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# The Effects of Cold Stress on Respiration of Diaphragm Muscle

E. N. Mokhova and D. B. Zorov

*Department of Bioenergetics, Laboratory of Bioorganic Chemistry*
*Moscow State University, Moscow, USSR*

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*Abstract*

The mechanism of the stimulating effect of short-term cold exposure of animal on tissue respiration has been studied. Oxygen consumption by a piece of the rat diaphragm muscle, fixed in a frame and put into a polarographic cell, was measured with an oxygen electrode. It is found that 13 min exposure of the rat to an air temperature of +2° C induces (1) an increase in respiration rate in the diaphragm tissue, (2) a decrease in stimulation of respiration by dinitrophenol (DNP) and (3) a decrease in the sensitivity of respiration to amytal. The maximal respiration rate observed in the presence of 40 μM DNP is unaffected. Acclimation for two weeks of rats at +2° C does not influence the measured parameters. Effects similar to those produced by short-term cold exposure can be obtained by treatment of the cold acclimated animals with norepinephrine. In non-acclimated rats, norepinephrine is ineffective. The effects of cold exposure (or norepinephrine) can be reproduced on the diaphragm from a non-treated animal by the addition of 0.2 mM oleate or 10 mM pyruvate to the incubation medium. All effects of cold exposure or norepinephrine *in vivo*, or oleate and pyruvate *in vitro* are inhibited by oubain or replacement of $Na^+$ by $Li^+$ in the incubation mixture. The role of fatty acids and Na,K ATP-ase in the thermoregulatory responses of the tissue respiration is discussed.

In mammalia, muscles not only take part in shivering but also in non-shivering thermogenesis induced by a decrease in the ambient temperature. The latter response involves stimulation of the muscle tissue respiration occurring without corresponding increase in contraction activity [1].

Skulachev and co-workers have formulated the hypothesis of thermoregulatory uncoupling by fatty acids in muscle tissue [2, 3]. They

showed an increase in the concentration of non-esterified fatty acids in skeletal muscles during the short-term cold exposure. The increased level of fatty acids was found also in muscle mitochondria isolated from cold treated animals. The P/O ratio of these mitochondria proved to be much lower than that of non-treated animals [4].

Beyer showed that cold acclimation of rats resulted in an increase in the respiration of the diaphragm pieces and in the lowering of the sensitivity of respiration to amytal [5]. On this ground, Beyer suggested that thermogenesis should be due not only to the uncoupling, but also to the activation of a non-phosphorylating, amytal resistant, pathway of oxidation.

In the present work, we have studied the influence of cold exposure on the respiration of diaphragm pieces of rat. The respiration changes brought about by cold exposure or norepinephrine treatment were found to be in accordance with the suggestion that the increase in respiration is connected with the growth in the level of fatty acids. Some indications of the involvement of Na,K ATP- ase in the phenomenon of the stimulation of muscle tissue respiration on brief cold exposure were obtained.

## *Methods*

Female Wistar rats weighing 100-150 g were used. The schedules of temperatures at which the animals were kept are shown in Table I. Norepinephrine was injected intramuscularly at a concentration of 0.5 mg/kg. The dissected diaphragm was kept in a cold ($\sim +6^\circ$ C) moist chamber. From one diaphragm from four to six preparations were obtained; the weight of each was about 10 mg. The edges of the diaphragm pieces were clamped between two Teflon or Perspex frames, under slight stretching. The frames were put into a polarographic cell. The incubation medium (NaCl-145 mM, KCl-5.6 mM, $NaH_2PO_4$-1 mM, $Na_2HPO_4$-5 mM) was saturated with oxygen at atmospheric pressure, 28° C, pH 7.5. The oxygen uptake was measured with a Clark-type oxygen electrode in a magnetically stirred cell.

