

A Cell-based High-throughput Assay System Reveals Modulation of Oxidative and Nonoxidative Glucose Metabolism due to Commonly Used Organic Solvents

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Key words

- glucose
- lactate
- reactive oxygen species
- mitochondria
- respiration
- adenosine triphosphate
- metabolism

Abstract

A 96-well format screening system was generated to quantify changes in nonoxidative glucose metabolism and oxidative pyruvate metabolism. D-Glucose uptake from the supernatant media was quantified by the glucose oxidase method, and L-lactate production of cells was quantified by the lactate dehydrogenase method applied on supernatant media. Mitochondrial membrane potential was quantified using tetramethylrhodamine methyl ester (TMRM) fluorescence, and reactive oxygen species (ROS) formation was determined by quantification of dihydrodichlorofluorescein fluorescence. Adenosine triphosphate (ATP) content of myocytes was determined using the luciferin reaction, and cellular respiration was quantified using com-

mercially available, precoated microtiter plates. These six assays were used to determine the putative influence of organic solvents, namely dimethyl sulfoxide (DMSO), ethanol, methanol, and N-methylpyrrolidone (NMP) at concentrations of 0.01, 0.1, 1.0, and 5.0% (vol/vol), respectively, on glucose and pyruvate metabolism after 4 and 24 hours. In summary, all solvents induced significant changes in regard to one or several of the parameters evaluated, affecting cellular glucose uptake, glycolysis, mitochondrial metabolism, or oxidative phosphorylation. Accordingly, this comprehensive HTS evaluation should enable researchers to choose specific organic solvents on a rational basis to avoid nonspecific effects in cultured cells and tissue culture based experimental setups.

Introduction

Mitochondria are sub-cellular organelles, where conversion of nutrient intermediates into readily available energy equivalents takes place. This involves a process called oxidative phosphorylation (OXPHOS) generating adenosine triphosphate (ATP). Recent scientific evidence has linked mitochondrial dysfunction to several human diseases, including cancer [1], neurodegenerative disorders [2], and type 2 diabetes mellitus [3–5]. Hence, high-throughput assay systems might be required to evaluate chemical compounds in regard to putative modulation of mitochondrial metabolism. Preferably, such an assay system would also analyze metabolic events prior to mitochondrial nutrient oxidation, namely glycolysis as the main source of pyruvate, a crucial precursor of acetyl-CoA, and hence required to maintain Krebs cycle activity.

Previous evidence tentatively suggests that organic solvents commonly required to apply chemical compounds to cell-based assay systems might influence mitochondrial metabolism [6–8]. Nevertheless, to our best knowledge this issue has never been systematically addressed.

We describe here the adaptation of previously established methods to quantify six parameters of nonoxidative and oxidative glucose metabolism to a 96-well-based scale, namely D-glucose uptake, L-lactate production, mitochondrial membrane potential ($\Delta\Psi_m$), ROS formation, respiration, and lastly ATP content. These assays are used to quantify the effects of organic solvents, namely dimethyl sulfoxide (DMSO), ethanol, methanol, and N-methylpyrrolidone (NMP) on these six metabolic parameters. The findings suggest relevant changes by all four solvents, while alterations caused by DMSO and ethanol are somewhat less pronounced, indicating that DMSO or ethanol should be considered the solvents of choice to determine the majority of parameters of oxidative glucose metabolism.

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received 06.02.2007

accepted 18.06.2007

Bibliography

DOI 10.1055/s-2007-1004542

Horm Metab Res 2008;

40: 29–37

© Georg Thieme Verlag KG

Stuttgart · New York

ISSN 0018-5043

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Materials and Methods

Reagents

The reagents and materials used are listed below together with the names of the suppliers: Dulbeccos modified eagle medium (DMEM, glucose free, Cambrex Bio Science, Belgium, Vervies) supplemented with 10 mmol/l D-Glucose (Applichem GmbH, Germany, Darmstadt) concentration; fetal bovine serum (FBS, Biochrom AG, Germany, Berlin); horse serum (HS, Invitrogen GmbH, Germany, Karlsruhe); trypsin (Sigma-Aldrich GmbH, Germany, Steinheim); penicillin/streptomycin mix (Biochrom AG, Germany, Berlin); bovine insulin (Sigma-Aldrich); carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, Sigma-Aldrich); *tert*-butyl hydroperoxide (TBHP, Sigma-Aldrich); oligomycin from Streptomyces diastatochromogene, as a mixture of the components A, B and C (Sigma-Aldrich); DMSO (Applichem); ethanol (Carl Roth GmbH Co KG, Germany, Karlsruhe); methanol (Carl Roth); NMP (Sigma-Aldrich); CellTiter Glo Assay Kit (Promega, Germany, Mannheim); glucose oxidase (GO) Assay Kit (Sigma-Aldrich); L-lactic acid Assay (R-biopharm, Germany, Darmstadt); tetramethylrhodamin methyl ester (TMRM; Sigma-Aldrich); 2',7'-dihydrodichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich); 96-well plates (BD Bioscience Oxygen Consumption System; BD Bioscience, Germany, Heidelberg).

Cell culture

L6 muscle cells [9–12] were obtained from LGC Promochem (Wesel, Germany) (local ATCC representative) and maintained as a strictly subconfluent myoblast monolayer culture in DMEM containing 10 mmol/l of D-glucose, 10% (vol/vol) FBS and 1% (vol/vol) antibiotic-antimycotic solution (100 U/ml penicillin, and 10 µg/ml streptomycin) ('propagation medium') in a humidified atmosphere of 5% CO₂ at 37 °C. For experiments, myoblasts were plated in 96-well plates at a density of 1 × 10⁴ cells per well. Spontaneous differentiation of myoblasts and fusion into myotubes was induced by reducing the serum content to 2% (vol/vol) HS ('differentiation medium') as previously described [13]. The differentiation medium was changed every 48 hours, myotubes were used for experiments on days 7–9 after plating.

