European Journal of Pharmacology 818 (2018) 335–342

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Full length article

The cardioprotective effects of (-)-Epicatechin are mediated through arginase activity inhibition in a murine model of ischemia/reperfusion

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ARTICLE INFO

Keywords:
Ischemia/reperfusion injury
Epicatechin
Arginase
Nitric oxide synthase

ABSTRACT

The production of nitric oxide (NO) by nitric oxide synthases (NOS) depends on the bioavailability of L-arginine as NOS competes with arginase for this common substrate. As arginase activity increases, less NO is produced and adverse cardiovascular consequences can emerge. (-)-Epicatechin (EPI), the most abundant flavonoid in cacao, has been reported to stimulate endothelial and neuronal NOS expression and function leading to enhanced vascular function and cardioprotective effects. However, little is known about the effects of EPI on myocardial arginase activity. The aim of the present study was to determine if EPI is able to interact and modulate myocardial arginase and NOS expression and activity. For this purpose, *in silico* modeling, *in vitro* activity assays and a rat model of ischemia/reperfusion injury were used. *In silico* and *in vitro* results demonstrate that EPI can interact with arginase and significantly decrease its activity. *In vivo*, 10 days of EPI pretreatment reduces ischemic myocardium arginase expression while increasing NOS expression and phosphorylation levels. Altogether, these results may partially account for the cardioprotective effects of EPI.

1. Introduction

Cardiovascular diseases including acute myocardial infarction (AMI) are the leading cause of death worldwide (Pagidipati and Gaziano, 2013). Current treatment for AMI caused by a coronary artery occlusion is the restoration of blood flow (i.e., reperfusion) by means of a percutaneous intervention. Early revascularization rescues the myocardium at risk and reduces infarct size, which improves AMI-associated morbidity and mortality. However, reperfusion itself can damage myocardium, a phenomenon referred to as ischemia-reperfusion (I/R) injury. Several mechanisms have been linked to this phenomenon, including oxidative stress, calcium overload, inflammation and endothelial dysfunction (Hadi et al., 2005). Endothelial dysfunction is characterized by the reduced bioavailability of nitric oxide (NO) and has been identified as a therapeutic target. Myocardial NO bioavailability can be compromised by several factors, including decreased expression and/or altered activity of endothelial NO synthase (eNOS), augmented production of reactive oxygen species (ROS) (e.g., anion superoxide [O2-]), and/or insufficient substrate availability (i.e., L-arginine) (Hadi et al., 2005).

One of the mechanisms that reduces NO availability in the setting of I/R injury, is an increase arginase (Ar) activity. Arginase is an enzyme that competes with NOS for the common substrate, L-arginine. Under normal conditions, NOS metabolizes L-arginine into L-citrulline, producing NO in the process. Ar metabolizes L-arginine into L-ornithine and urea (Yang and Ming, 2014). Of note, an increased activity of Ar has been reported in several conditions associated with endothelial dysfunction, including diabetes mellitus and atherosclerosis, and is thought to contribute to disease pathophysiology (Romero et al., 2008; Berkowitz et al., 2003; Ryoo et al., 2006, 2008; Kan et al., 2015). Two different isoforms of Ar (1 and 2) have been described that differ mainly in their intracellular localization. Ar 1 is an enzyme expressed primarily in liver, endothelium, smooth muscle cells and cardiomyocytes. Ar 2 is localized in mitochondria of multiple tissues, including heart and

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http://dx.doi.org/10.1016/j.ejphar.2017.11.007
Received 24 May 2017; Received in revised form 3 November 2017; Accepted 6 November 2017
Available online 07 November 2017

vasculature (Yang and Ming, 2014).

Compounds that increase NO availability by decreasing its metabolism or increasing its blood levels (e.g., NO donors, Ar inhibitors) to physiological levels have been shown to confer protection against I/R injury. In this regard, flavonoids, particularly (-)-Epicatechin (EPI), the most abundant flavanol in cacao, has been reported to inhibit Ar activity in cultured endothelial cells, thus increasing NO production (Schnorr et al., 2008). Recently, we demonstrated that EPI is able to reduce myocardial damage that was induced by permanent coronary occlusion or I/R in rats (measured 48 h and 3 weeks after I/R) (Yamazaki et al., 2008) by ~ 50%. We also reported that EPI induced NO synthesis through the activation of eNOS in endothelial cells (Ramirez-Sanchez et al., 2010). The aim of the present study was to determine if EPI is able to interact and modulate myocardial Ar as well as to modify its levels and those of NOS. For this purpose, *in silico* modeling, *in vitro* activity assays and a rat model of I/R injury were used.

