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# Dimethylbiguanide Inhibits Cell Respiration via an Indirect Effect Targeted on the Respiratory Chain Complex I*

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**We report here a new mitochondrial regulation occurring only in intact cells. We have investigated the effects of dimethylbiguanide on isolated rat hepatocytes, permeabilized hepatocytes, and isolated liver mitochondria. Addition of dimethylbiguanide decreased oxygen consumption and mitochondrial membrane potential only in intact cells but not in permeabilized hepatocytes or isolated mitochondria. Permeabilized hepatocytes after dimethylbiguanide exposure and mitochondria isolated from dimethylbiguanide pretreated livers or animals were characterized by a significant inhibition of oxygen consumption with complex I substrates (glutamate and malate) but not with complex II (succinate) or complex IV (*N,N,N′,N′*-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD)/ascorbate) substrates. Studies using functionally isolated complex I obtained from mitochondria isolated from dimethylbiguanide-pretreated livers or rats further confirmed that dimethylbiguanide action was located on the respiratory chain complex I. The dimethylbiguanide effect was temperature-dependent, oxygen consumption decreasing by 50, 20, and 0% at 37, 25, and 15 °C, respectively. This effect was not affected by insulin-signaling pathway inhibitors, nitric oxide precursor or inhibitors, oxygen radical scavengers, ceramide synthesis inhibitors, or chelation of intra- or extracellular $Ca^{2+}$. Because it is established that dimethylbiguanide is not metabolized, these results suggest the existence of a new cell-signaling pathway targeted to the respiratory chain complex I with a persistent effect after cessation of the signaling process.**

Mitochondria are intracellular organelles devoted mainly to energy metabolism (ATP production) that also play a pivotal role in the onset of cell death (1, 2). The regulation of such functions is essential and has been well characterized in isolated mitochondria, whereas much less is known in intact cells. Short term regulation of intact cell respiration has been established with $Ca^{2+}$ and is related to the $Ca^{2+}$-dependent mitochondrial dehydrogenases that regulate the supply of substrates to the respiratory chain (3). It has been reported that lipopolysaccharide *plus* interferon-γ can persistently inhibit respiratory chain complex IV in intact astrocytes (4) and that activation of glutamate receptors induces a persistent inhibition of complexes II, III, and IV in intact neurons (5). Both inhibitions can be prevented by nitric-oxide synthase inhibitors. Furthermore, it has been shown that prolonged direct exposure to nitric oxide (NO)[1] in intact J774 cells leads to a persistent inhibition of respiratory chain complex I, whereas inhibition of complex IV was reversible (6).

Dimethylbiguanide (metformin) is an oral antihyperglycemic drug widely used in the treatment of type-II diabetes (7–10), the action mechanism of which remains largely unknown (see Refs. 11 and 12 for review). Dimethylbiguanide inhibits hepatic gluconeogenesis, possibly through a decrease in the cytosolic ATP/ADP ratio (13). Although it has been long known that biguanides inhibit respiration in intact cells, dimethylbiguanide is 10 times less potent than phenethylbiguanide (phenformin) and has no direct effect on isolated mitochondria (12–15). Therefore the mechanism by which high concentrations of dimethylbiguanide inhibit oxidative phosphorylation remained unclear.

The present results indicate that dimethylbiguanide decreases oxygen consumption and mitochondrial membrane potential in intact hepatocytes, whereas it has no effect on isolated mitochondria or on permeabilized hepatocytes. Contrary to the previously identified long term mitochondrial regulators (4–6, 16), the mitochondrial inhibitory effect of dimethylbiguanide is purely located on the respiratory chain complex I and does not affect the oxidative phosphorylation machinery downstream complex I. This effect is not affected by a variety of cell signaling inhibitors but is completely prevented when cells are incubated at 15 °C. Because dimethylbiguanide is not metabolized (17), these results suggest the existence of a new cell signaling pathway targeted to the respiratory chain complex I.

## MATERIALS AND METHODS

Hepatocytes were isolated according to the method of Berry and Friend (18) as modified by Groen *et al.* (19). Hepatocytes (final concentration 10 mg dry cells/ml) were incubated in closed vials at 37 °C in a shaking water bath (60 strokes/min) in 2.5 ml of Krebs-bicarbonate buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 1.3 mM $CaCl_2$, pH 7.4) saturated with a mixture of $O_2/CO_2$ (19:1 by volume). For some experiments incubations were performed simultaneously at 37, 25, and 15 °C.

Isolated hepatocytes were permeabilized using digitonin (6 μg/mg

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[1] The abbreviations used are: NO, nitric oxide; CCCP, carbonyl cyanide *p*-trichloromethoxyphenylhydrazone; $TPMP^+$, triphenylmethylphosphonium; TMPD, *N,N,N′,N′*-tetramethyl-1,4-phenylenediamine dihydrochloride; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester; BAPTA-AM, 1,2-bis(aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid.

