Effect of 2,4-dinitrophenol on the hypometabolic response to hypoxia of conscious adult rats

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Saiki, Chikako, and Jacopo P. Mortola. Effect of 2,4-dinitrophenol on the hypometabolic response to hypoxia of conscious adult rats. J. Appl. Physiol. 83(2): 537–542, 1997.—During acute hypoxia, a hypometabolic response is commonly observed in many newborn and adult mammalian species. We hypothesized that, if hypoxic hypometabolism were entirely a regulated response with no limitation in O2 availability, pharmacological uncoupling of the oxidative phosphorylation should raise O2 consumption (V\textsuperscript{O2}) by similar amounts in hypoxia and normoxia. Metabolic, ventilatory, and cardiovascular measurements were collected from conscious rats in air and in hypoxia, both before and after intravenous injection of the mitochondrial uncoupler 2,4-dinitrophenol (DNP). In hypoxia (10% O2 breathing, 60% arterial O2 saturation), V\textsuperscript{O2}, as measured by an open-flow technique, was less than in normoxia (~80%). Successive DNP injections (6 mg/kg, 4 times) progressively increased V\textsuperscript{O2} in both normoxia and hypoxia by similar amounts. Body temperature slightly increased in normoxia, whereas it did not change in hypoxia. The DNP-stimulated V\textsuperscript{O2} during hypoxia could even exceed the control normoxic value. A single DNP injection (17 mg/kg iv) had a similar metabolic effect; it also resulted in hypotension and a drop in systemic vascular resistance. We conclude that pharmacological stimulation of V\textsuperscript{O2} counteracts the V\textsuperscript{O2} drop determined by hypoxia and stimulates V\textsuperscript{O2} not dissimilarly from normoxia. Hypoxic hypometabolism is likely to reflect a regulated process of depression of thermogenesis, with no limitation in cellular O2 availability.

METHODS

The study was performed on adult male Sprague-Dawley rats and was approved by the Animal Ethics Committee of this institution. The body weight of the rats ranged between 193 and 227 g (mean 204 ± 3 (SE) g). One group of animals was used for the metabolic response to successive administration of DNP in normoxia or hypoxia (hypoxic hypoxia). A second group was the object of respiratory and cardiovascular measurements in normoxia, followed by hypoxia before and after a single injection of DNP.

All measurements were performed on conscious animals, in the afternoon after the morning preparation, and at an ambient temperature of 15°C, i.e., in moderately cold conditions.

Animal preparation. In the early morning, the animal was prepared under general anesthesia (breathing halothane from an open circuit). Once anesthesia was induced, atropine sulfate was given (0.01 mg sc). After a small incision was made in the neck, a polyethylene catheter (PE-50, total volume 0.1 ml) filled with saline and heparin (100 U/ml saline) was introduced into the superior vena cava via the right jugular vein. This catheter was used for blood sampling and DNP injection. A second catheter (total volume 0.12 ml) similarly filled with heparin was introduced via the tail artery and was used for blood sampling and monitoring of mean arterial blood pressure (MAP) (31). The preparation was terminated within 1 h. The animal was returned to the...
cage, and within a few hours its behavior suggested full recovery. Data were collected in the afternoon.

Measurements. All measurements were performed on conscious animals loosely restrained in a cylindrical container made of a metal net that prevented back turning. A fine tungsten-constantan thermocouple (model DP30, Omega) was inserted 6 cm into the colon, and its temperature measurement taken as representative of body temperature (Tb). The restrainer was placed into a cylindrical 1.8-liter Plexiglas chamber for measurements of gaseous metabolism ([V˙O2, CO2 production (V˙CO2)], as well as minute expiratory ventilation (Ve). The venous and arterial catheters emerged outside the chamber. Both were connected to syringes via a three-way stopcock for blood sampling, and the arterial catheter was connected to a pressure transducer (model 1290C, Hewlett-Packard) for MAP and heart rate measurements. The sampling procedure and MAP recording were as previously described in detail (31).

The chamber temperature was preset at 15°C by adjusting the temperature of a water jacket surrounding the chamber. At this temperature, normoxic thermogenesis was expected to be increased, thus magnifying the hypometabolic response to hypoxia.

