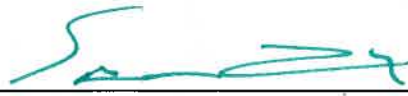


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Is HIF-1 $\alpha$  Required for V-ATPase-Dependent  
Regulation of Glycolytic Enzymes?

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## **Introduction:**

Vacuolar-ATPase (V-ATPase) is frequently up-regulated in tumors and tumor cell lines where it contributes to tumorigenic phenotypes, including the Warburg Effect (e.g. the phenomenon where glycolysis is preferred over oxidative phosphorylation (Vander Heiden et al. 2009)). The preferential use of glycolytic metabolism favored by tumor cells may be caused by at least two factors (Vander Heiden et al. 2009). First, the metabolic intermediates generated by the citric acid (TCA) cycle are used in a variety of anabolic pathways in the cell, and may supply a ready source of intermediate building-blocks for biosynthetic processes. Second, hypoxia observed in some tumors may limit the amount of oxygen for oxidative phosphorylation. V-ATPase up-regulation in tumors and tumor cell lines could possibly be occurring in order to address the increased concentration of  $H^+$  caused by the increase in glycolysis (Sennoune and Martinez 2012). Given the preference of tumor cells for glycolysis they are ideal for studying the effects of V-ATPase inhibition on glycolytic metabolism.

V-ATPase is an ATP-dependent proton pump that generates and sustains proton gradients throughout the endomembrane system (Forgac 2007). V-ATPase is composed of two domains:  $V_0$  and  $V_1$  (Forgac 2007). The  $V_0$  domain is embedded in the membranes of the endomembrane system, and is responsible for the transportation of  $H^+$  (Forgac 2007). The  $V_1$  domain is located in the cytoplasm and is responsible for the catalytic hydrolysis of ATP (Forgac 2007). Enzymes such as the regulator of ATPase of vacuoles and endosomes (RAVE) in yeast, phosphofructokinase (PFK-1), glycolytic enzymes, and glucose levels regulate V-ATPase activity by promoting assembly of  $V_0V_1$  (Parra et al. 2014).

Exposure to V-ATPase-specific inhibitors, Concanamycin A (CCA) or Bafilomycin A (Baf), increases expression of several glycolytic enzymes in breast tumor cell lines. Studies using the transcription-inhibitor Actinomycin D suggest that the induction of glycolytic genes observed when V-ATPase is inhibited is dependent upon increased transcription (Kane and Parra 2000). Hypoxia-inducible factor (HIF-1 $\alpha$ ) is a transcriptional inducer of many glycolytic genes and increases transport of glucose into cells (Lee et al. 2004). Preliminary results from this study suggested HIF-1 $\alpha$  is induced by V-ATPase inhibition and is sufficient to induce transcription of glycolytic genes. I postulate that the induction of HIF-1 $\alpha$  observed after V-ATPase inhibition will increase glucose uptake and induce several glycolytic enzymes, thus completing a circle of biochemical regulation whereby V-ATPase inhibits glycolysis.

## **Methods:**

**Propagation of Breast Tumor Cell Lines:** Cultured cancer cells (BT474, MCF-7, T47D) were exposed to V-ATPase inhibitors, Concanamycin A (CCA) and Bafilomycin A (Baf) at 5 and 10nM, or Actinomycin D (ActD) at 5 ug/ml which restricts all transcription. To test the importance of HIF-1 $\alpha$ , cells were treated with Dimethyloxalylglycine, N-(Methoxyoxoacetyl)-glycine methyl ester (DMOG) which stabilizes HIF-1 $\alpha$ .

**Endosome/Lysosome pH Measurement:** In order to test the effect of V-ATPase on endosome/lysosome pH levels, cultured cancer cells were stained with pyranine (HPTS) 24 hours prior to a 1 hour exposure to Baf or CCA. Cells were then collected using trypsin to separate them from the culture vessel. A Horiba Jobin Yvon Fluoromax-4 Spectrofluorometer was then used to excite the molecules at 458 nm and 405 nm and gather emissions at 515 nm. Emissions were measured against a standard curve created from measurements of cells prepared at controlled pHs (Figure 1).