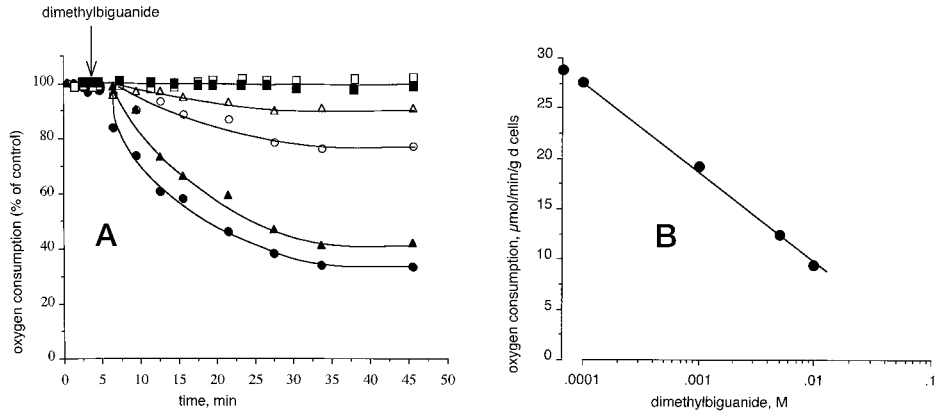


FIG. 1. **Effect of dimethylbiguanide on oxygen uptake in intact isolated hepatocytes, permeabilized hepatocytes, and isolated liver mitochondria.** *Panel A*, time course. Hepatocytes (approximately 10 mg of dry cells/ml) were incubated in closed vials at 37 °C in 2.5 ml of Krebs-bicarbonate buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 1.3 mM $CaCl_2$, pH 7.4) saturated with a mixture of $O_2/CO_2$ (19:1 by volume) supplemented with 20 mM lactate, 2 mM pyruvate, 4 mM octanoate, and different concentrations of dimethylbiguanide: *filled circles,* 10 mM; *filled triangles*, 5 mM; *open circles*, 1 mM; and *open triangles*, 0.1 mM. Permeabilized hepatocytes (*open squares*) or isolated mitochondria (2 mg of protein/ml, *filled squares*) were incubated in closed vials at 37 °C in a medium containing 250 mM sucrose, 20 mM Tris-HCl, 1 mM EGTA, 5 mM $P_i$, 5 mM glutamate, 1 mM malate, and 10 mM dimethylbiguanide. Dimethylbiguanide was added after 3-min incubation, and where indicated, oxygen consumption rate was measured. Results are expressed as percentage of oxygen uptake obtained in the absence of dimethylbiguanide for similar incubation times. *Panel A* illustrates one typical experiment, similar results were obtained in three others. *Panel B*, dose dependence. Hepatocytes were incubated as described in the legend for *panel A*. Oxygen consumption rate was measured after 30 min of incubation. Results are mean ± S.E. ($n$ = 15, 5 rats). *Error bars* lie within the symbols.

dry cells, 2 min, at room temperature) as described in Ref. 20. Cell membrane permeabilization was always evidenced by the lack of trypan blue exclusion.

Two different approaches were used to study the effect of dimethylbiguanide on liver mitochondria. In one case, mitochondria were prepared from rat liver previously perfused for 30 min with Krebs-Bicarbonate medium containing or not 10 mM dimethylbiguanide. In the second case pharmacological (sublethal) dose of dimethylbiguanide (60 mg/100 g of body weight) dissolved in saline solution buffer (NaCl, 0.9%) or saline solution alone were injected subcutaneously 30 min before sacrifice. When using the latter method, plasma concentration of dimethylbiguanide at the sacrifice was determined by high performance liquid chromatography (3.6 ± 0.3 mM, $n$ = 4). After liver perfusion or *in vivo* subcutaneous injection, liver mitochondria were then prepared according to Klingenberg and Slencza (21) and resuspended in a medium containing 250 mM sucrose, 1 mM EGTA, 20 mM Tris-HCl, pH 7.2.

Water-soluble fraction and nonpolar soluble fraction of livers were obtained as follows: rat livers were first isolated and perfused during 30 min at 37 °C with Krebs-bicarbonate buffer saturated with a mixture of $O_2/CO_2$ (19:1 by volume) supplemented or not with 10 mM dimethylbiguanide. Livers were then homogenized in a small volume of Krebs-bicarbonate buffer and centrifuged at 2000 g for 10 min then supernatant and pellet were separated. The supernatant was the soluble fraction, and the pellet lipids were extracted with chloroform/methanol (2:1) as described elsewhere (22, 23) and evaporated under nitrogen stream. Final liver nonpolar soluble fraction was resuspended in $Me_2SO$ and kept in the dark at −80 °C until used.

Oxygen consumption rate was measured polarographically in a stirred oxygraph vessel thermostated at 37 °C and equipped with a Clark oxygen electrode. For the study of the temperature effect, we performed prolonged incubations of up to 3 h at 15, 25, or 37 °C in the medium described above supplemented with 20 mM dihydroxyacetone and 4 mM octanoate in the presence or not of 10 mM dimethylbiguanide. After 0, 30, 60, 120, and 180 min of incubation, 1 ml of the suspension was removed from the vial and placed in the oxygraph vessel containing 1 ml of the same medium saturated with $O_2/CO_2$ gas mixture and thermostated at 37 °C. This procedure permitted to adjust precisely the temperature of the suspension to 37 °C in less than 2 min before recording oxygen consumption rate.

