

Metformin inhibits mitochondrial permeability transition and cell death: a pharmacological *in vitro* study

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Abbreviations: AMPK, AMP-activated protein kinase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CsA, cyclosporin A; JO_2 , oxygen consumption rate; Met, metformin; PTP, permeability transition pore; ROS, reactive oxygen species; tBH, *tert*-butyl hydroperoxide; TMPD, *N, N, N', N'*-tetramethyl-1,4-phenylenediamine.

ABSTRACT

Metformin, a drug widely used in the treatment of type-2 diabetes, has recently received attention by new findings regarding its mitochondrial and cellular effects. In this work the effect of metformin was investigated in cultured cells from a human carcinoma derived cell line (KB cells) on respiration, complex 1 activity, mitochondrial permeability transition, cytochrome c release and cell death. Metformin significantly decreased respiration both in intact cells and after permeabilization. This was due to a mild and specific inhibition of the respiratory chain complex 1. In addition, metformin significantly prevented mitochondrial permeability transition both in permeabilized cells, as induced by calcium, and in intact cells, as induced by the glutathione-oxidizing agent *tert*-butyl hydroperoxide. This effect was equivalent to that of cyclosporin A, the reference inhibitor. Finally, metformin impaired *tert*-butyl hydroperoxide-induced cell death, as assessed by trypan blue exclusion, propidium iodide staining and cytochrome c release. We propose that metformin prevents the permeability transition-related commitment to cell death in relation to its mild inhibitory effect on complex 1, which is responsible for a decreased probability of mitochondrial permeability transition.

Key words: Metformin, mitochondria, complex 1, PTP, oxidative stress, cell death

INTRODUCTION

The dramatic increase in the prevalence of type-2 diabetes is a leading health problem worldwide, which occurs in both developed and developing countries [1, 2]. It is now believed that hyperglycemia is not only a marker, but also a causal event responsible *per se* for most of the deleterious consequences of this disease. Hyperglycemia, which is the major metabolic abnormality in type-2 diabetes [3], has also been recently emphasized as an important factor in the prognosis of intensive care patients with inflammation-related insulin resistance [4]. Regarding the deleterious effect of hyperglycemia, Brownlee proposed unifying hypothesis based on superoxide overproduction from the mitochondrial electron transport chain as a consequence of hyperglycemia-related increased glycolysis [5]. Hence, if reducing hyperglycemia as early and as profoundly as possible remains the cornerstone for the treatment of diabetes [6], decreasing reactive oxygen species (ROS)-related glucose toxicity at the cellular level may represent an additional attractive perspective.

Among the different drugs used for the treatment of type-2 diabetes, metformin is widely used [7-9] since it lowers glucose by increasing muscle glucose uptake [10] and decreasing hepatic glucose production [11, 12]. However, its cellular mechanism of action is still poorly understood. Recently, Zhou *et al.* demonstrated that metformin activated the AMP-activated protein kinase (AMPK) both in hepatocytes and skeletal muscle [13]. This finding is of importance since AMPK is involved in the regulation of both glucose production and fatty acid oxidation, although the link between metformin and AMPK activation is not yet clear since, it does not seem to change the intracellular AMP:ATP ratio [14, 15]. Recently, we reported that metformin also mildly inhibits the respiratory chain in liver cells [16]. This effect, which is exclusively located on the respiratory chain complex 1, occurs only in intact cells, but not in isolated mitochondria nor in permeabilized cells and it vanishes at low temperature [16].

Studies over the last decade have greatly emphasized the central role of mitochondria, besides its prominent function in cellular energy metabolism, in several other major processes including the control of cell death. Although the exact mechanism relating mitochondria and cell death still requires clarification, it is likely that the mitochondrial Permeability Transition Pore (PTP) is involved via the release of cytochrome c [17]. Moreover, there is other evidence to suggest that a PTP-independent pathway involving Bcl-2 family proteins may also contribute to cytochrome c release from the mitochondrial intermembrane space to the

cytosol. Both mechanisms, *i.e.* PTP-dependent and independent, could potentially contribute to the commitment to cell death [18].

The molecular nature of the PTP is still unknown, but its modulation by several physiological factors has been widely studied [17]. Among these, Ca^{2+} is certainly the most important inducer, while matrix pH, transmembrane electrical potential, Mg^{2+} , Pi, cyclophilin D, oxidative stress, and adenine nucleotides are also effective regulators [17, 19]. In addition, cyclosporin A (CsA) is regarded as a specific reference inhibitor of the PTP. Recently we reported that the PTP is also modulated by the electron flux through respiratory chain complex 1 [17, 19]. This was initially proposed because a different amount of Ca^{2+} was necessary to induce the permeability transition according to the nature of the respiratory substrates, *i.e.* glutamate versus succinate. This observation, together with other considerations [20], led us to propose that the respiratory chain complex 1 may be part of the PTP [17, 19, 20]. By investigating the effects of complex 1 inhibitor rotenone, we found a significant inhibition of PTP associated with the prevention of cell death [21].

In the light of the mitochondrial effect of metformin on the respiratory chain [16], we hypothesized that this drug, by inhibiting complex 1, modulates the mitochondrial permeability transition and thus prevents the cell death due to PTP-related cytochrome c release.

MATERIALS AND METHODS

Materials and products.

Cells from an oral squamous carcinoma cell line, KB cells [22], were maintained in exponential growth phase using RPMI 1640 culture medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. These cells were purchased from ATCC (reference: CCL-17). Calcein-acetomethoxyl ester and Calcium Green-5N were from Molecular Probes while monoclonal antibodies were from Pharmingen. Metformin was a gift from Merck-Lipha. All other chemicals were purchased from Sigma.

Measurement of oxygen consumption rate in intact cells.

KB cells (10^7 cells/ml) were incubated in closed vials in a shaking water bath in 2.5 ml RPMI 1640 medium saturated with a mixture of O₂/CO₂ (19:1). Incubations were performed at 37°C unless otherwise indicated (15°C), with or without 10 mM metformin. After 30 minutes, 2 ml of the suspension was removed from vials and placed in a stirred oxygraph vessel thermostated at 37°C and equipped with a Clark oxygen electrode. Oxygen consumption rate was first measured in the absence of any addition, then 1.25 µM rotenone, 0.5 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 3.8 µM myxothiazol and 1 mM *N, N, N', N'*-tetramethyl-1,4-phenylenediamine (TMPD) plus 5 mM ascorbate were successively added, as indicated.

Measurement of oxygen consumption rate in permeabilized cells.

