Effects of aerobic and anaerobic metabolic inhibitors on avian intrapulmonary chemoreceptors

Jason Q. Pilarski, Irene C. Solomon, Delbert L. Kilgore Jr., and Steven C. Hempleman

Effects of aerobic and anaerobic metabolic inhibitors on avian intrapulmonary chemoreceptors. Am J Physiol Regul Integr Comp Physiol 296: R1576–R1584, 2009. First published March 18, 2009; doi:10.1152/ajpregu.90608.2008.—Birds have rapidly responding respiratory chemoreceptors [intrapulmonary chemoreceptors (IPC)] that provide vagal sensory feedback about breathing pattern. IPC are exquisitely sensitive to CO2 but are unaffected by hypoxia. IPC continue to respond to CO2 during hypoxic and even anoxic conditions, suggesting that they may generate ATP needed for signal transduction anaerobically. To assess IPC energy metabolism, single-cell action potential discharge and acid-base status were recorded from 26 pentobarbital-anesthetized Anas platyrhynchos before and after intravenous infusion of the glycolytic blocker iodoacetate (10–70 mg/kg), mitochondrial blocker rotenone (2 mg/kg), and/or mitochondrial uncoupler 2,4-dinitrophenol (5–15 mg/kg). After 5 min exposure at the highest dosages, iodoacetate inhibited IPC discharge 65% (15.9 ± 0.3 s−1 to 5.5 ± 0.3 s−1, P < 0.05), rotenone inhibited discharge 80% (12.9 ± 0.5 s−1 to 2.6 ± 0.6 s−1, P < 0.05), and 2,4-dinitrophenol inhibited discharge 99% (14.0 ± 0.3 s−1 to 11.3 ± 0.3 s−1, P < 0.05). These results suggest that IPC utilize glucose, require an intact glycolytic pathway, and metabolize the products of glycolysis to CO2 and H2O by mitochondrial respiration. The small but significant effect of 2,4-dinitrophenol suggests that ATP production by glycolysis may be sufficient to meet IPC energy demands if NADH can be oxidized to NAD experimentally by uncoupling mitochondrial or physiologically by transient lactate production. A model for IPC spike frequency adaptation is proposed, whereby the rapid onset of phasic IPC discharge requires ATP from anaerobic glycolysis, using lactate as the electron acceptor, and the roll-off in IPC discharge reflects transient acidosis due to intracellular lactic acid accumulation.

glycolysis; oxidative phosphorylation; NAD; ATP; action potential; ionic homeostasis; energy; spike frequency adaptation; respiration; Anas platyrhynchos

INTRAPULMONARY CHEMORECEPTORS (IPC) are vagal afferents that respond to tidal CO2 fluctuations in avian lungs and help control the rate and depth of breathing (2, 4, 10, 13, 14, 25). IPC are notable for their inverse sensitivity to CO2, insensitivity to O2, and their rapid stimulus response. Because they are stimulated by the low lung PCO2 during inspiration and inhibited by the high lung Pco2 during expiration, IPC discharge rate oscillates with every breath (28). Most IPC are also sensitive to the rate of CO2 change within each breath cycle, which is rapid in birds (12, 21).

Recent work on IPC has focused on cellular mechanisms that underlie CO2 signal transduction (3, 6, 16, 20, 21, 23, 26, 30). However, relatively little is known about IPC energy metabolism, specifically how these responsive respiratory chemoreceptors generate sufficient ATP to maintain their rapid intrabreath firing rates. Interestingly, despite the location of IPC receptor endings close to high PO2 levels at the intrapulmonary blood-gas interface (19), experiments indicate that IPC are remarkably insensitive to hypoxia and even to short-term anoxia (7, 10, 15). Accordingly, it has been proposed that IPC energy supply might be anaerobic (21), but the question has never been directly addressed and is the subject of the present study.

Some of the earliest studies testing the stimulus modality of IPC suggests that their discharge frequency is unaffected by intracellular disruptions in oxidative metabolism. Close intrarterial injection of sodium cyanide (NaCN, 10–50 μg), a traditional test of carotid body O2 sensitivity, has no effect on IPC (10, 15), whereas it produces rapid depolarization of carotid body glomus cells (29). Although NaCN experiments suggest IPC signal transduction is O2 independent, it is not clear that the small, transient NaCN boluses affected oxidative phosphorylation enough to disrupt ATP metabolism. Along similar lines, it has been shown that intravenous administration of mitochondrial uncoupler 2,4-dinitrophenol (DNP) does not affect breathing movements that are supposedly controlled by IPC (7). Although these observations support the idea of independence of IPC CO2 chemotransduction from ATP production by oxidative phosphorylation, the results were inferred from reflex effects on respiratory movements and not from direct electrophysiological recordings of IPC discharge. Other lines of evidence also suggest that IPC are relatively insensitive to disruptions of oxidative phosphorylation. Pulmonary arterial ligation during lung ventilation with nitrogen/CO2 gas mixtures does not alter IPC discharge rates during 10 min of anoxic exposure (21). However, these experiments may not have produced complete pulmonary anoxia, because the vertebral venous circulation can provide systemic blood flow to the avian lung independent of the pulmonary circulation (9).