Measurement of mitochondrial membrane potential in intact cells was performed as described in Ref. 24. Briefly, after determination of mitochondrial and cellular volumes using $^3H_2O$ plus [$^{14}$C]mannitol and $^3H_2O$ plus [$^{14}$C]carboxymethyl-inulin respectively, mitochondrial and plasmic membrane potentials were determined by measuring accumulation of [$^3$H]TPMP$^+$ and $^{36}Cl^-$, respectively.

Lactate, pyruvate, 3-hydroxybutyrate, and acetoacetate were measured enzymatically as described in Ref. 25 on sample of cell suspension previously quenched in $HClO_4$ (4% mass/volume final concentration) and neutralized with KOH (2 M)/MOPS (0.3 M). Intramitochondrial and cytosolic NADH/NAD$^+$ ratios were calculated assuming thermodynamic equilibrium with 3-hydroxybutyrate/acetoacetate and lactate/pyruvate ratios, respectively (26).

ATP and ADP were measured by chemoluminescence on mitochondrial and cytosolic spaces previously separated using the digitonin fractionation method (27, 28).

For complex I assay, mitochondria (0.5 mg/ml) were incubated in a 1 mM EGTA, 20 mM Tris-HCl, pH 7.2, solution (to break inner membrane by hypoosmotic shock) in the presence of 500 μM NADH and 5 mM KCN. Complex I activity was assessed by the oxidation rate of NADH (measuring absorbance at 340 nm in a Uvikon-Kontron 941-plus spectrophotometer equipped with thermostatic control and magnetic stirring) after addition of 100 μM decylubiquinone as electron acceptor.

Complex III plus IV activity was assessed by measuring oxygen consumption with decylubiquinol (300 μM) as the respiratory substrate in presence of rotenone (1.25 μM).

Pyruvate, digitonin, and ADP were purchased from Roche Molecular Biochemicals; phospholipase C inhibitor U73122 from Calbiochem; octanoate from Janssen; collagenase type IV, lactate, myxothiazol, CCCP, TMPD, rotenone, wortmannin, LY294002, PD98059, L-NAME, L-arginine, decylubiquinone, BAPTA-AM, EGTA, fumosine B1, L-cycloserine, β-DL-alanine, and all other reagents from Sigma-Aldrich. Dimethylbiguanide was a gift from Merck-Lipha Co. Decylubiquinol was prepared as described in Ref. 29 by chemical reduction of decylubiquinone with sodium borohydride.

Results are expressed either as typical experiment or as indicated as mean ± S.E. of the number of incubation from at least three rats. Statistical analyses were made using analysis of variance followed by Fisher's protected least significant difference post hoc test (Stat View®, Abacus concepts, Inc., Berkley, CA, 1992).

## RESULTS

*Inhibition of Oxygen Uptake by Dimethylbiguanide Requires Intact Cells*—The data shown in Fig. 1 illustrate the inhibitory effect of dimethylbiguanide on oxygen consumption in isolated hepatocytes with respect to dimethylbiguanide concentration and incubation time. As shown in *panel A*, the inhibition induced by dimethylbiguanide was already observed after 5 min of cell incubation, whereas the maximal effect was reached after 20–30 min whatever the dimethylbiguanide concentration used, *i.e.* 0.1, 1, 5, and 10 mM. Conversely, incubation of permeabilized hepatocytes (*panel A*, *filled squares*) or isolated liver mitochondria (*panel A, open squares*) with 10 mM dimethylbiguanide did not affect respiratory rate over 30 min. *Panel B* shows a dose-dependent decrease in the respiratory rate of intact cells for dimethylbiguanide concentrations between 0.1

TABLE I

*Comparative effects of dimethylbiguanide, myxothiazol, or rotenone on cellular energy metabolism*

Hepatocytes were incubated as described in the legend to Fig. 1 in a medium supplemented with 20 mM dihydroxyacetone and 4 mM octanoate in the absence of inhibitor or in the presence of either 10 mM dimethylbiguanide, 0.15 $\mu$M myxothiazol, or 0.52 $\mu$M rotenone, each of these inhibitors concentrations leading to a similar inhibition of respiration. After 30 min of incubation cellular respiratory rate ($JO_2$), *in situ* mitochondrial membrane potential ($\Delta\Psi$), cytosolic (cyto) and mitochondrial (mito) ATP/ADP ratios, lactate/pyruvate ratio (Lac/Pyr), and 3-hydroxybutyrate/acetoacetate ratio (3-OH/AcAc) were measured. Results are expressed as mean ± S.E. ($n$ = 15, five rats). Statistical comparisons were made using analysis of variance (Fisher PLSD test) by using Statview software®.

