β2-Adrenoceptor agonists reduce the decline of rat diaphragm twitch force during severe hypoxia

H. F. M. van der Heijden, L. M. A. Heunks, H. Folgering, C. L. A. van Herwaarden, and P. N. R. Dekhuijzen

Department of Pulmonary Diseases, University Hospital Nijmegen, 6500 HB Nijmegen, The Netherlands

Van der Heijden, H. F. M., L. M. A. Heunks, H. Folgering, C. L. A. van Herwaarden, and P. N. R. Dekhuijzen. β2-Adrenoceptor agonists reduce the decline of rat diaphragm twitch force during severe hypoxia. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L474–L480, 1999.—The aim of the present study was to investigate the in vitro effects of the short-acting β2-adrenoceptor agonist salbutamol and the long-acting β2-adrenoceptor agonist salmeterol on hypoxia-induced rat diaphragm force reduction. In vitro diaphragm twitch force (Pt) and maximal tetanic force (Po) of isolated diaphragm muscle strips were measured for 90 min during hyperoxia (tissue bath Po2 83.3 ± 0.9 kPa and PCO2 3.9 ± 0.1 kPa) or severe hypoxia (Po2 7.1 ± 0.3 kPa and PCO2 3.9 ± 0.1 kPa) in the presence and absence of 1 µM salbutamol or 1 µM salmeterol. During hyperoxia, salbutamol and salmeterol did not significantly alter the time-related decreases in Pt and Po (to ~50% of initial values). Salbutamol had no effects on Pt or the Pt-to-Po ratio. Salmeterol treatment significantly reduced Pt and increased the Pt-to-Po ratio during hyperoxia (P < 0.05 compared with control value). Hypoxia resulted in a severe decrease in Pt (to ~30% of initial value) and Po after 90 min. Both salbutamol and salmeterol significantly reduced the decline in Pt during hypoxia (P < 0.05). The reduction in Po was not prevented. Salbutamol increased Pt rapidly but transiently. Salmeterol had a delayed onset of effect and a longer duration of action. Addition of 1 µM propranolol (a nonselective β2-adrenoceptor antagonist) did not alter Pt, Po, or the Pt-to-Po ratio during hypoxia but completely blocked the inotropic effects of salbutamol and salmeterol, indicating that these effects are dependent on β2-adrenoceptor agonist-related processes.

Dysfunction of the respiratory muscles frequently occurs in patients with severe chronic obstructive pulmonary disease (COPD) (20). Alterations like hypoxia, hypercapnia, and electrolyte disorders are important factors that may impede respiratory muscle function (2, 9, 16, 26, 27, 29). In patients with severe COPD, chronic hypoxia may be present, often in combination with hypercapnia. Furthermore, during exacerbations of their disease, acute-on-chronic respiratory failure may occur. Hypoxia significantly affects respiratory muscle function; it reduces in situ diaphragm contractility (2), in vitro diaphragm twitch force (26), and in vitro isotonic and isometric diaphragm fatigue resistance (24). Also, in healthy subjects, fatigue resistance (measured by inspiratory resistive breathing) is reduced by hypoxia (13).

Therefore, pharmacological interventions that (partly) reverse these deleterious effects of hypoxia on respiratory muscle function are of clinical importance. β2-Adrenoceptor agonists are of special interest because these drugs are already widely used in the treatment of bronchoconstriction and exacerbations of asthma or COPD. Furthermore, previous studies (1, 32) have shown that β2-adrenoceptor agonists like salbutamol and terbutaline can improve normal rat diaphragm contractile properties under optimal in vitro conditions. These inotropic effects are increased by foreshortening (31) and are also present during fatigue (8, 19, 30) and in vivo metabolic acidosis (12). The effects of β2-adrenoceptor agonists on diaphragm contractile properties during hypoxia have not been investigated. Whether the new long-acting β2-adrenoceptor agonists like salmeterol have similar inotropic effects on in vitro diaphragm contractility is not known.

Because β2-adrenoceptor agonists like salbutamol have an inotropic effect on in vitro (1, 31, 32) and in vivo (8, 12, 19, 30) diaphragm contractile properties, we hypothesized that the observed decrease of twitch force induced by hypoxia (26) is reversed by these drugs. In addition, we hypothesized that salmeterol will have a sustained effect during hypoxia in comparison to salbutamol and that the effects of these β2-adrenoceptor agonists can be blocked by propranolol (a nonselective β2-antagonist).