D-Glucose uptake and L-lactic acid production

Prior to D-glucose uptake and L-lactate production experiments, L6 myotubes were placed onto 0.2% media for 16 hours. Cells were incubated with respective solvents at 37 °C in an atmosphere of 5% CO₂. To stop incubation, cells were placed on wet ice. The supernatant media were immediately removed and centrifuged at 2000 × g for 10 minutes at 4 °C to eliminate detached cells. The supernatant was immediately collected, frozen at –20 °C and D-glucose concentration was determined using a colorimetric kit in which glucose oxidase converts D-glucose to D-gluconic acid and hydrogen peroxide. In the second step, hydrogen peroxide and reduced *o*-dianisidine reacts to form oxidized *o*-dianisidine in the presence of peroxidase. Sulfuric acid oxidizes *o*-dianisidine to a more stable colored product and absorbance is measured at 540 nm [14]. For L-lactic acid measurement, samples were obtained as described above. Subsequently, the supernatant was kept for 20 minutes at 70 °C, cooled to room temperature, and L-lactic acid concentration was determined using a photometric kit in which L-lactate dehydrogenase converts L-lactic acid to pyruvate. The absorbance of NADH formed in this reaction is proportional to the L-lactic acid con-

centration in the supernatant [15]. D-Glucose uptake and L-lactic acid production were determined from the exact same samples. L6 myotubes were incubated for 4 and 24 hours with a final concentration of 100 nanomol/l of bovine insulin as a positive control.

All absorbance, fluorescence, and luminescence measurements were performed using a microplate fluorometer NOVOstar (BMG Labtech, Offenburg, Germany). This equipment was used for all subsequent assays as well. For D-glucose assays, a wavelength of 540 nm was used. For L-lactate assays, a wavelength of 340 nm was used.

Mitochondrial membrane potential (ΔΨ_m)

Mitochondrial membrane potential was determined using TMRM, a lipophilic, cationic fluorescent indicator. TMRM is rapidly taken up by mitochondria proportionally to the inner membrane potential, hence fluorescence intensity at a wavelength of 590 nm is directly proportional to ΔΨ_m of living cells [16]. For assessment of ΔΨ_m, L6 myotubes were pre-treated with the appropriate solvents in media containing 2% of HS at 37 °C and 5% CO₂. At the end of incubation period, TMRM was added to differentiation medium at a final concentration of 150 nanomol/l, and cells were incubated for additional 5 minutes at room temperature. After loading, cells were washed five times with PBS, and fluorescence intensity was read immediately with an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

As a positive control, cells were treated with CCCP, a mitochondrial uncoupler, which depolarizes the inner mitochondrial membrane, and thus compensatorily induces mitochondrial respiration and cellular oxygen uptake at a final concentration of 10 µmol/l for 4 and 24 hours.

Determination of reactive oxygen species (ROS)

Quantification of ROS formation was essentially performed according to the method of Wang and Joseph [17]. L6 myotubes were loaded with 2',7'-dihydrodichlorofluorescein diacetate (H₂-DCF-DA; 100 µmol/l final concentration) in differentiation medium for 30 minutes at 37 °C in an atmosphere of 5% CO₂. H₂-DCF-DA is a nonfluorescent fluorescein derivative, which emits fluorescence after oxidation to DCF-DA. Fluorescence intensity is directly proportional to intracellular ROS formation. After loading with H₂-DCF-DA, cells were washed once with PBS and test compound were added. Fluorescence intensity was read immediately (0h) and after different incubation periods at 37 °C under CO₂ atmosphere (5% or 4l/min within the fluorometer). Fluorescence intensity was read at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

As a positive control, TBHP was used at a final concentration of 50 µmol/l TBHP to induce oxidative stress. Fluorescence intensity was read immediately, after 4 hours, and after 24 hours of incubation. Basal ROS content at 0 hours was subtracted from values of 4 hours and 24 hours time point to calculate dynamic ROS accumulation.

Oxygen consumption

To quantify oxygen consumption, the BD™ Oxygen Biosensor System (BD™ OBS) was used. BD™ OBS is a standard 96-well microplate irreversibly coated with the oxygen-sensitive material, tris(1,7-diphenyl-1,10-phenanthroline)ruthenium(II) chloride, whose fluorescence intensity varies inversely with the oxygen concentration [18]. L6 myoblast were used for oxygen consumption assay. For all experiments cells were harvested

and plated at a density of 1×10^4 cells per well in propagation medium (10% FBS). For adaptation, L6 myoblasts were incubated for 60 minutes at 37 °C in an atmosphere of 5% CO₂. Appropriate solvents were added and microtiter plates were coated with a self-adhesive foil to avoid further contact with oxygen. Fluorescence intensity was read with an excitation wavelength of 485 nm, and an emission wavelength of 612 nm immediately after adding solvents, and again after defined incubation periods. Oxygen concentrations were calculated from BD™ OBS fluorescence signal by applying the Stern–Volmer theorem [19] as suggested by the supplier. Subsequently oxygen consumption rate (OCR) was computed from initial oxygen concentration using a variation of a model derived from Fick's Law by Mamchaoui and Saumon [20].

As a positive control, cells were treated with CCCP, a mitochondrial uncoupler, which depolarizes the inner mitochondrial membrane and thus induces compensatory mitochondrial respiration and cellular oxygen uptake at a final concentration of 10 μmol/l CCCP for 4 and 24 hours.

ATP formation

L6 myocytes were pre-treated with solvents in differentiation medium. At the end of incubation period, cells were placed on wet ice for 5 minutes, and cellular ATP was quantified by using a luciferase-based kit for cellular extracts in which luciferase converts luciferin in the presence of ATP and oxygen to oxyluciferin [21]. The emitted luminescence signal is directly proportional to ATP content of cells. Oligomycin, a selective inhibitor of F₀/F₁-ATPase was used as positive control at a final concentration of 20 mg/ml for 4 and 24 hours.

