Methylglyoxal (MG) (pyruvaldehyde) is a reactive carbonyl compound produced in glycolysis. MG can form covalent adducts on proteins resulting in advanced glycation end products that may alter protein function. Here we report that MG covalently modifies the mitochondrial permeability transition pore (PTP), a high conductance channel involved in the signal transduction of cell death processes. Incubation of isolated mitochondria with MG for a short period of time (5 min), followed by removal of excess free MG, prevented both ganglioside GD3- and Ca2+-induced PTP opening and the ensuing membrane depolarization, swelling, and cytochrome c release. Under these conditions MG did not significantly interfere with mitochondrial substrate transport, respiration, or oxidative phosphorylation. The suppression of permeability transition was reversible following extended incubation in MG-free medium. Of the 29 physiological carbonyl and dicarbonyl compounds tested only MG and its analogue glyoxal were able to specifically alter the behavior of the PTP. Using a set of arginine-containing peptides, we found that the major MG-derived arginine adduct formed, following a short time exposure to MG, was the 5-hydro-5-methylimidazol-4-one derivative. These findings demonstrate that MG rapidly modifies the PTP covalently and stabilizes the PTP in the closed conformation. This is probably due to the formation of an imidazolone adduct on an arginine residue involved in the control of PTP conformation (Linder, M. D., Morkunaite-Haimi, S., Kinnunen, P. J. K., Bernardi, P., and Eriksson, O. (2002) J. Biol. Chem. 277, 937–942). We deduce that the permeability transition constitutes a potentially important physiological target of MG.

Methylglyoxal (MG)1 is a reactive dicarbonyl compound that is formed during glucose metabolism. MG binds covalently to proteins resulting in the formation of advanced glycation end products (AGEs), which are involved in several pathological processes, including cellular proliferative disorders (1) and diabetes mellitus (2). Increased formation of AGEs owing to hyperglycemia is a key factor in the development of diabetic complications such as microvascular disease and atherosclerosis (2). Inhibition of glycation reactions slows the progression of diabetic vascular disease manifestations (3, 4).

During glucose metabolism triose phosphates may undergo either spontaneous (5) or enzyme-facilitated decomposition to yield MG (6, 7). The active site of triose phosphate isomerase harbors a flexible loop surrounding enzyme-bound reaction intermediate. This loop flickers continuously between open and closed states leading to a small but constant leakage of the unstable intermediate enediololate phosphate (8) that immediately undergoes phosphate elimination to yield MG. Triose phosphate isomerase is a very efficient catalyst that is present at high concentration in tissues (9, 10), and therefore significant amounts of MG and MG-modified proteins may be produced (11).

At physiological concentrations MG primarily targets the arginine residues of proteins (12), resulting initially in the formation of reversible adducts. These adducts may consequently undergo a series of rearrangements that yield several possible end-products that contain either imidazolone- or pyridine-based ring systems (13–15). MG is known to target several proteins involved in the regulation of cell growth and differentiation (16–19), although the coupling between MG-induced alterations and subsequent cellular effects remains under investigation.

Mitochondria play an important role in programmed cell death by releasing proteins from the intermembrane compartment, where they are normally confined, to cytosol and nucleus (20). These proteins include cytochrome c and Smac/DIABLO, which activate caspases, endonuclease G that induces DNA fragmentation, and apoptosis-inducing factor, which promotes...
caspase-independent chromatin condensation and DNA fragmentation (21). The release of these pro-apoptotic proteins may be triggered by mitochondrial permeability transition (22). This event is induced by upstream apoptotic signals such as formation of the ganglioside GD3 (23-25) and mitochondrial Ca\(^{2+}\) uptake (22). Permeability transition is due to opening of the permeability transition pore (PTP) (22), which under normal cell life remains closed (26). In the open conformation the PTP permits free diffusion of solutes with a molecular mass of <1.5 kDa across the mitochondrial membrane. Permeability transition leads to mitochondrial depolarization and equalization of matrix and cytosolic ion and metabolite concentrations. Concomitant osmotic swelling of the mitochondrial matrix may lead to rupture of the outer membrane and release of proteins from the intermembrane compartment. According to the prevalent model, permeability transition pores are composed of the adenine nucleotide translocator, the voltage-dependent anion channel, and mitochondrial matrix cyclophilin (27, 28).

Ca\(^{2+}\) uptake does not elicit permeability transition in isolated mitochondria after treatment with the synthetic dicarbonyl compounds phenylglyoxal (PGO) or 2,3-butanedione (BAD) (29–32). These compounds react specifically with the guanidino group of arginine, indicating that modification of an arginine residue by PGO or BAD stabilizes the PTP in its closed conformation. Interestingly, modification with the PGO analogue OH-PGO results in an arginine adduct that induces the open conformation of the PTP (32). The profound effect of arginine modification demonstrates that structural rearrangements of an arginine residue are of key importance for the control of the PTP conformation, although the location of that arginine residue is still unclear. The possibility that physiological PTP regulators act via that site prompted us to investigate whether the natural dicarbonyl compound MG was capable of inducing changes in the function of the PTP.

