to the greater number of mitochondria-rich and/or highly oxidative diaphragm muscle fibers in the younger animals (13).
Our studies gave rise to \( P_0 \) values in mature animals similar to those previously reported (e.g., Ref. 1). However, our \( P_0 \) values for young animals were significantly lower. This may have been due in part to the use of two animal groups with significantly different body weight, different muscle fiber composition (13), different histochemical structure (21), and nonuniformity of weight, different muscle fiber composition (13), difference in the treatment of respiratory failure. Doxapram increases central respiratory drive by stimulating peripheral arterial chemoreceptors in addition to central respiratory neurons (23). Almitrine is known to act via the peripheral arterial chemoreceptors, raising carotid sinus nerve output and minute ventilation (4, 19). It also improves gas exchange by enhancing hypoxic pulmonary vasoconstriction (14). The in vitro preparation used in this study would seem to exclude many of these effects.

The mechanism by which doxapram affects the directly stimulated diaphragm in our experiments is unclear. It has been tested on the neuromuscular junction in the rat phrenic nerve diaphragm preparation used by Pollard et al. (23), who found that it has a presynaptic facilitatory effect. However, in the presence of partial neuromuscular block, it produces a seemingly postjunctional inhibitory effect, albeit only when it is given at very high doses. Because the direct diaphragm stimulation in this study is independent of neuromuscu-

This form of fatigue has been shown to mimic the metabolic changes produced by peripheral fatigue in vivo, i.e., depletion of glycogen stores and phosphocreatine levels as well as a drop in intracellular pH and a rise in intracellular phosphate (Pi). It is thought that this acidosis and increased Pi inhibit the actin-myosin interaction giving rise to contractile failure. Reactive oxygen intermediates seemed to be implicated in this form of fatigue (25, 26). Rapid- and slow-onset fatigue protocols in the isolated rat diaphragm have also been documented by Kolbeck and Nosek (17). High-frequency fatigue protocols seem to bring about an impulse propagation block across the sarcolemma and do not seem to involve reactive oxygen intermediates (25). Rochester (29) suggested that the maximum recovery from high-frequency fatigue is relatively fast (~30 min), compared with maximum recovery from low-frequency fatigue, which does not occur until at least 1 h or more has elapsed after the fatigue. The results of this study tend to agree with this observation, namely, the younger animals reach a peak recovery after 180 min and the older animals after 210 min. This prolonged time period necessary for recovery may imply that damage occurs to subcellular organelles or membranes, although the high recovery rates suggest that this damage is minimal. Also, it is known that fatiguing muscle fibers undergo metabolite accumulation and loss of calcium homeostasis. This may directly inhibit contractile protein interactions and thereby depress muscle function especially in the mature muscle fibers (25). The larger CSA of the mature muscle fibers must also be taken into account, whereby oxygen diffusion may be impaired to a greater extent. The in vitro preparation used in this study would not have been able to replenish the muscle fiber energy requirements or take away metabolites to the same extent as the in vivo diaphragm. Nevertheless, we feel that the very large effect of almitrine (100% recovery of the twitch tension in many cases) indicates that little damage may have occurred to the individual fibers.

Doxapram and almitrine have previously been used in the treatment of respiratory failure. Doxapram increases central respiratory drive by stimulating peripheral arterial chemoreceptors in addition to central respiratory neurons (23). Almitrine is known to act via the peripheral arterial chemoreceptors, raising carotid sinus nerve output and minute ventilation (4, 19). It also improves gas exchange by enhancing hypoxic pulmonary vasoconstriction (14). The in vitro preparation used in these studies would seem to exclude many of these effects.

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lar junction activity, our results would seem to be in contrast to the work of Pollard et al. However, it is interesting to note that at very high doses (40 µg/ml) doxapram did enhance baseline twitch and tetanic tension.