After 30 minutes of pre-incubation as described above, intact KB cell suspensions were centrifuged and the cell pellets carefully resuspended in KCl medium (125 mM KCl, 20 mM Tris-HCl, 1 mM EGTA, 5 mM Pi-Tris, pH 7.2) containing 200 µg/ml digitonin. Cells were permeabilized for 2 minutes at 37°C and then the suspension was removed from the vial and placed in the oxygraph as described above. As indicated, either 5 mM glutamate-Tris plus 2.5 mM malate-Tris or 5 mM succinate-Tris plus 0.5 mM malate-Tris plus 1.25 µM rotenone were added. Oxygen consumption rate was measured before and after the successive additions of 1 mM ADP-Tris, 0.75 µg/ml oligomycin, 0.5 µM CCCP, 3.8 µM myxothiazol and 1 mM TMPD plus 5 mM ascorbate.

Measurement of complex 1 and citrate synthase activities.

After a pre-incubation in RPMI 1640 with or without 10 mM (30 minutes) or 100 μ M (24 hours) metformin, KB cell suspensions were centrifuged and the cell pellets were resuspended in cold buffer containing 40 mM KCl, 250 mM sucrose, 2 mM EGTA, 20 mM Tris, pH 7.2 and 200 μ g/ml digitonin. Following a 5 min-incubation on ice, cells were spun down (12,000 *g* for 10 minutes) to eliminate possible cytosolic contaminating enzyme activities. The permeabilized KB cells were then carefully washed and resuspended either in the above buffer devoid of digitonin for assay of complex 1, or in a lysis buffer (100 mM KH_2PO_4 , 2 mM EDTA, 1 mM DTT, pH 7.3 and 0.1% triton X-100) for assaying citrate synthase activity. Protein concentrations were measured using the bicinchoninic acid protein assay kit (Pierce, Rockford, Illinois, USA).

Citrate synthase activity was measured by the procedure of Srere [23], while complex 1 activity was determined fluorimetrically in a Kontron SFM23 spectrofluorometer by monitoring NADH oxidation with excitation and emission wavelengths set at 340 and 460 nm, respectively. In brief, permeabilized cells (8×10^6) were placed in 800 μ l H_2O in a well-stirred glass cuvette for 2 minutes at 30°C in order to break mitochondrial membranes by hypotonic shock. 200 μ l of Tris solution (50 mM, pH 8.0) containing 150 μ M NADH was then added for one minute and the reaction was started by adding 100 μ M decylubiquinone as final electron acceptor. Rotenone-sensitive complex 1 activity was obtained after subtraction of the remaining signal in the presence of 6 μ M rotenone.

Determination of permeability transition in permeabilized cells.

Intact KB cells (5×10^6) were incubated for 30 minutes with or without 10 mM metformin as described above. The cells were then centrifuged and resuspended in medium containing 250 mM sucrose, 10 mM MOPS, 1 mM Pi-Tris and 50 μ g/ml digitonin (pH 7.35) and placed in a spectrofluorometer glass cuvette continuously stirred and thermostated at 25°C. After 2 minutes, cells were permeabilized and 1 μ M CsA or vehicle was also added to the medium as indicated. After signal stabilization, successive 10 μ l of 1 mM Ca^{2+} pulses were added at two-minute intervals until opening of PTP, as indicated by the release of Ca^{2+} in the medium. Measurements of Ca^{2+} were carried out fluorimetrically with a PTI Quantmaster C61 spectrofluorometer. Free Ca^{2+} was measured in the presence of 0.25 μ M Calcium Green-5N with excitation and emission wavelengths set at 506 and 532 nm, respectively.

Determination of permeability transition in intact cells.

Calcein staining in KB cells was achieved after cells (5×10^4) were grown for 48 hours on 22 mm diameter round glass cover slips and exposed for 15 minutes at 37°C to PBS medium supplemented with 5 mM glucose, 0.35 mM pyruvate, 1 mM CoCl_2 and 1 μM calcein-acetomethoxyl ester as described [24]. After loading, cells were washed free of calcein and CoCl_2 and further incubated 20 minutes at 37°C in PBS/glucose/pyruvate medium supplemented with either 10 mM metformin, 1 μM CsA or vehicle. For low concentration of metformin, KB cells were first pre-incubated for 24 hours with or without metformin (100 μM) before the calcein and CoCl_2 loading step. Cover slips were then mounted on the stage of an inverted microscope and PTP opening was achieved by adding 50 μM *tert*-butyl hydroperoxide (tBH), a glutathione oxidizing agent. Changes in cellular fluorescence were quantified using the NIH image software. The intensity of the fluorescence of 10 cells was followed in time after tBH addition.

Determination of cellular death.

KB cells (2×10^7) were pre-incubated in Petri dishes with either 10 mM metformin, 1 μM CsA or vehicle for 30 minutes or after 24 hours pre-incubation at 37°C with or without 100 μM metformin. Cells were washed with PBS before subsequent exposure to 0.2 mM tBH for 45 minutes. Cells were then again washed with PBS and incubated at 37°C for 6 or 24 hours in a complete RPMI medium. Cytotoxicity was evaluated either by staining necrotic cells with 20 $\mu\text{g/ml}$ propidium iodide or by using a trypan blue (5%) exclusion assay.

Cellular images were acquired at 25°C with a Nikon TE200 microscope, which was equipped for epifluorescent illumination and included a xenon light source (75W) and a 12-bit-digital-cooled CCD camera (SPOT-RT, Diagnostic Instruments). For calcein fluorescence, $488 \pm 5/525 \pm 10$ nm excitation/emission filter settings were used, and images were collected every minute with a constant exposure time using a 60X/1.40 Plan Apo oil immersion objective (Nikon). For detection of propidium iodide, five randomly selected fields were acquired from each Petri dish using an excitation/emission cube of $550 \pm 10/580$ longpass and a ELWD 20X/0.45 Plan Fluor objective (Nikon). The corresponding bright field images were also acquired, and the two channels were overlaid using the appropriate function of the SPOT™ 3.0.6 software.

Cytochrome c was assessed in both mitochondrial and cytosolic spaces after KB cells were fractionated using the digitonin method [25]. Cytosolic (3 μg) and mitochondrial (15 μg)

proteins were separated by SDS-PAGE in 10% (w/v) polyacrylamide gels in MES buffer, followed by Western blotting. Membranes were probed with a monoclonal antibody against cytochrome c (1 µg/ml) clone 7H8.2C12 and developed with a secondary goat-antimouse horseradish peroxidase-labeled antibody followed by chemiluminescent detection. Quantification was carried out using the NIH image software.

