Acidosis & 2-Hydroxyglutarate Signaling by Mitochondria

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ABSTRACT

2-Hydroxyglutarate (2HG) is an epigenetic regulator, with potential roles in hypoxic/ischemic signaling. While D-2HG is an oncometabolite generated from α-ketoglutarate (αKG) by mutant isocitrate dehydrogenase (IDH), L-2HG is generated in hypoxic cells by lactate dehydrogenase (LDH) and malate dehydrogenase (MDH). Since acid pH is a common feature of hypoxia, we hypothesized pH may regulate cell 2HG levels. In HEK293T cells, cytosolic acidification under normoxia moderately elevated 2HG, and boosting substrate αKG levels further stimulated this. Studies on isolated LDH and MDH revealed 2HG generation by both enzymes was stimulated by acid. For isolated IDH, rather than generating 2HG, reductive carboxylation of αKG to isocitrate was stimulated by acid. Acid also inhibited the mitochondrial L-2HG removal enzyme L-2HG dehydrogenase (L-2HGDH). Using cells stably expressing (12x) L-2HGDH to disrupt 2HG signaling, we found 2HG is necessary for acid induced HIF activation. In addition we synthesized a cell-permeable L-2HG dimethyl ester to investigate potential 2 HG protective signaling, and found it to be protective in a perfused heart model of ischemia-reperfusion injury. These results lead to a prediction that pH alters substrate preference by α-ketoacid dehydrogenases, and in this regard we found that acid pH permits LDH to use oxaloacetate as a substrate to produce malate (i.e. to perform a reaction usually catalyzed by MDH). Finally, α-hydroxybutyrate is an important biomarker of mitochondrial dysfunction, and our data suggest that acid may play a role in driving α-HB generation from α-ketobutyrate. In summary, acidosis is an overlooked mediator of 2-Hydroxyacid signaling by mitochondria.

TL/DR: Acid pH drives 2-HG generation in normoxia. Dimethyl-2-HG protects against ischemia-reperfusion injury.

RESULTS

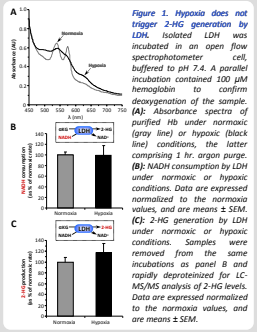

Figure 1. Hypoxia does not trigger 2-HG generation by LDH. Isolated LDH was incubated in an open flow spectrophotometer cell, buffered to pH 7.4. A parallel incubation contained 100 μM hemoglobin to confirm deoxygenation of the sample. **(A):** Absorbance spectra of purified Hb under normoxic (gray line) or hypoxic (black line) conditions, the latter comprising 1 hr. 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.

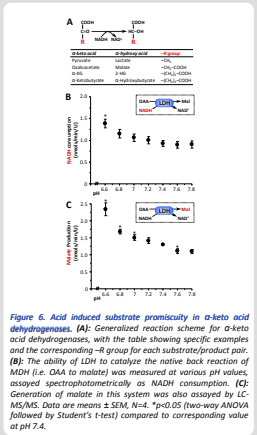

Figure 6. Acid induced substrate promiscuity in α-keto acid dehydrogenases. (A): Generalized reaction scheme for α-keto acid dehydrogenases, with the table showing specific examples and the corresponding –R group for each substrate/product pair. **(B):** The ability of LDH to catalyze the native back reaction of MDH (i.e. OAA to malate) was measured at various pH values, assayed spectrophotometrically as NADH consumption. **(C):** Generation of malate in this system was also assayed by LC-MS/MS. Data are means ± SEM, N=4. *p<0.05 (two-way ANOVA followed by Student's t-test) compared to corresponding value at pH 7.4.

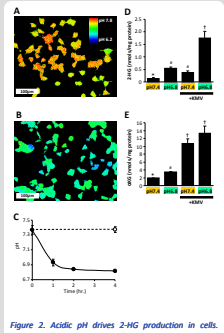

Figure 2. Acidic pH drives 2-HG production in cells. HEK293 cells were maintained at pH 7.4, or incubated under conditions to acidify the cytosol (ammonia plus amiloride analog EIPA). **(A/B):** Pseudocolored 490nm/440nm dual excitation ratiometric images of BCECF fluorescence, showing intracellular pH for cells at pH 7.4 (panel A), or pH 6.5 with EIPA (panel B). **(C):** 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, ± the α-KGDH inhibitor KMV. **(E):** Cellular α-KG levels measured by LC-MS/MS following incubation at different cellular pH levels, ± 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.