The purpose of the present study, therefore, was to investigate the effects of short- and long-acting β2-adrenoceptor agonists (salbutamol and salmeterol, respectively) on rat diaphragm contractile properties during severe hypoxia in vitro and to compare these effects with hypoxic conditions. To ascertain that these effects are mediated via β2-adrenoceptor-related processes, we investigated whether propranolol blocked the effects of salbutamol and salmeterol during hypoxia.

Methods

Study design. The effects of treatment with β2-adrenoceptor agonists on in vitro rat diaphragm contractile properties were studied either during standard optimal in vitro conditions (hyperoxia) or during severe hypoxia. In both conditions, the effects of salbutamol or salmeterol treatment were studied and compared with those of the untreated control group.

General procedures. Adult male outbred Wistar rats aged 16–18 wk and with a mean weight of 376 ± 5 (SE) g were...
used. The animals were housed under standard conditions and were fed ad libitum.

The rats were anesthetized with pentobarbital sodium (70 mg/kg ip; Narcovet, Opharma, Arnhem, The Netherlands). A tracheotomy was performed, and a polyethylene cannula was inserted. The animals were mechanically ventilated with 100% oxygen. The diaphragm and adherent lower ribs were quickly excised after a combined laparotomy and thoracotomy, and they were immediately submerged in cooled, oxygenated Krebs solution at pH = 7.4. This Krebs solution consisted of 137 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM KH₂PO₄, 24 mM NaHCO₃, 7 mM glucose, and 25 µM d-tubocurarine (Sigma, Bornem, Belgium).

From the central costal region of the right hemidiaphragm, two rectangular strips were dissected parallel to the long axis of the muscle fibers. Silk sutures were tied firmly to both ends. The strips were suspended in two tissue baths containing Krebs solution, maintained at 37°C, and perfused with a gas mixture containing 3% O₂-5% CO₂-92% N₂ (hypoxia). The diaphragm and adherent lower ribs were quickly excised after a combined laparotomy and thoracotomy, and the strips were weighed. Cross-sectional area of the muscle fibers. Silk sutures were tied firmly to both ends. The strips were suspended in two tissue baths containing Krebs solution, maintained at 37°C, and perfused with a gas mixture containing 3% O₂-5% CO₂-92% N₂ (hypoxia). The diaphragm and adherent lower ribs were quickly excised after a combined laparotomy and thoracotomy, and the strips were weighed. Cross-sectional area

The various combinations of oxygen supply and treatments were investigated in random order; the investigator (H. v. d. Heijden) was blinded with regard to treatment throughout the experiment. This study was approved by the Animal Experiments Committee of the University of Nijmegen, and it was performed in accordance with the Dutch National Guidelines for Animal Care.

Data analysis. Treatment effects were determined with two-way analysis of variance (ANOVA) in a repeated-measures design, with treatment and oxygen state (hypoxia or hyperoxia) as grouping variables. Because significant interactions were found between the two factors, i.e., treatment, the absence or presence of hypoxia, and repeated force measurements, treatment effects were subsequently analyzed in a repeated-measures design within the hypoxic and hyperoxic groups. Post hoc analysis (Student-Newman-Keuls test) was used to compare differences in P₀ and the Pt-to-P₀ ratio in independent treatment groups. Results were considered significant at P < 0.05. The SPSS package (version 6.1.3, Chicago, IL) was used for statistical analysis. All data are expressed as means ± SE.

RESULTS

Verification of tissue bath hypoxia. Perfusion of the tissue baths with the hypoxic gas mixture significantly reduced the P₀ in the Krebs solution to ~7 kPa compared with ~84 kPa in the hyperoxic group (P < 0.001; Table 1). No differences were found in pH or Pco₂ between hypoxia and hyperoxia, and no differences were found in P₀, Pco₂, or pH between treatment groups.

Diaphragm strip dimensions and specific forces. Average diaphragm strip length ranged from 21.8 ± 0.5 to

| Table 1. Tissue bath Krebs solution pH, Pco₂, and P₀ |
|-----------------|-----------------|-----------------|
|                 | Hyperoxia       | Hypoxia         |
| n               | 24              | 38              |
| pH              | 7.45±0.004      | 7.49±0.004      |
| Pco₂ (kPa)      | 3.92±0.05       | 3.96±0.03       |
| P₀ (kPa)        | 83.75±0.92      | 7.07±0.25*      |

Values are means ± SE after 90 min of tissue bath perfusion; n, no. of diaphragm muscle strips. *Significantly different from hyperoxic group, P < 0.001 by Student’s t-test.
23.0 ± 0.4 mm. Diaphragm muscle strip weight ranged from 39.7 ± 1.4 to 49.5 ± 3.4 mg. No significant differences were found between treatment groups for either strip length or strip weight (one-way ANOVA with subsequent Student-Newman-Keuls post hoc test). The initial \( P_t \) and initial maximal \( P_o \) are listed in Table 2. These measurements were performed at the end of the thermoequilibration period, before the start of treatment and under standard hyperoxic conditions. No differences were found between any treatment groups in the pretreatment period (one-way ANOVA).

