Modification of Permeability Transition Pore Arginine(s) by Phenylglyoxal Derivatives in Isolated Mitochondria and Mammalian Cells

STRUCTURE-FUNCTION RELATIONSHIP OF ARGININE LIGANDS*  

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Mitochondrial permeability transition is a crucial event in many forms of cell death (1). Permeability transition is a consequence of the opening of the permeability transition pore (PTP), a high conductance inner membrane channel (2, 3). The PTP is usually closed during normal cell life (4, 5) but can be triggered to open by an increase in matrix Ca\(^{2+}\) (2, 3) and by specific cell death signals such as ganglioside GD3 (6, 7) or arachidonic acid (8). Permeability transition promotes cell death by inducing a cellular bioenergetic crisis and by causing the release of mitochondrial proapoptotic proteins that activate caspases and degrade DNA (9). Opening of the PTP is inhibited by mitochondrial cyclophilin ligands, such as cyclosporin A (CsA) and sanglifehrin A (SfA) (10). In addition, the opening and closing of the PTP is modulated by adenine nucleotide carrier ligands (2, 3). Collectively, these findings have led to a model in which the PTP is formed by an interaction between the adenine nucleotide carrier (ANT), cyclophilin and the voltage-dependent anion channel (1, 11–13). The involvement of the voltage-dependent anion channel is supported by the identification of a novel selective high affinity PTP inhibitor, compound Ro 68-3400 (14). However, the recent finding that mitochondria from ANT knock-out mice, lacking both ANT isoforms, undergo CsA-sensitive permeability transition (15) suggests that ANT is not an essential component of the PTP.

Previously, we have studied PTP regulation using amino acid-specific covalent modification. Employing histidine- and cysteine-specific covalent reagents, we identified three separate sites through which matrix pH and the NADH/NAD\(^{+}\) ratio can influence the opening and closing of the PTP (16, 17). Using phenylglyoxal (PGO), 4-hydroxyphenylglyoxal (OH-PGO), 2,3-butanedione, and methylglyoxal, which form stable adducts with guanidino groups, we have also detected reactive arginine residue(s) on the PTP (8, 18–21). The arginine adducts formed by methylglyoxal, PGO, and 2,3-butanedione stabilize the closed conformation of the PTP, preventing triggering signals from inducing PTP opening, whereas the arginine adduct formed by OH-PGO results in PTP opening even in the absence of triggering signals (20). This suggests that the glyoxal-reactive target arginine(s) is functionally coupled to the opening and closing mechanism of the pore (20). Interestingly, methylglyoxal, and other phenylglyoxal derivatives, react covalently with arginine residue(s) on the mitochondrial permeability transition pore (PTP). In this study, we have investigated how the binding of a panel of synthetic phenylglyoxal derivatives influences the opening and closing of the PTP. Using both isolated mitochondria and mammalian cells, we demonstrate that the resulting arginine-phenylglyoxal adduct can lead to either suppression or induction of permeability transition, depending on the net charge and hydrogen bonding capacity of the adduct. We report that phenylglyoxal derivatives that possess a net negative charge and/or are capable of forming hydrogen bonds induced permeability transition. Derivatives that were overall electroneutral and cannot form hydrogen bonds suppressed permeability transition. When mammalian cells were incubated with low concentrations of negatively charged phenylglyoxal derivatives, the addition of oligomycin caused a depolarization of the mitochondrial membrane potential. This depolarization was completely blocked by cyclosporin A, a PTP opening inhibitor, indicating that the depolarization was due to PTP opening. Collectively, these findings highlight that the target arginine(s) is functionally linked with the opening/closing mechanism of the PTP and that the electric charge and hydrogen bonding of the resulting arginine adduct influences the conformation of the PTP. These results are consistent with a model where the target arginine plays a role as a voltage sensor.
glyoxal is constantly formed during glucose metabolism (22). The intracellular concentration of methylglyoxal is increased during hyperglycemia, and this leads to the formation of methylglyoxal-induced protein modifications, advanced glycation end products (AGEs), which disrupt the function of target proteins (22). AGEs are involved in several pathological processes, including diabetes mellitus and cellular proliferative disorders (23). We have proposed that methylglyoxal-induced disruption of the PTP function contributes to the development of these pathological conditions (21).

