European Journal of Pharmacology 652 (2011) 15–22

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

The β-adrenoceptor agonist isoproterenol promotes the activity of respiratory chain complex I and lowers cellular reactive oxygen species in fibroblasts and heart myoblasts

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ARTICLE INFO

Article history:
Received 11 August 2010
Received in revised form 20 October 2010
Accepted 3 November 2010
Available online 29 November 2010

Keywords:
β-adrenoceptor agonists
Isoproterenol
Complex I
Reactive oxygen species cellular balance
Mitochondria

ABSTRACT

A study is presented on the effect of the β-adrenoceptor agonist isoproterenol on mitochondrial oxygen metabolism in fibroblast and heart myoblast cultures. Isoproterenol treatment of serum-limited fibroblasts and proliferating myoblasts results in the promotion of mitochondrial complex I activity and decrease of the cellular level of reactive oxygen species. These effects of isoproterenol are associated with cAMP-dependent phosphorylation of complex I subunit(s). Addition of okadaic acid, inhibitor of protein phosphatase(s), reverses the decline of complex I activity in serum-limited fibroblast cultures and activates the complex in proliferating myoblast cultures. The effects of isoproterenol on complex I activity and reactive oxygen species balance can contribute to the therapeutic effect of the drug.

1. Introduction

Isoproterenol, a synthetic derivative of norepinephrine, is used as a therapeutic agent for its inotropic effect in supporting heart rate, myocardial contractility (Strodtbeck and Theorell, 2002) and the dilating action on vascular and bronchial smooth muscle (Ball et al., 1991). These effects are due to specific stimulation by isoproterenol of β_1 and β_2 adrenoceptors respectively, which activate plasma membrane adenylyl cyclase with elevation of the cytosolic level of cyclic AMP (cAMP) (Smiley et al., 1998). Investigations from different laboratories have shown that the cAMP-dependent protein kinase (PKA) exerts a regulatory effect on the functional capacity of respiratory chain complexes and controls the cellular level of oxygen free radicals (Papa et al., 2008; Acin-Perez et al., 2009; Papa et al., 2010). In cells there are different pools of PKA. Cytosolic PKA which is activated by hormone and neurotransmitter receptors of the plasma membrane associated with adenylyl cyclase (Cooper et al., 1995) and PKA localized in the mitochondrial inner compartment (Sardanelli et al., 2006), activated by bicarbonate dependent soluble adenylyl cyclase (Acin-Perez et al., 2009). Cytosolic and mitochondrial PKA (Wong and Scott, 2004; Sardanelli et al., 2006; Papa et al., 2008) and phosphatase(s) (Signorile et al., 2002) have been shown to regulate the biogenesis (Ryu et al., 2005; De Rasmo et al., 2009, 2010) and activity of respiratory chain complexes (Papa et al., 2008; Helling et al., 2008; Acin-Perez et al., 2009).

The mammalian complex I (E.C. 1.6.5.3) is made up of 45 subunits (Carroll et al., 2006). Seven are encoded by the mitochondrial genome, the others by nuclear genes (Hirst et al., 2003). Fourteen subunits are conserved from prokaryotes to humans (Hirst et al., 2003; Yagi and Matsuno-Yagi, 2003; Brandt, 2006). Some supernumerary are essential for the assembly of the complex (Scacco et al., 2003; Scheffler et al., 2004), for others regulatory function is under investigation (Carroll et al., 2003; Papa et al., 2008).

It has been found that the activation of the cAMP cascade by cholera toxin or dibutyryl-cAMP promotes the activity of complex I and lowers reactive oxygen species in different mammalian cell cultures including Balb/c3T3 mouse fibroblasts, C2C12 mouse myoblasts, Skin human fibroblasts, NHDF neonatal human fibroblasts, Hela cells, Skin fibroblasts with NDUFS1 pathological mutations, and retroviral-vector transfected human bone marrow mesenchymal cells (Piccoli et al., 2008; Papa et al., 2010). Immunoblotting with specific antibodies against the synthetic carboxy-terminal segment, with the RVSTK phosphorylation site, of NDUFS4 shows that activation of complex I activity is associated with the phosphorylation of this subunit (Scacco et al., 2000; Technikova-Dobrova et al., 2001).

