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The cardioprotective effect of sildenafil is mediated by the activation of malate dehydrogenase and an increase in the malate-aspartate shuttle in cardiomyocytes

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ABSTRACT

Recent evidence has shown the cardioprotective effect of PDE5 inhibition in myocardial ischemia/reperfusion injury, heart failure and cardiac hypertrophy. To investigate the biochemical changes that occur during PDE5 inhibition in cardiac cells, this study assessed the metabolic profile of the HL1 cell line, a murine atrial cell line with adult cardiomyocyte properties. After one hour of treatment with sildenafil, glycolysis was moderately but selectively stimulated, unlike the pentose phosphate pathway and the Krebs cycle. Moreover, malate and a-Ketoglutarate accumulated, paralleled by a decrease in aspartate and glutamate. Interestingly, increased activity of malate dehydrogenase (MDH) was also detected in these cells after sildenafil treatment. Thus, we hypothesized that sildenafil stimulates the malate-aspartate shuttle (MAS) with the final effect of transferring electrons and protons from glycolysis-derived cytosolic NADH into the matrix for use by the electron transport chain, using malate as an electron carrier. Through this metabolic modification, sildenafil may counteract what is often observed in ischemia, i.e. reduced MAS flux as well as a dramatic acceleration of glycolysis, which switches to lactate production. Additionally, the results observed in HL1 cells were also found in isolated mouse hearts. The documented metabolic alteration in cardiomyocytes upon treatment with sildenafil occurred by stimulating cGMP production, which did not activate PKG (cGMP-PKG signaling), since the addition of DT-2, a PKG inhibitor, did not block malate accumulation and increased MDH activity. Conversely, the addition of chelerythrine, a PKC inhibitor, counteracted both malate accumulation and MAS activation, supporting previous evidence that, upon the addition of sildenafil, some PKC isoforms may be implicated in cardioprotection (cGMP-PKC signaling). Interestingly, an increase in cGMP, driven by sildenafil, another cGMP stimulator such as nitroprusside (SNP), or a C-type natriuretic peptide (CNP) which does not inhibit PDE5, led to MAS stimulation and increased MDH activity.

1. Introduction

Sildenafil citrate (Viagra) is the first oral agent approved for the treatment of erectile dysfunction in men [1], and was recently approved for the management of pulmonary arterial hypertension, showing a potential benefit in the early phases of inflammation and vascular remodeling in this disease [2]. In addition to this effect, a number of preclinical studies have shown that sildenafil has a powerful protective effect against several clinical scenarios, including myocardial ischemia/reperfusion injury, heart failure and cardiac hypertrophy [3].

Sildenafil is a selective inhibitor of phosphodiesterase-5 (PDE-5), which hydrolyzes cyclic nucleotides (cGMP) into the inactive linear form (5′-GMP), thereby regulating both the duration and the amplitude of cyclic nucleotide signaling. cGMP regulates a broad array of physiological processes in the cardiovascular system, including cardiac contractility and cardiac and vascular remodeling [4]. cGMP exerts its physiological action through PKG (cGMP-dependent protein kinase), which in turn phosphorylates

Abbreviations: MDH, malate dehydrogenase; MAS, malate-aspartate shuttle; SNP, nitroprusside; CNP, C-type natriuretic; HPLC, high pressure liquid chromatography; TCA, tricarboxylic acid cycle; GSNO, S-Nitrosoglutathione.

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several proteins, including cardiac troponin I, the L-type Ca2+ channel, phospholamban and titin. cGMP activates PKG, which opens the mitoK-ATP that prevents the loss of ionic gradients and allows for continued ATP production and calcium transport [5–7] In addition to the well-documented interrelationship between cGMP-mediated signaling and PKG (cGMP-PKG signaling), there is substantial experimental evidence that another intracellular signal transduction pathway controlling cardiac protection by ischemic preconditioning, [8–11] involving the activation of PKC [12,13] and changes to mitochondrial function (cGMP-PKC signaling). A detailed examination of ouabain signaling suggests that the terminal cytosolic kinases are Src and PKC [14], and that they act in tandem to phosphorylate a p38 MAP kinase residing in the mitochondrial outer membrane (MOM)[15]. Thus, there is more than one signaling pathway capable of inducing mitoK-ATP opening and cardioprotection. Notably, the PKCε and the PKCδ isoforms have both been implicated in cardioprotection, as translocation of activated PKCε and PKCδ to the membrane fraction has been detected in preconditioned hearts [16]. These isoforms translocate to the membrane fraction, followed by activation of the Raf1-MEK1/2-p44/p42 MAPK signaling cascade, phosphorylation of STAT 1/3, and upregulation of STAT-dependent genes, including COX-2 [17]. It is not clear whether an interrelationship between these two different signaling pathways (cGMP-PKG and cGMP-PKC) exists. However, it is remarkable that both signaling pathways begin at the plasma membrane and carry messages to intracellular structures, including mitochondria, indicating that the mitochondrion is one of the final targets of both signaling pathways. Emerging research has indicated that the cytosolic signal is generally delivered to the MOM in the form of a phosphorylation event by PKG, since PKG cannot cross the MOM18. The signal is transmitted across the intermembrane space to PKC1, which is associated with mitoK-ATP, causing the latter to open with subsequent triggering of increased production of H_2O_2 and the activation of a second pool of PKC1 that stimulates a pathway that ultimately terminates in the inhibition of mitochondrial permeability transition MPT [18]. On the contrary, regarding cell metabolism, it cannot be excluded that some PKC isoforms may act independently of PKG signaling in cardiac metabolism.