Several experimental findings indicate a decrease in the electric potential difference (ΔΨ) across the inner mitochondrial membrane leads to an increase in the probability of the PTP being open, suggesting that the PTP directly senses the ΔΨ. In isolated Ca²⁺-primed mitochondria, a partial depolarization of the ΔΨ is sufficient to open the PTP (24, 25). Consistently, in patch-clamping experiments of mitoplasts, the PTP is more likely to be open at low ΔΨ (26). The mechanism for voltage sensing by voltage-dependent ion channels is based on the movement and reorientation of positively charged amino acids in the transmembrane electric field (27). Based on (i) the effect of arginine modification on PTP conformation and (ii) the exquisite sensitivity of PTP opening and closing to small structural differences between the arginine-reactive compounds, we have proposed that the arginine(s) is located on a putative voltage-sensing element of the PTP (19, 20). Covalent modification of the voltage-sensing arginines is expected to exert a large influence on sensor orientation and therefore on pore conformation. However, the resulting sensor orientation, opened or closed, is determined by an interplay between the steric and electronic properties of the resulting arginine adduct.

In this study, we have used a set of synthetic phenylglyoxal derivatives to investigate how arginine modification alters PTP function in isolated mitochondria and mammalian cells. These phenylglyoxal derivatives differ only in the functional groups attached to the 2-, 3-, and/or 4-position of the phenyl ring. This allowed us to compare the specific physicochemical properties of the attached groups with their effects on PTP opening and closing. We found that arginine modification by these derivatives could lead either to induction or suppression of permeability transition. A physicochemical analysis revealed that negative charge and/or an ability to form hydrogen bonds was a common property of phenylglyoxal derivatives that induced permeability transition. Incubation of mammalian cells with negatively charged phenylglyoxal derivatives resulted in an increased probability of the PTP being open, and this correlated with a decrease in cell viability. These findings are consistent with a model where the target arginine(s) plays a role in the PTP voltage sensing and indicate that covalent arginine modification can affect the regulation of the PTP in intact cells.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—The structural formulas of the phenylglyoxal derivatives used in this study are shown in Fig. 1. OH-PGO and MeO-PGO were synthesized from 4-hydroxycetophenone and 4-methoxyacetophenone by oxidation with selenium dioxide (28). Identification of the reaction product was performed using 1H NMR and 13C NMR spectra. NO-PGO and Flu-4FF were purchased from Molecular Probes, Inc. (Eugene, OR). CamOH-PGO, Cl-PGO, 2,4-diF-PGO, F-PGO, Me-PGO, and Mor-PGO were from SynChem. CSa and SFA were gifts from Novartis. All other chemicals were purchased from Sigma except where otherwise noted.

**Chemical Reactivity of Phenylglyoxal Derivatives with Arginine**—The chemical reactions of PGO and OH-PGO with N-acetyllarginine and peptideylarginine have been characterized (29, 30). No information is available about the reaction products formed between peptideylarginine and the following phenylglyoxal derivatives: Me-PGO, F-PGO, Cl-PGO, CamOH-PGO, 2,4-diF-PGO, Mor-PGO, and NO-PGO. Using a set of synthetic test peptides (NRVYHHPFHL, NRVYHHPF, RVYHHPF, and YGGFMRF), we investigated whether these phenylglyoxal derivatives can react covalently with the guanidine moiety of peptideylarginine. To induce the formation of the arginine adduct, 100 μM test peptide was allowed to react with 2 mM of phenylglyoxal derivative in 10 mM Hepes-KOH, pH 8.0, for 60 min at room temperature. The reaction was stopped by the addition of trifuoroacetic acid and cooling to +4 °C. The reaction mixture was desalted using a Zip Tip C18 silica bead micro-column (Millipore Corp.). Peptides were eluted with 60% acetonitrile in 0.1% trifuoroacetic acid and mixed with an equal volume of saturated α-hydroxyaceticacid in 33% acetonitrile in 0.1% trifuoroacetic acid.