Statistics.

Results are expressed as mean \pm S.E.M. and statistically significant differences were assessed by ANOVA followed by Fisher's protected least-significant differences (PLSD) post-hoc test or by paired or unpaired Student's t-test (StatView®, Abacus concepts, Inc., Berkeley, California, 1992) as indicated.

RESULTS

Specific inhibition of respiratory chain complex 1 by metformin in KB cells.

As shown in Table 1, 10 mM metformin significantly inhibited respiration in intact KB cells by 53% ($p < 0.01$), while rotenone led to a stronger inhibition ($p < 0.01$) regardless of the presence of metformin (89% and 79% respectively with or without metformin). Metformin also inhibited respiration when cells were uncoupled by CCCP (58%, $p < 0.01$), indicating that the inhibition was not related to mitochondrial adenine nucleotide phosphorylation. Finally, the lack of effect of metformin in the presence of TMPD-ascorbate, to assess cytochrome oxidase activity, indicated that metformin did not affect this complex of the respiratory chain. The inhibitory effect on respiration was not present when cells were incubated with metformin at 15°C prior the determination of oxygen consumption, which was followed at 37°C (data not shown).

The respiratory effect of metformin in intact cells was further investigated after digitonin permeabilization of the plasma membrane, allowing investigation of oxidative mitochondrial phosphorylation pathway *in situ*. Mean values of myxothiazol-sensitive oxygen consumption obtained from repeated experiments are given in Table 2. With glutamate-malate as respiratory substrates, metformin inhibited oxygen consumption (33%, $p < 0.01$), which persisted in the presence of ADP (State 3, 48%, $p < 0.01$) or in an uncoupled state (CCCP addition, 36%, $p < 0.01$). In state 4, (*i.e.* non-phosphorylating conditions obtained in the presence of oligomycin, an inhibitor of F_0 subunit of ATP synthase), the inhibition by metformin did not reach a significant level. As already found in intact cells (see Table 1), metformin did not affect the respiratory rate in the presence of TMPD-ascorbate. Furthermore, metformin affected none of these parameters with succinate and malate as respiratory substrates (Table 2).

These results led us to postulate that metformin affects mitochondrial respiration in KB cells by inhibiting respiratory chain complex 1, as already found in liver cells or in *Xenopus laevis* oocytes [16, 26]. In order to confirm this hypothesis, rotenone-sensitive NADH decylubiquinone reductase activity was measured in metformin-treated and control KB cells after cell permeabilization and osmotic shock. Table 3 summarizes the results obtained after short-time - high concentration and long-time - low concentration pre-incubation of the drug and clearly shows that, in both situations (10 mM or 100 μ M), metformin inhibited rotenone-

sensitive oxidation of NADH without altering citrate synthase activity. The maximal effect of metformin was obtained at 10 mM (- 34%, $p < 0.01$) and remained significant, even at a concentration close to the therapeutic range (100 μ M, - 24%, $p < 0.05$).

Metformin prevents mitochondrial PTP opening in both permeabilized and intact KB cells.

The regulation of PTP opening by calcium was studied by determining the amount of calcium required for inducing a permeability transition, as demonstrated by a calcium release to the medium from digitonin-permeabilized cells. Figure 1A shows typical experiments and Figure 1B contains the data of repeated experiments. As compared to controls, metformin significantly increased the calcium requirement for achieving the permeability transition (27%, $p < 0.01$), an effect only slightly lower (NS) than that of the reference inhibitor CsA (38%, $p < 0.01$). Since the effect of metformin on respiration was temperature dependent in KB cells (not shown), as found in rat liver cells [16], we studied in permeabilized cells the effect of metformin on calcium retention in permeabilized cells after exposure to metformin at low temperature (15°C). Although the low temperature did not affect basal or CsA-inhibited calcium retention, the metformin effect was completely abolished, indicating that in KB cells it was also temperature dependent.

The effect of metformin on the regulation of the permeability transition was also investigated in intact cells, where PTP opening was achieved using the glutathione-oxidizing agent, tBH. PTP opening was directly assessed by measuring mitochondrial permeability to calcein and cobalt [24]. Upon PTP opening, cobalt diffuses from the cytoplasm into the mitochondrial matrix, thus quenching the calcein-related fluorescence in this compartment. Hence, after a transition of permeability has occurred, intracellular fluorescence is progressively decompartmentalized and quenched. As shown in Figure 2, this occurred in cells after addition of tBH. Indeed, the cellular heterogeneity of fluorescence was less marked after exposure to tBH alone (Figure 2A, left panels from top to bottom) and this effect was associated with a quenching of fluorescence, which was already significant after 3 minutes and more pronounced after 5 and 10 minutes (Figure 2B). The cellular heterogeneity and intensity of fluorescence persisted for up to 10 minutes with either metformin or CsA (Figures 2A). Furthermore, the effect of tBH on the fluorescence quenching was significantly less and delayed with 10 mM metformin while it was completely prevented over 10 minutes with 100 μ M metformin, an effect equivalent to that of CsA (Figures 2B). From these data, we

conclude that metformin prevents mitochondrial permeability transition, both in intact or permeabilized KB cells, and that this effect is not different from that of CsA, the reference inhibitor of the PTP.

Prevention of tBH-induced cellular death by metformin.

The effect of metformin on cellular death was first investigated by using propidium iodide staining, allowing evaluation of the number of dead cells. As shown in Figure 3A, exposure to tBH resulted in an increase in the percentage of cells stained by the dye. The magnitude of the effect was rather limited when observed only 6 hours after tBH exposure (Figure 3A, black bars), however a large and significant effect of this exogenous oxidizing agent was obtained when observed after a longer period. Interestingly, the detrimental effect of tBH was completely prevented by metformin, whatever the time and concentration used (100 μ M or 10 mM), and by CsA. The protective effect on cell death was also assessed by trypan blue exclusion. As shown on Figure 3B, metformin (100 μ M and 10 mM) or CsA significantly protected KB cells against cell death as induced by tBH.

Lastly, the effect on cell death was confirmed by determining the release of cytochrome c from mitochondrial intermembrane space to the cytoplasm. This criterion is recognized, together with other factors, as a proapoptotic event in the commitment to cell death. Cytochrome c content of both mitochondrial and cytosolic spaces was assessed by western blotting (Figure 4A shows a typical experiment). We found that although cytochrome c was hardly detectable in the cytoplasm of control cells, tBH exposure resulted in a significant increase in cytosolic levels (Figure 4B). This increase was completely prevented by metformin (100 μ M or 10 mM) or CsA. None of the different conditions affected the mitochondrial content of cytochrome c, indicating that only a very small fraction was released into the cytoplasm.

