Original Article

Glucose and Its Metabolites Have Distinct Effects on the Calcium-Induced Mitochondrial Permeability Transition

(mitochondrial permeability transition pore / diabetes mellitus / oxidative stress / glycolysis / methylglyoxal)

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Abstract. Mitochondrial production of reactive oxygen species (ROS) due to up-regulated glucose oxidation is thought to play a crucial, unifying role in the pathogenesis of chronic complications associated with diabetes mellitus. Mitochondrial permeability transition (MPT) is an interesting phenomenon involved in calcium signalling and cell death. We investigated the effects of glucose and several of its metabolites on calcium-induced MPT (measured as mitochondrial swelling) in isolated rat liver mitochondria. The presence of glucose, glucose 1-phosphate (both at 30 mM) or methylglyoxal (6 mM) significantly slowed calcium-induced mitochondrial swelling. Thirty mM glucose also resulted in a significant delay of MPT onset. In contrast, 30 mM fructose 6-phosphate accelerated swelling, whereas glucose 6-phosphate did not influence the MPT. The calcium binding potentials of the three hexose phosphates were tested and found similar. *In vitro* hydrogen peroxide production by mitochondria respiring on succinate in the presence of rotenone was independent of mitochondrial membrane potential and increased transiently during calcium-induced MPT. Inhibition of MPT with cyclosporine A resulted in decreased mitochondrial ROS production in response to calcium. In contrast, inhibition of MPT by methylglyoxal was accompanied by increased ROS production in response to calcium. In conclusion, we confirm that methylglyoxal is a potent inhibitor of MPT. In addition, high levels of glucose, glucose 1-phosphate and fructose 6-phosphate can also affect MPT. Methylglyoxal simultaneously inhibits MPT and increases mitochondrial ROS production in response to calcium. Our findings provide a novel context for the role of MPT in glucose sensing and the cellular toxicity caused by methylglyoxal.

Introduction

Diabetes mellitus, including the associated long-term cardiovascular, renal, retinal and neurological complications, is one of the leading causes of morbidity and mortality worldwide (Roglic et al., 2005). Mitochondrial production of reactive oxygen species (ROS) has recently been recognized as an important factor in the pathogenesis of long-term diabetic complications (Nishikawa et al., 2000). According to the proposed, unifying mechanism, the increased production of ROS in the mitochondria induced by hyperglycaemia results in the inhibition of glyceraldehyde 3-phosphate dehydrogenase (Brownlee, 2005). Subsequent accumulation of upstream glycolytic intermediates is thought to bring about hyperglycaemia-associated tissue damage via the activation of the major relevant pathways: the polyol pathway, the protein kinase C activation pathway, the hexosamine pathway and production of advanced glycation end products (AGEs).

It is unclear, however, whether increased intracellular glucose oxidation by itself necessarily leads to overproduction of ROS in the mitochondria. In an attempt to further elucidate the basis for this relationship, we considered the possible involvement of the mitochondrial permeability transition (MPT). This phenomenon is caused by the rearrangement of certain inner mitochondrial membrane proteins into a 'mega-channel' that allows the passage of all low-molecular-weight solutes and collapses the proton-motive force (Hunter et al., 1976; Hunter and Haworth, 1979; Gunter and Pfeiffer, 1990; Bernardi et al., 2006). The opening of the MPT

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Abbreviations: AGEs – advanced glycation end products, BSA – bovine serum albumin, CPS – counts per second, FCCP – carbonyl cyanide p-trifluoromethoxyphenylhydrazone, HB – homogenization buffer, MPT – mitochondrial permeability transition, ROS – reactive oxygen species.

pore is triggered by calcium and modulated by numerous other agents. For example, MPT is enhanced by mitochondrial depolarization, phosphate and ROS; on the other hand, MPT is inhibited by low matrix pH, adenine nucleotides and cyclosporine A (Halestrap, 2009). Transient 'flickering' of the MPT pore is likely to play a role in cellular calcium homeostasis and calcium signalling (Miller, 1998), whereas persistent MPT that causes mitochondrial swelling, outer membrane rupture and release of cytochrome c, has been widely implicated in various modes of cell death, especially necrosis (Armstrong, 2006; Bernardi et al., 2006; Kroemer et al., 2007).