In order to shed light on the molecular mechanism by which sildenafil influences cell metabolism, we investigated changes in the metabolic profile induced by sildenafil in HL1 cells, with the final aim of understanding the modifications induced in healthy cells and consequently simulating the molecular mechanism by which sildenafil exerts a protective effect in ischemic cardiomyocytes. The data show that, in HL1 cells, sildenafil induces increased activity of malate dehydrogenase and, as a consequence, stimulates the malate-aspartate shuttle (MAS). Together with inhibition of the Krebs cycle and moderate stimulation of glycolysis, this metabolic modification induced by sildenafil counteracts the metabolic alterations observed in ischemic cardiomyocytes and thus exerts a cardioprotective effect. Finally, the stimulated signaling pathway is discussed, i.e. an increase in cGMP was observed upon PDE5 inhibition, which in some way affected malate dehydrogenase (MDH) and in turn stimulated the MAS. This stimulation occurred through PKC, as it was interrupted by the addition of chelerythrine, a potent inhibitor of PKC, but not through PKG, since the MAS was still activated following treatment with DT-2, an inhibitor of PKG.

2. Material and methods

2.1. Chemicals

The PDE5 inhibitor sildenafil was purchased from Pfizer (New-York, USA) and used at a final concentration of 1 μM. The PKC inhibitor chelerythrine chloride was purchased from Sigma Aldrich (St. Louis, MO, USA) and used at a final concentration of 2 μM. The PKG inhibitor DT-2 was purchased from Sigma Aldrich (Sigma Aldrich, CA, USA) and used at a final concentration of 10 μM. Sodium nitroprusside (SNP) was purchased from Sigma Aldrich and used at a final concentration of 100 μM. C-type natriuretic peptide (CNP) was purchased from Sigma Aldrich (Sigma Aldrich, CA, USA) and used at a final concentration of 10 μM.

2.2. Cell culture

HL1 cells, kindly provided by Dr. Simona Nanni (Catholic University, Rome, Italy), were cultured in Claycomb medium (Sigma Aldrich, CA, USA) supplemented with 10% v/v FBS (Sigma Aldrich), 0.2 mM norepinephrine, 2 mM L-glutamine, 1 U/ml penicillin and 1 μg/mL streptomycin solution (Sigma Aldrich,) as previously described. Cells were grown at 37 °C in an atmosphere of 5% CO_2 and 95% air at a relative humidity of approximately 95%. Treatment of HL1 cells with sildenafil (1 μM) was carried out for 1 h. Treatment with 100 μM sodium nitroprusside (SNP) or 100 μM C-type natriuretic peptide (CNP) was carried out for 1 h. Prior treatment with 10 μM DT-2 or 2 μM chelerythrine chloride, before adding 1 μM sildenafil, was performed for 15 min. The inactivation of MDH using butanedione was performed in 0.1 M borate/boric acid, pH 7.2. Experiments against malate dehydrogenase inactivation by butanedione have been carried out with 1 or 12 mM NADPH, AMP, or nicotinamide added before 4 mM butanedione in the inactivation mixture.

2.3. Animal models

Twelve-week-old CD1 male mice were purchased from Jackson Laboratories and handled according to Italian law (D.L. 2010/63EU). Sildenafil (1.6 mg/kg, in a water solution) was administered i.p. daily, for 3 weeks. Appropriate vehicle controls were performed. All experiments were performed in accordance with Italian law (D.L. 2010/63EU), the study was approved by the Sapienza University's Animal Research Ethics Committee and by the Italian Ministry of Health (165/2016-PR). Criteria for euthanasia were based on an independent assessment by a veterinarian according to Association for Assessment and Accreditation of Laboratory Animal Care of Laboratory Animal Care guidelines. Nine mice were sacrificed by cervical dislocation and hearts were collected for metabolomics analysis. Organs were dissected within few minutes, carefully patted with lint free tissue paper, cut into small pieces, and placed into pre-labeled tubes. Tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until extraction.

2.4. Metabolite extraction

$1 * 10^6$ cells from each treatment was mixed with 200 μL of methanol:acetonitrile (50:50 v/v). Methanol and acetonitrile were purchased from Sigma (Sigma Aldrich, CA, USA). Samples were vortexed for 30 min at max speed at 4 °C and then centrifuged at 16000*g* for 15 min at 4 °C. Supernatants were collected for subsequent metabolomic analysis, while insoluble debris was discarded. Metabolites from each heart mice were extracted from 50 mg of meat. Samples were crushed in a mortar containing liquid nitrogen and extracted in 1 ml of ice cold methanol:acetonitrile:water (50:30:20). Samples were vortexed for 30 min at max speed at 4 °C and then centrifuged at 16,000*g* for 15 min at 4 °C. Supernatants were collected for subsequent metabolomic analysis, while insoluble debris was discarded.