Glucose metabolism: impact and significance on intracellular Ca^{2+} regulation

Introduction

Glucose not only serves as a universal energy source for living cells, but also – and maybe this is not trivial for a lot of us - influences many intracellular signaling pathways. These pathways are involved e.g. in nutrient sensing, diabetic complications, stress response, post-translational modifications. My goal was to get a better view on the impact of glucose and its metabolites on one of the key elements of these intracellular signaling pathways: the $[Ca^{2+}]_i$ regulation.

Increased level of glucose most of the time is associated with deleterious effects. However, there are situations where high glucose and consequently high metabolite levels can be beneficial. In all probability, the same pathways that are deleterious during a long term hyperglycemia also might cause the beneficial effect in an acute stress response. Glucose-Insulin-Potassium (GIK) infusions e.g. are well known by invasive care specialists in the therapy of acute myocardial ischemia. Apart from the obvious function as ATP source, the underlining mechanism of the beneficial effect of glucose involves signaling pathways, one of them is the attenuation of the stress induced Ca^{2+} elevation.

Out of the 4 major known (diabetic) pathomechanisms (polyol pathway, AGE – advanced glycation endproducts, Protein kinase C activation, and hexosamine pathway) my thesis focuses on the hexosamine biosynthesis pathway (HBP). 2-4% of the glucose enters the HBP through the glutamine-fructose-6P aminotransferase (GFAT) which catalyses the L-glutamine + D-fructose 6-phosphate = L-glutamate + D-glucosamine 6-phosphate reaction. Adding glucosamine directly to the cells bypasses the GFAT and enhances the flux through the HBP. The endproduct of the HBP is UDP-glcNAc (UDP-N-acetyl-glucosamine) which among other things serves as a source of protein-O-linked N-acetylglucosamine (also called: O-glcNAc or O-GlcNAcylation).

The addition of O-GlcNAc to nuclear and cytoplasmic proteins, which is catalyzed by O-GlcNAc-transferase (OGT), is a dynamic and abundant posttranslational modification that has increasingly been recognized as an important regulatory mechanism in signal transduction and that also may be especially important in mediating the cellular stress response. O-glcNAc links to the serine, threonine OH-groups which means that on many proteins O-glcNAc competes with phosphorylation. The number of identified proteins capable of posttranslational O-glycosylation is quickly growing, including a wide range of proteins, such as NF-κB, annexin, endothelial nitric oxide synthase, αB-crystallin, OGT, α-tubulin, c-myc, and heat shock protein. Increased levels of O-glycosylation have been implicated in a range of cellular processes, including the development of insulin resistance in muscle, hyperglycemia-induced apoptosis, and impaired excitation-contraction coupling. Recently, it also was shown that increased protein O-Glc-NAcylation occurs in a range of different cells in response to stress, suggesting that activation of this pathway may be a component of an endogenous cell survival pathway.

In order to test the effect of increased HBP flux on cellular stress and on it's central mediator: Ca^{2+} we applied AngII induced stress on isolated neonatal cardiomyocytes. AngII, through its receptor, couples to the Gq protein to stimulate PLC, generating two secondary messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The major target of DAG is PKC, whereas IP3 triggers an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) through IP3 receptors located in the sarcoplasmic reticulum. In most cell types, the IP3-generated $[Ca^{2+}]_i$ elevation is a consequence of an initial release from the endo-, sarcoplasmic reticulum followed by a subsequent influx of extracellular Ca^{2+} into the cytoplasm. This latter process is termed store-operated or capacitative Ca^{2+} entry (CCE). The intracellular targets of

$[Ca^{2+}]_i$ are numerous and include, e.g., CaM-regulated kinases, calcineurin, PKC, MAPK, cytosolic phospholipase A2, and proteases.

The goal of my study was to test the hypothesis that the impact of the HBP on the response of neonatal cardiomyocytes to the IP3-generating agonist AngII is mediated by an increase in protein O-GlcNAcylation that alters the regulation of $[Ca^{2+}]_i$. I found that glucosamine increased HBP flux in isolated neonatal cardiomyocytes, resulting in increased O-GlcNAc modification of proteins and attenuated AngII-induced $[Ca^{2+}]_i$ elevation and also attenuated the CCE. I also demonstrate that independent of the HBP, up- or down regulation of protein O-GlcNAcylation directly influenced $[Ca^{2+}]_i$. These data demonstrate for the first time a direct link between protein O-GlcNAcylation and cardiomyocyte Ca^{2+} homeostasis, which may represent a novel mechanism for the regulation of cardiomyocyte function under normal and stress conditions.

