Effects of glucose and its metabolites on calcium-induced mitochondrial permeability transition

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Introduction

Diabetes mellitus represents one of the leading causes of morbidity and mortality worldwide. Mitochondrial production of reactive oxygen species (ROS) has recently emerged as a critical factor in the pathogenesis of long-term diabetic complications. According to the unifying mechanism of these complications proposed by M. Brownlee [1], hyperglycemia-induced overproduction of ROS in mitochondria leads to inhibition of glyceraldehyde 3-phosphate dehydrogenase, and subsequent accumulation of upstream glycolytic intermediates activates all the known major pathways of hyperglycemic tissue damage, namely, the polyol pathway, protein kinase C activation, hexosamine pathway and production of the advanced glycation end products (AGEs). (Fig. 1)

In this scenario, however, there is no obvious reason why increased intracellular glucose oxidation *per se* should lead to overproduction of mitochondrial ROS. We consider possible involvement of the mitochondrial permeability transition (MPT).

MPT phenomenon consists of rearrangement of certain inner mitochondrial membrane proteins, creating a 'megachannel' that allows passage of all low-molecular-weight solutes and collapses the proton-motive force. The MPT pore opening is triggered by calcium, and modulated by numerous other agents: e.g. it is potentiated by mitochondrial depolarization, phosphate, and ROS, but inhibited by low matrix pH, adenine nucleotides, and cyclosporine A. Transient 'flickering' of the MPT pore is likely to play a role in cellular calcium homeostasis and calcium signaling, while a long-lasting MPT causing mitochondrial swelling, outer membrane rupture and release of cytochrome c has been widely implicated in various modes of cell death, especially necrosis.

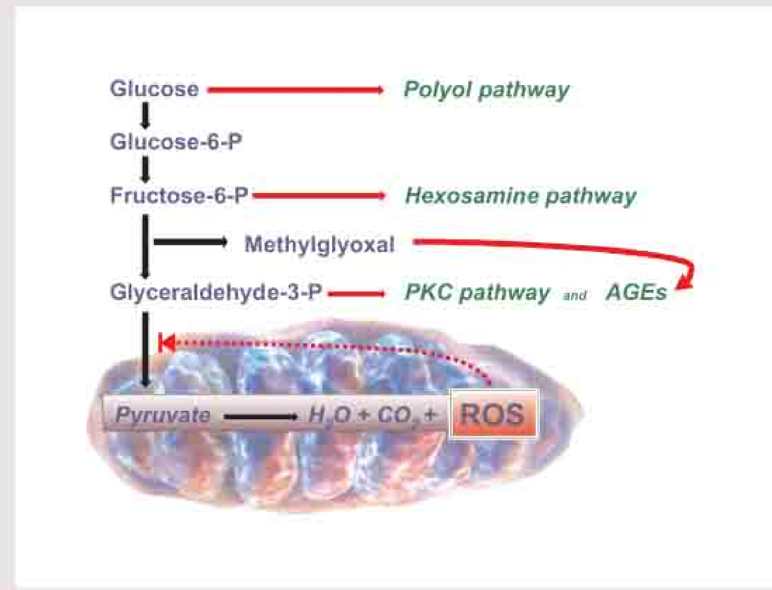


Figure 1: The scheme of glycolysis, mitochondrial overproduction of reactive oxygen species and major pathways of hyperglycemic damage.

Methods

Isolation of rat liver mitochondria: Male Wistar rats 4-7 months old were used for all experiments. The protocol employed was approved by the Committee for Work with Laboratory Animals at the First Faculty of Medicine, Charles University. All animals were fed in the time of sacrifice, and the time of the start of an experiment was kept constant. The rat was anesthetized with ether, exsanguinated by aortal puncture, and one liver lobe was quickly transferred to ice-cold 10 mM Tris/HCl buffer pH 7.4 containing 250 mM sucrose and 0.5 mM EDTA (homogenization buffer, HB). The subsequent isolation of mitochondria was performed at 4 °C. As soon as possible after the isolation, total mitochondrial protein was measured by the biuret method utilizing bovine serum albumin (BSA) as a standard. The mitochondrial suspension was then supplemented with BSA 1 g/L. The mitochondria were allowed to rest on ice for 3 hours before spectrofluorimetric measurements, and used within subsequent 6 hours.