Gaseous metabolism was measured by an open flow system, following the same procedure described previously (6, 31). Briefly, a polarographic O2 analyzer (Beckman OM-11) and an infrared CO2 analyzer (Beckman LB-2) monitored the O2 and CO2 concentrations of a gas delivered through the chamber at a constant flow rate (900–1,050 ml/min) maintained by a calibrated flowmeter. Data were acquired and displayed on a computer monitor every 5 s. VeO2 and VcO2 were computed from the average inflow-outflow difference in gas concentration over a time interval, multiplied by the flow. The error introduced by a respiratory quotient (RQ) <1, for the O2 and CO2 concentrations used in the present study, was small, ranging from almost zero for VcO2 to 6% for VeO2 in the worst case of RQ = 0.7 in normoxia (5). Therefore, no RQ correction was considered necessary. VeO2 and VcO2 are presented, normalized by the weight of the animal in kilograms at STPD conditions.

The breathing pattern was monitored by the barometric technique, after complete sealing of the chamber for the period of ~100 breaths (~1 min), and the oscillations of chamber pressure were recorded on paper at a speed of 10 mm/s. From the record and the appropriate correction factors, breathing frequency (f) and tidal volume (Vt) were determined with the help of a graphics tablet connected to a computer, from which pulmonary ventilation (Ve = f·Vt) was calculated (23).

Blood samples were collected anaerobically (~0.25 ml/sample) and immediately analyzed for blood gases (PO2, PCO2, and pH at the rat’s Tc) with a blood-gas analyzer (Instrumentation Laboratory System 1302, with repeated calibration before the measurements), and for hemoglobin (Hb) concentration (g/100 ml) and arterial O2 saturation (SatO2, in %) with a hemoximeter (OSM2b, Radiometer). Additional venous blood (~0.2 ml) was sampled in normoxia and hypoxia + DNP for the purpose of measuring lactate concentration. The deproteinized sample (1:2 vol/vol in 8% perchloric acid) was centrifuged (2,700 revolutions/min for 20 min at 4°C). The supernatant was assayed spectrophotometrically following standard enzymatic procedures (lactate kit 826, Sigma Chemical, St. Louis, MO).

From the above data, arterial and venous O2 contents (CaO2, Cvo2, respectively, in ml O2/100 ml blood) were calculated from the corresponding O2 saturation, as (SatO2/100)·Hb·1.34. Cardiac output (CO; in ml·kg⁻¹·min⁻¹) was calculated from the Fick principle as

\[
\text{CO} = \frac{\text{VeO}_2}{\text{CaO}_2 - \text{Cvo}_2} \times \frac{100}{\text{SV}}
\]

from which O2 delivery (DO2) (DO2 = CO·CaO2), stroke volume (SV) (SV = CO/heart rate), and systemic vascular resistance (SVR) index (SVR index = MAP/CO in mmHg·ml⁻¹·kg⁻¹·min⁻¹) were also computed. Pulmonary volumes (Ve, Ve) are presented at BTCP condition.

The hypoxic gas (10% O2) was prepared by blending air and pure N2 with flowmeters. DNP (Sigma Chemical) was prepared fresh as a 0.6 or 1.2% solution in phosphate-buffered saline (PBS) at pH 7.4.

Protocols. Experiments were conducted in the afternoon, once the animal was resting quietly, usually ~30 min after its placement in the metabolic chamber.

A first set of measurements in six rats was obtained for the purpose of establishing the effect of progressive dosages of DNP in either normoxia (n = 3) or hypoxia (n = 3). The normoxic group was exposed to air followed by injection of 6 mg/kg DNP every 20 min, for a total of four injections (i.e., a total of 24 mg DNP/kg). In the hypoxic group, data were first collected in air, followed by hypoxia (10% O2). DNP was then injected four times, as for the normoxic group, while hypoxia was maintained. Metabolic data were collected on each condition.

A second set of rats (n = 6) was studied in normoxia (30–60 min) followed by hypoxia (30 min). As hypoxia was maintained, PBS was then injected iv, and, after an additional 20–30 min, DNP was injected in a single dose (17 mg/kg). Injection of PBS was done to test for possible effects of the DNP vehicle. The DNP dosage was chosen on the basis of the results of the first set of experiments. The volumes injected (0.25–0.3 ml) were then flushed with 0.4–0.5 ml of saline. Each period (air, hypoxia, hypoxia + PBS, and hypoxia + DNP) lasted ~20–30 min, and data were collected toward the end of each condition. To test the possibility of a further increase in VeO2, a second DNP injection of the same dosage was administered at the end of the measurements in four rats of this group.