Effects of \( \beta_2 \)-adrenoceptor agonist treatment during hyperoxia. Salbutamol treatment did not significantly alter repetitive diaphragm \( P_t \) during hyperoxia. Also, when expressed as a percentage of the initial \( P_t \), no significant overall effect was found (Fig. 1, Table 2). Salbutamol did not affect \( P_o \) and the \( P_t \)-to-\( P_o \) ratio either during hyperoxia (Table 2). However, significant interactions were found between salbutamol treatment and time by repeated-measures analysis of \( P_t \) and \( P_t \)-to-\( P_o \) ratio \((P < 0.01)\). This may indicate that salbutamol initially increased \( P_t \) (and \( P_t \)-to-\( P_o \) ratio) but that this effect was not sustained throughout the experiment (Fig. 1).

Salmeterol did not significantly alter repetitive \( P_t \) generation during hyperoxia (Fig. 1). However, \( P_o \) was significantly reduced by salmeterol treatment, both expressed as specific force and as a percentage of initial \( P_o \) \((P < 0.05)\; \text{Table 2}\). This resulted in a significantly increased \( P_t \)-to-\( P_o \) ratio \((P < 0.05)\; \text{Table 2}\). Significant interactions were found between salmeterol treatment and time by repeated-measures analysis of \( P_t \) and \( P_t \)-to-\( P_o \) ratio \((P < 0.001)\). After 90 min of hyperoxia, \( P_o \) was significantly lower in salbutamol-treated diaphragm strips compared with those from control animals (Table 2). The \( P_t \)-to-\( P_o \) ratio was increased compared with that in control strips \((P < 0.01)\; \text{Table 2}\). Again, a significant interaction was found between salbutamol treatment and time \((P < 0.001)\). Post hoc analysis showed that the \( P_t \)-to-\( P_o \) ratio was significantly increased at all time points during the experiment (Table 2).

Table 2. Diaphragm \( P_t \), \( P_o \), and \( P_t \)-to-\( P_o \) ratio at 30-min intervals during hyperoxia and hypoxia in vitro

<table>
<thead>
<tr>
<th></th>
<th>Pretreatment</th>
<th>30 Min</th>
<th>60 Min</th>
<th>90 Min</th>
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<tr>
<td></td>
<td>Hyperoxia</td>
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<td></td>
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<td>-Propranolol</td>
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<td>( P_t ), N/cm²</td>
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<tr>
<td>Control</td>
<td>9.2 ± 0.4</td>
<td>8.2 ± 0.2</td>
<td>8.9 ± 0.4</td>
<td>7.3 ± 0.5</td>
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<td>%Baseline</td>
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<tr>
<td>Salbutamol</td>
<td>7.9 ± 0.5</td>
<td>8.3 ± 0.2</td>
<td>8.0 ± 0.2</td>
<td>6.7 ± 0.6</td>
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<tr>
<td>%Baseline</td>
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<tr>
<td>Salmeterol</td>
<td>9.0 ± 0.3</td>
<td>8.5 ± 0.2</td>
<td>7.9 ± 0.4</td>
<td>6.9 ± 0.4</td>
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<tr>
<td>%Baseline</td>
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<td>( P_t )-to-( P_o ) ratio</td>
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<td>Control</td>
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<td>Salmeterol</td>
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Values are means ± SE. During hyperoxia, twitch force \((P_t)\) was not significantly altered (Fig. 1); however, during hypoxia, \( P_t \) (both in N/cm² and as \%baseline) was significantly increased in salbutamol- and salmeterol-treated diaphragm strips \((P < 0.01)\; \text{by repeated-measures ANOVA; Figs. 1 and 2}\). Propranolol reduced \( P_t \) when expressed as \%baseline during hypoxia \((P < 0.001)\). In salbutamol plus propranolol- and salmeterol plus propranolol-treated strips, \( P_t \) (in N/cm²) was significantly reduced during hypoxia \((P < 0.05)\). *Significant difference compared with control treatment found in post hoc analysis \((P < 0.05)\). During hyperoxia, twitch force \((P_t)\) was significantly reduced in salmeterol-treated strips \((P < 0.05)\; \text{by repeated-measures ANOVA}\). During hypoxia, significant effects were found in salmeterol plus propranolol-treated strips only when expressed as \%baseline \((P < 0.05)\). *Significant difference compared with control treatment found in post hoc analysis \((P < 0.05)\). During hyperoxia, \( P_t \)-to-\( P_o \) ratio was significantly altered in salmeterol-treated strips \((P < 0.05)\; \text{by repeated-measures ANOVA}\). During hypoxia, significant effects were found for \( P_t \)-to-\( P_o \) ratio in salbutamol \((P < 0.01)\), salmeterol \((P < 0.01)\), and salmeterol plus propranolol-treated strips \((P < 0.05)\; \text{all by repeated-measures ANOVA})\. *Significant difference compared with control group found in post hoc analysis \((P < 0.05)\).
During hypoxia, salmeterol treatment significantly increased $P_t$ (in N/cm²) compared with that in control strips ($P < 0.05$; Fig. 2). $P_o$ and the $P_t$-to-$P_o$ ratio were not affected by the coadministration of propranolol (Table 2).