For every diaphragm the initial respiration rate ($V_0$), the respiration rate after adding 40 $\mu$M DNP ($V_{DNP}$) and that after subsequently adding 2 mM amytal ($V_{am}$) were measured. The respiration rate for all groups, in the presence of 40 $\mu$M DNP was maximal. We found that, to avoid hypoxia, the $PO_2$ value had to exceed 300 mm Hg. In these conditions the respiration rate in the presence of DNP does not change after decreasing the oxygen concentration as a result of its being consumed by the diaphragm in the incubation medium. The electrode drift was lower than 20 nAtoms O/min. Palmytic acid was dissolved in alcohol at a concentration of 20 mM. DNP was recrystallized in water. Oleic acid was

TABLE I. Effects of cooling and norepinephrine injection on diaphragm respiration rates

| Date | Group N, N | Treatment | $V_o$ | $V_{DNP}$ | $V_{DNP}/V_o$ | $V_{am}/V_{DNP}$ |
|---|---|---|---|---|---|---|
| October | I | 2 weeks at 28°C | 6.9 ± 0.6 (9) | 13.0 ± 1.1 (9) | 1.87 ± 0.06 (9) | 0.58 ± 0.03 (9) |
| | IV | 2 weeks at 2°C, 3 hours at 22°C, 13 min at 2°C | 11.9 ± 0.8 (9)[a] | 15.9 ± 1.2 (9) | 1.40 ± 0.03 (9)[a] | 0.76 ± 0.07 (9)[a] |
| | V | 2 weeks at 2°C, 3 hours at 22°C, norepinephrine, 13 min at 22°C | 11.7 ± 1.0 (9)[a] | 15.8 ± 1.1 (9) | 1.41 ± 0.04 (9)[a] | 0.82 ± 0.06 (9)[a] |
| January-February | I | 2 weeks at 28°C | 6.6 ± 0.4 (9) | 12.3 ± 0.4 (9) | 1.93 ± 0.04 (9) | 0.62 ± 0.03 (9) |
| | II | 2 weeks at 2°C | 6.2 ± 0.5 (8) | 12.6 ± 0.6 (8) | 2.03 ± 0.09 (8) | 0.58 ± 0.04 (8) |
| | III | 2 weeks at 2°C, 3 hours at 22°C | 6.7 ± 0.5 (10) | 12.5 ± 0.6 (10) | 1.94 ± 0.05 (10) | 0.55 ± 0.03 (10) |
| | IV | 2 weeks at 2°C, 3 hours at 22°C, 13 min at 2°C | 8.6 ± 0.5 (10)[a] | 12.5 ± 0.6 (10) | 1.48 ± 0.03 (10)[a] | 0.74 ± 0.03 (10)[a] |
| | VI | 2 weeks at 28°C, 13 min at 2°C | 8.5 ± 0.4 (9)[a] | 12.1 ± 0.5 (9) | 1.47 ± 0.04 (9)[a] | 0.75 ± 0.02 (9)[a] |
| May-June | I | 2 weeks at 28°C | 6.2 ± 0.4 (7) | 15.9 ± 1.2 (7) | 2.51 ± 0.17 (7) | 0.36 ± 0.05 (7) |
| | IV | 2 weeks at 2°C, 3 hours at 22°C, 13 min at 2°C | 8.8 ± 0.8 (7)[a] | 15.8 ± 1.5 (7) | 1.79 ± 0.04 (7)[a] | 0.55 ± 0.03 (7)[a] |
| | VII | 2 weeks at 28°C, norepinephrine | 6.5 ± 0.6 (7) | 16.1 ± 1.7 (7) | 2.48 ± 0.20 (7) | 0.44 ± 0.05 (7) |

[a] The difference between the data of this group and group I is significant at a level of $\alpha < 0.01$

$V_o$, initial respiration rate; $V_{DNP}$, respiration rate after adding 40 μM DNP; $V_{am}$, respiration rate after adding 2 mM amytal in the presence of 40 μM DNP.

Rates in nAtoms O/min mg wet wg. Values are means ± s.e. (number of rats).

dissolved in a small amount of alcohol, neutralized with NaOH and diluted with water to 20 mM.