In this study we have identified the mitochondrial PTP as a novel target of MG. Brief incubation of isolated rat liver mitochondria with MG led to complete suppression of both ganglioside GD3- and Ca\(^{2+}\)-induced permeability transition. This also induced inhibition of cytochrome c release. Suppression of permeability transition by MG could be reversed by extended incubation in MG-free media. These findings demonstrate that MG induced a reversible covalent PTP modification, most likely an imidazoline derivative on an arginine residue involved in the control of the PTP conformation (29–32). The results of this study raise the possibility that MG reacts with the PTP under physiological conditions and that the altered PTP regulation perturbs the cell death program.

**EXPERIMENTAL PROCEDURES**

**Chemical Modification of Mitochondria**—Preparation of liver mitochondria from male Wistar rats was performed as described previously (30). Mitochondria (1 mg of protein/ml) were preincubated in modification medium containing 250 mM sucrose, 100 mM EGTA, 10 mM Hepes-KOH, pH 7.8, for 5 min without MG or 5 mM MG in a closed chamber at 34 °C. The modification reaction was terminated by adjusting the pH to 6.8 with Hepes, cooling to 4 °C, and mitochondria were sedimented by centrifuging at 8000 × g for 5 min. Mitochondria were resuspended at a concentration of ~50 mg of protein/ml in 250 mM sucrose, 100 mM EGTA, and 10 mM Hepes-KOH, pH 7.4. Unless otherwise stated experiments were carried out at room temperature in assay medium containing 125 mM KCl, 5 mM succinate, 5 mM Pi-Tris, 2 mM Mg\(^{2+}\), 5 mM EGTA, 2 mM rotenone, 10 mM Hepes-Tris, pH 7.4. In one experimental series MG was substituted by PGO. Oxygen consumption was measured using a Clark electrode (Yellow Springs Instruments).

**PTP Assays**—Permeability transition was assayed by measuring matrix potential, Ca\(^{2+}\) transport and swelling. Medium [Ca\(^{2+}\)] was measured using the fluorescent indicator dye Fluoro-4FF (ex, 494 nm; em, 516 nm). Membrane potential was measured by TMRR fluorescence (ex, 550 nm; em, 575 nm), and swelling was monitored as a decrease in mitochondrial light scattering at 540 nm. Measurements were performed using a 96-well Tecan Spectrafluor plus plate reader or a Perkin-Elmer Life Sciences luminescence spectrometer LS50B. In swelling experiments, measured light scattering (I) was normalized by setting the initial scattering (I\(_0\)) of the mitochondrial suspension to one unit. Permeability transition was quantified using the initial swelling rate following addition of Ca\(^{2+}\). The initial swelling rate for mitochondria preincubated in the absence of MG was set to 100% permeability transition. The initial swelling rate for mitochondria preincubated in the absence of MG but supplemented with 1 μM Ca\(^{2+}\) was set to 0% permeability transition. GD3 was dissolved to 5 mM in water and sonicated in a water bath for 5 min before use. To test compounds containing the carbonyl group for effects on the PTP, mitochondria were incubated at 1 mM of protein/ml for 15 min at room temperature in assay medium supplemented with the compound of interest at the following concentrations: 10, 30, 100, and 300 mM, 1, 3, 10, 30, 100, and 330 mM, and 1, 3, and 10 mM. Mitochondria were diluted five times by addition of assay medium, and the effect of the compounds on the PTP was assessed following addition of Ca\(^{2+}\). Permeability transition was quantified as rate of swelling.

**Cytochrome c Release**—Release of cytochrome c was determined by immunoblotting. The mitochondrial suspension was removed from the photometer cuvette, cooled to +4 °C degrees and centrifuged for 3 min at 21,000 × g. The pellet and the supernatant were separated, and proteins were precipitated by addition of 10% trichloroacetic acid. Precipitated proteins were separated by SDS-PAGE using 12% gels and electrotransferred to a polyvinylidene fluoride membrane. Immunoblotting was performed using a monoclonal cytochrome c antibody (Zymed Laboratories) and visualized using the ECL system (Amersham Biosciences).