**Molecular Mass Spectra of the Peptides**—MALDI-TOF mass spectra of the peptides were recorded on a Bruker Autoflex spectrometer using the reflector device in positive mode. Calibration of the spectrometer was performed using the peaks of peptides with known masses. All of the tested phenylglyoxal compounds reacted with the peptide YGGFMRF, resulting in the formation of stable products (Fig. 2). The molecular masses of the products indicated that these phenylglyoxal derivatives were bound to the guanidine residue through imidazole formation. These phenylglyoxal derivatives also reacted with the other three test peptides in a similar way.

**Physicochemical Properties of Phenylglyoxal Derivatives**—Dipole moments were calculated from equilibrium geometry using standard semiempirical AM1 wave functions provided in GAMESS (31). Log P values were calculated for the nonionic species using KowWin version 1.67 (www.syrres.com/esc/est_kowdemo.htm). The surface activity profiles of the phenylglyoxal derivatives (the surface tension versus concentration isotherms) were measured in modification medium containing 250 mM sucrose, 10 mM succinate, 10 mM Hepes-KOH, pH 8.0, 100 μM EGTA, 1 μM rotenone, supplemented with 10% MeSO, using a Delta-8 instrument (Kibron Inc.) as described (32). The apparent air/water partition coefficient was determined from the isotherms as described (33). The pκ of the OH group of OH-PGO and CamOH-PGO was 7.50 and 7.15, respectively, as determined by the absorbance spectra of the protonated (λmax at 282 and 278 nm) and deprotonated (λmax at 335 and 333 nm) molecules. Titration was performed with 1 mM NaOH in assay medium containing 250 mM sucrose, 10 mM Hepes-Tris, pH 7.4, 5 mM succinate, 5 mM P-Tris, 2 mM Mg²⁺, 5 μM EGTA, 1 μM rotenone, supplemented with 20 mM phosphorous acid.

**Isolation of Mitochondria**—Male Wistar rats were prepared as described (18). The freshly isolated mitochondria (1 mg of protein/ml) were preincubated in modification medium with or without the individual phenylglyoxal derivatives for 15 min at +25 °C. The modification reaction was terminated by adjusting the pH to 6.8 with Hepes buffer and cooling to +4 °C. Mitochondria were separated from the reaction mixture containing residual free reagent by centrifugation at 1000 g for 5 min. Mitochondria were resuspended at a concentration of 50 mg of protein/ml in assay medium. Sequential modification experiments were performed as described (20).

**Assessment of Permeability Transition in Isolated Mitochondria**—Permeability transition was induced either by a single addition of 60 μM Ca²⁺ or by repeated additions of 15 μM Ca²⁺ until fast Ca²⁺ release was assayed. Permeability transition was assayed by measuring Ca²⁺ transport, mitochondrial swelling, and ΔΨ. Medium (Ca²⁺) was measured using the fluorescent indicator dye Fluo-4-FF, which becomes fluorescent upon Ca²⁺ binding (excitation, 494 nm; emission, 516 nm). Mitochondrial swelling was measured as a decrease in turbidity at 540 nm. ΔΨ was indirectly measured by TMRE accumulation, which is sensitive to the transmembrane potential (excitation, 550 nm; emission, 575 nm). Measurements were performed using a PerkinElmer Life Sciences LS50B luminescence spectrometer. PTP opening was quantified by the swelling rate and Ca²⁺ threshold, as described (21, 33).

**Cell Culture**—MH1C1 rat hepatoma cells were grown in Ham’s F-10 (Invitrogen) nutrient mixture supplemented with 40 mM NaHCO₃, 10% fetal calf serum, 30 μg/ml penicillin, and 50 μg/ml streptomycin. HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) medium supplemented with 2 mM glutamine, 10% fetal calf serum, 30 μg/ml penicillin, and 50 μg/ml streptomycin. Cells were kept at +37 °C in a humidified atmosphere containing 5% CO₂. To induce arginine modification, cells were incubated in a culture medium supplemented with the phenylglyoxal derivatives for 12 h. The remaining derivative was removed from the culture medium after the incubation, and cell culture was assessed using a resazurin assay according to the manufacturer’s instructions. After 3 h, the ratio of reduced to oxidized resazurin was measured using a microplate reader (Spectracount; Packard) at the wavelength couple 620–540 nm.