| | $JO_2$ | $\Delta\Psi$ | ATP/ADP (cyto) | ATP/ADP (mito) | Lac/Pyr | 3-OH/AcAc |
|---|---|---|---|---|---|---|
| | *% of control* | *mV* | | | | |
| Control | 100 ± 0 | −175 ± 3 | 7.3 ± 0.5 | 1.7 ± 0.1 | 19.8 ± 0.3 | 0.18 ± 0.001 |
| Dimethylbiguanide | 54 ± 2[a] | −141 ± 5[a] | 2.5 ± 0.2[a] | 0.5 ± 0.1[a] | 24.4 ± 0.3[a] | 0.38 ± 0.003[a] |
| Myxothiazol | 56 ± 9[a] | −154 ± 5[a] | 3.1 ± 0.3[a,b] | 0.7 ± 0.1[a,b] | 26.0 ± 0.5[a] | 0.25 ± 0.003[a,b] |
| Rotenone | 50 ± 7[a] | −149 ± 5[a] | 3.4 ± 0.3[a,b] | 0.9 ± 0.1[a,b,c] | 38.3 ± 0.7[a,b,c] | 0.44 ± 0.003[a,c] |

[a] $p < 0.001$ versus control.
[b] $p < 0.001$ *versus* dimethylbiguanide.
[c] $p < 0.01$ *versus* myxothiazol.

and 10 mM after 30 min of incubation. This finding confirms that dimethylbiguanide has no direct effect on the mitochondrial oxidative phosphorylation pathway (12) and indicates that the cellular integrity is required for an effect of dimethylbiguanide on respiration to be observed.

Studies in the pharmacokinetics of dimethylbiguanide have established that it is totally excreted in an unchanged form (17, 30–32), and so far, no dimethylbiguanide metabolite has been evidenced. However, to investigate the possibility that the respiratory effect of dimethylbiguanide might be due to some putative metabolite, we incubated control mitochondria in the presence of water-soluble fraction or of nonpolar soluble fraction extracts prepared as described under "Materials and Methods." Irrespective of the added fraction, the respiratory rate of mitochondria energized with glutamate and malate (see below) was not affected (data not shown). Although a short-lived intermediate cannot be excluded, the lack of effect of both polar and of nonpolar fractions indicate that the dimethylbiguanide effect was not related to the accumulation of a stable cellular factor(s) and may suggest the existence of a more integrated signaling pathway.

We next studied the consequences of the dimethylbiguanide-induced inhibitory effect on the energy metabolism of intact hepatocytes. As shown in Table I, dimethylbiguanide decreased the mitochondrial membrane potential ($\Delta\Psi$) measured *in situ* by approximately 30 mV and dramatically reduced ATP/ADP ratio in both cytosolic and mitochondrial compartments. Furthermore, the lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios, which are in thermodynamic equilibrium with cytosolic and mitochondrial NADH concentrations, respectively (26), were significantly increased by dimethylbiguanide. This data indicates that the respiratory inhibition is not due to a decrease in the supply of energy substrates but rather suggests that the inhibition is related to respiratory chain function. Identical results were obtained when respiration was inhibited in similar proportion with standard respiratory inhibitors such as rotenone or myxothiazol (see Table I). It is noteworthy that the lactate/pyruvate ratio was significantly higher with rotenone than with either dimethylbiguanide or myxothiazol exposures.

*Persistence of the Dimethylbiguanide Effect after Drug Removal in Both Permeabilized Hepatocytes and Isolated Mitochondria*—To investigate the reversibility of the dimethylbiguanide-induced respiratory effect, hepatocytes were incubated 30 min with 10 mM dimethylbiguanide, then washed (three times) and re-incubated in the absence of dimethylbiguanide. Under such conditions, the respiratory effect was seen to persist for over 30 min after removal of the drug (data not shown). In addition, hepatocytes permeabilized after 30 min of preincubation in the presence of 10 mM dimethylbiguanide, as well as liver mitochondria isolated from either *ex vivo* perfused liver with dimethylbiguanide, or *in vivo* dimethylbiguanide-treated rats, were all characterized by a clear respiratory inhibition when incubated in the presence of glutamate and malate as respiratory substrates (states 4 and 3 and uncoupled state, see Fig. 2, *panels A–C*). Thus, in addition to the finding that dimethylbiguanide-induced effect on respiration requires cells to be intact another striking feature is its persistence after cell permeabilization or mitochondria-isolation procedures.

*Complex I as the Sole Target of the Dimethylbiguanide-induced Cellular Respiratory Inhibition*—Contrary to observations in the presence of glutamate and malate, respiration of either dimethylbiguanide-exposed permeabilized hepatocytes or mitochondria isolated from pretreated livers or animals was unchanged in the presence of succinate (Fig. 2, *panels D–F*) or TMPD/ascorbate (Fig. 2, all *panels*), indicating that respiratory chain complexes II, III, and IV were not affected by dimethylbiguanide pretreatment. Moreover, because the phosphorylating respiratory rate (*i.e.* after addition of ADP) was not affected when succinate was the substrate (Fig. 2, *panels D–F*), it can be concluded that neither the ATP carrier nor the F1F0-ATPase are affected by dimethylbiguanide pretreatment.

