Metformin Prevents High-Glucose–Induced Endothelial Cell Death Through a Mitochondrial Permeability Transition-Dependent Process

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Hyperglycemia-induced oxidative stress is detrimental for endothelial cells, contributing to the vascular complications of diabetes. The mitochondrial permeability transition pore (PTP) is an oxidative stress–sensitive channel involved in cell death; therefore, we have examined its potential role in endothelial cells exposed to oxidative stress or high glucose level. Metformin, an antihyperglycemic agent used in type 2 diabetes, was also investigated because it inhibits PTP opening in transformed cell lines. Cyclosporin A (CsA), the reference PTP inhibitor, and a therapeutic dose of metformin (100 μmol/l) led to PTP inhibition in permeabilized human microvascular endothelial cells (HMEC-1). Furthermore, exposure of intact HMEC-1 or primary endothelial cells from either human umbilical vein or bovine aorta to the oxidizing agent *tert*-butylhydroperoxide or to 30 mmol/l glucose triggered PTP opening, cytochrome *c* decompartmentalization, and cell death. CsA or metformin prevented all of these effects. The antioxidant *N*-acetyl-L-cysteine also prevented hyperglycemia-induced apoptosis. We conclude that *1*) elevated glucose concentration leads to an oxidative stress that favors PTP opening and subsequent cell death in several endothelial cell types and *2*) metformin prevents this PTP opening–related cell death. We propose that metformin improves diabetes-associated vascular disease both by lowering blood glucose and by its effect on PTP regulation. *Diabetes* 54:2179–2187, 2005

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AMPK, AMP-activated protein kinase; BAEC, bovine aortic endothelial cell; CsA, cyclosporin A; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HMEC-1, human microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; NAC, *N*-acetyl-L-cysteine; PTP, permeability transition pore; ROS, reactive oxygen species; tBH, *tert*-butylhydroperoxide.

Diabetes is a worldwide leading cause of morbidity and mortality, and the management of chronic hyperglycemia remains a major therapeutic concern. Angiopathy represents a major complication of diabetes that determines the quality of life and life expectancy of diabetic patients. The prevalence of vascular complications has been clearly related to the degree of glycemic control, indicating that abnormally high blood glucose is a crucial risk factor for endothelial cell damage (1). Recently, compelling evidence has been provided that *1*) onset and progression of diabetes and its complications are closely associated with oxidative stress (2,3) and *2*) generation of reactive oxygen species (ROS) by the mitochondrial respiratory chain promotes seemingly independent biochemical pathways involved in the pathogenesis of hyperglycemia-induced angiopathy (4,5).

Mitochondria, albeit mainly devoted to energy metabolism, are also major actors in cell signaling functions including the commitment to cell death (6,7). Several intermembrane space proteins such as cytochrome *c*, AIF, SMAC/diablo, endonuclease G, and Omi/HtrA2, which have no pro-apoptotic activity when they remain inside mitochondria, promote cell death once released into the cytosol (8). There is evidence to suggest that two different pathways may make the outer mitochondrial membrane permeable to these pro-apoptotic proteins. One relies on outer membrane channel(s) involving Bcl-2 family proteins (9), whereas the other is due to the opening of an inner membrane channel: the permeability transition pore (PTP). Several drugs known to inhibit PTP opening have been shown to potently inhibit cell death in response to many cytotoxic insults, which strongly supports PTP opening as an important step in the cell suicide program (10,11).

In the last few years, functional links between PTP opening and respiratory chain complex I have been reported (12,13). In particular, it has been shown that the complex I inhibitor rotenone was more potent than the classical PTP inhibitor cyclosporin A (CsA) at preventing PTP opening in U937 and KB cells (14). Moreover, rotenone has been shown to prevent ROS-induced cell death (14–17), strongly suggesting that complex I inhibition may represent a critical turning point of apoptotic pathways. However, because of its toxicity, rotenone has no therapeutic potential for preventing cell death in clinical medicine, whereas serious side effects of the

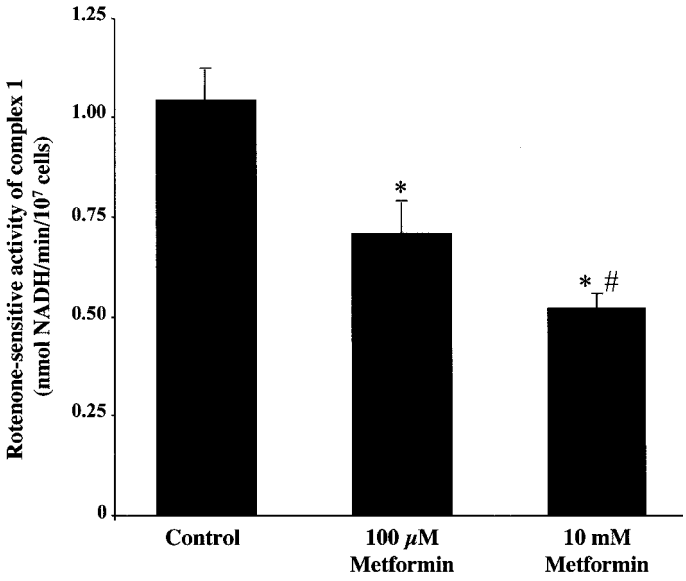

FIG. 1. Dose-dependent effect of metformin on rotenone-sensitive complex I activity in HMEC-1. HMEC-1 were incubated overnight in the presence or absence (control) of the indicated concentrations of metformin. Rotenone-sensitive NADH decylubiquinone reductase activity was measured after cell permeabilization and osmotic shock. In these conditions, 6 μmol/l rotenone blocked NADH conversion by 85 ± 6%, which indicates quantitative dependence on complex I. Each bar represents the mean ± SE of five separate experiments. *$P < 0.01$ compared with control cells. #$P < 0.05$ compared with 100 μmol/l metformin.

immunosuppressive drug CsA preclude its routine use in long-term disease such as hyperglycemia-induced angiopathy. On the other hand, it has been recently shown that metformin, an antidiabetic agent having a well-established glucose-lowering effect (18), also partly inhibits the respiratory chain complex I in various cell types (19–21). Importantly, a large prospective clinical survey has suggested that the beneficial effect of metformin on the incidence of diabetes complications was not only related to its action on blood glucose normalization (22). Therefore, we hypothesized that the slight inhibitory effect of metformin on complex I could be responsible for a subsequent prevention of PTP opening-induced cell death.

In the present work, we demonstrate that oxidative stress induces PTP opening in cultured micro- and macrovascular endothelial cells, which leads to cytochrome *c* release and subsequent cell death. Furthermore, therapeutic concentrations of metformin inhibit PTP opening and prevent endothelial cell death induced by either the direct oxidizing agent *tert*-butylhydroperoxide (tBH) or hyperglycemia.