Protein determination

Protein content was measured photometrically by using the Bradford method [22] briefly by lysing cells with 1 M NaOH on the 96-well plate, and determining protein content in an aliquot of the lysate at a wavelength of 600 nm. This assay was applied to separate control plates for each metabolic assay before starting the experiments (0 h) and after finishing the treatments (4 h and 24 h).

ATP and ΔΨ_m were normalized to protein content at the respective endpoints since they reflect a momentary state. D-Glucose uptake, L-lactic acid formation, oxygen consumption rate, and ROS formation were normalized to the area under the protein curve calculated by using protein concentrations determined before starting the respective experiment, and after finishing the respective determinations.

Statistical analysis

Values are expressed as means ± standard deviation. Statistical analyses of all parameters were performed by Mann–Whitney U-Test. All calculations were performed with SPSS Version 13.0. Differences were considered as statistically different when $p \leq 0.05$.

Results

Six metabolite assays were modified as described in Materials and Methods to quantify parameters of oxidative and nonoxidative glucose metabolism employing L6 myocytes in a 96-well scale suitable for HTS applications. To determine the biochemical effects of different organic solvents on cell metabolism and

ROS formation, L6 cells were incubated with DMSO, ethanol, methanol, and NMP at final concentrations of 0.01%, 0.1%, 1% and 5% for 4 and 24 hours. Glucose uptake, L-lactic acid production, respiration, ROS, ΔΨ_m, and ATP formation were determined as described in Materials and Methods.

Firstly, appropriate positive controls (described in Material and Methods, data not shown) were used to reveal functionality of all assays. For D-glucose uptake, insulin was used as a positive control at a concentration of 100 nanomol/l and induced an induction by $97.3 \pm 14.8\%$ of D-glucose uptake after 4 hours and induction by $78.3 \pm 8.1\%$ after 24 hours. For L-lactate production, insulin was used as a positive control at a concentration of 100 nanomol/l and induced an induction by $15.1 \pm 8.3\%$ of L-lactate production after 4 hours and an induction by $43.4 \pm 20.3\%$ after 24 hours. For ΔΨ_m, the mitochondrial uncoupler CCCP was used as a positive control at a concentration of 10 μmol/l and induced a reduction by $42.9 \pm 8.2\%$ of ΔΨ_m after 4 hours and a reduction by $78.2 \pm 12\%$ after 24 hours. For ROS formation, the membrane-permeable hydrogenperoxide derivative *tert*-butyl hydroperoxide (TBHP) was used as a positive control at a concentration of 50 μmol/l, and caused an induction by $155.3 \pm 13.4\%$ of ROS accumulation after 4 hours and an induction by $90.3 \pm 12.6\%$ after 24 hours. For oxygen consumption, the mitochondrial uncoupler CCCP was used as a positive control at a concentration of 10 μmol/l, and caused an induction by $395.3 \pm 26.3\%$ of respiration after 4 hours and an induction by $61 \pm 12.6\%$ of respiration after 24 hours. Finally, for ATP content, the F₀/F₁-ATPase inhibitor oligomycin was used as a positive control at a concentration of 20 μg/ml, and induced a reduction by $62.2 \pm 4\%$ of ATP content after 4 hours and a reduction by 100% of ATP content after 24 hours. Taken together, these findings so far indicate that all assays are capable of quantifying the respective metabolic state, since they show an adequate reaction of the respective metabolic state to the appropriate positive control substances.

Secondly, effects of different organic solvents, DMSO, ethanol, methanol, and NMP at concentrations of 0.01, 0.1, 1.0 and 5.0% on L6 myocyte protein content reflecting cell growth were tested (● Fig. 1). DMSO showed no significant effects on protein content after 4- as well as 24-hour treatment (● Fig. 1A). Ethanol had strongest influence on protein content. Especially after long time treatment with ethanol, a significant concentration dependent reduction of protein content was observed, while 4-hour treatment with 5% ethanol induces a significant increase of protein content (● Fig. 1B). Methanol had only minor influence on protein content, where a final concentration of 1% of this solvent increases protein content after short time incubation (● Fig. 1C). NMP significantly reduces protein content per well only if used at high concentration (5%) (● Fig. 1D). The lack of effect following DMSO treatment should be noted, since all subsequent assays are normalized to protein content of the respective 96-well plates. Hence only for DMSO a lack of influence on relative metabolite content can be assumed.

Thirdly, D-glucose uptake was quantified as described above in the presence of organic solvents, namely DMSO, ethanol, methanol, and NMP at concentrations of 0.01, 0.1, 1.0, and 5.0%. Because of cumulative decrease of glucose content in the supernatant medium over the entire incubation period, D-glucose uptake was normalized to the area under the protein curve (● Fig. 2A–D, β-panels) as described Materials and Methods. This is contrasted by results obtained by normalization to protein content at the end of experiments (● Fig. 2A–D, α-panels) yielding potentially

