Meclizine Inhibits Mitochondrial Respiration through Direct Targeting of Cytosolic Phosphoethanolamine Metabolism*§

Received for publication, May 27, 2013, and in revised form, October 9, 2013. Published, JBC Papers in Press, October 19, 2013, DOI 10.1074/jbc.M113.489237

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Background: Previous studies have shown that meclizine inhibits respiration in intact cells, but not in isolated mitochondria, via an unknown mechanism.

Results: Meclizine directly inhibits PCYT2 (CTP:phosphoethanolamine cytidylyltransferase). Inhibition of PCYT2 by meclizine led to rapid accumulation of its substrate, phosphoethanolamine, which is itself an inhibitor of mitochondrial respiration. Our work identifies the first pharmacologic inhibitor of the Kennedy pathway by directly inhibiting the Kennedy pathway of phosphatidylethanolamine biosynthesis. Metabolic labeling and in vitro enzyme assays confirmed direct inhibition of the cytosolic enzyme CTP:phosphoethanolamine cytidylyltransferase (PCYT2). Inhibition of PCYT2 by meclizine led to rapid accumulation of its substrate, phosphoethanolamine, which is itself an inhibitor of mitochondrial respiration. Our work identifies the first pharmacologic inhibitor of the Kennedy pathway, demonstrating that its biosynthetic intermediate is an endogenous inhibitor of respiration, and provides key mechanistic insights that may facilitate repurposing meclizine for disorders of energy metabolism.

Conclusion: Meclizine attenuates mitochondrial respiration by directly inhibiting the Kennedy pathway of phosphatidylethanolamine biosynthesis.

Significance: We identified a novel molecular target of meclizine, an over-the-counter antinausea drug, raising possibilities for new clinical applications.

Although mitochondrial respiration is crucial for cellular energetics and redox balance, during certain pathological conditions, respiration can actually contribute to pathogenesis (1). Attenuation of mitochondrial respiration has been proposed as a therapeutic strategy in a number of human disorders, including ischemia-reperfusion injury, neurodegeneration, autoimmune disease, and cancer (2). Many naturally occurring as well as synthetic compounds targeting the mitochondrial respiratory chain are available, but their clinical utility is limited by their narrow therapeutic index (3); thus, there is an unmet need for discovering new classes of drugs that can safely modulate mitochondrial respiration.

We recently identified meclizine, an over-the-counter antinausea drug, in a “nutrient-sensitized” chemical screen aimed at identifying compounds that attenuate mitochondrial respiration (4). In vivo follow-up studies demonstrated that meclizine could be protective against heart attack (4), stroke (4), and neurodegeneration (5) in animal models. Meclizine is a first generation pipеразине class of H1-antihistamine that has been in use for decades for prophylaxis against nausea and vertigo (6). Like many H1-antihistamines, meclizine has anticholinergic activity (7), and it has also been shown to target constitutive androstane receptors (8).

Meclizine inhibition of respiration had not been reported before, and unlike classical respiratory inhibitors (e.g. antmycin and rotenone), meclizine did not inhibit respiration in isolated mitochondria but rather only in intact cells (4). We previously reported that this effect was independent of its antihistaminergic and anticholinergic activity (4), but the precise mechanism remained unknown. Curiously, the kinetics of meclizine-mediated inhibition of respiration were on the time scale of minutes (4), which is far too fast for a transcriptional mechanism but slower than direct inhibitors of the respiratory chain, suggesting that the inhibition arose from a potentially novel mechanism perhaps through intracellular accumulation...
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of a meclizine-derived active metabolite or by perturbing metabolism.

To gain insights into the mechanism of meclizine action, we performed global metabolic profiling of meclizine-treated cells to detect alterations in intracellular metabolites of intermediary metabolism. Metabolic profiling revealed a sharp increase in intracellular levels of phosphoethanolamine (PEtn), an intermediate in the CDP-ethanolamine (Etn) Kennedy pathway of phosphatidylethanolamine (PE) biosynthesis. Follow-up biochemical experiments confirmed the direct inhibition of CTP:phosphoethanolamine cytidylyltransferase (PCYT2), a rate-limiting enzyme of the CDP-Etn Kennedy pathway. The inhibition of PCYT2 results in the buildup of its substrate, PEtn, which itself directly inhibits mitochondrial respiration. Our work thus identifies a novel molecular target of meclizine and links the CDP-Etn Kennedy pathway to mitochondrial respiration.

