ATP-sensitive K⁺ channel blocker glibenclamide and diaphragm fatigue during normoxia and hypoxia

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Van Lunteren, Erik, Michelle Moyer, and Augusto Torres. ATP-sensitive K⁺ channel blocker glibenclamide and diaphragm fatigue during normoxia and hypoxia. J. Appl. Physiol. 85(2): 601–608, 1998.—The role of ATP-sensitive K⁺ channels in skeletal muscle contractile performance is controversial: blockers of these channels have been found to not alter, accelerate, or attenuate fatigue. The present study reexamined whether glibenclamide affects contractile performance during repetitive contraction. Experiments systematically assessed the effects of stimulation paradigm, temperature, and presence of hypoxia and in addition compared intertrain with intraintrain fatigue. Adult rat diaphragm muscle strips were studied in vitro. At 37°C and normoxia, glibenclamide did not significantly affect any measure of fatigue during continuous 5- or 100-Hz or intermittent 20-Hz stimulation but progressively prolonged relaxation time during 20-Hz stimulation. At 20°C and normoxia, neither force nor relaxation rate was affected significantly by glibenclamide during 20-Hz stimulation. At 37°C and hypoxia, glibenclamide did not significantly affect fatigue at 5-Hz or intertrain fatigue during 20-Hz stimulation but reduced intraintrain fatigue and prolonged relaxation time during 20-Hz stimulation. These findings indicate that, although ATP-sensitive K⁺ channels may be activated during repetitive contraction, their activation has only a modest effect on the rate of fatigue development.

diaphragm; skeletal muscle; potassium; ATP-sensitive K⁺ channels; contraction; temperature

ATP-SENSITIVE K⁺ channels (KATP) are found in high density in many tissues, including skeletal muscle (7). In intact skeletal muscle fibers these channels are generally closed under resting normoxic conditions (2, 3, 15). However, KATP open under conditions of low ATP concentration ([ATP]), low intracellular pH, and metabolic poisoning (2, 7, 9, 22). It has been postulated that KATP become activated during repetitive muscle contraction especially during high-intensity contractions and/or under hypoxic stress, which thereby contributes to K⁺ efflux and the development of skeletal muscle fatigue (2, 7).

In support of the above postulate, studies utilizing openers of KATP generally concur that these agents accelerate fatigue, especially under hypoxic conditions (12, 28, 30). In contrast, studies utilizing blockers of KATP have found variable effects on fatigue: many studies have found no significant effect on fatigue (5, 12, 16, 28, 30), although an improvement (13, 14, 30) and a worsening (6) of fatigue have also been reported. The methodology of these studies varies considerably with respect to temperature, stimulation paradigm, presence of hypoxia, and data-analysis strategies, which may potentially account for some of the discrepant findings.

The purpose of the present study was to reexamine the issue of whether blocking KATP affects muscle performance during repetitive contractions leading to fatigue by 1) systematically addressing effects of stimulation paradigm, temperature, and presence of hypoxia; 2) comparing intertrain with intraintrain fatigue; and 3) assessing the rate of muscle relaxation, which is known to slow during fatigue (10, 11, 16, 19, 26). We found that the KATP blocker glibenclamide significantly improves intraintrain but not intertrain fatigue but only under hypoxic and not normoxic conditions and that it slows rate of muscle relaxation during fatigue under both normoxic and hypoxic conditions but not at low temperature. These findings indicate that KATP may be activated during repetitive contraction, especially during higher intensity contractions and/or under hypoxic conditions.

METHODS

Male Sprague-Dawley rats (250–350 g) were anesthetized with intraperitoneal urethan (1–1.5 g/kg), the diaphragms were removed surgically, and two to four small strips (diameter ~1–1.5 mm) were cut per animal, with care taken to preserve the attachment of the muscle to the central tendon and ribs. The muscle strips were mounted in physiological solution at optimal length and were stimulated via platinum electrodes by using a pulse width of 1 ms and supramaximal voltages (Grass Instruments, West Warwick, RI). The aerated (95% O₂-5% CO₂) physiological solution contained (in mM) 135 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgSO₄, 1 NaH₂PO₄, 15 NaHCO₃, and 11 glucose, with the pH adjusted to 7.35–7.45. Bath temperature was controlled at 20 or 37°C by circulating water of the appropriate temperature through the outer jacket of the tissue baths (Radnoti Glass, Monrovia, CA). Twitch forces of ~0.5 kg/cm² are obtained with this methodology in rat diaphragm (25). Force records were digitized, collected online with a computer (Axotape software, Axon Instruments, Foster City, CA), and stored for later data analysis. Drugs and reagents were obtained from Sigma Chemical (St. Louis, MO). Glibenclamide was dissolved as a 2 mM stock solution in 0.05 M NaOH, the proper volume of which was added to the bath to produce a final concentration of 100 μM (16).