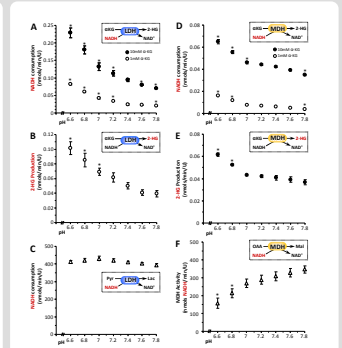

Figure 3. pH dependency of 2-HG generation by LDH or MDH. The 2-HG synthetic activity of isolated LDH or MDH was assayed spectrophotometrically as NADH consumption (panels A,D) or by direct LC-MS/MS assay of 2-HG formation (panels B,C). Insets to each panel show reaction schemes, with the measured parameter (y-axis of graph) highlighted in red. **(A):** NADH consumption at various pH values, for LDH with 1 mM (white circles) or 10 mM (black circles) α-KG. **(B):** 2-HG production at various pH values, for LDH with 1 mM α-KG. **(C):** Native LDH activity (pyruvate to lactate) at various pH values, assayed as NADH consumption. **(D):** NADH consumption at various pH values, for MDH with 1 mM (white symbols) or 10 mM (black symbols) α-KG. **(E):** 2-HG production at various pH values, for MDH with 10 mM α-KG. **(F):** Native MDH activity (oxaloacetate to malate) at various pH values, assayed as NADH consumption. (Note: MDH activity was measured in the reverse direction due to thermodynamic constraints). All data are means ± SEM, N>3. *p<0.05 (two-way ANOVA followed by Student's t-test) compared to corresponding value at pH 7.4.

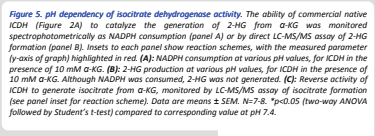

Figure 5. pH dependency of isocitrate dehydrogenase activity. The ability of commercial native ICDH (Figure 2A) to catalyze the generation of 2-HG from α-KG was monitored spectrophotometrically as NADPH consumption (panel A) or by direct LC-MS/MS assay of 2-HG formation (panel B). Insets to each panel show reaction schemes, with the measured parameter (y-axis of graph) highlighted in red. **(A):** NADPH consumption at various pH values, for ICDH in the presence of 10 mM α-KG. **(B):** 2-HG production at various pH values, for ICDH in the presence of 10 mM α-KG. Although NADPH was consumed, 2-HG was not generated. **(C):** Reverse activity of ICDH to generate isocitrate from α-KG, monitored by LC-MS/MS assay of isocitrate formation (see panel inset for reaction scheme). Data are means ± SEM. N=7-8. *p<0.05 (two-way ANOVA followed by Student's t-test) compared to corresponding value at pH 7.4.

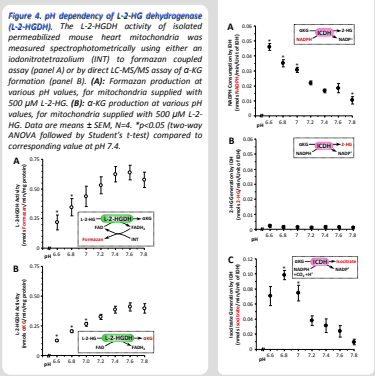

Figure 4. pH dependency of L-2-HG dehydrogenase (L-2-HGDH). The L-2-HGDH activity of isolated permeabilized mouse heart mitochondria was measured spectrophotometrically using either an iodonitrotetrazolium (INT) to formazan coupled assay (panel A) or by direct LC-MS/MS assay of α-KG formation (panel 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. *p<0.05 (two-way ANOVA followed by Student's t-test) compared to corresponding value at pH 7.4.

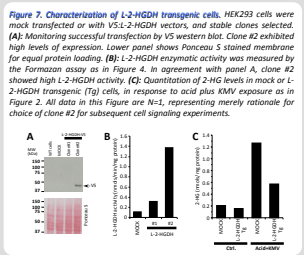

Figure 7. Characterization of L-2-HGDH transgenic cells. HEK293 cells were mock transfected or with V5-L-2-HGDH vectors, and stable clones selected. **(A):** Monitoring successful transfection by V5 western blot. Clone #2 exhibited high levels of expression. Lower panel shows Ponceau S stained membrane for equal protein loading. **(B):** L-2-HGDH enzymatic activity was measured by the Formazan assay as in Figure 4. In agreement with panel A, clone #2 showed high L-2-HGDH activity. **(C):** Quantitation of 2-HG levels in mock or L-2-HGDH transgenic (Tg) cells, in response to acid plus KMV exposure as in Figure 2. All data in this Figure are N=1, representing merely rationale for choice of clone #2 for subsequent cell signaling experiments.

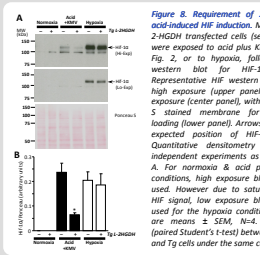

Figure 8. Requirement of 2-HG for acid-induced HIF induction. Mock & L-2-HGDH transfected cells (see Fig. 7) were exposed to acid plus KMV as in Fig. 2, or to hypoxia, followed by western blot for HIF-1α. **(A):** Representative HIF western blots at high exposure (upper panel) or low exposure (center panel), with Ponceau S stained membrane for protein loading (lower panel). Arrows indicate expected position of HIF-1α. **(B):** Quantitative densitometry from 4 independent experiments as in panel A. For normoxia & acid plus KMV conditions, high exposure blots were used. However due to saturation of HIF signal, low exposure blots were used for the hypoxia condition. Data are means ± SEM, N=4. *p<0.05 (paired Student's t-test) between mock and Tg cells under the same condition.