The goal of the present study was to test and clarify the nature of IPC energy metabolism by using pharmacological inhibitors to block specific metabolic pathways for intracellular ATP production. We used iodoacetate (IAA) to block glycolysis, rotenone to block mitochondrial electron transport, and DNP to uncouple oxidative phosphorylation from mitochondrial electron transport. Because IPC function seems to be unusually tolerant to oxygen deprivation, our specific aim was to test the hypothesis that glycolysis alone is necessary and sufficient for IPC energy requirements. Understanding energy metabolism in neurons, especially those that are resistant to cellular damage during oxygen deprivation, is critical in understanding biological phenomena and also clinical pathophys-
iological situations, such as ischemia and apnea. Although data show that virtually all nervous tissue is dependent on glucose metabolism for long-term survival, the ability of neurons and glia to tolerate low oxygen and high glycolytic activity can vary widely (24), especially among different taxa (5).

Our results suggest that glycolysis is necessary, but not sufficient, for normal IPC function. IPC require functionality of both aerobic and anaerobic metabolic machinery to support CO2 signal transduction. However, our data also suggest that ATP production by glycolysis might be sufficient to meet IPC energy demands if NADH can be oxidized to NAD+, such as by pyruvate reduction to lactate. Some of the results of this study have been presented previously in abstract form (27).

MATERIALS AND METHODS

General Surgical Preparation

Adult mallard ducks (Anas platyrhynchos, n = 26), body mass 1.0–1.4 kg, of either sex were studied in accordance with “Guiding principles for research involving animals, and human beings” (1), using protocols approved by the Institutional Animal Care and Use Committee at Northern Arizona University. Animals were anesthetized into a deep surgical plane with ~35 mg/kg pentobarbital sodium administered intravenously through a butterfly catheter inserted into the pedal vein. A second polyethylene catheter was inserted in the brachial vein for supplemental pentobarbital sodium dosages (3.5–5.0 mg/kg) as needed, and for infusion of metabolic inhibitors. A thermometer probe was inserted into the esophagus to the level of the heart, and body temperature was regulated to 39 ± 2°C using a circulating water bath and hot water-filled bags placed around the animal. Electrocardiograms were monitored using a Grass P511K AC preamplifier joined to a Grass AM5 audio amplifier and Hitachi analog oscilloscope.

Birds were intubated with a silicone cuffed endotracheal tube, the interclavicular air sac was opened, and humidified gas was passed continuously and unidirectionally through the lungs with a Cameron Instruments GF-1 mass flow controller. Unidirectional mixed gas flow rates during the surgical preparation were set to 1 l/min of 21% O2 and 79% N2, to which pure CO2 was added at the endotracheal tube to bring inspired CO2 to 3%. During neural recording protocols, the gas flow was set to 2 l/min of 21% O2 and 79% N2, and pure CO2 was added at the endotracheal tube with the mass flow controller to produce inspired CO2 between 1% and 7% as needed. Inspired CO2 could also be stepped between any two levels using the mass flow controller. Unidirectional ventilation and deep surgical anesthesia prevented all spontaneous breathing movements in the animals.

Neural Recording

The left vagus nerve was exposed in the neck, raised several millimeters onto a dissecting stage, and covered in a mineral oil pool. A portion of the vagus was freed from its nerve sheath and epineurium, and single extracellular recordings were made from the severed ends of fine vagal filaments placed in contact with a platinum-iridium monopolar electrode. Electrical activity of individual filaments was referenced to an Ag-AgCl indifferent electrode on the nerve sheath a few millimeters away. The electrical signal was measured through a Grass HIP high-impedance differential probe and amplified with a Grass P511K AC preamplifier coupled to an AM-5 audio amplifier. Only recordings from clearly identifiable single neurons were accepted for this study, and single neurons were selected based on the reproducible shape and amplitude of their action potentials using a slope/height window discriminator (Haer). A digital pulse triggered by each action potential was logged and timed by a dedicated microcomputer sampling at 14,500/s (18). Analog signals from the preamplifier were band pass filtered at 100–3,000 Hz, notch filtered at 60 Hz, visualized on the oscilloscope, and recorded by pulse code modulation on a Vetter VHS 4-channel recorder.

