

*This paper is dedicated to the 60th anniversary of the foundation
of Institute of Physiology, Czechoslovak Academy of Sciences, Prague*

Biguanides Inhibit Complex I, II and IV of Rat Liver Mitochondria and Modify Their Functional Properties

**Z. DRAHOTA^{1,3}, E. PALENICKOVA^{2,4}, R. ENDLICHER³, M. MILEROVA¹,
J. BREJCHOVA¹, M. VOSAHLIKOVA¹, P. SVOBODA¹, L. KAZDOVA², M. KALOUS⁴,
Z. CERVINKOVA³, M. CAHOVA²**

¹Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic, ²Center for Experimental Medicine, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, ³Department of Physiology, Medical Faculty, Charles University, Hradec Kralove, Czech Republic, ⁴Department of Cell Biology, Faculty of Science, Charles University, Prague, Czech Republic

Received June 24, 2013

Accepted September 10, 2013

On-line November 1, 2013

Summary

In this study, we focused on an analysis of biguanides effects on mitochondrial enzyme activities, mitochondrial membrane potential and membrane permeability transition pore function. We used phenformin, which is more efficient than metformin, and evaluated its effect on rat liver mitochondria and isolated hepatocytes. In contrast to previously published data, we found that phenformin, after a 5 min pre-incubation, dose-dependently inhibits not only mitochondrial complex I but also complex II and IV activity in isolated mitochondria. The enzymes complexes inhibition is paralleled by the decreased respiratory control index and mitochondrial membrane potential. Direct measurements of mitochondrial swelling revealed that phenformin increases the resistance of the permeability transition pore to Ca²⁺ ions. Our data might be in agreement with the hypothesis of Schäfer (1976) that binding of biguanides to membrane phospholipids alters membrane properties in a non-specific manner and, subsequently, different enzyme activities are modified *via* lipid phase. However, our measurements of anisotropy of fluorescence of hydrophobic membrane probe diphenylhexatriene have not shown a measurable effect of membrane fluidity with the 1 mM concentration of phenformin that strongly inhibited complex I activity. Our data therefore suggest that biguanides could be considered as agents with high efficacy but low specificity.

Key words

Metformin • Phenformin • Mitochondrial enzymes • Mitochondrial permeability transition pore • Membrane fluidity

Corresponding author

Monika Cahova, Centre of Experimental Medicine, Institute for Clinical and Experimental Medicine, Vídeňská 1958/9, 140 21 Prague, Czech Republic. Fax: +420 23605 3027. E-mail: monika.cahova@ikem.cz

Introduction

Metformin (N,N-dimethylimidodicarbonimidic diamide) has been used for the treatment of type 2 diabetes since late 50's in most European countries and in the United States (Bailey and Turner 1996, Campbell *et al.* 1992). Metformin is currently the first drug of first choice in type 2 diabetes treatment and it is highly efficient in lowering blood glucose levels. Initially, it was suggested that one of its key actions is the stimulation of the muscle glucose uptake (Goodarzi and Bryer-Ash 2005, Klip and Leiter 1990). Recently, there is a growing body of evidence suggesting that the primary function of metformin is to decrease hepatic glucose production, mainly by inhibiting gluconeogenesis (Hundal *et al.* 2000). Numerous reports indicate that metformin

treatment leads to the diminution of ATP reserves in the liver (Argaud *et al.* 1993, Owen *et al.* 2000), decreased ATP/ADP ratio and to a reduction in cellular energy charge. Quite reasonably, AMP-activated protein kinase (AMPK) was therefore considered to be a major mediator of the glucose-lowering effects of metformin (Boyle *et al.* 2010, Zhou *et al.* 2001). Surprisingly, Foretz *et al.* (2010) demonstrated that the hypoglycemic effect of metformin was fully maintained in mice lacking AMPK. Moreover, they showed that the control of hepatic glucose production by metformin is linked to the inhibition of gluconeogenesis in response to a decrease in hepatic energy charge. In spite of long-lasting metformin application in clinical practice, its precise mechanism of action is still unknown (Campbell *et al.* 1996, Gonzalez-Barroso *et al.* 2012).

The primary cellular target for metformin is believed to be complex I of mitochondrial oxidative phosphorylation. However, the data obtained on hepatocytes, mitochondria or cultured fibroblasts showed that high metformin concentrations (10 mM) and long-term preincubation (2-24 hours) are required for demonstration of its inhibitory effect on oxidation of NADH-dependent substrates and also its direct effect on complex I (NADH dehydrogenase) activity was not completely confirmed (Owen *et al.* 2000). These data indicate that the inhibition of mitochondrial respiratory chain complex I may be one, but not the only, effect of metformin. Regarding the mechanism of action it has become likely that the hypoglycemic effect of biguanides results from a combination of a number of modulations of membrane-linked metabolic reactions, depending on the particular experimental conditions.

In our last study (Palenickova *et al.* 2011), we have shown that 10 mM metformin inhibits oxidation of glutamate + malate or palmitoylcarnitine + malate by about 60 % both in homogenate and in mitochondria after 3-5 min preincubation and that cell integrity is not required for its action. A significant inhibitory effect (about 20 %) can be detected also at 2.5-5 mM concentration; however, these concentrations are much above those detected in blood after *in vivo* application as well.

Phenformin, another biguanide, has qualitatively similar effects as metformin (Owen *et al.* 2000) but it is a more potent inhibitor of mitochondrial oxidation and it has been excluded from clinical practice because of an unacceptable incidence of severe lactate acidosis. Nonetheless, it is highly eligible for experimental studies

focused on biguanide mechanisms of action due to its more pronounced inhibitory effect at lower concentrations.

Under physiological conditions metformin and phenformin only can exist in the positively charged protonated form. The positively charge on both drugs could account for their generation of positive surface potential at phospholipid membranes and their accumulation within matrix of mitochondria. Phenformin is more hydrophobic, lipid-soluble molecule, than metformin. The phenyl and ethyl groups on phenformin make it to permeate biological membranes readily. While phenformin is accumulated transitorily in liver, kidney, pancreas and muscle, metformin is concentrated mainly in the walls of esophagus, stomach, duodenum and salivary glands as well as in kidney. (Owen *et al.* 2000, Schäfer 1983)

In this paper, we thus evaluated the phenformin effect on activity of mitochondrial enzymes, on respiratory control index, on mitochondrial membrane potential and on function of mitochondrial membrane permeability transition pore. We further focused on the question whether the biguanides inhibitory effect is dependent on cell structural integrity.