The finding that the dimethylbiguanide-induced respiratory inhibition only occurred in the presence of complex I substrate strongly suggests that complex I was the selective target of this process. To further examine this question we functionally isolated complex I (NADH quinone oxidoreductase, EC 1.6.99.3) from the remaining part of the respiratory chain using decylubiquinone (oxidized quinone) as complex I electron acceptor, in the presence of KCN. As shown Fig. 3, *panel A*, electron transfer through complex I was inhibited in liver mitochondria isolated from liver previously perfused with dimethylbiguanide as compared with that of mitochondria isolated from a liver perfused in absence of dimethylbiguanide. On the other hand when decylubiquinol (reduced quinone) was used as complex III electron donor, no difference was observed between the dimethylbiguanide-treated and the nontreated group (Fig. 3, *panel B*), confirming previous results obtained with succinate. Similar results were obtained when using mitochondria isolated from *in vivo* dimethylbiguanide-treated rats (data not shown). It can thus be concluded that the treatment by dimethylbiguanide *in vivo* or *in vitro* (in intact isolated hepatocytes) affects the mitochondrial respiratory chain specifically at the complex I site.

*Dimethylbiguanide-induced Respiratory Inhibition Is Temperature-dependent*—In our attempt to clarify the mechanism by which dimethylbiguanide affects complex I in intact cells, we studied the effects of the incubating temperature performing prolonged incubations of up to 3 h at 15, 25, or 37 °C in the presence or not of dimethylbiguanide or of respiratory chain

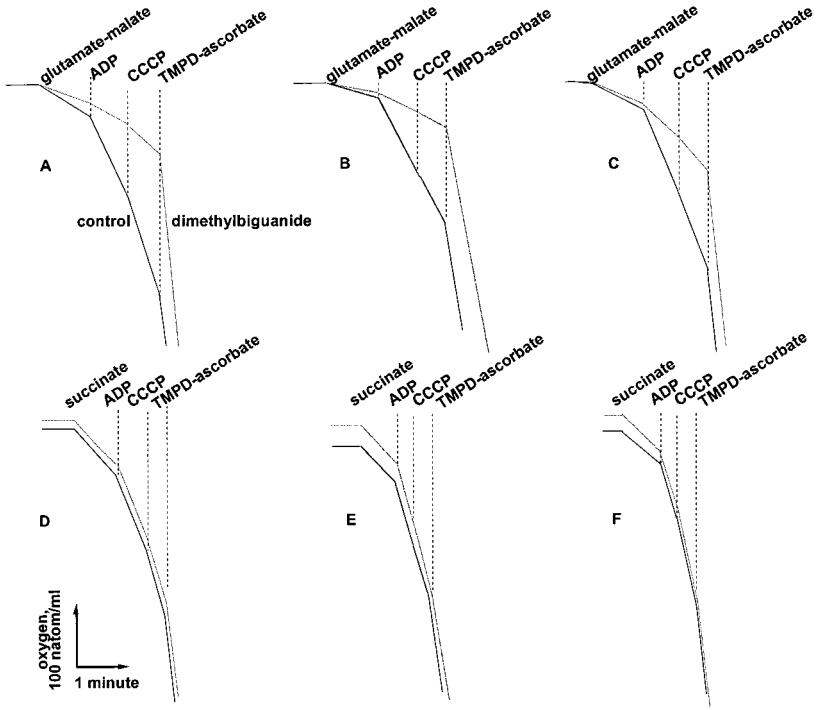


FIG. 2. **Consequence of dimethylbiguanide pretreatment on oxygen uptake of permeabilized hepatocytes or isolated mitochondria.** The incubation medium contained 250 mM sucrose, 1 mM EGTA, 5 mM $P_i$, 20 mM Tris-HCl, pH 7.4, 37 °C. Experiments were started by the addition of permeabilized hepatocytes obtained by digitonin treatment of intact liver cells (see "Materials and Methods") previously incubated 30 min with or without 10 mM dimethylbiguanide (*panels A* and *D*), of mitochondria isolated from *ex vivo* perfused liver for 30 min with a medium containing or not 10 mM dimethylbiguanide (*panels B* and *E*), or of mitochondria isolated from *in vivo* dimethylbiguanide-treated rat (*panels C* and *F*). In this latter case and for the typical experiment shown in *panels C* and *F*, plasma dimethylbiguanide concentration at the sacrifice was 3.6 mM. Where indicated 5 mM glutamate and 1 mM malate (*panels A–C*) or 5 mM succinate plus 1.25 μM rotenone (*panels D–F*), 1 mM ADP, 0.36 μM CCCP, and 1 mM TMPD plus 5 mM ascorbate were added. One typical experiment is presented (*black line*, control; *gray line*, dimethylbiguanide); similar results were obtained in four other different preparations.