MPT research has focused primarily on the role of this phenomenon in pathophysiology of myocardial ischaemia-reperfusion, brain ischaemia, neurodegeneration, liver diseases and organ transplantation (Bernardi et al., 2006). In comparison, the relationship between MPT and diabetes has remained a relatively unexplored topic. Limited data from studies using animal models suggest that diabetes may render mitochondria either more or less susceptible to MPT induction. Mitochondria isolated from the livers of streptozotocin-treated rats (Kristal et al., 1996) or the hearts of Goto-Kakizaki diabetic rats (Oliveira et al., 2001) were reported to be more resistant to MPT induction, whereas mitochondria isolated from the hearts of streptozotocin-treated rats (Oliveira et al., 2003) or the kidneys of the Goto-Kakizaki rats (Oliveira et al., 2004) were observed to be more susceptible to MPT. Methylglyoxal, a by-product of glycolysis and the main precursor of AGEs in the body, effectively inhibits the opening of the MPT pore by forming a reversible, covalent linkage with certain critical arginine residues on the pore proteins (Speer et al., 2003). Interestingly, cyclosporine A is known to be diabetogenic, and this adverse effect appears to be due to inhibition of MPT in pancreatic β-cells rather than immunosuppression (Düfer et al., 2001).

In this study, we utilized mitochondria isolated from the livers of healthy rats because, similarly to pancreatic β-cells and other cells that are most vulnerable to hyperglycaemia, liver cells do not regulate glucose entry (Brownlee, 2005). MPT was induced by addition of calcium in the presence of 'normal' (5 mM) and 'diabetic' (30 mM) concentrations of glucose or other metabolites that could be obtained commercially and do not directly serve as mitochondrial fuels, namely glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate. We included the known MPT blocker methylglyoxal (Speer et al., 2003) and the fructose metabolite glyceraldehyde, which has also been reported to inhibit the MPT in isolated rat liver mitochondria (Irwin et al., 2002). In addition to the possible effects of the listed substances on the MPT pore opening, we also examined the consequences of MPT inhibition on mitochondrial ROS production.

Material and Methods

Materials

Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and Calcium Green-5N hexapotassium salt were purchased from Molecular Probes (Invitrogen Corp., Carlsbad, CA). Bovine serum albumin (BSA, fatty acid free) and cyclosporine A were obtained from MP Biomedicals (Irvine, CA). Glucose and dipotassium salts of glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate as well as DL-glyceraldehyde, methylglyoxal, type XII horseradish peroxidase and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Isolation of mitochondria from rat liver

All Wistar rats used for our experiments were male and 4–7 months old. The protocol employed was approved by the Committee for Work with Laboratory Animals at the First Faculty of Medicine, Charles University in Prague. Rats were anaesthetized with ether and exsanguinated by aortic puncture, and one lobe of the liver was immediately transferred to ice-cold homogenization buffer (HB; 10 mM Tris-HCl pH 7.4, 250 mM sucrose, 0.5 mM Na_2K_2-EDTA). Mitochondria were isolated from the lobe at 4 °C according to a previously published protocol (Lai and Clark, 1979; Nedergaard and Cannon, 1979). The chopped tissue was homogenized in four volumes of HB with a motor-driven Teflon/glass homogenizer at 100 rpm, followed by dilution with an equal volume of HB. The resulting 10% homogenate was centrifuged at 2,000 *g* for 3 min; the supernatant was collected and spun again at 2,000 *g* for another 3 min. The resulting supernatant was centrifuged at 12,500 *g* for 8 min, and the mitochondria-containing pellets were resuspended and pooled in a minimal volume of HB. Soon after isolation, the concentration of total mitochondrial protein was determined using the biuret method with BSA as a standard. The mitochondrial suspension was then supplemented with 1 mg ml^{-1} BSA, and the volume of the suspension was adjusted with HB to a mitochondrial protein concentration of 10 mg ml^{-1}. The mitochondria were allowed to rest on ice for 3 hours prior to spectrofluorimetric measurements and were used within the subsequent 6 h.

Measurements of mitochondrial swelling and hydrogen peroxide release

Spectrofluorimetric measurements were performed using a Fluoromax-3 instrument (HORIBA Jobin Yvon., HORIBA Scientific, Kyoto, Japan) and quartz cuvettes with stirring at 37 °C. The recording medium contained 0.25 mg ml^{-1} mitochondrial protein, 0.025 mg ml^{-1} BSA, 0.25 mM Tris, 12.5 μM Na_2K_2-EDTA, 20 mM Hepes-KOH pH 7.3, 91.25 mM sucrose, 79.5 mM KCl, 1 mM $KHPO_4$, 5 mM sodium succinate and 1 μM rotenone. When examining the effects of different concentrations of glucose or glucose-related metabolite, the total saccharide concentration (and osmolarity if the extra K^+ is

not counted) was held constant by adjusting the concentration of sucrose in the recording medium. The stock solutions of Amplex Red reagent, rotenone and cyclosporine A were made in dimethyl sulphoxide (DMSO; final concentration 0.1–0.3% v/v). Because of its low water solubility, cyclosporine A was pre-mixed with the mitochondrial suspension, and both components were added together to the recording medium.