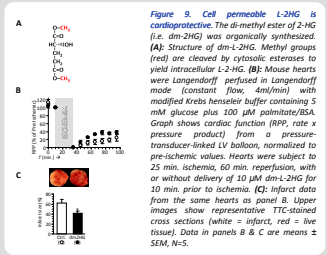

Figure 9. Cell permeable L-2HG is cardioprotective. The di-methyl ester of 2-HG (i.e. dm-2HG) was enzymatically synthesized. **(A):** Structure of dm-L-2HG. Methyl groups (red) are cleaved by cytosolic esterases to yield intracellular L-2-HG. **(B):** Mouse hearts were Langendorff perfused in Langendorff mode (constant flow, 4ml/min) with modified Krebs henseleit buffer containing 5 mM glucose plus 100 μM palmitate/BSA. Graph shows cardiac function (RPP, rate x pressure product) from a pressure-transducer-linked LV balloon, normalized to pre-ischemic values. Hearts were subject to 25 min. ischemia, 60 min. reperfusion, with or without delivery of 10 μM dm-L-2HG for 10 min. prior to ischemia. **(C):** Infarct data from the same hearts as panel B. Upper images show representative TTC-stained cross sections (white = infarct, red = live tissue). Data in panels B & C are means ± SEM, N=5.

DISCUSSION & CONCLUSIONS

Exposure of cells to hypoxia is known to stimulate L-2-HG generation by LDH [1,2]. However, **Figure 1** shows this is not the case for isolated LDH enzyme. Ergo, another change in hypoxic cells must be the trigger for 2-HG generation. We hypothesized this may be acidic pH (from lactic acid generated by anaerobic glycolysis). **Figure 2** shows that cytosolic acidification alone (by inhibiting H^+ export by NHE-1) is sufficient to stimulate 2-HG generation. Increasing the availability of substrate α-ketoglutarate (with the α-KGDH inhibitor KMV) further enhances acid-stimulated 2-HG generation. **Figure 3** shows that acidic pH stimulates 2-HG generation by purified LDH and MDH, with no effect on their native enzymatic reactions. **Figure 4** shows the activity of L-2-HG dehydrogenase [3] was inhibited by acidic pH. **Together these data suggest a coordinated metabolic response to elevate L-2-HG in response to acidosis.**

Another metabolic reaction stimulated by hypoxia is reversal of isocitrate dehydrogenase [4] (i.e., reductive carboxylation of α-KG to citrate), and **Figure 5** shows this reaction is also stimulated by acidic pH (not surprising since the reaction consumes a proton). Examining the common reaction mechanism of α-keto acid dehydrogenases (**Figure 6A**) leads to a hypothesis that acid pH may allow larger –R groups into the substrate binding pocket of LDH. An ensuing prediction, is that LDH should be able to catalyze the MDH reaction (OAA to malate) under acidic conditions. **Figure 6B/C** shows this is indeed the case. These findings, along with the well-known occurrence of 2-hydroxybutyrate as a biomarker of mitochondrial dysfunction [5,6] (commonly associated with metabolic acidosis) suggest that **α-keto to α-hydroxy acid conversion is a common metabolic signature of acidosis.**

2-HG is an epigenetic signal, and a known inhibitor of α-KG dependent dioxygenases [7,8] including: JmjC domain histone demethylases, TET 5-methylcytosine hydroxylases, AlkB homolog DNA/RNA demethylases, and EGLN prolyl-hydroxylases that regulate hypoxia inducible factor (HIF). It is also known that acid pH can activate HIF in normoxia [9]. To test the role of 2-HG in this signaling pathway, we generated a cell line stably over-expressing L-2HGDH (**Figure 7**), and these cells showed blunted 2-HG generation with acid+KMV. Furthermore, **Figure 8** shows that acid induction of HIF was abrogated in L-2HGDH over-expressing cells. **These data indicate a role for 2-HG in acid induction of HIF.**

We previously showed 2-HG is generated in the heart under the cardioprotective paradigm of ischemic preconditioning [10]. Thus we hypothesized 2-HG may play a protective role against ischemia-reperfusion (IR) injury. **Figure 9** shows that **a cell-permeable di-methyl-L-2HG ester conferred cardioprotection in a perfused mouse heart model of IR injury.** Signaling targets engaged by 2-HG to afford this protection are currently under investigation.

References: [1] Intlekofer (2015) *Cell Metab* 22:304-11. [2] Oldham (2015) *Cell Metab* 22:291-303. [3] Rzem (2004) *PNAS* 101:16849-54. [4] Wise (2011) *PNAS* 108:19611-6. [5] Butler (2013) *Aging Cell* 12:130-8. [6] Thompson (2015) *Cell Rep* 13:981-9. [7] Xu (2011) *Cancer Cell* 19:17-30. [8] Su (2016) *Curr Opin Chem Biol* 30:52-60. [9] Mekhail (2004) *Nat Cell Biol* 6:642-7. [10] Nadtochiy (2015) *J Mol Cell Cardiol* 88:64-72. **Funded by NIH R01 HL-071158**