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Acidic pH is a Metabolic Switch for 2-Hydroxyglutarate Generation

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Running Title: *Acid & 2-hydroxyglutarate*

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ABSTRACT

2-hydroxyglutarate (2-HG) is an important epigenetic regulator, with potential roles in cancer and stem cell biology. The D (R) enantiomer (D-2-HG) is an *oncometabolite* generated from α-ketoglutarate (α-KG) by mutant isocitrate dehydrogenase (ICDH), while L (S) 2-HG is generated by lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) in response to hypoxia. Since acidic pH is a common feature of hypoxia, as well as tumor and stem cell microenvironments, we hypothesized that pH may also impact cellular 2-HG levels. Herein we report that cytosolic acidification under normoxia moderately elevated 2-HG in cells, and boosting endogenous substrate α-KG levels further stimulated this elevation. Studies with isolated LDH-1 and MDH-2 revealed that generation of 2-HG by both enzymes was stimulated several-fold at acidic pH, relative to normal physiologic pH. Furthermore, acidic pH was found to inhibit the activity of the mitochondrial L-2-HG removal enzyme L-2-HG dehydrogenase. We conclude that acidosis is an important and previously overlooked regulator of 2-HG levels, with implications for 2-HG signaling.

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INTRODUCTION

The field of cancer biology has long been rapt by the notion of a cancer-specific metabolic phenotype, perhaps most famously embodied in the "Warburg effect", wherein glycolytic metabolism predominates in cancer cells despite O_2 availability and largely intact mitochondrial respiratory function (1).

A prominent feature of the cancer metabolic phenotype (reviewed in (2)) is an elevated level of the small metabolic acid 2-hydroxyglutarate (2-HG), derived from the TCA cycle intermediate α-ketoglutarate (α-KG) (3). The D (R) enantiomer of 2-HG (D-2-HG) was shown to be generated by mutant forms of isocitrate dehydrogenase (ICDH) that are associated with a variety of cancers including aggressive gliomas (4). In addition, more recently the L (S) enantiomer (L-2-HG) was shown to be generated under hypoxic conditions by lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) (5;6). We have also reported elevated D/L-2-HG levels in the heart following ischemic preconditioning (7).

In addition to synthesis, 2-HG levels are regulated by a pair of dehydrogenases that convert 2-HG back to α-KG (i.e., L-2-HGDH and D-2-HGDH). Mutations in these enzymes manifest as the hydroxyglutaric acidurias, devastating inherited metabolic diseases with symptoms including epilepsy and cerebellar ataxia (8-11). However, the importance of these dehydrogenases in regulating 2-HG levels in other settings is not clear.

The downstream signaling roles of D-2-HG in cancer biology and of L-2-HG in hypoxia or stem cell biology, are thought to be mediated by epigenetic effects (12-14), owing to competitive inhibition of the α-KG-dependent dioxygenase superfamily of enzymes. This includes the JmjC domain-containing histone demethylases, the TET 5-methylcytosine hydroxylases, the EGLN prolyl-hydroxylases, and the AlkB homologous family of DNA demethylases (15-18). As such, 2-HG is a potentially important link between metabolism and epigenetic signaling.

A common feature of hypoxia, as well as the tumor and stem cell microenvironments, is metabolic acidosis. However, the role of pH in regulating 2-HG formation and disposal has not been considered. We tested the hypothesis that acidic pH is a regulator of 2-HG metabolism at the cellular, mitochondrial, and isolated enzyme levels. Our results indicate that acidic pH can independently drive elevated 2-HG levels, and we propose that pH regulation of 2-HG may have important implications for 2-HG signaling in hypoxia and other settings.

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EXPERIMENTAL PROCEDURES

See also supplemental methods. All reagents including lactic dehydrogenase (LDH, L3916) and malic dehydrogenase (MDH, M2634) were purchased from Sigma (St. Louis, MO) unless otherwise stated. All experiments were performed a minimum of 3 times. For data in Figures 2 and 3 using commercial enzymes, "N" refers to a single measurement, with multiple measurements under each condition completed across several days with multiple aliquots of material (i.e., technical replicates). As such, statistics were not calculated for these data. For data in Figure 1 each "N" represents a different biological sample preparation on a different day. Statistical significance between groups was calculated using one-way ANOVA with post-hoc Student's t-test.

Enzyme activities were assayed in 25 mM potassium phosphate buffer at 37°C, adjusted to specific pH values as indicated in the figures. Conversion of α-KG to L-2-HG (mediated by LDH or MDH) and was monitored as the oxidation of NADH (0.1 mM) spectrophotometrically at 340 nm. Where appropriate, pO_2 in the cuvet was adjusted by flowing humidified argon through the headspace of the reaction for 60 min., with hypoxia verified by the spectrum of hemoglobin in solution. L-2-HG formation was also confirmed by LC-MS/MS analysis of the cuvet reactions. Metabolites were analyzed using reverse phase chromatography with an ion-paring reagent in a Shimadzu HPLC coupled to a Thermo Quantum triple-quadrupole mass spectrometer (7). Data were analyzed using MzRock machine learning tool kit (http://code.google.com/p/mzrock/), which automates analysis of targeted metabolomics data based on chromatographic retention time, whole molecule mass, collision energy, and resulting fragment mass. Metabolite concentrations were determined from standard curves constructed using known concentrations of metabolites.