**Mass Spectrometry**—For MALDI-TOF mass spectrometry of MG-derived arginine adducts 100 μM test peptide (NRYIVHHPHF, KYVET, pEWPRQIPP, YGGFMRF) was allowed to react with 2 mm MG in 10 mM Hepes-KOH, pH 8.0, at 34 °C. The reaction was stopped by adding 0.1% trifluoroacetic acid and cooling to 4 °C. The reaction mixture was desalted using a Zip Tip C18 silica bead microcolumn (Millipore). Peptides were eluted with acetonitrile/0.1% trifluoroacetic acid 1:2 and mixed with an equal volume of saturated ammonium citrate (1:1) and applied on the target. MALDI-TOF mass spectra were recorded on a Bruker Autoflex spectrometer using the linear detector in positive mode. Calibration of the machine was performed using the peaks of α-HCA and peptides of known masses. The rate constant value for formation of MG-derived arginine adducts was estimated from the relative peak intensity of native peptide and its MG derivatives after reaction for 1 min. For mass analysis of the GD3 ganglioside 0.5 nmol was mixed with 1 μl of 1 mM 2,4,6-trihydroxyacetophenone in acetoni- trile/20 mM ammonium citrate (1:1) and applied on the target. Mass spectra were recorded using the linear detector in negative mode.

**Electron Microscopy**—Fixation of mitochondria was performed by 1% glutaraldehyde directly to the suspension. Embedding, sectioning, and staining were performed as described previously (30). Sections were viewed in a Jeol 1200 transmission electron microscope at a magnification of ×10,000.

**Chemicals**—Ca\(^{2+}\) was a gift from Novartis, OH-PGO was from Pierce, 3-deoxyglucosone was from Toronto Research Chemicals, TMRM and Fluor-4FF were from Molecular Probes, and ganglioside GD3 was from Calbiochem. Analysis of GD3 by MALDI-TOF demonstrated that the C-2 N-fatty acyl-sphingosine moiety was a C18 fatty acid in 9%, C19 in 15%, C20 in 25%, C21 in 31%, and C22 in 20%. Other chemicals were from Sigma. 4′-Pyridyl 5-carboxylate was regenerated from its 2,4-dinitrophenylhydrazine derivative as described previously (33).

**RESULTS**

We first studied the effect of MG on permeability transition induced either by the ganglioside GD3 or Ca\(^{2+}\). Mitochondria were preincubated for 5 min in the presence or absence of 2 mM MG followed by removal of free MG by centrifugation. These mitochondria were then incubated for 30 min in assay medium containing 25 μM GD3 or 10 μM Ca\(^{2+}\), whereupon mitochondria were processed for ultrastructural analysis by transmission electron microscopy and for analysis of cytochrome c release by immunoblotting. The results, shown in Fig. 1, demonstrated that both GD3 and Ca\(^{2+}\) caused extensive swelling and cytochrome c release in mitochondria preincubated in the absence of MG. Supplementing the medium with Ca\(^{2+}\), a selective high affinity inhibitor of permeability transition, prevented mito-
Sections were viewed at a magnification of glutaraldehyde followed by embedding, thin-sectioning, and staining. Indicate cytochrome c release by MG treatment on the PTP, and not via these factors, investigating the effect of following compounds: 25 μg/mL of protein/mL. The mitochondrial suspensions were supplemented with the following compounds: 25 μg/mL GD3, 25 μg/mL GD3 plus 1 μg/mL CsA, 10 μg/mL Ca2+, or 10 μg/mL Ca2+ plus 1 μg/mL CsA. The suspensions were incubated at room temperature for 30 min, whereupon mitochondria were sedimented by centrifugation. Aliquots of the supernatant and pellets were taken for SDS-PAGE and immunoblotting of cytochrome c. S and P indicate cytochrome c detected in the supernatant and pellet, respectively. Chemical fixation of mitochondria was performed using 1% glutaraldehyde followed by embedding, thin-sectioning, and staining. Sections were viewed at a magnification of ×10,000. The scale bar is 1 μm.

Mitochondrial swelling and cytochrome c release, showing that both effects were due to permeability transition. Likewise, preincubation of mitochondria with MG completely prevented GD3- and Ca2+-induced swelling and cytochrome c release. These findings demonstrated that brief MG treatment effectively suppresses mitochondrial permeability transition.

The onset of permeability transition is dependent on Ca2+ uptake and production of Δψ by substrate oxidation under the conditions used in Fig. 1. We verified that MG acted directly on the PTP, and not via these factors, investigating the effect of MG treatment on Δψ and Ca2+ transport. The membrane potential-sensitive dye TMRM was used to measure Δψ. Ca2+ uptake was assessed by measuring medium [Ca2+] using the membrane impermeable dye fluo-4FF, which becomes fluorescent upon Ca2+ binding. Mitochondrial swelling was monitored as a decrease in light scattering. First we studied the effect of GD3 on Δψ and swelling (Fig. 2). Mitochondria preincubated in the absence or presence of MG were suspended in assay medium, and 10 μM Ca2+ was added after 2 min. This added led to a rapid accumulation of TMRM, regardless of whether MG had been present or not during preincubation, showing that MG did not interfere with the production of Δψ by substrate oxidation (Fig. 2A). However, in mitochondria preincubated in the absence of MG the addition of 25 μg/mL GD3 caused a release of TMRM, indicating a drop in Δψ (trace a). Addition of GD3 also induced a decrease in light scattering indicating that these mitochondria underwent swelling (Fig. 2B, trace a). Supplementing the medium of these mitochondria with CsA (trace b) or using MG-treated mitochondria (trace c) prevented both the GD3-induced drop in Δψ and swelling.