Diaphragm muscle strips were allowed to equilibrate and subsequently underwent twitch stimulation at 0.1 Hz for 3 min. Muscle strips were accepted for study only if twitch force varied by no more than 5% during the 3-min baseline period. Six separate experiments were performed, the conditions of which are summarized in Table 1. Muscle strips were randomized across arms of a given experiment but not across experiments. That is, muscle strips were randomized to receive drug or no drug under a given set of experimental conditions; assignment of muscle strips was not randomized...
across all six experimental conditions. Care was taken to ensure that muscle strips from a given animal were assigned to both drug and no drug. Each muscle strip was used only once. Experiments A–C and E–F were conducted at 37°C, whereas experiment D was conducted at 20°C. After the baseline period, glibenclamide (100 µM) or vehicle (containing an equal volume of 0.05 M NaOH) was added to the bath for all experiments, which was followed by an equilibration period of 4 min. For experiments E–F only, the gas with which the solution was aerated was subsequently switched to 95% N2:5% CO2 followed by an equilibration period of 4 min. Bath oxygen tension was measured in some of the hypoxia studies with a dissolved-oxygen meter (ISO-2, World Precision Instruments, Sarasota, FL) and averaged 3.8 ± 0.8% at the end of the 4-min equilibration period. The muscles were stimulated at 0.1 Hz to monitor twitch tension during all of the above. Finally, the muscle strips underwent one of three stimulation paradigms: continuous 5-Hz stimulation (experiments A and E), intermittent 20-Hz stimulation (train duration 0.33 s, with 1 train delivered every second) (experiments B, D, and F), or continuous 100-Hz stimulation (experiment C). Only limited studies were done at 20°C because it would be highly unusual for mammalian muscle to be contracting in vivo at this cold a temperature, whereas tissue hypoxia and alterations in motoneuronal firing frequency can be seen under a variety of circumstances.

Force records were analyzed offline with use of manually positioned cursors displayed on the computer screen. Isometric twitch tension was measured in grams and subsequently normalized for each muscle strip to the average of the last three isometric twitch contractions of the (predrug) baseline period. Normalization was performed to minimize the confounding effects of interstrip variability in size and hence baseline force and to reduce the influences of slight variations in dissection technique affecting baseline force. This method of normalization is consistent with approaches used by us (24, 25, 27) and others (30) in studies of KATP channel blockers. Other studies of KATP blockers have normalized force to postdrug, prefatigue values (e.g., 13, 16, 28), which is similar to the present approach in that KATP blockers in the concentrations used generally have minimal effects on baseline force. Intratrain fatigue was assessed during 20-Hz stimulation by measuring the force at the end of the 330-ms-long train and expressing this as a percentage of the maximum force within the same tetanus (force-330) (26; as modified from Ref. 16). During 0.1- and 5-Hz stimulation, contraction time was assessed as the amount of time for twitch force to reach its peak, and half relaxation time was assessed as the amount of time for twitch force to decay to one-half of the peak value. During 20-Hz trains, contraction time was assessed from the first twitch of the train, and relaxation time was assessed from the decay in force at the end of the train.

All values presented are means ± SE. Statistical analysis of the effects of glibenclamide on prefatigue isometric twitch kinetics was performed with the unpaired t-test. Statistical analysis of the effects of glibenclamide on peak force, force-330, and half relaxation times during fatigue runs was performed with two-way ANOVA for repeated measures, followed in the event of a significant ANOVA by the Newman–Keuls test. The criterion for statistical significance was set at P < 0.05 (2 tailed).

