

Neuroprotective Role of Antidiabetic Drug Metformin Against Apoptotic Cell Death in Primary Cortical Neurons

Mohamad-Yehia El-Mir · Dominique Detaille · Gloria R-Villanueva · Maria Delgado-Esteban · Bruno Guigas · Stephane Attia · Eric Fontaine · Angeles Almeida · Xavier Leverve

Received: 15 July 2007 / Accepted: 15 August 2007 / Published online: 27 November 2007
© Humana Press Inc. 2007

Abstract Oxidative damage has been reported to be involved in the pathogenesis of diabetic neuropathy and neurodegenerative diseases. Recent evidence suggests that the antidiabetic drug metformin prevents oxidative stress-related cellular death in non-neuronal cell lines. In this report, we point to the direct neuroprotective effect of metformin, using the etoposide-induced cell death model. The exposure of intact primary neurons to this cytotoxic insult induced permeability transition pore (PTP) opening, the dissipation of mitochondrial membrane potential ($\Delta\Psi_m$), cytochrome *c* release, and subsequent death. More importantly, metformin, together with the PTP classical inhibitor cyclosporin A (CsA), strongly mitigated the activation of this apoptotic cascade. Furthermore, the general antioxidant *N*-acetyl-L-cysteine also prevented

etoposide-promoted neuronal death. In addition, metformin was shown to delay CsA-sensitive PTP opening in permeabilized neurons, as triggered by a calcium overload, probably through its mild inhibitory effect on the respiratory chain complex I. We conclude that (1) etoposide-induced neuronal death is partly attributable to PTP opening and the disruption of $\Delta\Psi_m$, in association with the emergence of oxidative stress, and (2) metformin inhibits this PTP opening-driven commitment to death. We thus propose that metformin, beyond its antihyperglycemic role, can also function as a new therapeutic tool for diabetes-associated neurodegenerative disorders.

Keywords Cytochrome *c* release · Etoposide · Metformin · Neuronal apoptosis · Mitochondrial permeability transition pore

El-Mir and Detaille have contributed equally to this work.

M.-Y. El-Mir · G. R-Villanueva
Departamento de Fisiología y Farmacología,
Universidad de Salamanca,
Salamanca, Spain

D. Detaille · B. Guigas · S. Attia · E. Fontaine · X. Leverve (✉)
INSERM U884 Bioénergétique Fondamentale et Appliquée,
BP 53X, 38041 Grenoble Cedex, France
e-mail: Xavier.Leverve@ujf-grenoble.fr

D. Detaille · B. Guigas · S. Attia · E. Fontaine · X. Leverve
Université Joseph Fourier,
Grenoble, France

M. Delgado-Esteban
Departamento de Bioquímica y Biología Molecular,
Universidad de Salamanca,
Salamanca, Spain

A. Almeida
Hospital Clinico Universitario de Salamanca,
Salamanca, Spain

Abbreviations

$\Delta\Psi_m$	mitochondrial membrane potential
AMPK	AMP-activated protein kinase
CsA	cyclosporin A
NAC	<i>N</i> -acetyl-L-cysteine
PI	propidium iodide
PTP	permeability transition pore
TMRM	tetramethyl-rhodamine methyl ester

Introduction

Neuropathy represents a major complication for diabetes sufferers affecting approximately 50% of patients across the world (Pirart 1977; Dyck et al. 1991), yet its pathogenesis remains relatively poorly understood. Oxidative stress has been implicated in a variety of neurological disorders

including Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis diseases (Andersen 2004). It is generally agreed that the human brain, which contains high amounts of polyunsaturated lipids and catecholamins, is characterized by an elevated oxidative metabolism, while endogenous levels of antioxidant enzymes such as catalase are relatively low. Consequently, the brain may be an important target for free radical attacks leading to lipid peroxidation and/or protein nitration events, as well as the initiation of programmed neuronal death (Bolanos et al. 1997; Yuan and Yankner 2000).

An increasing number of studies places mitochondrial dysfunction at the heart of the disease, most notably in vascularization and the central nervous system (Green and Reed 1998; Duchen 2004). The intrinsic apoptotic cascade is regulated by the Bcl-2 family proteins or by the mitochondrial permeability transition pore (PTP). In the latter, PTP opens under conditions of stress, and the outer mitochondrial membrane ruptures locally, triggering the release of apoptogenic proteins into the cytosol, such as cytochrome *c*, an apoptosis-inducing factor, Smac/Diablo, procaspases, and endonuclease G (Kroemer and Reed 2000). Because several drugs known to inhibit PTP opening have also been reported to prevent cellular death in response to a number of cytotoxic insults, one may infer that PTP opening is a pivotal event in the apoptotic pathway.

Different therapeutic approaches have been investigated to overcome neurodegenerative diseases (Chong et al. 2005). Some of them consist of the use of drugs that act directly on the mitochondrial function, such as cyclosporin A (CsA), the PTP reference blocker (Mattson and Kroemer 2003). However, serious side effects of this immunosuppressive agent should preclude its routine use in clinical medicine. Over the past few years, we have reported that PTP can be modulated by the electron flux through the respiratory chain complex I (Fontaine et al. 1998; Chauvin et al. 2001). We have recently demonstrated that the antidiabetic drug metformin prevents PTP opening and subsequent cell death in various endothelial cell types when exposed to a high glucose level (Detaille et al. 2005). Moreover, a mild inhibition of complex I activity, already found in other non-neuronal cells (El-Mir et al. 2000; Guigas et al. 2004; Detaille et al. 2005), was accompanied by a decreased probability of mitochondrial permeability transition in permeabilized endothelial cells.

In the light of these new findings, we hypothesized that metformin can interrupt the apoptotic cascade in primary neurons when exposed to an oxidative stress. This is of great clinical interest because metformin, beyond its antihyperglycemic function, can also act as a pharmacological strategy in the treatment of neurodegenerative

diseases and diabetes-associated neuropathy, provided that metformin can be made to pass the blood–brain barrier.

In the current study, we show that metformin, at a therapeutic dose, has a substantial neuroprotective effect on etoposide-induced cell death in primary cortical neurons. This effect is mediated, at least in part, by inhibition of PTP opening and cytochrome *c* release.

Materials and Methods

Materials and Products

Metformin was a gift from Merck-Santé (Lyon, France). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture and Drug Treatments

Primary cortical neurons from embryonic day 17 Wistar rats were prepared as described elsewhere (Almeida et al. 2004). Dissociated cell suspensions were seeded in poly-D-lysine-coated culture dishes containing Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, then incubated at 37°C under a humidified atmosphere (5% CO₂). On day 2 after plating, the medium was replaced with fresh DMEM supplemented with 5% horse serum plus 20 mM D-glucose, and on day 4 of culture, cytosine arabinoside (10 μM) was added to prevent non-neuronal proliferation. Neurons, which were approximately 99% Map2 positive (Diaz-Hernandez et al. 2007), were used by days 7–8 when neuronal death was induced by etoposide (20 μM) for 45 min. Before this treatment, primary cultures were incubated in the absence or presence of 1 μM CsA for 30 min, 100 μM metformin for 24 h, or 100 μM *N*-acetyl-L-cysteine (NAC) for 24 h.

