FEBS Letters 578 (2004) 224–228 FEBS 29022

Mitochondrial activation and the pyruvate paradox in a human cell line

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Received 13 October 2004; accepted 25 October 2004

Available online 16 November 2004

Edited by Vladimir Skulachev

Abstract Pyruvate promotes hyperpolarization of the inner mitochondrial membrane. However, in isolated mitochondria, pyruvate could participate in a futile cycle leading to mitochondrial depolarization. Here, we investigated this paradox in intact human cells by measuring parameters reflecting mitochondrial activation in response to 1 mM pyruvate and 5 mM glucose. NAD(P)H levels were elevated similarly by both substrates. Conversely, pyruvate induced a first transient phase of mitochondrial depolarization before the establishment of the expected sustained hyperpolarization. This correlated with kinetics of cytosolic ATP levels exhibiting a first phase decrease followed by an increase. Therefore, pyruvate transiently depolarizes mitochondria and reduces ATP in intact cells.

Keywords: Pyruvate; Mitochondrial membrane potential; Cytosolic ATP; UCP2

1. Introduction

Mitochondria play essential physiological roles in cells and principally produce the bulk part of cellular ATP through oxidative metabolism. Under aerobic conditions, the majority of ATP used to maintain cellular homeostasis results from oxidation of the glycolytic product pyruvate. Pyruvate transport into mitochondria is also important for fatty acid synthesis, metabolism of some amino acids, and anaplerosis on the whole [1]. Additional roles of pyruvate include its antioxidant effect and cardioprotective activity, illustrated by reversal of the mitochondrial permeability transition [2]. Pyruvate, like other monocarboxylates, is efficiently transported through the plasma membrane via a proton-linked mechanism mediated by monocarboxylate transporters (MCT); see review [3]. On the other hand, transport into the mitochondria is still poorly characterized [4]. Recently, mitochondrial pyruvate carrier (MPC) was identified in *Saccharomyces cerevisiae* as a 41.9 kDa protein belonging to the mitochondrial carrier family [5]. However, the corresponding MPC has not been identified so far in mammalian mitochondria [6]. Pyruvate is not only a mitochondrial substrate for pyruvate dehydrogenase but also for citric acid cycle anaplerosis. Moreover, pyruvate was shown in isolated mitochondria to participate in a futile cycle, resulting in mitochondrial uncoupling and concomitant decrease in electrochemical proton gradient [7]. In this model, cytosolic pyruvate anion would be taken up by the pyruvate carrier together with a proton into mitochondrial matrix. Then, uncoupling protein (UCP), such as UCP1 expressed in brown adipose tissue [8], would pump pyruvate anion back to the cytosol towards the positive mitochondrial proton gradient, thereby completing the futile cycle [7]. Therefore, each pyruvate cycle results in the transfer of one proton from the cytosol to the mitochondria, thereby reducing mitochondrial membrane potential. Other members of the mitochondrial carrier family include UCP2, expressed in human and rodent tissues such as spleen, lung, intestine, white adipose tissue and immune cells [9], and UCP3, expressed mainly in skeletal muscles [8].

Mitochondrial pyruvate metabolism through dehydrogenase activity primarily leads to $NADH + H^+$ generation. NADH is the main carrier of reducing equivalents in mitochondria and can be reoxidized by complex I of the electron transport chain. This results in proton pumping out of the mitochondrial matrix and hyperpolarization of the mitochondrial membrane. The ultimate task of the respiratory chain is then to form ATP at the expense of the established proton gradient.

Extrapolated from studies on isolated mitochondria, pyruvate could both depolarize and hyperpolarize the mitochondrial membrane according to mechanisms of transfer and metabolism, respectively. In order to clarify the consequences of such dual effects on mitochondrial activation in intact cells, we measured NAD(P)H levels, mitochondrial membrane potential, and ATP levels in the human osteosarcoma cell line 143B. To our knowledge, it is not known which member of the uncoupling protein family is expressed in 143B cells. Therefore, we analyzed by RT-PCR whether UCP2 or UCP3 is expressed in 143B cells. Pyruvate stimulations, compared to glucose responses, revealed paradoxical effects of transient depolarization of the mitochondrial membrane, accompanied by a drop in cytosolic ATP levels, preceding metabolic activation.