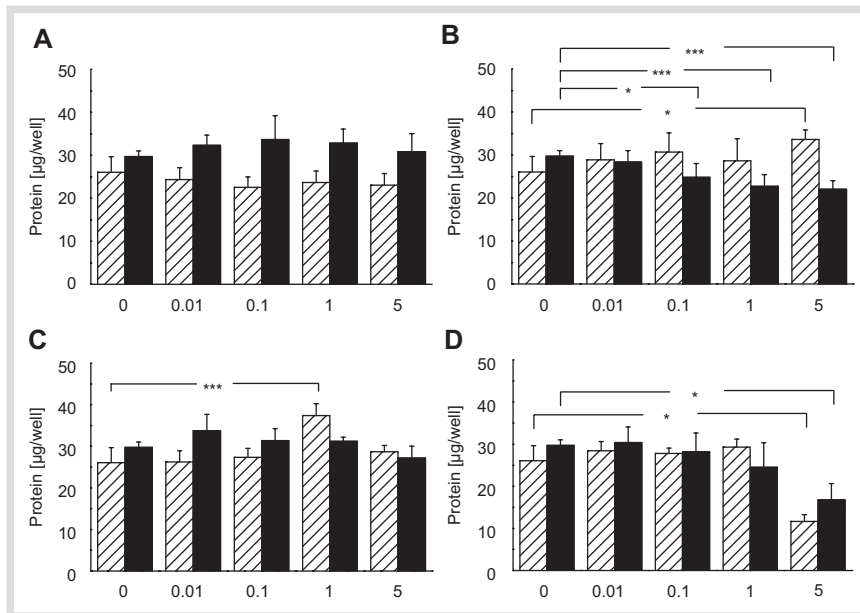


Fig. 1 Myocyte protein content following exposure to increasing concentrations of organic solvents. The following criteria applies to all subsequent figures: Panels depict DMSO (A), ethanol (B), methanol (C) and NMP (D). Striped bars represent 4-hour treatment; black bars depict 24-hour treatment. Results are given as means \pm standard deviation, $n=8$. Significant differences to control values are expressed as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$ and **** $p \leq 0.001$.

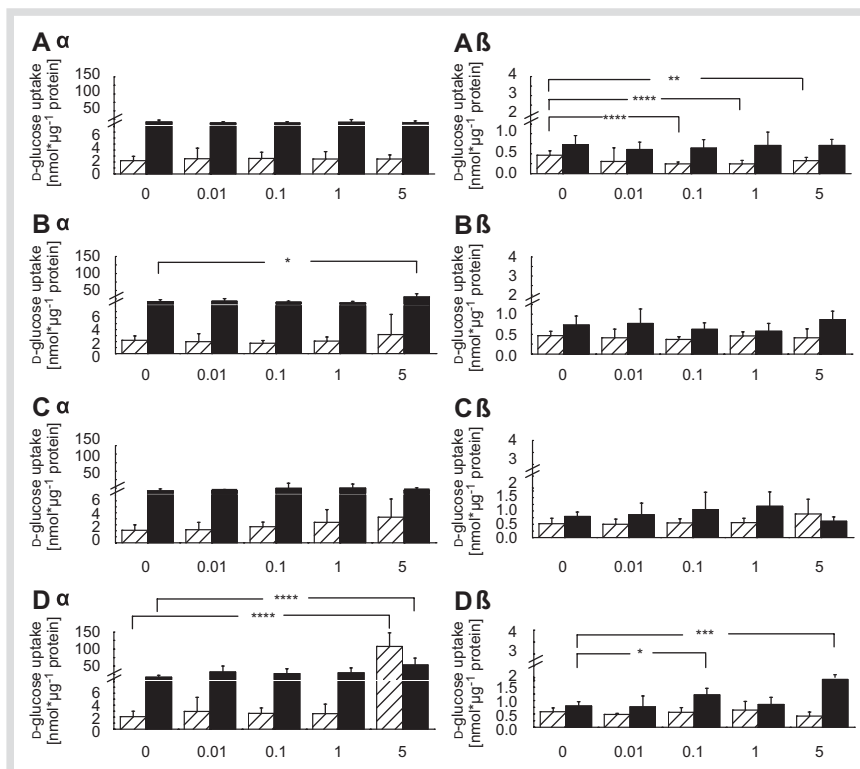


Fig. 2 D-Glucose uptake of myocytes following exposure to increasing concentrations of organic solvents. Results were either normalized to protein content at the end of experiment (α sub-panels), or normalized to the area under the protein curve between the respective time points (β sub-panels). For further legend, see **Fig. 1**.

erroneous effects on D-glucose uptake of myocytes. Accordingly, DMSO induces a slight but significant reduction of D-glucose uptake after 4-hour treatment on using 0.1–5% of this compound. No DMSO effect on D-glucose uptake was observed after the 24 hours incubation period (**Fig. 2A β**). Ethanol does not influence D-glucose uptake of L6 myocytes (**Fig. 2B β**). Methanol does not affect D-glucose uptake of L6 myocytes significantly, although methanol between 0.01 and 1% tends to induce a concentration dependent increase in D-glucose uptake after long time treatment (**Fig. 2C β**). NMP increases the D-glucose uptake after 24-hour treatment significantly at concentrations higher

than 0.1% while short time incubation has no significant effect (**Fig. 2D β**).

Subsequently, L-lactate production was quantified as described above in the presence of organic solvents, namely DMSO, ethanol, methanol, and NMP at concentrations of 0.01, 0.1, 1.0, and 5.0%. Because of the accumulation of L-lactic acid over incubation period, L-lactic acid formation was normalized to the area under the protein curve as described in Materials and Methods (**Fig. 3**). After 4-hour treatment, DMSO at very low concentration (0.01%) increases the L-lactic acid formation of myocytes slightly, whereas high DMSO concentrations (5%) cause a decrease of L-lactic acid formation. After a long term DMSO

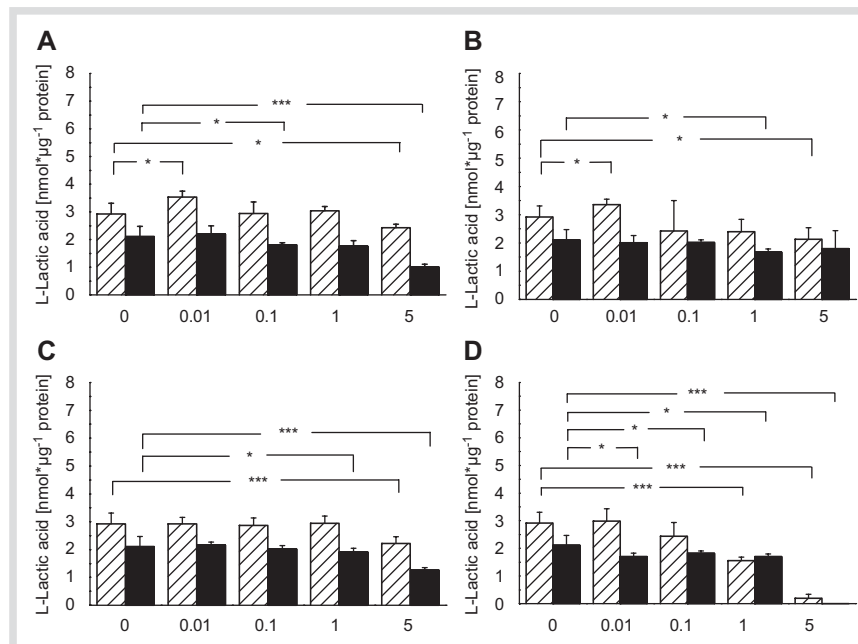


Fig. 3 L-Lactic acid formation of myocytes following exposure to increasing concentrations of organic solvents. For further legend, see **Fig. 1**.