Quantification of Cell Death by Flow Cytometry

Apoptosis analyses were performed using a commercially available kit (Molecular Probes, Eugene, OR), based on the double-stain system with Alexa Fluor-conjugated annexin V and propidium iodide (PI). Data acquisition (~10,000 cells) was carried out in a FACScalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) equipped with a 15-mW argon ion laser tuned at 488 nm, using the CellQuest software (Becton-Dickinson Biosciences). The analyzer threshold was adjusted on the forward light scatter channel (cell size) to exclude noise and most of the subcellular debris. Data were plotted as a function of fluorescence intensity FL-1 (annexin V) vs FL-3 (PI), and the Annexin V⁻/PI⁻ population was regarded as normal healthy cells.

Citrate Synthase and Complex I Activities Assay

Confluent monolayers of cortical neurons were incubated for 24 h in the absence or presence of 100 μ M metformin. Neurons were trypsinized, pelleted, then immediately resuspended in a cold buffer (40 mM KCl, 20 mM Tris-HCl, 250 mM sucrose, and 2 mM ethylene glycol tetraacetic acid at pH 7.2) containing 200 μ g/mL digitonin. After a 5-min incubation on ice, cell lysates were spun down at 12,000 \times *g* for 10 min to eliminate cytosolic-contaminating enzymes. Permeabilized neuronal cells were carefully washed and resuspended in appropriate buffers for the assay of complex I and citrate synthase as reported previously (Guigas et al. 2004). Both enzyme activities were expressed as nanomoles per minute per milligram of protein, the protein content being measured by the Pierce BCA Micro Protein Assay kit (Rockford, IL).

Determination of Mitochondrial Permeability Transition in Permeabilized Neurons

Neurons were incubated or not with metformin as described above. Cells were permeabilized immediately before use by incubation for 2 min at 25°C in a buffer (10 mM Tris-*N*-morpholino)propanesulfonic acid, 250 mM sucrose, 1 mM Pi-Tris, and 50 μ M ethylenediamine tetraacetic acid at pH 7.35) containing 5 mM succinate and 100 μ g/mL digitonin. The measurement of calcium retention capacity was carried out fluorimetrically using a PTI Quantamaster C61 spectrofluorimeter in the presence of 0.25 μ M Calcium Green (Molecular Probes) with excitation and emission wavelengths set at 506 and 527 nm, respectively. After signal stabilization, pulses of 10 μ M calcium were added every 2 min until the pore opening as indicated.

Calcein/Cobalt Assay for PTP Opening in Intact Neurons and Evaluation of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

We applied herein the method described by Petronilli et al. (1999), with some minor modifications, to study mitochondrial permeability transition in individual live cells. Cortical neurons were cultured for 6 days on coated glass coverslips and exposed for 45 min at 37°C to phosphate-buffered saline (PBS) supplemented with 15 mM glucose, 0.5 mM pyruvate, 2 mM CoCl₂, and 1 μ M calcein acetomethoxyl ester (Molecular Probes). After coloaded, cells were washed free of calcein/cobalt, then incubated in a PBS/glucose/pyruvate medium for an additional 30 min. Coverslips were mounted on the stage of a confocal microscope, and PTP opening was triggered by the direct addition of etoposide. Images were collected every minute with a constant

exposure time. To assess mitochondrial membrane potential, intact cortical neurons were loaded with the potentiometric dye tetramethyl-rhodamine methyl ester (TMRM) by immersion in a 50 nM solution. After their incubation with CsA or metformin, cells were allowed to equilibrate with TMRM for 20 min and then imaged in real time under a confocal microscope after the addition of etoposide.

Detection of Cytochrome *c* Release by Immunocytochemistry

Cortical neuron cultures on glass coverslips were fixed with 3.7% paraformaldehyde in PBS for 20 min at room temperature. After rinsing with PBS, the cells were permeabilized in PBS with 0.2% Triton X-100 for 5 min, and incubated for 45 min with a blocking solution (2% bovine serum albumin in PBS). They were then incubated for 2 h with a mouse monoclonal antibody against cytochrome *c* (clone 6H2.B4, Pharmingen, San Diego, CA) diluted 1:250 in blocking buffer. After three washes with PBS, the secondary antibody (Molecular Probes, 1:500 of goat oregon green-conjugated anti-mouse in blocking buffer) was applied for 1 h in the dark. After final washes, a mixture composed of 0.2 M Tris-HCl, pH 7.8, 90% glycerol, and 2.3% 1,4-diazobicyclo-[2.2.2]-octane (anti-fading agent) was added, and the cells were then imaged with a confocal microscope.

Confocal Imaging

Cell imaging was accomplished by a LEICA TCS SP2 confocal microscope equipped with an argon laser emitting a strong line at 488 nm, and two helium–neon lasers emitting lines at 543 and 633 nm, respectively. Neurons were viewed with an X63 Plan Apo water immersion objective (numerical aperture 1.20). The excitation and emission wavelengths for calcein and oregon green were 488 and 525 nm, respectively. For the detection of TMRM fluorescence, 568-nm excitation and 585-nm longpass emission filter settings were used. Neurons, randomly chosen with phase-contrast inverted microscopy, were scanned by the confocal microscope at 512 \times 512-pixel resolution. Generally, eight separate fields of view were scanned by glass coverslips. To obtain more information about mitochondria structure after calcein/cobalt staining, each native image was deconvoluted using the ImageJ 1.36b software (National Institutes of Health, USA).

Statistics

Results are presented as means \pm S.E.M. The statistical significance of differences was analyzed using the Student

t test (unpaired observations). Values were considered to be different from one other when *p* values were less than 0.05.

Results

Metformin Prevents Etoposide-induced Cell Death in Rat Primary Cortical Neurons

The antitumor drug etoposide is a well-established cytotoxic reagent that triggers apoptosis in cancer and normal cells (Nakajima et al. 1994; Karpinich et al. 2002). This partly proceeds from an increased generation of free radicals (Pham and Hedley 2001; Kurosu et al. 2003) and/or mitochondrial dysfunction, notably at the PTP level (Robertson et al. 2000; Custodio et al. 2002; Karpinich et al. 2006). In accordance with our recent works highlighting the protective effect of the antidiabetic drug metformin against oxidative stress-linked death (Guigas et al. 2004; Detaille et al. 2005), we first explored the efficiency of metformin on etoposide-induced neuronal death. To discriminate the features of apoptotic/necrotic from healthy neurons in stressed primary cultures, we conducted a typical flow cytometric analysis, giving demonstrative dot plots as shown in Fig. 1a–f. The exposure of neurons to etoposide resulted in a three- to fourfold increase in the percentage of dead cells (Fig. 1b). Importantly, double staining indicated that most of the damaged cells were annexin V-positive, i.e., apoptotic, and that the number of necrotic cells was rather low. This deleterious effect was substantially prevented by metformin in a dose-dependent manner after neurons were preincubated with this antidiabetic for 24 h (Fig. 1c,d,g). The maximal response of metformin was achieved at 1–10 mM, but a significant protection was already provided at 100 μ M, i.e., at a concentration close to the physiological range. It was therefore decided that this low dosage would be used for subsequent experiments. It is interesting to note that the PTP inhibitor CsA also protected neurons from etoposide-induced lethality (Fig. 1e,g). Moreover, the addition of the general antioxidant NAC markedly reduced the number of dead cells (Fig. 1f,g), confirming that the neurotoxicity of etoposide is partially related to oxidative stress. Otherwise, neither metformin (even at high concentrations) nor CsA and NAC affected the viability of neurons under control conditions (Fig. 1g).

