Effects of Aerobic and Anaerobic Metabolic Inhibitors on Avian Intrapulmonary Chemoreceptors

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Abstract  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 i.v. 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 dinitrophenol inhibited discharge 19% (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 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 mitochondria, or physiologically by transient lactate production. A model for IPC spike frequency adaptation (SFA) 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.

Key Words  glycolysis, oxidative phosphorylation, NAD, ATP, action potential, ionic homeostasis, energy, spike frequency adaptation, respiration, Anas platyrhynchos.
Introduction

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 intra-breath 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 (8, 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 intra-arterial 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 IPC energy metabolism 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
pathophysiological 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 suggests 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), 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 sodium pentobarbital administered intravenously through a butterfly catheter inserted into the pedal vein. A second polyethylene catheter was inserted in the brachial vein for supplemental sodium pentobarbital dosages (3.5-5.0 mg/kg) as needed, and for infusion of metabolic inhibitors. A thermistor 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 mm 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 mm 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/second (18). Analog signals from the preamplifier were band pass-filtered at 100-3000 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 CO$_2$ was electronically stepped between 0% and 7% at 11 second (cycle) intervals. IPC activity was identified by its nearly immediate response to ventilatory changes in CO$_2$, its inverse CO$_2$ sensitivity, and its insensitivity to O$_2$ stimuli. When an IPC was located, either 10 cycles or 2048 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.*, iodoacetate, rotenone, and 2,4 dinitrophenol).

**Iodoacetate (IAA) Infusion.** The effects of glycolytic inhibition on IPC discharge were tested by *i.v.* infusion of 10, 30 and 70 mg/kg sodium iodoacetate (Na-IAA; Sigma-Aldrich, Inc., St. Louis, MO, USA) into the brachial vein catheter over a period of approximately 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 1N NaOH. In two animals, prior to infusing Na-IAA, we infused a pH 7.4 Na-Acetate solution (0.38 mmole/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 *i.v.* infusion of 2 mg/kg
rotenone (n=6 animals). A concentrated (10x) rotenone stock solution was prepared by
dissolving 200 mg rotenone in 10ml 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 minutes, 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.

**2,4 Dinitrophenol infusion.** To test the effects of uncoupling mitochondrial
electron transport from oxidative phosphorylation, we infused 5-15 mg/kg 2, 4
dinitrophenol (Sigma-Aldrich, Inc., St. Louis, MO, USA). 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 1g 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. Then, 5 mg/kg DNP in same vehicle was 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 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 on-line 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 HSD test with automatic adjustment of α to maintain a 0.05 type-I error rate (JMP-IN v4, SAS Inc.). P-values ≤ 0.05 were considered significant.

Blood Acid-Base Measurements and Analysis. In 8 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 pHₐ were analyzed using a Radiometer ABL-500 blood gas system. Blood buffer curve intercepts (A) and slopes (B) were quantified by linear regression of pHₐ vs. log₁₀ PaCO₂ for each treatment.
\[ pH_a = A + B(\log_{10}PaCO_2) \] 

Eq. 1

ANCOVA was used to test for treatment effects on blood buffer slopes and intercepts. In addition, standard bicarbonate (HCO_3^{\text{std}}, in mmole/L) was calculated for each treatment using the measured blood buffer curves to determine plasma pH\_a at 40 mmHg PaCO_2, and the Henderson-Hasselbalch equation to determine plasma HCO_3^{\text{std}} \ (11):

\[ [\text{HCO}_3^{\text{std}}] = \alpha PCO_2 \times 10^{(pH_a-pK_a)}. \] 

Eq. 2

Here, \( \alpha \) was 0.03 mmole/L/mmHg, the physical solubility of CO_2 in plasma, pK_a was 6.07, the base 10 logarithm of the K_a for the reaction CO_2+H_2O\leftrightarrow HCO_3^- + H^+, and pH\_a was calculated from the blood buffer curve (Eq. 1) at 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 to 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 Iodoacetate (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 mmole/kg).

IAA inhibited IPC CO₂ step responses in a dosage-dependent manner: 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, Figs. 2A, 2B, 2C, 2D). This suggests that the infusion of 0.38 mmole/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 average (± SE) discharge rates occurring during the CO₂ stimulus step cycle are shown in Figure 1C. Average discharge rates were plotted against PaCO₂ values sampled at the corresponding maximum and minimum of the CO₂ step stimulus, giving dynamic CO₂ sensitivity lines. Relative to control, IAA significantly reduced the average 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 if the IAA effects could be explained by metabolic acidosis, average maximum and minimum IPC discharge rate during control and IAA treatment were plotted against
plasma pH values at the maxima and minima of PCO2 steps. The sensitivity of IPC expressed as a function of pH was still significantly depressed by IAA (Fig. 1D), suggesting that IAA effect on IPC discharge involved more than just metabolic acidosis.