Statistical analysis. All values are presented as means ± SE. Comparison of the dose-VeO2 curves between normoxia and hypoxia (protocol 1) was done by unpaired t-test of the mean values at the corresponding dosages as well as of the intercepts and slopes of the two linear regression equations. For protocol 2, significant differences between mean data were assessed by repeated-measurements analysis of variance, followed by three post hoc contrasts with Bonferroni’s limitation (air-hypoxia, hypoxia-PBS, PBS-DNP). In all cases, a significant difference was considered at a level of P < 0.05.

RESULTS

Successive DNP injections. During air breathing, progressive iv injections of 6 mg/kg DNP, up to a total dosage of 24 mg/kg, resulted in corresponding increases in VeO2, from 34 to 51 ml·kg⁻¹·min⁻¹ (Fig. 1), the slope of the DNP-VeO2 curve being significantly greater than zero (P < 0.01). Tb also increased, from 37.9 ± 0.3 to 39.0 ± 0.2°C.

Hypoxia decreased VeO2 by ~7 ml·kg⁻¹·min⁻¹, and Tb decreased by almost 2°C. During hypoxia, as in air, DNP administration increased VeO2 and by similar amounts. At the largest dosages, DNP-stimulated VeO2 during hypoxia exceeded the control normoxic value.
The hypoxic DNP-V˙O2 relationship had a similar slope (P > 0.05) and significantly lower intercept (P < 0.01) than the corresponding curve in air (Fig. 1). During hypoxia, unlike normoxia, DNP administration did not significantly change Tb (from 35.8 ± 0.5°C before DNP to 35.5 ± 0.5°C after the last DNP injection).

Single injection. A second set of experiments was performed on six rats, during air breathing followed by hypoxia, the latter before and after injection of PBS and DNP. The DNP dosage (17 mg/kg iv) was chosen on the basis of the results of the first set of measurements. Hypoxia resulted in the expected drop in V˙O2 to ~75% of normoxia (Fig. 2), a value that was not affected by injection of the DNP-vehicle PBS. DNP injection, on the other hand, significantly raised hypoxic V˙O2, on average by 7 ml·kg⁻¹·min⁻¹ (Fig. 2). Tb decreased with hypoxia and continued to decrease after PBS and DNP injections.

The corresponding data concerning metabolic, ventilatory, and cardiovascular variables, including blood gases and derived parameters, are summarized in Tables 1 and 2. Hypoxia resulted in hyperventilation, with a doubling of V˙E/V˙O2 and a drop in arterial Pco2 (PaCO2) of ~10 Torr, mild hypotension, and no changes in CO. PBS injection had no significant effect on most parameters, the only exception being PaCO2, which further decreased by 2 Torr.

DNP raised V˙E, in parallel with the increase in V˙O2, i.e., with no significant change in V˙E/V˙O2. However, the stimulatory effect on V˙T exceeded that on f, resulting in a further decrease in PaCO2 (~3 Torr). MAP dropped to 62 mmHg. Because this was not compensated by a major increase in CO, peripheral vascular resistance after DNP decreased further, to ~55% of the normoxic value. Venous lactate in hypoxia after DNP injection was increased to approximately four times the normoxic value.

At the end of the measurements, a second injection of DNP was administered in four rats. It resulted in a further increase in V˙O2 to ~43 ± 1 ml·kg⁻¹·min⁻¹.

**DISCUSSION**

The main finding of this study was that in conscious rats DNP increased V˙O2 during hypoxic hypometabolism, and the increase was similar to that observed in normoxia. The observations that hypoxic V˙O2 could be pharmacologically stimulated to levels even exceeding the normoxic value and that the sensitivity of V˙O2 for DNP during hypoxia was similar to that during normoxia (same slope of DNP-V˙O2 curve, Fig. 1) would seem to conclusively exclude the possibility that in hypoxia cellular metabolism is limited by O2 availability. Thus the results are compatible with the hypothesis and support the notion that the hypometabolic response to hypoxia reflects a regulated inhibition of some of the processes requiring O2. These processes, in both newborn and adult mammals, are mostly represented by various forms of thermogenic mechanisms.

The validity of this interpretation will be considered within the discussion of other results.