Metformin Inhibits Respiratory Chain Complex I Activity in Primary Neurons

We next investigated the mechanism by which metformin induced this neuro-protection. Previous studies have reported that metformin induced a mild and specific

inhibition of the mitochondrial respiratory chain complex I in rat liver cells (El-Mir et al. 2000), human endothelial cells (Detaille et al. 2005), and in a cancer cell line (Guigas et al. 2004). These data allowed us to postulate that metformin can also alter the mitochondrial function in primary neurons by selectively inhibiting the respiratory chain complex I. To test this hypothesis, the activity of rotenone-sensitive reduced nicotinamide adenine dinucleotide (NADH) decylubiquinone reductase was measured in control and metformin-treated neurons after cell permeabilization. Results from Table 1 show that metformin significantly inhibited rotenone-sensitive oxidation of NADH without altering citrate synthase activity.

Metformin Increases the Ca²⁺-buffering Capacity of Mitochondria in Permeabilized Neurons

It is widely accepted that mitochondrial PTP plays an important role as a mediator of cell injury and death (Kroemer and Reed 2000). Moreover, we recently reported that metformin prevented the PTP opening-mediated commitment to cell death, probably because of its mild inhibitory effect on complex I (Guigas et al. 2004; Detaille et al. 2005). Because both CsA and metformin produced a pronounced neuroprotective effect (Fig. 1g), we further tested whether PTP regulation influenced the ultimate death outcome. With successive additions of calcium pulses, digitonin-permeabilized neurons took up and retained Ca²⁺ until the final, greater, increase in calcium green dye fluorescence, indicative of the permeability transition induced by a calcium overload (Fig. 2a). When permeabilized cortical neurons are directly incubated with the reference inhibitor CsA, an approximately twofold amount of calcium was required to achieve PTP opening (Fig. 2b,d). In parallel, metformin significantly increased this quantity of calcium by 50% (Fig. 2c,d), also indicating a clear-cut inhibition of the pore opening.

Metformin Prevents PTP Opening and $\Delta\Psi$ m Collapse in Intact Neurons

To identify PTP opening in living cells, an elegant technique based on the distribution of the fluorescent molecule calcein was devised by Petronilli et al. (1999), then adapted by others, notably on primary cultures of neuronal cells (Gillissen et al. 2002). In brief, a selective mitochondrial labeling was achieved after quenching calcein fluorescence in the cytoplasm by cobalt. The regulation of PTP was thus assessed in intact neurons by monitoring the redistribution of calcein fluorescence between mitochondrial and cytosolic compartments after challenging neurons, preincubated or not with metformin or CsA, with etoposide. Before any addition, cellular

Figure 1 Effects of metformin, CsA and NAC on etoposide-induced neuronal death. Primary neurons were either cultured in a drug-free medium (a) or incubated with 20 μM etoposide in the absence (b) or presence of metformin at the indicated concentrations (c, d) or CsA (e) or NAC (f). Cells were then stained with Alexa Fluor-conjugated annexin V and PI and were then analyzed by flow cytometry. The distribution of early apoptotic (lower right), late apoptotic (upper right), necrotic (upper left), and viable (lower left) cells is shown. g Percent of neuronal cells showing a positive staining for each experimental condition. Each bar represents mean±SEM of four independent experiments. Asterisk, $p < 0.001$ vs control. Number sign, $p < 0.05$ vs etoposide alone; double number sign, $p < 0.001$ vs etoposide alone