**DISCUSSION**

The present study shows that both short-acting and long-acting $\beta_2$-adrenoceptor agonists improve diaphragm muscle $P_t$ generation during severe hypoxia in vitro. This partially restored the hypoxia-induced decline in $P_t$. The significant interactions found between treatment and time in these repeated force measurements indicate that the magnitude of these effects varied during the experiment. Salbutamol had a more rapid onset of action compared with salmeterol, but salmeterol had a longer duration of action. Toward the end of the protocol, salbutamol reduced $P_o$ during hypoxia and the decline in $P_t$ also appeared to be stronger. In contrast, neither salbutamol nor salmeterol affected $P_t$ during hyperoxia. Salmeterol decreased $P_o$ during hyperoxia, whereas salbutamol had no effect on $P_o$. Finally, the inotropic effects of both salmeterol and salbutamol were blocked by proprano-
loll, indicating that these processes are dependent on β₂-adrenoceptor-mediated processes.

Severity of hypoxia. In the present in vitro experiments, Krebs solution PO₂ was reduced to ~7 kPa to induce hypoxia compared with ~84 kPa in the hyperoxic group. Because oxygenation of the diaphragm muscle strips in these experiments is dependent solely on diffusion of oxygen into the core region of the strips, this partial pressure can be considered as severe hypoxia. Besides, at in vitro experimental conditions similar to our hyperoxia experiments (PO₂ ~84 kPa), significant hypoxia may be present in centrally situated muscle fibers when the critical radius of ~0.6 mm is exceeded at 37°C (23). These authors showed that at temperatures higher than 25°C, P₁ and P₀ in both the soleus and extensor digitorum longus muscles decreased with the duration of in vitro incubation. This was accompanied by a temperature-dependent depletion of glycogen content in the central portions of these muscles (23). However, in contrast to whole muscle preparations, diaphragm strip thickness was well within this critical radius (31, 32). In previous studies of preparations, diaphragm strip thickness was well within muscles (23). However, in contrast to whole muscle preparations, diaphragm strip thickness was well within this critical radius (31, 32). In previous studies of preparations, diaphragm strip thickness was well within this critical radius (31, 32).

In the present in vitro experiments, diaphragm muscle strips were directly stimulated and neuromuscular transmission was blocked by the addition of β-tubocurarine to the Krebs solution. Also, alterations in muscle blood flow in response to acute hypoxia are unlikely to be involved in these in vitro experiments. Therefore, downregulation of oxidative enzymes such as succinate dehydrogenase or cytochrome-c oxidase may be important mechanisms in the reduction of force generation induced by acute hypoxia during the present experiments. This downregulation may be the result of ROS formation and is encountered after repetitive diaphragm stimulation (15, 29). ROS and scavengers of ROS are increasingly implicated in modulating contractile properties in skeletal and respiratory muscles. In skeletal muscles, a low level of ROS is essential for excitation-contraction coupling and is obligatory for optimal contractile function (21). However, whether ROS formation is altered by β₂-adrenoceptor agonist treatment is not known.

