Effects of High Glucose on IGF-I–mediated Growth Signaling and Metabolic Responses in Renal Cell Carcinoma

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ABSTRACT Louisiana has the highest annual incidence of renal cancer for both men and women in the United States. Additionally, the state has the second highest mortality from type-2 diabetes in the nation, with a steadily increasing prevalence of 10.3% for the adult population as of 2010. Previous studies have shown that metabolic factors commonly seen in type 2 diabetic patients, including obesity, hypertension and hyperglycemia, are associated with an increased risk of renal cancer, and type 2 diabetes has been independently associated with an increased risk of renal cell cancer in women. However, studies disagree over the degree of excess risk involved, and the reason for increased risk of renal cell carcinoma (RCC) associated with type-2 diabetes is not well understood. This study aims to shed light on the relationship between these two diseases that both greatly impact the health of the Louisiana population.

Specifically, this study examines the extent to which elevated glucose affects growth, survival, signaling and metabolism of renal cancer, using the murine renal cancer model (Renca). Our results demonstrate that serum stimulated monolayer growth of the Renca cells is almost 2-fold higher when cells are maintained in the presence of high glucose (40mM) in comparison to normal glucose conditions (5mM). This increase in cell number was caused by a shift from G1 to S/G2M phase of the cell cycle, rather than by an increase in cell survival. In agreement with these growth-promoting effects of high glucose, Renca cells also demonstrated a metabolic shift from mitochondrial respiration to glycolysis and demonstrated slightly higher glycolytic capacity in the presence of high glucose.

To further investigate which growth-promoting pathway is affected by the high glucose in Renca cells, we first evaluated the Insulin-like growth factor 1 (IGF-I) signal transduction pathway. We have selected this pathway since previous reports indicate that kidney tumors are often characterized by elevated expression and activity of this growth promoting signaling system. Indeed, our immuno-histochemical labeling of clear cell carcinoma of the kidney demonstrates several fold higher immunoreactivity for insulin receptor substrate 1 (IRS-1) compared to the tumor-free area of the same kidney specimen. Interestingly, mitochondrial membrane potential (ΔΨm) measurements indicate that IGF-1 treatment improves mitochondria polarization of Renca cell, decreasing the known deleterious effects of elevated glucose on mitochondria function. Further *in vitro* analyses demonstrated that in serum starved Renca cells, IRS-1 is constitutively phosphorylated on tyrosine residues. Next, we evaluated the effects of high glucose concentrations on IGF-1 induced signaling responses. Interestingly, two major signaling branches, which originate from the activated IGF-1 receptor (IGF-IR) *i.e.* Akt and MAP kinases, Erk1/Erk2 were highly activated when stimulation with IGF-1 was administered in the presence of 40mM glucose in comparison to 5mM glucose.

IN CONCLUSION Our results indicate growth-promoting properties of high glucose in Renca cells, which may depend on hyper-activation of the IGF-IR signaling. Further experiments are required to verify if indeed a positive correlation exists between high-incidence of renal cell carcinoma and elevated blood glucose levels.

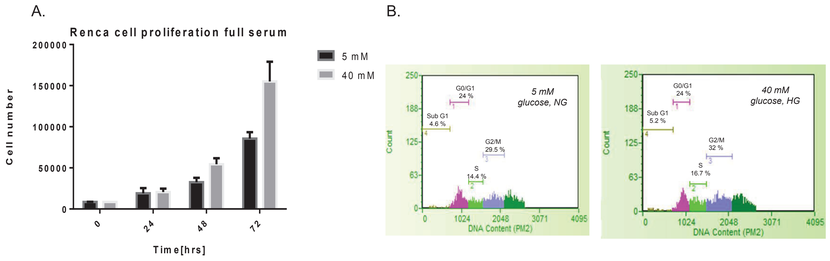

Figure 1. Effects of different glucose concentration on proliferation (A) and cell cycle distribution (B) of Renca cells. **(A):** Renca cells were cultured in full serum conditions for five days in the presence of normal (5mM, NG) or high glucose (40mM, HG) concentrations. Cells exposed to high glucose proliferated more rapidly than cells exposed to normal glucose. The greatest difference between cell populations was seen after 72 hours of growth. This data supports the hypothesis that high glucose exposure confers a growth-advantage to Renca cells. For this experiment 8,000 cells were plated per condition, and for each time point cells were harvested and counted using trypan blue exclusion test. ***(B):*** *Cell cycle distribution for Renca cells grown in serum and exposed to different glucose concentration (5mM, 40mM).* The growth advantage seen in cells exposed to high glucose (A) is partially explained by a shift in cell populations form G1 to S/G2M phases of the cell cycle, after 48 hrs exposure to high glucose. Briefly, aliquots of $2.5x10^5$ cells/ml were fixed in 70% ethanol; cells were centrifuged, labeled with Propidium iodide/RNaseA solution and evaluated by GUAVA easyCyte 8HT flow cytometer using CellCycle software included in guavaSoft 1.1.