DISCUSSION

The data presented in this work indicate that metformin inhibits complex 1 of the mitochondrial respiratory chain in a specific and temperature-dependent manner in KB cells, a finding in agreement with other results obtained in rat liver cells [16] or in *Xenopus laevis* oocytes [26]. However, the main finding presented here is that metformin decreases mitochondrial PTP opening and prevents the release of cytochrome c, this being associated with a decreased occurrence of cell death upon addition of the glutathione-oxidizing agent tBH. Such result is of interest when considering the clinical use of metformin as antidiabetic agent, since this effect was observed not only at 10 mM, a high pharmacological concentration, but also at a value close to the therapeutic range (100 μ M).

Use of KB cells as experimental model for studying the effect of metformin on cell death.

The KB cell line was used in this study as an experimental model to investigate the effect of metformin on the relationship between respiratory chain complex 1, PTP regulation and cell death. Since KB cells are very flat, the pictures obtained from epifluorescence microscopy allow an accurate assessment of changes in fluorescence distribution of calcein following oxidative stress induction. In a previous work, we have shown that mitochondria from KB cells exhibit a regulation by rotenone of the PTP following either a calcium challenge (after permeabilization) or oxidative stress (tBH addition to intact cells) [21]. Hence, KB cells represent a suitable model to investigate the effects of metformin on mitochondria and cell death.

Cellular action of metformin.

Despite recent progresses [13, 14, 16], the cellular action of metformin is still not fully understood. In this work we have used two concentrations: a high saturating one (10 mM) in order to investigate a maximal effect on respiratory chain complex 1, and a lower one corresponding to the therapeutic range (100 μ M). From previous reports, it is clear that the mitochondrial effect of metformin occurs only when the drug is administered to intact cells but not to isolated mitochondria or permeabilized cells [16, 26]. Similar results were also found with KB cells (data not shown). However, when the cells were permeabilized after metformin exposure, the mitochondrial effect is still present, indicating that the putative mitochondrial change persisted. The lack of effect of metformin on isolated mitochondria has

been challenged by data showing that metformin can directly inhibit complex 1 [27, 28]. However, this effect was only observed at very high concentrations ($K_{0.5} = 79$ mM) and after very long incubation times at 8° (225-400 min), contrasting with the rapid effects (20 min) observed in intact cells [16]. The requirement of intact cells for achieving the mitochondrial inhibition together with its complete suppression at low temperature, and to the failure to find any interference between metformin and several inhibitors of the main signaling pathways, led us to suggest that metformin could act via a plasma-membrane related event. This was further supported by data obtained in *Xenopus laevis* oocytes, where it was shown that a direct microinjection of metformin in the cytosol had no effect, whereas injection of liposome-encapsulated metformin inhibited complex 1 [26]. The mitochondrial effect was specifically located on complex 1 in KB cells, as in rat liver cells or *Xenopus laevis* oocytes. The inhibitory effect was moderate, as the highest metformin concentration used (10 mM) resulted in a significantly lower inhibition compared with rotenone.

Recently metformin was shown to activate AMPK, although its precise mechanism is unclear [13-15]. If AMPK represents a primary cellular target of the drug, metformin could affect cell death via a first activation of this kinase, which might also be responsible for the inhibitory effect on the respiratory chain. Indeed, it cannot be excluded that AMPK might phosphorylate one of the components of complex 1 [29], leading to its inhibition and subsequent events affecting PTP regulation, cytochrome c release and ultimately cell death. However such putative mechanism cannot be the consequence of a direct interaction because AMPK appears to be located in the cytoplasm and nucleus of cells [30] and therefore would presumably be inaccessible to the inner mitochondrial membrane complex 1. An indirect mechanism involving a cascade of phosphorylation events of some proteins located in the outer mitochondrial membrane could be proposed. Recent findings show that the voltage-dependent anion channel (VDAC) might be implicated in the regulation of mitochondria-mediated cell death by direct [31] or indirect modulation [32, 33] of its conformation. While a direct link between AMPK and VDAC has never been demonstrated, a modification of hexokinase (HK) binding to VDAC via phosphorylation of one of the protein binding sites of the channel by AMPK (or a downstream kinase) or due to an indirect pathway could also be envisaged. However, the previous finding that metformin inhibits complex 1 in isolated mitochondria or disrupted tissues, only when exposed to a large concentration and/or a long incubation time [27, 28], do not favor a direct involvement of AMPK.

Lastly, it cannot be excluded that AMPK activation by metformin might not be the result of a direct causal effect of the drug but rather the consequence of its mitochondrial effect by an unknown downstream mechanism that remains to be elucidated. However, our recent data indicates that AMPK activation by AICA riboside does not prevent the tBH-related induction of cell death in KB cells (unpublished data) as metformin does.

Metformin inhibits the mitochondrial permeability transition and cell death.

Under the conditions described here, metformin modulated the PTP similarly to the previous finding concerning rotenone [21]. This was shown in permeabilized cells, where metformin modulated PTP opening following a calcium challenge. PTP is regulated by NADH, ADP and the mitochondrial membrane potential, among other factors [17]. These parameters are potentially affected by respiratory chain inhibition by metformin. However, in the experiments presented in Figure 1 investigating the effect of metformin on calcium retention, the permeabilized cells were incubated with succinate as substrate, a condition where metformin does not affect the respiratory chain (see Table 1). Hence, the significant increase in calcium retention presented in Figure 1 is related to the inhibition of complex 1 by metformin but not to its consequence on oxidative phosphorylation. Interestingly, metformin also inhibited PTP opening in intact cells, although in this case such phenomenon was related to tBH-induced oxidative stress and not to calcium exposure. As described by Petronilli et al., the quenching of the intracellular fluorescent probe calcein by cobalt could be proposed as direct evidence of PTP opening in intact cells [24]. Of note, these effects of metformin, even at the lowest concentration, are equivalent to those obtained with CsA, and metformin appears to be as potent as the reference modulator of the PTP, *i.e.* CsA. The prevention of tBH-related cytochrome c release in the cytoplasm and of cell death by metformin is also very significant, and again a low concentration of metformin appears as potent as CsA (see Figure 2). Involvement of the PTP in the commitment to cell death was proposed from the findings that CsA was a protective agent in several models of cell death [34-37]. Other data further support this view, such as early mitochondrial depolarization [38] preceding cytochrome c release [39] or the inhibition of cellular death by rotenone [21]. The present data clearly account also for such an effect of PTP modulation by complex 1 inhibition on regulation of cell death, metformin being a novel agent of this regulation.