**Quantitative PCR:** RNA levels of glycolytic enzymes were used to quantify the effects of given treatments. RNA was isolated using Roche's High Pure RNA Isolation Kit following the manufacturer's protocol and quantified using a NanoDrop. RNA was reverse transcribed into complementary DNA (cDNA) using the Ambion RetroScript kit. Quantitative PCR (QPCR) was performed in a Roche Light Cycle 480 II using Sybr Green dye and the  $\Delta/\Delta$  Ct method and compared to an internal control,  $\beta$ -Glucuronidase (GUSB). Data gathered from these processes are normalized and compiled into figures 2, 4, 5, and 7.

**Protein Immunoblots (Westerns):** Whole cell lysates were collected under the conditions described (Figures 3, 6, and 9) and total protein was quantitated using bicinchoninic acid (BCA) assay. Western blots were performed with 8% SDS electrophoresis gels, primary antibodies at a 1:1000 dilution in 5% milk- Tris-Buffered Saline and Tween 20 (TBST), and secondary antibodies at a 1:2000 dilution in 5% milk-TBST. These were used to evaluate HIF-1 $\alpha$ , LDHA, hexokinase 2, and PGK2 protein levels (done by Y. Licon-Munoz and Y. Ordonez-Suarez).

**Statistical Analysis:** Data were analyzed using student t-test and ANOVA in Microsoft's Excel, version 2010. Results deemed significant had a P-value less than 0.05.

### **Results:**

When V-ATPase was inhibited by both Baf and CCA in both BT474 and T47D cell lines, endosomal/lysosomal pH was found to increase significantly (Figure 1). This shows that V-ATPase is used by BT474 and T47D cells to pump protons out of the cytosol and into endosomes/lysosomes.

When V-ATPase was inhibited separately by Baf and CCA in cell lines BT474, T47D, and MCF-7, QPCR data showed significant increases in glycolytic enzyme mRNA throughout the glycolysis pathway (Figure 2). Although the amount of mRNA increase varied between enzyme and cell line, the data imply functioning V-ATPase has an inhibitory effect on glycolytic mRNA levels. The Western blots associated with V-ATPase inhibition through CCA also show an increase in Hexokinase 2, Phosphoglycerate kinase (PGK), and Lactate dehydrogenase A (LDHA) protein levels in

MCF-7 cells (Figure 3). Similar Western results were obtained in T47D cells (not shown).

The measured degradation rates of Hex2 and GLUT1 mRNA between ActD and ActD plus CCA test groups were compared. The rates were found to be close enough to suggest that a transcription factor is involved in the mechanism rather than a stabilizing molecule (Figure 4).

When V-ATPase is inhibited with Baf or CCA, HIF-1 $\alpha$  was shown to increase in cell lines BT474, T47D, and MCF-7 (Figure 5). Western blots of the same tests showed that the increased mRNA was also translated into proteins (Figure 6).

An increase of HIF-1 $\alpha$  alone was found to increase the mRNA of glycolytic enzymes GLUT1, Hex2, and LDHA (Figure 7). Western blots of the same tests showed that the increased mRNA was also translated into proteins (Figure 8).