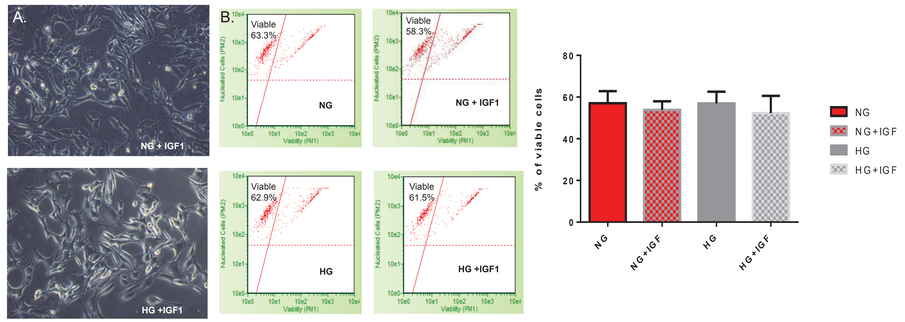

Figure 2. Effect of high glucose and IGF-1 on Renca cell morphology (A) and survival in suspension cultures (B). Contrast phase images show morphology of exponentially growing monolayer cultures of Renca cells cultured with two glucose concentration (5mM and 40 mM), and images were taken using camera equipped Olympus microscope (original magnification 10x). No morphological differences were observed between Renca cells exposed to normal (5mM) and high (40mM) glucose concentrations. (B) Cell viability of Renca cells growing in suspension on Poly-Hema dishes were evaluated by ViaCount assay based on membrane permeability (Millipore) adopted for the use with the GUAVA easyCyte 8HT flow cytometer (Millipore). The Guava/ViaCount software were used for data analysis and quantification according to the manufacturer recommendations (Millipore). We expected increase of survival in the presence of IGF1 and HG based on the growth advantage observed in Fig 1. However, the percent of viable cells was similar across all conditions, indicating that elevated glucose and IGF-1 does not promote increased survival. Histogram represent data from 3 repetitions of ViaCount assay.

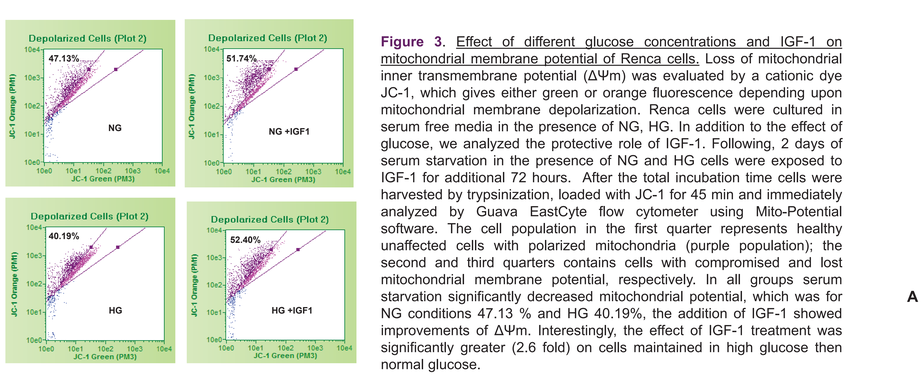

Figure 3. Effect of different glucose concentrations and IGF-1 on mitochondrial membrane potential of Renca cells. Loss of mitochondrial inner transmembrane potential (ΔΨm) was evaluated by a cationic dye JC-1, which gives either green or orange fluorescence depending upon mitochondrial membrane depolarization. Renca cells were cultured in serum free media in the presence of NG, HG. In addition to the effect of glucose, we analyzed the protective role of IGF-1. Following, 2 days of serum starvation in the presence of NG and HG cells were exposed to IGF-1 for additional 72 hours. After the total incubation time cells were harvested by trypsinization, loaded with JC-1 for 45 min and immediately analyzed by Guava EastCyte flow cytometer using Mito-Potential software. The cell population in the first quarter represents healthy unaffected cells with polarized mitochondria (purple population); the second and third quarters contains cells with compromised and lost mitochondrial membrane potential, respectively. In all groups serum starvation significantly decreased mitochondrial potential, which was for NG conditions 47.13 % and HG 40.19%, the addition of IGF-1 showed improvements of ΔΨm. Interestingly, the effect of IGF-1 treatment was significantly greater (2.6 fold) on cells maintained in high glucose then normal glucose.

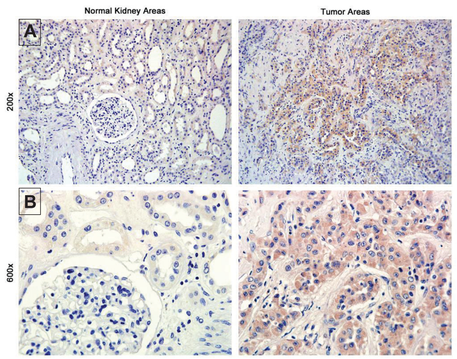

Figure 4. Immunohistochemical evaluation of insulin receptor substrate (IRS-1) accumulation in kidney carcinoma and normal kidney clinical samples. (**A**) Consecutive sections from a case of kidney carcinoma immunolabeled for insulin receptor substrate (IRS-1) demonstrate overall distribution pattern of this protein. In a non-affected area of normal kidney tissue we observed low levels of IRS-1. In contrast, the level of IRS-1 in tumor areas was much higher. **(B)** Higher magnification images from areas of normal kidney and tumor corroborate these findings. IRS-1 immunolabeling is significantly lower in the normal kidney area in comparison to the tumor area.
Since the IGF-1 signal transduction pathway has been shown to be upregulated in several kidney tumors, it is reasonable to speculate that it could be stimulated even more under high glucose conditions, leading to increased proliferation of these renal cancer cells.