EXPERIMENTAL PROCEDURES

Metabolite Profiling—Metabolite profiling was performed on MCH58 fibroblasts following treatment with 50 μM meclizine or vehicle control for 5 h using methods similar to those described previously (9). Briefly, low passage MCH58 cells were cultured on 6-cm tissue culture dishes in 4 ml of culture medium to 90% confluence and a final yield of 1 × 10⁶ cells. For assessment of intracellular metabolites, medium was aspirated from the above tissue culture dishes, and cells were gently washed with 4 ml of phosphate-buffered saline (PBS) to ensure complete removal of residual medium metabolites. After removal of PBS, cellular metabolism was quenched with immediate addition of 1 ml of precooled (−80 °C) methanol extraction solution (80% methanol, 20% H₂O). Cells were scraped in extraction solution, vortexed, and centrifuged, and the resulting supernatant was collected and stored at −80 °C. At the time of measurement, 100 μl of supernatant was diluted 1:1 with methanol extraction solution. The resulting solution was evaporated under nitrogen, the samples were reconstituted in 60 μl of high performance liquid chromatography (HPLC) grade water, and metabolites were assessed. Six biological replicates were assessed for each group. Analyses of endogenous metabolites were performed using a liquid chromatography-tandem mass spectrometry (LC-MS) system composed of a 4000 QTRAP triple quadrupole mass spectrometer (AB Sciex) coupled to three Agilent 1100 binary HPLC pumps (Agilent Technologies) and an HTS PAL autosampler (LEAP Technologies) equipped with three injection ports and a column selector valve. Three multiplexed chromatographic methods were configured for the analyses of each sample. LC method 1 used a Luna Phenyl-Hexyl column (Phenomenex) with a linear gradient of water/acetonitrile/acidic acid (initial proportions, 100:0:0.001; final proportions, 10:90:0.001). LC method 2 used a Luna NH₂ column (Phenomenex) with a linear gradient using acetonitrile/water containing 0.25% ammonium hydroxide and 10 mm ammonium acetate (acetonitrile/water proportions were 80:20 at the beginning of the gradient and 20:80 at its conclusion). LC method 3 used a Synergi Polar-RP column (Phenomenex) and gradient elution with 5% acetonitrile, 5 mM ammonium acetate (mobile phase A) and 95% acetonitrile, 5 mM ammonium acetate (mobile phase B). MS data were acquired using multiple reaction monitoring in both the positive (LC method 1) and negative (LC methods 2 and 3) ion modes. During the development of this method, authentic reference compounds were used to determine LC retention times and to tune multiple reaction monitoring transitions. Metabolite quantification was performed by integrating peak areas for specific multiple reaction monitoring transitions using MultiQuant software (version 1.1; AB Sciex), and all integrated peaks were manually reviewed for quality. Extraction and quantification procedures were optimized prior to assessment of samples in this study to ensure measured intracellular and medium metabolites were within the linear range of detection. Confirmation of the PEtn peak was performed using an exogenous PEtn standard (Sigma P0503). The relative quantification of PEtn in MCH58 skin fibroblasts or mouse striatal cells (STHdhQ7/Q7) was also confirmed using hydrophilic interaction liquid chromatography (10).

Determination of the Intracellular Concentration of PEtn and Phosphocholine (PCho)—Intracellular concentrations of PEtn and PCho in meclizine-treated MCH58 fibroblasts and PCYT2 knockdown cells were determined as follows. MCH58 fibroblast cells were seeded into a 6-well plate (0.2 × 10⁶ cells/well). After 20 h of growth, three wells were treated with 50 μM meclizine, and the three remaining wells were treated with DMSO for ~5 h. Cells were scraped and collected in methanol extraction solution (80% methanol, 20% H₂O), and PEtn and PCho levels were quantified by LC-MS using commercially available standards. Cell number and mean cellular diameter of MCH58 cells were determined using a Beckman Coulter Z-series cell counter in a parallel plate, which was subsequently used to calculate the intracellular concentration of PEtn and PCho.