### RESULTS

Normoxic conditions (37°C). Under normoxic conditions in nonfatigued muscle, glibenclamide (100 µM) did not significantly affect isometric twitch contraction or half relaxation times, although there was a nonsignificant trend for the latter to be prolonged (Table 2). In response to repetitive stimulation, glibenclamide did not significantly affect peak force over time during 5-, 20-, or 100-Hz stimulation under normoxic conditions at 37°C (Fig. 1). Force-330, an evaluation of the ability of the muscle to maintain force during the plateau phase within the same tetanic stimulation (evaluated during 20-Hz trains), was slightly but not significantly improved by glibenclamide under normoxic conditions (Fig. 2). However, the extent to which the half relaxation time progressively prolonged during repetitive stimulation was augmented significantly by glibenclamide during 20-Hz and to a lesser extent 5-Hz stimulation (Fig. 3).

Effects of lowering temperature (normoxia). Effects of lowering temperature to 20°C were assessed during fatigue produced by 20-Hz stimulation. At this temperature, glibenclamide had no significant effect on baseline twitch kinetics (Table 2), peak force over time (Fig. 4, left), force-330 (Fig. 4, middle), or rate of relaxation (Fig. 4, right).

Effects of hypoxia (37°C). Glibenclamide had no significant effect on isometric twitch contraction and half relaxation times under hypoxic conditions (Table 2).
2). The change in peak force over time during 5- and 20-Hz stimulation was not affected significantly by glibenclamide under hypoxic conditions, although there was a trend for peak force to be improved by glibenclamide during 20-Hz stimulation (Fig. 5). In contrast to during normoxia, force-330 (assessed during 20-Hz trains) was improved significantly by glibenclamide during hypoxia (Fig. 6). The extent to which relaxation rate slowed during repetitive 20-Hz stimulation was generally more prominent during hypoxia than during normoxia, and this was augmented significantly by glibenclamide during 20- but not 5-Hz stimulation (Fig. 7).

DISCUSSION

Methodological issues. There are a number of agents that block K$_{ATP}$, several of which have been used in previous studies of muscle contractility. Glibenclamide was chosen for the present study on the basis of two major considerations. First, glibenclamide at the concentration used in this study (100 µM) blocks rat skeletal muscle K$_{ATP}$ but not voltage-gated K$^+$ channels or Ca$^{2+}$-activated K$^+$ channels (17); comparable data on sensitivity and specificity in rat skeletal muscle are not available for the other K$_{ATP}$ blockers. However, tolbutamide affects muscle excitability, suggesting that it may have effects in addition to blocking K$_{ATP}$ (5). Thus it was felt best to pick the agent for which specificity for K$_{ATP}$ was best established in rat skeletal muscle. Second, glibenclamide has been used in the majority of previous studies examining muscle fatigue and K$_{ATP}$. Among eight studies, six used glibenclamide (6, 12–14, 16, 30), two used glyburide (5, 28), and one study each used phentolamine (30), ciclazindol (30), and tolbutamide (5). It is easier to compare the present data with other data by choosing the agent used most commonly in previous studies (glibenclamide) rather than another agent (e.g., tolbutamide, glyburide, ciclazinol).

Light and French (17) examined the sensitivity to glibenclamide of K$_{ATP}$ reconstituted from rat skeletal muscle. They noted a concentration for one-half inhibition of open probability ($K_i$) of 3–5 µM and found that a dose of 10–100 µM was sufficient to fully eliminate visible channel openings. The value for $K_i$ in rat muscle is higher than that of mouse muscle (K$_i$ of 3–5 nM) (1). Light and French (17) also found that 100 µM glibenclamide had no effects on voltage-gated or Ca$^{2+}$-activated K$^+$ channels, suggesting good specificity for K$_{ATP}$ (consistent with studies in other tissues). A glibenclamide concentration of 100 µM was chosen over 10 µM in the present study for several reasons. First, a concentration of glibenclamide was desired that would definitely block K$_{ATP}$ so that any absent effects of glibenclamide...
on fatigue could not be attributed to a concentration
that was possibly too low. Second, the present study
used muscle strips, whereas French and Light (17)
studied biplanar layers; a concentration higher than
the minimal amount needed would ensure that an
adequate concentration of drug would reach the center
of the muscle strip. Third, a concentration of 100 µM
was used in the most detailed previous study of gliben-
clamide and muscle fatigue (which also included data
on action potentials) (16) so that direct comparisons
could most easily be made by using the same drug
concentration.

