Functional mitochondria are required for O$_2$ but not CO$_2$ sensing in immortalized adrenomedullary chromaffin cells


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ADRENOMEDULLARY CATECHOLAMINE (CAT) release in response to perinatal stressors plays a vital role in the adaptation of the neonate to extraterrestrial life (20, 21). These stressors, including low O$_2$ (hypoxia), elevated CO$_2$ (hypercapnia), and low pH (acidity), accompany intermittent breathing and asphyxia experienced by the newborn (11) and directly stimulate adrenomedullary chromaffin cells (AMC) prior to acquire functional splanchnic innervation. The downstream mechanisms of O$_2$ sensing in these AMC involve hypoxic inhibition of a variety of K$^+$ channels, leading to or facilitating membrane depolarization, voltage-gated Ca$^{2+}$ entry, and CAT secretion (2, 8, 13, 14, 23–25). Increases in CO$_2$/H$^+$ also cause membrane depolarization and CAT secretion in these cells via activation of a resting cation conductance as well as K$^+$ channel inhibition (15, 19). Consequently, the combined effects of low O$_2$ and increased CO$_2$/H$^+$ during perinatal asphyxia contribute to a robust adrenomedullary secretory response that aids critically in the transformation of the lung to an air-breathing organ and in the regulation of cardiac contractility (11, 15, 19, 20).

The upstream mechanisms by which chromaffin cells in the adrenal medulla, as well as their counterparts in the carotid body, sense acute hypoxia have been the subject of intense investigation and controversy (12, 22). While heme proteins of the mitochondrial electron transport chain (ETC) have long received strong support as candidates for the O$_2$ sensor, there is evidence for the involvement of extra-mitochondrial proteins including NADPH oxidase, hemeoxygenase-2, and AMP-kinase in the mediation of the hypoxic response (10). In the case of adrenal chromaffin cells, the mitochondrial ETC has been proposed as the site for the O$_2$ sensor based primarily on the use of pharmacological ETC blockers including the complex I blocker, rotenone, which was found to mimic and occlude the effects of hypoxia (8, 14, 22). However, general concern about the lack of specificity of these ETC blockers, as well as the demonstration that rotenone may block a putative extra-mitochondrial O$_2$ sensor in the carotid body (17), raises questions about the mitochondrial origin of the O$_2$ sensor.

More definitive studies on the O$_2$-sensing mechanisms in adrenal chromaffin cells can be greatly facilitated through the use of immortalized cell lines because their genetics can be conveniently manipulated. Also advantageous is a cell line that faithfully reproduces O$_2$-sensing properties characteristic of the native cell. An attractive candidate for this role is the v-myc adrenal-derived HNK1$^+$ immortalized chromaffin cell line (MAH) derived from fetal rat adrenal medulla (1). Recent studies from this laboratory have demonstrated that MAH cells express several O$_2$-sensing properties of neonatal rat AMC, including hypoxic regulation of similar K$^+$ channel subtypes as found in native cells (4). In the present study, we use wild-type (WT) MAH cells and mutant MAH cells, devoid of a functional ETC due to defective mitochondrial DNA ($\rho^0$ cells), to show that functional mitochondria are indeed required for O$_2$ sensing by chromaffin cells. Several independent assays of O$_2$ sensitivity were used to support this conclusion and involved whole cell recordings of K$^+$ currents and membrane potential, ratiometric fura-2 intracellular Ca$^{2+}$ (Ca$_i$) measurements, and carbon fiber amperometric determination of CAT secretion. We further show that the ability of rotenone to mimic hypoxia in these cells is dependent on functional mitochondria. Finally, we provide evidence for the first time that MAH cells can also act as CO$_2$ sensors and express the CO$_2$ marker carbonic anhydrase II (CA II). However, unlike hypoxia-sensing, CO$_2$ sensing occurs in mutant $\rho^0$ MAH cells and is therefore independent of functional mitochondria.

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**MATERIALS AND METHODS**

WT MAH cells. WT MAH cells (a generous gift from Dr. Laurie Doering) were grown in modified L-15/CO2 medium supplemented with 0.6% glucose, 1% penicillin-streptomycin, 10% fetal bovine serum, and 5 μM dexamethasone (4). All cultures were grown in a humidified atmosphere of 95% air-5% CO2 at 37°C. Cultures were fed every 1–2 days and split every 3–4 days. To passage cells, the culture medium was removed and 0.25% trypsin was added to detach cells from the culture substrate. The resulting cell suspension was pelleted by centrifugation, the supernatant discarded, and the pellet resuspended in fresh medium. Cells were then plated at a density of approximately 2 × 104 cells/ml onto standard 35-mm culture dishes, which had been previously coated with poly-d-lysine and laminin to promote cell adhesion.

Mitochondria-deficient p0 MAH cells. To produce cultures of MAH cells deficient in functional mitochondria (p0 MAH cells), WT cells were grown in modified L-15/CO2 medium supplemented with 0.6% glucose, 1% penicillin-streptomycin, 10% fetal bovine serum, 5 μM dexamethasone, 10 mM sodium pyruvate, 2 mM uridine, and 200 ng/ml ethidium bromide, to inhibit mitochondrial function and division (18). After incubation with ethidium bromide for 3 wk, cells were treated with 20 μM rotenone or 20 μM myxothiazol to select for cells deficient in functional mitochondria. Ethidium bromide prevented replication of mitochondrial DNA, and the surviving cells lacked a functional ETC (see below). p0 MAH cells were fed every 1–2 days.

