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Screening of protein kinase inhibitors and knockdown experiments identified four kinases that affect mitochondrial ATP synthesis activity

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ABSTRACT

Mitochondrial ATP synthase, a major ATP supplier in respiring cells, should be regulated in amount and in activity to respond to the varying demands of cells for ATP. We screened 80 protein kinase inhibitors and found that HeLa cells treated with four inhibitors exhibited reduced mitochondrial ATP synthesis activity. Consistently, knockdown of their target kinases (PKA, PKCδ, CaMKII and smMLCK) resulted in a decrease in mitochondrial ATP synthesis activity. Among them, mitochondria of smMLCK-knockdown cells contained only a small amount of ATP synthase, while the α- and β-subunits of ATP synthase were produced normally, suggesting that smMLCK affects assembly (or decay) of ATP synthase.

1. Introduction

In mammalian cells under aerobic conditions, mitochondrial ATP synthases (often called F_oF_1-ATP synthases) produce most of the cellular ATP in a process driven by a proton motive force, which is built up by respiratory complexes. The amount and activity of ATP synthase in cells must be regulated in response to the cells' changing energy state, but the framework of this regulatory system is largely unknown. Recently, the involvement of protein kinase in the regulation of mitochondrial oxidative phosphorylation has been recognized. One of the subunits of respiratory complex I is phosphorylated by protein kinase A (PKA) and is recruited to mitochondria [1,2]. The δ-subunit of ATP synthase is phosphorylated by platelet-derived growth factor stimulation [3]. In this study, by adopting a newly developed high-throughput microplate assay for mitochondrial ATP synthesis [4], we have screened a library of 80 compounds that have been known to inhibit protein kinases, and knocked-down the selected inhibitor-target protein kinases. The results suggest that smooth muscle type myosin light chain kinase (smMLCK) helps to maintain the population of ATP synthase in mitochondria and hence the ATP synthesis activity. The involvement of PKA, protein kinase Cδ, (PKCδ), and calcium/calmodulin-dependent protein kinase 2 (CaMKII) in regulation of ATP synthesis activity is also indicated.

2. Materials and methods

2.1. Cell culture and materials

HeLa cells were purchased from the Health Science Research Resources Bank (JCRB9004). They were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical) supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin. The kinase inhibitor library was obtained from Enzo Life Sciences International (#2832A). Antibodies against ATP5A (A21350) and ATP5B (A21351) were purchased from Molecular Probes; the antibody for the voltage-dependent anion channel (VDAC) was from Abcam (ab16816); anti-smMLCK antibody was from SIGMA–ALDRICH (M7905); and anti-PKA antibody was from Cell Signaling (#4782). The HRP-linked anti-mouse and anti-rabbit antibodies were from GE Healthcare (NA931 and NA934). FuGENE HD transfection reagent was from Roche Applied Science. Glass bottom 35-mm dishes were from IWAKI.

Abbreviations: PKC, protein kinase C; PKA, protein kinase A; CaMKII, calcium/calmodulin-dependent protein kinase 2; miRNA, microRNA used for knockdown; smMLCK, smooth muscle type myosin light chain kinase; MASC assay, mitochondrial activity of SLO-permeabilized cells assay; VDAC, voltage-dependent anion channel of mitochondria

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2.2. ATP synthesis activity, viability and membrane potential

HeLa cells were passaged on 96-well microplates at 5000 cells/well and cultivated for 6 h. The culture medium was changed to Dulbecco's modified Eagle's medium containing an inhibitor (staurosporin at 100 nM and others at 10 μM) and, after an 18 h incubation, living cells were counted by MTT assay (Cell Counting Kit-8; Dojindo) and mitochondrial ATP synthesis was measured by MASC (Mitochondrial Activity of Streptolysin O-permeabilized Cells) assay [4]. The MASC assay is a newly developed method for high-throughput screening of mitochondrial ATP synthesis. Briefly, the cytoplasmic membranes (but not mitochondrial membranes) of cells passaged on a 96-well white microplate were permeabilized by streptolysin O, and their cytosolic components were washed out. Luciferase, luciferin, 0.1 mM ADP and 5 mM Pi were added and the reaction was started by addition of 10 mM succinic acid. When the direct effect of the inhibitors on mitochondria was examined, the inhibitor was not included in the culture medium but added at the time of the start of MASC assay (addition of succinic acid). Luciferin emission was monitored by a luminometer (Berthold Tech.). After the measurement, cells were washed twice, the proteins in each well were solubilized by sodium dodecylsulfate, and the amount of proteins was measured. The ATP synthesis rate per unit protein amount was compared. To assess mitochondrial membrane potential, HeLa cells were passaged at 7.7×10^4 cells per 35 mm glass-bottom dish and incubated with the inhibitors described above. The dishes were set in a microchamber on a fluorescent microscope at 37 °C under 5% CO_2 in air, and tetramethylrhodamine ethylester (TMRE) was added at 50 nM (final concentration). After a 10 min incubation, the potential-dependent mitochondrial accumulation of TMRE was observed by fluorescence of TMRE.