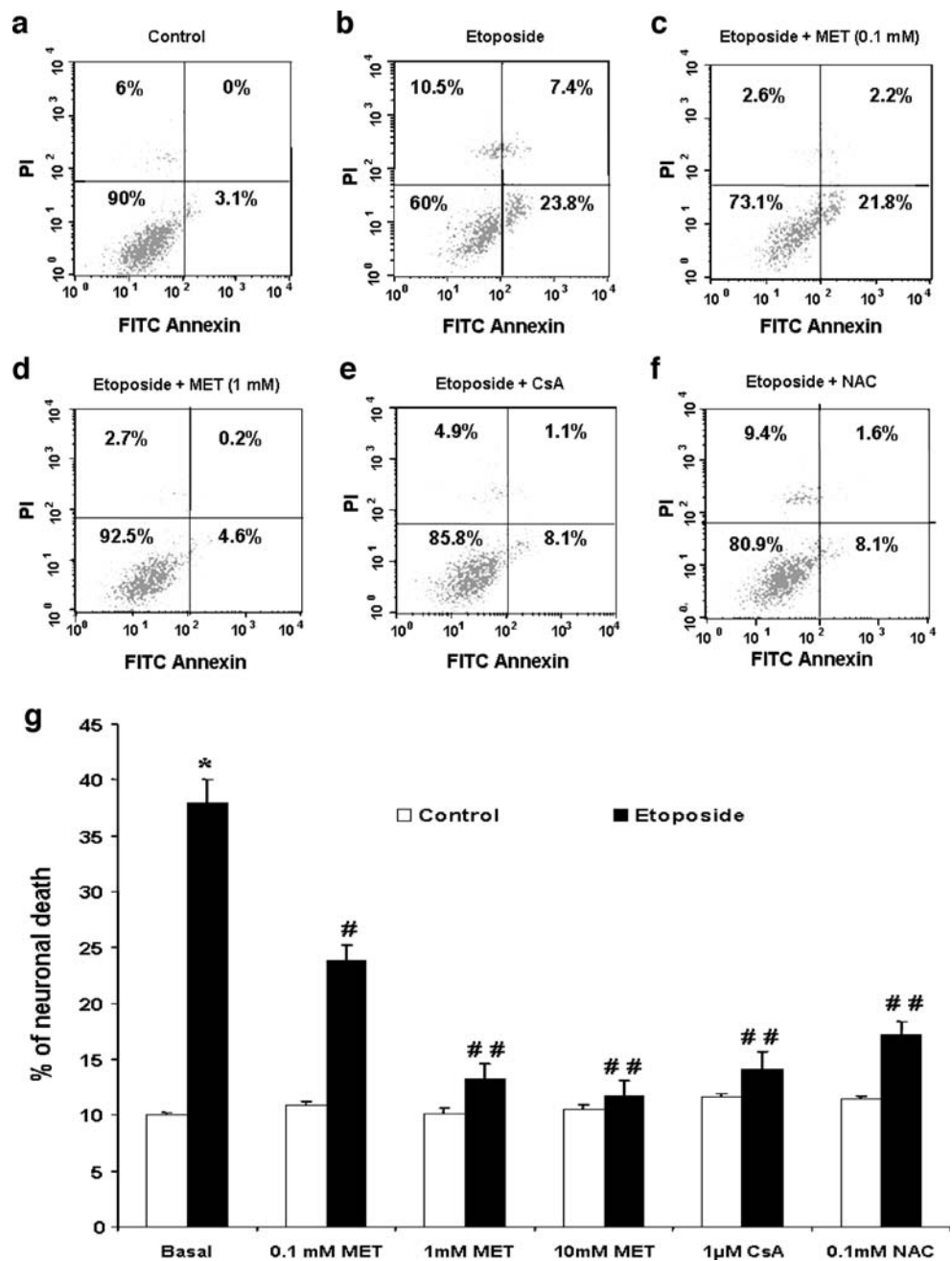


Table 1 Effect of 100 μM metformin on both mitochondrial-isolated complex I and citrate synthase activities in cultured primary neurons

	Control	Metformin (6 h)	Metformin (24 h)
Citrate synthase activity (pmol/min/mg protein)	34.1±1.8	33.7±3.8	33.2±2.7
Rotenone-sensitive activity of complex I (nmol NADH/min/mg protein)	3.54±0.25	3.29±0.22	2.78±0.22*
nmol NADH/unit of citrate synthase	104.9±6.8	98.0±4.0	83.4±2.3*

Neurons were incubated for 6 or 24 h in the absence (control) or presence of 100 μM metformin. Permeabilized cells were used for the determination of enzyme activities as described in “Materials and Methods.” Data are means±SEM values of four different culture preparations. * $p < 0.001$ compared with control cells

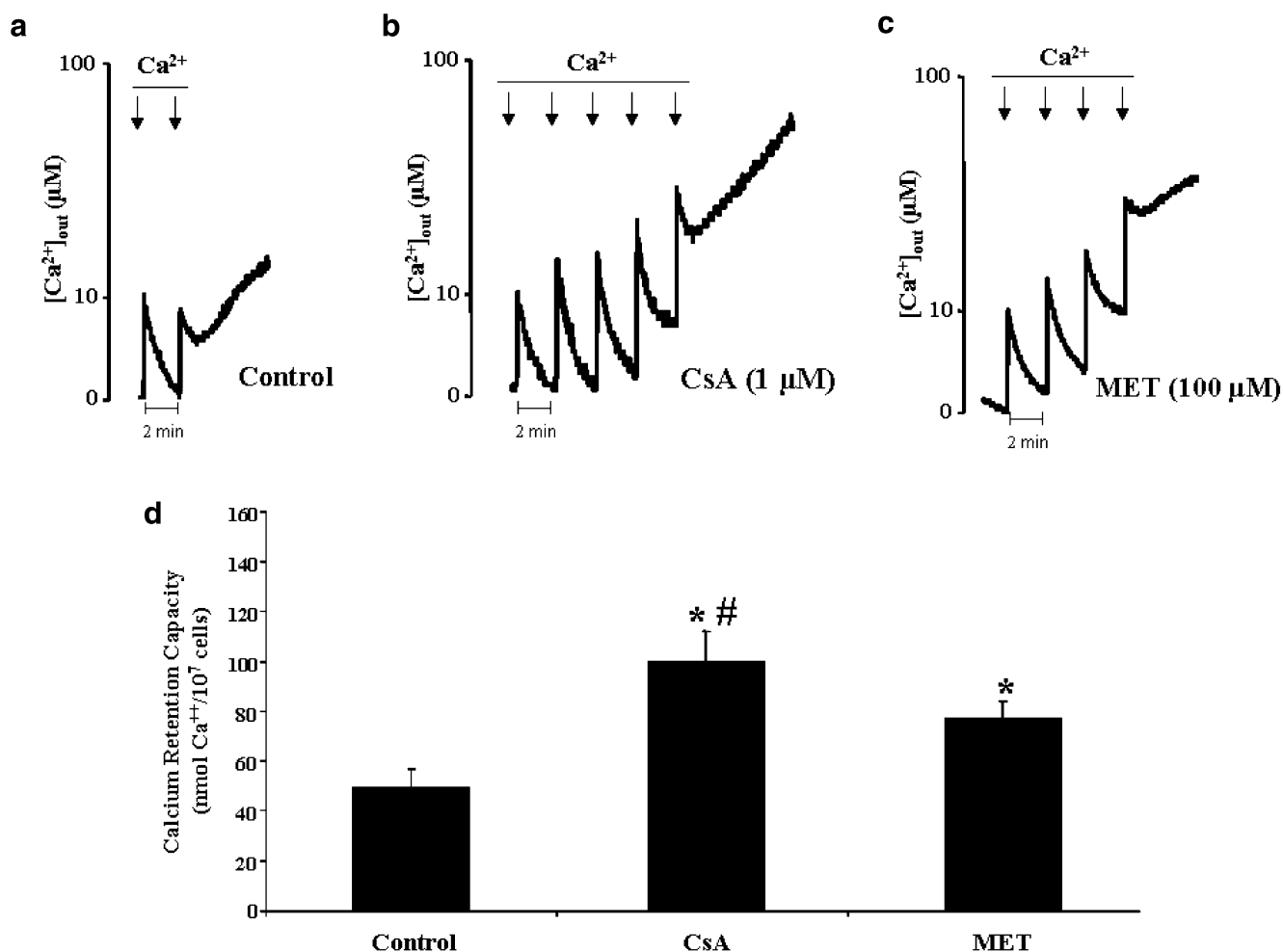


Figure 2 Effects of CsA and metformin on PTP opening in permeabilized neurons. Control cells (**a**), cells directly exposed to 1 μM CsA (**b**), or cells cultured in the presence of 100 μM metformin (**c**) were digitonin-permeabilized in a medium supplemented with succinate and calcium green. As indicated, pulses of 10 μM Ca⁺⁺ were

added every 2 min. **d** Quantitative analysis of the calcium retention capacity for each experimental condition. Each bar represents mean ± SEM of four separate experiments. Asterisk, $p < 0.001$ vs control. Number sign, $p < 0.05$ vs metformin