L-2-HG dehydrogenase activity was assayed (10) in mitochondria isolated from male C57BL6/J mouse hearts as previously described (19). Mitochondria were snap-frozen in liquid N_2 and stored at -80°C. Mitochondrial protein (0.3 mg/ml) was incubated in 20 mM HEPES buffer supplemented with 0.85 mM $MgCl_2$, 0.05 % (vol/vol) Triton X-100, and 1.5 mM iodonitrotetrazolium (ACROS organics). After 5 min., 500 µM L-2-HG was added, and the reaction monitored spectrophotometrically at 500 nm (ε=19300 cm^{-1} M^{-1}) for 60 min. At the end of each run, *de-novo* α-KG formation was confirmed by LC-MS/MS.

HEK-293 cells (ATCC, Manassas VA) were maintained in DMEM (Life Technologies, Grand Island NY) with 25 mM D-glucose, 4 mM L-glutamine, 0.1 mM sodium pyruvate, 10 % heat-inactivated FBS

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(Life Technologies) and 100 µg/ml penicillin-streptomycin (Life Technologies), in 5% CO_2 at 37°C. Cells were seeded at 10^6 cells per 75 cm^2 flask, 24 hr. prior to experiments.

Cells were incubated in bicarbonate-free DMEM with 4 mM L-glutamine, 0.1 mM pyruvate, 10 mM glucose, and 10 mM HEPES (adjusted to pH 7.4 or 6.5) at 37°C. Intracellular acidification (20) was achieved by supplementing pH 6.5 media for 15 min. with 30 mM NH_4Cl (ACROS organics) and 10 µM 5-(N-Ethyl-N-isopropyl) amiloride (EIPA), followed by re-incubation in pH 6.5 media with EIPA alone, for the duration of the experiment (20 hr). Where appropriate, the α-KGDH inhibitor ketomethylvalerate (KMV) was present at 20 mM. For cellular metabolite analysis (2-HG and α-KG levels) a bolus of 80 % (vol/vol) methanol at -80°C was added after incubations, and cells serially extracted with the same, followed by analysis by LC-MS/MS as for enzyme assays above. In a separate series of incubations, intracellular pH was monitored by fluorescence microscopy (Nikon Eclipse TE2000-S) using the ratiometric fluorescent pH indicator 2',7'-Bis-(2-Carboxyethyl)-5-6-carboxyfluorescein-acetoxymethyl ester (BCECF-AM, Invitrogen, Eugene OR) (21). This protocol brought intracellular pH to 6.8 within 2 hrs. (Figure 4C).

RESULTS & DISCUSSION

We investigated the role of pH as an independent variable in the regulation of 2-HG levels in HEK293 cells, using an ammonium plus 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) pH clamp system (20). This system was capable of depressing and maintaining intracellular pH to 6.8 within 2 hrs. (Figures 1A–C). Although this degree of acidification drove a 4-fold elevation in 2-HG[1] levels (Figure 1D, left two bars), this was somewhat lower than the >8-fold elevation reported in the same cell type in response to hypoxia (5;6).

Since 2-HG generation depends on the availability of substrate α-KG, which is elevated in hypoxia (6), we hypothesized that α-KG may be limiting in the acidic pH condition. As such, we found that inhibiting the TCA cycle enzyme α-KGDH with ketomethyvalerate (KMV) boosted cellular α-KG levels by ~5-fold regardless of pH (Figure 1E). Furthermore, while KMV alone drove a small increase in 2-HG levels at pH 7.4 (Figure 1D), the combination of KMV plus acidic pH yielded an overall 13-fold increase in 2-HG levels. Together these data indicate that reproducing the acidic pH and elevated α-KG

[1]Note – The methodology used herein was incapable of distinguishing between L and D 2-HG. However, since the D enantiomer is primarily found in cancers associated with ICDH mutations, while the L enantiomer is primarily made by LDH and MDH under hypoxia, we assume that the bulk of 2-HG measured herein was the L enantiomer.

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levels seen in hypoxia is sufficient to increase 2-HG in cells. Acidosis may therefore be an important variable that drives 2-HG elevation in hypoxia.

Operating under a reasonable assumption that the 2-HG observed in cells was the L-isomer (see footnote[1]), we next investigated the mechanism of acid-induced 2-HG elevation, by examining the effects of pH on the enzymes that generate and remove this metabolite – namely LDH, MDH, and 2-HGDH. The purity of isolated LDH (bovine heart, Sigma #L3916, primarily LDH-1) and MDH (porcine heart, Sigma #M2634, primarily MDH-2) were verified by gel electrophoresis (Figure S1A). A spectrophotometric assay for the generation of 2-HG from α-KG and NADH (Figure 2A inset) was performed across a range of pH values spanning 6.6–7.8 (i.e. encompassing the pH values studied in cells), and revealed that NADH consumption was approximately 2.5-fold faster at pH 6.8 than at pH 7.4 (Figure 2A). In agreement with the cell data (Figure 1D), the rate was also [α-KG] dependent, being faster at 10 mM than at 1 mM α-KG. The spectrophotometric data were confirmed by LC-MS/MS measurement of 2-HG generation (Figure 2B), with approximate molar equivalents of 2-HG produced across the pH range 6.6–7.8 (Figure S1B, r^2 0.97).