Mitochondrial swelling was monitored by changes in the intensity of light scattering at 504 nm. The plot of MPT progression measured by light scattering was observed to assume a sigmoid (S-shaped) function (Hunter and Haworth, 1979). Consequently, we employed two approaches to facilitate quantitative analysis. Linear regression of the near-linear portion of each trace, usually occurring between 30-120 s after calcium addition (but may be earlier and shorter if MPT progresses more rapidly), was used to determine the rate of MPT progression. The rates calculated were equivalent to the initial swelling rates previously reported by Speer et al. (2003). In addition to linear regression analysis, each trace was subjected to calculation of its first derivative to determine its inflection point; the result of this analysis was the time elapsed between calcium addition and the maximal swelling rate. This approach is analogous to that employed by Hunter and Haworth (1979) and Kristal et al. (1996), which involves measuring the half-maximal swelling; however, it should be noted that these two methods are not equivalent because true MPT sigmoid curves can be asymmetric.

Production of hydrogen peroxide was indirectly measured by generation of the fluorescent species resorufin (λex = 571 nm; λem = 585 nm) resulting from the oxidation of Amplex Red reagent (5 μM) in the presence of 1 U ml^{-1} horseradish peroxidase (Mohanty et al., 1997; Tretter et al., 2007). To calculate rates of hydrogen peroxide production from the resorufin fluorescence data, the slope was calculated for simple linear traces; if a trace was non-linear, its first derivative was calculated to determine the maximal transient rate.

Measurement of Ca^{2+} levels in recording medium

To determine the extent of calcium sequestration by phosphorylated glucose derivatives in recording medium, we used the same recording medium as described above with two exceptions: the substitution of mitochondrial suspension with BSA (1 mg ml^{-1} in HB) and the addition of 8 μM of Calcium Green-5N hexapotassium salt. After calcium chloride was added (final concentration 20 μM), the fluorescence of a calcium-bound probe (λex = 506 nm; λem = 531 nm) was recorded at 37°C. Free Ca^{2+} in the medium was calculated using the formula

$$[Ca^{2+}]_{free} = K_d \times [(F - F_{min}) / (F_{max} - F)]$$

where the variables are defined as follows: F, probe fluorescence at experimental Ca^{2+} levels; F_{min}, probe fluorescence in the absence of Ca^{2+} (no calcium chloride added, plus 500 μM EDTA); F_{max}, probe fluorescence in the presence of saturating (500 μM) Ca^{2+}; and K_d, the dissociation constant. K_d = 14 μM (Molecular Probes, Invitrogen Corp., 2005, Long-Wavelength Calcium Indicators. Product information. http://products.invitrogen.com/ivgn/product/C3737).

Results

The isolated rat liver mitochondria were initially stabilized with EDTA (chelates calcium) and BSA (binds fatty acids, known as MPT inducers). The mitochondria developed a stable inner membrane potential when in recording medium containing succinate and rotenone, as detected with the JC-1 probe (not shown). The addition of 20 μM $CaCl_2$ (yielding theoretical 7.5 μM free Ca^{2+} ions over the residual EDTA) consistently induced rapid swelling of the mitochondria, which was completely inhibited by cyclosporine A (Fig. 1 A), indicating that the phenomenon is a genuine MPT event. Similar measurements were conducted in the presence of 5 mM and 30 mM glucose, glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate and also in the presence of 1 and 6 mM glyceraldehyde and methylglyoxal (Fig. 1). Compared to the rate in the absence of metabolite, the initial swelling rate was considerably slower in the presence of 30 mM glucose, 30 mM glucose 1-phosphate or 6 mM methylglyoxal. On the other hand, the swelling rate was significantly faster (relative to the control) in the presence of 30 mM fructose 6-phosphate (Fig. 2A). Unlike the other metabolites, 30 mM glucose delayed the onset of MPT in response to calcium (Fig. 2B).

The induction of MPT generally requires previous accumulation of calcium ions in the mitochondrial matrix due to the action of a mitochondrial Ca^{2+} uniporter. As a result, any treatment that interferes with calcium uptake into the mitochondria, such as mitochondrial depolarization, Ruthenium Red or calcium chelation, will inhibit MPT. Because glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate are expected to bind calcium ions, we tested whether the differences between their calcium-sequestering abilities might account for their varying effects on MPT. However, our measurements of free Ca^{2+} levels in the recording medium supplemented with hexose phosphates (Table 1) revealed that all three sugar phosphates possess a similar, rather weak affinity for calcium. Thus, calcium chelation cannot explain their divergent effects on MPT.