**PCR determination of p0 status.** Confirmation of p0 status in mutant MAH cells was obtained by examining the expression of mitochondrial DNA-encoded cytochrome oxidase I (COX I) subunit gene of complex IV (rat mitochondrial genome sequence 5161–5700). Primer sequences were derived from GeneBank accession number J05318, using the program GeneFisher. Primer sequences were as follows: (forward) 3′ TGGAGGCTGAGGAATAG and (reverse) 5′ AAT-CTACGGATACTCCGGAGCA. PCR amplification of the β-actin gene was used as a control with the following primers: (forward) 3′ CCTAGTGGTGTGCTGTCATGC and (reverse) 5′ GAAGATCCTGACCGAGCCTG.

**Quantitative RT-PCR.** RNA from MAH cell cultures was extracted with the RNeasy Mini Kit (Qiagen). RNA was quantified in an RiboGreen Assay (Molecular Probes). Reverse transcription was carried out on 100 ng of RNA using Superscript III (Invitrogen) and random primers (100 ng). Quantitative PCR was carried out with the Absolute QPCR SYBR Green Mix (ABGene) and analyzed with a Stratagene MX3000P machine. Gene-specific primers were designed using GeneFisher software and were synthesized by The Central Facility of the Institute for Molecular Biology and Biotechnology (MOBIX, McMaster University, Hamilton, ON, Canada). The following primers were used and listed as gene amplified, sequence (forward, reverse), and melt temperature: Lamin A/C: 5′-GAATGCTGCTCTTGCAGAAC-3′ and 5′-GTGAGTTGCGGCTTGCAC-3′, 55°C; CA II: 5′-GCAGTACAAGAAGGAGGACA-3′ and 5′-TGCGGTGGCAGGTCACAG-3′, 50°C; CA I: 5′-ACCAGCAGAGAAGGATGC-3′ and 5′-GTGCTGACGGTGGGACTAC-3′, 57°C; CA I: 5′-GGACGAGCGTG-3′ and 5′-GACCGAGCGTG-3′, 55°C; and 5′-CTACGGATACCCCAGCA. PCR amplification of the actin gene was used as a control with the following primers: (forward) 3′ CCTAGTGGTGTGCTGTCATGC and (reverse) 5′ GAAGATCCTGACCGAGCCTG.

**Electrophysiology.** Whole cell recordings from WT and p0 MAH cells were obtained with the nystatin perforated-patch technique as previously described (4, 24). In voltage-clamp experiments, cells were held at −60 mV and step depolarized to the indicated test potential (between −100 mV and +80 mV in 10-mV increments) for 100 ms at a frequency of 0.1 Hz. In some cases, cells were held at −60 mV and were ramped from −80 to +50 mV for 500 ms at a frequency of 0.1 Hz. Currents were filtered at 5 kHz, digitized at 10 kHz, and stored on computer for later analysis. Capacitative transients were minimized by analog means.

In these experiments, the pipette solution contained (in mM) 95 K gluconate, 35 KCl, 5 NaCl, 2 CaCl2, 10 HEPES, at pH 7.2, and nystatin (400 μg/ml). In some cases, the external bathing solution contained (in mM) 135 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 glucose, and 10 HEPES, at pH 7.4. Solutions were changed via a gravity fed perfusion system. Hypoxic solutions (PO2 15–20 mmHg) were generated by bubbling N2 (gas) in a reservoir that was surrounded by a warm water bath (37°C) to minimize temperature artifacts. The temperature of the bathing solution at the recording site was approximately 37°C. The PO2 was measured in the chamber with a dissolved PO2 reader (WPI ISO2). In experiments designed to test the effects of CO2, bicarbonate-buffered extracellular solutions were used. Normocapnic (control) extracellular solution consisted of a bicarbonate/CO2-buffered saline of the following composition (in mM): 115 NaCl, 24 NaHCO3, 5 KCl, 2 CaCl2, 1 MgCl2, and 10 glucose at pH 7.4 maintained by bubbling 95% air-5% CO2. For isohydric hypercapnia (10% CO2), the pH was kept constant at 7.4 by elevating NaHCO3 to 48 mM (equimolar NaCl substituted). Solutions were made hypoxic by bubbling a 5% CO2-95% N2 gas mixture. The culture was perfused via gas-impermeable Tygon tubing, and excess solution was removed by vacuum suction. Data acquisition and analysis were performed with either an Axopatch 1D or Multiclamp 700 amplifier in combination with a Digidata 1200 or Digidata 1322A interface (respectively) and pCLAMP 9.2 software (Axon Instruments). Current densities were calculated by dividing the evoked current by cell capacitance.

Carbon fiber amperometry. Cyt secretion from MAH cells was monitored with carbon fiber amperometry after the culture dish was placed on the stage of Zeiss Axioskop 2 upright microscope equipped with a ×40 water immersion objective. The culture was perfused under gravity with bicarbonate-buffered extracellular solution, bubbled with 95% air-5% CO2 (pH 7.4; see above) at 37°C. In some experiments, high K+ (30 mM) solutions were used after equimolar substitution for NaCl. Hypoxic solution (PO2 = 15–20 mmHg) was obtained by continuously bubbling with a 5% CO2-95% N2 gas mixture (see above). Catecholamine secretion was monitored with ProCFE low noise carbon fiber electrodes (electrode diameter 5 μm; Dagan) connected to a CV 238U headstage and an Axopatch 200B amplifier set at 800 mV. Data acquisition and analysis were performed with Clampfit 9.2 (Axon Instruments); currents were filtered at 100 Hz, digitized at 250 Hz, and stored on a personal computer. Charge of individual secretory events was calculated by integrating the area under each spike, and total secretion during stimulus application was plotted as the cumulative charge (in fC). Events smaller than 3 pA were excluded from the analysis, and spike frequency was calculated as the number of spike events/min. Secretory events were compared with Student’s t-test, and the level of significance was set at P < 0.05. Unless otherwise noted, the data are expressed as means ± SE.