The native activity of LDH (i.e., conversion of pyruvate to lactate) was unaffected by pH (Figure 2C); unsurprising for an enzyme that typically drives metabolic acidosis. Fractionally, the 2-HG generating capacity of LDH was at least 4 orders of magnitude lower than its native activity (Figure 2A vs. 2C), indicating that acidic pH unmasks a latent 2-HG generating activity in LDH without impacting its native activity. This observation also suggests that the generation of 2-HG is not a quantitatively important metabolic sink for NADH.

As an control, the effect of hypoxia on the ability of isolated LDH to generate 2-HG at pH 7.4 was also studied. Hypoxia was verified by measuring the spectrum of oxy- vs. deoxy- hemoglobin in the cuvet (Figure S2A). In contrast to the situation seen in cells (5;6), no effect of pO_2 on 2-HG generation by isolated LDH was seen (Figures S2B and C). These data suggest that hypoxic stimulation of 2-HG generation by LDH is not a property of the isolated enzyme, but rather it requires the intact hypoxic cell environment, likely including acidosis.

Similar to LDH, the generation of 2-HG by MDH was also found to be higher at pH 6.8 than at pH 7.4 (Figure 2D and 2E). As for LDH, spectrophotometric and LC-MS/MS data for MDH correlated well (Figure S1C, r^2 0.98), with approximate molar equivalents of 2-HG produced across the pH range 6.6–7.8. Notably, unlike LDH (Figure 2C) the native activity of MDH was itself pH sensitive, being inhibited ~30% at pH 6.8 versus pH 7.4 (Figure 2F). At first glance, this result might suggest an acid-induced

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activity switch, away from native and toward 2-HG-generation. However, it should again be noted that the 2-HG generating activity of MDH was at least 3 orders of magnitude lower than native activity. Although 2-HG generation has been termed a "metabolic error" and the 2-HG dehydrogenases are referred to as metabolite repair enzymes (11), our data suggest that the 2-HG synthetic capacity of MDH unmasked by acidic pH is fractionally small, and does not represent a major diversion of carbon away from the TCA cycle.

Comparing LDH and MDH, at the pH range seen in cells (6.8 vs. 7.4), and at the same α-KG level (10 mM), the relative acid-induced stimulation of 2-HG generation was slightly larger for LDH (2-fold) vs. MDH (1.3-fold). In addition, the pH response of LDH appeared to initiate at slightly more neutral levels, whereas the MDH response was flatter until more acidic pH values were attained. These data, plus the >3-fold greater specific activity for 2-HG generation by LDH vs. MDH, suggest that LDH may be a quantitatively more important source of 2-HG in response to acid stimulus. Although the effect of pH on 2-HG generation by isocitrate dehydrogenase was not tested (owing to difficulties in obtaining a sufficiently pure enzyme preparation), native ICDH is known to preferentially carboxylate α-KG to citrate under hypoxic conditions (22), suggesting it is an unlikely source of 2-HG in hypoxia.

Finally, since cellular 2-HG levels are also regulated by specific dehydrogenases, we tested the effects of pH on mitochondrial L-2-HGDH activity, using both a formazan-linked colorimetric assay (10) and LC-MS/MS to quantify α-KG generation from the substrate L-2-HG. Both assays correlated well (Figure S1D, r^2 0.98), and showed that L-2-HGDH activity was inhibited by acidic pH (Figure 3). Together with the data in Figure 2 this result indicates that acidic pH both promotes the formation of L-2-HG and inhibits its removal, suggesting a coordinated metabolic response to elevate L-2-HG in response to acid. We conclude that pH is an important determinant of 2-HG levels, and that acidosis plays a key role in the L-2-HG elevation observed in hypoxia.

These findings may hold important therapeutic implications for the hydroxyglutaric acidurias. Notably lactic acidosis has been reported in some 2-HG aciduric patients (23), and it could be speculated acidotic episodes may trigger 2-HG aciduria. As such, careful management of pH to avoid acidosis, for example by treatment with dichloroacetate, (24) may be an overlooked therapeutic approach in these patients.

Downstream signaling by 2-HG is thought to be mediated by inhibition of α-KG-dependent dioxygenases, such as the EGLN prolyl-hydroxylases that regulate Hif (15). It is therefore notable that

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acidic pH alone can activate Hif-1α (25), and it is tempting to speculate this may be due to stimulation of 2-HG production via the mechanisms outlined herein.

Although acidic pH is often associated with the high glycolytic metabolism of cancer cells, the regulation of pH in cancer is complex, with external pH (pH_e) tending toward acidicty (26), while intracellular pH (pH_i) is often more alkaline (27). Furthermore, although exposure to acidic pH_e may also play a role in promoting "stemness" (28), there has been much recent controversy regarding the effects of acid on stem cell phenotype (29). Thus, while it could be speculated that an elevation in 2-HG levels can transmit a pH signal to the epigenetic machinery that underlies cancer or stem cell phenotype, such a signaling pathway remains to be elucidated at the intracellular level. Although beyond the scope of this study, genetic modulation of 2-HG levels by manipulating 2-HGDH enzymes may provide novel insights to the role of this metabolite in linking metabolism, pH, and downstream signaling events.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest with regard to the content of this paper. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

CONTRIBUTIONS

PSB, SMN, KWN, JM and DF conceived the study. SMN, JM and XS performed experiments. SMN prepared the figures. PSB wrote the manuscript, with input and editing by KWN, JM and DF.