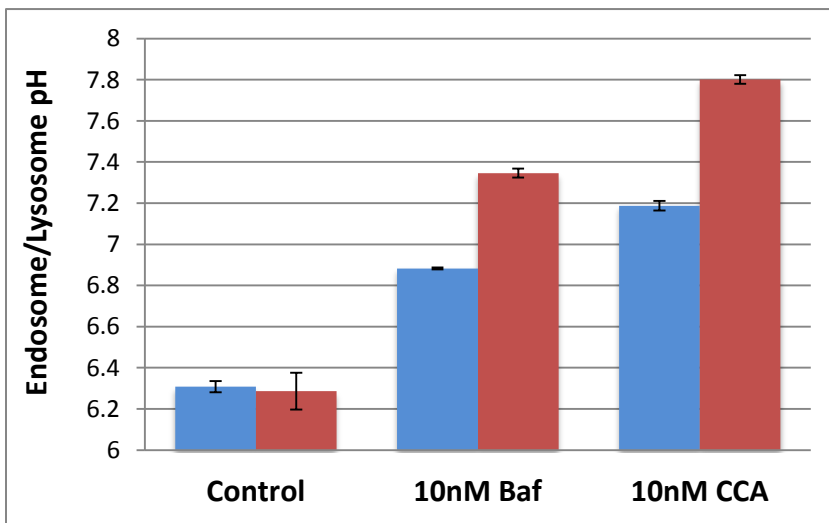


Figure 1: Endosomal/lysosomal pH measurement using HPTS in BT474 (blue) and T47D (red) cells. Bars represent the mean pH of lysosomes/endosomes in given cell lines after one hour of exposure to Baf or CCA. Error bars are standard deviations between triplicate samples.

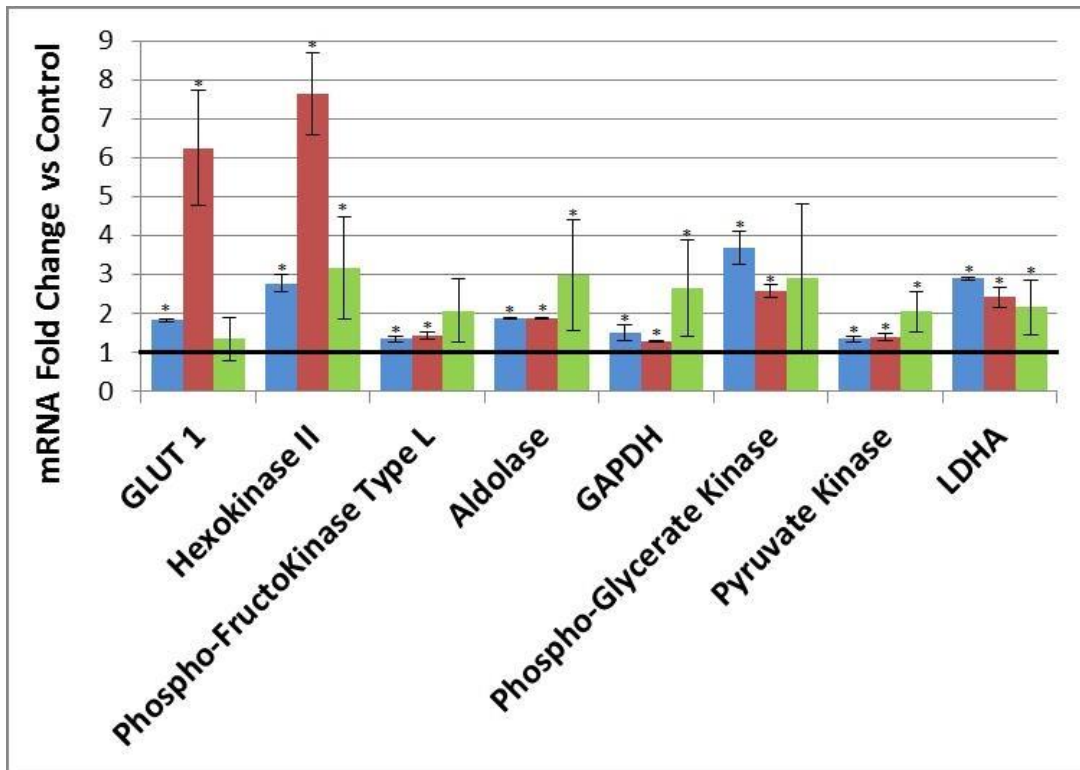


Figure 2: The glycolytic enzyme mRNA expression fold change after treatment with 10nM CCA for 24 hours. Bars represent the fold change from control at one. Blue, red, and green bars represent cell lines BT474, T47D, and MCF-7, respectively. Error bars are standard deviations from triplicate samples. Significant differences to the control are marked with “\*” symbol. The thick black line indicates the position of the control. Similar observations were found with 10nM Baf for 24 hours (data not shown).