2. Materials and methods

2.1. Cell culture

The human osteorsarcoma cell line 143B.TK$^-$ (143B) was cultured in a humidified atmosphere containing 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and antibiotics. For all experiments, cells were seeded in 24-well plates and cultured 3–4 days before use.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCCP carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; KRBH, Krebs–Ringer bicarbonate HEPES; MCT, monocarboxylate transporters; MPC, mitochondrial pyruvate carrier; UCP2, uncoupling protein 2; 143B, human osteorsarcoma cell line 143B.TK$^-$

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.10.088

2.2. Fluorescence and luminescence measurements

Metabolic parameters were measured using either fluorescence or bioluminescence. The 24-well plates were transferred in a thermostated (37 °C) plate-reader set either in the fluorescence or luminescence mode (Fluostar Optima, BMG Labtechnologies, Offenburg, Germany). Individual wells of 24-well plate were recorded sequentially over 20 or 30 min periods and test compounds were added at the indicated times through automated built-in microinjectors.

2.3. NAD(P)H

NAD(P)H autofluorescence was monitored in Krebs–Ringer bicarbonate Hepes buffer (KRBH buffer; composition in mM: 135 NaCl, 3.6 KCl, 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, and 10 HEPES, pH 7.4) with excitation and emission filters set at 340 and 460 nm, respectively [10]. After stabilization of the signal for 10 min in KRBH, 5 mM glucose or 1 mM pyruvate was administered as indicated. NAD(P)H autofluorescence was normalized to pre-stimulation period by setting the fluorescence as 100%. Control of maximal fluorescence changes was performed after the addition of 5 μM rotenone plus 2 μM antimycin-A, added as inhibitors of the electron transport chain at complexes I and III, respectively.

2.4. Mitochondrial membrane potential ($\Delta\psi_m$) measurements

Mitochondrial membrane potential was measured following cell preincubation in glucose-free medium for 2 h, essentially as described previously [10,11]. The cells were then incubated in glucose-free KRBH containing 10 μg/ml of rhodamine-123 (Molecular Probes, Eugene, OR) for 20 min and washed once with KRBH before starting the experiment in KRBH. The $\Delta\Psi_m$ was monitored with excitation and emission filters set at 485 and 520 nm, respectively [10]. Glucose, pyruvate, and then the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were added to each well at the indicated times. In the present study, the term hyperpolarization of the inner mitochondrial membrane refers to as mitochondrial state 3 respiration, as phosphorylation occurs.

2.5. Cytosolic ATP levels in living 143B cells

Cytosolic ATP levels were monitored in cells expressing the ATP-sensitive bioluminescent probe luciferase, 1 day after transduction with the specific AdCAG-Luc viral construct [12,13]. Following the preincubation steps as described above, the 24-well plates were transferred in the plate-reader in the luminometer mode. As a substrate for luciferase, KRBH was supplemented with 500 μM beetle luciferin (Promega, Madison, WI). Following a 5-min period in glucose-free KRBH, cells were stimulated with the indicated pyruvate or glucose concentrations and 2 mM NaN_3 was added at the end as a mitochondrial poison.

2.6. UCPs expression analysis in 143B cells by reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from 143B cells using TRIzol® reagent (Invitrogen AG, Basel, Switzerland). Contaminating genomic DNA was discarded by DNase treatment (Qiagen AG, Hombrechtikon, Switzerland) before RNA re-extraction by acid phenol:chloroform and ethanol precipitation. Total RNA (2 μg) was reverse-transcribed to cDNA using SuperScript™ reverse transcriptase and random primers (Invitrogen AG). Primers used to amplify UCP2 and UCP3 were designed based on GenBank sequences (UCP2: HSU76367; UCP3 shorter form: NM_022803, UCP3 longer form: NM_003356) and synthesized by Microsynth (Balgach, Switzerland). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control housekeeping gene. GAPDH primers were designed based on GenBank sequence (BC023632). Negative controls were conducted using primers added to RNA reverse transcription (-RT). PCR amplification conditions were the following: initial denaturation (2 min at 94 °C) followed by 40 cycles of 1 min at 94 °C, 1 min at 53 °C, and 1 min at 72 °C with a final extension for 7 min at 72 °C. PCR products were run in a 2% (w/v) agarose gel containing ethidium bromide and a 50 bp step ladder used as a marker. The RT-PCR was performed with three independent RNA preparations.