Radiolabeling of Kennedy Pathway Intermediates—Radiolabeling of CDP-Etn Kennedy pathway intermediates was performed as described previously (11). Briefly, MCH58 fibroblasts were cultured in the presence of 50 μM meclizine for 5 h to 60% confluence at which point [¹⁴C]EtN (0.5 μCi/dish) was added, and cells were further incubated for 24 h. Lipids were extracted using the method of Bligh and Dyer (12). The total radioactivity from the water/methanol phase and that of the lipid phase were measured separately. [¹⁴C]PE was separated from the lipid phase using TLC in a solvent consisting of methanol/chloroform/ammonia (65:35:5). EtN, PEtn, and CDP-Etn were separated from the water/methanol phase using TLC in a solution of methanol, 0.5% NaCl, ammonia (50:50:5). The separated [¹⁴C]-labeled compounds were measured by scintillation counting.

PCYT2 Enzyme Assay—PCYT2 activity was assayed as described previously with minor modifications (13). Briefly, a 50-μl mixture of 20 mM Tris-HCl buffer, pH 7.8, 10 mM MgCl₂, 5 mM DTT, 650 μM CTP, 650 μM unlabeled PEtn, and 65 μM [¹⁴C]EtN was incubated with 0.4 μg of purified PCYT2 at 37 °C for 15 min. Reactions were terminated by boiling for 2 min. Meclizine was added at the indicated concentration in the reac-

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5 The abbreviations used are: PEtn, phosphoethanolamine; PE, phosphatidylethanolamine; PCho, phosphocholine; PCYT2, CTP:phosphoethanolamine cytidylyltransferase; Etn, ethanolamine; OCR, oxygen consumption rate; ECAR, extracellular acidification rate.
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Determination of IC$_{50}$ for PEtn Inhibition of Mitochondrial Oxygen Consumption—MitoXpress oxygen-sensitive probe (Luxcel Biosciences, Ireland) was used to monitor glutamate/malate-driven respiration of mouse kidney mitochondria in 96-well plate format (16). Each well contained glutamate/malate (11.5 mM), 20 µl of MitoXpress probe (dry probe was dissolved in 1 ml of water), mitochondria (0.5 mg/ml), and PEtn (0–50 mM) in 200 µl of total volume of buffer A. Two injections of ADP (487 µM final) were made using the injector of an EnVision 2104 plate reader (PerkinElmer Life Sciences). Time-resolved fluorescence was measured with the following settings: delay and gate times, 70 and 30 µs, respectively. Data analysis was performed using SigmaPlot 12.3. Briefly, slopes of basal respiration (state 2) and ADP-activated respiration (state 3) were taken, and the data were normalized against maximum respiration with no PEtn.

PE Determination—MCH58 fibroblasts were treated with 50 µM meclizine or DMSO for 5 h. Mitochondria were isolated from ~1.5 g of cells (wet weight) using the Mitochondria Isolation kit for cultured cells (Abcam MS852). Total phospholipids were extracted from whole cells or isolated mitochondria (1.5 mg of mitochondrial protein) as described previously (17). Briefly, phospholipids were extracted in 3 ml of chloroform/methanol (2:1) by shaking for 1 h followed by addition of 600 µl of 0.9% NaCl solution and vortexing for an additional 15 min. The aqueous and organic layers were separated via centrifugation (300 × g for 5 min) followed by an additional wash with 500 µl of H$_2$O. The bottom layer (organic phase) was evaporated to dryness under N$_2$ gas. The resulting lipid film was resuspended in 90 µl of 2:1 chloroform/methanol and separated on silica gel 60 TLC plates (EMD Millipore 1.05721.0001) using a solvent system consisting of chloroform/acetic acid/methanol/water (75:25:5:2.2). The TLC plates were then dried and exposed to iodine vapor to visualize phospholipid spots. The identity of each spot was verified using phospholipid standards (Sigma PH9-1KT). Individual spots were scraped, and phospholipid phosphorus was quantified using the method of Bartlett (18).