RESEARCH DESIGN AND METHODS

Cell culture conditions. The immortalized human dermal microvascular endothelial cell line HMEC-1 (23) was a generous gift from Dr. J.J. Feige (CEA, Grenoble). HMEC-1 were maintained in MCBD 131 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 2 mmol/l L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, 10 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone. Human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs) were supplied by Dr. P. Huber (CEA, Grenoble). HUVECs were seeded in 0.2% gelatin-coated Petri dishes containing M199 medium, 20% heat-inactivated FBS, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 0.25 μg/ml amphotericin B. BAECs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 2 mmol/l L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. All cell types were grown to confluence and maintained at 37°C in a humidified atmosphere (5% CO_2). Cells were harvested after trypsinization with 0.05% trypsin-0.02% EDTA by centrifugation at 1,000 rpm for 10 min at 30°C and then counted with a Mallassez chamber. Passage numbers of cells used in experiments were HMEC-1, 40–60; HUVECs, 2–5; and BAECs, 4–8.

Assay of isolated mitochondrial complex I. Confluent monolayers of HMEC-1 were incubated overnight in the presence or absence of 100 μmol/l or 10 mmol/l metformin. Cells were harvested and immediately resuspended in a cold solution consisting of 20 mmol/l Tris, pH 7.2, 40 mmol/l KCl, 250 mmol/l sucrose, 2 mmol/l EGTA, and 200 μg/ml digitonin. After a 5-min incubation on ice, cells were spun down (10,000 rpm for 10 min) to eliminate possible cytosolic contaminating enzyme activities. The permeabilized cells were carefully washed and resuspended in the above buffer devoid of digitonin before determining complex I activity as previously described (24). In brief, 2×10^7 permeabilized cells were placed in 800 μl H_2O in a well-stirred glass cuvette for 2 min at 30°C to break mitochondrial membranes by hypotonic shock. Next, 200 μl Tris solution (50 mmol/l, pH 8.0) containing 50 μmol/l NADH was added for 1 further min, and the reaction was started by adding 50 μmol/l decyl-ubiquinone as a final electron acceptor. NADH oxidation rate was measured fluorimetrically (excitation–emission, 340–460 nm). The rotenone-sensitive complex I activity was obtained after subtraction of the remaining signal in the presence of 6 μmol/l rotenone.

Determination of permeability transition in permeabilized HMEC-1. Endothelial cells (1×10^7) were incubated overnight in serum-free MCDB 131 medium alone or in the presence of 100 μmol/l metformin. Cells were permeabilized immediately before use by incubation under stirring for 2 min at 25°C in a medium containing 10 mmol/l MOPS (pH 7.35), 250 mmol/l sucrose, 1 mmol/l Pi-Tris, 5 mmol/l succinate, 50 μmol/l EDTA, and 100 μg/ml digitonin. Measurement of Ca^{2+} was carried out fluorimetrically at 25°C using a PTI Quantamaster C61 spectrofluorimeter in the presence of 0.25 μmol/l Calcium Green-5N (Molecular Probes, Eugene, OR), with excitation and emission wavelengths set at 506 and 527 nm, respectively.

Determination of permeability transition in intact human or bovine endothelial cells. For calcein/cobalt staining $5–8 \times 10^4$ endothelial cells were grown for 2 days on 22-mm diameter glass coverslips and exposed for 20 min at 37°C to PBS medium supplemented with 5 mmol/l glucose, 0.35 mmol/l pyruvate, 1 mmol/l $CoCl_2$, and 1 μmol/l calcein acetomethoxyl ester (Molecular Probes) as described by Petronilli et al. (25). After loading, cells were washed free of calcein plus cobalt and incubated for another 30 min in warm PBS/glucose/pyruvate mixture. Coverslips were mounted on the stage of an inverted fluorescence microscope, and PTP opening was triggered by adding 100 μmol/l tBH. Images were collected every minute with a constant exposure time.

Cell death induction. HMEC-1, HUVECs, or BAECs, either incubated overnight in the presence of 100 μmol/l metformin or incubated for 30 min in the presence or absence of 1 μmol/l CsA, were exposed to tBH for 45 min and then washed and incubated with fresh complete culture medium for 6 h at 37°C. Cytotoxicity was evaluated by staining dead cells with annexin V. For induction of cell death by glucose, cells were exposed to 5.5 mmol/l glucose (control cells) or to 30 mmol/l glucose for 24 to 72 h. The medium was changed every day. Where indicated, cells were also exposed to 5.5 mmol/l glucose + 25 mmol/l mannitol or to 5.5 mmol/l D-glucose + 25 mmol/l L-glucose (osmotic controls).

Detection of cytochrome *c* release by immunocytochemistry. HMEC-1 and HUVECs cultured on glass coverslips were fixed in 3.7% paraformaldehyde/PBS for 20 min, permeabilized in 0.2% Triton X-100 for 5 min, and blocked in 2% BSA/PBS (blocking buffer) for 1 h. Human endothelial cells were next stained for the detection of cytochrome *c*, using a suitable secondary antibody (goat anti-mouse IgG conjugated with fluorescein isothiocyanate [FITC] or Oregon green), as described elsewhere (26).

Cell imaging. Cell images were acquired at 25°C with a Nikon TE200 microscope equipped for epifluorescent illumination (xenon light source, 75 W) and associated with a 12-bit digital-cooled charged-couple device camera (SPOT-RT, Diagnostic Instruments). For calcein and cytochrome *c* fluorescence, 488 ± 5/525 ± 10 nm excitation/emission filter settings were applied, using a 60×/1.40 Plan Apo oil immersion objective (Nikon). For detection of FITC-annexin V, at least five randomly selected fields were inspected from each culture plate, using the 488 ± 5/525 ± 10 nm excitation/emission filter settings and a ELWD 20×/0.45 Plan Fluor objective (Nikon). The corresponding bright-field negatives were also acquired, and the two channels were accordingly overlaid using the appropriate function of the SPOT 3.0.6 software.

Statistics. Results are presented as means ± SE. Statistical significance of differences was analyzed using Student's *t* test (paired and unpaired observations). Values were considered different from each other when *P* values were <0.05.