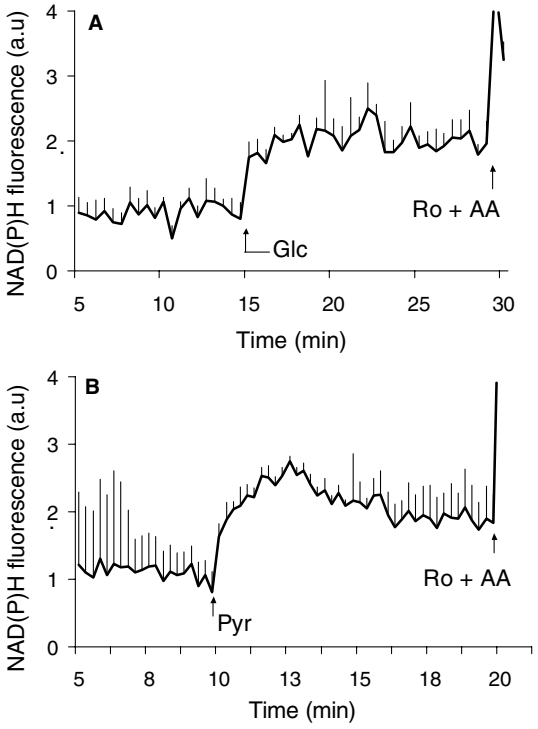

Fig. 1. Effects of glucose or pyruvate on NAD(P)H in 143B cells. (A) Cells were stimulated at the indicated time with 5 mM glucose (Glc) and complexes I and III of the electron transport chain were blocked 15 min later by the addition of 5 μM rotenone plus 2 μM antimycin-A (Ro + AA). (B) Cells were stimulated with 1 mM pyruvate (Pyr) followed by Ro + AA treatment. Values are means + S.E. of five independent experiments.

3. Results

3.1. NAD(P)H

Glucose (5 mM) stimulation evoked rapid and sustained elevations of NAD(P)H relative levels (Fig. 1A). Additive to glucose stimulation, blockage of complexes I and III of the electron transport chain by rotenone/antimycin-A further increased NAD(P)H levels, thereby revealing the mitochondrial origin of the signal. When the cells were stimulated with 1 mM pyruvate (Fig. 1B), progressive increases in NAD(P)H levels were observed, reaching a peak after 2–3 min. Subsequently, there was a slow decay in NAD(P)H signal before the establishment of a plateau phase 5–6 min after pyruvate addition. Amplitudes and kinetics of the responses to glucose and pyruvate were similar. These results indicate that mitochondrial metabolism was the major contributor of NADH generation.

3.2. Mitochondrial membrane potential in 143B cells

Stimulation of 143B cells with 5 mM glucose resulted in hyperpolarization of the mitochondrial membrane (Fig. 2A). This was revealed by deflection of the rhodamine-123 signal 20–25% below basal values recorded in the absence of glucose. Added subsequently, the protonophore FCCP (1 μM) rapidly dissipated the electrochemical proton gradient established under state 3 respiration conditions. The $\Delta\Psi_m$ was saturated at 5 mM glucose, since higher concentrations did not produce further hyperpolarizations (not shown). Remarkably, exposure to