Methods

Animals

Male Wistar rats (6-8 months old) were kept in a temperature-controlled environment (25 ± 2 °C) with a 12 h light/dark cycle on standard laboratory diet and tap water supply *ad libitum*. All the experiments were conducted in accordance with the Animal Protection Law of the Czech Republic and according to the Institute for Clinical and Experimental Medicine Animal Care and Use Committee.

Preparation of rat liver homogenate and isolation of liver mitochondria

Liver mitochondria were prepared by differential centrifugation as described by Bustamante *et al.* (1977) with some modifications. Rat liver tissue was homogenized at 0 °C by a teflon-glass homogenizer as a 10 % homogenate in medium containing 220 mM mannitol, 75 mM sucrose and 1 mM HEPES, pH 7.2. For homogenization, 0.5 g/l fatty acid free bovine serum albumin (BSA) and 1 mM EGTA were added. Homogenate was centrifuged for 10 min at 800 g. The supernatant was filtered through nylon mesh and

centrifuged for 10 min at 8 000 g. Pelleted mitochondria were re-suspended in the medium without BSA and EGTA and washed twice by 10 min centrifugation at 8 000 g. The final mitochondrial pellet was re-suspended in the medium without BSA and EGTA to a concentration of 20-30 mg protein/ml. Mitochondrial proteins were determined according to the method of Bradford (1976) using BSA as a standard.

Determination of mitochondrial respiration

Oxygen consumption was measured at 30 °C using Oxygraph-K2 (OROBOROS, Austria). Measurements were performed in 2 ml of K-medium containing 80 mM KCl, 10 mM Tris-HCl, 3 mM MgCl₂, 1 mM EDTA, 5 mM KH₂PO₄, pH 7.4. The rate of oxygen consumption was calculated using Oroboros DatLab-4 software and expressed as nmol oxygen/s/mg protein.

Determination of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was determined using safranin O dye according to Akerman and Wikstrom (1976). Fluorescence changes were measured in 3 ml of K-medium containing 3 μM safranin, Ex 495 nm/Em 586 nm wavelengths, 30 °C, and using ISS-Vinci software in ISS Inc.-PC1 Spectrofluorimeter (Illinois U.S.A.).

Determination of mitochondrial swelling

Mitochondrial swelling was measured as described before (Drahota *et al.* 2012) as a decrease of mitochondrial suspension absorbance at 520 nm after the addition of Ca²⁺. The measurements were carried out at 30 °C using a Shimadzu spectrophotometer. The swelling medium contained 125 mM sucrose, 65 mM KCl, 5 mM succinate, 10 mM HEPES and 1 mM KH₂PO₄, pH 7.2. Mitochondria were added to yield an absorbance of approximately 1. After 1 min of incubation, CaCl₂ solution was added to the suspension of mitochondria. The decrease in the absorbance was determined at 0.1-min intervals for 5 min. The extent of swelling was calculated as the difference of the optical density at the beginning and the end of the measurement period. Maximum swelling rate was calculated from the curve obtained after derivation of the original swelling curve and expressed as absorbency change per 0.1-min interval. This derivation was used as a source to obtain the third parameter of swelling process – the time at which the maximum swelling rate was reached after calcium

addition. These three parameters than can better characterize the effects of biguanides on the kinetics of the swelling process.

Determination of anisotropy of fluorescence hydrophobic membrane probe diphenylhexatriene

The steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence was measured as described previously (Brejchova *et al.* 2011). Frozen-thawed rat liver mitochondria were labelled with DPH by the fast addition of 1 mM DPH in acetone to the mitochondrial suspension (0.1 mg protein/ml, final concentration 1 μM) under mixing. After 30 min at 25 °C, DPH fluorescence was measured at Ex 365 nm/Em 425 nm wavelengths with a ISS PC1 spectrofluorometer and the steady-state anisotropy (r_{DPH}) determined by ISS Vinci software. Both excitation and emission spectra of 1 μM DPH were determined in the presence of increasing concentrations of phenformin with the aim of detecting a possible quenching effect on DPH fluorescence.

Results

Phenformin inhibits complex I of mitochondrial respiratory chain in a dose-dependent manner

As previously reported (El-Mir *et al.* 2000, Owen *et al.* 2000, Palenickova *et al.* 2011), metformin inhibits complex I of mitochondrial respiratory chain. In this study, we focused our attention on the action of other representative of biguanide family, phenformin, on rat liver mitochondria. In order to obtain detailed information about the effect of phenformin on different respiratory chain components, we determined the oxygen consumption of isolated liver mitochondria using different substrates known to provide reducing equivalents specifically to the particular mitochondrial complexes. Figure 1 demonstrates the typical result of the oxygen consumption curve by rat liver mitochondria shortly (5 min) incubated with 1 mM phenformin after sequential additions of NADH- and flavoprotein-dependent substrates compared with control, i.e. untreated sample. When using only glutamate and malate as substrates, the respiration was very low and we did not observe any difference between phenformin-treated and control mitochondria. In contrast, when the respiratory chain was coupled to ATP synthesis by the addition of ADP, we discovered a strong inhibitory effect of phenformin on glutamate and malate respiration. Added

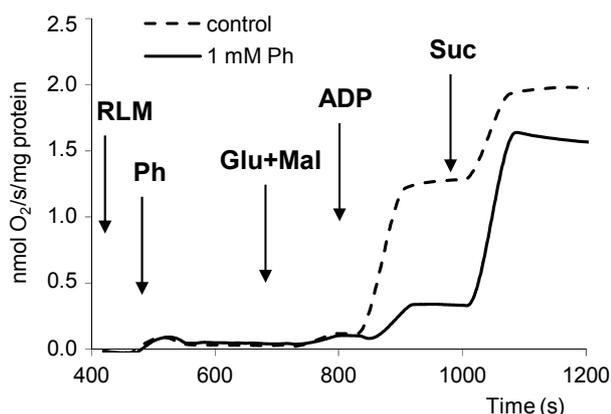


Fig. 1. Oxygraphic measurement of rat liver mitochondria respiration *in vitro*. Oxygen consumption measurement is described in Methods. Dashed trace indicate the respiration of rat liver mitochondria (RLM) incubated in the absence of phenformin (control), solid trace indicate the respiration of mitochondria to which 1 mM phenformin (Ph) was added where indicated. Other additions to both curves were: 10 mM glutamate and 2.5 mM malate (Glu+Mal), 1.5 mM ADP and 10 mM succinate (Suc). Mitochondrial protein concentration was 0.2 mg/ml. This figure represents a typical result of three independent experiments.

succinate then significantly increased mitochondrial respiration in both control and phenformin-treated mitochondria but the respiration rate of both substrates was still lower in the presence of phenformin compared to controls (Fig. 1).