Considering that (i) most of the deleterious complications of diabetes seem to be due to glucose-induced ROS production by the respiratory chain [40]; (ii) that exogenous oxidative

stress-related cell death is mediated by PTP opening [21], and (iii) that inhibition of complex 1 prevents PTP opening [21], we propose that metformin, which acts as a mild complex 1 inhibitor, prevents oxidative stress related cytochrome c release and commitment to cell death. This finding may represent an important new direction in the treatment of hyperglycemia-related detrimental effects. If hyperglycemia-related deleterious consequences are linked to superoxide overproduction at the level of the mitochondrial respiratory chain [5], while mitochondrial superoxide generation, or exogenous oxidative stress, lead to apoptosis, inhibition of PTP opening may efficiently prevent the effect of hyperglycemia. Hence, if metformin prevents PTP opening upon exogenous oxidative stress as well as its consequence on apoptosis, this may suggest that metformin also prevents hyperglycemia-induced cellular death. This finding may open a new direction for the treatment of hyperglycemia-related complications: besides the necessity for lowering blood glucose, it is possible to diminish the mitochondria-related toxicity of hyperglycemia.

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Table 1. Inhibition of oxygen uptake by metformin in intact KB cells.

	<i>JO</i> ₂	
	natom.min ⁻¹ . 10 ⁶ cells ⁻¹	
	Control	Metformin
No addition	1.9 ± 0.1	0.9 ± 0.1*
+ Rotenone	0.4 ± 0.1	0.1 ± 0.0*
+ CCCP	2.6 ± 0.2	1.1 ± 0.1*
+ TMPD-a	10.0 ± 0.3	10.5 ± 0.5

KB cells (10⁷ cells/ml) were incubated in closed vials at 37°C in RPMI 1640 medium saturated with a mixture of O₂/CO₂ (19:1) with or without 10 mM metformin. After 30 minutes, oxygen uptake (*JO*₂) was measured before and after the successive additions of 1.25 μM rotenone, 0.5 μM CCCP, 3.8 μM myxothiazol and 1 mM TMPD plus 5 mM ascorbate. Myxothiazol-sensitive oxygen uptake was calculated and the results are expressed as mean ± S.E.M. (n = 5 five different preparations, each assay being performed in triplicate). *, *p*<0.01 *versus* control.

Table 2. Inhibitory effect of metformin on oxygen uptake in permeabilized KB cells.

	<i>J</i> O ₂			
	natom.min ⁻¹ . 10 ⁶ cells ⁻¹			
	Glutamate / Malate		Succinate / Malate	
	Control	Metformin	Control	Metformin
No addition	1.5 ± 0.1	1.0 ± 0.1*	2.4 ± 0.2	2.4 ± 0.2
+ ADP	5.0 ± 0.2	2.6 ± 0.2*	7.5 ± 0.3	7.7 ± 0.4
+ Oligomycin	1.0 ± 0.1	0.8 ± 0.1	1.5 ± 0.3	1.6 ± 0.3
+ CCCP	6.1 ± 0.5	3.9 ± 0.3*	8.2 ± 0.6	8.4 ± 0.6
+ TMPD-a	11.2 ± 0.3	11.5 ± 0.3	10.4 ± 0.3	10.7 ± 0.3

Permeabilization of KB cells was achieved after intact cells had been previously incubated for 30 minutes with or without 10 mM metformin. Digitonin was added to intact cells (see Materials and Methods) in a medium containing 125 mM KCl, 20 mM Tris-HCl, 1 mM EGTA, 5 mM Pi-Tris, pH 7.2, 37°C. Permeabilized cells were then incubated in the presence of either 5 mM glutamate plus 2.5 mM malate or 5 mM succinate plus 0.5 mM malate plus 1.25 µM rotenone. Oxygen uptake (*J*O₂) was determined before and after the successive additions of 1 mM ADP-Tris, 0.75 µg/ml oligomycin, 0.5 µM CCCP, 3.8 µM myxothiazol and 1 mM TMPD plus 5 mM ascorbate. Myxothiazol-sensitive respiration was calculated and is expressed as mean ± S.E.M. (n = 5 five different preparations, each assay being performed in triplicate). *, *p*<0.01 versus control.

Table 3. Inhibitory effect of metformin on isolated mitochondrial complex 1 in permeabilized KB cells.

	Control	Metformin (10 mM - 30')	Metformin (100 µM – 24 h)
Rotenone-sensitive activity of complex 1 (nmol NADH.min ⁻¹ .mg protein ⁻¹)	3.8 ± 0.5	2.5 ± 0.5**	2.9 ± 0.5*
Citrate synthase activity (pmol.min ⁻¹ .mg protein ⁻¹)	42.6 ± 8.5	41.7 ± 8	40.4 ± 11.3
Enzyme activities ratio (nmol NADH/Unit citrate synthase)	90.3 ± 7.4	59.9 ± 4.1**	73.2 ± 7.5*

Permeabilization of KB cells was achieved after intact cells had been previously preincubated in the absence or presence of 10 mM or 100 µM metformin. Digitonin was added to intact cells in a medium containing 250 mM sucrose, 40 mM KCl, 2 mM EGTA, 20 mM Tris, pH 7.2 (see Materials and Methods). Complex 1 activity was measured after hyposmotic shock-induced mitochondrial membrane rupture and NADH oxidation was then monitored in the simultaneous presence of 150 µM NADH and 100 µM decylubiquinone, before and after addition of 6 µM rotenone. Citrate synthase activity was measured after lysis of permeabilized KB cells in a buffer (100 mM KH₂PO₄, 2 mM EDTA, 1 mM DTT, pH 7.3) containing 0.1% triton X-100. Each enzyme activity and the corresponding ratio of both were calculated and expressed as mean ± S.E.M. (n = 4 different experiments, each assay being performed in duplicate). *, $p < 0.05$; **, $p < 0.01$ *versus* control.

FIGURE LEGENDS

Figure 1. Metformin delays permeability transition in permeabilized KB cells.