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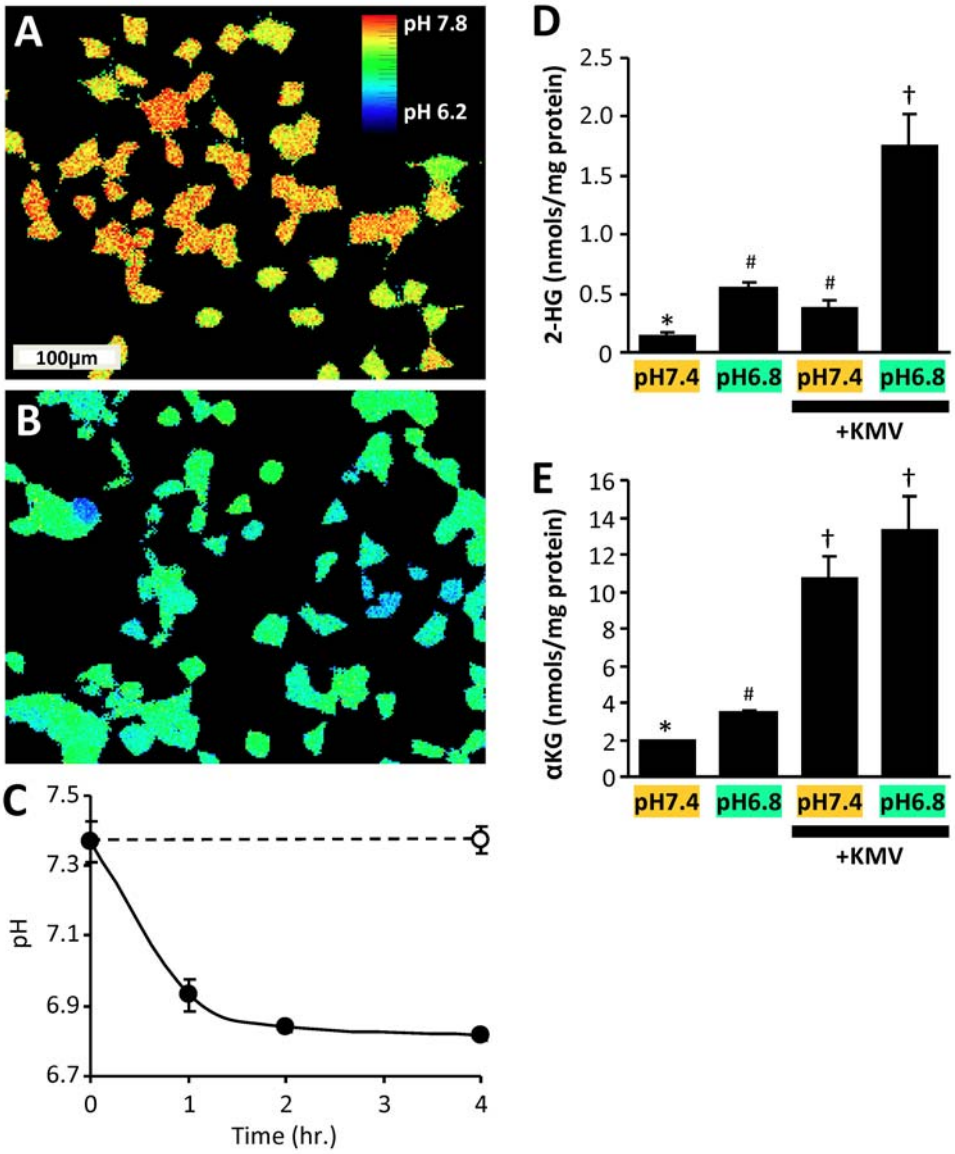

Figure 1. Acidic pH drives 2-HG elevation in cells. HEK293 cells were maintained at pH 7.4, or incubated under conditions to acidify the cytosol (see methods). **(A/B):** Pseudocolored 490nm/440nm dual excitation ratiometric images of BCECF fluorescence, showing intracellular pH for cells maintained at pH 7.4 (panel A), or pH 6.5 (panel B). **(C):** Quantitative time course of cellular acidification. Data are means ± SD, N=3. **(D):** Cellular 2-HG levels measured by LC-MS/MS following incubation at different cellular pH levels in the absence or presence of the α-KGDH inhibitor KMV. **(E):** Cellular α-KG levels measured by LC-MS/MS following incubation at different cellular pH levels in the absence or presence of KMV. Data are means ± SEM, N=6. Different symbols above bars (* # †) indicate statistically significant differences (two-way ANOVA and Student's t-test) between groups – i.e. bars with the same symbol are not different.