**Neural Responses of IPC to Rotenone.** Figure 3B shows the effects of rotenone on the mean IPC response to step changes in inspired CO2 (n=6). Vehicle controls given immediately prior to the dose of rotenone had no significant effects on IPC discharge (Table 2 and Fig 3B). However, rotenone (2 mg/kg) caused profound inhibition of the IPC CO2 step response. In all IPC, neural discharge started to decrease about 2 min after rotenone infusion. After 5 min, IPC discharge responses to CO2 steps were significantly depressed, as shown in Figure 3B and Table 2. For example, average discharge rate (±SE) over the CO2 stimulus cycle was 13.6±0.8 Hz with vehicle control, and 2.9±0.9 Hz with rotenone at 5 min (Table 2, drug treatment effects column). About fifteen min after rotenone infusion, most IPC were completely inhibited (silenced) and unresponsive to CO2 stimuli. ANOVA (Table 2) showed that IPC discharge rate was significantly affected by both CO2, and rotenone treatment, and that the interaction of CO2*rotenone was significant.

Average (±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 Figure 3C. Average 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 to vehicle control, rotenone significantly reduced the average sensitivity (slope) of IPC discharge to PaCO2 by depressing discharge rate at low PaCO2 (Fig. 3C). To determine if the rotenone effects could be
explained simply by the metabolic acidosis caused by rotenone (Fig. 3A), average maximum and minimum IPC discharge rate during control and rotenone treatment were plotted against plasma pH values at the maxima and minima of PCO₂ 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 2,4 Dinitrophenol.* 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 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).

Average (± SE) discharge rates during the CO₂ stimulus step cycle with the vehicle control and following 10-15 mg/kg DNP infusion are shown in Figure 4C. Average min-max 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 average 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 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 average 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 ionic 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 ability of neurons to tolerate hypoxia/anoxia and generate ATP by glycolysis varies widely within the CNS (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.

**Iodoacetate Treatment.** In these experiments, IAA significantly depressed IPC discharge rate and IPC sensitivity to CO$_2$ steps in a dosage 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 other many 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 CO$_2$ 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 iodoacetate 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 Figs. 2A, B, 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 remain 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. 1D), 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, 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 NAD availability (Fig. 5). This slows or halts aerobic ATP production and forces cellular energy supply to be produced by anaerobic glycolysis. The resulting glycolytic activity leads to a metabolic acidosis through lactate accumulation, and metabolic acidosis and depression of IPC discharge observed here are 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+H⁺ 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 HCO$_3^-$/Cl$^-$ anion exchangers (30) and amiloride-sensitive Na$^+$/H$^+$ antiports (16) participate in modulating IPC activity during step changes in CO$_2$, thus lending support to this idea. In addition, several experiments indicate that IPC are sensitive to rapid intravenous infusions of HCl or NaHCO$_3$, 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.
Perspective and Significance. Despite reports of IPC tolerance to hypoxia, ischemia, and anoxia (8, 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 suggests 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 (non adapting) IPC may rely predominantly on ATP produced by aerobic metabolism to maintain their slower responding, 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 may reflect transient acidosis due to intracellular lactate accumulation, which is then corrected by slower aerobic respiration that metabolizes lactate to CO2, or by lactate simply diffusing away from the IPC sensory ending.

The above hypothesis is analogous to the differential metabolism of vertebrate twitch skeletal muscle fibers. Muscle fiber types are categorized histologically by the metabolic machinery they possess, and functionally by the influence of their 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.
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References

**Figure Legends**

Figure 1. Panel A: *In-vivo* blood buffer curves measured during control (n=8 animals) and following 70 mg/kg (0.38 mmole/kg) sodium iodoacetate (IAA) infusion (n=3 animals). Linear regression of plasma pH vs. log PaCO₂, with 95% confidence intervals showed IAA produced a significant metabolic acidosis (p<0.05, also Table 1). Panel B: Cycle triggered stimulus histograms of instantaneous IPC discharge (mean ± SE) vs. time under control conditions and after successive infusions of 10 mg/kg and 70 mg/kg IAA (n=6 IPC), with timing of CO₂ step stimulus indicated. IAA at 70 mg/kg (and 30 mg/kg, not shown), but not 10 mg/kg, reduced discharge rates (see also Table 2). Panel C: Mean (±SE) maximum and minimum IPC discharge rates during the CO₂ step stimulus cycle plotted vs. inspired PCO₂. Panel D: Mean (±SE) maximum and minimum IPC discharge rates during the CO₂ step stimulus cycle plotted vs. plasma pH (see text).