## *Results and Discussion*

The effects of cold exposure and norepinephrine treatment on the respiration rate are shown in Table I. It is seen that a short cold exposure of rats previously acclimated to cold (Group IV) resulted in some increase in the initial respiration rate ($V_0$). The rate after addition of DNP was not changed. In the cold-exposed animals, sensitivity of DNP-stimulated respiration to amytal ($V_{am}/V_{DNP}$) was found to decrease. The increase in the respiration is due neither to the activation of extramitochondrial respiratory pathways nor to the increase in the number of mitochondria, as $V_{DNP}$ is unchanged.

The difference between the respiration rates of the control preparations and those after cold exposure of cold adapted rats remained constant in the 2-10 mM amytal concentration range (Fig. 1).

It is known that the injection of norepinephrine produces the same effect on the *in vivo* respiration of the cold acclimated rats as does a short cold stress [6]. As it is seen from Table I, injection of norepinephrine into cold acclimated rats changed the respiration of the diaphragm preparation to the same extent as short-term cold exposure (group V). In rats acclimated to +28° C, norepinephrine was ineffective (group VII). Norepinephrine is known to stimulate lypolysis in brown fat, which increases during cold acclimation of rats. Perhaps, differences in the responses of diaphragm respiration to norepinephrine of the cold- and warm-acclimated rats were due to the corresponding differences in the amount of the brown fat.

It is noteworthy that changes in diaphragm respiration induced by the short-term cold exposure were observed in both cold- (group IV) and warm- (group VI) acclimated animals. Apparently, in warm-acclimated rats, cold stress induced some effects which do not require brown fat and norepinephrine involvement. These effects may be connected with the fatty acids whose concentration in the plasma increases after the first cooling [7].

The respiration rate of cold-acclimated rats which were not exposed to cold stress conditions before the measurements (group II and III) was about the same rate as that of the control group, acclimated to 28° C. This observation can be compared with data by Hannon *et al.* who showed that an increase in the oxidation of fatty acids in tissues occurs only after a short cold exposure of rats, but not after their living for a long time at a low temperature [8]. It should be also mentioned that it is cold stress but not cold acclimation which increases free fatty acids level in the blood plasma [7].

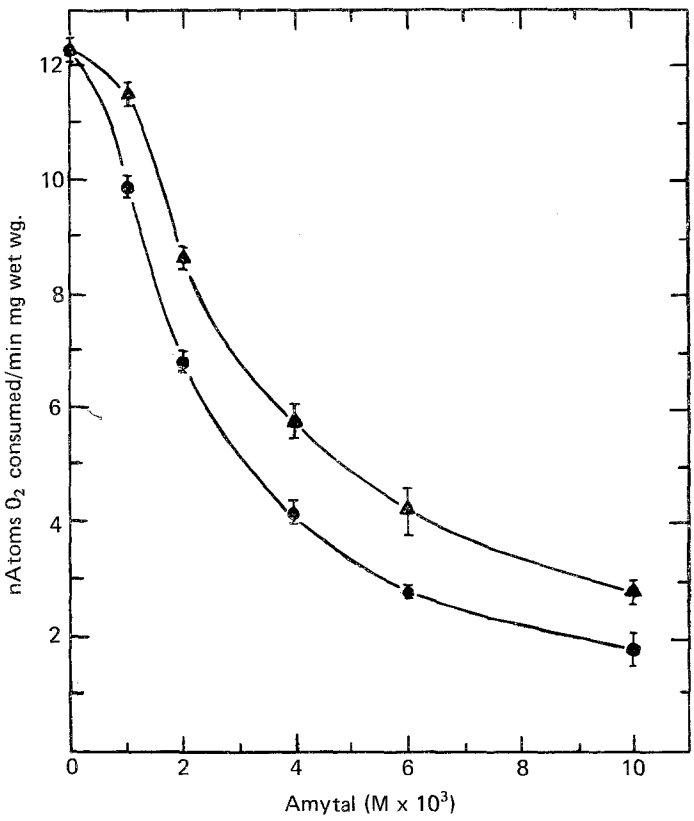


Figure 1. The sensitivity of the diaphragm muscle respiration to amytal. Incubation mixture (145 mM NaCl, 5.6 mM KCl, 5 mM $NaH_2PO_4$, 1 mM $Na_2HPO_4$, 40 $\mu$M DNP, pH 7.5, 28° C) ▲—▲— the respiration of diaphragm preparations from cold adapted rats after short cold exposure, ●—●— the respiration of preparations from control rats. Mean ± s.e.