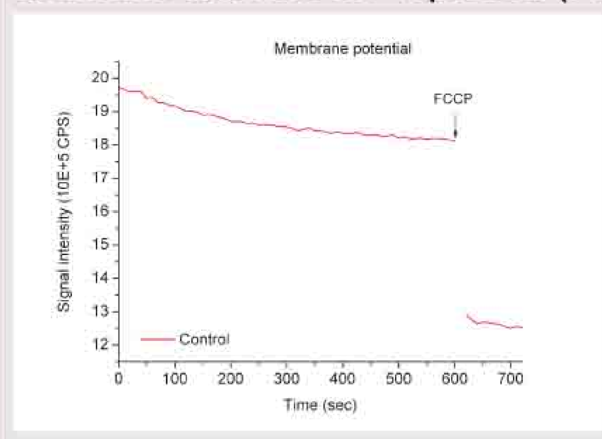
Measurement of mitochondrial swelling, inner membrane potential and hydrogen peroxide release: All the spectrofluorimetric measurements were performed on Fluoromax 3 instrument (HORIBA Jobin-Yvon S.A.S., France) in quartz cuvettes with magnetic stirrer at 37 °C. The mitochondrial swelling was measured as light scatter at 504 nm. For assessment of the inner membrane potential the incubation mixture with mitochondria was placed to the cuvette holder for two minutes (energization), then the JC-1 probe was added to 0.56 μM, and its red fluorescence (488/593 nm), was followed. Mitochondrial production of hydrogen peroxide was detected as oxidation of the Amplex Red reagent (5 μM) to fluorescent resorufin (571/585 nm) in the presence of 1 U/mL horseradish peroxidase.

Aims

In this study, we have chosen the mitochondria isolated from normal rat liver. The MPT was induced by calcium in the presence of 'normal' (5 mM) and 'diabetic' (30 mM) concentration of glucose (Glc), or its metabolites glucose 6-phosphate (Glc 6-P), glucose 1-phosphate (Glc 1-P), and fructose 6-phosphate (Fru 6-P). We included the known MPT blocker methylglyoxal (MGO), and the fructose metabolite glyceraldehyde (Gra). In addition to the possible effects of listed substances on the MPT pore opening, we also examined the consequences of MPT inhibition for mitochondrial ROS production.

Results

During the whole measurement, mitochondria built stable inner membrane potential with succinate (+rotenone), as detected with the JC-1 probe. (Fig. 2)



Glc 6-P can regulate the MPT pore through binding to hexokinase or glucokinase that physically associate with the outer mitochondrial membrane in many cell types, but not in the liver – isolated mouse liver mitochondria were reported to lack glucokinase, which is in agreement with absent effect of Glc 6-P in our experiments. (Fig. 5)

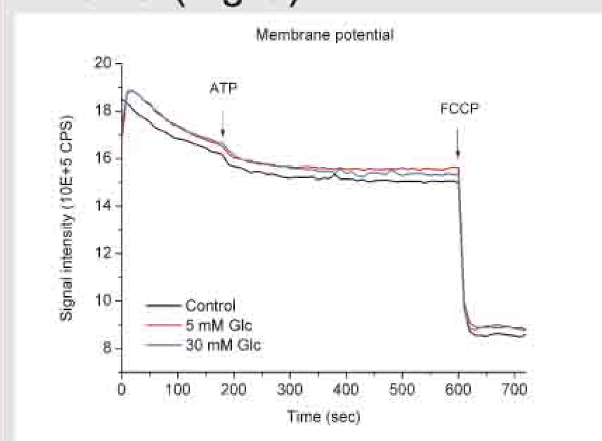


Figure 5: The absent effect of glucose with addition of Mg-ATP on the inner membrane potential indirectly signalizes absence of glucokinase in our mitochondrial preparation.

Addition of 20 μM calcium chloride consistently induced rapid swelling of mitochondria, completely inhibitable by cyclosporine A (Fig. 3 A), which qualifies the phenomenon as the genuine MPT.

The initial swelling rate (30-120 sec. after Ca addition) was significantly slower with 30 mM glucose, 30 mM Glc 1-P and 6 mM MGO, but significantly faster in the presence of 30 mM Fru 6-P (Fig. 4A). Unlike the other effective metabolites, the 30 mM glucose rather delayed the onset of MPT in response to calcium (Fig. 4B).