Measurements During Control and Metabolic Inhibition

We tested vagal filaments for IPC activity as lung CO2 was electronically stepped between 0% and 7% at 11-s (cycle) intervals. IPC activity was identified by its nearly immediate response to ventilatory changes in CO2, its inverse CO2 sensitivity, and its insensitivity to O2 stimuli. When an IPC was located, either 10 cycles or 2,048 action potentials, whichever occurred first, were recorded on tape and computer for each treatment level, usually in duplicate. In all experiments, only one IPC was recorded from each animal and each animal received only one drug treatment (i.e., IAA, rotenone, or DNP).

IAA infusion. The effects of glycolytic inhibition on IPC discharge were tested by intralesional infusion of 10, 30, and 70 mg/kg sodium IAA (Na-IAA; Sigma-Aldrich, St. Louis, MO) into the brachial vein catheter over a period of ~1–2 min (n = 6 animals). IPC discharge was recorded 5 min after completing drug infusion. Stock IAA solutions were made by mixing IAA with normal saline, and adjusting the pH to 7.4 using 1 N NaOH. In two animals, to prevent infusion of Na-IAA, we infused a pH 7.4 Na-acetate solution (0.38 mmol/kg), which was the molar concentration equivalent of 70 mg/kg IAA. Na-IAA and Na-acetate are both weak acids, but only Na-IAA inhibits glycolysis. Na-acetate was given as a negative control to distinguish effects of glycolytic inhibition from general effects of weak acetic acids.

Rotenone infusion. The effects of inhibiting mitochondrial electron transport and oxidative phosphorylation on IPC discharge were tested by intralesional infusion of 2 mg/kg rotenone (n = 6 animals). A concentrated (10×) rotenone stock solution was prepared by dissolving 200 mg rotenone in 10 ml of 1 part DMSO and 1 part 1,2-propanediol (Sigma-Aldrich). Just before use, the rotenone stock solution was diluted 1:10 with normal saline, and then 1.0–1.4 ml of diluted rotenone (concentration = 2 mg/ml) were infused into the brachial vein catheter over a period of 2 min (n = 6). The drug was allowed to distribute for 5 min, and then IPC discharge was recorded. Prior to receiving the rotenone infusion, all IPC first received a vehicle control infusion of 1.0–1.4 ml of diluent containing the same concentration of DMSO, 1,2-propanediol, and saline, but without the rotenone. IPC response to vehicle control was measured 5 min after vehicle infusion, just as described for rotenone treatment.

DNP infusion. To test the effects of uncoupling mitochondrial electron transport from oxidative phosphorylation, we infused 5–15 mg/kg DNP (Sigma-Aldrich). A DNP stock solution was prepared by dissolving 2 g DNP and 1 g NaHCO3 in 100 ml of 35% aqueous ethanol (17). A vehicle control solution without DNP was prepared from 1 g NaHCO3 dissolved in 100 ml of 35% ethanol. Prior to infusion into the animal, stock solutions (both DNP containing, and vehicle control) were diluted 1:4 with normal saline. The first treatment was infusion of the vehicle control solution into the brachial vein catheter over a period of 2 min, followed by 5 min for equilibration, and then recording of IPC neural response to CO2 step stimuli. DNP (5 mg/kg) in the same vehicle was then infused intravenously over a period of 2 min, followed by 5 min for equilibration and then measurement of IPC neural response to CO2 steps. One or two additional doses of DNP (up to a cumulative total of 10–15 mg/kg) were given, and the neural recordings were repeated. Body temperature increased with DNP treatment in a dose dependent manner, even with the heating pad turned off, thermal blankets removed, and addition of ice bags beside the animal. Rising body temperature was an indication of the mitochondrial uncoupling action of DNP and assured that a physiologically effective dosage of DNP had been administered (22).
Analysis of Single-Unit Neural Activity

Differences in dynamic IPC discharge between control and drug treatments were analyzed with online cycle-triggered histograms of IPC action potential discharge vs. time during the CO2 step (18, 23). Two-way ANOVA with repeated measures was used to quantify differences in dynamic IPC discharge between the control and treatments (main effects: time during CO2 cycle and drug treatments). Post hoc multiple comparisons were made using Tukey’s honestly significant difference test with automatic adjustment of α to maintain a 0.05 type-I error rate (JMP-IN version 4; SAS). P ≤ 0.05 were considered significant.