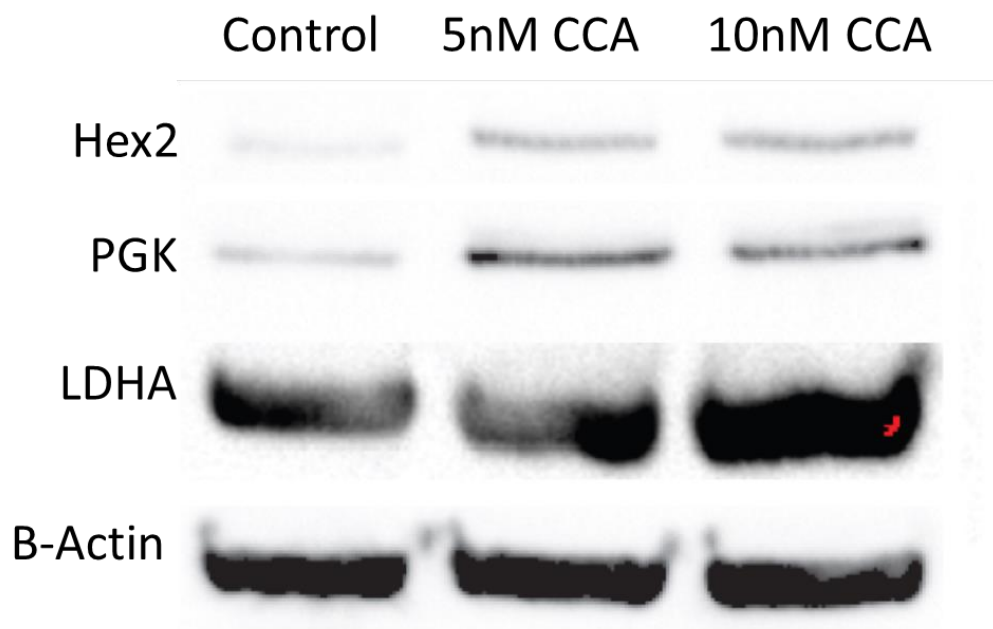


Figure 3: Glycolytic gene protein immunoblot of Hexokinase 2 (Hex2), PGK, (LDHA), and B-Actin after MCF-7 cells were treated with CCA. B-Actin was used as the control for measuring relative protein levels. Similar results were found in T47D cells (not shown).