We then examined the consequences of MPT inhibition by glucose 1-phosphate and methylglyoxal on mitochondrial ROS production. Whereas depolarization due to MPT might decrease ROS production, swelling and the resultant release of cytochrome c likely increases oxidative stress by disrupting the respiratory chain (Bernardi et al., 2006; Tretter et al., 2007). In fact, not all modes of mitochondrial ROS production are depend-

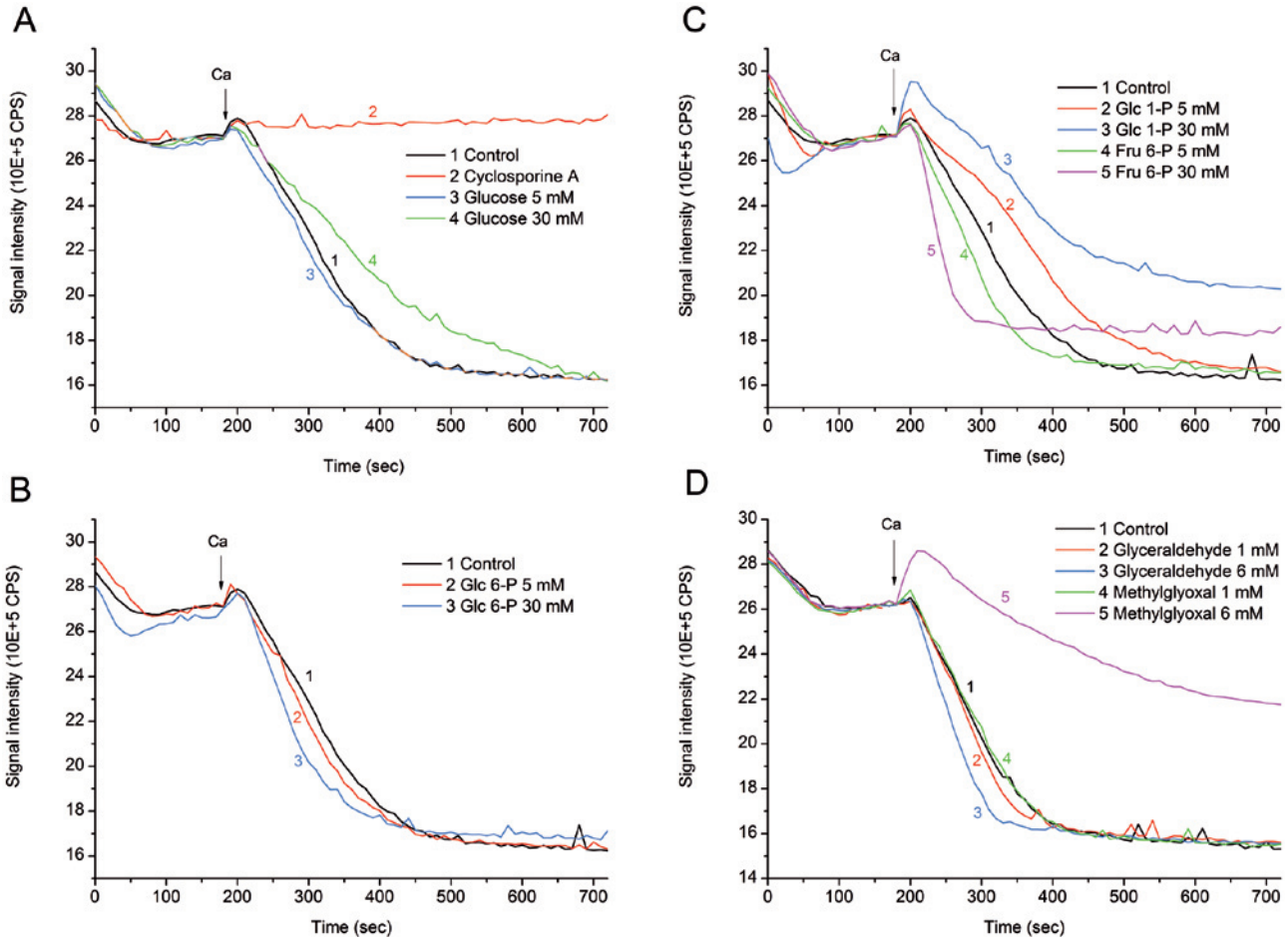

Fig. 1. Induction of MPT by calcium, measured as swelling of rat liver mitochondria in the presence of glucose, glucose 6-phosphate (Glc 6-P), glucose 1-phosphate (Glc 1-P), fructose 6-phosphate (Fru 6-P), glyceraldehyde, methylglyoxal or cyclosporine A. Swelling was assessed by monitoring light scattering at 504 nm. Representative traces are shown.