calcein fluorescence was highly compartmentalized, corresponding to the mitochondrial space (Fig. 3a, 0 min). Indeed, calcein is allowed to permeate throughout the cell, but only the cytosolic labeling vanished with the simultaneous loading of cobalt, leaving the intact mitochondria brightly stained. After etoposide addition, a decompartmentalization of fluorescence at the neural cell endings was rapidly evidenced, leading to a pronounced decrease in mitochondrial staining (Fig. 3a, 10 min), which reflected PTP opening. However, this effect seems to depend on subcellular localization because the calcein signal appears to be enhanced in the vicinity of the nucleus after etoposide. After the deconvolution of native images, we consistently noted that the perinuclear region of etoposide-treated neurons housed a subpopulation of mitochondria, which are thicker and larger than in control cells (Fig. 3a, insets). Likewise, when looking at the evolution of $\Delta\Psi_m$,

an almost identical phenomenon was recorded. The first organelles injured by etoposide are those present in the cell endings, exhibiting a dramatic drop of $\Delta\Psi_m$ as assessed by reduced TMRM staining, while central mitochondria tend to maintain their membrane potential (Fig. 3b, top). When neurons were previously exposed to the two PTP inhibitors, CsA (Fig. 3, middle) or metformin (Fig. 3, bottom), all the damaging effects of etoposide were fully abrogated. Indeed, no or minor changes in calcein fluorescence were detected, and its cellular repartition was remarkably preserved (Fig. 3a). In addition, the mitochondrial integrity was significantly restored by both compounds, as evidenced by the constancy of TMRM signal intensity over a timescale similar to that applied for etoposide alone (Fig. 3b), suggesting that metformin was as effective as CsA in the prevention of PTP opening-driven neuronal death.

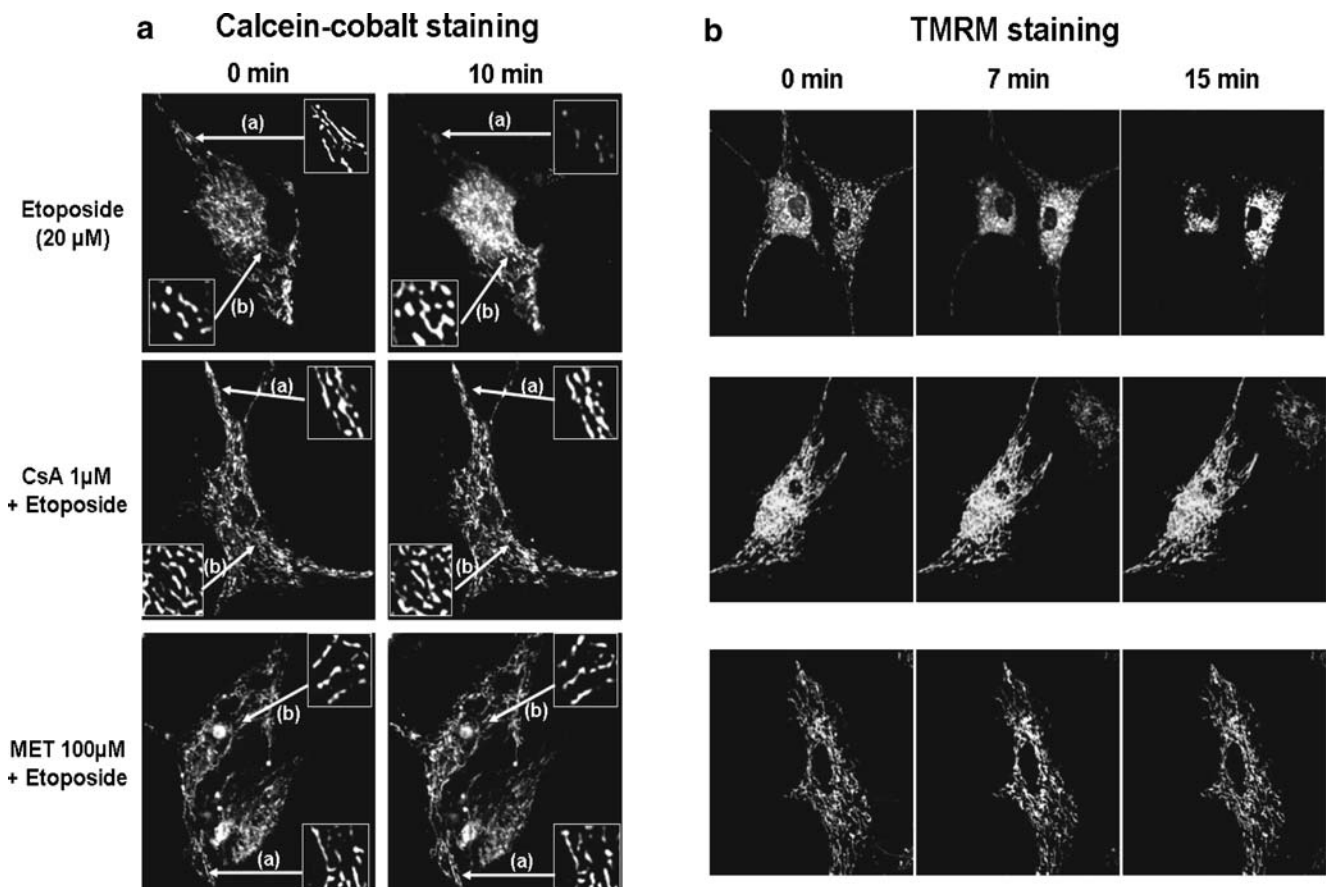


Figure 3 Effects of CsA and metformin on etoposide-induced PTP opening and subsequent loss of $\Delta\Psi^m$ in intact neurons. **a** Cells were coloaded with 1 μ M calcein plus 2 mM cobalt, then exposed to 20 μ M etoposide. Images were collected every minute with an inverted confocal microscope, using a 63 \times water immersion objective. Control cells (*top*) and cells incubated in the presence of CsA (*middle*) or metformin (*bottom*) are shown at 0 and 10 min after the addition of etoposide. Similar results were obtained in five other cell preparations.

Structural details of mitochondria localized in nerve endings (*arrow a*) or in the cellular body (*arrow b*) after deconvolution are depicted in the insets. **b** Cells were loaded with 50 nM TMRM for 20 min before exposure to etoposide, and the images were then collected as before. Control cells or cells incubated in the presence of CsA or metformin are shown at 0, 7, and 15 min after the addition of etoposide. Similar results were obtained in four other cell preparations

Metformin Prevents the PTP-mediated Release of Cytochrome *c*

Because the escape of cytochrome *c* together with other factors from the mitochondrial intermembrane space is a critical process in the apoptotic cascade (Kroemer and Reed 2000), we finally investigated the distribution of cytochrome *c* in primary neurons, cultured under the same conditions as described above, then transiently exposed to etoposide for 45 min (Fig. 4). In control neurons, cytochrome *c* was confined within mitochondria, which appeared as thin filaments around the nucleus, a staining pattern in total agreement with those of calcein and TMRM (Fig. 3). However, after etoposide addition, cytochrome *c* diffused out of some mitochondria, which did not retain their initial filamentous morphology. The ratio of cells exhibiting a cytochrome *c* leakage was roughly similar to that of annexin V-stained cells under similar conditions (Fig. 1). It is interesting to note that metformin and CsA

prevented this etoposide-induced cytochrome *c* delocalization, confirming that PTP inhibition provided a powerful mean of reducing neuronal apoptosis.