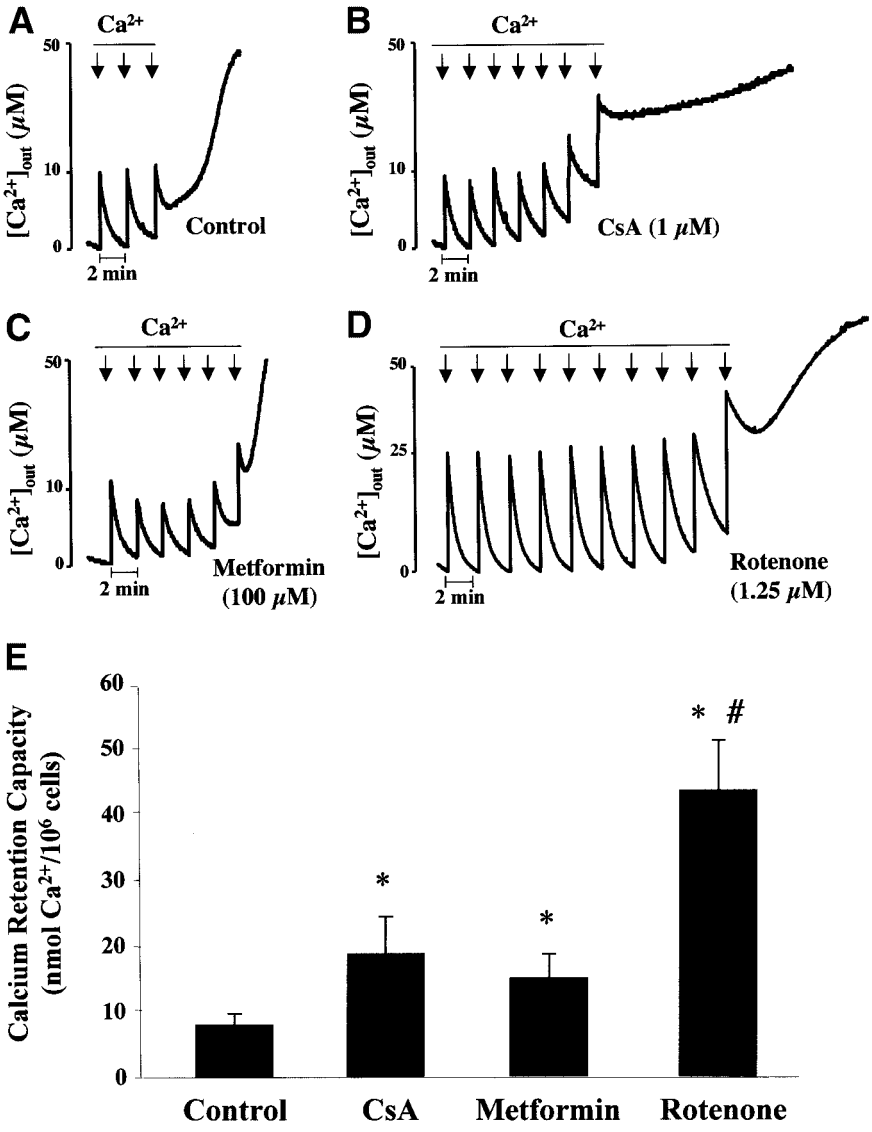

FIG. 2. Effects of CsA, metformin, or rotenone on PTP opening in permeabilized HMEC-1 cells. Control cells (*A*), cells incubated overnight in the presence of 100 μmol/l metformin (*C*), or cells directly exposed to 1 μmol/l CsA or 1.25 μmol/l rotenone (*B* and *D*, respectively) were digitonin permeabilized in a medium supplemented with 0.25 μmol/l Calcium Green, and mitochondria were energized with 5 mmol/l succinate. Where indicated, pulses of 10 μmol/l Ca^{2+} (or 25 μmol/l Ca^{2+} in the case of rotenone) were injected every 2 min. *E*: Quantitation of the calcium retention capacity for each experimental condition. Each bar represents the mean ± SE of six separate experiments. **P* < 0.01 compared with control cells. #*P* < 0.05 compared with 100 μmol/l metformin.

RESULTS

Metformin induces complex I inhibition in human endothelial cells. In light of recent literature documenting an inhibitory effect of metformin on respiratory chain complex I in *Xenopus laevis* oocytes (19), rat liver cells (20), and KB cells (21), we have investigated this phenomenon with more relevant cell types in the context of diabetes, such as human endothelial cells. As shown in Fig. 1, metformin induced a significant and dose-dependent inhibition of the rotenone-sensitive complex I activity in HMEC-1 cells. It must be noted however that metformin remained a mild inhibitor of complex I even at high (saturating) concentration (10 mmol/l) and was unable to completely block electron flux through complex I. Interestingly, a lower dose of metformin (100 μmol/l) also significantly inhibited complex I activity, and therefore this concentration close to the therapeutic range was then used in this work.

Metformin modulates PTP opening in permeabilized and intact endothelial cells. We next tested whether complex I inhibition by metformin regulated PTP opening in HMEC-1. PTP opening was induced by addition of Ca^{2+} pulses on digitonin-permeabilized cells. Under this protocol, HMEC-1 took up and retained Ca^{2+} until induction of permeability transition, as assessed by calcium release (Fig. 2*A*). As expected, the reference PTP inhibitor CsA increased the amount of calcium required for PTP opening (Fig. 2*B*). Interestingly, metformin (Fig. 2*C*) also inhibited PTP opening with an efficacy similar to that of CsA. It must be noted that rotenone (Fig. 2*D*) was more potent than CsA at preventing PTP opening in HMEC-1, as previously observed with U937 and KB cells. As shown in Fig. 2*E*, these effects were significant.

PTP opening was also investigated in situ in three different vascular endothelial cell types (HMEC-1, HUVEC, and BAEC) by monitoring the distribution of calcein fluorescence after challenging intact cells by tBH, an oxidizing agent known to induce PTP opening in living cells (21,27,28). Before any addition, cellular calcein fluorescence was highly compartmentalized, corresponding to the mitochondrial space (Fig. 3, 0 min). After tBH addition, a progressive decompartmentalization associated with a gradual decrease in signal intensity was obtained. Upon permeability, cobalt diffuses from the cytosol into mitochondria, thus quenching calcein-related fluorescence in this compartment. The intracellular decompartmentaliza-