**Fura-2 Ca2+ measurements.** Intracellular Ca2+ was monitored with the fluorescent Ca2+ indicator fura-2 AM. Cells were first plated into central wells of modified culture dishes, in which a central hole was drilled before a glass coverslip was attached to the underside. Cultures were loaded with 5 μM fura-2 AM for 30 min at 37°C, then rinsed (3 times) with extracellular solution, and allowed to deesterify for 30 min before use. Ratiometric Ca2+ measurements
were obtained with a Nikon Eclipse TE2000-U inverted microscope equipped with a filter switching lambda DG-4 high-speed optical filter changer, a Hamamatsu ORCCA-ET digital CCD camera, and a Nikon S-Fluor ×40 oil immersion objective lens with a numerical aperture of 1.3. Dual images (340- to 380-nm excitation, 510-nm emission) were collected, and pseudocolor ratiometric images were monitored during the experiments by using Simple PCI software (version 5.3, Compix). The imaging system was standardized with a two-point calibration, using Ca²⁺-free solution and Ca²⁺ solution (39 µM) obtained from Molecular Probes (F-6774). The parameters used for the two-point calibration include the dissociation constant of fura-2 ($K_d$ 224 nM), the ratio values for the (−) and (+) concentration standards ($R_{\text{min}} =$ 0.026 and $R_{\text{max}} =$ 4.4) and β-value of 5.6. [Ca²⁺]i (in nM) was calculated according to the equation previously described (6). All experiments were performed at 37°C. Cells were continuously

![wt MAH cells](image1) ![ρ₀ MAH cells](image2)

Fig. 1. Expression of characteristic markers in wild-type (WT) and ρ₀ MAH cells. Phase contrast micrographs of WT (A) and ρ₀ (B) MAH cells are shown. Positive immunoreactivity against tyrosine hydroxylase (TH), was obtained in both WT (C) and ρ₀ (D) MAH cells with the aid of a FITC-conjugated secondary antibody. Similarly, both WT (E) and ρ₀ (F) MAH cells were immunopositive for carbonic anhydrase II (CA II), visualized with a FITC-conjugated secondary antibody. WT MAH cells showed significant uptake of the mitochondrial fluorescent probe rhodamine 123 (Rh 123; G), whereas ρ₀ MAH cells showed weak Rh 123 uptake (H), consistent with the absence of a functional electron transport chain (ETC). Confirmation of the ρ₀ status was obtained during PCR amplification of the mitochondria-encoded cytochrome oxidase I (COX I) gene, a subunit of complex IV. The COX I gene was absent in ρ₀ MAH cells but present in WT MAH cells (I). Both WT and ρ₀ MAH cells expressed the genome-encoded β-actin gene (I).
perfused with 5% CO₂-bicarbonate-buffered extracellular solution as described above. Rhodamine 123 staining. WT and p⁰ MAH cells were grown in wells of modified 35-mm dishes (see above) for 24 h and were then treated with 10 μg/ml of rhodamine 123 for 10 min at room temperature. Cells were then rinsed and examined with a Nikon Eclipse TE2000-U inverted microscope with ×100 oil immersion objective (Nikon). Images were captured using a Hamamatsu ORCA-ER digital CCD camera using Simple PCI software (version 5.3).

Drugs. All solutions containing drugs were made fresh on the day of the experiments. Drugs were obtained from Sigma-Aldrich unless otherwise stated.

Immunocytochemistry. MAH cells were grown in the central wells of modified 35-mm Nunc dishes as previously described (4). The well was formed by drilling a central hole (~1 cm in diameter) in the dish and attaching a glass coverslip to the underside. Medium was removed, and the cells were washed two times in 3 ml phosphate-buffered saline (PBS). Cells were then fixed with 3 ml of 5% acetic acid-95% methanol at 20°C for 60 min, and the solution was replaced with 2 ml PBS. Samples were then washed three times with PBS, before the addition of 30 μl of primary antibody followed by incubation for 24 h at 4°C. The following primary antibodies were used at the dilutions indicated: anti-tyrosine hydroxylase, 1:1,000 (Millipore); anti-carbonic anhydrase, 1:50 (Biogenesis); anti-Kv1.2, 1:50; anti-Kv1.5, 1:50; and anti-Ca²⁺-dependent K⁺ or BK 1:100 (Alomone). Following incubation, the primary antibody solution was removed, and the samples were washed three times in PBS. Secondary antibody, conjugated with FITC or Texas red (Jackson Immunoresearch) (as indicated) was diluted in PBS (1:50) and incubated for 1 h at room temperature shielded from light. After removal of the solution, the samples were washed three times in PBS. Vecta-shield was then added to the dishes to prevent photobleaching. Control experiments, in which primary antibody was omitted from the first incubation step, were also performed. In the case of the BK, Kv1.2, and Kv1.5 channel antibodies, an additional control involved preincubating the primary antibody with excess blocking peptide overnight at 4°C (at 3 g of fusion peptide per 1 g of antibody), before addition to the cells. The samples were visualized with a Zeiss inverted microscope (IM 35) equipped with epifluorescence, as well as fluorescein and rhodamine filter sets. Images were acquired using a digital camera with Northern Eclipse software and were saved in TIFF format.