So, the effects described in Table I (an increase in the respiration rate of the diaphragm, a decrease in the stimulation of respiration by DNP and a decrease in the sensitivity of respiration to amytal) seems to occur under conditions under which one may expect an increase in the uptake of fatty acids by the muscles.

In the further experiments, the action of adding fatty acids *in vitro* to diaphragm preparations from control animals has been studied. It was found that addition of 0.2 mM palmitic acid did not influence the initial respiration rate but induced a decrease in the sensitivity of respiration to amytal. After adding 2.8 mM amytal to the preparation from the control

group we obtained $V_{am}/V_{DNP} = 0.43 \pm 0.04$ (7) and in the presence of palmitic acid $V_{am}/V_{DNP} = 0.66 \pm 0.03$ (7). Addition of 0.2-0.3 mM sodium oleate resulted in the enhancement of initial respiration and of the $V_{am}/V_{DNP}$ ratio. These changes were of the same extent as those induced by cold exposure of the animal (Table II). The increase in the oleate concentration to 1 mM did not cause any additional stimulation.

TABLE II. Effect of oleate on diaphragm respiration rates

| | $V_o$ | $V_{DNP}/V_o$ | $V_{am}/V_{DNP}$ |
|---|---|---|---|
| Control | 5.4 ± 0.3 (10) | 2.17 ± 0.08 (10) | 0.39 ± 0.03 (10) |
| Control + 0.2 mM oleate | 8.9 ± 0.3 (10) $\alpha < 0.01$ | 1.50 ± 0.04 (10) $\alpha < 0.01$ | 0.52 ± 0.02 (10) $\alpha < 0.01$ |
| Control + 0.2 mM oleate + 0.1 mM oubain | 7.4 ± 0.4 (10) | 1.81 ± 0.05 (10) | 0.46 ± 0.01 (10) |

$V_o$, initial respiration rate; $V_{DNP}$, respiration rate after adding 40 μM DNP; $V_{am}$, respiration rate after adding 2 mM amytal in presence of 40 μM DNP.

Rates in nAtoms O/min mg wet wg. Values are means ± s.e. (number of rats).

We found that addition of 10 mM pyruvate induced effects which were similar to those of oleate, i.e. an increase in the initial respiration rate and a decrease in the sensitivity of respiration to amytal, the respiration rate in the presence of DNP was not changed. The influence of pyruvate on the respiration of a preparation from cold exposed rats was less pronounced than on one from control rats (Table III).

Addition of pyruvate does not result in changes in the respiration rate in the presence of 0.2-0.3 mM oleate. In other experiments it was found that addition of 10 mM 3-0-methyl-d-glucose, instead of pyruvate or oleate, also stimulated the initial respiration of the diaphragm and decreased the amytal inhibition.

One of the hypotheses explaining the mechanism of the cold-induced stimulation of respiration proposes an activation of Na,K ATP-ase [9]. We supposed that in our case the primary cause of the increase of the initial respiration rate is the activation of Na,K ATP-ase by the increase in the concentration of some transported substrates [10, 11]. In a number of papers it was shown that some substrates were transported into the cell with Na [12]. So, the increase in the transport of some substrates leads to the increase in Na level in cells, and hence to the Na,K ATP-ase activation. We studied the influence of some agents inhibiting Na,K ATP-ase activity. The results of these experiments are summarized in Tables II, IV and V. Both oubain addition and replacement of $Na^+$ by