After 30 minutes of pre-incubation in RPMI 1640 with or without 10 mM metformin at 37°C or 15°C, 5×10^6 KB cells were added in a medium containing 250 mM sucrose, 10 mM MOPS, 1 mM Pi-Tris, 25°C (pH 7.35). The medium was supplemented with 5 mM succinate-Tris, 0.25 μ M Calcium Green-5N followed by the addition of vehicle (*Panel A*, traces a, b, d and e) or 1 μ M CsA (*Panel A*, traces c and f). Experiments were started 3 minutes after permeabilization with 50 μ g/ml digitonin. Where indicated, 10 μ l of 1 mM Ca^{2+} pulses were added every 2 minutes (*arrows*) until opening of PTP, as observed by the release of Ca^{2+} into the medium. Typical experiments on 37°C and 15°C pre-incubated KB cells are shown in *Panel A* (left and right respectively). Comparison of the effect of pre-incubation with or without 10 mM metformin at different temperature on the Ca^{2+} retention capacity of permeabilized KB cells are presented in *Panel B* (black bars, 37°C ; open bars, 15°C) as mean \pm S.E.M. from five different experiments. *, $p < 0.05$ versus control ; †, $p < 0.05$ versus 37°C, ANOVA followed by Fisher's PLSD post hoc test and unpaired Student's t-test.

Figure 2. Metformin prevents tBH-induced PTP opening in intact KB cells.

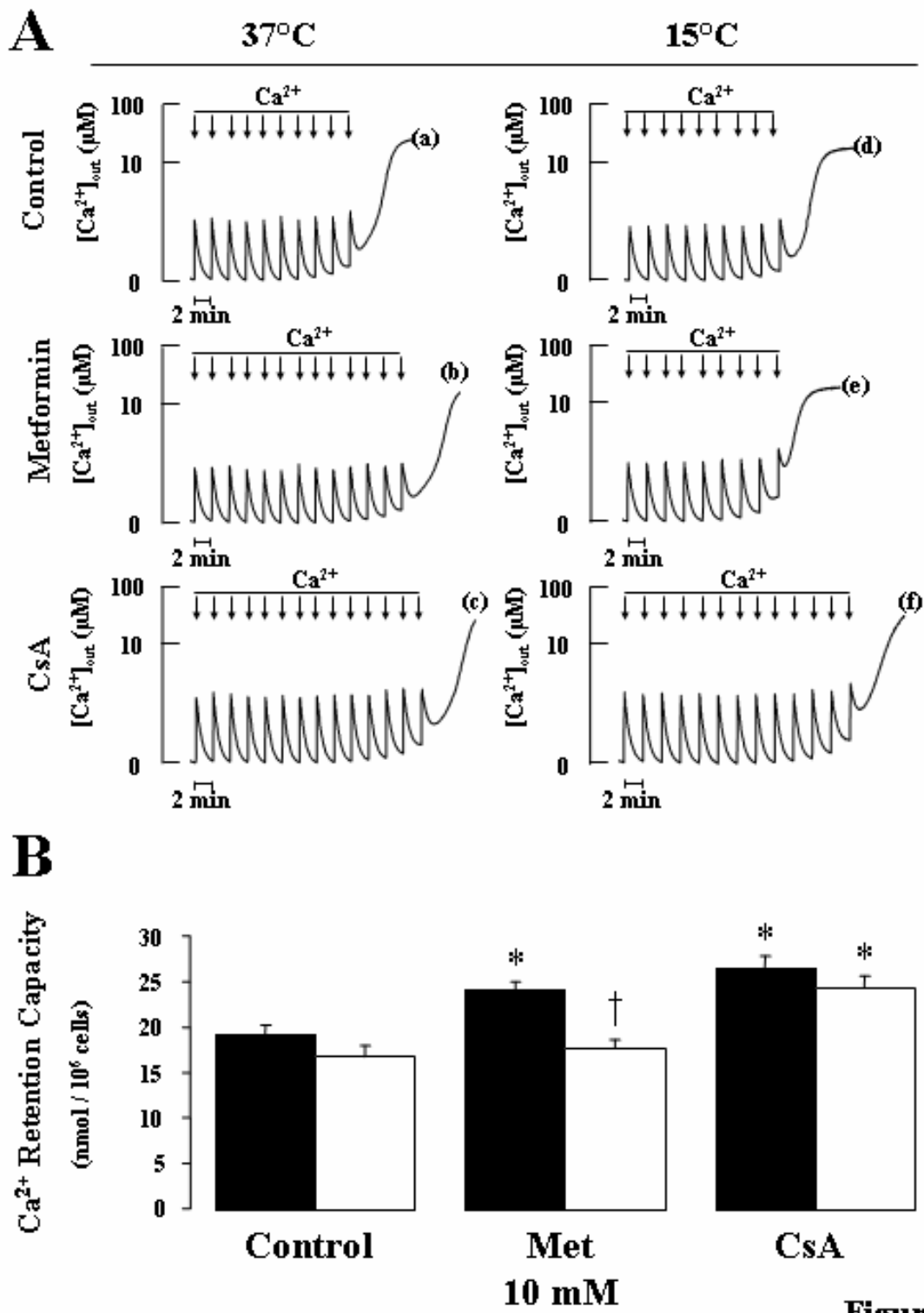
KB cells (5×10^4) were grown 48 h on glass cover slips and loaded for 15 minutes at 37°C with 1 μ M calcein-acetomethoxyl ester in a PBS medium supplemented with 5 mM glucose, 0.35 mM pyruvate and 1 mM CoCl_2 . Vehicle, 1 μ M CsA or 10 mM metformin were added after calcein loading and post-incubated for 20 minutes at 37°C. When low concentration of metformin were investigated, cells were first pre-incubated for 24-h with or without 100 μ M metformin. Images were collected at 1 minute intervals with an inverted microscope using a 60x oil immersion objective, before and after addition of 50 μ M *tert*-butyl hydroperoxide (tBH). The same cells are shown at 0 minute (immediately after tBH addition), then 3, 5 and 10 minutes later. Figure 3A represents a typical experiment. Figure 3B represents the quantification of the fluorescence intensity by the NIH image software. Light intensity of 10 different cells was followed 3, 5 and 10 min after tBH addition in four different cell preparations for each condition: tBH (●), 100 μ M metformin (□), 10 mM metformin (■), CsA (△) ; Results are expressed as mean \pm S.E.M. and represent the percentage of change from initial value, *i.e.* before tBH addition (t_0). *, $p < 0.05$, statistical comparisons between each value and its own reference at t_0 was achieved by using paired Student's t-test.

Figure 3. Metformin inhibits tBH-induced cell death.