In the present study, glibenclamide was dissolved in
NaOH as a stock solution before it was added to the
bath. Glibenclamide (and glyburide) do not dissolve
readily in water or saline. Previous studies of K_ATP
blockers and muscle contraction have dissolved gliben-
clamide and glyburide in either DMSO (28, 30) or
NaOH (6, 16). The latter was chosen for the present
study because DMSO affects free radicals and thereby
muscle contractile performance and, hence, could have
greater confounding effects than a slight increase in pH
induced by NaOH. Control and drug-treated muscle
strips had an equal amount of NaOH added to the bath
so that any potential effects of acid-base changes would
be similar.

An incubation period for glibenclamide of 4 min was
used in the present study. Light et al. (16) examined
effects of glibenclamide (100 µM) on muscle action
potential repolarization at a temperature of 20°C. They
found that action potential repolarization was slowed
by fatigue and that glibenclamide further slowed action
potential repolarization. Furthermore, the mean val-
ues of the half repolarization time after fatigue in the

Fig. 3. Diaphragm half relaxation time over the course
of repetitive 5- (A) and 20-Hz (B) stimulation in the
presence and absence of glibenclamide (100 µM) under
normoxic conditions and a temperature of 37°C. Values
are means ± SE. *Significant differences between gliben-
clamide and no drug, P < 0.05.

Fig. 4. Effect of glibenclamide (100 µM) on diaphragm
during 20-Hz stimulation under normoxic conditions
and a temperature of 20°C. Values are means ± SE.
Changes in peak force (left), force-330 (middle), and half
relaxation time (right) are indicated. Peak force was
normalized to the value for force immediately before
addition of drug or no drug, as described in METHODS.
Glibenclamide had no significant effects on peak force
(P = 0.81), force-330 (P = 0.94), or half relaxation time
(P = 0.36).
presence of glibenclamide were the same whether the drug was applied 60 min before fatigue or 60 s before the end of fatigue. The latter data suggest a fast rate of diffusion and a fast onset of action of glibenclamide (=60 s) in skeletal muscle tissue. Light et al. studied muscle fiber bundles with diameters of 1–1.5 mm, which is the same size used in the present study. They used a temperature of 20°C, whereas the present study used a temperature of 37°C; diffusion and onset of drug action should be faster at the higher temperature. Based on these data, 4 min should be sufficiently long for equilibration after drug addition.

Effects of glibenclamide on muscle contraction. Effects of $K_{ATP}$ blockers on muscle fatigue have been assessed by utilizing a variety of stimulation frequencies, ranging from 0.2 to 140 Hz (5, 6, 12, 13, 16, 28, 30). In addition, one study used a spontaneously breathing model to test diaphragm fatigue (14), in which motoneuronal firing frequency was not assessed but would be expected to vary among motor units and over time. During muscle contraction, $K^+$ efflux and $Na^+$ influx and the resultant alteration in transmembranous $K^+$ and $Na^+$ concentration gradients may lead to sarcolemmal depolarization, especially in the T tubules in which diffusion of ions is slower than at the outer surface of the muscle (21, 29). Much of the $K^+$ efflux during

Fig. 5. Alterations in peak diaphragm force over time during repetitive 5- (A) and 20-Hz (B) stimulation in presence and absence of glibenclamide (100 µM) under hypoxic conditions and a temperature of 37°C. Force values are means ± SE and are normalized to the value for twitch force immediately before addition of drug or no drug as described in METHODS. There were no significant effects of glibenclamide during 5-Hz stimulation, but there was a nonsignificant trend for force to improve during 20-Hz stimulation ($P = 0.07$).

Fig. 6. Effects of glibenclamide (100 µM) on ability of diaphragm to maintain force during plateau phase within same tetanic stimulation (force-330) during 20-Hz stimulation under hypoxic conditions and a temperature of 37°C. Values are means ± SE. Force-330, a measure of intratrain fatigue, was determined by evaluating force of the last contraction in the train as percentage of maximum tetanic force of the same tetanic contraction at each time point. *Significant differences between glibenclamide and no drug, $P < 0.05$.