**Microscopy**—For light microscopy, cells were seeded onto glass coverslips in 6-well plates and incubated overnight. The coverslips were mounted on the stage of a Zeiss Axiovert 100TV inverted microscope equipped with a mercury lamp (100 watts). ΔΨ was measured using the
fluorescent indicator dye TMRM. The excitation filter wavelength was 546 ± 5 nm, the emission filter wavelength was 580 ± 15 nm, and the dichroic mirror cut-off wavelength was 560 nm. Images were acquired using a 12-bit CCD camera and analyzed using the MetaFluor imaging software (Molecular Probes, Eugene, OR). Before the measurement, cells were incubated for 30 min at 37 °C in Hanks’ balanced salt solution (Sigma) supplemented with 20 nM TMRM and 1.6 μM cyclosporin H. Unlike CaA, cyclosporin H does not inhibit the PTP, whereas both compounds inhibit the multidrug resistance pump, and therefore cyclosporin H was used to optimize TMRM loading as detailed (34). Mitochondrial clusters were defined as regions of interest, and background fluorescence was measured from fields without cells. Sequential digital images were acquired every 0.5–2 min for 60 min, and the average fluorescence intensity of all of the selected regions of interest and of the background was recorded and stored for subsequent analysis.

RESULTS

To investigate the structure-function relationship of arginine-glyoxal adducts upon PTP opening and closing, we used a set of phenylglyoxal derivatives that differ only in the functional groups attached to the phenyl ring (Fig. 1). All of these phenylglyoxal derivatives can react with peptidylarginine and form stable adducts as evidenced by the molecular mass of the resulting reaction products (Fig. 2). We then investigated the effects of the phenylglyoxal derivatives on the PTP in isolated rat liver mitochondria, and Fig. 3 shows our findings with Me-PGO. Mitochondria were preincubated with or without 2 mM Me-PGO and subsequently resuspended in assay medium, and permeability transition was induced by a single addition of 60 μM CaCl₂. Control mitochondria that had been incubated without Me-PGO took up CaCl₂ from the medium (Fig. 3A, trace a). The addition of CaCl₂ to the assay medium can be seen as an increase in the Fluo-4FF fluorescence at 3 min. As the control mitochondria took up the added CaCl₂, the Fluorescein diacetate fluorescence decreased. However, these mitochondria subsequently released CaCl₂ back into the medium and underwent large amplitude swelling as indicated by the large decrease in turbidity (Fig. 3, A and B, traces a). Concomitantly, the ΔΨ diminished (Fig. 3C, trace a), indicating that deenergization had occurred. Supplementing the assay medium of control mitochondria with 1 μM CsA (Fig. 3, all panels, traces b) or SCA (data not shown) completely inhibited CaCl₂ release, swelling, and the ΔΨ decrease, indicating that these events were due to PTP opening. In contrast, mitochondria that had been incubated with Me-PGO were able to retain the added CaCl₂ in the matrix throughout the experiment (Fig. 3A, trace c). Me-PGO-treated

FIG. 1. Structural formulas of phenylglyoxal derivatives. For clarity, hydrogen atoms (-H) on the phenyl ring are not shown. The pKₐ of the OH group of OH-PGO and CamOH-PGO was 7.50 and 7.15, respectively.

| Phenylglyoxal Derivative | R₂ | R₃ | R₄ |
|--------------------------|----|----|----|
| PGO                      | -  | -  | -  |
| Me-PGO                   | -Ch| -  | -  |
| MeO-PGO                  | -OCH| -  | -  |
| F-PGO                    | -  | -F | -  |
| 2,4-diF-PGO              | -F | -F | -  |
| 3,4-diF-PGO              | -F | -F | -  |
| Cl-PGO                   | -Cl| -  | -  |
| NO-PGO                   | -N+O| -  | -  |
| OH-PGO                   | -OH| -  | -  |
| CamOH-PGO                | -OCH| -  | -  |
| Mor-PGOG                 | -N+O| -  | -  |