Discussion

Results of the present study provide strong evidence of a direct neuroprotective effect of metformin. By using cortical neurons in a primary culture under etoposide treatment, we found that metformin delays or inhibits PTP opening, reverses the dissipation of $\Delta\Psi^m$, and prevents cytochrome *c* release. Furthermore, this sequence of mitochondrial events is associated with a reduction in cell death. These findings are of interest because the beneficial effect of metformin was observed at a dose that is close to the clinically evidenced therapeutic range of serum concentrations.

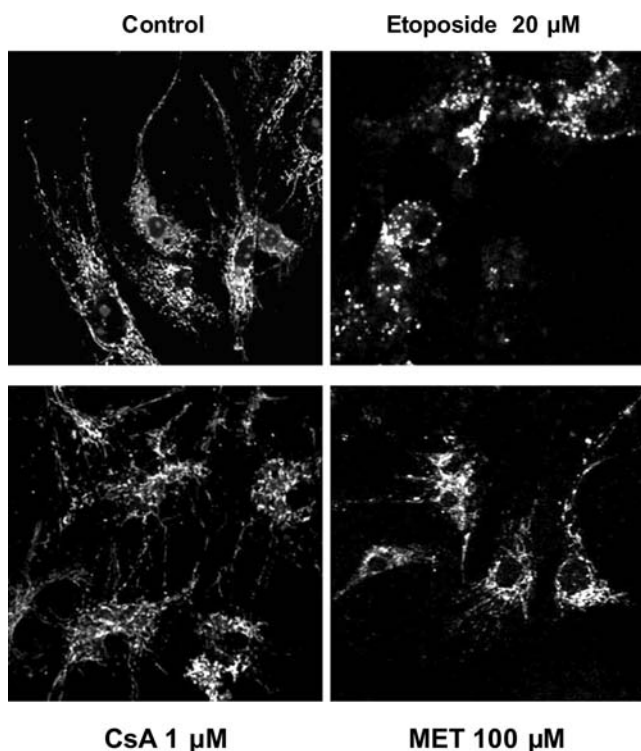


Figure 4 Effects of CsA and metformin on cytochrome *c* redistribution in primary neurons after etoposide exposure. Control cells or cells incubated in the presence of CsA or metformin were submitted to 20 μM etoposide for 45 min, then cultured overnight in a fresh culture medium before fixing and staining with the anti-cytochrome *c* antibody. *Control* denotes neuronal cells not exposed to etoposide but incubated under similar conditions. Similar results were obtained in four other cell preparations

Etoposide was used as the neuronal death trigger, as this procedure has been previously validated to be suitable for the investigation of apoptotic cell death and neuroprotective effects in cortical neurons (Nakajima et al. 1994; Delgado-Esteban et al. 2007). The etoposide-promoted stress is responsible for the commitment of cortical neurons into an apoptotic program involving a mitochondrial dysfunction, as described in previous studies (Custodio et al. 2002; Kurosu et al. 2003; Barrett et al. 2006; Karpinich et al. 2006). Indeed, both PTP opening, as evidenced in intact primary neurons by a significant fading of mitochondrial calcein fluorescence (Fig. 3a), and leakage of cytochrome *c* into the cytoplasm detected by immunolabeling (Fig. 4) are key steps in the mitochondrion-driven cell death pathway. Intriguingly, in our experimental setting, we noticed that etoposide altered mitochondria in different ways according to their location inside the cortical neuron. Mitochondria at the cell periphery or in the vicinity of nerve endings were rapidly damaged and virtually disappeared from the focal plane, while mitochondria within the cellular body (near the nucleus) underwent important morphological changes (insets of Fig. 3a, top). Irrespective of the early activation process by etoposide and which notwithstanding merits a

more detailed investigation, such injured cells are ultimately prone to die. This is well demonstrated when monitoring the progressive loss of mitochondrial membrane potential $\Delta\Psi\text{m}$, as indicated by a marked reduction in TMRM signal (Fig. 3b, top). Although the cobalt-quench method for PTP opening in live cells was not routinely applied for neurons, this overall drop in $\Delta\Psi\text{m}$ (in spite of a slightly increased local fluorescence around the nucleus, as previously seen for calcein) allowed us to further substantiated the involvement of PTP in etoposide-induced cytotoxicity. Most importantly, metformin appeared to be as potent as CsA (the PTP reference inhibitor) in preventing all the harmful effects of etoposide leading to the loss of mitochondrial function after pore opening: the collapsed $\Delta\Psi\text{m}$, the release of cytochrome *c*, and the drastic modifications in the shape of some mitochondria. Our observations show that this last event is not commonly observed and raises the question as to whether PTP is solely responsible for this particular mitochondrial dysfunction. Finally, because antioxidant NAC also inhibited etoposide-triggered neuronal death (Fig. 1), mitochondrial membrane depolarization, and cytochrome *c* release (data not shown), this suggests that the adverse effects of etoposide in cultured primary neurons are at least partly attributable to the occurrence of an oxidative stress, as outlined elsewhere (Pham and Hedley 2001; Gilman et al. 2003; Kurosu et al. 2003).

Taken together, these data confirm our previous work demonstrating a protective effect of two PTP inhibitors (CsA and metformin) on endothelial cell death induced by the glutathione-oxidizing agent *tert*-butyl hydroperoxide or by an elevated glucose level (Detaille et al. 2005). On the other hand, the current study contrasts with the view that etoposide-mediated death is only dependent upon the proapoptotic proteins of the Bcl-2 family but not upon the PTP (Wei et al. 2001). Two recent publications examining the permeability transition in mitochondria from cyclophilin D knockout mice actually argue against a role for the CsA-sensitive PTP in apoptosis regulated by Bax and Bid (Baines et al. 2005; Schinzel et al. 2005). In fact, there is some uncertainty in the literature about the mechanism that controls cytochrome *c* release in response to DNA-damaging agents such as etoposide, as the distinct pathways accounting for its cytotoxic effect are unlikely to be mutually exclusive because of differing experimental conditions in terms of the duration of drug exposure, dose of etoposide applied, and the cell model used (Robertson et al. 2000). As noted by Forte and Bernardi (2005), we must be very cautious of the belief that PTP may participate in cell death pathways solely in response to a restricted set of insults. It is also important to mention that our results do not exclude the contribution of proteins belonging to the Bcl-2 family because PTP opening was recently found to be

an initiating event in the stimulation of Bax translocation to mitochondria during neuronal apoptosis (Precht et al. 2005).