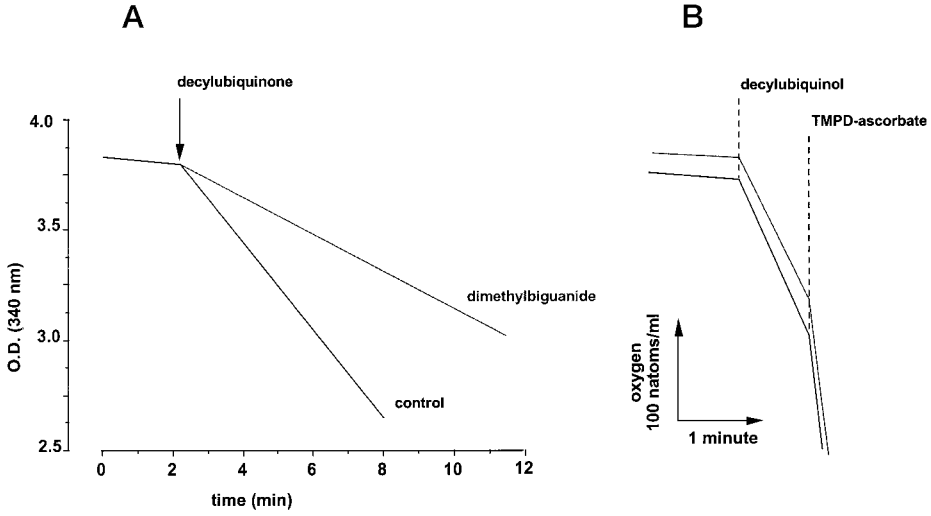


FIG. 3. **Consequence of dimethylbiguanide pretreatment on complex I and complexes III–IV activities.** The incubation mediums contained 500 μM NADH, 1 mM EGTA, 5 mM KCN, 20 mM Tris-HCl, pH 7.4, 25 °C (*panel A*) or 250 mM sucrose, 1.25 μM rotenone, 1 mM EGTA, 5 mM $P_i$, 20 mM Tris-HCl, pH 7.4, 25 °C (*panel B*). Experiments were started after addition of mitochondria isolated from liver perfused 30 min with a medium containing or not 10 mM dimethylbiguanide (not shown). Where indicated, either 100 μM decylubiquinone (*panel A*) or 300 μM decylubiquinol (*panel B*) were added. NADH oxidation (*panel A*) or oxygen consumption rate (*panel B*) were followed as described under "Materials and Methods." Results represent one typical experiment, similar results were obtained in three other different preparations. Moreover similar results were also obtained when mitochondria were isolated from *in vivo* dimethylbiguanide-treated rat (data not shown).

inhibitors (rotenone and myxothiazol). Cell incubations were performed at the adequate temperature and at the indicate time a sample of the suspension was removed from the vial and placed in an oxygraph vessel thermostated at 37 °C to quickly adjust the temperature of the suspension to 37 °C before recording oxygen consumption rate (see "Materials and Methods"). As shown in Fig. 4, incubations of up to 3 h at 15 °C (*panel A*) exhibited no effect of dimethylbiguanide, whereas incubations at 25 and 37 °C showed a decreased respiration by 20 and 50%, respectively. It is striking that the maximal dimethylbiguanide-induced inhibition was reached after 30 min of incubation and remained stable thereafter indicating that amplitude but not kinetics of the response is temperature-dependent. Regardless of the incubation temperature, rotenone

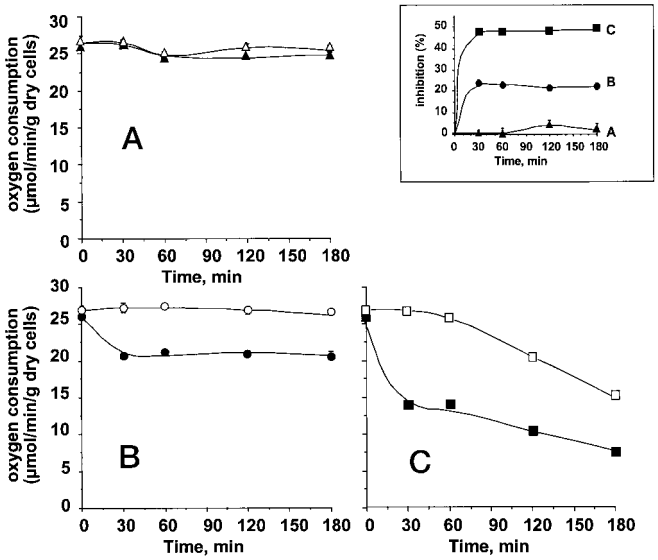


FIG. 4. **Effect of temperature on dimethylbiguanide-induced respiratory inhibition.** Hepatocytes were incubated at 15 °C (*panel A*), 25 °C (*panel B*), or 37 °C (*panel C*) in the medium described in the legend to Fig. 1 supplemented with 20 mM dihydroxyacetone and 4 mM octanoate in the absence (*open symbols*) or in the presence of 10 mM dimethylbiguanide (*filled symbols*). After 0, 30, 60, 120, and 180 min of incubation, a sample of the suspension was quickly removed from the vial and placed in an oxygraph vessel thermostated at 37 °C permitting to adjust the temperature of the suspension precisely to 37 °C before oxygen consumption rate was recorded. Results are expressed as mean ± S.E. ($n = 12$, three rats). *Inset* shows the percentage of inhibition respectively at 15, 25, and 37 °C. Statistical comparisons were made using analysis of variance; dimethylbiguanide significantly decreased oxygen consumption rate at 25 and 37 °C ($p < 0.001$) but not at 15 °C.

and myxothiazol were seen to be identically effective at either 15, 25, or 37 °C leading to conclude that the effect of rotenone and myxothiazol were almost not temperature-dependent (data not shown).