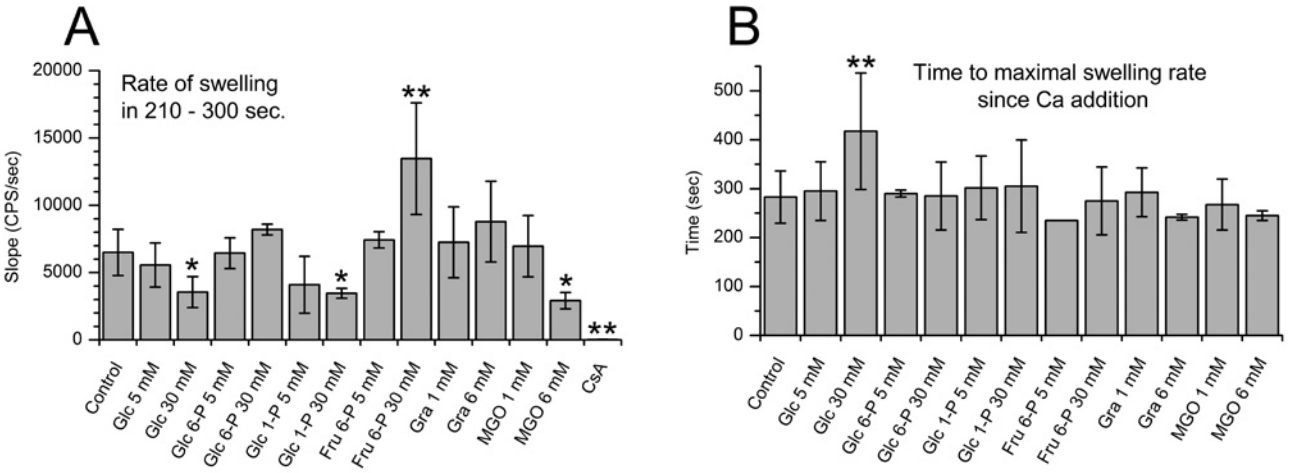

Fig. 2. Quantitative analysis of the effects of glucose (Glc), glucose 6-phosphate (Glc 6-P), glucose 1-phosphate (Glc 1-P), fructose 6-phosphate (Fru 6-P), glyceraldehyde (Gra), methylglyoxal (MGO) or cyclosporine A (CsA) on calcium-induced MPT. **A**: Determination of the initial swelling rate by linear regression of kinetics following addition of calcium (at 180 s) as described in the Methods section. **B:** Data is the same as in A, but the progression of MPT was measured as the time elapsed between calcium addition and achievement of the maximal swelling rate (computed from the first derivative of each trace). Combined data from measurements of three mitochondrial preparations are expressed for each metabolite (N = 2–4) or control (N = 23) as mean ± SD. ANOVA with Dunnett post-test was used for statistical evaluation; * $P < 0.05$; ** $P < 0.01$.

Table 1: Determination of free Ca^{2+} ion concentration after addition of 20 μM $CaCl_2$ to the recording medium in the presence of phosphorylated glucose derivatives

Metabolite	*Metabolite concentration*	*Free Ca^{2+} (μM)*[1]
None (control)	0	10.5 ± 1.1[2]
Glucose 6-phosphate	5 mM	8.0 ± 0.1
	30 mM	4.5 ± 0.3
Glucose 1-phosphate	5 mM	7.8 ± 0.3
	30 mM	3.5 ± 0.3
Fructose 6-phosphate	5 mM	8.0 ± 0.2
	30 mM	4.2 ± 0.03

[1] The free calcium ion concentration was calculated using the measured fluorescence change at 531 nm of a low-affinity probe, Calcium Green-5N, as described in the Methods section. Data from two measurements are presented in the form of mean ± SD.
[2] The recording medium contained 12.5 μM of EDTA; under the control condition, a measured Ca^{2+} concentration above the theoretical value of 7.5 μM revealed the presence of extra Ca^{2+}, possibly contributed by glassware and reagent impurities.

ent on an existing potential across the inner mitochondrial membrane. Potential-dependent ROS production in mitochondria respiring on succinate mainly results from a reversal in electron flow from complex III to complex I; the reversed flow is inhibited by rotenone (Tretter et al., 2007). Accordingly, hydrogen peroxide release from our rat liver mitochondria energized by succinate in the presence of rotenone rather *increased* after depolarization with the protonophoric uncoupler FCCP, while without rotenone the outcome was the exact opposite (Fig. 3). Calcium-induced MPT resulted in a transient increase in hydrogen peroxide release that roughly coincided with swelling and was not markedly affected by the presence of glucose 1-phosphate (Fig.