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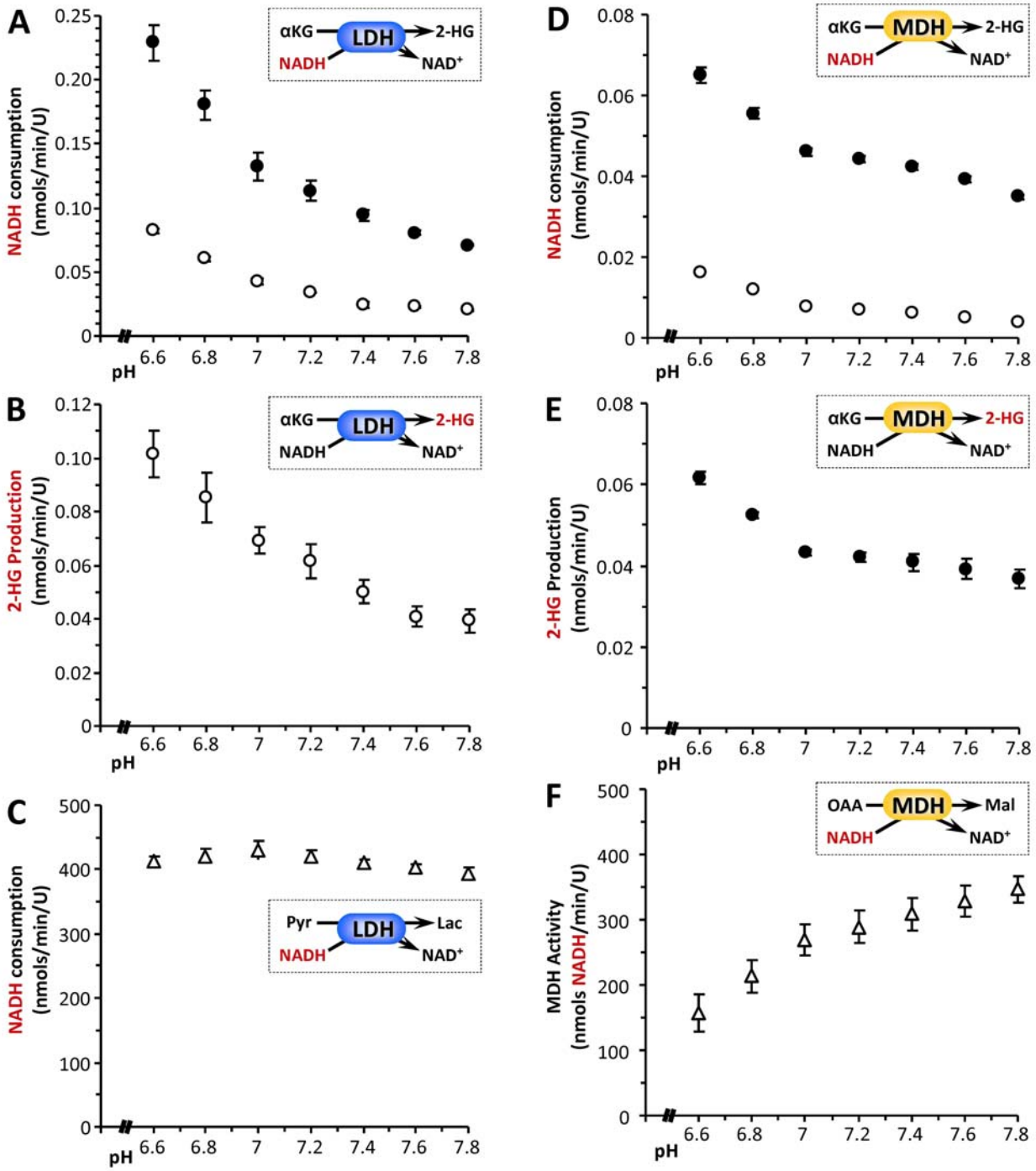

Figure 2. pH dependency of 2-HG generation by lactic or malic dehydrogenases. The 2-HG synthetic activity of isolated LDH was assayed spectrophotometrically as NADH consumption (panel A) or by direct LC-MS/MS assay of 2-HG formation (panel B). Insets to each panel show reaction schemes, with the measured parameter (y-axis of graph) highlighted in red. **(A):** NADH consumption at various pH values, for LDH in the presence of 1 mM (white circles) or 10 mM (black circles) α-KG. **(B):** 2-HG production at various pH values, for LDH in the presence of 1 mM α-KG. **(C):** Native pyruvate to lactate converting activity of LDH at various pH values, assayed spectrophotometrically as NADH consumption. Similar experiments were also performed with isolated MDH: **(D):** NADH consumption at various pH values, for MDH in the presence of 1 mM (white symbols) or 10 mM (black symbols) α-KG. **(E):** 2-HG

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production at various pH values, for MDH in the presence of 10 mM α-KG. (Note – with MDH, the higher concentration of α-KG was necessary due to the low sensitivity of LC-MS/MS based 2-HG assay). **(F):** Native MDH activity (oxaloacetate to malate) at various pH values, assayed spectrophotometrically as NADH consumption. (Note – MDH activity was measured in the reverse direction, i.e., oxaloacetate to malate, due to thermodynamic constraints as detailed in the methods). All data are means ± SEM, N>3.