Figure 2. Panel A: *In-vivo* blood buffer curves measured during control (n=8 animals) and following 0.38 mmole/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). Panel B: Cycle triggered stimulus histograms of instantaneous IPC discharge (mean ± SE) vs. time under control conditions and 0.38 mmole/kg sodium acetate (n=2 IPC), with timing of CO₂ step stimulus indicated. No effect of acetate on discharge was observed. Panel 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.
Panel D: Mean (±SE) maximum and minimum IPC discharge rates during the CO2 step stimulus cycle plotted vs. plasma pH (see text).

Figure 3. Panel 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). Panel 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. Panel C: Mean (±SE) maximum and minimum IPC discharge rates during the CO2 step stimulus cycle plotted vs. inspired PCO2. Panel D: Mean (±SE) maximum and minimum IPC discharge rates during the CO2 step stimulus cycle plotted vs. plasma pH (see text).

Figure 4. Panel A: In-vivo blood buffer curves measured during control (n=8 animals) and following 10-15 mg/kg 2,4 dinitrophenol (DNP) infusion (n=3 animals). Linear regression of plasma pH vs. log PaCO2, with 95% confidence intervals showed DNP did not significantly affect acid base balance. Panel B: Cycle triggered stimulus histograms of instantaneous IPC discharge (mean ± SE) vs. time under control conditions and after infusion of 10-15 mg/kg DNP (n=6 IPC), with timing of CO2 step stimulus indicated. DNP had a small inhibitory effect on discharge (also see Table 2 and Fig. 6). Panel C: Mean (±SE) maximum and minimum IPC discharge rates during the CO2 step stimulus
cycle plotted vs. inspired PCO$_2$. Panel D: Mean (±SE) maximum and minimum IPC discharge rates during the CO$_2$ step stimulus cycle plotted vs. plasma pH (see text).

Figure 5. Metabolic pathways involved in glycolysis and aerobic respiration, and the sites of action of inhibitors used here. IAA blocks glycolysis at the glyceraldehyde 3-phosphate dehydrogenase step. Rotenone blocks both electron transport and oxidative phosphorylation at complex I in the mitochondrial cytochrome respiratory chain, preventing the oxidation of reduced 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.

Figure 6. Six IPC were studied before and after DNP administration. Three of the IPC were “phasic” in their CO$_2$ response, and exhibited pronounced spike frequency adaptation. The three other IPC were “tonic” in their response, and had little or no spike frequency adaptation. Only the “tonic” IPC were significantly affected by DNP (see text for discussion).
Table 1. Regression: Effect of Metabolic Inhibitors on Blood Buffer Curves (plasma pH vs. log PaCO2). Model Equation: pHa = A*(log10PaCO2) + B

<table>
<thead>
<tr>
<th>Experiment (# of animals)</th>
<th>Slope ± SE ΔpH/Δlog_{10}PCO₂</th>
<th>Intercept ± SE (pH at PCO₂=1 mmHg)</th>
<th>R²</th>
<th>Standard [HCO₃⁻] ± SE @ PCO₂ = 40 mmHg (Calculated)</th>
<th>Base Excess relative to control (Calculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=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>
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<td>Iodoacetate (n=3)</td>
<td>-0.589 ± 0.041</td>
<td>8.222 ± 0.056</td>
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<td>19.4 ± 0.5 mM</td>
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<td>-0.587 ± 0.026</td>
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Table 2. ANOVA: Effect of Metabolic Inhibitors on Dynamic IPC Discharge Pattern. Repeated measures two-way ANOVA (Main effects: IPC, Drug, CO2; Interaction: Drug*CO2). Alphabet letters different from control indicate significant difference of indicated treatment using Tukey HSD post-hoc test (multiple comparisons controlled to keep overall \( \alpha = 0.05 \)).