**Tb.** Previous investigations have indicated that the hypermetabolic action of DNP could cause a major increase in Tb, possibly even affecting the animal's survival. In this study, despite the major increase in V˙O2, we observed only a 1°C increase in Tb during air breathing, and no increase during hypoxia. A species difference could be a factor, because Tb in the conscious rat increased only ~1.5°C even when "treated with the maximum nonfatal dose of DNP" (3). Rats, compared with the more commonly used dogs, have a greater body surface-to-volume ratio, which, in combination with the low ambient temperature, the hyperpnea, and the important decrease in vascular resistance, must have favored heat dissipation. In some studies in dogs, the use of anesthesia could have
compromised their thermoregulatory mechanisms. Indeed, Williams et al. (33) mentioned that if the dog was not anesthetized, DNP injection did not result in hyperthermia.

A number of indirect considerations (22), including experiments of artificial reawarming of hypoxic newborns (28, 30), have indicated that the drop in Tb during experiments of artificial rewarming of hypoxic newborns (28, 30) have indicated that the drop in Tb during hypoxia is the effect, not the cause, of the hypoxic hypometabolism. The fact that V\textsubscript{O2} had a similar response to DNP in normoxia and hypoxia, despite Tb being ~2°C lower in hypoxia, extends this notion, indicating that the hypoxic change in Tb does not have any sizable effect on V\textsubscript{O2} sensitivity.

Ventilatory responses. Increases in normoxic V\textsubscript{O2}, of which the most studied are cold-induced thermogenesis and muscle exercise, are accompanied by parallel changes in V\textsubscript{E} (4, 22). Pharmacological increases in V\textsubscript{O2} by use of mitochondrial uncouplers are also accompanied by corresponding increases in V\textsubscript{E}, with perfect or nearly perfect isocapnic conditions (13, 16, 18, 20, 29). When DNP was administered to the hypoxic rats, we also found a parallel increase in V\textsubscript{E} and V\textsubscript{O2}, i.e., constancy of V\textsubscript{E}/V\textsubscript{O2}, indicating that hypoxic hypometabolism does not hinder this remarkable association. However, we did observe a small drop in P\textsubscript{aCO2} (~3 Torr), suggesting a slightly disproportionate increase in alveolar ventilation compared with V\textsubscript{E}. The hypotension may have provided an additional ventilatory drive (27). In addition, the DNP-vehicle PBS was reported to stimulate V\textsubscript{E} in dogs (33), and we did observe a small drop in P\textsubscript{aCO2} with injection of PBS alone. Finally, it is known that DNP, in addition to its generalized hypermetabolic effect, directly stimulates the activity of the carotid body (26). One effect of the

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<th>Table 1. Metabolic, ventilatory, and cardiovascular parameters</th>
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Values are means ± SE; n = 6. PBS, phosphate-buffered saline; DNP, 2,4-dinitrophenol; V\textsubscript{O2}, oxygen consumption; V\textsubscript{CO2}, CO\textsubscript{2} production; Tb, body temperature; V\textsubscript{T}, tidal volume; f, breathing rate; V\textsubscript{E}, pulmonary expiratory ventilation; CO, cardiac output; HR, heart rate; SV, stroke volume; MAP, mean arterial pressure; DO\textsubscript{2}, O\textsubscript{2} delivery; SVRI, systemic vascular resistance index. *Significant difference from immediately preceding treatment; repeated-measurements analysis of variance (ANOVA) with post hoc Bonferroni’s limitations; P < 0.05.

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<th>Table 2. Blood values</th>
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<td>P\textsubscript{vCO2}, Torr</td>
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<td>Venous pH</td>
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<td>Cv\textsubscript{O2}, vol%</td>
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<td>Lactate, mM</td>
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<td>Arteriovenous difference</td>
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<td>CO\textsubscript{2}, vol%</td>
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Values are means ± SE; n = 6 rats. PBS, phosphate-buffered saline; DNP, 2,4-dinitrophenol; P\textsubscript{aO2}, arterial O\textsubscript{2} pressure; P\textsubscript{aCO2}, arterial CO\textsubscript{2} pressure; Sa\textsubscript{O2}, arterial O\textsubscript{2} saturation; Ca\textsubscript{O2}, arterial O\textsubscript{2} content; Hb, hemoglobin; P\textsubscript{vO2}, venous O\textsubscript{2} pressure; P\textsubscript{vCO2}, venous CO\textsubscript{2} pressure; Sv\textsubscript{O2}, venous O\textsubscript{2} saturation; Cv\textsubscript{O2}, venous O\textsubscript{2} content; C\textsubscript{O2}, O\textsubscript{2} content. *Significant difference from immediately preceding treatment; repeated measurements ANOVA with post hoc Bonferroni’s limitations. P < 0.05. †Lactate was measured only in normoxia and hypoxia + DNP; data were analyzed statistically by paired t-test.
modest hyperventilation after DNP was the increase in PO₂ from 32 to 36 Torr (Table 2). This lessening of the hypoxemia with DNP was, however, too minuscule for an appreciable contribution to the reversal of the hypoxic hypometabolism.

