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 compartmentalization, 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

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...
The immortalized human dermal microvascular endothelial cells, which leads to cytochrome c release and subsequent cell death. Furthermore, therapeutic concentrations of metformin inhibit PTP opening and prevent endo-

ditions 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 MCDB 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 and bovine aortic endothelial cells (BAECs) were supplied by Dr. P. Huber (CEA, Grenoble). HUVECs were seeded in 0.2% gelatin-coated Petri dishes and bovine aortic endothelial cells (BAECs) were supplied by Dr. P. Huber (CEA, Grenoble). HUVECs were seeded in 0.2% gelatin-coated Petri dishes and bovine aortic endothelial cells (BAECs) were supplied by Dr. P. Huber (CEA, Grenoble). HUVECs were seeded in 0.2% gelatin-coated Petri dishes and bovine aortic endothelial cells (BAECs) were supplied by Dr. P. Huber (CEA, Grenoble).
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. 2A). As expected, the reference PTP inhibitor CsA increased the amount of calcium required for PTP opening (Fig. 2B). Interestingly, metformin (Fig. 2C) also inhibited PTP opening with an efficacy similar to that of CsA. It must be noted that rotenone (Fig. 2D) was more potent than CsA at preventing PTP opening in HMEC-1, as previously observed with U937 and KB cells. As shown in Fig. 2E, 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 decompartmentalization-
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.
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tBH but incubated under similar conditions. Similar results were
indicating that PTP inhibition also prevented tBH-induced
release and cell death. Because tBH treatment led to a dramatic
increase in the percentage of apoptotic cell death what-
ever the endothelial cell type studied. This harmful effect of tBH was fully prevented by either CsA or metformin. As shown in Fig. 5B, 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. 6A). 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. 6B).

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.

As shown in Fig. 5A, 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. 5B, 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.

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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. 6A) 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 hyperglyce-
mia 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 endothe-

tial cell death promoted by a high glucose level (Fig. 6A).
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 met-
formin prevents tBH-induced cell death suggests that met-
formin 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 pre-
vented 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. 6A)
or primary cells (Fig. 6B), 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 inhib-
itors 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 experi-
mental 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 metabo-
lized (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. 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.
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-aminimidazole-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.
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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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 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.