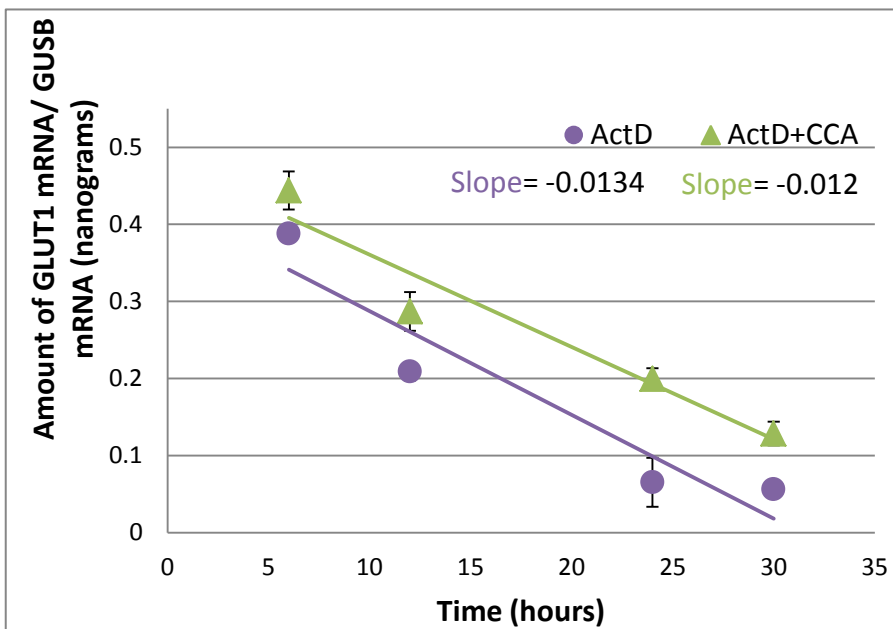
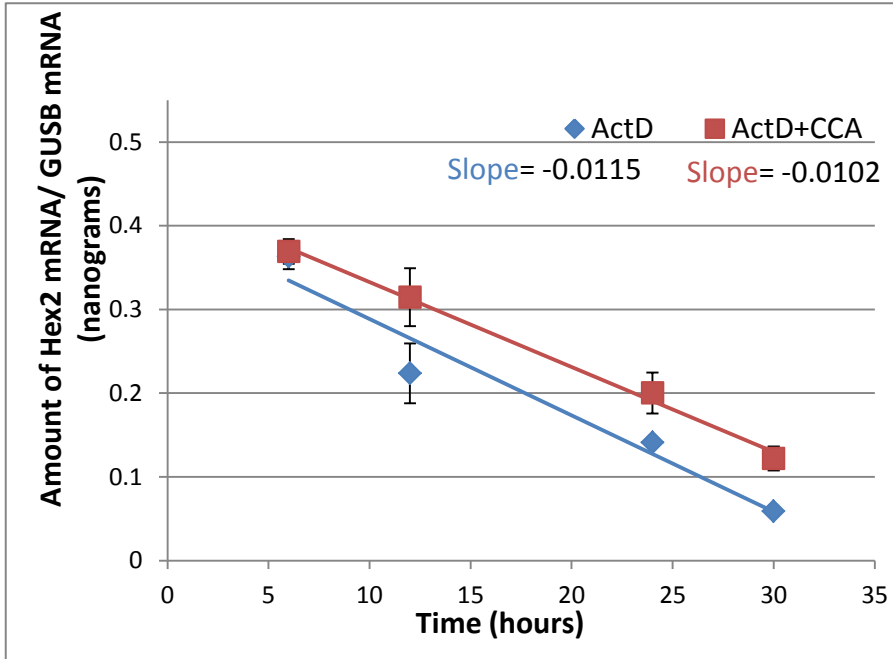


Figure 4: The rate of Hexokinase 2 (top) and GLUT1 (bottom) mRNA degradation in MCF-7 cells treated with Actinomycin D (ActD) or ActD and Concanamycin A (CCA).

Blue diamonds and purple circles represent cells treated with ActD (5ug/ml). Red squares and green triangles represent cells treated with ActD and CCA (10nM). Error bars are standard deviations between triplicate samples. Similar results were found in the cell line T47D (not shown).