As we have shown previously (Palenickova *et al.* 2011), the effect of metformin on glutamate + malate + ADP respiration was dose dependent with the maximum inhibition to 30 % at 10 mM metformin, EC_{50} was 5 mM. When we have measured under the same experimental conditions inhibitory effect of phenformin, maximum inhibition to 30 % of control values was obtained at 1 mM concentration and EC_{50} was 0.25 mM (Fig. 2A). At 1 mM phenformin glutamate respiration was inhibited to 30 %, after addition of succinate to 55 % only (Fig. 2A, B). Respiratory control index (RCI) was calculated as a ratio of oxygen consumption with and without ADP. In coupled mitochondria, phenformin decreased RCI in dose-dependent manner (Fig. 2C) and the course of the inhibitory curve was in parallel to its effect on glutamate + malate + ADP oxidation (Fig. 2A). We may thus conclude that both biguanides have the same inhibitory effect on glutamate oxidation and on the decrease of respiratory control, however, at different concentrations. Succinate oxidation under these experimental conditions was not much inhibited and could thus partially compensate the inhibition of glutamate + malate + ADP oxidation (Fig. 2B).

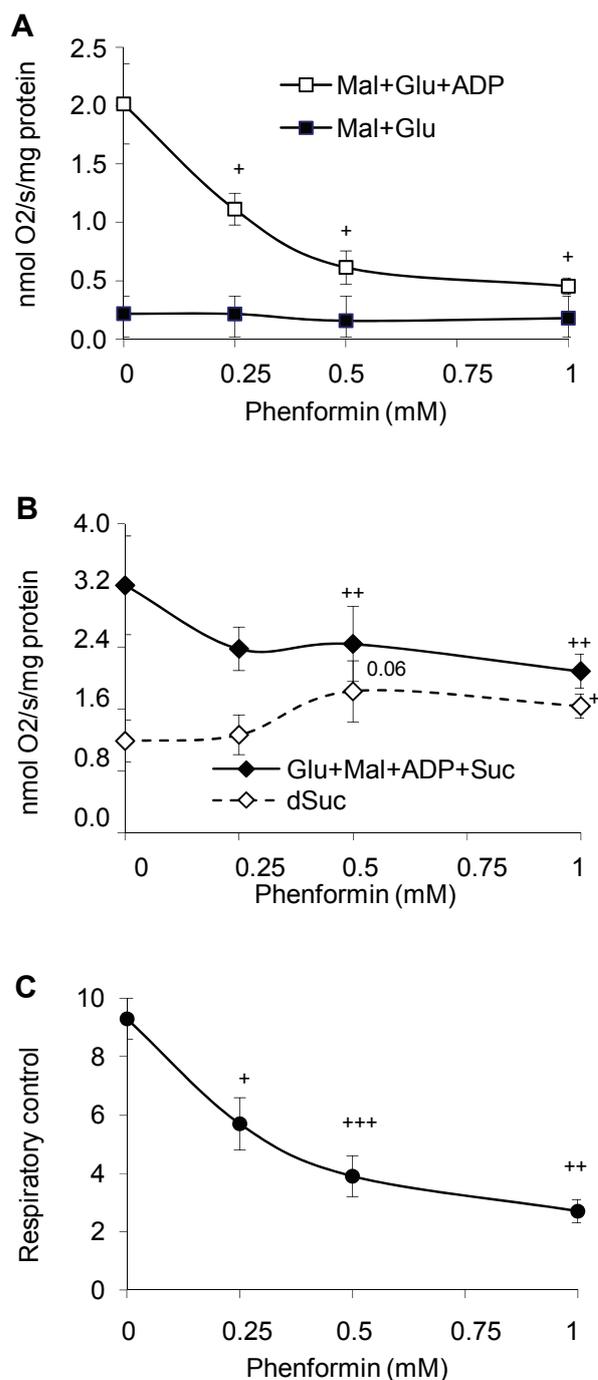


Fig. 2. Dose-dependent effect of phenformin on the oxidation of glutamate + malate and glutamate + malate + ADP (A), on the FADH-dependent (B) substrates and respiratory control index (C) in rat liver mitochondria. Respiration with glutamate + malate (black squares) (A) and glutamate + malate + ADP (white squares) as substrates (B). The effect of phenformin on glutamate + malate + ADP + succinate respiration (black rhombus) and on the increase of respiration induced by addition of succinate after glutamate + malate + ADP (white rhombus). dSuc was calculated as the difference of the oxygen consumption before and after the addition of succinate to glutamate + malate + ADP. (C) Respiratory control index (RCI) is calculated as a ratio of oxygen consumption with and without ADP. Data are given as mean of three independent experiments \pm S.E.M. $p < 0.05$; $++ p < 0.01$; $+++ p < 0.001$ phenformin-treated vs. control mitochondria.