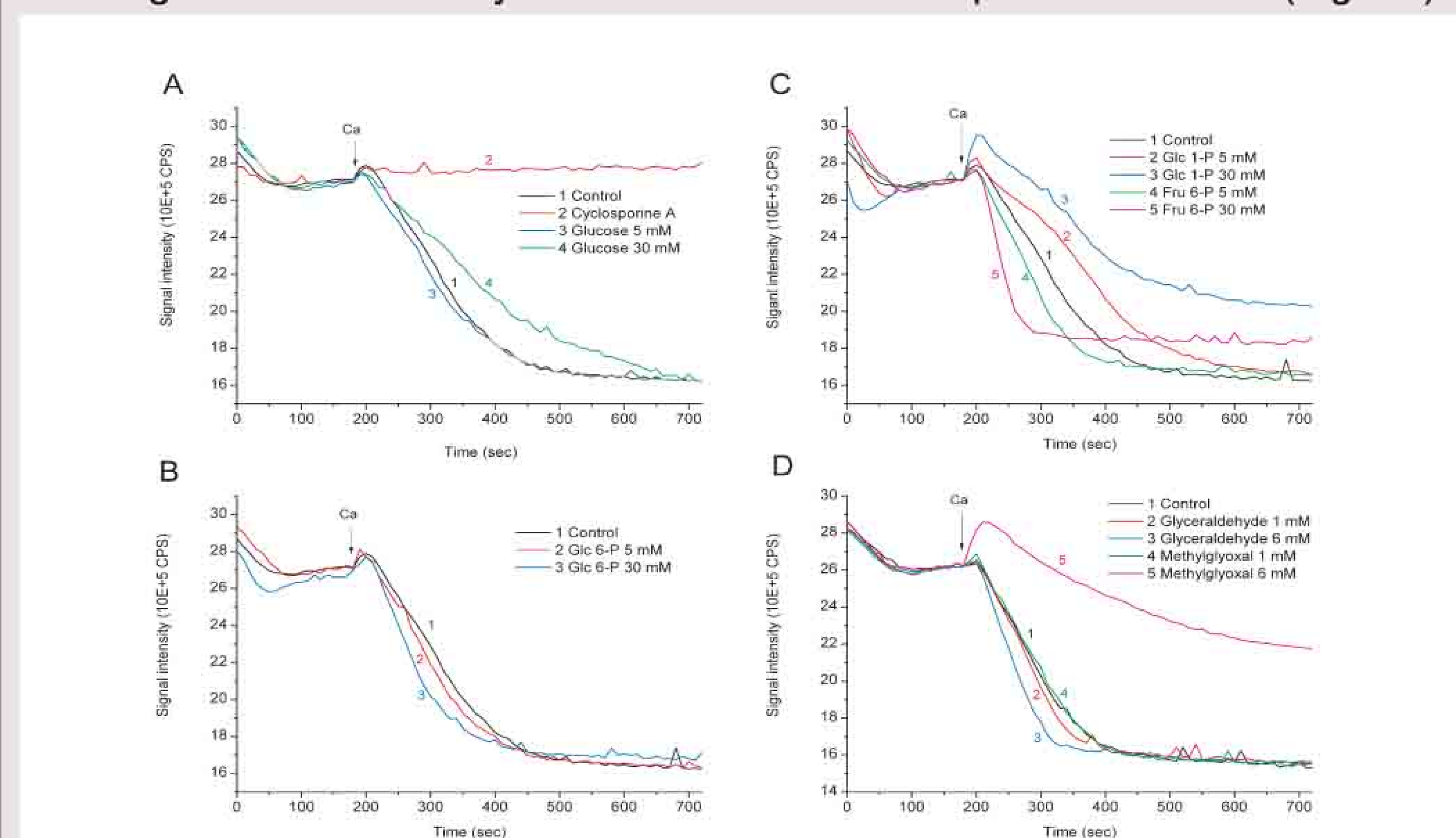


Figure 3: Effect of glucose, its metabolites, and cyclosporine A on calcium-induced MPT measured as swelling of the rat liver mitochondria, representative traces.