Fig. 7. Diaphragm half relaxation time over the course of repetitive 5- (A) and 20-Hz (B) stimulation in presence and absence of glibenclamide (100 µM) under hypoxic conditions and a temperature of 37°C. Values are means ± SE. Half relaxation times were difficult to quantify accurately when force values became very small toward the end of fatiguing stimulation (see Fig. 5) and hence are reported only for the first 2 min of the 3-min stimulation period. *Significant differences between glibenclamide and no drug, $P < 0.05$. Contraction time was not affected significantly by glibenclamide ($P = 0.39$).
contraction occurs via delayed rectifier K$^+$ channels (29), although K$_{ATP}$ has been postulated to contribute to the K$^+$ efflux under conditions of depleted intracellular [ATP] and concomitant acidosis (8, 21). The Na$^+$-K$^+$-ATPase will restore the membranous ion gradients back to normal, but at high rates of muscle contraction the active transport is overwhelmed (4). Thus the role of K$^+$ channels in regulating muscle fatigue is believed to be most prominent during intense muscle activation. Hence K$^+$ channel blockers should improve high-frequency more than low-frequency fatigue and intra-train more than intertrain fatigue. That one of the studies with the greatest beneficial effects of K$_{ATP}$ blockade on fatigue used a low stimulation rate of 0.25 Hz (13) is therefore surprising and may very well reflect other methodological differences (e.g., use of an in vivo preparation in which vascular or other systemic effects of glibenclamide may have contributed to the findings) compared with the other studies of K$_{ATP}$ blockers (5, 6, 12, 16, 28, 30).

In the present study we found no significant effects of glibenclamide on fatigue during continuous 5- or 100-Hz stimulation or on intertrain fatigue during intermittent 20-Hz stimulation under normoxic or hypoxic conditions, consistent with all of the other in vitro studies of glibenclamide and fatigue (5, 12, 16, 28, 30). The only in vitro study reporting an improvement of intertrain fatigue with K$_{ATP}$ blockers noted a modest improvement in fatigue with ciclazinol but not with glibenclamide (30), suggesting that ciclazinol may be a more effective blocker of K$_{ATP}$ or may have additional effects in addition to blocking K$_{ATP}$ (e.g., blocking other K$^+$ channels). In the present study we found no significant effects of glibenclamide on intratrain fatigue during normoxia (although there was a trend toward improvement), but we found an attenuation of intratrain fatigue during hypoxia. The former finding (normoxic conditions) is consistent with two previous studies (5, 16), neither of which, however, examined intratrain fatigue under hypoxic conditions. The present finding of glibenclamide significantly attenuating only intratrain fatigue and only during hypoxia suggests that the contribution of K$_{ATP}$ to fatigue is small and is limited to conditions expected to lead to profound ATP depletion and/or intracellular acidosis.

The rate of muscle relaxation slows with fatigue and especially does so under hypoxic conditions (10, 11, 16, 19, 26). Two previous studies have found that glyburide and glibenclamide slow the rate of action potential repolarization in resting and fatigued muscle (5, 16). Surprisingly, the rate of muscle relaxation was not found to be affected by glibenclamide in either resting or fatigued muscle in a previous study despite changes in action potential repolarization rate (16). Data on muscle relaxation rate were not provided for glyburide (5), nor have other studies of glibenclamide and fatigue reported values for rate of muscle relaxation. The present data concur with those of Light et al. (16), who found that that K$_{ATP}$ blockade does not significantly alter rate of relaxation of resting muscle. In contrast to Light et al., we found a slowing of relaxation rate by glibenclamide during fatigue produced during 20-Hz stimulation under both normoxic and hypoxic conditions. These data suggest that K$_{ATP}$ may be activated during fatiguing stimuli but to an insufficient extent to affect peak force production. This is consistent with a previously proposed explanation for glibenclamide not affecting fatigue but delaying the recovery from fatigue (16).