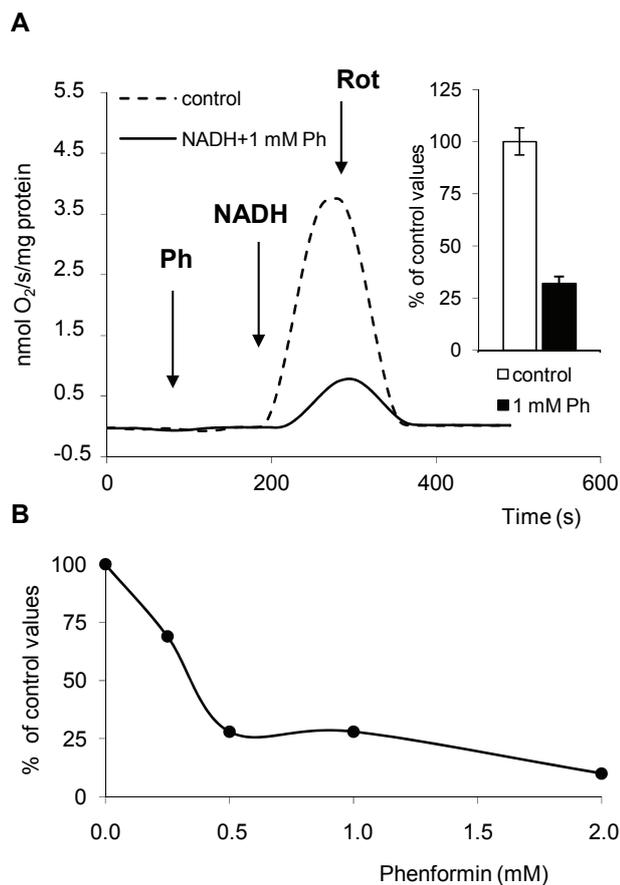


Fig. 3. The effect of phenformin on rotenone-sensitive NADH oxidation by frozen-thawed liver mitochondria. **(A)** The effect of 1 mM phenformin on the NADH oxidation in the absence (dashed line) or in the presence (solid line) 1 mM phenformin (Ph). 100 μ M NADH and 2 μ M rotenone (ROT) were added as indicated. This figure represents a typical result of three independent mitochondrial preparations. The extent of the inhibitory effect of Ph in % of control values is shown in the insert. **(B)** Concentration-dependent effect of phenformin on NADH oxidation.

Phenformin inhibitory effect on NADH oxidation

The observed biguanide-induced inhibition of NADH-dependent substrates oxidation may be explained either by the inhibition of dehydrogenases producing NADH or by inhibition of complex I (NADH-oxidoreductase), i.e. by diminution of electron flux from complex I through the respiratory chain. In order to distinguish between these possibilities, we directly measured NADH oxidation using frozen-thawed liver mitochondria as in this model NADH had free and direct access to complex I. Figure 3A demonstrates that in the presence of 1 mM phenformin rotenone-sensitive NADH oxidation in disrupted mitochondria is decreased to the same extent as the respiration of glutamate + malate + ADP in intact mitochondria. Furthermore, the curve describing the dependence of O₂ consumption on the phenformin concentration (Fig. 3B) had a similar value of

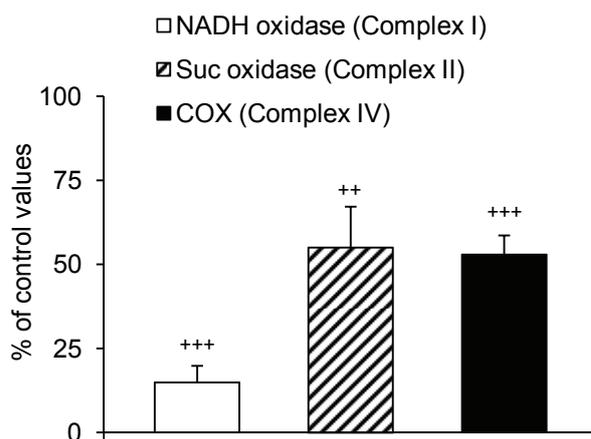


Fig. 4. Effect of phenformin on the oxidation of complex I, complex II and complex IV substrates by rat liver mitochondria *in vitro*. The effect of 6 mM phenformin was determined on substrates of NADH oxidase (complex I), succinate oxidase (complex II) and cytochrom c oxidase (complex IV). Medium was supplemented with 0.15 mM uncoupler 2,4-dinitrophenol and 0.02 mM cytochrom c. Data are given as mean of five independent experiments \pm S.E.M. ++ $p < 0.01$; +++ $p < 0.001$ phenformin-treated vs. control mitochondria.

50 % inhibition (0.25 mM) as for glutamate + malate + ADP oxidation (Fig. 2A). These data indicate that phenformin site of action is complex I.

Phenformin in high concentration inhibits not only complex I but also other complexes of mitochondrial respiratory chain

In our previous study, we were not able to find any inhibitory effect of metformin on complex II at concentrations required for the maximum inhibition of complex I (Palenickova *et al.* 2011). In contrast, phenformin is a 10 times more potent inhibitor of complex I compared with metformin and also exhibits some inhibitory action on succinate oxidation when complex I is fully inhibited (Fig. 2B). We have therefore tested the inhibitory effect of higher concentrations of phenformin on succinate and cytochrome c oxidases and we found that at 6 mM concentration phenformin displays significant inhibitory effect not only on complex I but also on complex II and IV (Fig. 4). Our data thus have shown that biguanides do not specifically inhibit complex I activity, but also other complexes of the mitochondrial respiratory chain.

Phenformin inhibitory effect on mitochondrial membrane potential is dependent on complex I function

As demonstrated in Figure 5, phenformin inhibits glutamate + malate oxidation in uncoupled as in

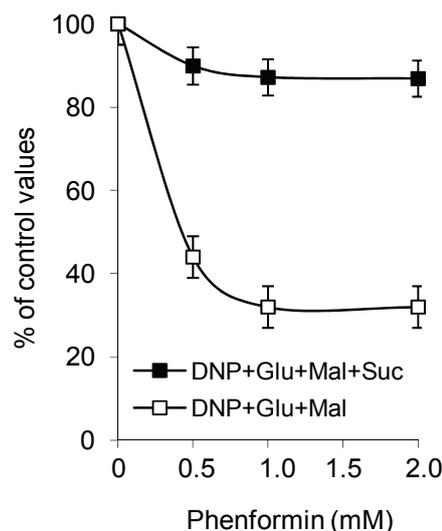


Fig. 5. Effect of phenformin on the oxidation of FADH-dependent and NADH-dependent substrates by uncoupled rat liver mitochondria. The inhibition of oxidation of glutamate + malate (Glu+Mal, white squares) and for the increase of respiration induced by succinate addition (black squares) after phenformin treatment is shown. 0.15 mM 2,4-dinitrophenol (DNP) was added prior to the other substrates in order to dissipate the electron transport from oxidative phosphorylation. Data are expressed in % of control, i.e. untreated mitochondria, values and are given as means from two independent experiments.