TABLE III. Effect of pyruvate on diaphragm respiration rates

| | 2 weeks at 28°C | | Significance of differences | 2 weeks at 28°C, 13 min at 2°C | | Significance of differences |
|---|---|---|---|---|---|---|
| | without pyruvate | with pyruvate | | without pyruvate | with pyruvate | |
| $V_o$ | 6.6 ± 0.4 (7) | 9.3 ± 0.5 (7) | $\alpha < 0.01$ | 8.5 ± 0.4 (7) | 10.0 ± 0.8 (7) | |
| $V_{DNP}$ | 12.3 ± 0.4 (7) | 12.6 ± 0.9 (7) | | 12.1 ± 0.5 (7) | 13.8 ± 0.8 (7) | |
| $V_{DNP}/V_o$ | 1.93 ± 0.04 (7) | 1.39 ± 0.03 (7) | $\alpha < 0.01$ | 1.47 ± 0.04 (7) | 1.31 ± 0.07 (7) | |
| $V_{am}/V_{DNP}$ | 0.62 ± 0.03 (7) | 0.71 ± 0.02 (7) | $0.01 < \alpha < 0.02$ | 0.75 ± 0.02 (7) | 0.80 ± 0.03 (7) | $0.02 < \alpha < 0.05$ |

$V_o$, initial respiration rate.
$V_{DNP}$, respiration rate after adding 40 $\mu$M DNP.
$V_{am}$, respiration rate after adding 2 mM amytal in presence of 40 $\mu$M DNP.
Rates in nAtoms O/min mg wet wg. Values are means ± s.e. (number of rats).

$Li^+$ in the incubation medium decreases respiratory responses induced by cold stress (Tables IV and V). In other experiments it was shown that oubain addition decreased also the respiratory responses induced by oleate (Table II) and pyruvate *in vitro*.

All the data described above were obtained with the frames that press the diaphragm edges only slightly. When these frames were replaced by frames that clamp the diaphragm edges tightly, no effect of cold exposure and substrate treatment could be observed. With tightly clamping frames we had $V_{DNP}/V_0 = 2.18 \pm 0.28$ (9) for preparations of the control group and $V_{DNP}/V_0 = 2.32 \pm 0.39$ (6) for preparations of rats after cold exposure. For the slightly clamping frames the corresponding values are $V_{DNP}/V_0 = 1.83 \pm 0.09$ (13) and $V_{DNP}/V_0 = 1.28 \pm 0.03$ (13). Similar results were obtained when studying the action of pyruvate on the respiration of the diaphragm preparation of control rats. Using slightly clamped frames, we obtained $V_{pyr}/V_0 = 1.60 \pm 0.05$ (7), and for tightly clamping frames $V_{pyr}/V_0 = 0.99 \pm 0.02$ (7). If we made the injury on the latter preparation, the effect of pyruvate appeared, but it was rather small: $V_{pyr}/V_0 = 1.30 \pm 0.07$ (7). The effects under study are not due to pyruvate diffusion through the damaged muscle fibres. In all cases the addition of 0.1 mM oubain abolishes all the pyruvate effects.

Summarizing the above results one can conclude that a rather wide range of factors affecting diaphragm muscle *in situ* or during incubation in the polarographic cell induce similar changes in the respiratory system of this tissue, i.e. an increase in the respiration rate and decrease of both stimulation of respiration by DNP and inhibition by amytal. Among those factors are cold stress, and the administration of norepinephrine *in vivo*, and *in vitro* treatment of diaphragm with oleate, pyruvate or 3-0-methyl-d-glucose. All responses seem to require the Na,K ATP-ase to be in the working state. Stimulation of respiration and decrease of its DNP-induced enhancement might be explained by activation of the Na,K ATP-ase due to symport of $Na^+$ and some substances into the muscle cell [12]. Apparently, this was the case in experiments with 3-0-methyl-d-glucose, which like glucose is transported through the muscle cell membrane, but can not be metabolized. It is probable that activation of Na,K ATP-ase results in stimulation of phosphorylating oxidation because of increase in the ADP and inorganic phosphate levels. However, it is obscure why such an effect caused a decrease in the amytal inhibition of respiration. It follows from our data that to decrease the sensitivity of diaphragm respiration to amytal it is enough to increase the level of some transported substrates (metabolized or unmetabolized).