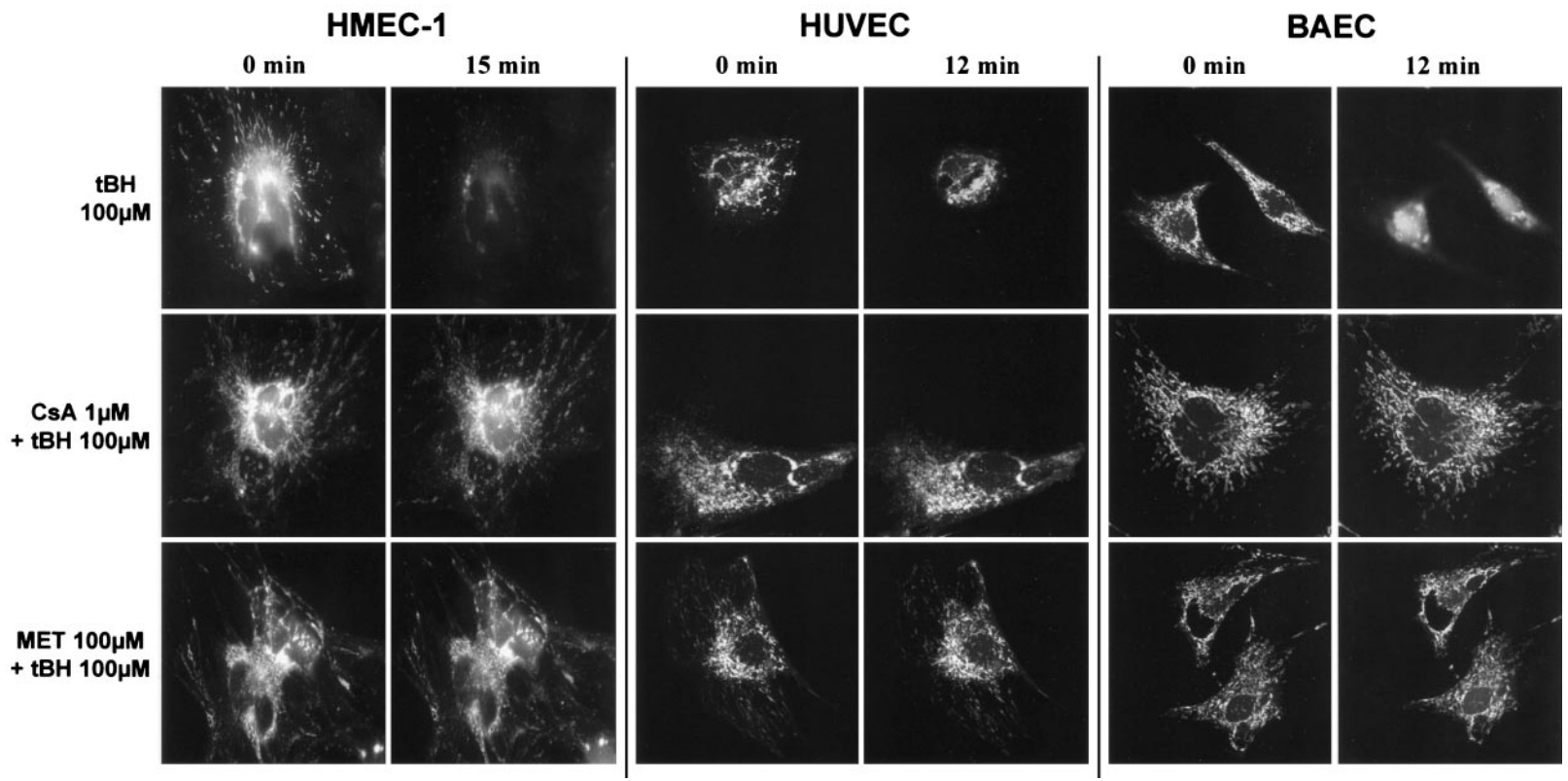

FIG. 3. Effects of CsA or metformin on tBH-induced PTP opening in intact endothelial cells. HMEC-1, HUVECs, and BAECs were loaded for 15 min with 1 μmol/l calcein and 1 mmol/l cobalt and exposed to 100 μmol/l tBH. Images were collected every minute with an inverted microscope, using a 60× oil immersion objective. Control cells (*top*), cells incubated in the presence of CsA, and metformin (MET) overnight-incubated cells are shown at 0 min and 12–15 min after tBH addition. Similar results were obtained in four other cell preparations.

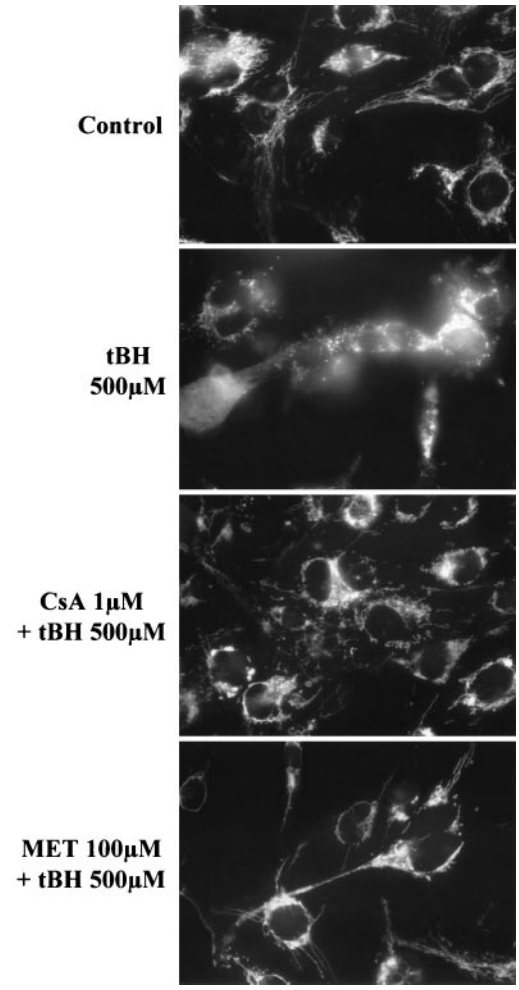

FIG. 4. Effects of CsA or metformin on cytochrome *c* distribution in HMEC-1 exposed to tBH. Control cells, cells incubated in the presence of CsA, and metformin (MET) overnight-incubated cells were exposed to 500 μmol/l tBH for 45 min and then cultured in fresh complete culture medium for 14 h before fixing and staining with anti-cytochrome *c* antibody. Control denotes endothelial cells not exposed to tBH but incubated under similar conditions. Similar results were obtained in four other cell preparations.

tion was completely achieved within 10–15 min. When endothelial cells were previously exposed to CsA or metformin, the cellular heterogeneity of fluorescence was remarkably preserved over this time period (Fig. 3). These results clearly indicated that metformin was as effective as CsA at preventing oxidative stress-related PTP opening in both transformed (HMEC-1) and primary (HUVECs and BAECs) endothelial cells.

Metformin prevents tBH-related cytochrome *c* release and cell death. Because the release of cytochrome *c* from mitochondrial intermembrane space is a key process in the commitment to cell death, we further investigated cytochrome *c* distribution in HMEC-1 treated or not treated with CsA or metformin and transiently exposed to 500 μmol/l tBH (Fig. 4). Cytochrome *c* in control cells was confined within mitochondria, which appeared as thin filaments around the nucleus. After tBH addition, cytochrome *c* diffused out of mitochondria, which appeared as spherical structures. CsA or metformin completely prevented the tBH-induced delocalization of cytochrome *c*, indicating that PTP inhibition also prevented tBH-induced cytochrome *c* release.