*Dimethylbiguanide-induced Effect Does Not Involve Standard Cell Signaling Pathways*—In the light of recent findings showing nitric oxide to induce a persistent inhibition of the respiratory chain (4–6, 16), we also carried out a series of experiments using NO synthase inhibitor (L-NAME) or NO precursor (L-arginine). As shown in Table II, neither inhibition nor stimulation of the NO pathway affected the dimethylbiguanide-induced effect.

Reactive oxygen intermediates (mainly superoxides and hydroxyl radicals) have been shown to inhibit mitochondrial respiration (33–36). To evaluate the role of oxygen radicals on the dimethylbiguanide-induced effect, oxygen radical scavengers were added to the incubation medium 20 min before addition of the drug. Neither ascorbate (a superoxide scavenger), mannitol (a hydroxyl radical scavenger), nor catalase (hydrogen peroxide scavenger) succeeded in preventing the effect of dimethylbiguanide on cellular respiration (Table II).

Although the cellular mechanism of action of dimethylbiguanide remains largely unknown, results from several studies pointed to a connection between dimethylbiguanide and cellular insulin signaling (30, 31, 37–40). Activation of the insulin receptor tyrosine kinase and tyrosine phosphorylation of intracellular substrates are important steps in insulin signaling (41, 42). However, inhibition of the phosphatidylinositol 3-kinase pathway by specific inhibitors such as wortmannin (43, 44) or LY294002 (45, 46) and of the MAP kinase pathway by PD98059 (47) did not affect the dimethylbiguanide-induced inhibition of respiration (Table II), leading us to exclude these two pathways in the signaling effects of dimethylbiguanide.

TABLE II
*Lack of effect of cell-signaling inhibitors on dimethylbiguanide-induced inhibition of cellular respiration*

Hepatocytes were incubated in the medium described in the legend to Fig. 1 with 1.3 mM free calcium. Insulin-signaling pathways inhibitors (wortmannin, 1 µM; LY29002, 50 µM; or PD98059, 20 µM), nitric-oxide synthase inhibitor (L-NAME, 1 mM), nitric oxide precursor (L-arginine, 2 mM), reactive oxygen species scavengers (ascorbate, 10 mM; mannitol, 10 mM; or catalase, 500 units/ml), different extracellular free calcium concentrations (0 mM + 1 mM EGTA, 2.6 and 4 mM), intracellular $Ca^{2+}$ chelator (BAPTA-AM, 0.4 mM), phospholipase C inhibitor (U73211, 25 µM) or ceramide synthesis inhibitors (fumosine B1, 5 µM; L-cycloserine, 1 mM; β-DL-alanine, 2.5 mM) were added 20 min before 10 mM dimethylbiguanide addition. Oxygen consumption rate was measured after 30-min incubation. Results are mean ± S.E. ($n \geq 9$ for at least three rats). Whatever the experimental conditions, dimethylbiguanide always significantly inhibited the respiratory rate by 40–50% ($p < 0.001$), and no significant difference was found concerning the extent of the inhibitory effect among the various conditions.

| | $JO_2$ | | |
|---|---|---|---|
| | Control | Dimethylbiguanide | $JO_2$ control |
| | *µmol $O_2$/min/g dry cells* | | *%* |
| Control ($n = 42$) | 29.4 ± 0.5 | 11.6 ± 0.3 | 60.4 ± 1.1 |
| Wortmannin ($n = 9$) | 27.8 ± 0.6 | 11.0 ± 0.7 | 60.6 ± 1.0 |
| LY29002 ($n = 9$) | 27.5 ± 0.6 | 9.9 ± 0.6 | 63.9 ± 2.4 |
| PD98059 ($n = 9$) | 27.9 ± 0.6 | 9.2 ± 0.5 | 66.9 ± 1.9 |
| L-Name ($n = 9$) | 30.3 ± 2.1 | 10.0 ± 0.3 | 66.6 ± 0.9 |
| L-Arginine ($n = 9$) | 29.1 ± 1.9 | 9.7 ± 0.4 | 65.9 ± 1.5 |
| Ascorbate ($n = 9$) | 26.8 ± 0.4 | 13.1 ± 0.4 | 51.0 ± 1.5 |
| Mannitol ($n = 9$) | 31.8 ± 0.4 | 15.5 ± 0.2 | 51.0 ± 1.6 |
| Catalase ($n = 9$) | 31.9 ± 0.3 | 15.8 ± 0.5 | 50.1 ± 1.6 |
| 0 mM $Ca^{2+}$ ($n = 9$) | 27.3 ± 0.5 | 11.3 ± 0.5 | 58.3 ± 2.3 |
| 2.6 mM $Ca^{2+}$ ($n = 9$) | 27.5 ± 0.5 | 11.1 ± 0.6 | 59.2 ± 2.4 |
| 4 mM $Ca^{2+}$ ($n = 9$) | 27.4 ± 0.5 | 10.9 ± 0.5 | 60.0 ± 2.2 |
| BAPTA ($n = 9$) | 26.9 ± 0.4 | 13.9 ± 0.3 | 51.7 ± 1.2 |
| U73211 ($n = 9$) | 27.3 ± 0.2 | 13.8 ± 0.5 | 50.6 ± 1.8 |
| Fumosine B1 ($n = 9$) | 30.5 ± 1.1 | 14.9 ± 0.3 | 49.0 ± 3.6 |
| L-Cycloserine ($n = 9$) | 30.3 ± 1.0 | 15.1 ± 0.3 | 49.9 ± 0.9 |
| β-DL-Alanine ($n = 9$) | 30.1 ± 0.9 | 15.3 ± 0.2 | 50.6 ± 0.8 |