We then proceeded to study Δψ, Ca2+ transport and swelling following Ca2+ addition (Fig. 3). Mitochondria were suspended in assay medium, and 10 μM Ca2+ was added after 2 min. The increase in medium [Ca2+] resulted in an increase in fluo-4FF fluorescence (panel C). Three minutes later mitochondria were energized by the addition of 5 mM succinate. In mitochondria preincubated without MG, this addition caused rapid swelling as indicated by the decrease in light scattering (panel B, trace a). Consistently, these mitochondria failed to produce Δψ in the presence of Ca2+ as indicated by the lack of both TMRM and Ca2+ accumulation (panels A and C, trace a). However, supplementing the medium of these mitochondria with CsA prevented swelling, and the mitochondria were able both to maintain Δψ (panel A, trace b) and take up Ca2+ (panel C, trace b). As expected, succinate addition to MG-treated mitochondria resulted in an immediate production of Δψ (panel A, trace c), rapid Ca2+ uptake (panel C, trace c), and prevention of swelling. These results indicate that MG acts directly on the PTP and not on Δψ production or Ca2+ uptake.

This finding was further corroborated by measurements of respiration rate and Δψ production by ATP hydrolysis (Fig. 4). The results indicated that mitochondria retained their maximal respiration rates in the presence of ADP or the protonophoric uncoupler FCCP following preincubation with up to 2 mM MG (left panel). Similarly, preincubation with 2 mM MG had no effect on Δψ production by ATP hydrolysis as measured by the uptake of TMRM indicating that the function of the adenine nucleotide translocator and the ATPase was not affected by MG (right panel).
In these initial experiments we used a supraphysiological MG concentration. To investigate the physiological significance of our findings, it was necessary to determine the quantitative relationship between MG concentration and suppression of permeability transition. Therefore we preincubated mitochondria with varying concentrations of MG followed by permeabilization using Ca\(^{2+}\)/H\(_{11001}\) as the triggering signal. Permeability transition was quantified using the initial swelling rates, which were plotted against the preincubation concentrations of MG (Fig. 5). The plot indicates that the apparent \(K_{50}\) for PTP inhibition is \(600\) \(\mu\)M and that MG had already a significant effect at \(250\) \(\mu\)M.

Because MG reacts with proteins, resulting in both reversible and irreversible adducts, it was of interest to determine whether the effect of MG was reversible or not. To address this question we preincubated mitochondria with 2 mM MG or 2 mM PGO, which is known to form an irreversible adduct (30). Following the modification reaction and removal of free MG or PGO, mitochondria were incubated in assay medium at room temperature for up to 3 h whereupon permeability transition was measured by swelling. The results shown in Fig. 5 (inset) indicated that suppression of permeability transition by MG was transient and disappeared after 2 h at room temperature. As shown previously the effect of PGO was irreversible (30).

We then proceeded to investigate whether any physiological changes were associated with MG-induced suppression of permeability transition. The left panel depicts mitochondrial oxygen consumption measured using a Clark electrode. Mitochondria were preincubated with the indicated concentrations of MG followed by centrifugation and resuspension at a concentration of 0.2 mg of protein/ml in assay medium. Oxygen consumption was calculated from the decrease in medium oxygen concentration following the addition of succinate, ADP, and FCCP, respectively. Values are expressed as mean ± S.E. obtained for three mitochondrial preparations. The right panel shows membrane potential as measured in Fig. 2. ATP (1 mM) was added to energize mitochondria after 3 min. Dotted trace, mitochondria treated with 2 mM MG. Solid trace, mitochondria preincubated in the absence of MG.

**Fig. 3.** Suppression of Ca\(^{2+}\)-induced permeability transition by MG. Mitochondria were preincubated, centrifuged, and resuspended as in Fig. 1. Ca\(^{2+}\) (10 \(\mu\)M) was added after 2 min followed by succinate (5 mM) after 5 min. A, membrane potential as measured in Fig. 2. B, swelling as measured in Fig. 2. C, the Ca\(^{2+}\) concentration of the medium measured using the fluorescent dye Fluo-4FF. Traces a and b show mitochondria preincubated in the absence of MG, and trace c shows MG-treated mitochondria. The suspension was supplemented with 1 \(\mu\)M CsA in trace b.

**Fig. 4.** Effect of MG on mitochondrial respiration and production of \(\Delta\psi\) by ATP hydrolysis. The left panel depicts mitochondrial oxygen consumption measured using a Clark electrode. Mitochondria were preincubated with the indicated concentrations of MG followed by centrifugation and resuspension at a concentration of 0.2 mg of protein/ml in assay medium. The suspension was added to the electrode chamber, and succinate (5 mM) was added immediately followed by ADP (200 \(\mu\)M) after 3 min and FCCP (1 \(\mu\)M) after 6 min. The oxygen consumption was calculated from the decrease in medium oxygen concentration following the addition of succinate, ADP, and FCCP, respectively. Values are expressed as mean ± S.E. obtained for three mitochondrial preparations. The right panel shows membrane potential as measured in Fig. 2. ATP (1 mM) was added to energize mitochondria after 3 min. Dotted trace, mitochondria treated with 2 mM MG. Solid trace, mitochondria preincubated in the absence of MG.