Cardiovascular responses. A modest drop in MAP is known to occur in conscious rats when they are exposed to acute hypoxia (25, 31). A much more important hypotension developed after injection of DNP and was accompanied by a major decrease in SVR. The causative link between these events is not clear. Because Tₚ was not increased, it seems unlikely that the drop in vascular resistance was the effect of hyperthermia, although in hypoxia the thermoregulatory set point shifts to a lower value, and a Tₚ lower than in normoxia could still be perceived as an hyperthermic condition (22). DNP could have decreased the energetic efficiency of the heart, already challenged by the hypoxia. Indeed, DNP has been frequently used in the past as a model of myocardial failure (See Ref. 13 for review). In anesthetized dogs, administration of DNP decreased vascular resistance without a drop in MAP (13, 19), and the same was reported to occur in the isolated hindlimb preparation, both in normoxia and hypoxia (2). However, while the dogs were breathing 11% O₂, DNP injection was often lethal, and death was attributed to a precipitous fall in MAP, even after artificial cooling to control the hyperthermic problems mentioned earlier (13).

The drop in vascular resistance after DNP may suggest increased perfusion to peripheral tissues, e.g., hindlimb skeletal muscles, intestines, fat, and skin, possibly underperfused during hypoxia (15). In such a case, the increase in VO₂ after DNP injection during hypoxia could also be interpreted as the result of reestablishing full aerobic metabolism to hypoxic tissues that were, in effect, O₂ limited before DNP. At first glance, the finding that blood lactate was increased after DNP in hypoxia, with respiratory compensation of the metabolic acidosis (drop in PA₂, with constant pH; Table 2), may be taken in support of this view. However, an increase in blood lactate after DNP injection should be expected, because the stimulation of mitochondrial respiration far exceeds the available O₂ (e.g., Refs. 10, 19). Therefore, the increase in blood lactate is much more likely to be the direct effect of DNP on tissue metabolism, rather than the systemic result of reperfusion of anaerobic tissues. In the isolated hindlimb of anesthetized lambs, DNP administration increased skeletal muscle VO₂ even during stagnant hypoxia, when O₂ extraction seemed limited by O₂ availability (11). Finally, if the increase in VO₂ after DNP injection in hypoxia was mostly the result of reoxygenation of O₂-deprived tissues, the similarity in VO₂ sensitivity to DNP between normoxia and hypoxia (Fig. 1) would be a curious coincidence difficult to explain.

Other interventions. A few scattered data from previous studies are pertinent to the issue of metabolic stimulation during hypoxic hypometabolism. In conscious dogs made severely hypoxic by breathing 7% O₂, the endorphin-inhibitor naloxone stimulated Vₑ without changes in blood gases (32). The simplest interpretation is that naloxone raised hypoxic VO₂. Indeed, in two dogs in which VO₂ was measured, Schaeffer and Hadad (32) mentioned that hypoxia dropped metabolism by 50–70% and naloxone more than doubled hypoxic VO₂, with no changes in arterial gases. A comparison of data collected by Gonzalez and collaborators (8, 9) in conscious rats shows that, during acute hypoxia, resting VO₂ decreased ~15%, whereas it doubled during hypoxic exercise. Despite their paucity and anecdotal appearance, these data are in complete agreement with the information in the present study. Indeed, if hypoxic hypometabolism is the expression of selective inhibition of thermogenesis, without cellular O₂ limitation, it would seem likely that not only DNP but also other pharmacological or physiological interventions should result in an increase of hypoxic VO₂. It also follows that in hypoxia the hypometabolic state should not be assumed to remain constant, and interventions altering ventilatory or cardiovascular parameters may be mediated by its changes.

In conclusion, DNP stimulated VO₂ in conscious rats, not only during normoxia but also in hypoxia, and by similar amounts, despite the hypometabolic state of the hypoxic condition. The most likely interpretation is that during hypoxic hypometabolism cellular O₂ availability is not limited. Rather, hypoxic hypometabolism could indicate a selective, regulated inhibition of some processes requiring O₂. Among these processes, those pertinent to thermogenesis are probably the most important.

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542 2,4-DINITROPHENOL AND HYPOXIC HYPOMETABOLISM