It is also not clear for a diaphragm piece incubated *in vitro*, what transport processes are activated by cold or norepinephrine treatment *in vivo*. Maybe, it is transport of fatty acids released from intercellular space or from fat cells presenting in diaphragm into cells of the same

TABLE IV. Effects of ouabain on diaphragm respiration rates

| | 2 weeks at 28°C | 2 weeks at 28°C, 13 min at 2°C | Significance | 2 weeks at 28°C | 2 weeks at 2°C, 3 hours at 22°C, 13 min at 2°C | Significance |
|---|---|---|---|---|---|---|
| $V_o$ | 7.8 ± 1.1 (6) | 10.3 ± 1.6 (6) | $0.02 < \alpha < 0.05$ | 6.5 ± 0.6 (8) | 9.8 ± 1.0 (8) | $\alpha < 0.01$ |
| $V_{ouab.}$ | 7.5 ± 1.1 (6) | 8.0 ± 1.6 (6) | | 6.3 ± 0.5 (8) | 7.1 ± 0.8 (8) | |
| $V_{DNP}$ | 12.3 ± 1.2 (6) | 13.1 ± 2.2 (6) | | 12.9 ± 1.1 (8) | 12.9 ± 1.9 (8) | |
| $V_{am.}$ | – | – | | 5.6 ± 0.4 (8) | 6.6 ± 0.8 (8) | |
| $V_{ouab}/V_o$ | 0.95 ± 0.03 (6) | 0.76 ± 0.03 (6) | $\alpha < 0.01$ | 0.97 ± 0.02 (8) | 0.73 ± 0.02 (8) | $\alpha < 0.01$ |
| $V_{DNP}/V_o$ | 1.69 ± 0.10 (6) | 1.31 ± 0.05 (6) | $0.01 < \alpha < 0.02$ | 2.06 ± 0.12 (8) | 1.34 ± 0.12 (8) | $\alpha < 0.01$ |
| $V_{DNP}/V_{ouab.}$ | 1.72 ± 0.15 (6) | 1.69 ± 0.11 (6) | | 2.11 ± 0.12 (8) | 1.85 ± 0.05 (8) | |
| $V_{am}/V_{DNP}$ | – | – | | 0.43 ± 0.02 (8) | 0.51 ± 0.02 (8) | |

$V_o$, initial respiration rate; $V_{DNP}$, respiration rate after adding of 40 μM DNP; $V_{ouab.}$, respiration rate after adding of 0.1 mM ouabain; $V_{am.}$, respiration rate after adding of 2 mM amytal in presence of ouabain and DNP.
Rates in nAtoms O/min mg wet wg. Values are means ± s.e. (number of rats).

TABLE V. Effect of substituting $Li^+$ for $Na^+$ on diaphragm respiration rates

| | 2 weeks at 28°C | | 2 weeks at 2°C, 3 hours at 22°C, 13 min at 2°C | | Significance |
|---|---|---|---|---|---|
| | Na | Li | Na | Li | |
| $V_o$ | 8.7 ± 1.3 (8) | 8.9 ± 2.1 (7) | 11.8 ± 0.9 (8) | 9.8 ± 0.5 (7) | $0.02 < \alpha < 0.05$ |
| $V_{DNP}$ | 15.4 ± 1.3 (8) | 16.0 ± 1.8 (7) | 15.9 ± 1.1 (8) | 17.4 ± 0.8 (7) | |
| $V_{DNP}/V_o$ | 1.78 ± 0.06 (8) | 1.76 ± 0.11 (7) | 1.33 ± 0.03 (8) | 1.79 ± 0.07 (7) | $\alpha < 0.01$ |

$V_o$, initial respiration rate; $V_{DNP}$, respiration rate after adding of 40 μM DNP.
Rates in nAtoms O/min mg wet wg. Values are means ± s.e. (number of rats).

tissue. Perhaps the slight pressure exerted on the diaphragm edges or making the injury produces a permeability increase because of the swelling of the preparation. The effect may explain the difference between the two types of frames used in the above experiments.

In any case we may suggest that an increase in fatty acid and other substrate levels may activate the respiration by activation of Na,K ATP-ase. However, fatty acids may act as uncouplers or in some other manner. These possibilities concerning the mechanism of cold induced increase of the diaphragm respiration as well as the amytal effects in this tissue are now under investigation.

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