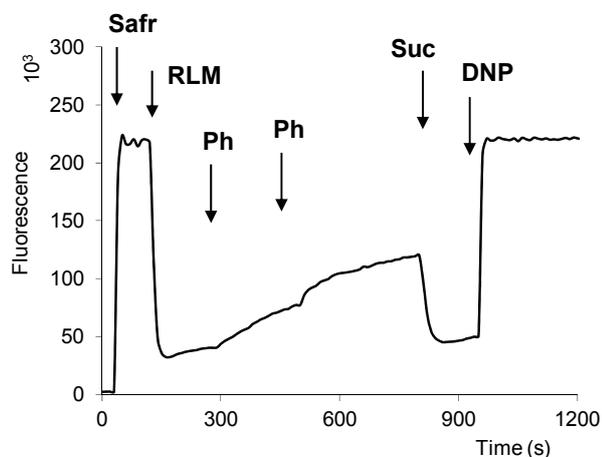


Fig. 6. Effect of phenformin on the mitochondrial membrane potential of rat liver mitochondria. Safranin O (Safr) was added to the K-medium containing 10 mM glutamate and 2.5 mM malate. Then intact rat liver mitochondria (RLM) were added (0.12 mg of protein/ml) followed by addition of 0.25 mM and 0.5 mM phenformin (Pf), 10 mM succinate (Suc) and 0.05 mM 2,4-dinitrophenol (DNP). The curve demonstrates a typical representative of three independent experiments.

coupled mitochondria (Fig. 2A). As the final step to confirm these findings, we measured the effect of phenformin on mitochondrial membrane potential. Membrane potential measured by fluorescent probe safranin O in the presence of glutamate and malate was slowly discharged by the addition of phenformin,

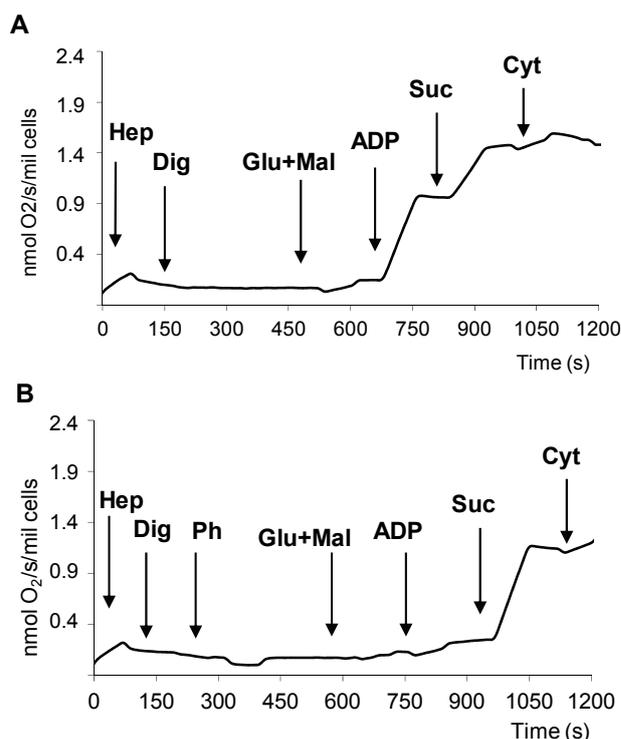


Fig. 7. Respiration of digitonin-permeabilised hepatocytes in the absence (A) and presence (B) of 1 mM phenformin. Hepatocytes (Hep; 2.5×10^5 cells/ml) were incubated in K-medium. Additions to both curves were as follows: 0.03 mg/ml digitonin (Dig), 1 mM phenformin (Ph, Fig. 7B), 10 mM glutamate and 2.5 mM malate (Glu+Mal), 1.5 mM ADP; 10 mM succinate (Suc) and 0.02 mM cytochrom c (Cyt). This figure represents a typical result of three independent experiments.

however, when succinate was added, the membrane potential was again recovered and could be released by an addition of uncoupler (Fig. 6). Taken together, we may conclude that phenformin has no uncoupling effect and impairment of glutamate + malate + ADP respiration and the decrease of the respiratory control index as well as the mitochondrial membrane potential is the consequence of the mitochondrial complex I inhibition.

Phenformin exhibits similar inhibitory effect both on isolated mitochondria and permeabilized hepatocytes

Several lines of evidence indicate that mitochondrial function may be modified in isolated mitochondria due to the destruction of mitochondrial filament structures as well as the disruption of contacts between mitochondria and other cell structures that may have an important role in their functional properties (Kondrashova *et al.* 2001, 2009). There are also discussions whether there are differences in biguanides action on intact cells and isolated mitochondria (El-Mir *et al.* 2000). We have consequently tested the inhibitory effect of phenformin on the activity of mitochondrial

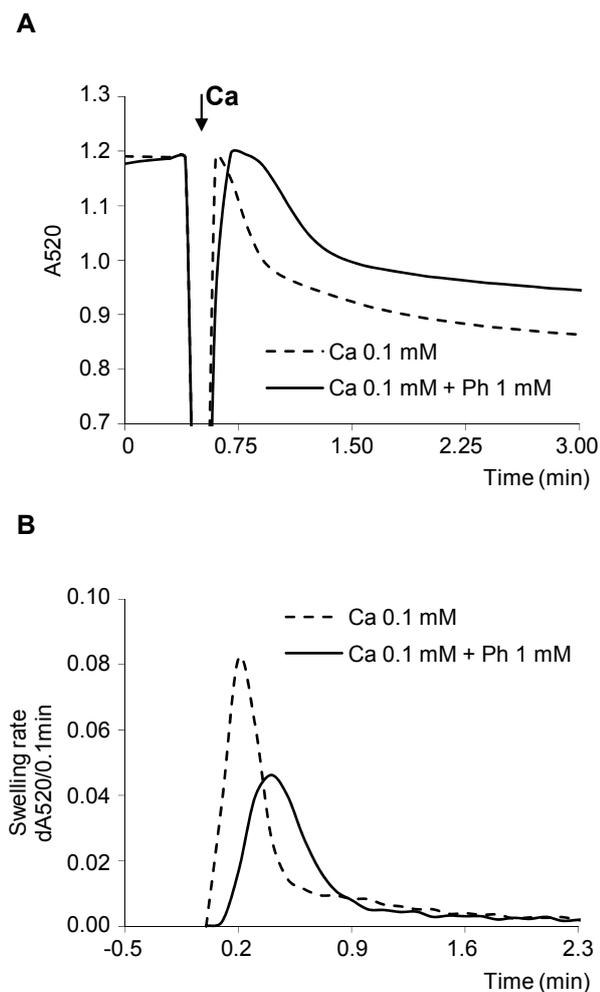


Fig. 8. Effect of phenformin on the calcium-induced swelling of rat liver mitochondria. Mitochondria were shortly (3 min) pre-incubated with 1 mM phenformin followed by the addition of 0.1 mM Ca^{2+} . The decrease of absorbance at 520 nm was detected in 0.1 min intervals. The extent of swelling (**A**) was calculated as the change in the optical density during the measurement period. Swelling rate (**B**) was calculated from the curves obtained after as the original data derivation. This figure represents a typical result of three independent experiments.