FIG. 2. MALDI-TOF mass spectra of phenylglyoxal derivatives bound to a test peptide. The peptide (YGGFMRF) was reacted with each phenylglyoxal derivative as described under “Experimental Procedures.” The reaction mixture was desalted and applied to the target. Mass spectra were acquired in the positive mode using the reflector detector. The phenylglyoxal derivatives used were as follows. 1, Me-PGO; 2, F-PGO; 3, MeO-PGO; 4, Cl-PGO; 5, 2,4-diF-PGO; 6, NO-PGO; 7, CamOH-PGO; 8, Mor-PGO. The peak at m/z 877.40 in the spectra is due to remaining underivatized peptide.

FIG. 3. Inhibition of CaCl₂-induced PTP opening by Me-PGO. Mitochondria were preincubated for 15 min with or without 2 mM Me-PGO. Mitochondria were then sedimented by centrifugation and resuspended in assay medium at a concentration of 1 mg of protein/ml. CaCl₂ (60 μM) was added after 2 min. A, CaCl₂ concentration of the medium measured using the fluorescent dye Fluo-4FF. B, swelling measured as a decrease in turbidity. C, ΔΨ measured using the fluorescent dye TMRM. All panels, traces a and b show mitochondria preincubated without Me-PGO; traces c show Me-PGO-treated mitochondria; in traces b, the suspension was supplemented with 1 μM CsA.
mitochondria remained energized, and swelling did not occur as indicated by the unchanged turbidity of the mitochondrial suspension (Fig. 3, all panels, traces c). These results show that brief Me-PGO treatment results in suppression of permeability transition. Since Me-PGO had no detrimental effect on the generation of ∆ψ or Ca²⁺ uptake, we conclude that Me-PGO acted directly on the PTP.

The results of similar measurements on isolated mitochondria incubated with the other phenylglyoxal derivatives are summarized in Table I. For comparison, we included data on PGO and OH-PGO (19, 20). In order to quantify the PTP opening/closing, we also determined the Ca²⁺ uptake threshold for PTP opening as described (33). To illustrate how Ca²⁺ uptake threshold measurements were performed, we show results for mitochondria incubated with either Me-PGO (Fig. 4A) or CamOH-PGO (Fig. 4B). After removal of excess phenylglyoxal derivative, mitochondria were suspended in the assay medium as described above, and pulses of 15 μmol of Ca²⁺ were added until Ca²⁺ release ensued. The results showed that mitochondria incubated without phenylglyoxal derivative (Fig. 4, A and B, traces a) were able to take up about 55 nmol of Ca²⁺/mg of protein from the medium before Ca²⁺ release occurred, whereas treatment with Me-PGO (Fig. 4A, trace b) increased the Ca²⁺ uptake threshold to 190 nmol of Ca²⁺/mg of protein. Supplemen-
ting the medium of control mitochondria with 1 μmol CsA or SfA increased the Ca²⁺ uptake threshold to over 200 nmol of Ca²⁺/mg of protein (data not shown). Therefore, Me-PGO treatment of mitochondria resulted in a 4-fold increase in the Ca²⁺ uptake threshold, showing that Me-PGO was almost as effective as the cyclophilin ligands for closing the PTP. In contrast, incubation with CamOH-PGO resulted in a dramatic decrease in the Ca²⁺ threshold. In this case, one single pulse of 15 μmol Ca²⁺ was sufficient to trigger Ca²⁺ release, compared with the four pulses for mitochondria incubated without phenylglyoxal derivative (Fig. 4B, traces a and b). The Ca²⁺ release from mitochondria incubated with CamOH-PGO remained fully sensitive to both 1 μmol CsA and SfA (Fig. 4B, traces c and d). This result demonstrates that CamOH-PGO acts selectively on the PTP and increases the likelihood of PTP opening in response to Ca²⁺. During incubation of isolated mitochondria with the phenylglyoxal derivatives CamOH-PGO, OH-PGO, and NO-PGO, energization by succinate was required to prevent spontaneous permeability transition (data not shown). Comparing the results of the phenylglyoxal derivatives, it is evident that rapid mitochondrial swelling correlated with a low Ca²⁺ uptake threshold, and vice versa. None of the phenylglyoxal derivatives had any major effects on the generation of ∆ψ or Ca²⁺ uptake at concentrations that effectively modify the PTP (data not shown).