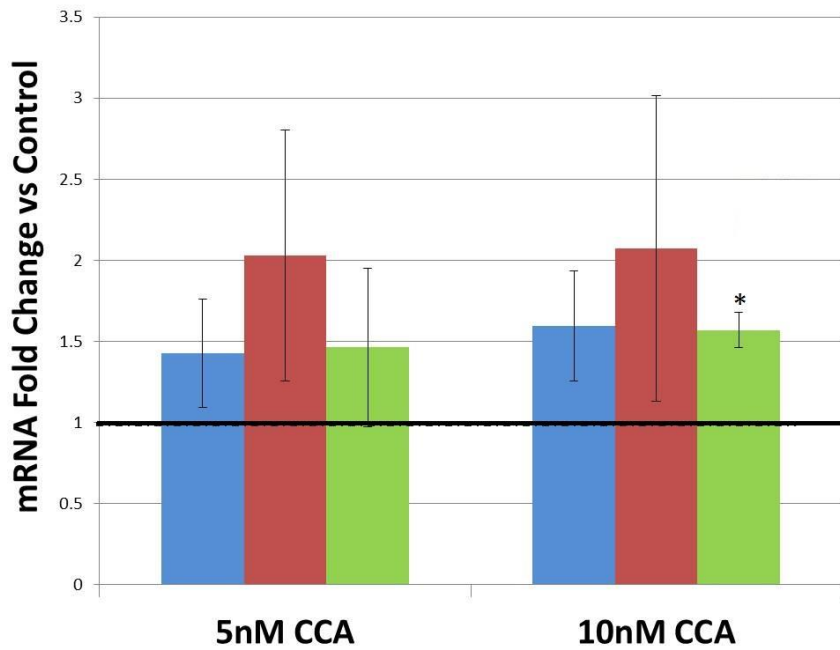


Figure 5. HIF-1 $\alpha$  mRNA fold change in BT474 (blue), T47D (green), and MCF-7 (red). Cells were treated for 24 hours. Bars represent an mRNA fold change from the control (thick black line). Error bars are standard deviations from triplicate analyses. Significant differences are marked with “\*” symbol. N=3

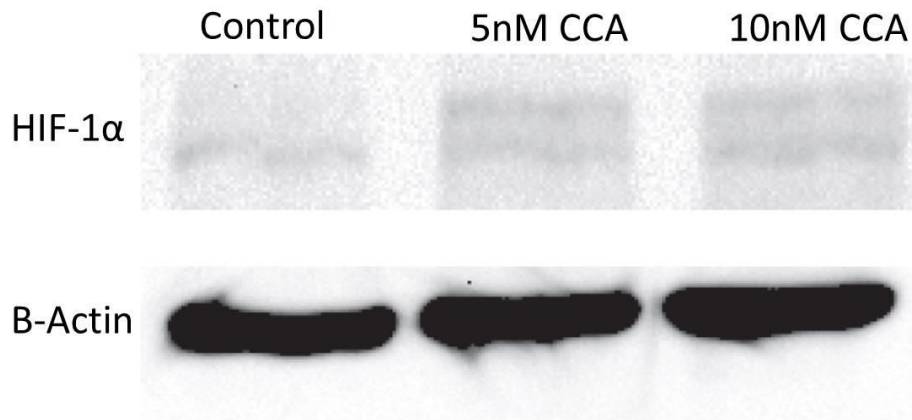


Figure 6: The increase of protein HIF-1α in MCF-7 cells after a 24 hour treatment in 5nM and 10nM CCA. Similar results were obtained in MCF-7 and shown in Figure 8.