**Fig. 5.** Concentration dependence and reversibility of MG-induced suppression of permeability transition. Mitochondria were incubated with the indicated concentrations of MG followed by centrifugation and resuspension at a concentration of 0.2 mg of protein/ml in assay medium. Permeability transition was assayed exactly as in Fig. 3B. Swelling was quantified using the initial decrease in light scattering as described under “Experimental Procedures.” Inset, mitochondria preincubated either with 2 mM MG (circles) or 2 mM PGO (triangles) were suspended in assay medium at a concentration of 0.2 mg of protein/ml at room temperature for the indicated time. Permeability transition was quantified as in the main figure. Values are expressed as mean ± S.E. obtained for three mitochondrial preparations.
carbonyl compound other than MG was able to suppress permeability transition. We selected candidate carbonyl compounds from the main pathways of carbonydrate, amino acid, and fatty acid metabolism and some aliphatic aldehydes formed in the metabolism of alcohols. Mitochondria were incubated with each compound at concentrations of up to 10 mM in modification medium, whereupon permeability transition was assayed by swelling, using Ca$^{2+}$ as the triggering signal. The results are presented in Fig. 6 and Table I. We first tested the α-oxoaldehydes glyoxal and 3-deoxyglucosone, both of which are implicated in the formation of AGEs. Data showed that, whereas glyoxal effectively suppressed permeability transition at an apparent $K_{50}$ of 2 mM, 3-deoxyglucosone was completely without effect under the same conditions. CsA inhibited permeability transition in the submicromolar concentration range. For comparison we also used the highly toxic cross-linker glutaraldehyde, which was effective in the submillimolar concentration range. Other carbonyls were analyzed in a similar way, plots were constructed as in Fig. 6, and the results of these titrations are presented in Table I. The data indicate that none of the other compounds specifically altered the behavior of the PTP, despite the high chemical reactivity of several of them.

In general, formation of significant amounts of MG-derived arginine adducts on proteins requires hours or even days (12, 13, 34). In contrast, our findings showed that complete suppression of permeability transition by MG required a reaction time of only a few minutes. Therefore, it was of interest to clarify the nature of the MG adduct formed after the short incubation time used in this study. We analyzed the products formed in the reaction of 2 mM MG with the following arginine-containing peptides: NRVYIHPFHL (A), RVYVHPF (B), pEWPRQIPP (C), and YGGFMRF (D). The crude reaction mixture containing peptides: NRVYIHPFHL (A), RVYVHPF (B), pEWPRQIPP (C), and YGGFMRF (D) was subjected to desalting in C18 reversed-phase Zip tips followed by MALDI-TOF mass spectrometry analysis. After incubation with MG for 1 h, no adduct could be detected on peptides A and B, but prominent additional peaks indicating an increase in molecular mass of 54 could be detected on peptides C and D. Peptide D was selected for studying the time dependence of the reaction, because it was the most reactive. In the absence of MG the native peptide D gave rise to a single peak at m/z 877.4 (M+H$^+$) (Fig. 7). After incubation for 1 min additional peaks appeared at m/z 931.7 corresponding to the 5-hydro-5-methylimidazol-4-one derivative, and at m/z 913.7 corresponding to the 5-methylimidazol-4-one derivative. Peaks corresponding to the expected molecular weight of pyrimidine- and tetrahydro-pyrimidine derivatives could not be detected. These findings demonstrate that the reaction between MG and peptidyl-arginine can proceed to completion within minutes and suggest that the predominating adduct formed under these conditions is the 5-hydro-5-methylimidazol-4-one derivative.

**DISCUSSION**

In this study we have characterized a novel target of MG: the mitochondrial permeability transition pore. We have demonstrated that incubation of isolated mitochondria with MG in the millimolar concentration range for a short time was sufficient to completely suppress permeability transition. Under these conditions MG had no significant side effects on mitochondrial substrate transport, respiration, or oxidative phosphorylation demonstrating that MG acted selectively on the permeability transition. The suppression of permeability transition persisted following removal of free MG indicating that MG had formed a covalent ligand on the PTP or on an associated regulatory protein. Nevertheless, extended incubation in the absence of MG reversed the effect of MG on permeability transition. Because MG reacts almost exclusively with arginine residues under these conditions (12, 13), we suggest that MG