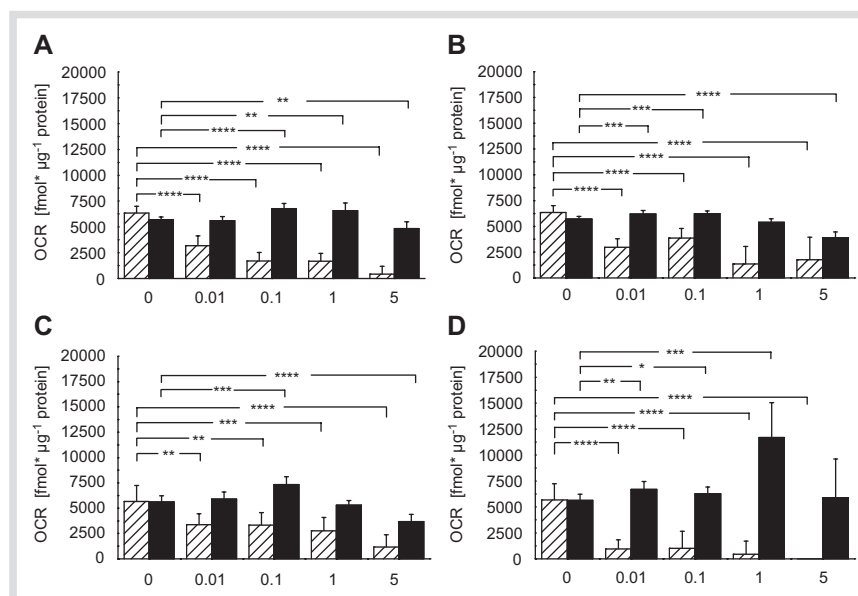


Fig. 4 OCR of myoblasts following exposure to increasing concentrations of organic solvents. For further legend, see **Fig. 1**.

treatment (24 h), a slight but significant reduction of L-lactic acid formation was observed on using 0.1% of this organic solvent. High DMSO concentrations (5%) cause a more pronounced decrease of L-lactic acid formation (**Fig. 3A**). Ethanol induces only small effects on L-lactic acid formation of L6 myocytes. After a short time incubation period, 0.01% ethanol increases L-lactic acid production slightly, whereas 5% ethanol reduces L-lactic acid formation. Long time incubation (24h) causes only a slight significant decrease of L-lactic acid formation on using 1% of this organic solvent (**Fig. 3B**). Methanol reduces L-lactic acid formation after long and short time treatment on using 1 and 5% of this solvent, whereas lower methanol concentrations have no effects on L-lactic acid production (**Fig. 3C**). NMP shows strongest influence on L-lactic acid formation of myocytes especially after a long time treatment. If the L-lactic acid production was reduced in a concentration dependent manner on using NMP concentrations of up to 0.1%, an NMP concentration of 5% can cause a 100% decrease of L-lactic acid formation of myocytes

(**Fig. 3D**). This increase in lactate production caused, albeit at a different degree, by all four solvents suggests an impairment of oxidative metabolism, which is compensated for by an increase in glycolytic flux.

Therefore, respiration was subsequently quantified as described above in the presence of organic solvents, namely DMSO, ethanol, methanol, and NMP at concentrations of 0.01, 0.1, 1.0, and 5.0%. Oxygen consumption rate (OCR) was calculated as described in Materials and Methods. Data were normalized to the area under the protein curve (**Fig. 4**). All four organic solvents produce a pronounced and concentration-dependent suppression of OCR after 4-hour treatment, where NMP was observed to be the most detrimental solvent (**Fig. 4D**); it represses OCR completely at high concentrations. After long time incubation period (24 h), OCR returns to control cell levels. DMSO concentrations from 0.1 to 1% cause a significant increase of OCR, whereas the highest concentration of DMSO rather reduces cellular respiration significantly (**Fig. 4D**). Similar