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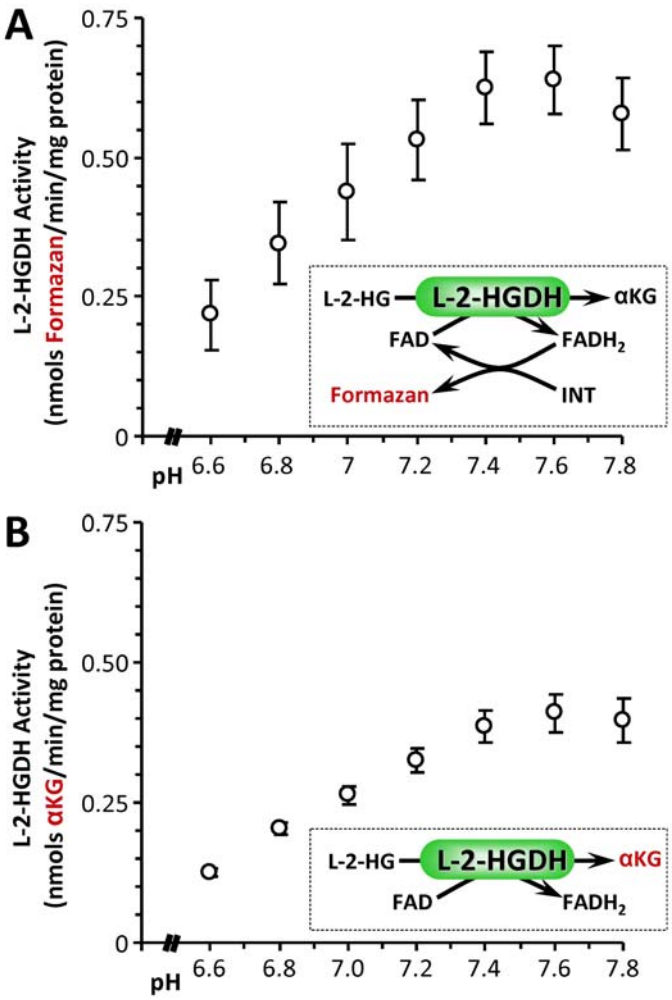

Figure 3. pH dependency of L-2-HG dehydrogenase (L-2-HGDH). The L-2-HGDH activity of isolated permeabilized mouse heart mitochondria was measured spectrophotometrically using either a iodonitrotetrazolium (INT) to formazan coupled assay (panel A) or by direct LC-MS/MS assay of α-KG formation (panel B). **(A):** Formazan production at various pH values, for mitochondria supplied with 500 µM L-2-HG. **(B):** α-KG production at various pH values, for mitochondria supplied with 500 µM L-2-HG. Data are means ± SEM, N=4.

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Supplemental Information for Nadtochiy *et al.*

"Acidic pH is a Metabolic Switch for 2-Hydroxyglutarate Generation"

DETAILED METHODS

All reagents including lactic dehydrogenase (LDH, L3916) and malic dehydrogenase (MDH, M2634) were purchased from Sigma (St. Louis, MO) unless otherwise stated. Male C57BL/6J mice were bred in-house from stocks obtained from Jax Labs (Bar Harbor, ME) and were maintained in a thermoneutral environment on a 12 hr. light/dark cycle with food and water available *ad libitum*. All experiments were in compliance with the NIH "Guide for the Care and Use of Laboratory Animals" and were approved by a local ethics committee (UCAR protocol #2007-087).

Enzyme activities were assayed in 25 mM potassium phosphate buffer at 37°C, adjusted to specific pH values as indicated in the figures. Conversion of α-KG to L-2-HG (mediated by LDH or MDH) and was monitored as the oxidation of NADH (0.1 mM) spectrophotometrically at 340 nm. L-2-HG formation was also confirmed by LC-MS/MS. Metabolites were analyzed using reverse phase chromatography with an ion-paring reagent in a Shimadzu HPLC coupled to a Thermo Quantum triple-quadrupole mass spectrometer (1). Data were analyzed using MzRock machine learning tool kit (http://code.google.com/p/mzrock/), which automates analysis of targeted metabolomics data based on chromatographic retention time, whole molecule mass, collision energy, and resulting fragment mass. Metabolite concentrations were determined from standard curves constructed using known concentrations of metabolites.

L-2-HG dehydrogenase activity was assayed (2) in mitochondria freshly isolated from mouse heart as previously described (3). Mitochondria were snap-frozen and stored at -80°C. Mitochondrial protein (0.3 mg/ml) was incubated in 20 mM HEPES buffer supplemented with 0.85 mM $MgCl_2$, 0.05 % (vol/vol) Triton X-100, and 1.5 mM iodonitrotetrazolium (INT, ACROS organics). After 5 min., 500 mM L-2-HG was added, and the reaction monitored spectrophotometrically at 500 nm (ε=19300 cm^{-1} M^{-1}) for 60 min. At the end of each run, *de-novo* α-KG formation was confirmed by LC-MS/MS.

HEK-293 cells (ATCC, Manassas VA) were maintained in DMEM (Life Technologies, Grand Island NY) supplemented with 25 mM D-glucose, 4 mM L-glutamine, 0.1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (Life Technologies) and 100 µg/ml penicillin-streptomycin (Life Technologies), in a 5 % CO_2 humidified 37°C incubator. For passaging, cells at 75-90% confluence were

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released by 3 min incubation with 0.25% trypsin-EDTA (Life Technologies, Grand Island NY), followed by collection in 5 volumes media and pelleting at 500 x g for 3 min. Cells were resuspended in 3 volumes media and enumerated in a hemocytometer after trypan blue staining. 10^6 cells were seeded in 75 cm^2 flasks 24 hrs. prior to experimental procedures.