Western blot analysis. Confluent cultures of WT and p⁰ MAH cells were trypsinized (0.25%) and pelleted by centrifugation. Cells were then washed three times in PBS. Cell pellets were then homogenized in 0.2 ml ice-cold buffer A (in mM: 20 HEPES, 20 KCl, 2 EDTA, 2 EGTA, and 2 DTT) containing 1 pellet of complete mini, EDTA-free protease inhibitor cocktail (Roche 1836170) and were placed on ice for 15 min. Then 6.25 μl of 10% NP40 was added to the homogenate and vortexed for 15 s. The resulting solution was then centrifuged at

![Outward currents](Fig. 2. Whole cell recordings of the effects of hypoxia on outward K⁺ current and membrane potential in WT MAH vs. p⁰ MAH cells. Hypoxia (Hox; Po₂ ~15 mmHg) caused reversible inhibition of outward K⁺ current in WT MAH cells at positive potentials (n = 15; A and C). Sample recordings at a step potential of +30 mV are shown in A, and current density (I) vs. voltage (V) plot is shown in C for 15 cells. In A, control traces (C) before hypoxia and after washout (W) are also shown. Holding potential was ~60 mV. In contrast, hypoxia had no effect on outward current in p⁰ MAH cells as shown in sample traces (B), and in the I-V plot (D; n = 16). Under current clamp, hypoxia depolarized WT MAH cells (E; n = 11) but had no effect on membrane potential in p⁰ MAH cells (F; n = 10).)
We used two independent assays to validate that mitochondrial function was impaired in ρ0 MAH cells. First, we tested for uptake of the fluorescent dye rhodamine 123, which is known to accumulate in functional mitochondria (7). Accordingly, whereas WT MAH cells displayed significant rhodamine 123 fluorescence following a 10-min exposure to the dye (Fig. 1G), ρ0 MAH cells showed little or no rhodamine 123 fluorescence (Fig. 1H). Second, we probed for the expression of a key subunit of the mitochondria-encoded cytochrome c oxidase gene (COX I) using PCR. As shown in Fig. 1I, WT MAH cells expressed COX I gene but ρ0 MAH cells did not; note, however, that both WT and ρ0 MAH cells expressed the β-actin gene (Fig. 1J). Taken together, these data confirm that the ρ0 MAH cells used in the present study were deficient in functional mitochondria.

Are functional mitochondria required for O2 sensing in MAH cells? Previous studies in this laboratory have shown that the O2 sensitivity of MAH cells involves hypoxia-induced inhibition of outward K+ current and membrane depolarization, via closure of several K+ channel subtypes (4). With the use of perforated-patch recording, outward K+ currents from both WT MAH and ρ0 MAH cells were monitored under voltage clamp. Exposure of WT MAH cells to acute hypoxia (P02 ~15 mmHg), caused inhibition of outward K+ current at more positive potentials (Fig. 2, A and C). For a voltage step to +30 mV, from an initial holding potential of ~60 mV, the mean outward current density decreased significantly from 46.1 ± 4.3 pA/pF under normoxia to 26.2 ± 5.2 pA/pF during hypoxia (n = 15; P < 0.05; Fig. 2C). In contrast, hypoxia had no effect on outward current in ρ0 MAH cells; for a step to +30 mV, the normoxic outward current density was 38.7 ± 6.3 pA/pF versus 36.3 ± 4.1 pA/pF in hypoxia (n = 16; Fig. 2, B and D).

We also compared the effects of hypoxia on membrane potential in WT and ρ0 MAH cells under current clamp. As illustrated in Fig. 2E, acute hypoxia depolarized the membrane potential in WT MAH cells from a mean resting level of −57 ± 5.1 mV to −49 ± 4.7 mV (n = 11), corresponding to...
ROLE OF MITOCHONDRIAL FUNCTION IN O₂ SENSING

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C950

wt MAH

A1

A2

A3

ρΟ MAH

B1

B2

B3

C

KV 1.2

KV 1.5

BK

Control

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a receptor potential of ~8 mV. In contrast, hypoxia had no effect on the resting potential of ρ₀ MAH cells, as illustrated in Fig. 2F; the mean resting potential was −55 ± 4.3 mV before and during hypoxia (n = 10). Taken together, these data support the idea that hypoxia sensitivity of MAH cells requires functional mitochondria.

To validate further the important role of mitochondrial function in O₂ sensing by MAH cells, we tested the effects of rotenone, a blocker of complex I of the ETC. We previously showed that rotenone mimicked and occluded the effects of hypoxia in primary neonatal adrenal chromaffin cells (22). In the present study, rotenone (1 μM) caused a reversible inhibition of outward current in WT MAH cells at more positive potentials (n = 10; Fig. 3A), similar to hypoxia (Fig. 2, A and B). In contrast, rotenone had no detectable effect on outward current in ρ₀ MAH cells (n = 12; Fig. 3B), consistent with the lack of complex I function in these mitochondria-deficient cells. These data also argue against a nonspecific effect of rotenone on K⁺ currents, of which several subtypes are expressed in both WT and ρ₀ MAH cells (see below).