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doi:10.1016/j.ejphar.2010.11.016

In the present paper we have studied the impact of isoproterenol-dependent activation of plasma membrane adenylyl cyclase and cytosolic PKA, on the activity of complex I and cellular reactive oxygen species, in fibroblast and heart-derived myoblast cultures. In both types of cell cultures isoproterenol-induced elevation of the cAMP level was associated with phosphorylation of the NDUFS4 subunit of complex I, marked stimulation of activity of the complex I and decrease in the level of reactive oxygen species.

2. Materials and methods

2.1. Cell cultures

Neonatal normal human dermal fibroblasts (NHDF-neo, Cambrex #CC-2509, East Rutherford, NJ, USA) were grown in the exponential phase in high glucose Dulbecco's modified Eagle's medium (DMEM) (EuroClone, Paignton, UK) supplemented with 10% fetal bovine serum (FBS), plus 2 mM glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin (Euroclone, Paignton, UK) at 37 °C, 5% CO_2. Where indicated once fibroblasts were at 75% confluence, the medium was replaced with DMEM with 0.5% fetal bovine serum and the cells were cultivated for 48 h (serum limited). Further conditions are specified in the legends to figures.

The H9c2 cell line, derived from embryonic rat heart, was purchased from American Type Culture Collection (A.T.T.C. #CRL1446, Manassas, VA). Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), plus 2 mM glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin at 37 °C, 10% CO_2. Further conditions are specified in the legends to figures.

2.2. Measurement of endogenous respiration rate in intact cells and substrate-supported respiration rate in digitonin-permeabilized cells

The respiratory activity was measured polarographically with a Clark-type oxygen electrode in a water-jacketed chamber (Hansatech Instruments, Norfolk, UK), magnetically stirred at 37 °C, essentially as described in Sgobbo et al. (2007).

Cells were collected by trypsinization and centrifugation and resuspended at $1–3\times10^6$ cells/ml in Buffer A (75 mM sucrose, 5 mM KH_2PO_4, 40 mM KCl, 0.5 mM EDTA, 3 mM $MgCl_2$, 30 mM Tris–HCl, pH 7.4), supplied with 0.3 mM P1,P5-di(adenosine-5′) pentaphosphate (Ap5A) (Sigma-Aldrich, St. Louis, MO) to prevent dissipation of ADP by adenylate kinase, for measurement of oxidative phosphorylation (OXPHOS) efficiency (P/O ratios). The cell suspension was transferred to the polarographic chamber and an aliquot was utilized for cell counting and protein determination. After permeabilization by digitonin, substrates and inhibitors of mitochondrial OXPHOS were added, when specified in the legends to figures, at the following concentrations: pyruvate (5 mM)/malate (2.5 mM), succinate (5 mM), rotenone (200 nM), and antimycin A (15 nM) (Sigma-Aldrich, St. Louis, MO). For OXPHOS efficiency measurement, the substrates pyruvate/malate and succinate were followed by the addition of 0.16 mM ADP and 0.08 mM ADP, respectively, to induce transient stimulation of oxygen consumption.

The P/O was calculated as the ratio between the amount (nanomoles) of added ADP and the oxygen (natoms) consumed during the ADP-induced state 3 respiration (Chance and Williams, 1955). The ATP production rates were calculated by multiplying the individual P/O ratio values by the double of the corresponding ADP-stimulated (state III) respiration rates (Cocco et al., 2009).