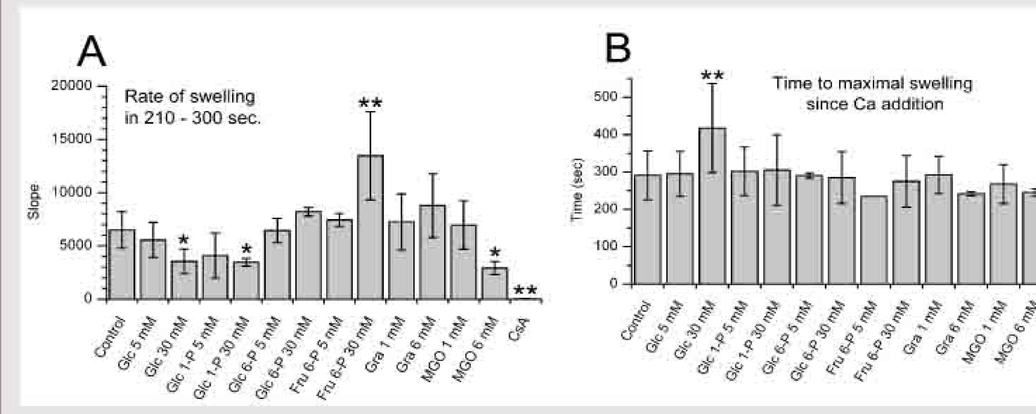


Figure 4: Effect of glucose, its metabolites, and cyclosporine A on calcium-induced MPT, quantitative evaluation of data.
A: The initial swelling rate obtained by linear regression of the kinetics between 210 sec. (Ca added in 180 sec.) and 300 sec., or less if the swelling reached completion too fast.
B: The same data, but progression of MPT evaluated as the time between Ca addition and the maximal swelling rate (read from the first derivative of each trace). Combined data from measurements with three mitochondrial preparations, mean ± SD, N=2-4 for each metabolite, for controls N=23. ANOVA with Dunnett post-test was used for statistical evaluation; *...p<0.05; **...p<0.01 compared to the controls.

Induction of MPT by calcium resulted in transient increase in H_2O_2 release that roughly coincided with the course of swelling and was not markedly affected by Glc 1-P (Fig. 6). In the presence of MGO the basal rate of ROS production appeared higher, but was comparable to control after correction for generation of ROS by MGO without mitochondria, probably by redox cycling the traces of transition metals (Fig. 6B, traces 4 and 5, Fig. 6C). Addition of Ca in the presence of MGO resulted in increased steady mitochondrial ROS production despite robust inhibition of swelling (Fig. 6). In contrast, complete inhibition of MPT by cyclosporine A significantly decreased ROS production (Fig. 6C).