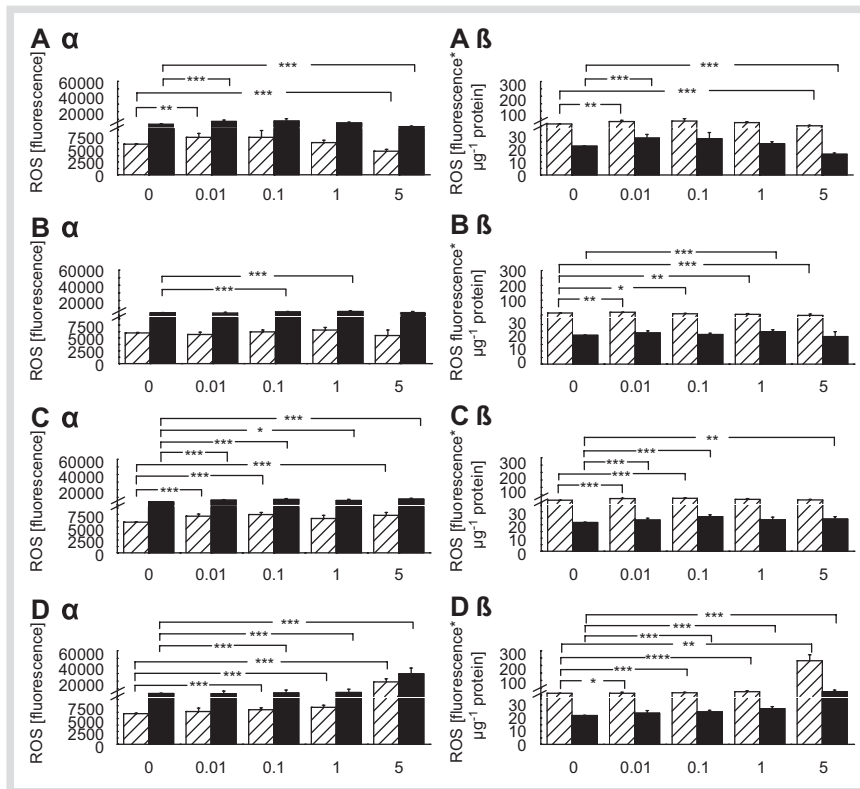


Fig. 5 ROS formation of myocytes following exposure to increasing concentrations of organic solvents. Results were either not normalized (absolute fluorescence) (α sub-panels), or normalized to the area under the protein curve between the respective time points (β sub-panels). For further legend, see **Fig. 1**.

results were obtained for ethanol and methanol after 24 hours of treatment (**Fig. 4C, D**), but the suppression of OCR at higher concentrations of alcohols was more pronounced in comparison to DMSO (**Fig. 4A**). Opposite to short-term observations, 24-hour incubation with NMP influences OCR most. At concentrations of up to 0.1%, NMP induces a slight increase of cellular respiration, but a more pronounced enhancement of OCR was observed on using 1% of NMP (**Fig. 4D**). Taken together, DMSO and alcohols cause a long-term increase of OCR at lower concentrations, while short-term treatment suppresses OCR independent of concentration. Whether this is, at least in part, caused by alcohols serving as a metabolic substrate remains to be evaluated. Accordingly, ROS formation was quantified as described above in the presence of organic solvents, namely DMSO, ethanol, methanol, and NMP at concentrations of 0.01, 0.1, 1.0, and 5.0%. Because of accumulation of ROS during the incubation period, dichlorofluorescein fluorescence intensity was normalized to the area under the protein curve (**Fig. 5 β**). This is contrasted by not normalized results (**Fig. 5 α**) yielding potentially erroneous effects of this cumulative parameter. DMSO at low (0.01%) and high (5%) concentrations had significant influence on ROS formation after both short time and long time incubation periods (**Fig. 5A β**). While 0.01% DMSO increases ROS formation, the highest DMSO concentration causes a significant reduction of ROS formation both after 4- and 24-hour treatment. Ethanol shows significant effects on ROS formation especially after short time treatment (**Fig. 5B β**). Ethanol at 0.01% increases ROS formation slightly whereas higher concentrations reduce myocyte ROS formation. Methanol causes a slight increase of ROS at concentrations of up to 0.1% after 4-hour treatment. Longer methanol incubation proves to be more effective, low concentrations (0.01 and 0.1% methanol) increase myocyte ROS formation, whereas 5% reduces intracellular ROS (**Fig. 5C β**). NMP was identified as the most effective solvent in regards to ROS induc-

tion (**Fig. 5D β**). All the concentrations evaluated increased ROS formation of myocytes after short incubation period; accordingly, 5% NMP causes a very high induction of ROS formation (351% enhancement compared to control cells). Similar effects were observed after a long time treatment, although induction of myocyte ROS formation by NMP was less pronounced. Of interest, only DMSO appears to be acceptable in regard to alterations of ROS formation in this assay system. Moreover, and since most publications do not normalize ROS formation, it should be noted that significant differences exist between absolute fluorescence intensity and intensities normalized to protein content (α -panels vs. β -panels). Next, $\Delta\Psi_m$ was quantified as described above in the presence of organic solvents, namely DMSO, ethanol, methanol, and NMP at concentrations of 0.01, 0.1, 1.0, and 5.0%. TMRM fluorescence intensity representing $\Delta\Psi_m$ was normalized to protein content at the end of the experiments (**Fig. 6 β**). This is contrasted by not normalized results (**Fig. 6 α**) yielding potentially erroneous effects. DMSO influences $\Delta\Psi_m$ especially after 24-hour treatment (**Fig. 6A β**); concentrations higher than 0.1% decrease $\Delta\Psi_m$ in a concentration dependent manner. Short time DMSO incubation has no significant effect on $\Delta\Psi_m$ with exception of the highest concentration used; 5% DMSO reduces $\Delta\Psi_m$. Ethanol has more pronounced effects on $\Delta\Psi_m$ both after short and long time incubation (**Fig. 6B β**). A 1.7- to 2.1-fold increase of $\Delta\Psi_m$ was observed up to a concentration of 0.1% ethanol after 4 and 24 hours, respectively. Higher concentrations were less effective. Strongest influence on $\Delta\Psi_m$ was observed after methanol treatment for 4 hours (**Fig. 6C β**). All except for the lowest concentrations of methanol used increased $\Delta\Psi_m$. Long time methanol treatment was less effective, but enhancement of $\Delta\Psi_m$ for concentrations of 0.01 to 1% methanol was observed. Since $\Delta\Psi_m$ is the driving force for ATP generation, it should be noted that a good correlation exists between ATP content