Blood Acid-Base Measurements and Analysis

In eight of the 26 animals, changes in blood pH before and after drug treatment were quantified from blood samples drawn anaerobically into heparinized 1 ml syringes from a carotid artery cannula. Blood PaCO2, and plasma pH were analyzed using a Radiometer ABL-500 blood gas system. Blood-buffer curve intercepts (A) and slopes (B) were quantified by linear regression of pH vs. log₁₀PaCO₂ for each treatment:

\[ \text{pH} = A + B(\log_{10}\text{PaCO}_2) \] (1)

ANOVA was used to test for treatment effects on blood-buffer slopes and intercepts. In addition, standard bicarbonate ([HCO₃]₀) in mmol/l was calculated for each treatment using the measured blood-buffer curves to determine plasma pH at 40 mmHg PaCO₂ and the Henderson-Hasselbalch equation to determine plasma HCO₃⁻ (11):

\[ [\text{HCO}_3^-] = \alpha\text{PCO}_2(10^{pH-\alpha K_a}) \] (2)

Here, \( \alpha \) was 0.03 mmol/l/mmHg, the physical solubility of CO₂ in plasma, \( pK_a \) was 6.07, the base 10 logarithm of the \( K_a \) for the reaction was \( \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \), and pH was calculated from the blood-buffer curve (Eq. 1) at \( \text{PCO}_2 = 40 \) mmHg for each specific treatment.

RESULTS

Blood Acid-Base Responses to Metabolic Inhibitors

Table 1 and Figs. 1A, 2A, 3A, and 4A summarize acid-base measurements during control and drug treatments. None of the drug treatments (IAA, acetate, rotenone, DNP) had a significant effect on slopes of the blood-buffer lines compared with control (\( P > 0.76 \) by ANCOVA), but some drug treatments did have significant effects on pH intercepts and calculated standard bicarbonate (\( P < 0.05 \) by ANCOVA).

Neural Responses of IPC to IAA and Acetate

Table 2 and Fig. 1B show the effects of IAA treatments on IPC response to step changes in inspired CO₂ (\( n = 6 \)). IAA was administered intravenously in normal saline as a pH-neutral solution at 10, 30, and 70 mg/kg. Two animals also received a pH-neutral solution of Na-acetate at the same molar dosage as 70 mg/kg IAA (i.e., 0.38 mmol/kg).

IAA inhibited IPC CO₂ step responses in a dosage-dependent manner. A dose of 10 mg/kg IAA was not different from control, but 30 mg/kg (not shown) and 70 mg/kg IAA caused significant dose-dependent inhibition of IPC discharge (Table 2 and Fig. 1B). ANOVA (Table 2) showed that IPC discharge rate was significantly affected by both the CO₂ step, and the IAA treatment, and that the interaction of CO₂*IAA was significant.

Sodium acetate insignificantly stimulated IPC discharge (\( P > 0.05 \)), had no significant interaction with CO₂ (CO₂*acetate, \( P > 0.05 \)), and had no discernable effect on acid-base status (Table 2, Fig. 2, A–D). This suggests that the infusion of 0.38 mmol/kg of weak acid does not explain the depression of IPC discharge produced by IAA, implicating IAA’s inhibiting effects on glycolysis.

The effect of 70 mg/kg IAA on mean ± SE discharge rates occurring during the CO₂ stimulus step cycle are shown in Fig. 1C. Mean discharge rates were plotted against PaCO₂ values sampled at the corresponding maximum and minimum of the CO₂ step stimulus cycle, giving dynamic CO₂ sensitivity lines. Relative to control, IAA significantly reduced the mean sensitivity of IPC discharge to PaCO₂ by depressing IPC discharge rate at low PaCO₂ more than at high CO₂ (\( P < 0.05 \), Fig. 1C). To determine whether the IAA effects could be explained by metabolic acidosis, mean maximum and minimum IPC discharge rate during control and IAA treatment were plotted against plasma pH values at the maxima and minima of PaCO₂ steps. The sensitivity of IPC expressed as a function of pH was still significantly depressed by IAA (Fig. 1D), suggesting that the IAA effect on IPC discharge involved more than just metabolic acidosis.

Table 1. Regression: effect of metabolic inhibitors on blood buffer curves (plasma pH vs. log PaCO₂)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. Animals</th>
<th>Slope ± SE, ΔpH/Δlog₁₀PaCO₂</th>
<th>Intercept ± SE, pH at PCO₂ = 1 mmHg</th>
<th>( R^2 )</th>
<th>Standard, [HCO₃] ± SE @ PCO₂ = 40 mmHg</th>
<th>Base Excess, Relative to Control†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>−0.619 ± 0.014</td>
<td>8.346 ± 0.017</td>
<td>0.97</td>
<td>23.1 ± 0.3 mM</td>
<td>0.0 mM</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>3</td>
<td>−0.589 ± 0.041</td>
<td>8.222 ± 0.056</td>
<td>0.91</td>
<td>19.4 ± 0.5 mM</td>
<td>−3.7 mM</td>
</tr>
<tr>
<td>Acetate</td>
<td>3</td>
<td>−0.628 ± 0.012</td>
<td>8.336 ± 0.016</td>
<td>0.99</td>
<td>21.8 ± 0.3 mM</td>
<td>−1.3 mM</td>
</tr>
<tr>
<td>Rotenone</td>
<td>2</td>
<td>−0.720 ± 0.074</td>
<td>8.366 ± 0.113</td>
<td>0.96</td>
<td>16.7 ± 0.6 mM</td>
<td>−6.4 mM</td>
</tr>
<tr>
<td>2,4-DNP</td>
<td>3</td>
<td>−0.587 ± 0.026</td>
<td>8.295 ± 0.036</td>
<td>0.97</td>
<td>22.7 ± 0.4 mM</td>
<td>−0.4 mM</td>
</tr>
</tbody>
</table>