Phosphoglucomutase:

Phosphoglucomutase (PGM) catalyzes the reversible conversion of glucose-6-P and glucose-1-P, thus a key enzyme linking glucose and galactose metabolisms. It has been shown recently that the deletion of this enzyme causes significant changes in both the Ca^{2+} signaling and the Ca^{2+} storage in yeast. Therefore, PGM is a useful tool to understand how glucose metabolism influence$[Ca^{2+}]_i$.

There are multiple isoforms of PGM, deletion of all is lethal. In humans, beside the classical PGM activity (Glc-1-P – Glc-6-P conversion), another PGM activity can also be measured that recognizes both 5C and 6C sugars as substrates. Most probably PGM3 is responsible for this. PGM3 (also called: phosphoacetyl-glucosamine-mutase) has both classical PGM activity and also glcNAc-6P → glcNAc-1P activity, the later being part of the HBP. PGM3 has a high sequence homology with PGM1, and also needs Mg^{2+} to function.

It has been shown that Li^+ inhibits PGM. This can be explained by the competition of Mg^{2+} and Li^+. Since Li^+ is used to treat bipolar patients but it's underlying mechanism is unknown, investigating the effect of Li^+ can not only help to understand the role of glucose metabolism in Ca^{2+} regulation but also might help to understand the molecular mechanisms involved in Li^+ treatment in maniac disorders.

Aims

- to test whether the hexosamine pathway regulates $[Ca^{2+}]_i$ homeostasis

- if hexosamine pathway is involved in Ca^{2+} regulation, which participants of it is responsible?

- through which process does the HBP modify Ca^{2+} regulation? Is CCE involved?

- Can distinct proteins be identified that are both involved in Ca^{2+} regulation and influenced by HBP?

- Since PGM3 is part of the HBP and PGM3 is closely related to PGM, can the inhibition of Li^+ cause any change in HBP?

Materials and Methods

Preparation of neonatal rat ventricular myocytes (**NRVM**): Primary culture of NRVMs was obtained from 2-5 days old neonatal Sprague-Dawley rats and after isolation by collagenase digestion, the cell suspensions from each digestion step were combined and centrifuged. The pellets were resuspended in DMEM:M199 (4:1), supplemented with 15% FBS, penicillin (100 U/mL) streptomycin (100 μg/mL) and arabinose C (10 μM). Finally, NRVM were plated densely on 6-well plates (2*106 cells/well) or on 4 chambered coverslips (0.3*106 cells/chamber)

Jurkat cells: the cells were kept in glucose free RPMI-1640 supplemented with either glucose or galactose and with or w/o Li^{+}.

Ca^{2+} imaging: Spontaneously beating NRVMs washed in HBSS and loaded with 3 μM Fluo3-AM (Molecular Probes) for 45 min at 37°C. After loading with Fluo-3, NRVMs were washed 3 times with dye-free HBSS and finally the buffer was replaced with fresh HBSS containing 1.2 mM $CaCl_2$ and 1.0 mM $MgSO_4$. Image acquisition was carried out at 37 °C on an Olympus IX70 inverted microscope.

Immunoblotting with CTD110.6: NRVMs or Jurkats were washed in ice-cold PBS, scraped and harvested in a modified RIPA buffer. Proteins were separated on 7.5% SDS-PAGE (27) and transferred to PVDF membrane. Blots were probed with CTD110.6, a monoclonal mouse IgM antibody that is highly specific for O-glycosylated proteins with no cross reactivity to similar carbohydrate antigens, in casein blocking buffer and followed with HRP conjugated mouse anti-IgM antibody. For developing, Pico chemiluminescent substrate was used.

Immunofluorescence microscopy: NRVMs were plated on coverslips as indicated above and the cells were fixed in 3% formaldehyde/PBS, permeabilized with 0.5% Triton X-100/PBS for 2 min. The cells were rinsed in PBS and blocked in 5% bovine serum albumin (BSA)/PBS for 5 min. and then incubated with the CTD110.6. After rinsing in PBS, the coverslips were incubated with the secondary antibody Alexa-Fluor 594-conjugated goat anti-mouse IgM. Image acquisition was performed with an Olympus IX70 inverted microscope.