KB cells (2×10^7) pre-incubated with either vehicle, 1 μ M CsA or 10 mM metformin for 30 minutes in a RPMI 1640 medium or after 24-h of pre-incubation with 100 μ M metformin, were exposed to 0.2 mM tBH for 45 minutes then washed with PBS and incubated at 37°C for 6 hours (black bars) or 24 hours (open bars) in a complete RPMI 1640 medium. Control denotes cells not exposed to tBH. *Panel A*: Quantification of the percentage of cell death stained with propidium iodide. *Panel B*: Percentage of cell death using the trypan blue exclusion assay. The results are expressed as mean \pm S.E.M. of 5-8 different experiments, more than 1000 cells were counted and analyzed during each assay. *, $p < 0.05$ versus control ; †, $p < 0.05$ versus 6 hours, ANOVA followed by Fisher's PLSD post hoc test.

Figure 4. Metformin prevents tBH-induced release of cytochrome c into the cytoplasm of KB cells.

Mitochondrial and cytosolic spaces of KB cells were separated using the digitonin fractionation method. Cytosolic (3 μ g) and mitochondrial (15 μ g) proteins were separated by SDS-PAGE in 10% (w/v) polyacrylamide gels in MES buffer, followed by Western blotting. One typical immunoblot of cytosolic (*Panel A, upper*) and mitochondrial (*Panel A, lower*) cytochrome c was represented. Cumulative data of 5 different experiments \pm S.E.M. of cytosolic (*Panel B*) and mitochondrial (*Panel C*) cytochrome c measurements was also shown. *, $p < 0.05$ versus control, ANOVA followed by Fisher's PLSD post hoc test.