Testing the Combined Effect of Meclizine and Etn on MCH58 Fibroblast ATP Levels—MCH58 fibroblasts were seeded at 10,000 cells/well in a 96-well plate in high glucose DMEM and allowed to grow overnight. After ~20 h, growth medium was replaced with 10 mm galactose medium containing different concentrations of meclizine (0, 12.5, 25, and 50 µM) and Etn (0, 0.2, 1, and 5 mM). The ATP levels in the cells were measured using CellTiter-Glo reagent (Promega) 24 h after the treatment.

RESULTS

Meclizine Treatment Results in the Elevation of PEtn across Multiple Mammalian Cell Types—To decipher the mechanism by which meclizine blunts respiration, we used a metabolomics approach because previous reports have shown that the technology can reveal targets of desired and undesired actions of drugs (19, 20). We performed mass spectrometry-based metabolic profiling of human immortalized skin fibroblasts (MCH58) cells treated with 50 µM meclizine for 5 h. Our choice of MCH58 immortalized human skin fibroblasts was guided by our previous study characterizing the effect of meclizine on mitochondrial energy metabolism (4). Of the 124 metabolites...
that were measured reproducibly across six biological replicates, the most striking change corresponded to the PEtn signal, which increased nearly 35-fold \( (p_{\text{adj}} < 0.005) \), well outside the background distribution (Fig. 1A and supplemental Table 1). We further confirmed the identity and up-regulation of PEtn levels using hydrophilic interaction liquid chromatography-mass spectrometry in human fibroblasts and mouse striatal cells (STHdhQ7/7) treated with DMSO or 50 \( \mu \)M meclizine for 5 h. Data are expressed as mean \( \pm \) S.D. \( (n = 6 \text { for fibroblasts}; n = 3 \text { for striatal cells}) \).

**The Pattern of CDP-Etn Kennedy Pathway Intermediates in Meclizine-treated Cells Is Consistent with PCYT2 Inhibition**—PEtn is an intermediate in the CDP-Etn Kennedy pathway of PE biosynthesis (21, 22). The mammalian CDP-Etn Kennedy pathway consists of three enzymatic steps. Etn is first phosphorylated by Etn kinase to PEtn, which is converted to CDP-Etn by PCYT2 (also referred to as ECT). Finally Etn phosphotransferase (CEPT1) catalyzes the CDP-Etn conversion to PE (Fig. 2A). To understand why PEtn levels rise with meclizine, vehicle and meclizine-treated cells were incubated with \([^{14}C]\)Etn for 24 h, and the incorporation of the radiolabel was measured in the intermediates of the CDP-Etn Kennedy pathway. With meclizine treatment, \([^{14}C]\)PEtn levels were increased, whereas radiolabel incorporation into downstream metabolites, CDP-Etn and PE, was decreased (Fig. 2B). These results raise the hypothesis that meclizine directly blocks PCYT2 enzymatic activity, which catalyzes the conversion of PEtn to CDP-Etn.

**Meclizine Is a Non-competitive Inhibitor of PCYT2**—To confirm whether meclizine directly inhibits PCYT2, we performed an \textit{in vitro} enzyme assay with purified recombinant mouse PCYT2 (13) in the presence of varying concentrations of meclizine. Meclizine inhibited PCYT2 enzyme activity in a dose-dependent manner (Fig. 3A), and the Lineweaver-Burke plot of the PCYT2 enzyme kinetics suggests a non-competitive inhibition with an approximate \( K_i \) of 31 \( \mu \)M (Fig. 3B).