K⁺ channel expression in ρ₀ MAH cells. Several K⁺ channel subtypes contribute to the O₂-sensing properties of neonatal adrenal chromaffin and MAH cells including large (BK) and small (SK) conductance Ca²⁺-activated K⁺, and delayed-rectifier (Kᵥ) K⁺ channels (4, 8, 23, 25). To confirm that the loss of O₂ sensitivity in ρ₀ MAH cells was not due to the lack of expression of these O₂-sensitive K⁺ channels, we used pharmacological blockers and immunocytochemistry. As shown in Fig. 4, outward K⁺ currents in both WT and ρ₀ MAH cells were inhibited by the selective SK and BK channel blockers apamin (100 nM; Fig. 4A) and iberiotoxin (IbTx, 100 nM; Fig. 4A2), respectively, as well as by the general Ca²⁺ channel blocker cadmium, which indirectly blocks Ca²⁺-dependent K⁺ channels (Fig. 4A3). For a voltage step to +30 mV, inhibition by IbTx was 30 ± 6.3% (n = 7) in WT MAH cells (Fig. 4A2) and 25 ± 5.7% (n = 10) in ρ₀ cells (Fig. 4B2). In the case of apamin, the inhibition was 18 ± 7.2% (n = 8) for WT MAH cells (Fig. 4A1) and 19 ± 5.3% (n = 10) for ρ₀ MAH cells (Fig. 4B1). The percent inhibition by 50 μM Cd²⁺ was 28 ± 6.8% (n = 10) for WT MAH cells (Fig. 4A3) and 26 ± 8.3% (n = 10) for ρ₀ MAH cells (Fig. 4B3). These data indicate that ρ₀ MAH cells express functional Ca²⁺-dependent K⁺ channels previously shown to mediate at least part of the O₂-sensing properties of chromaffin cells. In general, the percent inhibition due to the various K⁺ channel blockers was not significantly different between WT MAH and ρ₀ MAH cells. Further confirmation that WT and ρ₀ MAH cells expressed similar ion channel profiles was obtained from a comparison of the time to full activation of the outward K⁺ current at +30 mV; the mean time of 9.2 ± 1.1 ms for WT MAH cells was not significantly different (P > 0.05) from that of 8.7 ± 0.9 ms for ρ₀ MAH cells.

Additional tests were used to probe for K⁺ channel expression in ρ₀ MAH cells. In particular, because delayed-rectifier

Fig. 4. K⁺ channel expression in WT and ρ₀ MAH cells. Mean (±SE) current vs. voltage plots are shown for WT MAH cells exposed to control (C) and either the small-conductance Ca²⁺-activated K⁺ (SK) channel blocker, apamin (Apa; 100 nM, A1; n = 8), the large-conductance Ca²⁺-activated K⁺ (BK) channel blocker, iberiotoxin (IbTx; 100 nM, A2; n = 7), or the nonspecific blocker of Ca²⁺ channels, cadmium (Cd²⁺, 50 μM, A3; n = 10), which indirectly blocks Ca²⁺-dependent K⁺ channels. Insets, sample recordings at +30 mV; holding potential was −60 mV. Corresponding data for ρ₀ MAH cells are shown in B1–B3 (n = 10). Note that the ρ₀ MAH cells expressed similar Ca²⁺-dependent K⁺ currents as WT MAH cells. Error bars represent means ± SE. ρ₀ MAH cells also showed expression of Kv1.2, Kv1.5 subunits and the BK channel α-subunit, as determined by immunofluorescence (C). Staining was abolished in control experiments using blocking peptides (C, bottom).

K⁺ channels consisting of Kv1.2 and Kv1.5 subunits have also been implicated in O₂ sensing by MAH cells (4), we used immunocytochemistry to test for possible expression of these channels in ρ₀ MAH cells. As shown in Fig. 4C, ρ₀ MAH cells showed positive immunoreactivity for both Kv1.2 and Kv1.5 subunits. In concert with our electrophysiological studies showing the presence of IbTx-sensitive BK currents in ρ₀ MAH cells (Fig. 4B2), positive immunoreactivity was also observed with antibodies against the BK channel (Fig. 4C). In control experiments, immunostaining was abolished in each case following preincubation with the corresponding blocking peptide or omission of the primary antiserum (Fig. 4C, bottom). These data further indicate that loss of mitochondrial function in these ρ₀ MAH cells did not result in an overt loss of expression of K⁺ channel proteins that mediate O₂ sensitivity.