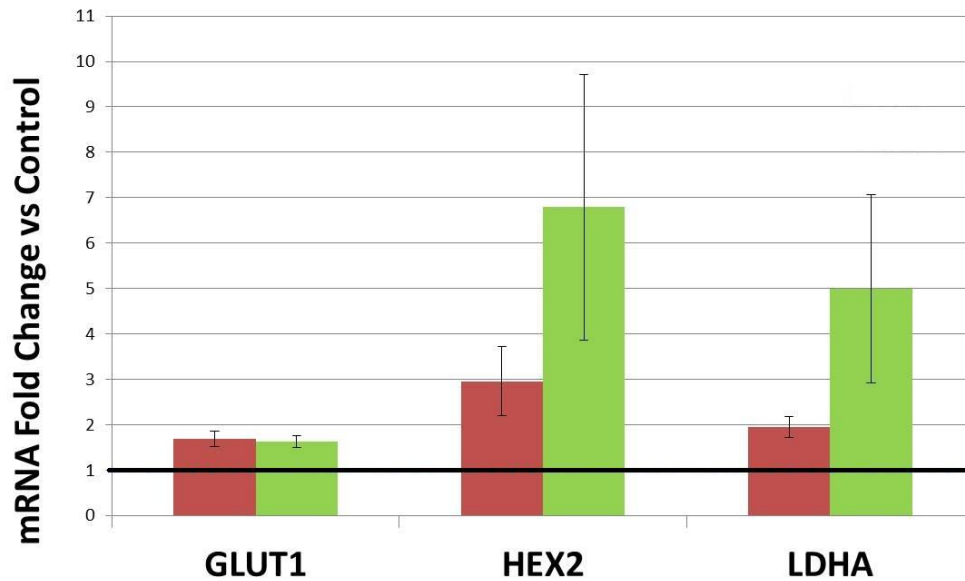


Figure 7: The bars represent fold change of glycolytic enzyme mRNA in MCF-7 (green) and T47D (red) cell lines after treatment with 1mM DMOG for 24 hours. Error bars are standard deviations between duplicate samples. The thick black line indicates the position of the control. N=2

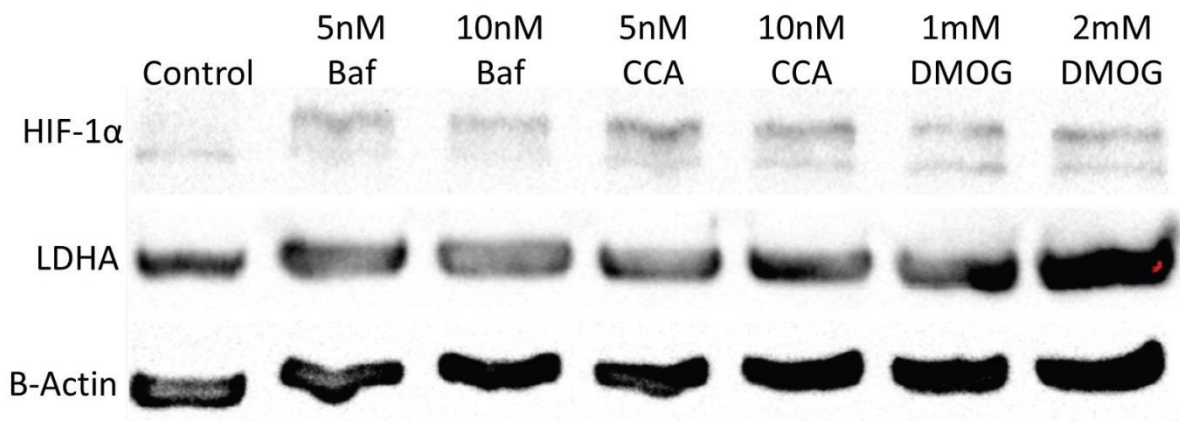


Figure 8: Western blot of protein HIF-1 $\alpha$ , LDHA, and Beta-Actin in MCF-7 cells after a 24 hour treatment of 5 and 10nM Baf and CCA and 1 and 2mM DMOG.

**Discussion:**

These results suggest that mRNA stability is unaltered by the CCA drug which increases glycolytic enzyme transcription. In turn, the presence of a transcription factor in the mechanism is apparent as the degradation of glycolytic mRNA is the same between the transcription inhibited experimental group and the additionally V-ATPase inhibited experimental group. If the increased glycolytic mRNA levels (Figure 2) were a result of mRNA stability, possibly induced in some way by V-ATPase activity, the experimental group with both transcription and V-ATPase inhibition would have a significantly faster degradation rate than the experimental group with just transcription inhibited (Figure 4). The transcription factor HIF-1 $\alpha$  is shown to be sufficient to induce V-ATPase dependent increase of glycolytic enzyme levels. Collectively the data suggest a feedback-loop between V-ATPase and glycolytic metabolism in tumor cells (Figure 9). Thus my hypothesis can be accepted. These results are meaningful in that they show a mechanism

by which current anti-HIF-1 $\alpha$  chemotherapeutics might work and also support the possible use of anti-V-ATPase drugs as a novel or adjuvant chemotherapeutic.