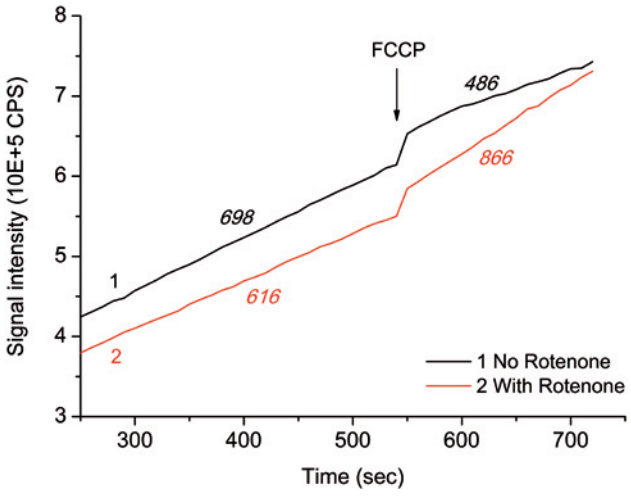

Fig. 3. Production of hydrogen peroxide by mitochondria respiring on succinate in the presence or absence of rotenone (1 μM). FCCP (5 μM) was used as an uncoupling agent. The number accompanying each trace is the slope (in CPS s^{-1}), which was obtained by linear regression analysis. The traces are representative plots from experiments using two different mitochondrial preparations.