MAH cells as O₂ sensors: nonrequirement for functional mitochondria. Neonatal adrenal chromaffin cells also act as CO₂ sensors, which elicit catecholamine secretion during elevated CO₂ or hypercapnia (15). This property depends on intracellular CA activity and involves high CO₂-induced inhibition of outward K⁺ current and membrane depolarization. Two CA isoforms, CA I and CA II, are expressed in neonatal adrenal chromaffin cells, but it is unclear whether one or both isoforms are required for CO₂ sensing (15; see also Fig. 5A). Here, we tested for the first time whether MAH cells can act as a suitable surrogate model for CO₂ sensing by adrenal chromaffin cells. Indeed, both WT MAH and ρ₀ MAH cells...
expressed mRNA for the CA II isoform (Fig. 5B), as well as the CA II protein (Fig. 1, E and F, and Fig. 5B). However, unlike neonatal chromaffin cells, they did not express mRNA for the CA I isoform (Fig. 5A). To test whether MAH cells are able to sense hypercapnia, whole cell recordings of ionic currents and membrane potential were obtained in bicarbonate-buffered medium. As illustrated in Fig. 6, A–D, isohydric hypercapnia (10% CO2; pH 7.4) caused inhibition of outward K+ current in both WT MAH and pO MAH cells at more positive potentials, as previously reported for neonatal rat adrenal chromaffin cells (15). For a voltage step to +30 mV, hypercapnia caused a significant reduction in outward K+ current density from 52.3 ± 4.7 to 33.8 ± 5.1 pA/pF (n = 15; P < 0.05). Interestingly, the combined effects of hypoxia and hypercapnia on outward K+ current in WT MAH cells were additive (Fig. 6C). Under current clamp, hypercapnia also induced a significant depolarization of 11.2 ± 4.9 mV in WT MAH cells (Fig. 4E); the membrane potential depolarized reversibly from a mean resting level of −45 ± 5.6 mV to −33.8 ± 4.9 mV (n = 10), as exemplified in Fig. 6E. Taken together, these data indicate that MAH cells possess several of the CO2-sensing properties previously described for neonatal adrenal chromaffin cells.

The mechanism of CO2 sensing in adrenal chromaffin cells involves acidification of the intracellular pH catalyzed by CA activity (15). Since there is no evidence for mitochondrial involvement, we predicted that CO2 sensing should be preserved in pO MAH cells. This was indeed the case, further confirming that the pO status did not lead to a generalized loss of sensory functions. Similar to WT MAH cells, isohydric hypercapnia (10% CO2; pH 7.4) caused inhibition of outward K+ current at positive potentials in pO MAH cells (Fig. 6, B and D). During a voltage step to +30 mV, this stimulus evoked a significant reduction in outward K+ current density from 49.6 ± 4.7 to 23.4 ± 3.3 pA/pF (n = 20; P < 0.001) in pO MAH cells. Additionally, isohydric hypercapnia induced membrane depolarization in pO MAH cells, as exemplified in Fig. 6F; the membrane potential reversibly depolarized from a mean resting level of −52.1 ± 5.8 mV in 5% CO2 to −43.7 ± 4.4 mV (n = 10) in 10% CO2, at constant extracellular pH. These data indicate that MAH cells act as CO2 sensors and that, in contrast with O2 sensing, functional mitochondria are not required for CO2 sensing. These results also suggest that CA II expression may be sufficient for CO2 sensing in MAH cells and that CA I expression is not necessary for this function.

Intracellular Ca2+ responses in MAH cells. To learn more about the suitability of MAH cells as a surrogate model for O2 and CO2 sensing in neonatal adrenal chromaffin cells, we investigated Ca2+ responses using ratiometric fura-2 spectrofluorimetry. Typically, elevations in Ca2+ precede CAT secretion in

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**Fig. 6.** Effects of isohydric hypercapnia and hypoxia on whole cell currents and membrane potential in WT vs. pO MAH cells. Isohydric hypercapnia (10% CO2; pH 7.4) caused reversible inhibition of outward K+ current in WT MAH cells, as shown in sample traces during steps to +30 mV (A) and in the current density (pA/pF) plot (C). As seen in HEPES-buffered medium (Fig. 2), hypoxia also inhibited outward K+ current in bicarbonate-buffered medium, and the combined application of hypoxia and hypercapnia (10% CO2) led to an additive response (C). Data represent means ± SE for step to +30 mV (n = 15); independent t-test. **P < 0.05, significantly different from normoxic control (Nox); ***P < 0.05, significantly different from hypoxia. Traces for control (C) and recovery after washout (W) in A were obtained under normocapnia (5% CO2; pH 7.4). In pO MAH cells, isohydric hypercapnia still produced significant inhibition (t-test; *P < 0.001) of outward K+ current as shown in sample traces at +30 mV (B) and in the current density plot (D; n = 14). In contrast, hypoxia had no effect on outward K+ current in bicarbonate-buffered medium (D; n = 14), as was the case in HEPES-buffered medium (Fig. 2). Under current clamp, hypercapnia (10% CO2; pH 7.4) induced a depolarizing receptor potential in both WT (E) and pO (F) MAH cells.
adrenal chromaffin cells exposed to various secretagogues, e.g., hypoxia (13, 14). In WT MAH cells, both hypoxia (Po2 ~15 mmHg) and hypercapnia (10% CO2; pH 7.4) caused a significant increase in Ca$_i$ (Fig. 7A). Robust increases in Ca$_i$ were also induced by the depolarizing stimulus high K$^+$ (30 mM) and by 10 μM nicotine (Fig. 7A), which presumably acts via binding to nicotinic acetylcholine receptors expressed in these cells (unpublished observations). Consistent with our electrophysiological studies demonstrating the failure of hypoxia to modulate outward currents and membrane potential in mitochondria-deficient p0 MAH cells (Fig. 2), hypoxia failed to evoke increases in Ca$_i$ in p0 MAH cells (Fig. 7B). The fact that the depolarizing stimulus high K$^+$, and other chemostimuli including hypercapnia (10% CO2) and 10 μM nicotine, did elicit rises in Ca$_i$ in these cells (Fig. 7B) suggested that the p0 phenotype did not cause any major perturbations in Ca$^{2+}$ entry pathways.