HPLC: The cells were precipitated with ice-cold 0.3 M perchloric acid (PCA). PCA was extracted from the supernatant with 2 volumes of 1:4 trioctylamine:freon mixture. Samples were loaded on an anion exchange HPLC column (Partisil 10 SAX, Beckman) and nucleotide sugars were detected at 262 nm using 2 mL/min flow rate and linear salt and pH gradient from 5 to 750 mM $(NH_4)H_2PO_4$ and from pH 2.8 to 3.7, respectively.

Results and discussion

We supposed that the HBP influences the response of cells to stress, therefore we investigated the role of HBP in $[Ca^{2+}]_i$ regulation focusing on the end-product of HBP; the protein O-glcNAc. We used the agonist AngII to induce stress. AngII has positive inotropic effect, and causes hypertrophy and apoptosis. One of the most important acute effects of AngII is to increase the basal Ca^{2+} levels in cardiomyocytes.

The effect of glucosamine on AngII induced stress: Glucosamine bypasses GFAT by entering directly to HBP, increasing the levels of UDP-glcNAc and consequently protein O-glcNAc. To prove that, we measured in NRVMs the UDP-glcNAc levels by HPLC, and the O-glcNAc levels by Western blot and immunofluorescence. As it turned out, a 5-10 exposure to glucosamine was enough to increase both UDP-glcNAc and O-glcNAc significantly (Fig. 2).

Labeling NRVMs in vivo with Ca^{2+} sensitive fluorescence dye the rapid changes in Ca^{2+} caused by AngII can be observed using a fluorescence microscope. We found that AngII increased the rate of the beating, on the other hand significantly increased the basal Ca^{2+} levels (Fig 1). With glucosamine pretreatment, this increase in basal Ca^{2+} levels was inhibited.

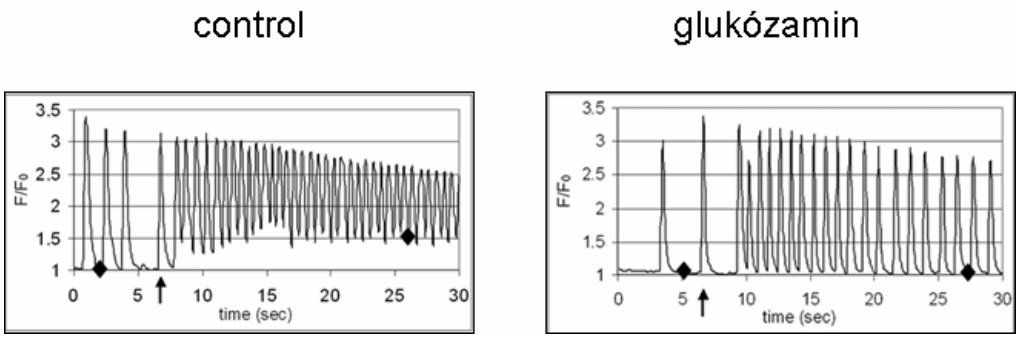

Fig. 1.: pretreatment with glucosamine for 5 min abolishes the effect of AngII (arrows) on NRVMs.

Since it was shown earlier that NRVMs exhibit CCE, we investigated whether glucosamine influences CCE. Treating NRVMs with thapsigargin (CCE-inducer drug) caused increased diastolic Ca^{2+} concentration, similarly to AngII. Pretreatment with glucosamine also blocked this thapsigargin induced increase.

The effect of O-glcNAc on AngII induced stress: Based on this result, HBP seems to be involved in Ca^{2+} regulation but the question remains which part of it is responsible? We found that glucosamine not only influenced $[Ca^{2+}]_i$ but also caused an increase in O-glcNAc. A large number of proteins were sensitive to glucosamine (Fig. 2).