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**TABLE I**

| Compound                  | $K_{50}$ (mM) | Swelling at 1 mM (%) | ±S.E. | n  |
|---------------------------|--------------|----------------------|-------|----|
| **2-Oxoaldehydes**        |              |                      |       |    |
| Glyoxal                   | 2            | 56.8                 | 9.7   | 6  |
| 3-Deoxyglucosone          | 115.2        | 16.9                 | 3     |    |
| **Carbohydrates and derivatives** |          |                      |       |    |
| D,L-Glyceraldehyde        | 101.4        | 3.2                  | 6     |    |
| D,L-Erythrose             | 95.4         | 16.7                 | 2     |    |
| D,L-Ribose                | 89.0         | 14.0                 | 2     |    |
| D,L-Galactose             | 130.9        | 20.3                 | 4     |    |
| Dihydroxyacetone          | 98.5         | 3.7                  | 4     |    |
| p-Erythryllose            | 87.5         | 22.4                 | 2     |    |
| d-Ribulose                | 105.5        | 23.7                 | 2     |    |
| D-Fructose                | 89.2         | 19.5                 | 2     |    |
| D-Glucaric acid           | 115.0        | 23.0                 | 4     |    |
| D-Glucose 6-phosphate     | 87.4         | 6.6                  | 6     |    |
| D-Fructose 6-phosphate    | 108.2        | 50.6                 | 6     |    |
| **Amino acid metabolites**|              |                      |       |    |
| Oxaloacetate              | 92.9         | 5.1                  | 6     |    |
| Pyruvate                  | 107.0        | 8.4                  | 2     |    |
| a-Ketoglutarate           | 113.7        | 9.1                  | 4     |    |
| p-Hydroxyphenylpyruvate   | 96.9         | 11.0                 | 6     |    |
| Phenylpyruvate            | 92.6         | 8.6                  | 6     |    |
| a-Ketobutyrate            | 92.4         | 7.5                  | 4     |    |
| a-Ketoadipate             | 94.2         | 1.8                  | 2     |    |
| 2-A-Pyrrolidine 5-carboxylic acid | 105.4 | 5.7                  | 3     |    |
| **Other aldehydes**       |              |                      |       |    |
| Formaldehyde (b)          | >10$^*$      | 78.4                 | 6.5   | 2  |
| Acetaldehyde (b)          | >10$^*$      | 76.0                 | 12.4  | 2  |
| Propionaldehyde           | 103.2        | 11.6                 | 4     |    |
| Glycoldehyde              | 97.6         | 8.4                  | 8     |    |
| Acetone                   | 78.9         | 12.3                 | 3     |    |
| Hydroxyacetone            | 124.0        | 19.3                 | 6     |    |
| Acetoacetic acid          | 124.5        | 9.5                  | 3     |    |
| Trans 2-hexenal           | 76.9         | 11.2                 | 4     |    |
| Glutaraldehyde            | 0.1$^*$      | 1.7                  | 1.4   | 2  |
| CaA                       | 0.2 μM       | <0.5                 | 2     |    |

$^*$ These compounds inhibited substrate transport and/or respiration.
$^*$ The initial swelling rate for mitochondria in the presence of CaA was set to 0% as described under "Experimental Procedures."
targets the same arginine residue(s) as the synthetic reagents PGO, BAD, and OH-PGO (29–32).

In these experiments we have used the ganglioside GD3 or Ca\(^{2+}\) as signals to induce permeability transition. In several cell types the ganglioside GD3 is formed as a consequence of tumor necrosis factor-a or Fas receptor activation (35) and functions as an apoptosis mediator by transferring from the plasma membrane to mitochondria (36), where it induces permeability transition (23–25). Influx of Ca\(^{2+}\) through the plasma membrane leading to extensive mitochondrial Ca\(^{2+}\) accumulation and subsequent permeability transition is of key importance in neuronal death (37). In all likelihood, mobilization of either GD3 or Ca\(^{2+}\) during cell death processes is essential for induction of mitochondrial permeability transition. Because MG suppressed both GD3- and Ca\(^{2+}\)-induced permeability transition, we conclude that the effect of MG is robust and not restricted to narrowly defined conditions.

To approach the question of whether MG can regulate the permeability transition \textit{in vivo} we devised an experimental approach based on the predicted behavior of MG in the cell. In the intracellular milieu, production of MG is limited, and therefore several factors contribute to determine on which proteins MG-derived adducts are formed. The net production of MG is undoubtedly the most important factor influencing the total number of proteins modified by MG. However, under conditions of limiting MG production the selection of individual targets is not a fortuitous event but is determined by the relative target reactivity, which in turn depends on the local chemical environment. If free and reversibly bound MG are in dynamic equilibrium (11), the extent of modification of different targets is expected to be proportional to their respective equilibrium constants. We therefore characterized the modification reaction regarding its concentration dependence, reversibility, and selectivity for the permeability transition. We also investigated whether physiological carbonyl compounds other than MG could modify the permeability transition.