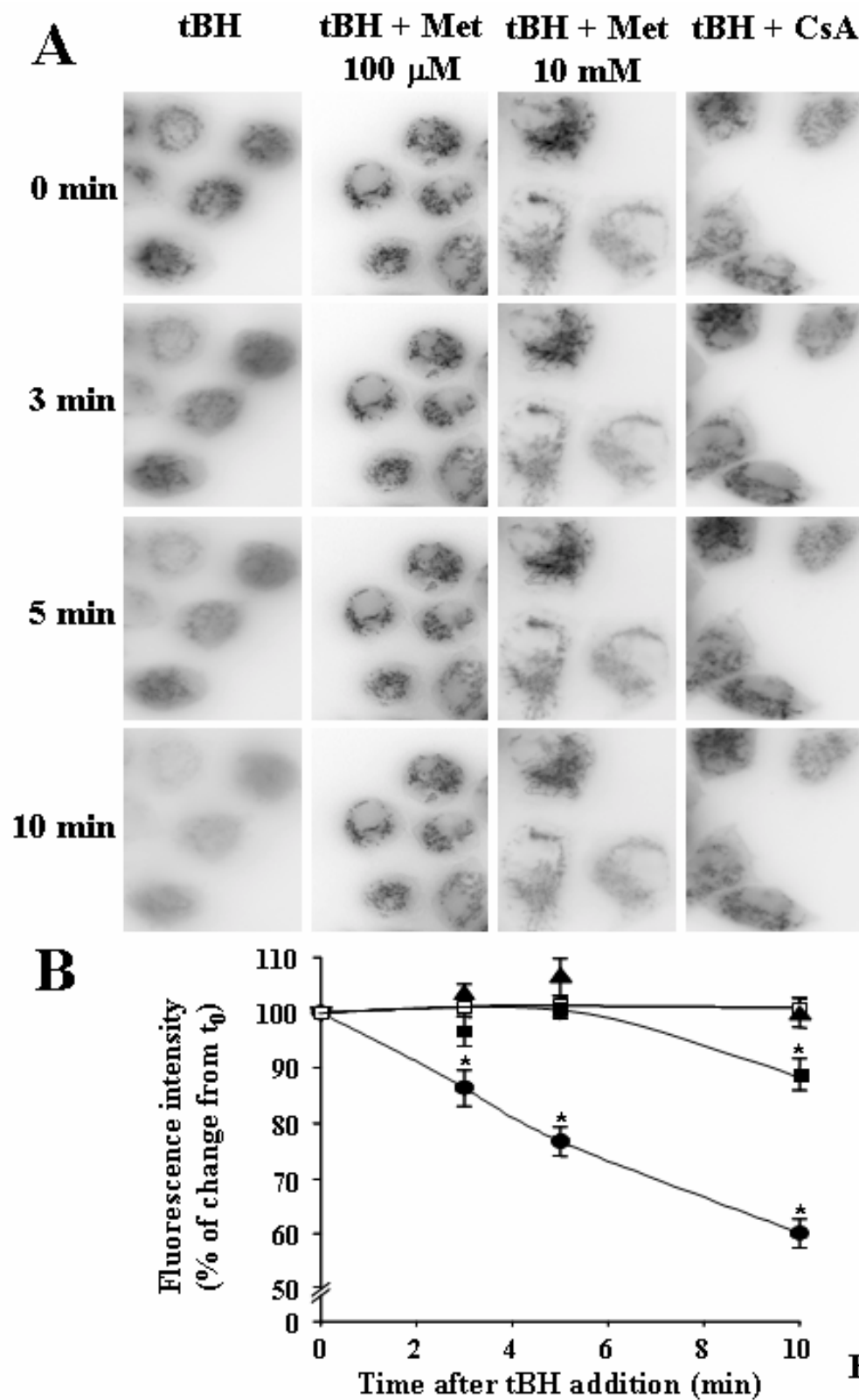


Figure 2

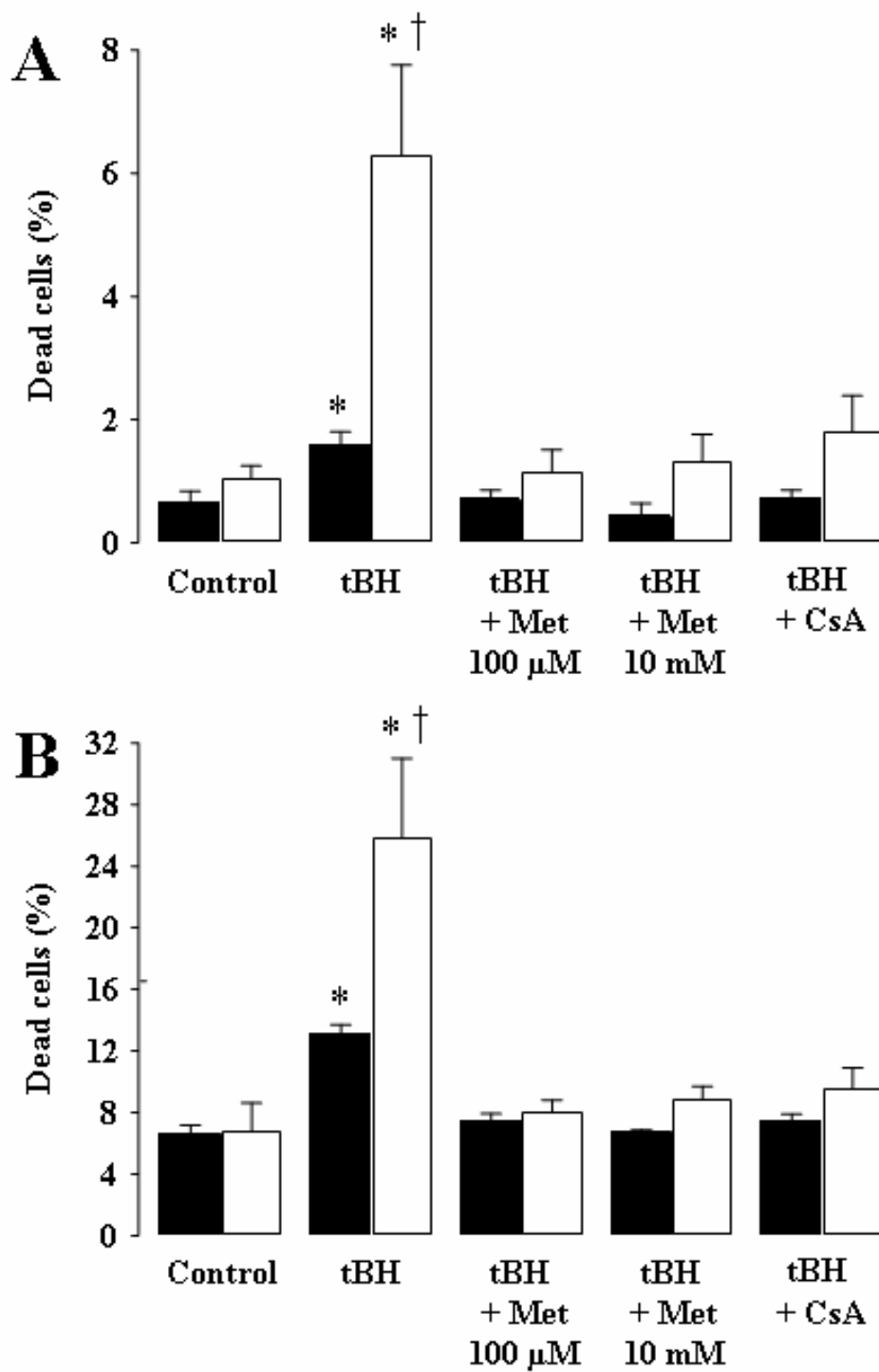


Figure 3

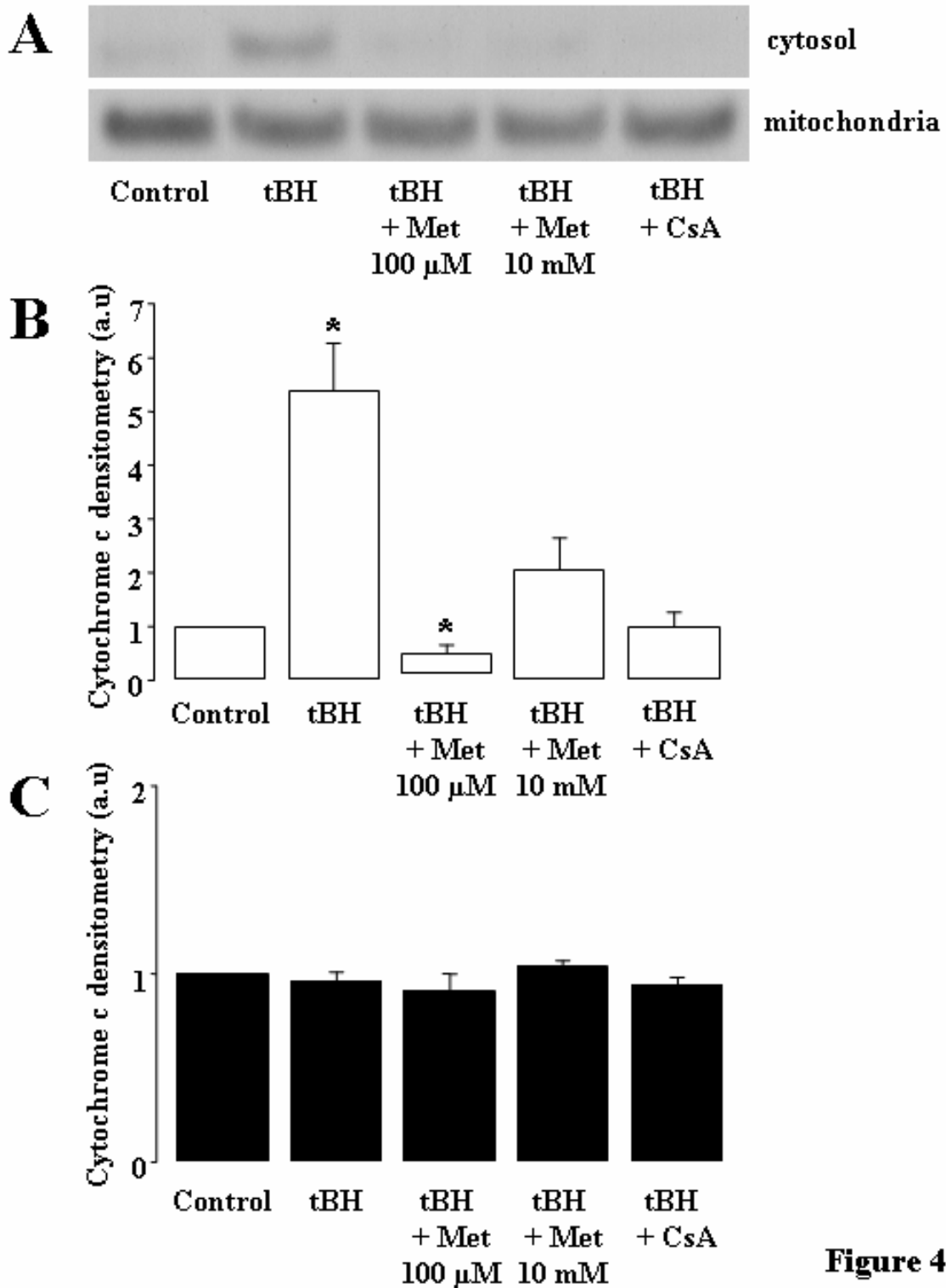


Figure 4