Although metformin is one of the most commonly used drugs in the treatment of type 2 diabetes, its precise mode of action on a molecular level remains to be fully understood. Several new intracellular targets have recently been described, among them adenosine monophosphate-activated protein kinase (AMPK; Zhou et al. 2001; Zou et al. 2004) and respiratory chain complex I (El-Mir et al. 2000; Guigas et al. 2004; Demaille et al. 2005), but the underlying mechanism is still elusive. At present, we lack conclusive evidence that supports the idea that AMPK activation by metformin, as a consequence or not of its action on mitochondrial respiratory chain, could be responsible for the whole of its therapeutic effects. From recent reports, it seems that metformin does not activate AMPK directly, nor does it affect its phosphorylation by upstream kinases in cell-free assays (Hawley et al. 2002). However, it inhibits complex I activity in isolated mitochondria or disrupted tissues, for an extended incubation (Owen et al. 2000) or a very high concentration (10–30 mM) of the antidiabetic drug (Brunmair et al. 2004). It is unlikely that the inhibition of mitochondrial complex I by metformin requires prior activation of AMPK. This was indeed recently confirmed in isolated rat liver cells from mice lacking both alpha 1 and alpha 2 AMPK catalytic subunits by the persistence of a specific inhibitory effect of metformin on the respiratory chain complex I (unpublished experiments). Nonetheless, the loss of the effect of metformin on AMPK in cells devoid of mitochondrial DNA (Zou et al. 2004) strongly argues for the involvement of the mitochondrion and therefore to the possible association of the molecular complex I in the mechanism of metformin action. Otherwise, based on the rotenone-sensitive NADH oxidation capacity, we were able to evidence a mild inhibition of respiratory chain complex I by metformin in primary neurons. As previously reported for human endothelial cells (Demaille et al. 2005), this kinetic constraint in complex I was accompanied by an inhibition of PTP opening in permeabilized neurons. Moreover, contrary to what is commonly observed with rotenone or other potent respiratory poisons, the mild reduction in mitochondrial complex I activity by metformin is not associated with a loss of cell viability. This observation is in accordance with our recent work showing that metformin inhibited the production of free radicals linked to the reverse electron transfer through complex I (Batandier et al. 2006).

It is significant that a 24-h preincubation was necessary to obtain the optimal response of metformin (Table 1). When this antidiabetic drug was administered to living neurons, it induced a partial inhibition of complex I, with

an effect persisting after cell permeabilization (data not shown). In agreement with our previous results (Leverve et al. 2003), and although there is no evidence that metformin is metabolized, we nevertheless proposed that it could indirectly alter the mitochondrial function via an original plasma membrane-mediated process. In the context of a potential neuroprotection in vivo, this hypothesis needs further clarification because the permeation of metformin across the blood–brain barrier remains to be investigated in greater depth. Provided that metformin can be made to pass this frontier, it may become another agent, which can be used in the treatment of diabetes-associated vascular and peripheral neuropathy and an important new therapeutical approach in neuro-degenerative diseases. In a less recent report (Rapin et al. 1988), metformin was found to afford protection against cerebral ischemia in rats; at the time, this result was unexplained because no positive effect on blood flow was recorded. Twenty years later, others demonstrated that metformin therapy significantly prolonged survival in a transgenic mouse model of Huntington's disease (Ma et al. 2007). As mitochondria from patients or mice with this neurodegenerative disorder have a decreased mitochondrial calcium retention capacity (Panov et al. 2002), the reported inhibitions of PTP and downstream mitochondrial signaling to cell death (Guigas et al. 2004; Demaille et al. 2005; the present study) represent a potential mechanism by which metformin may be protective. Our results also open the way to the design of new lipophilic derivatives of metformin that could cross the blood–brain barrier more easily while maintaining this cytoprotective effect.

In conclusion, metformin is able to exert an effective neuroprotective effect against etoposide-triggered apoptotic cellular death in primary cortical neurons of cultured rats. By reducing the mitochondria-related cytotoxicity of etoposide in cells isolated from the central nervous system, this antidiabetic agent may soon come to represent a new direction in the treatment of neurological complications associated with diabetes.

Acknowledgments The authors are very grateful to Drs. Juan P. Bolanos and Nicolas Wiernsperger for a stimulating discussion and to Mrs. MC Alguero Martín for her helpful technical assistance in flow cytometry. This work was partially supported by the JCyL (Grant SA062/03; Spain), INSERM, and Merck.

References

- Almeida, A., Moncada, S., & Bolanos, J. P. (2004). Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. *Nature Cell Biology*, 6, 45–51.
- Andersen, J. K. (2004). Oxidative stress in neurodegeneration: Cause or consequence? *Nature Medicine*, 10, S18–S25.