Model equation: pHₐ = A*(log₁₀(PaCO₂)) + B. 2,4-DNP, 2,4-dinitrophenol. †Calculated.
showed that the IPC discharge rate was significantly affected by both CO₂ and rotenone treatment, and that the interaction of CO₂*rotenone was significant.

Mean ± SE maximum and minimum IPC discharge rates during the CO₂ stimulus step cycle measured with vehicle control and with 2 mg/kg rotenone infusion are shown in Fig. 3C. Mean discharge rates were plotted against PₐCO₂, values sampled at the corresponding minimum and maximum CO₂ stimulus during the step, giving dynamic CO₂ sensitivity lines. Compared with vehicle control, rotenone significantly reduced the mean sensitivity (slope) of IPC discharge to PₐCO₂ by depressing discharge rate at low PₐCO₂ (Fig. 3C). To determine whether the rotenone effects could be explained simply by the metabolic acidosis caused by rotenone (Fig. 3A), mean maximum and minimum IPC discharge rates during control and rotenone treatment were plotted against plasma pH values at the maxima and minima of PₐCO₂ steps. The sensitivity of IPC to pH was still significantly depressed by rotenone (Fig. 3D), suggesting that the mechanism of rotenone’s action included more than just metabolic acidosis.

Neural Responses of IPC to DNP

Figure 4B shows the effects of DNP treatment on the IPC response to step changes in inspired CO₂ (n = 6). Vehicle controls given prior to DNP treatment in each IPC had no significant effects on IPC discharge responses (Table 2, Fig. 4B). DNP had no effect on IPC discharge at 5 mg/kg, but caused a small depression of overall mean IPC discharge rate, from 14.0 ± 0.3 Hz to 11.3 ± 0.3 Hz at the 10–15 mg/kg dose (Table 2). ANOVA showed that the IPC discharge rate was significantly affected by CO₂ (P < 0.001) and by DNP (P < 0.003), but that the interaction of CO₂*DNP was not significant (Table 2).

Mean ± SE discharge rates during the CO₂ stimulus step cycle with the vehicle control and following 10–15 mg/kg DNP infusion are shown in Fig. 4C. Mean minimum-maximum discharge rates were plotted against PₐCO₂ values sampled at the corresponding minimum and maximum CO₂ stimulus during the step, giving dynamic CO₂ sensitivity lines. DNP did not significantly change mean dynamic sensitivity of IPC to PₐCO₂ (Fig. 4C), or to pHₐ (Fig. 4D).

Thermogenic Effect of DNP

DNP infusion caused a time-dependent and dose-dependent increase in body temperature. This occurred despite efforts to cool the animals by removing heating pads and blankets, and by applying ice around the body. We tracked body temperature, relative to initial baseline readings (39 ± 2°C), in four animals. Linear regression showed that the mean rates of temperature increase were significant at both DNP dosages: 0.05 ± 0.02°C/min with 5 mg DNP/kg (n = 4 animals, P < 0.02), and 0.14 ± 0.04°C/min at 10–15 mg DNP/kg (n = 4 animals, P < 0.007). The significant thermogenic effect of DNP indicates that dosages were sufficient for uncoupling mitochondrial function.

DISCUSSION

Avian IPC receptor endings are surprisingly insensitive to physiological levels of hypoxia and even to short-term anoxia (7, 10, 15, 21). Because IPC are highly active neurons that
should have considerable ATP demand for homeostasis, we speculated that IPC ATP production may be anaerobic, despite their access to normal PO2 levels in the lung. This is of interest because the abilities of neurons to tolerate hypoxia/anoxia and generate ATP by glycolysis varies widely within the central nervous system (24), and identifying mechanisms that enable a neuron to withstand injury despite low oxygen is a matter of great clinical importance. Therefore, we tested the hypothesis that glycolysis alone is necessary and sufficient for IPC energy supply by measuring single-unit IPC CO2-related responses with and without pharmacological inhibitors that block specific intracellular ATP generating pathways. For these experiments, pathways involved in both anaerobic and aerobic ATP production were examined, with anaerobic glycolysis being blocked by IAA, mitochondrial electron transport being blocked by rotenone, and oxidative phosphorylation being uncoupled from mitochondrial electron transport by DNP. Our results show that IPC CO2 signal transduction is dependent on glucose for ATP production, but that anaerobic glycolysis does not appear sufficient by itself. Our main conclusion is that glycolysis followed by mitochondrial oxidative phosphorylation is typically required for normal IPC function.