As shown in Fig. 5*A*, tBH treatment led to a dramatic increase in the percentage of apoptotic cell death whatever the endothelial cell type studied. This harmful effect of tBH was fully prevented by either CsA or metformin. As shown in Fig. 5*B*, tBH cytotoxicity nearly plateaued above 100 μmol/l, and a 10-fold increase in tBH concentration (from 100 to 1,000 μmol/l) was only accompanied by a 1.5-fold increase in the percentage of dead HMEC-1.

Metformin or CsA prevent high-glucose–induced endothelial cell death. Because *1*) metformin was able to suppress tBH-induced cell death in micro- and macrovascular endothelial cells, *2*) tBH is a potent oxidant, and *3*) evidence suggests that hyperglycemia toxicity may be related to oxidative stress, we next investigated the effect of metformin on cell viability when HMEC-1, HUVECs, or BAECs were cultured under elevated glucose concentration. After either a 48- or 72-h incubation time, 30 mmol/l glucose led to a significant 3.5-fold rise in death of HMEC-1 (Fig. 6*A*). This action was unrelated to osmotic change because 5.5 mmol/l D-glucose plus 25 mmol/l mannitol or 5.5 mmol/l D-glucose plus 25 mmol/l L-glucose did not affect cell viability. Confirming that glucotoxicity is related to oxidative stress, addition of 10 mmol/l *N*-acetyl-L-cysteine (NAC) completely prevented the high-glucose–induced cell death. Furthermore, CsA or metformin fully blocked hyperglycemia-induced apoptotic cell death. Interestingly, either CsA or metformin prevented hyperglycemia-promoted apoptosis in HUVECs and BAECs (Fig. 6*B*).

Finally, we explored the relationship between cytotoxicity of glucose and cytochrome *c* release by studying cytochrome *c* compartmentalization in HMEC-1 and HUVECs cultured under a low or high D-glucose concentration for 36 h. As shown in Fig. 7, 30 mmol/l glucose induced cytochrome *c* release in some endothelial cells (~25–30%); this effect was prevented by CsA or metformin. The ratio of cells exhibiting a cytochrome *c* decompartmentalization was roughly similar to that of annexin V–stained cells in similar conditions (Fig. 6). Moreover, the nonmetabolizable compound L-glucose did not affect cytochrome *c* distribution, underlying the importance of glucose metabolism in glucose cytotoxicity (data not shown).

DISCUSSION

In this work, we show that metformin inhibits respiratory chain complex I activity in HMEC-1 as well as mitochondrial PTP opening in three endothelial cell types (HMEC-1, HUVECs, and BAECs), in which it prevents tBH- and hyperglycemia-induced cell death. We propose that the initial effect of metformin on complex I might be responsible for the vanishing of apoptotic events in cascade.

As already reported in other cells (19–21), metformin appears to be a mild inhibitor of complex I in HMEC-1, because it leads to a moderate inhibition even at high concentration compared with rotenone (85 vs. 40%, respectively). Concomitantly to its effect on complex I, metformin also inhibits PTP opening in HMEC-1 (Fig. 2). The fact that complex I inhibition led to PTP inhibition regardless of the inhibitor (rotenone or metformin) suggests that metformin inhibited PTP opening because it decreased electron flux through complex I. Consistent

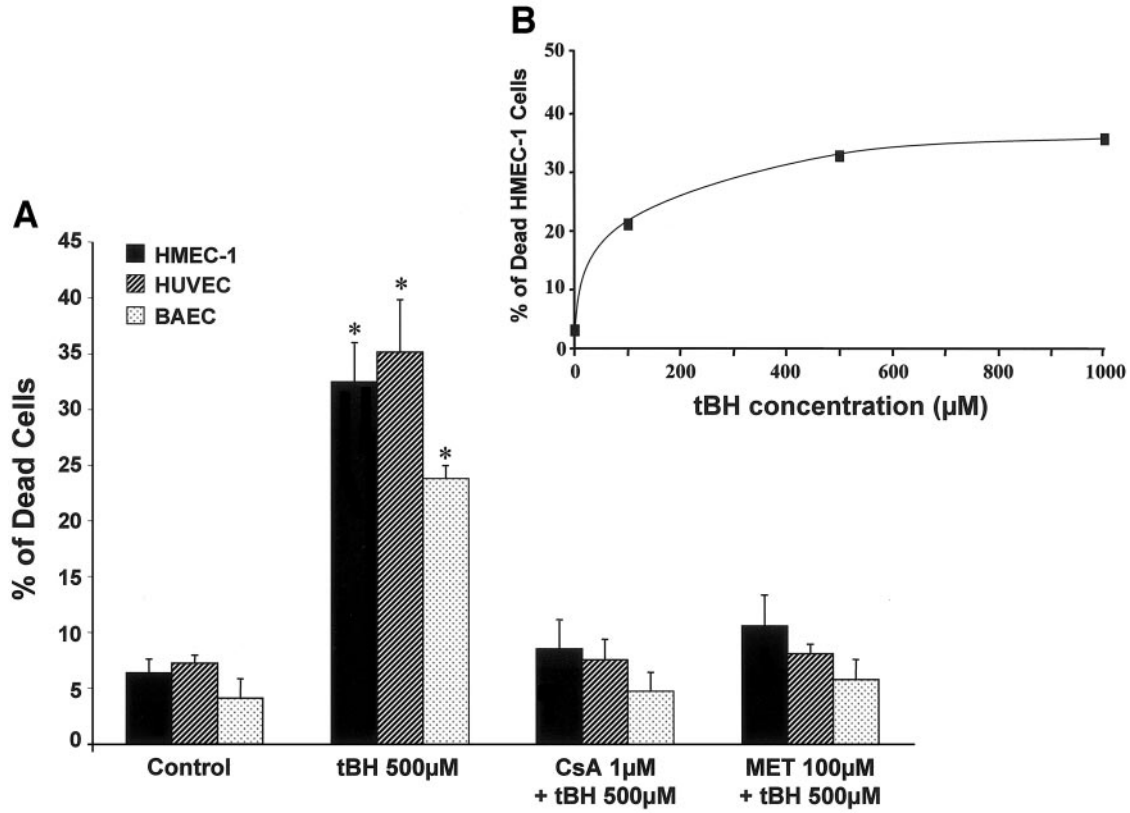

FIG. 5. Effect of CsA or metformin (MET) on tBH-induced cell death in HMEC-1, HUVECs, and BAECs. *A*: Control cells, cells incubated in the presence of CsA, and metformin overnight-incubated cells were exposed to tBH as described in the legend to Fig. 4. Cells were next incubated in fresh complete culture medium for 6 h before assessing cell death by FITC-annexin V staining. Each bar represents the mean ± SE of four experiments. *$P < 0.01$, **$P < 0.005$ compared with control cells. *B*: HMEC-1 were exposed to the indicated tBH concentrations and then incubated for 6 h before assessing cell death by FITC–annexin V staining. More than 500 cells were counted and analyzed during each assay.

with this proposal, the stronger the inhibition of complex I was, the higher the calcium retention capacity was (compare Figs. 1 and 2). Moreover, it can be noted that PTP opening, which is not regulated by complex I activity in liver cells, is not inhibited by metformin or rotenone in liver mitochondria (data not shown).