Almitrine may act like the agent aminophylline, a compound also reported to improve diaphragmatic contractility (2, 3, 18) and reverse diaphragmatic fatigue (12). It is thought that aminophylline increases diaphragmatic contractility by increasing intracellular calcium concentration (9) as well as changing high-energy Pi metabolism (15). Almitrine and doxapram may also increase intracellular calcium by either increasing calcium flow into the cell or preventing its

Fig. 3. Effect of doxapram on rate of recovery of twitch tension from fatigue protocol. Doxapram was applied continuously from 15 min before fatigue protocol. A: time course of recovery of twitch tension from fatigue over 90 min in group 1 control animals (○) and those treated with 2 (●), 10 (△), and 20 (▲) µg/ml doxapram. There was a 72% recovery at 90 min after fatigue run in control animals. Doxapram at concentrations of 10 and 20 µg/ml significantly increased recovery postfatigue (P < 0.01 and P < 0.001, respectively; Fisher’s test). B: time course of recovery of the twitch tension from fatigue over 90 min in group 2 control animals (○) and those treated with 2 (●), 10 (△), and 20 (▲) µg/ml doxapram. There was a 41.5% recovery at 90 min after fatigue run in control animals. Doxapram at concentrations of 10 and 20 µg/ml significantly increased recovery postfatigue (P < 0.001; Fisher’s test). Each point is mean ± SE; n = 5 observations except for controls (n = 10). Twitch tension is expressed as percentage of prefatigue control.

Fig. 4. Effect of almitrine on rate of recovery of twitch tension from fatigue protocol. Almitrine was applied continuously from 15 min before fatigue protocol. A: time course of recovery of twitch tension from fatigue over 90 min in group 1 control animals (○) and those treated with 3 (●), 12 (△), and 30 (▲) µg/ml almitrine. There was a 71% recovery at 90 min after fatigue run in control animals. All concentrations of almitrine gave rise to 100% recovery from fatigue (P < 0.001 for all; Fisher’s test). B: time course of recovery of twitch tension from fatigue over 90 min in group 2 control animals (○) and those treated with 3 (●), 12 (△), and 30 (▲) µg/ml almitrine. There was a 40.4% recovery at 90 min after fatigue run in control animals. There was a recovery of 95.6, 100, and 100% at concentrations of 3, 12, and 30 µg/ml respectively compared with prefatigue controls (P < 0.01; Fisher’s test). Each point is mean ± SE; n = 5 observations except for controls (n = 10). Twitch tension is expressed as percentage of prefatigue control.
uptake by the sarcoplasmic reticulum. Indeed, in our laboratories it was found that almitrine attenuates the inhibitory effect of dantrolene on twitch tension (unpublished observations). Fitts (11), in a recent comprehensive review, has focused attention on the mechanism of muscle fatigue at the cellular level. Recent studies have shown that almitrine can act directly on mitochondria, decreasing the cytosolic and mitochondrial ATP/ADP ratios and thus decreasing ATP utilization (20). Our present set of experiments provided no direct proof for these mechanisms of action. There is also increasing evidence that an increased production of reactive oxidant species occurs in exercising diaphragmatic muscle (10, 25). Reid et al. postulate that these species contribute to muscular fatigue in vitro (see also Ref. 10). Also, muscles that produce the most reactive oxidant species also show the greatest fatigue (27).

Almitrine has also been shown to reduce the adverse effects of cerebral ischemia in cats (7), which may be due to an oxygen free radical-scavenging effect (6, 8). Therefore, it would be of interest to discover whether almitrine had a similar mode of action in the fatigued diaphragm in our studies. Muscular fatigue has been shown to be attenuated by pretreatment with antioxidants such as N-acetylcysteine (NAC; 16, 28). In our laboratories it was found that the effects of NAC are not additive to those of almitrine on muscle fatigue (unpublished observations). The importance of these cellular effects of almitrine on diaphragm muscle fatigue remains to be established. Whether they can be attributed to the greater effect (decrease in time to recovery and increase in magnitude of recovery) of almitrine on fatigued muscle compared with doxapram is still unknown.

In summary, almitrine and doxapram, at doses that had no effect on baseline muscle contractility, both decreased the time to maximum recovery and increased the percent recovery from fatigue (in the case of almitrine to 100% of control). These significant effects may be due to increased intracellular calcium, altered ADP/ATP ratios (20), or oxygen free radical scavenging.

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