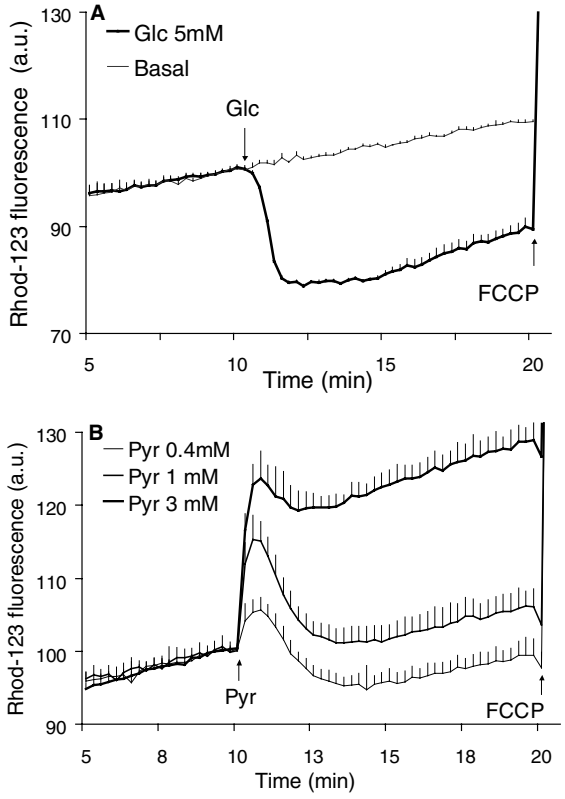

Fig. 2. Mitochondrial membrane potential ($\Delta\Psi_m$) in 143B cells. (A) Cells were either kept in glucose free KRBH (Basal) or stimulated with 5 mM glucose (Glc). Ten minutes after stimulation, $\Delta\Psi_m$ was collapsed by the addition of 1 μM FCCP. (B) Pyruvate (Pyr) at different concentrations (0.4, 1 and 3 mM) was added 10 min before FCCP. Values are means + S.E. of five (A) and one out of five (B) independent experiments.

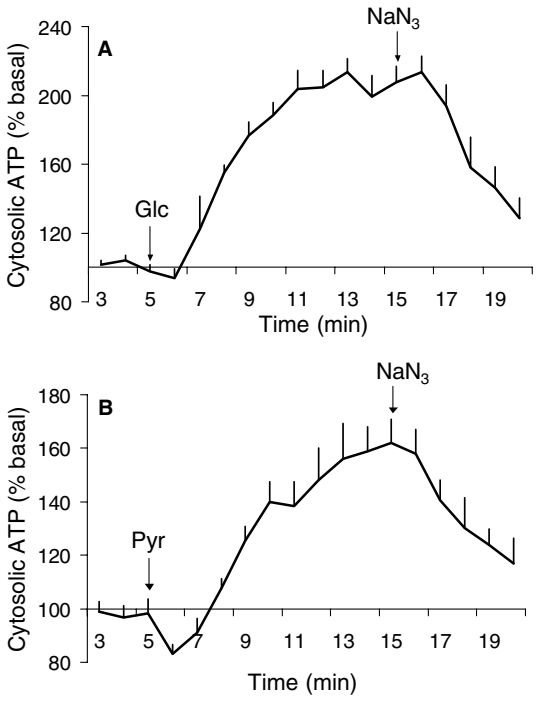

Fig. 3. Cytosolic ATP levels of 143B cells. Bioluminescence was monitored from 143B cells expressing luciferase. (A) Cells were stimulated with 5 mM glucose (Glc) and 10 min later 2 mM NaN_3 was added as an inhibitor of the respiratory chain. (B) Stimulation with 1 mM pyruvate (Pyr) followed by 2 mM NaN_3 addition 10 min later. Values are means + SE of four independent experiments.

pyruvate (Fig. 2B) induced a first transient depolarization phase of the mitochondrial membrane lasting 45–60 s. The amplitudes of pyruvate-evoked depolarization were dose dependent with signal rises of 5%, 15%, and 22% above baseline at 0.4, 1, and 3 mM pyruvate, respectively. These paradoxical depolarization phases were followed by the expected sustained hyperpolarization responses. The rate of hyperpolarization evoked by 1 mM pyruvate was about half the rate of glucose-induced hyperpolarization. As a control, FCCP dissipated the electrochemical proton gradient. Hyperpolarization was maximal already at 1 mM pyruvate and drastically reduced upon 3 mM pyruvate exposure. Therefore, at 3 mM pyruvate, activation of the electron transport chain could not efficiently compensate depolarization.