Because it has been shown that dimethylbiguanide interferes with cellular $Ca^{2+}$ homeostasis (48, 49), we also incubated hepatocytes with different free $Ca^{2+}$ concentrations (0, 2.60, and 4 mM) or in the presence of EGTA (1 mM) in $Ca^{2+}$-free medium. As shown in Table II, none of the higher $Ca^{2+}$ concentration conditions affected the dimethylbiguanide-induced effect observed at the physiological concentration (1.3 mM). Similarly, results also showed preincubation in the presence of BAPTA (an intracellular calcium chelator) did not affect the dimethylbiguanide effect. Inhibition of phospholipase C by U73211 had no effect on the dimethylbiguanide inhibitory effect. Hence, neither chelation of intra- or extracellular $Ca^{2+}$ nor inhibition of phospholipase C signaling pathway affected the dimethylbiguanide-induced inhibition of respiration (Table II).

Finally, we have tested the possibility that ceramides could be involved in this effect, although the inhibitory effect reported on mitochondrial respiration is located on complex III (50). As shown in Table II the ceramide synthesis inhibitors fumosine B1, L-cycloserine, and β-DL-alanine had no effect on the dimethylbiguanide-induced inhibition of respiration.

## DISCUSSION

In this study we have shown that dimethylbiguanide specifically inhibits respiratory chain complex I through an indirect mechanism that (i) does not operate through traditional cell signaling pathways but (ii) requires cells to be intact to be initiated and (iii) persists after removal of the drug or after isolation of the mitochondria. The amplitude, but not the kinetics, of this effect is temperature-dependent. Although the chain of cellular reactions triggering such a mitochondrial effect has not been identified and its physiological role remains unknown, we conclude that hepatocytes have a signaling pathway targeted to the respiratory chain complex I.

*Dimethylbiguanide Indirectly Affects Respiratory Chain Complex I*—Electron transfer through complex I can be modulated by numerous substances including poisons such as rotenone and physiological molecules such as NO (6, 51). Although the molecular mechanisms by which such compounds inhibit complex I are not totally resolved, a direct interaction between the inhibitors and the enzymatic complex is essential. The novelty of the results presented in this work is based on the finding that the dimethylbiguanide-induced complex I inhibition is not a consequence of a direct interaction with the respiratory chain, because dimethylbiguanide, which is known for being not metabolized, has no effect on isolated mitochondria.

Among the cell signaling pathways capable of mitochondrial regulation, we have clearly shown that neither the NO pathway nor $Ca^{2+}$ homeostasis are involved in the dimethylbiguanide-induced respiratory inhibition. The absence of a link between the dimethylbiguanide effect and NO pathway is not surprising considering that the NO effect seems to be related to a decrease in glutathione level, whereas dimethylbiguanide is known to increase the liver glutathione content (52, 53).

The finding that oxygen radical scavengers do not neutralize the dimethylbiguanide-induced effect suggests that oxygen radicals are not involved in this process. However, because complex I can be both a source and a target of oxygen radicals, this hypothesis cannot be definitively ruled out.

Dimethylbiguanide signaling effect could operate via ceramide formation, because it has been reported recently to inhibit respiration. But ceramide affects complex III conversely to the highly specific effect toward complex I reported here. Moreover the lack of effect of ceramide synthesis inhibitors (Fumosine B1, L-cycloserine, and β-DL-alanine) does not support this hypothesis.

*Mechanism of Mitochondrial Regulation by Dimethylbiguanide*—The finding that hydrophylic or lipophylic extracts of dimethylbiguanide-treated livers do not affect mitochondria strongly suggests that dimethylbiguanide does not simply induce an accumulation of stable natural compounds or putative metabolites. This conclusion associated with the compelling evidence that dimethylbiguanide inhibits respiration in intact cells lead us to propose that dimethylbiguanide acts via a complex signaling pathway, the first step of which may be an interaction between the drug and a membrane receptor. This hypothesis is supported by the observed logarithmic dose-dependent effect of dimethylbiguanide on cellular respiration (Fig. 1, *panel B*) such as seen for hormone-receptor interaction. Although, the temperature-dependent nature of this inhibition is difficult to explain, it could suggest an effect related to the physicochemical state of the plasmic membrane. Indeed, the temperature dependence of an enzymatic reaction is generally expected to influence the kinetics but not the final amplitude of the reaction. Considering the rapid onset (5 min) and the short time to maximal effect (20–30 min), the data are more consistent with a phosphorylation-dephosphorylation or a protein degradation mechanism.

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