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<tr>
<th>Experiment (# of IPC)</th>
<th>Model significance of Main Effects and Crossed Effects</th>
<th>Drug Treatments:</th>
<th>Drug Treatment Effects: Average Discharge Rate (sec(^{-1})) Mean ± SE</th>
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</table>
| **Control vs. Iodoacetate (n=6)** | IAA \((p<0.001)\)
CO2 \((p<0.001)\)
IAA*CO2 \((p<0.001)\) | Control
Iodoacetate 10 mg/kg
Iodoacetate 70 mg/kg | **Control**
15.9 ± 0.3 (a)
15.2 ± 0.3 (a)
5.5 ± 0.3 (b) |
| **Control vs. Acetate (n=2)** | Acetate \((p=0.45)\)
CO2 \((p<0.001)\)
Acetate*CO2 \((p=0.56)\) | Control
Acetate (0.38 mmole/kg) (equiv to 70 mg/kg IAA) | **Control**
6.6 ± 0.5 (c)
6.1 ± 0.5 (c) |
| **Control vs. Rotenone (n=6)** | Rotenone \((p<0.001)\)
CO2 \((p<0.001)\)
Rot*CO2 \((p<0.001)\) | Control
Vehicle Control
Rotenone 2 mg/kg | **Control**
12.9 ± 0.5 (d)
13.6 ± 0.5 (d)
2.6 ± 0.6 (e) |
| **Control vs. 2,4-DNP (n=6)** | DNP \((p<0.001)\)
CO2 \((p<0.001)\)
DNP*CO2 \((p=0.50)\) | Control
Vehicle Control
DNP 5 mg/kg
DNP 10-15 mg/kg | **Control**
14.0 ± 0.3 (f)
14.8 ± 0.3 (f)
14.1 ± 0.3 (f)
11.3 ± 0.3 (g) |
Iodoacetate

A

Plasma pH vs. PaCO2 (mmHg)

- Control
- Regression
- 95% CI

B

IPC Discharge Rate (Hz) vs. Time (sec)

- Control
- 10 mg/kg IAA
- 70 mg/kg IAA
- CO2 Step

C

Dynamic Min and Max Discharge Frequency (Hz) vs. Inspired PCO2 (mmHg)

- Control
- IAA 70 mg/kg

D

Dynamic Max and Min Discharge Frequency (Hz) vs. Plasma pH

- Control
- IAA (70 mg/kg)
Acetate

A

Plasma pH

PaCO$_2$ (mmHg)

B

IPC Discharge Frequency (Hz)

Time (sec)

C

Dynamic Min and Max Discharge Frequency (Hz)

Inspired PCO$_2$ (mmHg)

D

Dynamic Min and Max Discharge Frequency (Hz)

Plasma pH

Control

Regression

95% CI

Acetate

Regression

95% CI

Control

Regression

95% CI

Control

Acetate (Equivalent to 70 mg/kg IAA)

Control

Acetate (Equivalent to 70 mg/kg IAA)

Control

Acetate (Equivalent to 70 mg/kg IAA)
2,4 Dinitrophenol

A

Plasma pH

- Control
- Regression
- DNP
- 95% CI

PaCO₂ (mmHg)

B

IPC Discharge Rate (Hz)

- Control
- Vehicle Control
- DNP 5 mg/kg
- DNP 10-15 mg/kg
- CO₂ Step

Time (sec)

C

Dynamic Min and Max Discharge Frequency (Hz)

- Control
- DNP

Inspired PCO₂ (mmHg)

D

Dynamic Min and Max Discharge Frequency (Hz)

- Control
- DNP (10-15 mg/kg)

Plasma pH
Effect of 2,4 DNP on "Tonic" IPC (D3, D6, D7)

Time (sec) 0 5 10 15 20 25
IPC Discharge Rate (Hz) 0 10 20 30 40 50

Average 2,4-DNP Effect (units D1,D2,D3,D5,D6,D7)

- Control
- Vehicle Control
- DNP 5 mg/kg
- DNP 10-15 mg/kg
- CO2 Step

Effect of 2,4 DNP on "Phasic" IPC (D1, D2, D5)

Time (sec) 0 5 10 15 20 25
IPC Discharge Rate (Hz) 0 20 40 60 80 100

Effect of 2,4 DNP on "Tonic" IPC (D3, D6, D7)

Time (sec) 0 5 10 15 20 25
IPC Discharge Rate (Hz) 0 10 20 30 40 50
Table 1. Regression: Effect of Metabolic Inhibitors on Blood Buffer Curves (plasma pH vs. log PaCO2). Model Equation: $pHa = A \cdot (\log_{10}PaCO_2) + B$

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