3.3. Cytosolic ATP levels

Cytosolic ATP levels were monitored in 143B cells expressing luciferase. Cells responded to 5 mM glucose stimulation with a sustained 100% elevation of cytosolic ATP levels (Fig. 3A). Subsequent blockage of complex IV by the addition of 2 mM NaN_3 resulted in a drop in ATP levels. When the cells were exposed to 1 mM pyruvate (Fig. 3B), a transient decrease in cytosolic ATP levels during the first min was observed (-11%, $P < 0.05$). This was followed by sustained rise in ATP levels up to 62% above basal levels after 9–10 min pyruvate stimulation. Again, NaN_3 addition abolished subsequent mitochondrial ATP production. Exposure to 1 μM FCCP abrogated both transient drop and successive elevation of ATP evoked by pyruvate (not shown).

3.4. UCPs expression in 143B cells

We analyzed the putative expression of the two UCPs expressed in non-brown adipose tissues, namely UCP2 and UCP3. Agarose gel of RT-PCR products revealed that UCP2 is expressed in the human osteosarcoma 143B cell line (Fig. 4), as demonstrated by the amplification of a corresponding 190 bp fragment (lane UCP2). This positive band did not result from genomic DNA contamination, as evidenced by negative PCR using UCP2 primers in the absence of RT. UCP3 expression was not detected in 143B cells.

4. Discussion

It is commonly expected that both glucose and pyruvate stimulations lead to hyperpolarization of the inner mitochondrial membrane due to generation of electrochemical proton gradient. However, we observed that pyruvate induced a first transient phase of mitochondrial depolarization before the establishment of the expected sustained state 3 hyperpolarization. It has been previously reported that pyruvate exerts uncoupling activity in isolated mitochondria [7,14]. Our study demonstrates such phenomenon in intact human cells and its consequences on cytosolic ATP. As pyruvate is not able to cross the mitochondrial membrane due to its negative charge, it has to be taken up with a proton (symport) by a monocar-

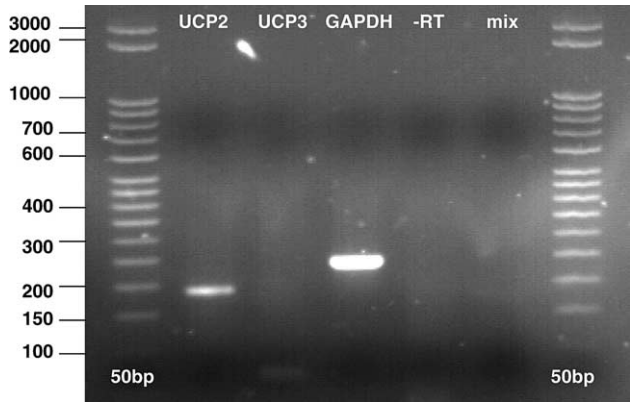

Fig. 4. UCPs expression in 143B cells. RT-PCR products using primers for UCP2, UCP3, and GAPDH were analyzed on agarose gel. RNA derived cDNA with UCP2 primers (lane *UCP2*), UCP3 primers (lane *UCP3*), and GAPDH primers (lane *GAPDH*). RNA not reverse transcribed with UCP2 primers (lane-*RT*) and RT mix solution without primers (lane *mix*).