The phenylglyoxal derivatives can be categorized into three

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**Table I**

**Effect of phenylglyoxal derivatives on the PTP and their physicochemical properties**

| Compound      | Swelling rate | Ca²⁺ threshold | Dipole moments (D) | Log P | Apparent log Kₐ/M | Hydrogen bond donor | Hydrogen bond acceptor |
|---------------|---------------|----------------|-------------------|-------|-------------------|---------------------|------------------------|
| PGO           | 12            | 487            | -2.1 0.0 -4.2 4.7 | 1.0   | 3.0               | No                  | No                     |
| Me-PGO        | 14            | 350            | 2.1 0.0 4.7 5.2  | 1.5   | 2.7               | No                  | No                     |
| MeO-PGO       | 36            | 183            | 0.8 0.0 2.4 2.5  | 1.0   | 2.2               | Weak                | No                     |
| P-PGO         | 12            | 550            | -2.0 0.0 -2.7 3.3 | 1.2   | ND                | No                  | No                     |
| 2,4-diP-PGO   | 32            | 250            | 3.1 0.0 -3.2 4.4 | 1.4   | 2.7               | No                  | No                     |
| Cl-PGO        | 25            | 237            | -2.0 0.0 -2.9 3.5 | 1.6   | 2.4               | No                  | No                     |
| NO-PGO        | 300           | 15             | 1.6 0.0 1.2 2.0  | 0.8   | 2.2               | No                  | Yes                    |
| OH-PGO        | 430           | 15             | -0.9 0.0 4.2 4.3 | 0.5   | 1.6               | Yes                 | Yes                    |
| (O⁻)PGO       | 430           | 15             | -2.6 0.0 -2.2 3.4 | ND    | ND                | ND                  | ND                     |
| CamOH-PGO     | 550           | 15             | -1.0 0.1 -2.0 2.2 | 1.6   | 2.3               | Yes                 | Yes                    |
| Cam(O⁻)PGO    | 550           | 15             | 0.1 -2.0 3.5 4.1 | ND    | ND                | ND                  | ND                     |
| Mor-PGO       | 100           | 100            | 0.0 -3.2 2.0 3.8 | 0.7   | 2.1               | No                  | Yes                    |
| CsA/SfA       | <5            | >450           |                   |       |                   |                     |                        |
| Control       | 100           | 100            |                   |       |                   |                     |                        |

a Since the sensitivity of the PTP to Ca²⁺ can vary between different mitochondrial preparations, the swelling rate and Ca²⁺ threshold are expressed in relative values as described under “Experimental Procedures.”

b Not determined.

c Determined for the prevailing equilibrium between the protonated and deprotonated form at pH 7.4.

d Log P not defined for ions.
groups: (i) those that suppressed permeability transition (Cl-PGO, 2,4-diF-PGO, F-PGO, Me-PGO, MeO-PGO, and PGO); (ii) those that induced permeability transition (CamOH-PGO, NO-PGO, and OH-PGO); and (iii) one (Mor-PGO) that had no effect on permeability transition. Similar to CamOH-PGO-induced PTP opening, NO-PGO- and OH-PGO-induced PTP opening was inhibited by CsA or SIA (data not shown). To understand why Mor-PGO did not influence the PTP conformation, we conducted a sequential modification experiment. Mitochondria were incubated with PGO, sedimented by centrifugation, and resuspended in Mor-PGO-containing medium. Subsequently, the mitochondria were sedimented and resuspended in assay medium. Alternatively, we first incubated with Mor-PGO and then with PGO. We then determined how these two treatment schemes affected the PTP, as in Fig. 3. The results showed that PTP closing occurred, regardless of whether PGO was used before or after Mor-PGO (data not shown), indicating that Mor-PGO did not react with the target arginine(s).