For experimental protocols cells were incubated in bicarbonate free DMEM containing 4 mM L-glutamine, 0.1 mM pyruvate, 5 mM glucose, and 10 mM Hepes, at 37°C; media was adjusted either to pH 7.4 or pH 6.5. Intracellular acidification was performed similar to (4). Briefly, cells were incubated for 15 min. at pH 7.4 in media supplemented with 30 mM ammonium chloride (ACROS Organics) plus 10 µM of 5-(N-Ethyl-N-isopropyl) amiloride (EIPA). Cells were then washed and incubated in pH 6.5 media plus EIPA alone, which brought intracellular pH level down to 6.7-6.8 in ~2 hrs. (Figure 1 main manuscript). For measurements of intracellular pH, cells were loaded with 2 µg/ml of the ratiometric fluorescent indicator 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein-acetoxymethyl ester (BCECF-AM, Invitrogen, Eugene OR) and incubated for 30 min. at +37°C. Cells were then washed once and fluorescence microscopy was performed using an Eclipse TE2000-S (Nikon, Avon MA) and data were analyzed using TILL Photonics Imaging System Software. In separate sets of experiments, media was replaced with pH 6.5 buffer and cells were placed into an anoxic chamber (Coy, Grass Lake, MI) for 20 hrs. at 0% O_2.

For metabolite analysis, cell incubation media was replaced with 4 ml of ice-cold 80% methanol. Cells were then incubated for 10 min. at -80°C, followed by harvesting and centrifugation at 1000xg for 5 min. The supernatant was collected and the pellet further extracted with 2 x 0.5ml of 80% methanol. Combined supernatants (5 ml) were dried under N_2 then renovated in 200 µl of 50% methanol, centrifuged at 10,000 x *g*, and the supernatant analyzed by LC-MS/MS.

For hypoxia experiments on isolated enzymes, an “open flow” apparatus was used, in which a spectrophotometer cuvet was fitted with a lid and stirring apparatus, allowing purging of the headspace with gas mixtures defined by mass-flow controllers (5). Gas mixtures were humidified to prevent sample evaporation, and successful achievement of anoxia after 1 hr. of purging with argon was verified by the spectrum of oxy/deoxy-hemoglobin (Hb, 100 µM) in a matching, neighboring cuvet. Oxy-Hb was prepared by dithionite reduction followed by gel filtration and incubation under 100% O_2 and white light.