enzymes in permeabilized hepatocytes. Under these conditions, mitochondria are still in contact with other intracellular particles and mitochondrial filament structures are preserved (Kondrashova *et al.* 2001, 2009). As shown in Figure 7, in the presence of 1 mM phenformin, we obtained the same inhibitory effects with isolated hepatocytes as with isolated mitochondria (see Fig. 1). Phenformin affected only the glutamate and malate oxidation in the presence of ADP similarly as in isolated mitochondria and the inhibition of electron flux through complex I was partly compensated by electron supply from succinate. No significant increase was observed after cytochrome c addition which confirmed

that the mitochondria were not damaged by digitonin. Similar data could be also obtained with liver homogenate (not shown). We may thus conclude that the inhibitory effect of phenformin can be detected under the same experimental conditions in liver hepatocytes, in homogenate as well as in isolated mitochondria.

Phenformin increases the resistance of mitochondrial permeability pore to Ca^{2+}

Several reports indicate that biguanides can modify the function of the mitochondrial permeability transition pore (Detaille *et al.* 2005, Guigas *et al.* 2004). These data have shown that in isolated cells, metformin can change the mitochondrial calcium retention capacity as well as the accompanying release of mitochondrial cytochrome c.

We have therefore tested the effect of phenformin on the calcium induced swelling on isolated mitochondria. Three parameters characterizing the kinetics of the membrane permeability pore function were evaluated – the extent of swelling, the maximum rate of swelling and the time interval between the calcium addition and the time, when the swelling rate reaches its maximum rate (Drahota *et al.* 2012). As demonstrated in Figure 8, the extent of swelling and the maximum rate of swelling after addition of 0.1 mM CaCl_2 decreased due to the phenformin treatment by 20 % from 0.36 to 0.29 A_{520} , the maximum swelling rate decreased by 44 % from 0.0819 to 0.0462 $dA_{520}/0.1 \text{ min}$ and the time interval preceding the maximum swelling rate after CaCl_2 addition was prolonged two-fold from 0.2 to 0.4 min. All these results confirm that phenformin can increase the mitochondrial transition pore resistance to calcium and influence mitochondrial membrane bound complex which is not involved in the energy transformation process.

The effect of phenformin on anisotropy of fluorescence of hydrophobic membrane probe DPH in liver mitochondria

Our previous data are in agreement with the hypothesis that modification of various mitochondrial functions by biguanides could be explained by their unspecific modifications of protein-lipid interactions (Schäfer 1976, 1983, Wiernsperger 1999). We have therefore tested to what extent inhibition of various mitochondrial enzymes and functions by phenformin could be explained by its non-specific change of the hydrophobic interior of the mitochondrial membrane. This has been performed by determination of steady-state anisotropy of DPH (r_{DPH}) fluorescence. Fluorescence of

Table 1. Effect of increasing concentrations of phenformin on fluorescence anisotropy of hydrophobic membrane probe DPH in frozen rat liver mitochondria.

Phenformin (mM)	0	0.125	0.25	0.5	1	2
Mean	0.199	0.197	0.201	0.198	0.200	0.197
s.e.	0.001	0.001	0.002	0.001	0.005	0.003

Mitochondria were labeled with DPH as described in methods and the effect of increasing concentrations of phenformin on anisotropy of DPH fluorescence was determined at Ex 365 nm/Em 425 nm wavelengths. The data are expressed as mean \pm standard error of means.

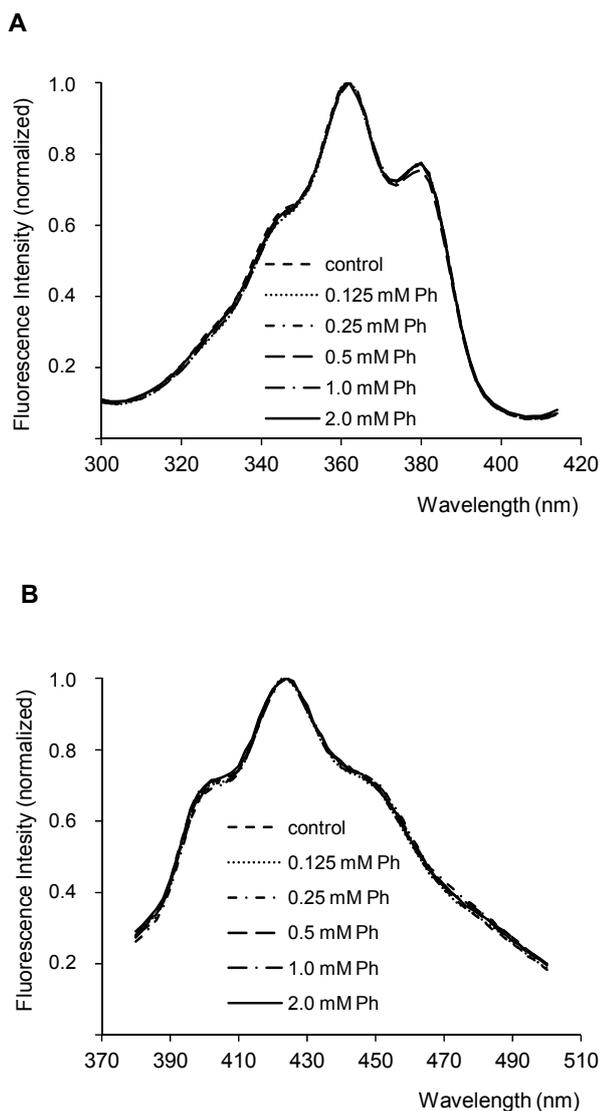


Fig. 9. Quenching of DPH fluorescence intensity by increasing concentrations of phenformin. DPH-labelled mitochondria were exposed to increasing concentrations of phenformin and the decrease of fluorescence intensity was determined up to the 2 mM concentration of this inhibitory agent. Fluorescence intensity values for each excitation wavelength (300–415 nm) were normalized as fraction of the fluorescence intensity of excitation maximum at 365 nm (**A**) and values for each emission wavelength (380–500 nm) were normalized as fraction of the fluorescence intensity of emission maximum at 425 nm (**B**). Both emission curve and a curve of excitation spectra represent 5 measurements with 0 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1.0 mM and 2.0 mM phenformin.

this highly hydrophobic probe is based exclusively on the membrane-bound signal and r_{DPH} reflects readily the changes of the hydrophobic membrane interior altered by temperature, hydrostatic and osmotic pressure, membrane lipid composition (cholesterol level, state of saturation of fatty acids, hydration), cell cycle, ageing and wide range of pharmacological interferences (Shinitzki 1984, Cossins and Sinenski 1984, de Laat *et al.* 1984, Hitzemann *et al.* 1984, Amler *et al.* 1986, 1990).