The mechanism by which altering K$^+$ channel conductance affects muscle relaxation rate is unlikely to be a direct effect on either the rate of Ca$^{2+}$ reuptake by the sarcoplasmic reticulum or the rate of Ca$^{2+}$ binding by parvalbumin. More likely, the effects of K$^+$ channels on the rate of relaxation are mediated by altering the rate of action potential repolarization. Normally, membrane potential repolarizes very quickly during an action potential. As a result, there is only a brief period of time during repolarization when there is continued Ca$^{2+}$ influx but Ca$^{2+}$ is simultaneously being taken back up by the sarcoplasmic reticulum and/or being bound to intracellular Ca$^{2+}$ buffers. If action potential repolarization is slowed (e.g., with K$^+$ channel blockade), this period can be prolonged, thereby slowing the rate at which intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) falls and hence slowing the rate of relaxation. If the degree of action potential repolarization slowing is small, it may not be sufficient to affect mechanical relaxation. This could explain why Light et al. (16) found action potential prolongation but no slowing of relaxation with glibenclamide. On the other hand, if the degree of action potential slowing is large, either contraction or relaxation time could be slowed depending on the kinetics of the changes in intracellular [Ca$^{2+}$] relative to the kinetics of actin-myosin interactions. As muscle fatigues, action potential repolarization slows and relaxation rate slows. Under these circumstances, effects of K$^+$ channel blockers on rate of relaxation may become more manifest, as was found for glibenclamide in the present study. This is consistent with previous studies of the K$^+$ channel-blocking aminopyridines, which do not slow relaxation rate in nonfatigued muscle but markedly augment slowing of relaxation rate as muscle undergoes fatiguing contractions (25, 26).

Effects of K$_{ATP}$ blockers have been assessed at 20°C (5, 16), 30°C (28), or 37–38°C (6, 12–14, 30). Studies at 20°C utilized frog muscle, whereas studies performed at 30–38°C utilized mammalian muscle so that the influence of temperature on muscle contractile responses to K$_{ATP}$ blockers cannot be inferred directly from previous work. Of note, however, is that both of the studies at a cool temperature found no effect of K$_{ATP}$ blockers on fatigue, whereas the studies at warmer temperatures have noted variable effects of K$_{ATP}$ blockers on fatigue. Ion channels are very sensitive to temperature, with rates of activation and deactivation having especially high values for Q$_{10}$ compared with peak current; furthermore, values for Q$_{10}$ may vary as a function of membrane potential (see, e.g., Refs. 20, 23). In the present study of 20-Hz stimulation during...
normoxia, we found that at neither warm nor cold temperature was there a significant effect of glibenclamide on either intertrain or intratrain fatigue. This suggests that differences among previous studies regarding whether fatigue is attenuated with glibenclamide are unlikely to be due to differences in muscle temperature. On the other hand, we found that, during fatiguing 20-Hz stimulation, glibenclamide led to an augmentation of the relaxation rate prolongation at 37°C but not at 20°C. Therefore, a low temperature may be higher during repetitive dynamic contractions, which could lead to augmentations of agents, and these increases are accompanied by prolongations of contraction time and for the aminopyridines a leftward shift in the force-frequency relationship (18, 24, 25, 27). Furthermore, the aminopyridines improve both peak force and maintenance of intratrain force during repetitive 20-Hz stimulation, reduce the neurotransmission failure contribution to fatigue, and augment the slowing of relaxation rate during fatigue (all tested under normoxic conditions) (24, 25). Therefore, K$_{\text{ATP}}$ appears to play a relatively small role compared with other K$^+$ channels (e.g., delayed rectifier K$^+$ channels) in regulating muscle contractile performance. However, there may be other conditions under which K$_{\text{ATP}}$ play a more prominent role in modulating muscle force. Specifically, ATP consumption may be higher during repetitive dynamic contractions (contractions associated with muscle shortening) than during isometric contractions, which could lead to a greater fall in intracellular ATP and hence a greater activation of K$_{\text{ATP}}$. To test this, future studies are needed to determine the extent to which glibenclamide modulates muscle shortening velocity during fatiguing isotonic contractions.

In conclusion, K$_{\text{ATP}}$ contributes to fatigue production during isometric contractions only under extreme conditions, such as intense stimulation during hypoxia. These channels are activated under less extreme conditions as evidenced by a prolonged relaxation time as fatigue develops, but the degree of activation is not sufficient to substantially decrease force production. Intense stimulation during hypoxia may result in an acidic intracellular environment that is partially depleted of ATP. Under these circumstances, ATP-dependent Na$^+$-K$^+$ pumps are no longer sufficient to restore normal transmembrane ion gradients so that K$^+$ efflux through K$_{\text{ATP}}$ may contribute to fatigue.

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