The fact that nonmetabolized L-glucose did not lead to cell death (Fig. 6*A*) and did not induce cytochrome *c* release (data not shown) indicates that the mitochondrion-driven apoptotic pathway is linked to glucose metabolism. It has been shown that glycolytic flux during hyperglycemia led to an overproduction of ROS at the level of the mitochondrial electron transport chain (29). The harmful effect of ROS on cells is widely appreciated, and findings from Fig. 5 confirm that exogenous oxidizing agent tBH alters cell viability irrespective of the vascular cell type. Furthermore, this study shows that glucotoxicity is not due to an osmotic stress (no effect of mannitol or L-glucose), whereas the antioxidant NAC prevents endothelial cell death promoted by a high glucose level (Fig. 6*A*). This implies that the adverse effects of hyperglycemia in cultured endothelial cells are at least partly attributable to the occurrence of an oxidative stress. The fact that metformin prevents tBH-induced cell death suggests that metformin may affect an event that follows ROS generation.

Among various effects of ROS on cell metabolism, ROS are recognized to favor PTP opening both in vitro and in intact cells. The fact that tBH-induced oxidative stress triggered PTP opening (Fig. 3), cytochrome *c* release (Fig. 4), and cell death (Fig. 5), whereas PTP inhibitors prevented all of these events, strongly suggests that oxidative stress toxicity in endothelial cells is mainly due to PTP opening. Because glucose cytotoxicity was prevented by PTP inhibitors in either an immortalized cell line (Fig. 6*A*) or primary cells (Fig. 6*B*), we propose that hyperglycemia induces PTP opening in endothelial cells, too. It must be noted that because of photobleaching and phototoxicity, long-exposure experiments with the calcein technique were precluded. Therefore, hyperglycemia-induced PTP openings were not directly visualized in intact vascular cells. Nonetheless, we herein documented that PTP inhibitors blocked cytochrome *c* release (Fig. 7), probably by modulating mitochondrial permeability. Collectively, these data tend, at best, to demonstrate that endothelial cells undergo programmed cell death when exposed to experimental conditions designed to mimic a diabetic state. Furthermore, the presence of apoptotic changes in this setting suggests a possible, but unproven, link between activation of ROS and death initiation through PTP opening.

If the preventive effect of metformin on PTP opening and cell death is clear, its primary cellular target remains partly unknown. When metformin, which is not metabolized (30), was administered to intact cells, it induced a partial inhibition of complex I in HMEC-1 (this study; Fig. 1), *Xenopus* oocytes (19), rat liver cells (20), and KB cells (21), with an effect persisting after cell permeabilization. Because *1*) microinjection of metformin in intact cells did not affect complex I, whereas liposome-encapsulated met-