Concentration dependence measurements showed that a significant suppression of permeability transition by MG could be observed already after 5 min of incubation at 250 \(\mu\text{M}\). This concentration of free MG is in excess of that detected in living systems. However, in cells the MG production goes on more or less constantly leading to an equilibrium between free and protein-bound MG, which is shifted far in the direction toward the bound form. Consistent with this, the total concentration of MG in cells can be as high as 310 \(\mu\text{M}\) (11). Accordingly, MG-induced suppression of permeability transition was reversible, indicating that the MG adduct slowly decomposes and restores the PTP to its native state, suggesting that the level of modification of the PTP is proportional to the prevailing MG concentration. Furthermore, MG did not affect other mitochondrial functions suggesting that the arginine of interest is exceptionally reactive and hence that MG targets primarily the permeability transition.

A functional significance is also suggested by the observation that MG and its structural analogue glyoxal were the only physiological carbonyl compounds, among a large number of related compounds tested that selectively suppressed the permeability transition. Most notably, the reactive \(\alpha\)-oxoaldehyde 3-deoxyglucosone, which is an important mediator of the formation of AGEs (38), completely lacked effect. Our results suggest that MG and glyoxal are the only physiological carbonyl compounds that form covalent ligands on the PTP. Because the reaction between MG and the PTP was fast, reversible, selective, and specific, this suggests the possibility that MG regulates the permeability transition \textit{in vivo}.

The PTP can also be modified by the synthetic MG analogues PGO and OH-PGO (30–32). The resulting uncharged PGO adduct strongly suppresses permeability transition, whereas the negatively charged OH-PGO adduct promotes permeability transition (32). This led us to propose that the effect of arginine modification on the PTP conformation is due to the electrical charge of the resulting adduct. To test this hypothesis we investigated the reaction product of MG and a set of arginine-containing test peptides under the same conditions as were used for incubating mitochondria. The molecular mass of the detected compounds indicated that the major products formed during the first minutes of the reaction were imidazolone derivatives. This finding is consistent with our hypothesis that uncharged adducts stabilize the closed conformation of the PTP. The results also demonstrated that the reactivity of the arginine-peptides with MG varied largely, probably owing to the local chemical environment of the respective arginine residues. The rate constant value for the formation of the 5-hydro-5-methylimidazol-4-one derivatives from the most reactive test peptide and MG was 1.5 \(\text{m}^{-1}\text{s}^{-1}\). This value is 100–1000-fold greater than that previously reported for the formation of the same derivative from N-acetylarginine and MG (13). These findings demonstrate that modification of arginine residues by MG can proceed at an unprecedented speed.

The major intracellular precursors of MG are the glycolysis intermediates dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (5–8). The former compound takes part in the glycerol phosphate shuttle, in which cytosolic NADH reduces dihydroxyacetone phosphate to glycerol 3-phosphate, which is then reoxidized by glycerol phosphate dehydrogenase of the inner membrane. Owing to this design the local concentration of triose phosphates is presumably elevated in the vicinity of mitochondria. The PTP thus constitutes a nearby target for MG formed from triose phosphates. Consequently, we propose that MG may react with the PTP in living organisms, leading to MG-induced suppression of permeability transition and alterations of the regulation of the mitochondrial apoptosis pathway. This mechanism may be important under conditions of enhanced flux through glycolysis where the rate of MG production is increased over the normal level. The flux through glycolysis is increased not only during diabetic hyperglycemia (39) but also in many malignant tumors (40).

The findings of this and other recent studies (16–19) suggest that MG-induced modification of specific target proteins may play a role in cell signaling inducing specific alterations in cell 

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behavior, as proposed previously (41). It is conceivable that these mechanisms operate at low MG concentration below the threshold of unspecific toxicity. The question of how specific MG-induced protein modifications are integrated in the regulation of cell signaling pathways should be the subject of future studies.

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Addendum—During review of this paper, we became aware of a study on the effect of MG on mitochondrial respiration in cardiac cells (Roy, S. S., Biswas, S., Ray, M., and Ray, S. (2003) Biochem. J. 372, 661–669).