Effects of hypoxia and hypercapnia on secretion in WT MAH and p0 MAH cells. Using carbon fiber amperometry, we monitored CAT secretion from both WT and p0 MAH cells to examine further the role of mitochondria in O2 and CO2 sensing. As illustrated in Fig. 8, A–D, acute hypoxia and isohydric hypercapnia stimulated quantal CAT secretion from WT MAH cells. These findings are reminiscent of those previously reported in neonatal AMC (13–15, 24). Additionally, exposure of WT MAH cells to high K$^+$ (30 mM) also stimulated CAT secretion (Fig. 8, A and C). In contrast, hypoxia failed to induce CAT secretion from mitochondria-deficient p0 MAH cells (Fig. 8, B and D), in agreement with the studies reported above indicating loss of O2 sensitivity in these cells. However, both hypercapnia and high K$^+$ stimulated CAT secretion from p0 MAH cells (Fig. 8, B and D), indicating that the secretory machinery was intact and confirming that CO2 sensing was independent of functional mitochondria. Additionally, basal secretion in WT MAH cells was significantly higher than that in p0 MAH cells. When the data for stimulus-evoked CAT secretion were analyzed relative to basal secretion (ΔCAT), hypoxia induced a significant increase in CAT secretion in WT MAH cells (ΔCAT = 7.33 ± 2.1 events/min); in contrast, in p0 MAH cells ΔCAT (0.4 ± 0.09 events/min) was not significantly altered, indicating the lack of a hypoxic response.

DISCUSSION

A major goal of the present study was to explore further the hypothesis that hypoxia-sensing by perinatal AMCs is critically dependent on functional mitochondria, the presumed site of the O2 sensor (8, 14, 22). This mechanism is biologically and clinically important since CAT secretion from these cells in response to hypoxia, one of several stressors associated with birth, is critical for the proper transition to extrauterine life (11, 20, 21). One key function of this hypoxia-induced CAT surge at birth is to stimulate pulmonary fluid reabsorption and surfactant secretion, thereby preparing the lung for air-breathing. To avoid overreliance on the use of mitochondrial blockers, whose lack of specificity has been a source of major concern, we adopted a genetic strategy where impairment of mitochondrial function was the result of defective mitochondrial DNA (mtDNA) encoded COX I whose lack of specificity has been a source of major concern, with the use of an immortalized adrenal chromaffin cell line derived from embryonic (day 14.5) rat sympathoadrenal progenitors (i.e., MAH cells; 1), which, importantly, are known to express several hypoxia-sensing pathways, including hypoxia to modulate outward currents and membrane potential in mitochondria-deficient p0 MAH cells (Fig. 2), hypoxia failed to evoke increases in Ca$_i$ in p0 MAH cells (Fig. 7B). The fact that the depolarizing stimulus high K$^+$, and other chemostimuli including hypercapnia (10% CO2) and 10 μM nicotine, did elicit rises in Ca$_i$ in these cells (Fig. 7B) suggested that the p0 phenotype did not cause any major perturbations in Ca$^{2+}$ entry pathways.
subunit gene and showed deficient uptake of the mitochondrial fluorescent probe rhodamine 123, failed to respond to hypoxia, in contrast with WT MAH cells. Moreover, we found for the first time that MAH cells were also CO₂ sensors, another recently characterized property of neonatal AMCs (15), although this property was independent of functional mitochondria. Indeed, the ability of mitochondria-deficient ρ₀ MAH cells to sense CO₂ was of interest since it ruled out the possibility of a generalized loss in sensory functions due to the mutation. Additionally, these mutant cells appeared healthy under phase contrast microscopy, with well-defined nuclei and nucleoli, and they expressed several phenotypic markers characteristic of WT cells including tyrosine hydroxylase and CA II.

Preservation of downstream targets of the O₂-sensing pathway in ρ₀ MAH cells. Although the mutant ρ₀ MAH cells appeared healthy, they grew more slowly than their WT counterparts, as expected, and were slightly smaller in size as revealed by whole cell capacitance measurements. It was conceivable, however, that the mutation affected the cells in more subtle ways such that downstream targets in the O₂-sensing pathway, rather than the O₂ sensor per se, were absent or poorly expressed. Several tests suggested that this possibility was unlikely. In particular, we found that several of the K⁺ channel subtypes known to be downstream targets for hypoxia modulation in WT MAH cells as well as in primary AMCs were functionally expressed in the mutant ρ₀ cells. These included both the iberiotoxin-sensitive large (BK) and apamin-sensitive small (SK) conductance Ca²⁺-dependent K⁺ channels, whose inhibition by hypoxia are thought to facilitate membrane depolarization, voltage-gated Ca²⁺ entry, and CAT secretion in chromaffin cells (2, 4, 8, 13, 24, 25). Because the hypoxia-induced depolarization in chromaffin cells may involve different K⁺ channels than those mediating the inhibition of outward current, these two measures were used as separate or distinct indices of hypoxia sensitivity in MAH cells in the present study. At least four different types of K⁺ currents (i.e., BK, SK, Kv, and ATP-sensitive K⁺) are known to be regulated by hypoxia in chromaffin cells (3, 4, 8, 9, 25), and of these, inhibition of the SK current appears to be important near the resting potential where these channels are active (8, 9). Because MAH cells also express T-type Ca²⁺ currents (3; unpublished observations), which are also known to be active at more negative potentials near rest, it is likely that they are the source of Ca²⁺ entry, leading to CAT secretion during hypoxia-induced depolarization in WT MAH cells.