In experiments represented by Figures 1, 2, and 7 where repetition is lacking, variation in cell line or a synonymous drug is used as supplementation for more robust results. Experiment results showing that the same effects are gathered in various cell lines and with the use of separate drugs suggest a higher probability of the pathway existing in tumor cells within a cancer patient.

The results of the glycolytic enzyme protein immunoblot on cell line T47D are not shown. Although the bands showed qualitative increase of protein levels, the bands appeared like “smiley faces” and would need to be redone before being acceptable for including in a study paper. The electric current running the blot is thought to have been inconsistent, causing the gel to run strangely. The HIF-1 $\alpha$  enzyme protein immunoblot in figure 6 shows a doublet band for HIF-1 $\alpha$ . This is believed to be caused by post-translational modifications of HIF-1 $\alpha$  (Powell et al. 2002).

Other areas where data was gathered but was not shown include: 10nM Baf on glycolytic enzyme mRNA levels in figure 2 and glycolytic mRNA degradation over time in T47D cells. This is in order to keep the paper concise.

Future experiments in Dr. Parra’s lab will test if HIF-1 $\alpha$  is necessary for V-ATPase dependent increase of glycolytic gene transcription in tumor cells and evaluate the relationship between V-ATPase and glycolytic metabolism in normal breast cells. These studies may help identify the mechanism of anti-HIF-1 $\alpha$  chemotherapeutics and

justify V-ATPase inhibitors as novel or adjuvant chemotherapeutics. Repetition of previous experiments will also be performed to show significance.

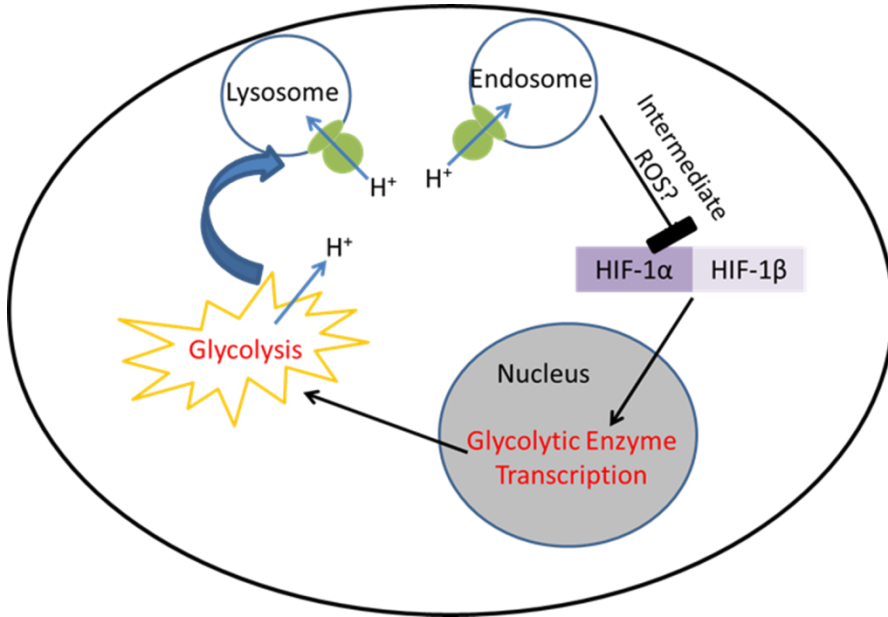


Figure 9: Glycolysis produces ATP and  $H^+$ , inducing V-ATPase assembly. When V-ATPase is inhibited, it is hypothesized intracellular pH change prevents HIF-1 $\alpha$  degradation, increasing HIF dependent glycolytic enzyme transcription. In turn this induces higher levels of glycolysis which would increase V-ATPase activity.

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