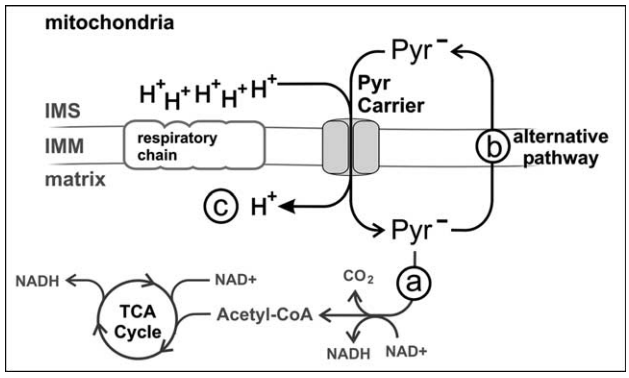

Fig. 5. Model for mitochondrial pyruvate cycle (adapted from [7]): as pyruvate is negatively charged and not able to cross the mitochondrial membrane, it is either taken up with a proton by a pyruvate carrier (symport) or exchanged by a hydroxide anion (antiport). Once pyruvate enters the mitochondrial matrix, it could follow the oxidative pathway (a). Alternatively, pyruvate could be driven through an alternative pathway, being pumped back into the intermembrane space (b), possibly through UCP2. Therefore, the complete cycle would promote the transient depolarization of the inner mitochondrial membrane (c).

boxylate transporter, such as MPC [5], or exchanged for a hydroxide (antiport). This would cause transient depolarization of the mitochondrial membrane as shown in this study. Consequently, oxidative phosphorylation might be transiently uncoupled when the electrochemical proton gradient across the inner mitochondrial membrane is utilized to drive solute transport, e.g., pyruvate.

Pyruvate stimulation evoked a first phase of lowered cytosolic ATP levels, lasting for approximately 1 min. This should be correlated with the observed first phase depolarization of the mitochondrial membrane. Kinetic comparisons revealed that concomitant with second phase state 3 hyperpolarization, ATP levels increased up to 62% above basal levels. As expected, ATP production upon glucose stimulation was higher (~50%) than in response to pyruvate, the latter bypassing glycolysis. The difference between glucose and pyruvate induced ATP generation was greater than the only theoretical cytosolic contribution, indicating important leak of glucose derived lactate. Glucose stimulation neither induced first phase mitochondrial membrane depolarization nor significant initial ATP level drop. This could be explained by the actual intracellular concentrations of glucose-derived pyruvate, probably dynamically lower than the one imposed by direct pyruvate addition.

NADH produced in the cytosol cannot be directly transferred to the mitochondria and, therefore, shuttle systems transport reducing equivalents carried by NADH + H^+ into the mitochondrial matrix [15]. The involvement of these shuttles might account for the immediate build up of NAD(P)H levels upon glucose stimulation compared to delayed pyruvate response. However, the predominant contribution of NADH signal was of mitochondrial origin as indicated by responses to inhibitors of the electron transport chain. Interestingly, there was no initial decrease of NAD(P)H levels upon pyruvate stimulation. This supports the concept that pyruvate-induced depolarization directly implicated the inner mitochondrial membrane rather than pyruvate catabolism.

Pyruvate can be oxidized in mitochondria, leading to citric acid cycle feeding, generation of reducing equivalents, and activation of the electron transport chain resulting in hyperpolarization of the inner mitochondrial membrane. However, pyruvate can also be transported by UCP1 [7], the latter acting as an anion uniporter and mild mitochondrial uncoupler. Taken as a whole, pyruvate is primarily transported into the mitochondria by specific carrier (e.g., MPC). Then, the pyruvate fraction that is not oxidized might leave the mitochondrial matrix via UCP1, thereby completing a futile cycle. These events would account for the paradoxical effect reported here, i.e., pyruvate-induced mitochondrial depolarization. We have identified here UCP2 expression in the human osteosarcoma cell line 143B. Possibly, UCP2 might participate in the pyruvate transport across the inner mitochondrial membrane, acting as an alternative pathway for non-oxidized pyruvate (Fig. 5). However, further studies should be conducted to investigate putative UCP2 contribution to pyruvate-induced transient mitochondrial depolarization.

We conclude that the electroneutral pyruvate uptake by the mitochondria accounts for a paradoxical transient depolarization of the inner mitochondrial membrane, associated with a drop in cytosolic ATP.

Acknowledgments: We are grateful to Blanca Rubi for discussions and to Guilherme A. Marson for illustrations. This study was supported by the Leenaards Foundation through a collaborative grant with Urs Ruegg (Lausanne). Supports from the Swiss National Science Foundation and the Max Cloetta Foundation are also acknowledged. The project was part of the Geneva Program for Metabolic Disorders.

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