We also demonstrated the presence of Kv1.2 and Kv1.5 K⁺ channel subunits in mutant ρ₀ MAH cells using immunocytochemistry. These subunits were proposed to contribute to a heteromeric Kv1.2/Kv1.5 delayed-rectifier-type K⁺ channel that also appears to be inhibited by hypoxia in WT MAH cells (4). Thus, several of the normal O₂-sensitive K⁺ channel subtypes were expressed in ρ₀ MAH cells, suggesting that the lack of O₂ sensitivity in these cells was not due to the absence of these key downstream targets. Although it was not possible in the present study to test for all the possible changes that could have been induced in ρ₀ MAH cells, it was clear that subsequent downstream steps in the hypoxia response pathway, i.e., those mediating voltage-gated Ca²⁺ entry and secretion, were also preserved in the mutant cells. Accordingly, application of high K⁺ depolarizing stimuli, or stimulation of nicotinic acetylcholine receptors with bath-applied nicotine, led to increases in Ca⁹ signals and/or CAT secretion in ρ₀ MAH cells. Taken together, these data suggest that the defect in the mutant cells leading to the loss of O₂ sensitivity occurs at an early upstream step that is critically dependent on a functional mitochondrial ETC.
Mitochondrial O2 sensor in adrenal chromaffin cells. The present study provides strong support for the hypothesis that the O2 sensor in adrenal chromaffin cells is located within the mitochondrial ETC or at an upstream site. It therefore substantiates, via a genetic approach, a similar conclusion reached in previous studies that used pharmacological ETC blockers (8, 14, 22). The ability of rotenone, a mitochondrial complex I blocker, to mimic and occlude the effects of hypoxia in perinatal adrenal chromaffin cells has been used to support the idea that complex I is, or is closely associated with, the O2 sensor (8, 22). In the present study, we found that rotenone did mimic hypoxia in inhibiting outward K+ current in WT MAH cells, and, consistent with mitochondria being the target for the drug’s action, rotenone had no effect in mutant p0 MAH cells. These data therefore do not support the thesis of a rotenone-sensitive, extra-mitochondrial O2 sensor in adrenal chromaffin cells, as has been proposed for rat carotid body O2 chemoreceptors (17). The signaling pathway that links the O2 sensor to K+ channel inhibition has been a source of controversy in a variety of O2-sensing cells including carotid body type I cells, neuroepithelial body cells, and pulmonary smooth muscle cells (26). Nevertheless, the more popular theories consider P02-induced changes in redox status or in the ADP/ATP ratio acting as the main intermediary signal. In the case of perinatal adrenal chromaffin cells, the link appears to be associated at least with a change in redox state, particularly a decrease in mitochondrial-derived reactive oxygen species (8, 22). However, the question as to whether cellular reactive oxygen species levels increase or decrease during acute hypoxia remains a controversial one in the O2-sensing field (26). In p0 MAH cells, the production of reactive oxygen species appears to be significantly impaired (unpublished observations), and this may play a role in the lack of a hypoxic response in these cells.

MAH cells as CO2 sensors. A novel finding in the present study was that MAH cells also functioned as CO2 sensors, indicating that this cell line expressed yet another property recently characterized in native chromaffin cells of the neonatal rat adrenal medulla (15). Because CO2 sensing was preserved in mutant p0 MAH cells, functional mitochondria were not required for its expression. This finding was not surprising since enzymatic activity of cytosolic CA was shown to mediate CO2 sensing in chromaffin cells of the adrenal medulla (15) and the developmentally related carotid body receptors (5, 16). In both organs, increased CO2 sensing is inhibited by membrane-permeable CA inhibitors and involves acidification of the intracellular pH (pHi) as a consequence of CA-catalyzed hydration of CO2. The acidic pHi in turn leads to inhibition of K+ conductance and may also cause activation of a resting cation conductance, at least in neonatal rat adrenal chromaffin cells (15). The net result of these acidic pHi-induced changes is enhanced membrane depolarization, leading to voltage-gated Ca2+ entry and CAT secretion. In the present study, isohydric hypercapnia (10% CO2; extracellular pH 7.4) caused inhibition of outward K+ current and a depolarizing receptor potential in both WT and p0 MAH cells. Moreover, this stimulus induced a rise in Ca2+ and CAT secretion as determined by ratiometric fura-2 measurements and carbon fiber amperometry, respectively. Interestingly, although neonatal rat adrenal chromaffin cells expressed two CA isoforms, i.e., CA I and CA II (15), we found that only the CA II isoform was expressed in WT and p0 MAH cells using a combination of RT-PCR, Western blot, and immunofluorescence techniques. These data suggest that of the two isoforms, CA II may be the more important isoform, and certainly sufficient for CO2 sensing in MAH cells.

In summary, these studies provide strong support for the idea that a functional mitochondrial ETC is essential for O2 sensing by perinatal adrenal chromaffin cells. Additionally, these studies further highlight the immortalized MAH cell line as an attractive model for perinatal adrenal chromaffin cells because they respond in similar ways as their native counterparts to two key stressors associated with birth, i.e., hypoxia and hypercapnia. This cell line should allow detailed molecular analyses of the regulatory mechanisms involved in these processes, which are critical during adaptation to extrauterine life.

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