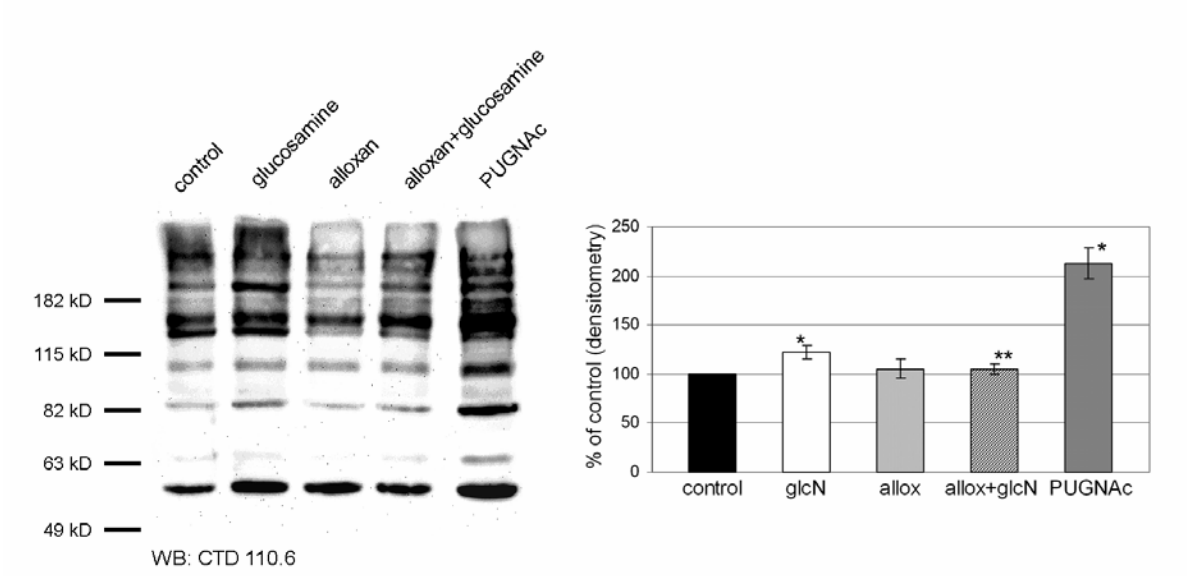

Fig. 2: left: CTD 110.6 western-blot, right: densitometry, statistical analysis of 3 independent experiments.

To prove that O-glcNAc directly influences $[Ca^{2+}]_i$ we used specific inhibitors: *PUGNAc* (O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenyl-carbamate) inhibits O-glcNAcase which removes N-acetyl-glucosamine from the Ser/Thr side chains of proteins and *alloxan* which inhibits OGT. Since only a part of UDP-glcNAc is used up for O-glcNAc, we haven't seen any significant changes in UDP-glcNAc levels. On the other hand, PUGNAc increased the O-glcNAc levels while alloxan prevented glucosamine to increase it. Therefore any changes seen in Ca^{2+} regulation has to be caused by O-glcNAc and not the metabolites of HBP. Pretreatment with alloxan didn't change the AngII caused Ca^{2+} increase, however it prevented the inhibitory effect of glucosamine. In contrast, PUGNAc mimicked the effect of glucosamine, abolishing the AngII induced Ca^{2+} elevation. To further specify the effect of O-glcNAc on $[Ca^{2+}]_i$, we used thapsigargin to test O-glcNAc on CCE. It resulted in the same outcome, PUGNAc prevented the CCE, whereas alloxan blocked the inhibitory effect of glucosamine.

The targets of O-glcNAc are numerous: transcription factors, cytoskeletal proteins, membrane proteins, kinases, etc. Supposedly the effect of O-glcNAc is not the result of the modification of one single protein but many others. However, the Trp (transient receptor potential) channel protein family, that is the prime candidate for the long sought CCE channel proteins and thus may be involved in the O-GlcNAc-mediated effects on Ca^{2+} homeostasis. Analysis of the protein sequence for Trp1, suggests a high affinity site for O-GlcNAc, close to the N-terminal region.

The effect of Li^+ on PGM and O-glcNAc: According to earlier result, PGM activity is closely related to Ca^{2+} homeostasis. Li^+ was found to effectively inhibit the activity of PGM which opens up the possibility to further investigate the link between glucose metabolism and Ca^{2+}. We propose that the missing link would be HBP and O-glcNAc. PGM3, part of the HBP pathway is a very similar protein to PGM therefore it could be either target for Li^+, or partly overtake the function of PGM in case of an inhibition. Interestingly, treating Jurkat cells with Li^+ caused an increase in O-glcNAc. This could be related to the assumption that blocking PGM (and possibly PGM3) caused a compensatory increased PGM3 expression which in turn elevated O-glcNAc. Our plan is to further investigate this phenomenon which eventually can lead also to better understand the underlying mechanism of Li^+ treatment in bipolar patients.

Publications:

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