- Baines, C. P., Kaiser, R. A., & Purcell, N. H., et al. (2005). Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature*, *434*, 658–662.
- Barrett, L. E., Van Bockstaele, E. J., Sul, J. Y., Takano, H., Haydon, P. G., & Eberwine, J. H. (2006). Elk-1 associates with the mitochondrial permeability transition pore complex in neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 5155–5160.
- Batandier, C., Guigas, B., & Detaille, D., et al. (2006). The ROS production induced by a reverse-electron flux at respiratory chain complex I is hampered by metformin. *J. Biomembr. Bioenerg.*, *38*, 33–42.
- Bolanos, J. P., Almeida, A., & Stewart, V., et al. (1997). Nitric oxide-mediated mitochondrial damage in the brain: Mechanisms and implications for neurodegenerative diseases. *Journal of Neurochemistry*, *68*, 2227–2240.
- Brunmair, B., Staniek, K., & Gras, F., et al. (2004). Thiazolidinediones, like metformin, inhibit respiratory complex I: A common mechanism contributing to their antidiabetic action. *Diabetes*, *53*, 1052–1059.
- Chauvin, C., De Oliveira, F., Ronot, X., Mousseau, M., Le, X., & Fontaine, E. (2001). Rotenone inhibits the mitochondrial permeability transition-induced cell death in U937 and KB cells. *Journal of Biological Chemistry*, *276*, 41394–41398.
- Chong, Z. Z., Li, F., & Maiese, K. (2005). Oxidative stress in the brain: Novel cellular targets that govern survival during neurodegenerative disease. *Progress in Neurobiology*, *75*, 207–246.
- Custodio, J. B., Cardoso, C. M., & Almeida, L. M. (2002). Thiol protecting agents and antioxidants inhibit the mitochondrial permeability transition promoted by etoposide: Implications in the prevention of etoposide-induced apoptosis. *Chemico-Biological Interactions*, *140*, 169–184.
- Delgado-Esteban, M., Martin-Zanca, D., Andres-Martin, L., Almeida, A., & Bolanos, J. P. (2007). Inhibition of PTEN by peroxynitrite activates the phosphoinositide-3-kinase/akt neuroprotective signaling pathway. *Journal of Neurochemistry*, *102*, 194–205.
- Detaille, D., Guigas, B., & Chauvin, C., et al. (2005). Metformin prevents high glucose-induced endothelial cell death through a mitochondrial permeability transition-dependent process. *Diabetes*, *54*, 2179–2187.
- Diaz-Hernandez, J. I., Moncada, S., Bolanos, J. P., & Almeida, A. (2007). Poly(ADP-ribose) polymerase-1 protects neurons against apoptosis induced by oxidative stress. *Cell Death and Differentiation*, *14*, 1211–1221.
- Duchen, M. R. (2004). Roles of mitochondria in health and disease. *Diabetes*, *53*, S96–S102.
- Dyck, P. J., Kratz, K. M., & Lehman, K. A., et al. (1991). The Rochester diabetic neuropathy study: Design, criteria for types of neuropathy, selection bias, and reproducibility of neuropathic tests. *Neurology*, *41*, 799–807.
- El-Mir, M. Y., Nogueira, V., Fontaine, E., Averet, N., Rigoulet, M., & Leverve, X. (2000). Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *Journal of Biological Chemistry*, *275*, 223–228.
- Fontaine, E., Eriksson, O., Ichas, F., & Bernardi, P. (1998). Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation by electron flow through the respiratory chain complex I. *Journal of Biological Chemistry*, *273*, 12662–12668.
- Forte, M., & Bernardi, P. (2005). Genetic dissection of the permeability transition pore. *Journal of Bioenergetics and Biomembranes*, *37*, 121–128.
- Gillessen, T., Grasshoff, C., & Szincz, L. (2002). Mitochondrial permeability transition can be directly monitored in living neurons. *Biomedicine & Pharmacotherapy*, *56*, 186–193.
- Gilman, C. P., Chan, S. L., Guo, Z., Zhu, X., Greig, N., & Mattson, M. P. (2003). p53 is present in synapses where it mediates mitochondrial dysfunction and synaptic degeneration in response to DNA damage, and oxidative and excitotoxic insults. *Neuro-molecular Medicine*, *3*, 159–172.
- Green, D. R., & Reed, J. C. (1998). Mitochondria and apoptosis. *Science*, *281*, 1309–1312.
- Guigas, B., Detaille, D., & Chauvin, C., et al. (2004). Metformin inhibits mitochondrial permeability transition and cell death: A pharmacological in vitro study. *Biochemical Journal*, *382*, 877–884.
- Hawley, S. A., Gadalla, A. E., Olsen, G. S., & Hardie, D. G. (2002). The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes*, *51*, 2420–2425.
- Karpinich, N. O., Tafani, M., Rothman, R. J., Russo, M. A., & Farber, J. L. (2002). The course of etoposide-induced apoptosis from damage to DNA and p53 activation to mitochondrial release of cytochrome c. *Journal of Biological Chemistry*, *277*, 16547–16552.
- Karpinich, N. O., Tafani, M., Schneider, T., Russo, M. A., & Farber, J. L. (2006). The course of etoposide-induced apoptosis in Jurkat cells lacking p53 and bax. *Journal of Cellular Physiology*, *208*, 55–63.
- Kroemer, G., & Reed, J. C. (2000). Mitochondrial control of cell death. *Nature Medicine*, *6*, 513–519.
- Kurosu, T., Fukuda, T., Miki, T., & Miura, O. (2003). Bcl6 overexpression prevents increase in reactive oxygen species and inhibits apoptosis induced by chemotherapeutic reagents in B-cell lymphoma cells. *Oncogene*, *22*, 4459–4468.
- Leverve, X. M., Guigas, B., & Detaille, D., et al. (2003). Mitochondrial metabolism and type-2 diabetes: A specific target of metformin. *Diabetes & Metabolism*, *29*, 6S88–6S94.
- Ma, T. C., Buescher, J. L., & Oatis, B., et al. (2007). Metformin therapy in a transgenic mouse model of Huntington's disease. *Neuroscience Letters*, *411*, 98–103.
- Mattson, M. P., & Kroemer, G. (2003). Mitochondria in cell death: novel targets for neuroprotection and cardioprotection. *Trends in Molecular Medicine*, *9*, 196–205.
- Nakajima, M., Kashiwagi, K., & Ohta, J., et al. (1994). Etoposide induces programmed death in neurons cultured from the fetal rat central nervous system. *Brain Research*, *641*, 350–352.
- Owen, M. R., Doran, E., & Halestrap, A. P. (2000). Evidence that metformin exerts its anti-diabetic effects through inhibition of complex I of the mitochondrial respiratory chain. *Biochemical Journal*, *348*, 607–614.
- Panov, A. V., Gutekunst, C.-A., & Leavitt, B. R., et al. (2002). Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nature Neuroscience*, *5*, 731–736.
- Pham, N.-U., & Hedley, D. W. (2001). Respiratory chain-generated oxidative stress following treatment of leukemic blasts with DNA-damaging agents. *Experimental Cell Research*, *264*, 345–352.
- Petronilli, V., Miotto, G., & Canton, M., et al. (1999). Transient and long-lasting openings of the mitochondrial permeability pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophysical Journal*, *76*, 725–734.
- Pirart, J. (1977). Diabetes mellitus and its degenerative complications: A prospective study of 4,400 patients observed between 1947 and 1973. *Diabetes & Metabolism*, *3*, 245–255.
- Precht, T. A., Phelps, R. A., & Linseman, D. A., et al. (2005). The permeability transition pore triggers Bax translocation to mitochondria during neuronal apoptosis. *Cell Death and Differentiation*, *12*, 255–265.
- Rapin, J. R., Lamproglou, I., Jacques, V., & Leponcin, M. (1988). Effects of metformin on metabolic indices of cerebral and peripheral ischemia. *Diabetes & Metabolism*, *14*(Suppl 4bis), 587–590.
- Robertson, J. D., Gogvadze, V., Zhivotovsky, B., & Orrenius, S. (2000). Distinct pathways for stimulation of cytochrome c release by etoposide. *Journal of Biological Chemistry*, *275*, 32438–32443.
- Schinzel, A. C., Takeuchi, O., & Huang, Z., et al. (2005). Cyclophilin D is a component of mitochondrial permeability transition and

- mediates neuronal cell death after focal cerebral ischemia. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 12005–12010.
- Wei, M. C., Zong, W. X., & Cheng, E. H., et al. (2001). Proapoptotic Bax and Bak: a requisite gateway to mitochondrial dysfunction and death. *Science*, 292, 727–730.
- Yuan, J., & Yankner, B. A. (2000). Apoptosis in the nervous system. *Nature*, 407, 802–809.
- Zhou, G., Myers, R., & Li, Y., et al. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *Journal of Clinical Investigation*, 108, 1167–1174.
- Zou, M. H., Kirkpatrick, S. S., & Davis, B. J., et al. (2004). Activation of the AMP-activated protein kinase by the antidiabetic drug metformin in vivo. Role of mitochondrial reactive nitrogen species. *Journal of Biological Chemistry*, 279, 43940–43951.