Surprisingly, we found that anisotropy of DPH fluorescence was unchanged at 1 mM and 2 mM concentration of phenformin (Table 1) under experimental conditions when the activity of complex I was strongly inhibited (Fig. 3 and 4). Simultaneously, determination of both emission and excitation spectra of DPH-labeled membranes indicated no significant change of intensity of DPH fluorescence which might obscure r_{DPH} data (Fig. 9).

Discussion

In our study, we present data indicating that biguanides (metformin and phenformin) seriously affects more than one aspect of mitochondrial function. Our conclusion is based on the following findings: 1) Phenformin, as well as metformin, inhibits mitochondrial respiration in dose-dependent manner, phenformin being 10 times more efficient than metformin; 2) both the phenformin and the metformin inhibitory effect is not dependent on cellular integrity but manifests itself in liver homogenates and isolated mitochondria as well as in permeabilized cells; 3) phenformin and metformin inhibit most effectively the activity of mitochondrial complex I but phenformin in higher concentration affects also complex II and complex IV; 4) in isolated mitochondria, phenformin decreases respiratory control index and releases the mitochondrial membrane potential; 5) phenformin *in vitro* decreases the sensitivity of mitochondrial Ca^{2+} -activated pore to calcium and reduces

the rate and extent of mitochondrial swelling.

From all the data presented in this study, we may conclude that the contradictory results relating to metformin inhibitory action on mitochondrial respiration resulted from its very low efficacy to affect mitochondrial complex I in *in vitro* experiments. On the contrary, another biguanide, phenformin, was known as a substance with many side effects when used for clinical treatments and as a more potent inhibitor in *in vitro* experiments. For this reason, for experimental studies, phenformin is evidently a better choice when we try to understand mechanism through which biguanides affect mitochondrial functions.

Guanidines and biguanides cause a wide variety of metabolic effects on many types of organisms and species and this rather extreme variability is mirrored by the heterogeneity of proposed explanations of their actions. Nevertheless, we can find several common features across all of the seemingly unrelated desired and undesired biguanide effects: 1) there are no biguanide-specific receptors which could transmit specific signals; 2) the glucose lowering effect of biguanides is not associated with the increased insulin secretion; 3) all blood glucose lowering biguanides exist only as positively charged species in physiological environment and 4) they bind readily to biological membranes.

It was proposed that membrane changes largely represent a common denominator explaining metformin effects on various systems involved in receptor signaling and related functions (Schäfer 1976, Wiernsperger 1999). Indeed, no effects of biguanides at therapeutic doses are known on soluble enzymes. As outlined in Schäfer (1983), the interaction of phospholipid membrane with biguanides may have several consequences on its physiological properties which may in turn induce a variety of disorders including changes at the membrane surface of ion activities, K_m of membrane enzymes, V_{max} of catalysis, protein conformation, receptor properties, fluidity of lipids, water structure at interphase, etc. Nevertheless, the exact character of this interaction remains unclear.

Based on our results, the direct interference of phenformin on the modification of hydrophobic lipid interior of mitochondrial membrane may be excluded as a mechanism of action of this potent inhibitory agent. The

possibility of interference with the polar-head group region of the membrane bilayer cannot be excluded at present time, but it is not to be expected as analysis of the effect of different salts on isolated plasma membranes prepared from the brain indicated no change of r_{DPH} values even at very high concentrations of NaCl or KCl (Vosahlikova, unpublished data). The role of the membrane surface-membrane water interface and the net-negative surface charge should be thus considered in this respect as a non-specific biophysical parameters increasing concentration of this inhibitor in the near vicinity of mitochondrial membrane and the changes of numerous enzyme activities participating in regulation of glucose metabolism and genesis of increased sensitivity of peripheral tissues to insulin (DeFronzo and Goodman 1995).

The data presented in our study indicate that biguanides act on various processes occurring in mitochondria. All these processes are connected with the inner mitochondrial membrane and thus the observed response of mitochondrial function to phenformin and metformin is compatible with the above mentioned concept. In fact, 36 years ago a paper entitled "Some new aspects on the interaction of hypoglycemia-producing biguanides with biological membranes" was published (Schäfer 1976). We thus propose that the conclusion of Prof. Schäfer that, "the specific consequences of biguanide – membrane phospholipids interactions provide a unique molecular basis for an understanding of the large variety of biguanide effects and explain their hypoglycemic effect as an accidental result rather than a principal of drug action" should be re-evaluated in further studies as it offers the most plausible explanation of the observed variety of biguanide effects.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This study was funded by grants no. ECRCZLL1204 and RVO67985823 and by MH CR-DRO ("Institute for Clinical and Experimental Medicine – IKEM, IN 00023001"), PRVOUK P37/02 and GACR (P207/12/0919).

References

AKERMAN KE, WIKSTROM MK: Safranin as a probe of the mitochondrial membrane potential. *FEBS Lett* **68**: 191-197, 1976.