**PCYT2 Knockdown Cells Partially Mimic the Meclizine Effect**—We next sought to phenocopy the effects of meclizine using RNAi against \textit{PCYT2}. We stably silenced \textit{PCYT2} in MCH58 human skin fibroblasts using five hairpins and focused on two hairpins that achieved 77 and 90% knockdown at the RNA level (Fig. 4A). We treated control and knockdown cells with \([^{14}C]\)Etn and quantified CDP-Etn Kennedy pathway intermediates. As expected, we observed an increase in \([^{14}C]\)PEtn levels and a decrease in downstream PCYT2 products CDP-\([^{14}C]\)Etn and \([^{14}C]\)PE, although the
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The current study provides the molecular basis for inhibition of respiration by meclizine, an over-the-counter antinausea and antivertigo drug. Using a combination of mass spectrometry, metabolic labeling, and in vitro biochemical assays, we found that PCYT2 is a direct target of meclizine. Our study highlights the use of metabolic profiling in deciphering mechanisms of drug action with important biological and clinical implications.

To our knowledge, this study is the first to pharmacologically link the Kennedy pathway of PE biosynthesis to mitochondrial energy metabolism and is consistent with a previous report showing that mice heterozygous for the Pcyt2 gene have reduced energy production from fatty acid oxidation (25). PE is an essential phospholipid present in eukaryotic membranes and is highly enriched in mitochondrial membranes. It has been shown that a modest reduction in PE in mammalian mitochondria impairs oxidative phosphorylation, reducing respiration (24). Therefore, we asked whether short term (5-h) inhibition of the Kennedy pathway of PE synthesis by meclizine causes a decrease in cellular and mitochondrial PE levels and contributes to reduced respiration. As shown in Fig. 6, A and B, we did not observe a significant decrease in the cellular or mitochondrial PE levels of human fibroblasts treated with meclizine, ruling out a reduction in PE as a cause for decreased respiration in vivo.

Inhibition of Respiratory Growth by Meclizine Is Accentuated through Addition of Ethanolamine—We originally identified meclizine as an agent that inhibits the growth of human skin fibroblasts grown in galactose versus glucose. It has long been known that when human cells are grown in galactose they are highly reliant on mitochondrial respiration. In the current study, we showed that in the presence of meclizine the phospholipid biosynthetic intermediate PEtn accumulated and directly inhibited mitochondrial respiration. Our model predicts that exogenous Etn should have an additive or synergistic effect with meclizine in inhibiting respiratory growth. Consistent with our model, we found that a combination of meclizine treatment with Etn supplementation synergistically reduced cellular ATP levels (Fig. 7).

DISCUSSION

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overlapping functions with cardiolipin (26), a mitochondrion-specific phospholipid that is essential for optimal respiration (27). PE is synthesized by multiple biochemical pathways (21, 22), including the phosphatidylserine decarboxylase-catalyzed mitochondrial pathway and the cytosolic/endoplasmic reticulum CDP-Etn Kennedy pathway. The mitochondrial pathway contributes the bulk of mitochondrial PE that is retained in this organelle and contributes to mitochondrial function (28, 29). Recently, it has been shown that a decrease in mitochondrial PE by deletion of phosphatidylserine decarboxylase in the yeast Saccharomyces cerevisiae results in reduced respiration (30). A similar reduction in mitochondrial respiration has been observed in mammalian cells where mitochondrial phosphatidylserine decarboxylase is depleted (24). However, the non-mitochondrial CDP-Etn Kennedy pathway of PE synthesis has never been linked to a respiratory defect in either yeast or mammalian cells.

How does direct inhibition of PCYT2 lead to attenuation of mitochondrial respiration? Two possibilities exist. 1) The accumulation of upstream metabolites (PEtn) could interfere with mitochondrial respiration, or 2) depletion of downstream metabolites (PE) could alter mitochondrial membrane structure, thereby inhibiting respiration. We favor the first hypothesis, which is supported by our observation that the intracellular concentration of PEtn in meclizine-treated fibroblasts increased to a level sufficient to inhibit mitochondrial respiration (Figs. 1D and 5, A and B). Notably, our in vitro data on PEtn inhibition of mitochondrial respiration is consistent with a previous study that showed that both Etn and PEtn inhibit respiration in isolated mitochondria (23). The second mechanism seems less likely because the bulk of mitochondrial PE is synthesized in situ by the action of phosphatidylserine decarboxylase. Moreover, we did not observe any decrease in cellular or mitochondrial PE of the meclizine-treated fibroblasts, discounting the second possibility (Fig. 6). The synthetic interaction between meclizine and Etn in MCH58 cells further buttresses the first model because addition of Etn to meclizine-treated cells exacerbated, rather than alleviated, the cell viability as measured by ATP levels (Fig. 7).