**IAA Treatment**

In these experiments, IAA significantly depressed IPC discharge rate and IPC sensitivity to CO2 steps in a dose-dependent manner above 10 mg/kg. IAA is a widely used inhibitor of glycolysis and affects glucose utilization at the glyceraldehyde 3-phosphate dehydrogenase step (Fig. 5). The simplest interpretation of our IAA results is that glucose is a required substrate for ATP production and normal sensory function in IPC, and alternative substrates (e.g., fats) cannot substitute for glucose. This finding is consistent with many other studies that show glucose to be the dominant metabolic fuel in the nervous system (24). However, IAA also caused metabolic acidosis, which could contribute to this depressant effect (see below for discussion).

**Metabolic Inhibition vs. Metabolic Acidosis with IAA**

Because IPC CO2 signal transduction critically depends on intracellular pH excitability is depressed by acidosis and stimulated by alkalosis (21), we considered the possibility that the depression of IPC discharge following IAA administration may result from changes in pH evoked by IAA, rather than metabolic inhibition. We tested this two ways: 1) by comparing responses of blood-buffer curves and IPC discharge to infusion of equimolar solutions of sodium IAA and sodium acetate, and 2) by looking at IPC discharge response as a function of blood pH during IAA and acetate infusion. Although infusion of IAA created a significant metabolic acidosis (decreasing both plasma pH and base excess, Table 1, Fig. 1A) infusion of an equimolar acetate control solution did not. Both acetate and IAA are weak acids and should have the similar effects on acid-base balance. As shown in Fig. 2, A–C, infusion of acetate also did not disturb IPC function, although equimolar IAA did. Therefore, it is likely that the effects of IAA are due to its glycolytic inhibition properties and not due to its intrinsic properties as a weak acid.

**Does IAA Inhibit IPC by Decreasing Intracellular pH Through Glycolytic Inhibition?**

IAA-induced glycolytic inhibition has been shown to increase (32) or decrease (31) intracellular pH, which likely depends on cell type or, more precisely, on the metabolic machinery of the cell under study and its demand for ATP. For example, in metabolically demanding tissue, such as astrocytes, glycolytic inhibition can lower intracellular pH secondarily by depleting energy stores and generating protons during ATP breakdown (31). On the other hand, in rod photoreceptors cells, IAA-induced glycolytic block alkalinizes intracellular pH by forcing oxidative metabolism to increase, which removes protons via respiratory chain reactions (32).

It is not known whether IAA secondarily changes intracellular pH in IPC. IPC receptor endings are embedded in the parabronchial mantle and are impossible to study intracellularly with present techniques without severe lung damage. However, if it is assumed that changes in extracellular pH track changes in intracellular pH during metabolic acid-base disturbances, then plasma pH could be used as a proxy for intracellular pH. If IAA simply depressed IPC by lowering intracellular and extracellular pH (e.g., by accumulating or depleting metabolic intermediates) IPC discharge frequency should re-

---

**Table 2. ANOVA: effect of metabolic inhibitors on dynamic intrapulmonary chemoreceptors discharge pattern**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. Animals</th>
<th>Model Significance of Main Effects and Crossed Effects</th>
<th>Drug Treatments</th>
<th>Drug Treatment Effects†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs. acetate</td>
<td>2</td>
<td>Acetate (P &lt; 0.001)</td>
<td>Control</td>
<td>15.9±0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO2 (P &lt; 0.001)</td>
<td>IAA, 10 mg/kg</td>
<td>15.2±0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IAA*CO2 (P &lt; 0.001)</td>
<td>70 mg/kg</td>
<td>5.5±0.3</td>
</tr>
<tr>
<td>Control vs. rotenone</td>
<td>6</td>
<td>Rotenone (P &lt; 0.001)</td>
<td>Control</td>
<td>6.6±0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO2 (P &lt; 0.001)</td>
<td>Acetate, 0.38 mmole/kg (equiv. to 70 mg/kg IAA)</td>
<td>6.1±0.5</td>
</tr>
<tr>
<td>Control vs. 2,4-DNP</td>
<td>6</td>
<td>DNP (P &lt; 0.001)</td>
<td>Control</td>
<td>12.9±0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNP*CO2 (P &lt; 0.001)</td>
<td>Vehicle Control</td>
<td>13.6±0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNP*CO2 (P = 0.50)</td>
<td>Rotenone, 2 mg/kg</td>
<td>2.6±0.6</td>
</tr>
</tbody>
</table>