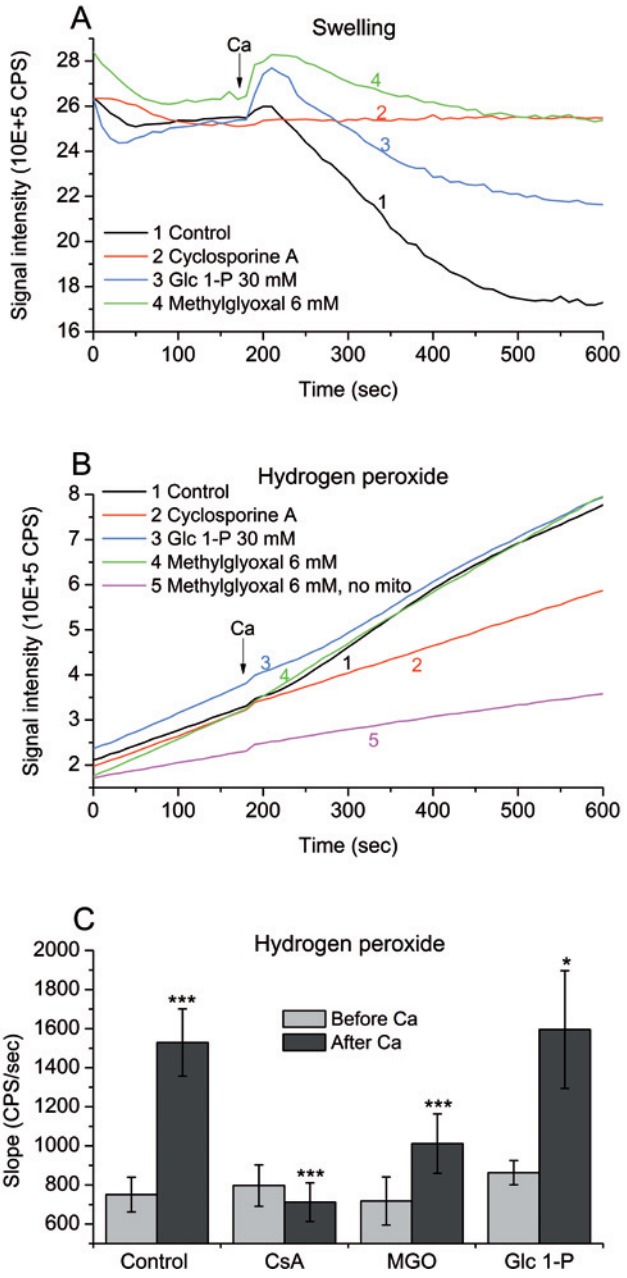

Fig. 4. Analysis of the effects caused by glucose 1-phosphate (Glc 1-P), methylglyoxal (MGO) and cyclosporine A (CsA) on mitochondrial ROS production during calcium-induced MPT. **A:** Time course of mitochondrial swelling in the recording medium for ROS detection. **B:** Representative traces of hydrogen peroxide release, detected as resorufin fluorescence at 585 nm. **C:** Quantitative determination of peroxide release rates. Linear regression was used in all cases, except for the control and for glucose 1-phosphate after calcium addition; for these cases, the maximal transient release of peroxide was determined following calculation of the first derivative of each trace. For methylglyoxal, the rate was obtained after subtraction of ROS production in the absence of mitochondria. Data represent duplicate measurements with two mitochondrial preparations (N = 4) and are presented in the form of mean ± SD. A paired *t*-test was used for comparisons of the rates before and after calcium stimulation; * $P < 0.05$; *** $P < 0.001$.

4). In the presence of methylglyoxal, the basal rate of ROS production by isolated mitochondria originally appeared to be elevated; however, after correction for ROS generation by methylglyoxal in the absence of mitochondria (probably due to redox cycling from trace transition metals), the rate was comparable to that of controls (Fig. 4B traces 4 and 5; Fig. 4C). Despite inhibition of mitochondrial swelling due to the presence of methylglyoxal, addition of calcium still resulted in increased and continuous mitochondrial ROS production (Fig. 4). In contrast, complete inhibition of MPT by cyclosporine A led to a significant decrease in ROS production (Fig. 4C).

Discussion

Our results show that glucose and some of its metabolic derivatives, namely glucose 1-phosphate, fructose 6-phosphate and methylglyoxal, significantly affect the course of calcium-induced MPT in isolated rat liver mitochondria.

Glucose was observed to significantly delay onset and progression of MPT. Even though the concentration required to produce these effects (30 mM) seems exceedingly high, previous studies modelling hyperglycaemic damage to cell cultures used the same glucose level (Detaille et al., 2005; Ksiazek et al., 2008). Moreover, if a cell cannot regulate glucose entry and fails to process glucose effectively, the cytosolic glucose concentration can be expected to approach the extracellular concentration. In human patients the glycaemia values reaching or even exceeding 30 mM would be expected only in acutely decompensated diabetes mellitus, especially in the nonketotic hyperosmolar coma where the glycaemia often exceeds 50 mM (Škrha et al., 2009). However, it is well-known in clinical practice that even self-monitoring by diabetic patients can occasionally reveal glycaemia over 20 mM.

To our knowledge, except for one report by Irwin et al. (2002) suggesting that glucose is ineffective in altering the course of MPT, the direct effect of glucose on MPT in isolated mitochondria had not been studied previously. Kristal et al. (1996) isolated mitochondria from livers of diabetic, streptozotocin-treated rats and observed that treatment with calcium phosphate delayed induction of MPT. This result was correlated with the hyperglycaemic state of the animals, suggesting that delayed induction of MPT may be the result of reduced calcium import by diabetic mitochondria (Grinblat et al., 1988). This conclusion is in agreement with our observation that MPT is delayed by elevated glucose levels, even though the two experimental setups were substantially different; whereas Kristal et al. (1996) compared the responses to calcium of mitochondria isolated from diabetic versus healthy animals, our investigation compared the response of healthy mitochondria to calcium in either the presence or absence of glucose. If mitochondrial calcium import is, in fact, impaired in diabetes (Grinblat et al., 1988; Kristal et al., 1996), then our data suggest that the presence of glucose might influence the mitochondrial calcium uniporter directly.

Glucose 6-phosphate can regulate the MPT pore by binding to hexokinase or glucokinase, both of which physically associate with the outer mitochondrial membrane in many cell types (Lemasters and Holmuhamedov, 2006). However, mitochondria isolated from mouse liver lack glucokinase (Bustamante et al., 2005), which might explain the lack of an effect due to glucose 6-phosphate in our experiments.

Glucose 1-phosphate is a substrate for glycogenesis rather than glycolysis and is likely found exclusively in the liver or muscle. Fructose 6-phosphate appears to be present in most cells and if the cytosol is flooded with glucose, fructose 6-phosphate should accumulate because phosphofructokinase, the enzyme that uses it as a substrate, is the rate-limiting step of glycolysis. The opposing effects of glucose 1-phosphate and fructose 6-phosphate on MPT have not been described previously; nevertheless, they are of uncertain physiological significance because the high concentrations of metabolite required to produce these effects are unlikely to exist *in vivo*. On the other hand, these observations might be (patho)physiologically relevant in certain congenital metabolic disorders in which glucose 1-phosphate or fructose 6-phosphate might accumulate, such as in glycogen storage diseases type I and VII, respectively.