REFERENCES
1. Thornalley, P. J. (1995) Crit. Rev. Oncol. Hematol. 20, 99–128
2. Brownlee, M. (2001) Nature 414, 813–820
3. Nakamura, S., Makita, Z., Ishikawa, S., Yasumura, S., Fujii, W., Yanagisawa, K., Kawata, T., and Koike, T. (1997) Diabetes 46, 895–899
4. Hammes, H.-P., Martin, S., Federlin, K., Geisen, K., and Brownlee, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 15555–15558
5. Phillips, S. A., and Thornalley, P. J. (1993) Eur. J. Biochem. 212, 101–105
6. Pompliano, D. L., Peyman, A., and Knowles, J. R. (1990) Biochemistry 29, 3186–3194
7. Richard, J. P. (1991) Biochemistry 30, 4581–4585
8. Williams, J. C., and McDermott, A. E. (1995) Biochemistry 34, 8309–8319
9. Shonk, C. E., and Boxer, G. E. (1964) Cancer Res. 24, 709–721
10. Srivastava, D. K., and Bernhard, S. A. (1987) Ann. Rev. Biophys. Biophys. Chem. 16, 175–204
11. Chaplin, F. R. W., Fahl, W. E., and Cameron, D. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5533–5538
12. Westwood, M. E., and Thornalley, P. J. (1995) J. Biol. Chem. 270, 12035–12040
13. Lo, T. W. C., Westwood, M. E., McLellan, A. C., Selwood, T., and Thornalley, P. J. (1994) J. Biol. Chem. 269, 32299–32305
14. Shipanov, I. N., Glomb, M. A., and Nagaur, R. H. (1997) Arch. Biochem. Biophys. 344, 29–36
15. Oya, T., Hattori, N., Mizuno, Y., Miyata, S., Maeda, S., Osawa, T., and Uchida, K. (1999) J. Biol. Chem. 274, 18492–18502
16. Sakamoto, H., Mashima, T., Yamamoto, K., and Tsuruo, T. (2002) J. Biol. Chem. 277, 45770–45775
17. Portero-Otín, M., Pampalona, R., Bellmunt, M. J., Ruiz, M. C., Prat, J., Salavray, R., and Negre-Salvayre, A. (2002) Diabetes 51, 1535–1542
18. Godbout, J. P., Pesavento, J., Hartman, M. E., Manson, S. R., and Freund, G. G. (2002) J. Biol. Chem. 277, 2554–2561
19. Van Herreweghe, F., Mao, J., Chaplin, F. W. R., Grooten, J., Gevaert, K., Vandekerckhove, J., and Vankompernolle, K. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 849–854
20. Hengartner, M. O. (2000) Nature 407, 770–776
21. Ya, S.-W., Wang, H., Potiras, M. F., Coombs, C., Bowers, W. J., Fedoroff, H. J., Poirier, G. G., Dawson, T. M., and Dawson, V. L. (2002) Science 297, 259–263
22. Bernardi, P. (1999) Physiol. Rev. 79, 1127–1155
23. Kristal, B. S., and Brown, A. M. (1999) J. Biol. Chem. 274, 23169–23175
24. Scorrano, L., Petronilli, V., Li, L., and Bernardi, P. (1999) J. Biol. Chem. 274, 25981–25985
25. Garcia-Ruiz, C., Coeli, A., Paris, R., and Fernández-Checa, J. C. (2000) FASEB J. 14, 847–858
26. Eriksson, O., Pollesello, P., and Geimonen, E. (1999) Am. J. Physiol. 276, C1297–C1302
27. Desagher, S., and Martinou, J.-C. (2000) Trends Cell Biol. 10, 369–377
28. Viera, H. L. A., Haouzi, D., El Hamel, C., Jaestot, E., Belzaag, A. S., Brenner, C., and Kroemer, G. (2000) Cell Death Differ. 7, 1146–1154
29. Eriksson, O., Fontaine, E., Petronilli, V., and Bernardi, P. (1997) FEBS Lett. 409, 361–364
30. Eriksson, O., Fontaine, E., and Bernardi, P. (1998) J. Biol. Chem. 273, 12669–12674
31. Scorrano, L., Penzo, D., Petronilli, V., Pagano, F., and Bernardi, P. (2001) J. Biol. Chem. 276, 12035–12040
32. Linder, M. D., Markunaite-Haimi, S., Kinnunen, P. K. J., Bernardi, P., and Eriksson, O. (2002) J. Biol. Chem. 277, 937–942
33. Mez, V. A., and Knox, W. E. (1976) Anal. Biochem. 74, 430–440
34. Riley, M. L., and Harding, J. J. (1995) Biochim. Biophys. Acta 1270, 36–43
35. De Maria, R., Lenti, L., Malsan, F., d’Agostino, F., Tomassini, B., Zeuner, A., Rippe, M. R., and Testi, R. (1997) Science 277, 1652–1655
36. Garcia-Ruiz, C., Coeli, A., Morales, A., Calvo, M., Enrich, C., and Fernandez-Checa, J. C. (2002) J. Biol. Chem. 277, 36443–36448
37. Mattson, M. P. (2000) Nat. Rev. Mol. Cell. Biol. 1, 120–129
38. Niwa, T. (1999) J. Chromatogr. B. 781, 23–36
39. Nishikawa, T., Edelstein, D., Du, X. L., Yamagashi, S.-I., Matsumura, T., Kaneda, Y., Yorek, M. A., Beebe, D., Oates, P. J., Hammers, H.-P., Giardino, I., and Brownlee, M. (2000) Nature 404, 787–790
40. Harris, A. L. (2002) Nat. Rev. Cancer 2, 38–47
41. Egyud, L. G., and Szent-Györgyi, A. (1966) Proc. Natl. Acad. Sci. U. S. A. 56, 203–207

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