IAA, iodoacetate. †Average discharge rate, (s–1) means ± SE. Repeated-measures two-way ANOVA (main effects: dynamic intrapulmonary chemoreceptors, drug, CO2; Interaction: drug *CO2). a,b,c,d,e,f,g Letters different from control indicate significant difference of indicated treatment using Tukey’s honestly significant difference post hoc test (multiple comparisons controlled to keep overall a = 0.05).
Fig. 2. Acetate infusion. A: In vivo blood-buffer curves measured during control (n = 8 animals) and following 0.38 mmol/kg sodium acetate infusion (n = 3 animals). Linear regression of plasma pH vs. log PaCO₂, with 95% confidence intervals showed no acid-base disturbance with acetate infusion (also see Table 1). B: cycle-triggered stimulus histograms of instantaneous IPC discharge (mean ± SE) vs. time under control conditions and 0.38 mmol/kg sodium acetate (n = 2 IPC), with timing of CO₂ step stimulus indicated. No effect of acetate on discharge was observed. C: mean ± SE maximum and minimum IPC discharge rates during the CO₂ step stimulus cycle plotted vs. inspired PCO₂. Control data points are completely hidden behind acetate data points. D: mean ± SE maximum and minimum IPC discharge rates during the CO₂ step stimulus cycle plotted vs. plasma pH (see text).

Fig. 3. Rotenone infusion. A: In vivo blood-buffer curves measured during control (n = 8 animals) and following 2 mg/kg rotenone infusion (n = 2 animals). Linear regression of plasma pH vs. log PaCO₂, with 95% confidence intervals indicated rotenone produced metabolic acidosis (P < 0.05, Table 1). B: cycle-triggered stimulus histograms of instantaneous IPC discharge (mean ± SE) vs. time under control conditions and 5 min after infusion of 2 mg/kg rotenone (n = 6 IPC), with timing of CO₂ step stimulus indicated. Rotenone depressed IPC discharge (P < 0.05, Table 2) and typically silenced discharge after 15 min. C: mean ± SE maximum and minimum IPC discharge rates during the CO₂ step stimulus cycle plotted vs. inspired PCO₂. D: mean ± SE maximum and minimum IPC discharge rates during the CO₂ step stimulus cycle plotted vs. plasma pH (see text).
main a single linear function of plasma pH. Given that the observed relationship between IPC discharge and plasma pH displays an altered sensitivity (slope and intercept) following IAA administration (Fig. 1), this suggests that the depression of IPC function is not explained by pH changes alone. When this analysis is coupled with the acetate negative control data (above), it suggests that IAA has metabolic effects on IPC that extend beyond altering pH, most likely through its well-known ability to disrupt glycolysis, glucose utilization, and ATP production.

DNP and Rotenone Treatments

DNP had a marked thermogenic effect and slightly depressed IPC function at the highest dosages but had no effect on acid-base status. Rotenone had large inhibitory effects on IPC function and produced a significant metabolic acidosis. Although both rotenone and DNP are mitochondrial poisons and block oxidative phosphorylation, their mitochondrial inhibitory mechanisms are different.

Rotenone blocks the electron transport chain at complex I and prevents oxidation of substrates by limiting NADH+H+ and preventing the formation of mitochondrial H+ gradient needed for oxidative phosphorylation. DNP, a proton ionophore, uncouples electron transport from oxidative phosphorylation, permitting electron transfer to continue through the cytochrome chain with oxidation of NADH+H+ to NAD+ but short circuiting the inner mitochondrial membrane proton gradient and oxidative phosphorylation.
consistent with a rotenone-induced metabolic acidosis as well as a rotenone-induced block of ATP supply.

DNP blocks oxidative phosphorylation by uncoupling electron transport from ATP synthesis (Fig. 5). Because the electron transport system can run freely when DNP is present, NADH + H+ from glycolysis can be readily recycled to NAD, glycolysis can produce pyruvate rather than lactate from glucose, the Krebs cycle can process acetyl-CoA, substrate level phosphorylation of ATP can occur, and O2 can be reduced to water. Unlike rotenone, DNP is less likely to produce acidosis, as the free running electron transport chain recycles reduced NADH and does not force glycolysis to use lactate as a final electron acceptor. These differences likely underlie the different degree of IPC functional loss with rotenone and DNP treatments. Rotenone both acidifies the cell and blocks ATP formation, whereas DNP blocks oxidative (but not glycolytic) ATP formation, and does not acidify the cell, a more benign condition for IPC function.