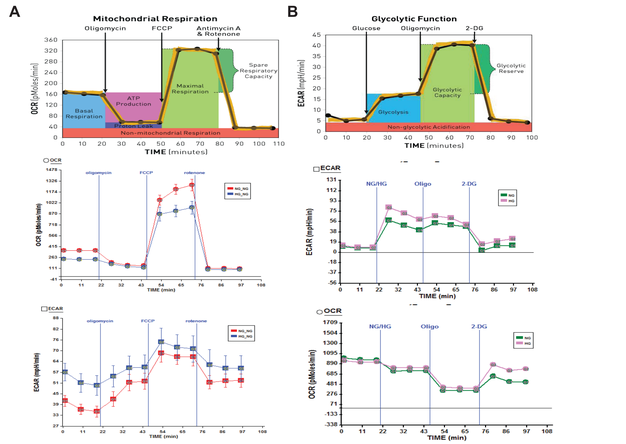

Figure 5. Effect of high glucose on metabolism of Renca cells. To evaluate metabolic effects of different glucose concentrations we employed XF24 Extracellular Flux Analyzer (*Seahorse Bioscience*) with integrated drug delivery system to evaluate basic metabolic requirements and metabolic responses characterizing Renca cells. **(A)** Mitochondrial respiration. We exposed Renca cells to 5mM and 40mM glucose to measure mitochondrial respiration as oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) as glycolysis. In normal glucose, Renca cells express higher level of basal respiration compared to high glucose and at the same time high glucose treatment significantly increased ECAR. In analyzed cells from both experimental groups oligomycin (inhibitor of ATP synthase) causes decrease in oxygen consumption rate (OCR); which at the same time results in elevated ECAR, to compensate for ATP deficit by increasing glycolysis. Subsequently, injection of FCCP (uncoupling factor) leads to the increase in OCR due to the uncoupling of the OxPhos from the ETC- **maximal respiration** and this value is lower for cells cultured in high glucose. Finally, injection of rotenone (inhibitor of mitochondrial complex I) causes OCR shut down by inhibition of complex I of ETC. **(B)** Glycolytic stress. We exposed glucose starved Renca cells to different glucose concentrations (5mM and 40 mM). Higher glucose concentration causes increase in the level of glycolysis, and subsequent injection of oligomycin showed a slightly higher glycolytic capacity compared to cells treated with normal glucose. At the end of the assay addition of the inhibitor of hexokinase, 2-deoxyglucose, leads to the shutdown of glycolysis. Based on these experiments Renca cells do not have additional glycolytic reserve above the basal high level of glycolysis.

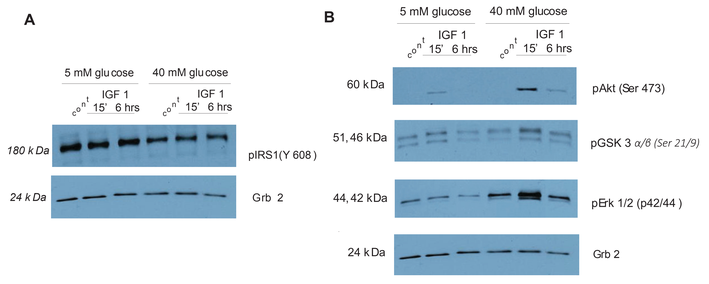

Figure 6. Effects of high glucose concentration on IGF-1signaling pathways in Renca cells. Western blot analyses showing levels of the phosphorylated downstream signaling molecules of IGF-IR: IRS-1 **(A);** Akt, GSK3 *α/β,* and Erk 1/2 **(B)** in Renca cells cultured in serum-free media for 48 hrs, and treated with IGF-1(50ng/ml) at indicated time points. The cells without treatment were used as controls (cont). **(A)** Renca cells are characterized by the presence of constitutively active IRS-1 (tyrosine phosphorylated at Y608) in serum-free media maintained at both glucose concentrations. **(B)** Higher levels of phosphorylated Akt, GSK3 *α/β,* and Erk 1/2, all downstream signaling molecules in the IGF-1 pathway, were detected in cells stimulated with IGF-1 in the presence of 40mM glucose compared to cells stimulated with IGF-1 in the presence of 5mM glucose. The highest level of activation for these proteins was observed after 15 min of IGF-1 stimulation. Levels of pIRS-1, pAkt, pGSK3 *α/β* and Erk 1/2 were normalized with the corresponding levels of Grb-2 (protein loading marker). These results are consistent with our hypothesis that hyper-activation of IGF-1 signaling may play a significant role in the growth advantage observed in Renca cells maintained in high glucose conditions.