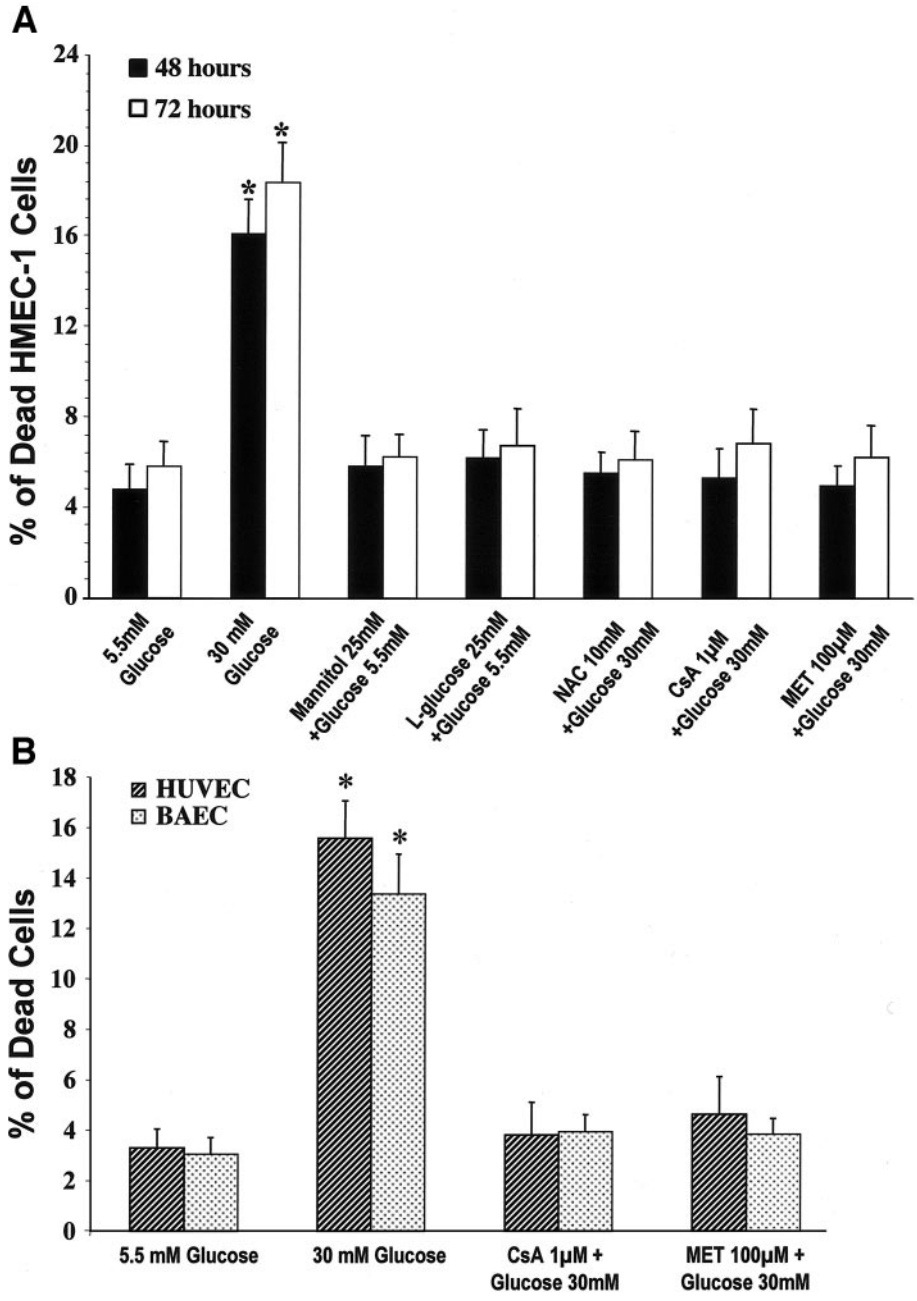

FIG. 6. Effect of CsA, metformin (MET), or NAC on hyperglycemia-induced endothelial cell death. *A*: HMEC-1 were incubated in a medium supplemented either with 5.5 mmol/l glucose, 30 mmol/l glucose, 5.5 mmol/l glucose plus 25 mmol/l mannitol, or 5.5 mmol/l glucose plus 25 mmol/l L-glucose. Where indicated, cells were incubated in the presence of NAC, CsA, or metformin. *B*: HUVECs and BAECs were incubated in a medium supplemented either with 5.5 mmol/l gluclose or 30 mmol/l glucose in the absence and presence of CsA or metformin. The percentage of apoptotic cell death was assessed after annexin V staining at 48 and 72 h (*A*) or at 48 h (*B*). Each bar represents the mean ± SE of six experiments. *$P < 0.01$ compared with all of the other conditions of incubation.

formin led to complex I inhibition in isolated mitochondria (19), and *2*) metformin-induced inhibition of complex I did not occur when cells were incubated at low temperature (20), we proposed that metformin could affect mitochondrial function via an original plasma membrane-related mechanism. By contrast, it has been reported in the literature that metformin is able to inhibit complex I in isolated mitochondria or disrupted tissues when exposed in a high concentration and/or for a long incubation time (31,32). Such apparently controversial results could be reconciled with the hypothesis that although a direct effect of metformin on complex I is possible, it seems to be considerably facilitated in intact cells (33).

Besides its effect on complex I, several reports during recent years (34,35) have shown that metformin activates AMP-activated protein kinase (AMPK). It is possible that metformin affects cell death by first activating AMPK, which would be responsible for the respiratory chain–related effects (complex I, PTP regulation, and cytochrome *c* release). Moreover, it has been shown that addition of the AMPK activator 5-aminoimidazole-4-carboxamide-riboside to endothelial cells prevents some of the metabolic effects of hyperglycemia preceding cell death (36). AMPK activation might affect the phosphorylation status of complex I, but it must be noted that AMPK is located in the cytoplasm and the nucleus, not in mitochondria. Alternatively, AMPK activation might affect hexokinase binding to the voltage-dependent anion channel, which is known to prevent cytochrome *c* release and to inhibit the commitment to cell death (37). However, evidence supporting this proposal is lacking. Conversely, it can be hypothesized that the modulatory effect of metformin on complex I may be responsible for the AMPK activation.

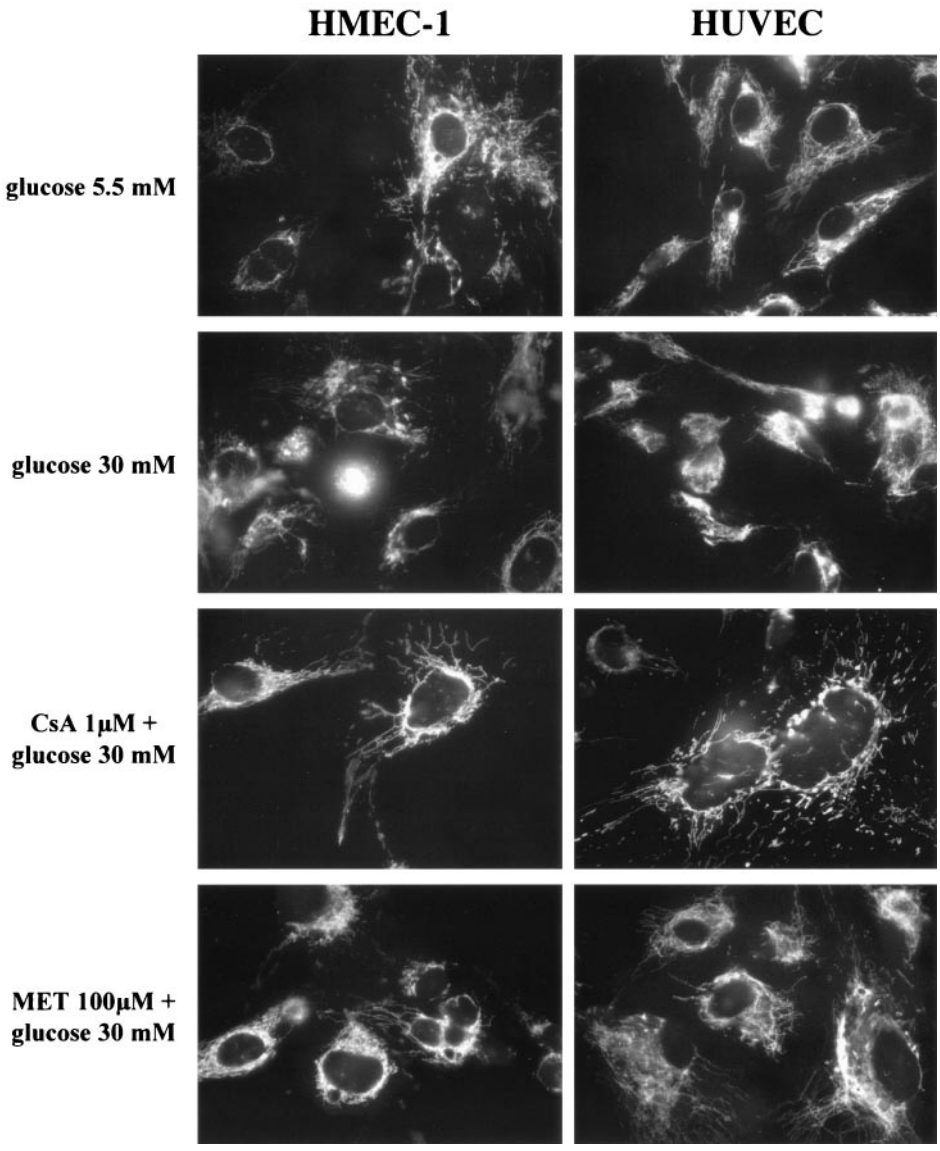

FIG. 7. Effects of CsA or metformin (MET) on cytochrome c distribution in human endothelial cells exposed to hyperglycemic stress. Immortalized (HMEC-1) or primary (HUVECs) endothelial cells were incubated in a medium supplemented wither with 5.5 mmol/l glucose or 30 mmol/l glucose in the absence or presence of CsA or metformin. After 36 h, cells were fixed and stained with anti-cytochrome *c* antibody. Similar results were obtained in four other cell preparations.