Effects of β₂-adrenoceptor agonist treatment. The present study shows that treatment with short- and long-acting β₂-adrenoceptor agonists reduced the hypoxia-induced decline in diaphragm P₀. The exact mechanism by which β₂-adrenoceptor agonists exert their inotropic effect on respiratory muscles is not fully understood. These mechanisms may involve excitation-contraction coupling of skeletal muscle. In earlier experiments, the inotropic effect of salbutamol was blocked by ryanodine, which indicates that this effect is most likely mediated by an improvement of sarcoplasmic reticulum (SR) Ca²⁺ release (31). This is in agreement with previous findings reported by Cairns and Dulhunty (4, 5) and Cairns et al. (6). They further showed that enhancement of sodium-pump activity, dihydropyridine-sensitive Ca²⁺ currents, glycolysis, and altered action potentials are unlikely to be involved in the mechanisms of action β₂-adrenoceptor agonists (4, 5).

These findings in mammalian skeletal muscle are in line with earlier experiments conducted with frog skeletal muscle, showing that adrenaline treatment potentiated P₀ by modulating calcium channels (10).

The decrease in P₀ found in the salmeterol-treated hyperoxic and salbutamol-treated hypoxic groups may
also found for propranolol alone when $P_t$ is expressed as a percentage of initial $P_t$. This is in agreement with an earlier report (17) in which a selective $\beta_2$-adrenoceptor antagonist reduced force generation in gastrocnemius muscle preparations. It has been suggested that this could be the result of a blockade of endogenous catecholamines (17). However, it is unlikely that such an effect was of importance in the present in vitro experiment. Alternatively, these findings may indicate changes in oxidative enzyme activity (14).

Methodological considerations. In the present study, alteration of tissue bath oxygenation and addition of $\beta_2$-adrenoceptor agonists and/or antagonists were performed simultaneously. These changes do not immediately exert their effect, and all parameters are likely to have different equilibration times. We did not include separate time frames for either hypoxia (diffusion time) or the onset of action of salbutamol or salmeterol in the diaphragm strips. The use of different time frames would certainly have affected force production because at 37°C, a time-related decrease of in vitro force production is present. In such an experimental design, the use of separate time-matched control groups would have been obligatory.

Not surprisingly, the present study shows that salbutamol has a more rapid onset of action and a larger effect on $P_t$ compared with those with salmeterol. This can be explained by differences in lipophilicity and a lower efficacy (partial agonist) of salmeterol compared with salbutamol (11). Furthermore, diaphragm $P_t$ was decreased further in both hypoxia and hyperoxia when salmeterol started to have an effect. This may have reduced the inotropic effect of salmeterol. However, the duration of action of salmeterol was longer compared with that of salbutamol, which is in agreement with its pharmacological properties (11).

In the present study, very high concentrations (1 µM) of salbutamol and salmeterol were used. At these high concentrations, salmeterol may have non-$\beta_2$-adrenoceptor properties due to its high lipophilicity (18). However, the experiments in which propranolol was added to salbutamol or salmeterol treatment show that these non-$\beta_2$-adrenoceptor-related processes did not play a role in the inotropic effects of salmeterol on diaphragm $P_t$ during hypoxia. In earlier studies (31, 32), salbutamol was shown to have significant inotropic effects at the clinically relevant concentration of 0.05 µM. Preliminary studies with salmeterol showed that lower concentrations had similar but less pronounced effects during hypoxia. To reach maximal effects and to simplify comparison between the two drugs, we chose the high concentration of 1 µM for both compounds.

Clinical relevance. In patients with severe COPD, chronic hypoxia can frequently be found, often in combination with hypercapnia. Furthermore, during exacerbations of their disease, acute hypoxia or acute-on-chronic hypoxia may be present. This is of particular interest because hypoxia may reduce diaphragm contractile properties in situ (2) and in vitro (26) and may reduce fatigue resistance in vitro (24) and in humans (13). Dysfunction of the respiratory muscles frequently occurs in patients with severe COPD (20). Metabolic changes like hypoxia, hypercapnia, and electrolyte disorders are important factors that may impede (the already compromised) respiratory muscle function (2, 9, 16, 26, 27, 29). In this clinical situation, $\beta_2$-adrenoceptor agonists are often used for bronchodilatation. Previous studies have shown that $\beta_2$-adrenoceptor agonists like salbutamol and terbutaline can improve diaphragm contractile properties under optimal in vitro conditions (1, 32) and after fatigue (8, 19, 30). The present study shows that the decrease in diaphragm contractility under hypoxic conditions can be partially prevented by the addition of $\beta_2$-adrenoceptor agonists in vitro. This might be of importance in the treatment of incipient or manifest respiratory muscle fatigue in COPD patients, but clinical studies are recommended to evaluate the effects of $\beta_2$-adrenoceptor agonists in these situations.
REFERENCES