2.3. Mitoplasts preparation

The cells (NHDF-neo and H9c2) were harvested from Petri dishes with 0.05% trypsin, 0.02% EDTA, pelleted by centrifugation at $500\times g$ and then resuspended in phosphate-buffered saline, pH 7.4 (PBS). The cell suspension was exposed for 10 min on ice to 2 mg of digitonin/mg cellular protein. The mitoplast fraction, obtained by digitonin cell disruption, was pelleted at $14\,000\times g$ and resuspended in PBS.

2.4. Enzymatic spectrophotometric assay

The isolated mitoplasts were exposed to ultrasound energy for 15 s at 0 °C. The NADH-UQ oxidoreductase activity was performed in 40 mM potassium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, in the presence of 3 mM KCN, 1 μg/ml antimycin, 200 μM decylubiquinone, using 30 μg of mitoplast proteins, by following the oxidation of 1.25–25 μM NADH at 360–374 nm ($\Delta\varepsilon = 2.01\ mM^{-1}\ cm^{-1}$). The activity was corrected for the residual activity measured in the presence of 1 μg/ml rotenone. Vmax values were obtained from Lineweaver–Burk plots as described in Scacco et al. (2000).

For citrate synthase activity, mitoplast proteins (20 μg), 0.5 mM Acetyl CoA and 5′5′ dithiobis-2 nitrobenzoate (DTNB) were added to Tris–HCl buffer (100 mM), pH 8.0. The reaction was started by the addition of 0.5 mM oxaloacetate and, the initial rate, measured following the reduction of DTNB at 419 nm ($\Delta\varepsilon = 163\ mM^{-1}\ cm^{-1}$).

Cytochrome *c* oxidase (complex IV) activity was measured by following the oxidation of ferrocytochrome c at 550–540 nm ($\Delta\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$). Enzymatic activity was estimated in 10 mM phosphate buffer, pH 7.4, using 20 μg of mitoplast proteins (Cooperstein and Lararow, 1951).

To determine the glutathione reductase and glutathione peroxidase activities, cells were scraped, suspended in 25 mM phosphate buffer pH 7.4, 5 mM $MgCl_2$, 0.25 mM Phenylmethanesulfonyl fluoride (PMSF) and exposed to ultrasound energy for 15 s at 0 °C. The glutathione reductase and glutathione peroxidase activities were assayed as described in Piccoli et al. (2006).

2.5. Protein phosphatases activity

Cells were scraped with 0.4 ml of phosphatase extraction buffer containing 20 mM imidazole–HCl, 2 mM EGTA, 2 mM EDTA, pH 7.0, in the presence of a proteases inhibitors cocktail (Sigma, Saint Louis, Missouri, USA). Cells were sonicated for 10 s and then centrifuged at $2000\times g$ for 5 min. The supernatants were used to assay the protein phosphatases activity, using a Ser/Thr phosphatase assay Kit (Millipore, Billerica, Massachusetts, USA).

2.6. cAMP assay

For cAMP assay the culture medium was removed and 1 ml of 0.1 M HCl was added to the cell layer. After 10 min at 37 °C, the lysed cells were scraped into Eppendorf tubes. The samples were centrifuged at $1300\times g$ for 10 min at 4 °C. The supernatants were used to determine cAMP concentration using a direct immunoassay kit (Assay Designs, Ann Arbor, Michigan,USA) as described by the manufacturer. Total protein concentration was determined by Bio Rad protein assay (BioRad, Milan, Italy). The cAMP level in the sample was normalized to the protein concentration and expressed as pmol/mg protein.

2.7. Measurement of total cellular ATP

The cells (H9c2) were grown in six-well plates as indicated in Section 2.1. Once the cells were at 75% confluence, where indicated, the cells were incubated for 35 min with 1 μM isoproterenol in the presence or absence of 5 μM oligomycin. After incubation the cells were harvested with 0.05% trypsin, 0.02% EDTA, pelleted by centrifugation at $500\times g$ and then resuspended in phosphate-buffered saline, pH 7.4 (PBS). The 10 000 cells were utilized to measure the ATP using a luciferin–luciferase reaction system (PROMEGA, Madison, Wisconsin, USA).