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SUPPLEMENTAL FIGURES

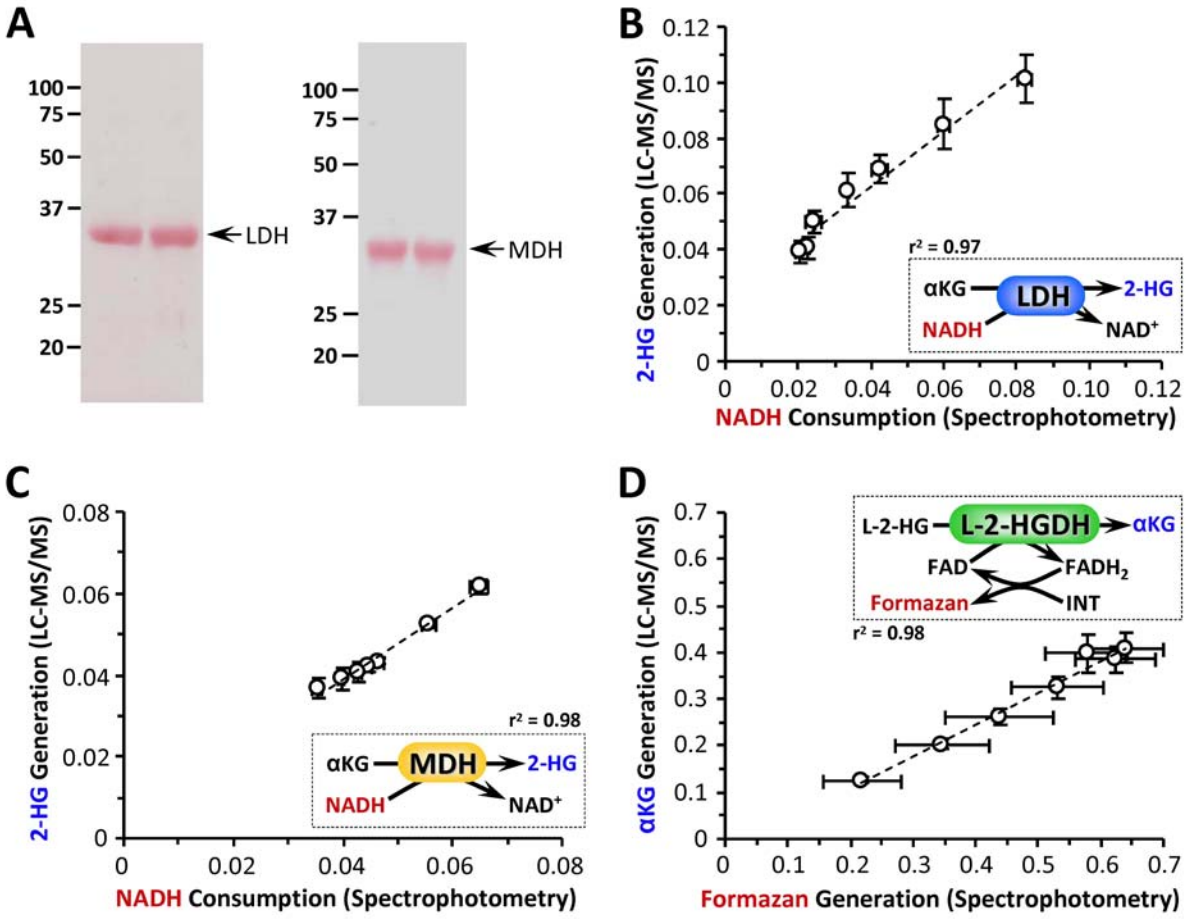

Figure S1. Enzyme purity and Assay Validation. (A): SDS-PAGE analysis of commercially obtained LDH (Sigma L3916) and MDH (Sigma M2634). Following electrophoresis on 12.5% SDS-PAGE, gels were transferred to nitrocellulose membranes and stained with Ponceau S. Numbers to the left of images show molecular weight markers, in kDa. Data are shown for two separate batches of material. Arrows to the right of images show expected position of band, for published molecular weight of the protein. **(B):** Comparison of NADH spectrophotometric assay vs. 2-HG LC-MS/MS assay, for the 2-HG generating activity of LDH (i.e., data from Figures 2A and 2B of main manuscript). Reaction is shown in the inset, with red/blue colored parameters matching appropriate axes on the graph. Data are means ± SEM. Dotted line shows linear regression curve fit with correlation coefficient shown alongside inset. **(C):** Comparison of NADH spectrophotometric assay vs. 2-HG LC-MS/MS assay, for the 2-HG generating activity of MDH (i.e., data from Figures 2D and 2E of main manuscript). Reaction is shown in the inset, with red/blue colored parameters matching appropriate axes on the graph. Data are means ± SEM. Dotted line shows linear regression curve fit with correlation coefficient shown alongside inset. **(D):** Comparison of Formazan spectrophotometric assay vs. α-KG LC-MS/MS assay, for the L-2-HG consuming activity of L-2-HGDH (i.e., data from Figures 3A and 3B of main manuscript). Reaction is shown in the inset, with red/blue colored parameters matching appropriate axes on the graph. Data are means ± SEM. Dotted line shows linear regression curve fit with correlation coefficient shown alongside inset.

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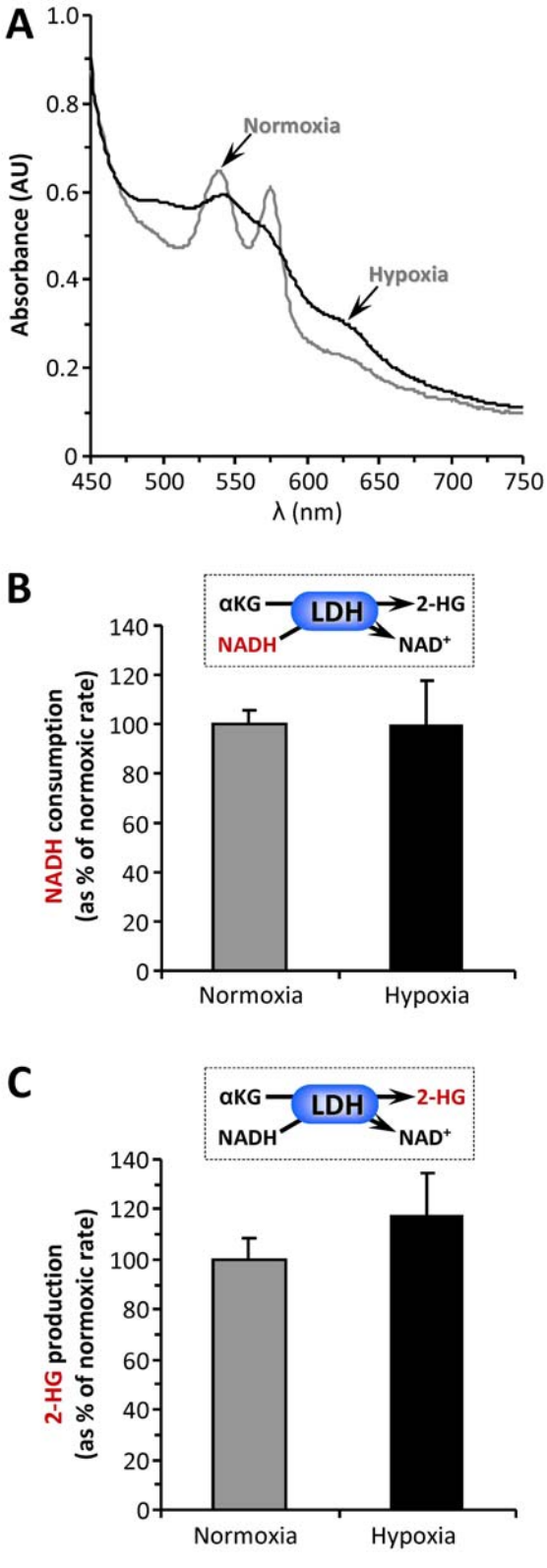

Figure S2. Effect of Hypoxia on 2-HG Generation by LDH. Isolated LDH was incubated in an open flow spectrophotometer cell, as detailed in the methods. A parallel incubation contained 100 µM hemoglobin, to confirm deoxygenation of the sample. **(A):** Absorbance spectra of purified Hb under normoxic (gray line) or hypoxic (black line) conditions, the latter comprising a 1 hr. of argon purge. **(B):** NADH consumption by LDH under normoxic or hypoxic conditions. Data are expressed normalized to the normoxia values, and are means ± SEM. **(C):** 2-HG generation by LDH under normoxic or hypoxic conditions. Samples were removed from the same incubations as panel B and rapidly deproteinized for LC-MS/MS analysis of 2-HG levels. Data are expressed normalized to the normoxia values, and are means ± SEM.