We then analyzed the physicochemical properties of the phenylglyoxal derivatives and compared these properties with their effects on the PTP (Table I). This analysis revealed that OH-PGO and CamOH-PGO, which are partially protonated and thus negatively charged at neutral pH, are PTP-openers. OH-PGO and CamOH-PGO (insets), the relationship between OH-PGO concentration and the survival of MH1C1 cells. B, inset, the relationship between CamOH-PGO concentration and the survival of HeLa cells.

FIG. 5. Effect of OH-PGO and CamOH-PGO on permeability transition and viability in mammalian cells. Cells were incubated with phenylglyoxal derivatives for 12 h as described under "Experimental Procedures." ΔΨ was measured using TMRM (main graphs), and cell viability was assessed using the resazurin assay (insets). A, main graph, MH1C1 cells incubated with 300 μM OH-PGO. B, main graph, HeLa cells incubated with 20 μM CamOH-PGO. The addition of 2 μg/ml oligomycin and 2 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) are indicated with arrows. Open squares indicate that 1 μM CsA was added immediately before the measurement. A, inset, the relationship between OH-PGO concentration and the survival of MH1C1 cells. B, inset, the relationship between CamOH-PGO concentration and the survival of HeLa cells.
PGO was able to increase the probability of PTP opening, as judged by the lack of effects by oligomycin on the \( \Delta \psi \) (data not shown). These findings show that inhibition of the ATPase did not reveal a latent PTP sensitization, suggesting that NO- PGO- and CamOH-PGO-induced cell death was not preceded by an increased probability of the PTP being open.

To assess whether the effects of the phenylglyoxal derivatives were cell-specific, we also studied their effects upon human cervical carcinoma HeLa cells. Interestingly, we found that CamOH-PGO, but neither OH-PGO nor NO-PGO, was able to induce PTP opening. Following incubation of HeLa cells at an LD\(_{50}\) of 20 \( \mu \)M CamOH-PGO (Fig. 5B, inset), the \( \Delta \psi \) depolarized upon the addition of oligomycin (Fig. 5B, main graph), and this effect could be completely prevented by adding CsA. Incubation at low concentrations (<LD\(_{50}\)) of OH-PGO, CamOH-PGO, or NO-PGO had no measurable effects on the PTP in MH1C1 or HeLa cells (data not shown). Taken together, these findings are consistent with the effects of OH-PGO and CamOH-PGO on isolated mitochondria and show that the PTP is a potentially important target for glyoxal derivatives in mammalian cells.

**DISCUSSION**

In this study, we have used a set of phenylglyoxal derivatives to investigate the effect of arginine modification on PTP opening and closing in isolated mitochondria and mammalian cells. Analysis of the structure-function relationship showed that negative charge and hydrogen bonding capacity was a common property of derivatives that induced PTP opening. We conclude that glyoxal compounds can regulate the PTP in intact cells and that this may lead to a disruption of the cell death program.

Using a series of test peptides, we demonstrated that all of the phenylglyoxal derivatives studied formed stable adducts on the arginine side chain. Thus, neither steric nor electronic factors of the different functional groups perturb the reaction between the glyoxal and the guanidino groups. Ensuring that our phenylglyoxal derivatives react with peptidylarginine was an essential prerequisite before studying their effects on PTP opening and closing. In isolated mitochondria, all of the phenylglyoxal derivatives except Mor-PGO had an effect on the PTP after a relatively short incubation time, showing that the functional groups in the 2-, 3-, and/or 4-position of the phenyl ring do not compromise the ability of the phenylglyoxal compounds to bind to the target PTP arginine(s). However, despite the reactivity of Mor-PGO with the test peptides, Mor-PGO did not react with the PTP target arginine(s), suggesting that the reaction was hindered by its bulky side chain (Fig. 1). Since none of the phenylglyoxal derivatives had detrimental effects on the generation of \( \Delta \psi \) or Ca\(^{2+}\) uptake, we conclude that they acted directly upon the PTP. Therefore, the finding that some phenylglyoxal derivatives (Cl-PGO, 2,4-diF-PGO, P-PGO, Me-PGO, MeO-PGO, and PGO) promote PTP closing, whereas others (CamOH-PGO, NO-PGO, and OH-PGO) induce PTP opening, can be explained by the differences in the physicochemical properties of the functional groups.