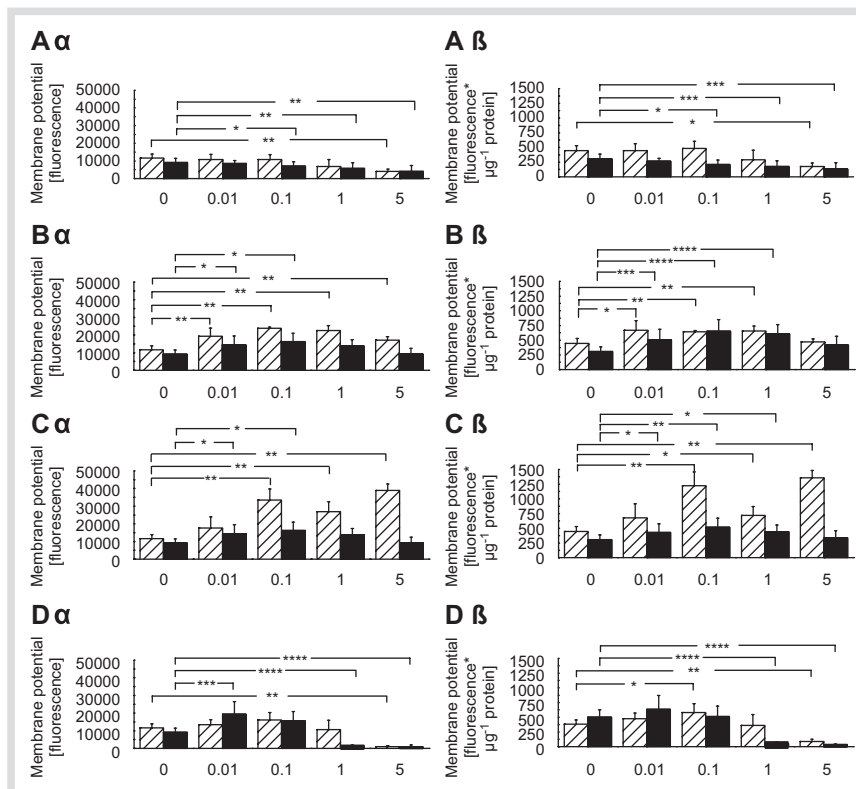


Fig. 6 $\Delta\Psi_m$ of myocytes following exposure to increasing concentrations of organic solvents. Results were either not normalized (absolute fluorescence) (α sub-panels), or normalized to protein content at the end of experiment (β sub-panels). For further legend, see **Fig. 1**.

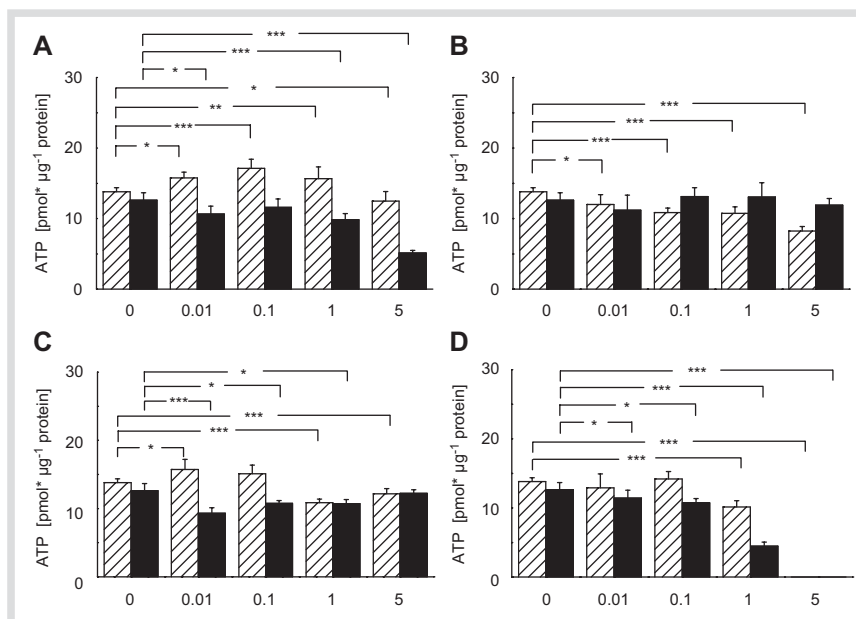


Fig. 7 ATP formation of myoblasts following exposure to increasing concentrations of organic solvents. For further legend, see **Fig. 1**.

(see below, **Fig. 7**) and $\Delta\Psi_m$ for DMSO and ethanol-treated cells. Especially in the case of methanol $\Delta\Psi_m$ increases while ATP content decreases, suggesting mitochondrial uncoupling due to methanol addition.

Lastly, ATP content was quantified as described above in the presence of organic solvents, namely DMSO, ethanol, methanol, and NMP at concentrations of 0.01, 0.1, 1.0, and 5.0%. ATP of myocytes was normalized to protein content (**Fig. 7**). In contrast to 24-hour treatment, DMSO induces a significant enhancement of myocyte ATP content after short incubation period by using lower concentrations (0.01–1%) (**Fig. 7A**). The highest

DMSO concentration tested reduces ATP concentration significantly after 4-hour treatment as well as after 24 hours. A significant concentration dependent decrease of ATP formation was observed after 4-hour ethanol treatment (**Fig. 7B**). Longer ethanol incubation has no effect on myocyte ATP formation. Short time methanol treatment cause a significant increase about $18.2 \pm 6.1\%$ on using 0.01% of this solvent, whereas higher concentrations (1 and 5%) reduce myocyte ATP formation slightly (**Fig. 7C**). The lowest methanol concentration tested shows strongest effects on ATP production following long time treatment ($26 \pm 6.2\%$ reduction compared to control), whereas

Table 1 Summary of effects of different organic solvents on nonoxidative glucose metabolism and oxidative pyruvate metabolism

	Protein content	D-Glucose uptake	L-lactic acid formation	Oxygen consumption	ROS	$\Delta\Psi_m$	ATP
DMSO	0	+	+	+++	+	+	+++
ethanol	++	0	+	+++	+++	+++	++
methanol	+	+	+	+++	+++	++++	+++
NMP	++	++	+++	++++	+++	++	++++

0 no, + weak, ++ intermediate, +++ strong, ++++ very strong influence

5% methanol has no effect on ATP content after 24-hour treatment (● Fig. 7C). NMP was observed to be the most detrimental organic solvent in regard to myocyte ATP formation, especially for long time treatment (● Fig. 7D). ATP content of myocytes was reduced in a concentration dependent manner by NMP, where the highest concentration of this solvent decreases ATP to undetectable concentrations after long time as well as short time incubation.