According to our model, meclizine itself has no effect on mitochondria but rather blocks PCYT2, leading to the accumulation of PEtn, which is itself an endogenous inhibitor of respiration. Although multiple lines of evidence support our model, questions still remain. First, the genetic depletion of PCYT2 by RNAi did not completely phenocopy the effect of meclizine on mitochondrial respiration, although this could be due to
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Incomplete knockdown and the lack of sustained accumulation of PEtn (Fig. 4E). The inability of genetic silencing to fully phenocopy drug treatment is not uncommon (31) and in principle could be due to the presence of residual enzyme activity of PCYT2. Second, overexpression of PCYT2 did not confer resistance to the effect of meclizine on respiration (data not shown), which could be due to tight regulation of its intracellular levels. Given that meclizine is known to target multiple cellular proteins (6–8), we cannot exclude additional mechanisms that may underlie its impact on respiration.

Regardless, we have clearly shown that meclizine inhibits PCYT2 and causes an increase in cytosolic PEtn to a level sufficient to inhibit mitochondrial respiration. To our knowledge, such a mechanism of respiratory inhibition has never been described before. The mechanism of PEtn-mediated inhibition of respiration appears to be distinct from canonical inhibitors of respiration, including inhibitors of electron transport (rotenone and antimycin), uncouplers (carbonyl cyanide 3-chlorophenylhydrazone and dinitrophenol), and ATP synthesis (oligomycin). PEtn addition to mitochondria resulted in reduced oxygen consumption, diminished membrane potential, and a decrease in NADH levels (Fig. 5), whereas treatment with rotenone or antimycin would have increased NADH levels, carbonyl cyanide 3-chlorophenylhydrazone would have increased oxygen consumption, and oligomycin would have
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increased membrane potential. The inhibition does not appear to be substrate-specific as we observed inhibition of respiration using complex I- or complex II-linked substrates. The precise mechanism by which accumulation of cytosolic PEtn inhibits respiration is currently not known, but our bioenergetics measurements suggest a mechanism whereby PEtn may interfere with the generation of reducing equivalents that feed into the respiratory chain.

Importantly, our work identifies for the first time an inhibitor of the CDP-Etn Kennedy pathway with therapeutic potential. This pathway has been implicated in a range of human disorders, including cancer (32) and ischemia-reperfusion injury of brain and heart (33), and infectious disorders, including African sleeping sickness and malaria (34, 35). Our own previous work has shown that meclizine, through blunting of respiration, is cytoprotective against ischemic injury to the brain and the heart (4) as well as polyglutamine toxicity observed in Huntington disease (5). Currently, effective therapeutics are not available for these disorders, making meclizine an attractive drug for repurposing. An intriguing question is to what extent the anti-vertigo and antinausea effects of meclizine may be occurring through targeting of PCYT2. A recent pharmacokinetics study on human subjects given an oral dose of 25 mg showed a peak plasma concentration of 80 ng/ml (~0.2 μM) (36), which is almost 70-fold below the minimum concentration required for inhibition of respiration in cell lines we have tested (4); thus, currently approved doses are unlikely to be active on mitochondrial respiration. We anticipate that identification of PCYT2 as a direct molecular target of meclizine may help to guide its clinical development for new uses. It is notable that PEtn, a direct molecular target of meclizine may help to guide its pharmacodynamics.

Acknowledgments—We thank Michelle Yu for assistance with measurements of bioenergetics. We thank Eric Shoubridge and Marcy MacDonald for providing MCH58 and mouse striatal cell lines, respectively.

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