Properties that influence the microenvironment around the target arginine(s) include electrical charge, dipole moment, polarity, and hydrogen bonding of the neighboring chemical groups. We found that OH-PGO and CamOH-PGO, which carry a net negative charge and can participate in hydrogen bonding, were PTP openers. In addition, NO-PGO, which can form hydrogen bonds but carries no net charge also induced PTP opening. We found that Cl-PGO, 2,4-diF-PGO, P-PGO, Me-PGO, and PGO, which have electroneutral side chains that do not form hydrogen bonds, close the PTP. Interestingly, we found that MeO-PGO, which is electroneutral, closed the PTP. However, MeO-PGO is a weak hydrogen bond acceptor, and this property can explain why MeO-PGO was the least potent of the PTP closing compounds. No correlation could be found between dipole moment, partition coefficient, or surface activity and effects on the PTP. However, the polarity values for all of the phenylglyoxal derivatives are within a narrow range, and therefore they are unlikely to contribute to the observed differences. Collectively, the observations of the present study indicate that the critical adduct properties are net electric charge and hydrogen bonding capacity.

Although the structure of the PTP remains unsolved, our results support a simple model for a voltage-dependent pore gate (Fig. 6). In voltage-dependent ion channels, positively charged amino acid residues play a key role in translating changes in membrane potential to conformational changes of the ion pore (27). We propose a PTP model based on the “voltage paddle” mechanism similar to that of the voltage-gated K\(^+\) channel (38). The reactive arginine is assigned to a hypothetical loop or helix protruding from the external channel surface, the orientation of which determines the conformation of the pore. In the absence of any arginine adduct, the arginine-containing loop reorientates according to the direction and strength of the electric field. At high \( \Delta \psi \), the positively charged sensor is pulled into the low dielectric membrane interior by the electric field. This orientation favors a closed pore conformation (upper row). Upon depolarization of the \( \Delta \psi \), the voltage sensor moves out of the membrane interior and is located in the aqueous phase forming hydrogen bonds. This orientation favors an open pore conformation (lower row). A negatively charged and/or hydrogen bond-forming arginine adduct would keep the voltage sensor in the aqueous phase irrespective of the magnitude of the \( \Delta \psi \) (left). This stabilizes an open pore conformation. An electroneutral and non-hydrogen bond-forming adduct would stabilize the position of the sensor inside the low dielectricity membrane interior irrespective of the magnitude of the \( \Delta \psi \) (right). This stabilizes a closed pore conformation. This model is based on the gating mechanism proposed by Jiang et al. (38).

![Fig. 6. Hypothetical model of the PTP gating.](image)
Regulation of Permeability Transition by Arginine Ligands

It is clear from our work that glycoly compounds have major effects on the function of the PTP in isolated mitochondria. However, the regulation of the PTP in intact cells may differ from that of isolated mitochondria. Therefore, we addressed whether the PTP target arginine(s) is modified by phenylglyoxal derivatives in intact mammalian cells. The partition coefficient and surface activity profiles suggested that the phenylglyoxal derivatives are cell membrane-permeable. However, several factors may affect the fate of the derivative when it reaches the cell interior. These include (i) the presence of additional potential targets and (ii) metabolic conversion, which may be different in different cell lines. Of particular concern is aromatic hydroxylation, which would turn a PTP closer (PGO) to a PTP opener (OH-PGO) (39). Therefore, these factors may explain the cell-specific response to different phenylglyoxal derivatives.

AGEs play a key role in the molecular mechanism of long term complications of diabetes (23). The most important intracellular precursor of AGEs is methylglyoxal, which reacts preferentially with arginine residues, causing altered function of target proteins (22). Small amounts of methylglyoxal are constantly formed as a side product from the reaction catalyzed by triose-phosphate isomerase (40), a process that is greatly accelerated during hyperglycemia, leading to an increased formation of AGEs. Since the PTP arginine(s) is responsive to phenylglyoxal derivatives, it is possible that the PTP is a target for methylglyoxal and/or its metabolic products. Experiments aimed at testing this hypothesis are under way in our laboratory.

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