Regardless of the exact mechanism by which metformin regulates complex I, this work shows that metformin prevents human-derived endothelial cell death induced by high glucose exposure. Such beneficial effects of metformin may partly explain its long-term protection on diabetes-related vascular complications (38). Besides any active attempts in lowering blood glucose, such properties in reducing high glucose toxicity might reveal valuable targets for novel therapies to fight diabetes more efficiently.

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REFERENCES

1. UK Prospective Diabetes Study Group: Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 352:837–853, 1998
2. Rosen P, Nawroth PP, King G, Moller W, Tritschler HJ, Packer L: The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab Res Rev* 17:189–212, 2001
3. Wiernsperger NF: Oxidative stress: the special case of diabetes. *Biofactors* 19:11–18, 2003
4. Du X, Matsumura T, Edelstein D, Rossetti L, Zsengeller Z, Szabo C, Brownlee M: Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest* 112:1049–1057, 2003
5. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787–790, 2000
6. Desagher S, Martinou JC: Mitochondria as the central control point of apoptosis. *Trends Cell Biol* 10:369–377, 2000
7. Nieminen AL: Apoptosis and necrosis in health and disease: role of mitochondria. *Int Rev Cytol* 224:29–55, 2003
8. Ravagnan L, Roumier T, Kroemer G: Mitochondria, the killer organelles and their weapons. *J Cell Physiol* 192:131–137, 2002
9. Sharpe JC, Arnoult D, Youle RJ: Control of mitochondrial permeability by Bcl-2 family members. *Biochim Biophys Acta* 1644:107–113, 2004
10. Bernardi P, Scorrano L, Colonna R, Petronilli V, Di Lisa F: Mitochondria and cell death: mechanistic aspects and methodological issues. *Eur J Biochem* 264:687–701, 1999
11. Petronilli V, Penzo D, Scorrano L, Bernardi P, Di Lisa F: The mitochondrial permeability transition, release of cytochrome *c* and cell death: correlation

with the duration of pore openings in situ. *J Biol Chem* 276:12030–12034, 2001
12. Fontaine E, Eriksson O, Ichas F, Bernardi P: Regulation of the permeability transition pore in skeletal muscle mitochondria: modulation by electron flow through the respiratory chain complex I. *J Biol Chem* 273:12662–12668, 1998
13. Fontaine E, Bernardi P: Progress on the mitochondrial permeability transition pore: regulation by complex I and ubiquinone analogs. *J Bioenerg Biomembr* 31:335–345, 1999
14. Chauvin C, De Oliveira F, Ronot X, Mousseau M, Leverve X, Fontaine E: Rotenone inhibits the mitochondrial permeability transition-induced cell death in U937 and KB cells. *J Biol Chem* 276:41394–41398, 2001
15. Dumont A, Hehner SP, Hofmann TG, Ueffing M, Droge W, Schmitz ML: Hydrogen peroxide-induced apoptosis is CD95-independent, requires the release of mitochondria-derived reactive oxygen species and the activation of NF-κB. *Oncogene* 18:747–757, 1999
16. Guidarelli A, Brambilla L, Clementi E, Sciorati C, Cantoni O: Stimulation of oxygen consumption promotes mitochondrial calcium accumulation, a process associated with, and causally linked to, enhanced formation of tert-butylhydroperoxide-induced DNA single-strand breaks. *Exp Cell Res* 237:176–185, 1997
17. Sestili P, Brambilla L, Cantoni O: Rotenone and pyruvate prevent the tert-butylhydroperoxide-induced necrosis of U937 cells and allow them to proliferate. *FEBS Lett* 457:139–143, 1999
18. Bailey CJ: Biguanides and NIDDM. *Diabetes Care* 15:755–775, 1992
19. Detaille D, Guigas B, Leverve X, Wiernsperger N, Devos P: Obligatory role of membrane events in the regulatory effect of metformin on the respiratory chain function. *Biochem Pharmacol* 63:1259–1272, 2002
20. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X: Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem* 275:223–228, 2000
21. Guigas B, Detaille D, Chauvin C, Batandier C, De Oliveira F, Fontaine E, Leverve X: Metformin inhibits mitochondrial permeability transition and cell death: a pharmacological in vitro study. *Biochem J* 382:877–884, 2004
22. UK Prospective Diabetes Study Group: Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet* 352:854–865, 1998
23. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ: HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol* 99:683–690, 1992
24. Rustin P, Chretien D, Bourgeron T, Gerard B, Rotig A, Saudubray JM, Munnich A: Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta* 228:35–51, 1994
25. Petronilli V, Miotto G, Canton M, Brini M, Colonna R, Bernardi P, Di Lisa F: Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophys J* 76:725–734, 1999
26. Rossé T, Olivier R, Monney L, Rager M, Conus S, Fellay I, Jansen B, Borner C: Bcl-2 prolongs cell survival after Bax-induced release of cytochrome *c*. *Nature* 391:496–499, 1998
27. Collins TJ, Berridge MJ, Lipp P, Bootman MD: Mitochondria are morphologically and functionally heterogeneous within cells. *EMBO J* 21:1616–1627, 2002
28. Lemasters JJ, Qian T, Bradham CA, Brenner DA, Cascio WE, Trost LC, Nishimura Y, Nieminen AL, Herman B: Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. *J Bioenerg Biomembr* 31:305–319, 1999
29. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
30. Bailey CJ, Turner RC: Metformin. *N Engl J Med* 334:574–579, 1996
31. Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R, Roden M, Gnaiger E, Nohl H, Waldhausl W, Furnsinn C: Thiazolidinediones, like metformin, inhibit respiratory complex I: a common mechanism contributing to their antidiabetic actions? *Diabetes* 53:1052–1059, 2004
32. Owen MR, Doran E, Halestrap AP: Evidence that metformin exerts its anti-diabetic effects through inhibition of complex I of the mitochondrial respiratory chain. *Biochem J* 348:607–614, 2000
33. Leverve XM, Guigas B, Detaille D, Batandier C, Koceir EA, Chauvin C, Fontaine E, Wiernsperger NF: Mitochondrial metabolism and type-2 diabetes: a specific target of metformin. *Diabete Metab* 29:6S88–6S94, 2003
34. Hawley SA, Gadalla AE, Olsen GS, Hardie DG: The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* 51:2420–2425, 2002
35. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE: Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108:1167–1174, 2001
36. Ido Y, Carling D, Ruderman N: Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation. *Diabetes* 51:159–167, 2002
37. Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N: Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev* 15:1406–1418, 2001
38. Wiernsperger NF: Metformin: intrinsic vasculoprotective properties. *Diabetes Technol Ther* 2:259–272, 2000