Glyceraldehyde, although previously reported to be an effective MPT inhibitor by Irwin et al. (2002), failed to affect the pore in our experiments. In the earlier study, MPT was induced by calcium in the absence of exogenous respiratory substrates, and the inhibition by glyceraldehyde was dependent on its oxidation by aldehyde dehydrogenase. The oxidation reaction alters the NADH/NAD^+ ratio, known as a powerful regulator of MPT. Such changes are extremely unlikely in our isolated mitochondria, because in the presence of rotenone succinate ensures that all NAD^+ is reduced to NADH.

Of all compounds tested, methylglyoxal was observed to be the most potent inhibitor of MPT. Speer et al. (2003) tested 29 physiological carbonyl compounds and reached the same conclusion. Although MPT has been widely implicated in cell death, it is also involved in physiological calcium signalling, the inhibition of which would be rather detrimental for the cell. For instance, MPT has been implicated in the glucose-sensing mechanism of β-cells (Düfer et al., 2001), and it is tempting to speculate that chronically elevated levels of methylglyoxal, and perhaps glucose, may interfere with this mechanism via inhibition of MPT and result in ineffective insulin secretion.

To produce significant inhibition of the MPT, our experiments required at least 6 mM methylglyoxal. Interestingly, Speer et al. (2003) used a different, more sophisticated protocol involving pre-incubation of mitochondria with methylglyoxal, and observed measurable effects with methylglyoxal concentrations as low as 250 μM. Even so, the methylglyoxal concentration inside the cell is thought to be well below that value, pos-

sibly as low as 2–4 μM (Rabbani and Thornalley, 2008). For this reason, the suggestion that even chronic exposure to methylglyoxal might have an effect on MPT *in vivo* continues to be met with scepticism (Rabbani and Thornalley, 2008). On the other hand, mild but constant production of methylglyoxal inside the cell should shift the equilibrium between the free and the protein-bound methylglyoxal considerably towards the protein-bound form (Speer et al., 2003). If the portion of methylglyoxal reversibly bound to cellular protein is included to the measurement, the total concentration of methylglyoxal inside the cell can be as high as 310 μM (Chaplen et al., 1998).

Admittedly, the concentrations of glucose and its metabolites that in our experiments *in vitro* produced any measurable effect on the MPT generally exceed the levels at which these substances occur *in vivo*. This may limit the applicability of our findings; however, a difference in time scale should also be taken into consideration. While the measurement with isolated mitochondria lasts several minutes, the development of diabetic complications takes years *in vivo*. Some of the metabolic or regulatory disturbances that are highly relevant for pathogenesis of diabetic complications can be extremely difficult to reveal *in vitro* on a compressed time scale, unless supra-physiological concentrations are used.

Interestingly, whereas both cyclosporine A and methylglyoxal inhibited swelling of mitochondria in our experiments, they produced opposite effects with respect to hydrogen peroxide release; after calcium addition, cyclosporine A decreased the rate of hydrogen peroxide release, whereas methylglyoxal increased this rate. The observed inhibitory effect of cyclosporine A may appear puzzling as this compound is also known as an inducer of oxidative and nitrosative stress (e.g., Redondo-Horcajo and Lamas, 2005). However, van der Toorn et al. (2007) examined the mechanism of cyclosporine A-induced oxidative stress in cultured cells and concluded that the source of ROS was extra-mitochondrial. In the same study, cyclosporine A actually inhibited both basal and calcium-induced ROS production in isolated pig liver mitochondria.

On the other hand, the observation that methylglyoxal selectively glycates specific components of complex III (Rosca et al., 2005; Rabbani and Thornalley, 2008), leading to an inhibition of respiration and a concomitant increase in ROS production, may explain its stimulation of hydrogen peroxide release. Our report is the first description of the effects of methylglyoxal on calcium-dependent ROS production associated with MPT inhibition. In our opinion, this effect may be relevant to the methylglyoxal toxicity. Programmed cell death can be regarded as the ultimate antioxidant defence of the body because it eliminates ROS-overproducing cells from tissues (Skulachev, 1996). Methylglyoxal may be able to prevent this ultimate defence through its inhibition of MPT (which would otherwise promote cell death) and its simultaneous enhancement of mitochondrial ROS production. Further studies are needed to elucidate whether and to what extent this mechanism of methylglyoxal toxicity contributes to tissue damage and the ensuing, long-term complications of diabetes.

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