- AMLER E, RAUCHOVA H, SVOBODOVA J, DRAHOTA Z: Regulation of glycerol 3-phosphate oxidation in mitochondria by changes in membrane microviscosity. *FEBS Lett* **206**: 1-3, 1986.
- AMLER E, JASINSKA R, DRAHOTA Z, ZBOROWSKI J: Membrane lateral pressure as a modulator of glycerol-3-phosphate dehydrogenase activity. *FEBS Lett* **271**: 165-168, 1990.
- ARGAUD D, ROTH H, WIERNSPERGER N, LEVERVE XM: Metformin decreases gluconeogenesis by enhancing the pyruvate kinase flux in isolated rat hepatocytes. *Eur J Biochem* **213**: 1341-1348, 1993.
- BAILEY CJ, TURNER RC: Metformin. *N Engl J Med* **334**: 574-579, 1996.
- BOYLE JG, SALT IP, MCKAY GA: Metformin action on AMP-activated protein kinase: a translational research approach to understanding a potential new therapeutic target. *Diabet Med* **27**: 1097-1106, 2010.
- BRADFORD MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254, 1976.
- BREJCHOVA J, SYKORA J, DLOUHA K, ROUBALOVA L, OSTASOV P, VOSAHLIKOVA M, HOF M, SVOBODA P: Fluorescence spectroscopy studies of HEK293 cells expressing DOR-Gi1alpha fusion protein; the effect of cholesterol depletion. *Biochim Biophys Acta* **1808**: 2819-2829, 2011.
- BUSTAMANTE E, SOPER JW, PEDERSEN PL: A high-yield preparative method for isolation of rat liver mitochondria. *Anal Biochem* **80**: 401-408, 1977.
- CAMPBELL PJ, CARLSON MG, HILL JO, NURJHAN N: Regulation of free fatty acid metabolism by insulin in humans: role of lipolysis and reesterification. *Am J Physiol* **263**: E1063-E1069, 1992.
- CAMPBELL RK, WHITE JR Jr, SAULIE BA: Metformin: a new oral biguanide. *Clin Ther* **18**: 360-371, 1996.
- COSSINS AR, SINENSKI M: Adaptation of membranes to temperature, pressure and exogenous lipids. In: *Physiology of Membrane Fluidity II*. SHINITZKI M (ed), CRC Press, Boca Raton, USA, 1984, pp 1-20.
- DEFRONZO RA, GOODMAN AM: Efficacy of metformin in patients with non-insulin-dependent diabetes mellitus. The Multicenter Metformin Study Group. *N Engl J Med* **333**: 541-549, 1995.
- DE LAAT SW, BLUEMINK JG, BOONSTRA J, MUMMERY CL, VAN DER SAAG PT, VAN ZOELLEN EJJ: Membrane fluidity in growth and differentiation; the neuroblastoma cell model. In: *Physiology of Membrane Fluidity II*. SHINITZKI M (ed), CRC Press, Boca Raton, USA, 1984, pp 21-52.
- DETAILLE D, GUIGAS B, CHAUVIN C, BATANDIER C, FONTAINE E, WIERNSPERGER N, LEVERVE X: Metformin prevents high-glucose-induced endothelial cell death through a mitochondrial permeability transition-dependent process. *Diabetes* **54**: 2179-2187, 2005.
- DRAHOTA Z, ENDLICHER R, STANKOVA P, RYCHTRMOC D, MILEROVA M, CERVINKOVA Z: Characterization of calcium, phosphate and peroxide interactions in activation of mitochondrial swelling using derivative of the swelling curves. *J Bioenerg Biomembr* **44**: 309-315, 2012.
- EL-MIR MY, NOGUEIRA V, FONTAINE E, AVERET N, RIGOLET 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.
- FORETZ M, HEBRARD S, LECLERC J, ZARRINPASHNEH E, SOTY M, MITHIEUX G, SAKAMOTO K, ANDREELLI F, VIOLLET B: Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest* **120**: 2355-2369, 2010.
- GONZALEZ-BARROSO MM, ANEDDA A, GALLARDO-VARA E, REDONDO-HORCAJO M, RODRIGUEZ-SANCHEZ L, RIAL E: Fatty acids revert the inhibition of respiration caused by the antidiabetic drug metformin to facilitate their mitochondrial beta-oxidation. *Biochim Biophys Acta* **1817**: 1768-1775, 2012.
- GOODARZI MO, BRYER-ASH M: Metformin revisited: re-evaluation of its properties and role in the pharmacopoeia of modern antidiabetic agents. *Diabetes Obes Metab* **7**: 654-665, 2005.
- 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.
- HITZEMANN RJ, HARROS RA, LOH HH: Synaptic membrane fluidity and function. In: *Physiology of Membrane Fluidity II*. SHINITZKI M (ed), CRC Press, Boca Raton, USA, 1984, pp 110-123.

- HUNDAL RS, KRSSAK M, DUFOUR S, LAURENT D, LEBON V, CHANDRAMOULI V, INZUCCHI SE, SCHUMANN WC, PETERSEN KF, LANDAU BR, SHULMAN GI: Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* **49**: 2063-2069, 2000.
- KLIP A, LEITER LA: Cellular mechanism of action of metformin. *Diabetes Care* **13**: 696-704, 1990.
- KONDRASHOVA MN, FEDOTCHEVA NI, SAAKYAN IR, SIROTA TV, LYAMZAEV KG, KULIKOVA MV, TEMNOV AV: Preservation of native properties of mitochondria in rat liver homogenate. *Mitochondrion* **1**: 249-267, 2001.
- KONDRASHOVA M, ZAKHARCHENKO M, KHUNDERYAKOVA N: Preservation of the in vivo state of mitochondrial network for ex vivo physiological study of mitochondria. *Int J Biochem Cell Biol* **41**: 2036-2050, 2009.
- OWEN MR, DORAN E, HALESTRAP AP: Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J* **348**: 607-614, 2000.
- PALENICKOVA E, CAHOVA M, DRAHOTA Z, KAZDOVA L, KALOUS M: Inhibitory effect of metformin on oxidation of NADH-dependent substrates in rat liver homogenate. *Physiol Res* **60**: 835-839, 2011.
- SCHÄFER G: Some new aspects on the interaction of hypoglycemia-producing biguanides with biological membranes. *Biochem Pharmacol* **25**: 2015-2024, 1976.
- SCHÄFER G: Biguanides. A review of history, pharmacodynamics and therapy. *Diabete Metab* **9**: 148-163, 1983.
- SHINITZKI M: Membrane fluidity and cellular functions. In: *Physiology of Membrane Fluidity I*. SHINITZKI M (ed), CRC Press, Boca Raton, USA, 1984, pp 1-52.
- WIERNSPERGER NF: Membrane physiology as a basis for the cellular effects of metformin in insulin resistance and diabetes. *Diabetes Metab* **25**: 110-127, 1999.
- 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.
-