2.3. MicroRNA construction and transfection

The microRNA (miRNA or miR) sequences were designed using an RNAi designer on-line tool (Invitrogen). Two different double-stranded oligo duplexes encoding the desired miRNA target sequences for PKCδ, α subunit of PKA, α isoform of CaMKII, and smMLCK were selected and cloned into pcDNA™6.2-GW/EmGFP-miR expression vector using a BLOCK-iT™ pol II miR RNAi Expression Vector Kit (Invitrogen). The miRNA target sequences were as follows: for PKCδ_miR-1, 5′-CCGCTTCAAGGTTCACAACTA-3′; PKCδ_miR-2, 5′-TGCCTTCGGAGGGAAATTGTA-3′; PKA_miR-1, 5′-GCCCACTTGGATCAGTTTGAA-3′; PKA_miR-2, 5′-TCTTTCCTCCC AGCAGCGTTT-3′; CaMKII_miR-1, 5′-AGTATGCTGCCAAGATCATCA-3′; CaMKII_miR-2, 5′-AAGGCCCAGTGATCCCATTAT-3′; smMLCK_miR-1, 5′-AATGCTGTGAGAGCCATTGGA-3′; and smMLCK_miR-2, 5′-CTATGTTAGTTGGCACCTTAA-3′. The sequence of the negative control without 5′ overhangs was as follows: 5′-GAAATGTACTG CGCGTGGAGACGTTTTGGCCACTGACGTCTCCACGCAGTACATTT-3′.

HeLa cells were transfected with miRNAs targeted to PKCδ, PKA, CaMKII or smMLCK using lipofection. After 96 h, ATP synthesis activities were measured by MASC assay, and cells were collected for immuno-staining electrophoresis and quantitative PCR.

2.4. Quantitative PCR

The mRNAs for PKCδ, CaMKII, and glyceroaldehyde three phosphate dehydrogenase (internal calibration control) were quantified by PCR. The total RNA was extracted with an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare), and cDNAs were synthesized with PrimeScript II reverse transcriptase using oligo(dT) primer (TAKARA). PCR was performed on a StepOnePlus™ instrument (Applied Biosystems) with SYBR Green Master Mix (Applied Biosystems) using the following primers: for PKCδ, forward 5′-ATGAGCCCCACCTTCTGTGA-3′ and reverse 5′-TTCTCCCGGCATT-TATGGTG-3′; CaMKII, forward 5′-CCTGCATGCACAGACAGGAG-3′ and reverse 5′-CCTCCGGAGAAGTTCCTGGT-3′; and glyceroaldehyde three phosphate dehydrogenase, forward 5′-GCTCTCCAGAA-CATCATCCC-3′ and reverse 5′-TTTCTAGACGGCAGGTCAGG-3′. The mRNA expressions were calculated according to the $\Delta\Delta C_t$ method.

2.5. Retroviral gene transduction

An miRNA-expressing retroviral vector was used for stable gene knockdown. The miRNA target sequences of smMLCK were cloned into pMX retroviral vector. Retroviruses were prepared and used to infect the target cells as described previously [4,5].

2.6. Electrophoresis

Clear Native PAGE was performed to analyze the amount of ATP synthase as described previously [6]. Briefly, whole cells (5 μg protein) were suspended with solubilization buffer (50 mM NaCl, 50 mM imidazole–HCl, pH 7.0, 2 mM 6-aminohexanoic acid,

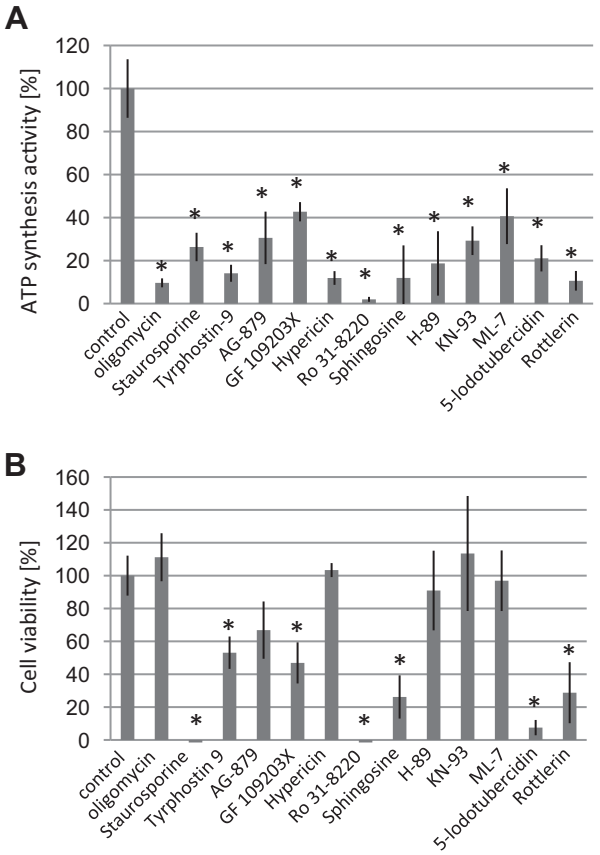

Fig. 1. (A) Mitochondrial ATP synthesis activities of HeLa cells treated with inhibitors of protein kinases. Cells were treated with each of the various inhibitors for 18 h and mitochondrial ATP synthesis activity was measured by MASC assay. The rate of ATP synthesis was compared with that of the control cells (treated with dimethysulfoxide) and only the results of the effective inhibitors are shown ($n = 4$, mean ± S.D., $^*P < 0.05$). Oligomycin is a specific inhibitor of ATP synthase. Targets of the inhibitors were as follows: staurosporine, pan-specific; tyrphostin-9, protein-tyrosine kinase activity of the PDGF receptor; AG-879, protein-tyrosine kinase activity of the TrkA; GF109203X, hypericin, Ro 31-8220 and sphingosine, protein kinase C (PKC); H-89, protein kinase A (PKA); KN-93, calcium/calmodulin-dependent protein kinase II (CaMKII); ML-7, myosin light chain kinase (MLCK); 5-Iodotubercidin, mitogen-activated protein kinase, adenosine kinase, casein kinases I and II, and insulin receptor kinase; Rottlerin, protein kinase Cδ (PKCδ). (B) Viability of HeLa cells after an 18 h treatment with the inhibitors ($n = 4$, mean ± S.D., $^*P < 0.05$). Details are described in Section 2.