Discussion

Previous observations suggest that organic solvents might influence mitochondrial metabolism [6–8]. Our data clearly indicate that commonly used solvents, namely DMSO, ethanol, methanol, and NMP in some cases modulate, that is, either stimulate or suppress nonoxidative and oxidative metabolism depending on concentrations applied and/or time of exposure.

DMSO is a hygroscopic, dipolar compound most frequently employed as a solvent in various *in vivo* and *in vitro* experiments. It is effective as transport medium and it has also been shown to improve absorption of, for example, insulin [23]. Yoon and colleagues [24] have shown that DMSO affects the metabolism and hepatotoxicity of several xenobiotics. Our data indicate that DMSO alone only mildly modulates mitochondrial metabolism of L6 myocytes. Especially high DMSO concentrations (5% vol/vol) suppress oxidative metabolism, as reflected by reduced ATP content, $\Delta\Psi_m$, and OCR in this experimental setup. These findings are in agreement with Velasco et al. [7], who observed decreased oxygen uptake *in vitro* in rat brain slices and heart slices. Other investigators have documented a comparable decrease of $\Delta\Psi_m$ in murine erythroleukemia cells at 12 and 24 hours after exposure to DMSO [25]. Low concentrations of DMSO (0.01–1%) exhibit mild and time dependent effects on nonoxidative and oxidative glucose metabolism of myocytes. ATP content of myocytes was found elevated at 4 hours, whereas longer treatment decreases $\Delta\Psi_m$ and thus reduces the energy store of myocytes significantly. It remains to be evaluated why oxygen uptake appears to be increased at the same time. Interestingly ROS formation increases significantly using DMSO at concentrations of 1% and below, although DMSO has been suggested to function as a hydroxyl radical scavenger [26]. Taken together, DMSO seems to exert toxic effects on mitochondria at high concentrations only without affecting cell growth, since protein content of myocyte was not altered.

Ethanol, the second organic solvent tested, is known as a potentially neurotoxic and/or teratogenic agent. In cultured fetal rat hepatocytes ethanol inhibits activity of NADH coenzyme Q reductase (complex I) and cytochrome c (complex IV) of respiratory chain in fetal rat hepatocytes [27] suggesting a direct influence on mitochondrial function. In our study, short time ethanol

treatment suppresses mitochondrial metabolism as recognized in a concentration-dependent reduction of OCR and ATP content of L6 myocytes, while nonoxidative metabolism remained widely unaffected. This is probably due to the described inhibitory effect of ethanol on complex I and complex IV of respiratory chain potentially causing a reduction in net ATP synthesis [27]. In cultured fetal rat hepatocytes ethanol was reported to induce oxidative stress via increase in cellular levels of ROS due to decreased levels of reduced glutathione (GSH) and hence antioxidative defense [28]. In this study, L6 myocytes showed elevated ROS levels when exposed to ethanol, especially for longer periods of time.

Next, possible effects of methanol on oxidative and nonoxidative metabolism were assayed. Methanol affects nonoxidative metabolism only at high concentrations (i.e., 5%) as seen by the significant reduction of lactic acid formation after 24 hours of treatment. Nevertheless, pronounced effects of this solvent were seen in regard to changes in oxidative metabolism. Specifically in comparison to DMSO and ethanol, methanol caused more pronounced changes in oxidative metabolism of L6 myocytes. Moreover, a remarkable increase of $\Delta\Psi_m$ in a concentration dependent manner especially at the 4-hour treatment was observed. This was dissipated from ATP synthesis strongly suggesting that methanol might inhibit ATP synthase. Accordingly, ROS formation in L6 myocytes was found to be increased at states of both 4 and 24 hours of methanol treatment. Furthermore, formic acid, the main metabolite of methanol, has been shown to act as an inhibitor of electron transport chain [29] and thus may also cause a reduction of ATP synthesis.

Lastly, the effects of NMP on oxidative and nonoxidative metabolism were analyzed. NMP is widely used as a vehicle for drugs or to facilitate their percutaneous absorption. Its acute toxicity *in vivo* is reported to be low [30]. Our results demonstrate a clear decrease in protein content as well as ATP and $\Delta\Psi_m$ of L6 myocytes on using NMP at concentrations greater than 1%, and these effects are more pronounced compared to other organic solvents tested for unknown reasons. Saillenfait et al. [31] reported developmental, maternal, and reproductive toxicity of NMP to rats while Lan and colleagues [32] even observed NMP as the most cytotoxic organic solvent to *Daphnia magna*. Cytotoxicity of NMP can be potentially explained by the induction of oxidative stress noticed as the remarkable increase of ROS 4 and 24 hours after NMP addition. On the other hand, NMP at concentrations higher than 1% decrease $\Delta\Psi_m$ and ATP content of myocytes, while OCR normalized to protein was unaltered, respectively, increased at 24 hours. Possibly, NMP uncouples OXPHOS from ATP synthesis and this is causing energy depletion of myocytes and subsequently stimulates glucose-uptake of myocytes to compensate the fall in ATP. NMP concentrations of up to 1% exhibit only weak effects on myocyte oxidative and nonoxidative metabolism with exception of OCR at short time exposition.

In summary, while all four organic solvents evaluated induce significant changes in regard to glucose uptake, glycolysis, mitochondrial metabolism and oxidative phosphorylation, DMSO and possibly ethanol (**Table 1**) might be considered the preferred option for quantification of nonoxidative and oxidative glucose metabolism in cultured myocytes. This study was initiated to find the ideal solvent for nonaqueous soluble substances. While experiments are usually carried out by comparing effects of dissolved substances with a solvent-treated control, it cannot be excluded that solvents *per se* prevent effects normally seen in biological systems. Especially for OCR and ATP formation, other solvents than those tested in this study may be desirable.

Acknowledgments

The excellent technical assistance of Beate Laube and Waltraut Scheiding is gratefully acknowledged. This work was supported in part by a grant from the German Bundesministerium für Wirtschaft und Technologie (Federal Ministry of Economy and Technology) (BMW grant no. IW051205).

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