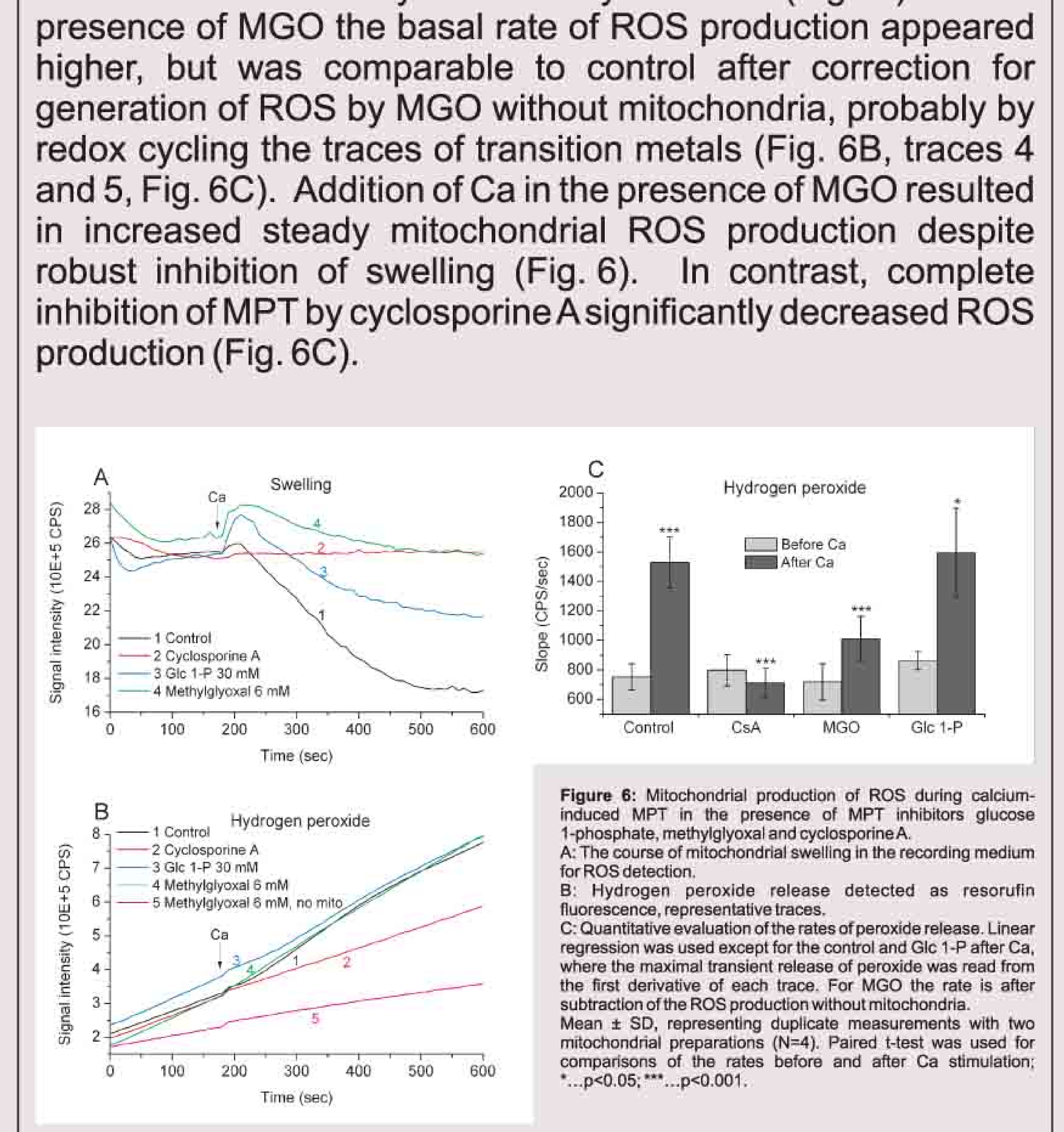


Figure 6: Mitochondrial production of ROS during calcium-induced MPT in the presence of MPT inhibitors cyclosporine A, glucose 1-phosphate, methylglyoxal and cyclosporine A.
A: The course of mitochondrial swelling in the recording medium for ROS detection.
B: Hydrogen peroxide release detected as resorufin fluorescence, representative traces.
C: Quantitative evaluation of the rates of peroxide release. Linear regression was used except for the control and Glc 1-P after Ca, where the maximal transient release of peroxide was read from the first derivative of each trace. For MGO the rate is after subtraction of the ROS production without mitochondria.
Mean ± SD, representing duplicate measurements with two mitochondrial preparations (N=4). Paired t-test was used for comparisons of the rates before and after Ca stimulation; *...p<0.05; ***...p<0.001.

Conclusions

- **Methylglyoxal (6 mM), glucose 1-P (30 mM) and glucose (30 mM) significantly inhibit calcium-induced MPT. In case of glucose, the effect can be described as a delayed onset of MPT.**
- **On the other hand, fructose 6-P (30 mM) significantly accelerates the calcium-induced MPT.**
- **Unlike cyclosporine A, methylglyoxal (6 mM) despite MPT inhibition increases ROS production by mitochondria after stimulation with calcium.**

Discussion

To the best of our knowledge, the effects of glucose on the MPT of isolated normal mitochondria have not been studied. The delayed MPT in the presence of glucose we observed fits well to the work of Kristal et al. [2], where the mitochondria isolated from liver of streptozotocin-treated rats displayed also a delayed induction of MPT by calcium phosphate, which correlated with hyperglycemia of the animals. It is explicable by the reduced calcium import to diabetic mitochondria. Our observations suggest that perhaps glucose can also influence the mitochondrial calcium uniporter directly.

The observed opposing effects of Glc 1-P and Fru 6-P on the MPT are novel, albeit of uncertain physiological significance as the 30 mM concentrations of these intermediates appear quite unlikely in vivo. Glyceraldehyde failed to affect the pore in our experiments, while reported as good MPT inhibitor by Irwin et al. [3].

From all the compounds we tested, MGO appears as the most potent inhibitor of MPT as described by Speer [4]. Although MPT has been widely implicated in cell death, it is also involved in physiological calcium signaling where its inhibition would not be beneficial. Notably, the MPT apparently plays a role in the normal glucose-sensing mechanism of the B-cells, and it is tempting to speculate that chronically elevated levels of MGO or perhaps even glucose may through MPT inhibition interfere with this mechanism and contribute to the inadequate insulin secretion.

MGO has been shown to glycate relatively selectively certain components of the complex III [5], leading to inhibition of respiration and concomitant increase in ROS production. The stimulation of peroxide release by calcium in presence of MGO described in this study (probably for the first time) can be based on a similar mechanism. This finding deserves a special attention. Programmed cell death can be regarded as the ultimate antioxidant defense of the body as it purifies the tissues from ROS-overproducing cells. In this context, if MGO inhibits MPT and simultaneously increases mitochondrial ROS production, it is certainly very bad alternative for the body, and as such would be sufficient – together with the unifying hypothesis – to explain the genesis of long-term diabetic complications.

References

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