Metabolic Inhibition vs. Metabolic Acidosis with Rotenone and DNP

Similar to IAA, rotenone caused a metabolic acidosis (Table 1, Fig. 3A), and had a profound depressant effect on IPC discharge. Yet, if IPC discharge were simply being depressed by acidosis, then IPC discharge frequency should remain a linear function of pH after rotenone infusion (3). Instead, the IPC discharge-plasma pH relationship after rotenone infusion was different from control. It had a reduced sensitivity to pH (Fig. 3D), suggesting that with rotenone, IPC function is depressed by both acidosis and by other mechanisms independent of pH, most likely the disruption of oxidative phosphorylation and ATP supply.

DNP caused a small depression of overall mean IPC discharge rate at the highest dosages, but did not cause a metabolic acidosis (Table 1, Fig. 4A). It seems likely that the main effects of DNP on IPC function result from a slightly diminished ATP production: oxidative phosphorylation is markedly decreased by DNP, but this is partly compensated by an increase in substrate level phosphorylation in glycolysis and the Krebs cycle.

Critique: Estimating Intracellular pH from Extracellular pH

Intracellular pH changes may be smaller or larger than plasma pH changes, thereby complicating analysis of inhibitors that caused marked acidosis (e.g., rotenone). Arguably, the main concern is underestimating acidic intracellular pH changes produced by metabolic inhibition, because this would lead to overestimating the importance of energy production pathways affected by the inhibitor. For example, rotenone had the greatest acidifying effect of inhibitors we tested (Fig. 3A). In the extreme, if rotenone acidified intracellular pH by 0.55 pH units, instead of 0.15 pH units as predicted by plasma pH, the IPC discharge vs. pH relationship after rotenone would be shifted much farther to the left in Fig. 3D, and acid pH might explain most of the observed IPC inhibition. At this extreme, the inferred importance of mitochondrial electron transport and oxidative phosphorylation on IPC function would be uncertain (it could be significant or not, possibly masked by the pH effect). However, as discussed below, we think that use of plasma pH is unlikely to produce errors of this magnitude.

The assumption that changes in intracellular pH can be inferred by changes in extracellular pH has been made previously for chronic metabolic acid-base disturbances in IPC (3). In applying the assumption to acute metabolic disturbances, we reasoned that because IPC express several acid-base transporters, they should be able to employ several mechanisms capable of rapidly modifying intracellular pH. Recent experimental evidence demonstrates that stilbene-sensitive HCO3−/Cl− anion exchangers (30) and amiloride-sensitive Na+−/H+ antiports (16) participate in modulating IPC activity during step changes in CO2, thus lending support to this idea. In addition, several experiments indicate that IPC are sensitive to rapid intravenous infusions of HCl or NaHCO3, which suggests that some degree
of acid-base transfer across IPC cell membranes probably occurs as soon as a pH disturbance commences. Taken together, these studies suggest that extreme deviations between intracellular and extracellular pH changes are unlikely in IPC, and that plasma pH is probably an acceptable proxy for intracellular pH in this experiment.

**Perspectives and Significance**

Despite reports of IPC tolerance to hypoxia, ischemia, and anoxia (7, 10, 15, 21), we showed that most IPC are dependent on mitochondrial respiration for energy metabolism. Nevertheless, the different effects of DNP on tonic vs. phasic IPC suggest that anaerobic glycolysis may have a role in IPC spike frequency adaptation. Except for DNP, all metabolic inhibitors used in this study affected phasic and tonic IPC responses in the same way and to the same extent. However, DNP inhibited tonic IPC and had almost no effect on phasic IPC (Fig. 6). These results suggest that tonic (nonadapting) IPC may rely predominantly on ATP produced by aerobic metabolism to maintain their slower responding and steadier discharge rates, whereas, rapidly adapting phasic IPC may make use of ATP from anaerobic glycolysis during their rapid onset response to changing CO2 stimuli, using lactate as the electron acceptor. The roll-off (adaptation) in IPC discharge seen in phasic IPC applies to phasic and tonic IPC.

More twitch fibers are rapidly contracting, fatigable cells that rely on aerobic respiration. More fast-machinery they possess and functionally by the influence of fiber types are categorized histologically by the metabolic properties on their contractile activity. Fast-twitch fibers are rapidly contracting, fatigable cells that rely on anaerobic glycolysis; slow-twitch fibers are slowly contracting, fatigue-resistant cells that rely on aerobic respiration. More study is needed to test whether a similar metabolic principle applies to phasic and tonic IPC.

**ACKNOWLEDGMENTS**

We thank Leslie B. Hempleman for technical assistance.

**GRANTS**

This study was supported, in part, by National Heart, Lung, and Blood Institute Grant R15-HL-087269-01 and National Science Foundation Grant IBN-0217815.

**REFERENCES**