2. Materials and methods

2.1. In Silico studies

A docking analysis of interactions between Ar and L-arginine and Ar with EPI was pursued as outlined below. The three-dimensional structure of Ar isoform 1 (pdb code 2AEB) was obtained from www.rcsb.org. The Discovery Studio Visualizer was used to add the Charmm force field. Polar hydrogen atoms were added, followed by Gasteiger charge calculation using Autodock tools (ADT) 1.5.4. The three dimensional structures of EPI and L-arginine were downloaded from Chem Spider (www.chemspider.com) and saved in protein data bank (pdb) format using Discovery Studio. Polar hydrogen atoms were added, the number of torsions were set and Gasteiger charges were assigned using Autodock tools (ADT) 1.5.4.

Docking analysis was performed in AutoDock Vina. A blind docking method was used with the coordinate of origin set at x = 11.946, y = 20.979 and z = 0.033, at the center of the protein. The box size was set at x = 70, y = 70 and z = 70. Docking simulations to analyze binding affinities and binding sites were run with the number of modes set to 8. The Discovery Studio was used to produce two-dimensional docking representations of the interactions.

To evaluate changes in L-arginine free energy (ΔG, kcal/mol) and aminoacid interactions with EPI, a pdbqt file was created using PyMolwin software. The docking was performed in AutoDock Vina, with the coordinate of origin at x = 2.017, y = − 7.457 and z = − 0.116. The box size was set at x = 70, y = 70 and z = 70.

2.2. Animals

Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee and carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, 2011, https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf) and Mexican official regulations (Diario official de la federacion. http://www.fmvz.unam.mx/fmvz/principal/archivos/062ZOO.PDF) Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250–300 g were used. Rats were randomly allocated into 3 groups. A sham group (n = 10) that was pre-treated with vehicle (water) by oral gavage. The I/R group (n = 10) that was pre-treated with vehicle for 10 days prior to coronary occlusion, and the I/R + EPI group (n = 10), which was pretreated for 10 days with 1 mg/kg/day of EPI (Yamazaki et al., 2008). Prior to surgery, animals were anesthetized via an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (30 mg/kg), intubated, and ventilated under positive pressure. A left thoracotomy was then performed. The left anterior descending coronary artery was occluded during 45 min and then released. The suture was left in place as a point of reference. Successful occlusion and reperfusion was verified by visual inspection of left ventricle color. The chest wall was closed in layers and animals were allowed to recover for 48 h. For biochemical analysis, the left ventricle free wall was visually identified, collected and flash frozen for subsequent analysis.

2.3. Ar activity assay

Under anesthesia, normal hearts were excised from male Sprague-Dawley rats (n = 5). Left ventricles (LV) were homogenized (0.1 g/mL) in solution A (sucrose 2 M, EDTA 0.01 M, HEPES 0.5 M; pH 7.4) and samples centrifuged for 10 min (800 g) at 4 °C. The supernatant fluid was again centrifuged for 10 min (8000 g) at 4 °C and the 2nd supernatant fluid was preserved as the cytoplasmic fraction. The precipitate was resuspended in solution B (sucrose 2 M, EDTA 0.01 M, Tris 0.5 M-H_2PO_4-50 mM; pH 7.4) and centrifuged for 10 min (10,000 g) at 4 °C. The 2nd precipitate was resuspended in 10 mL of solution C (sucrose 2 M, EDTA 0.01 M, Tris 0.5 M-H_2PO_4-50 mM, succinate 1 M; pH 7.4) to obtain the mitochondrial fraction. Protein concentration was determined via Lowry's method (Waterborg and Matthews, 1984).

In the absence and presence of EPI (1 μM), Ar 1 and Ar 2 kinetic activities were evaluated respectively in post-mitochondrial and mitochondrial LV fractions in triplicate. To evaluate Ar activity, 200 μg of protein in 25 mM Tris-HCl pH 7.4 and 5 mM $MnCl_2$ (adjusted volume to 100 μl) were activated at 55 °C for 10 min. The enzymatic reaction was started by the addition of 100 μl of 5, 10, 25, 50 and 80 mM L-arginine as substrate (pH 9.7). The solution was incubated at 37 °C for 60 min. The reaction was stopped by the addition of 200 μl of an acid mixture containing H_2SO_4, H_3PO_4 and H_2O (1:3:7). Next, 25 μl of a solution containing 9% α-isonitrosopropiophenone were added and heated at 100 °C for 45 min. The urea concentration was quantified by evaluating the OD at 540 nm in a microplate spectrophotometer (Biotek®, Synergy HT). Kinetic Ar activity levels were calculated by extrapolating the data onto urea standard curves.

A Michaelis–Menten saturation curve was generated by plotting the reaction rate against concentration. Maximal enzymatic rate (Vmax) and Michaelis constant (Km) were calculated using Graph Pad Prizm 6.0 software.

2.4. Western blots

LV samples were homogenized with a polytron in 30 μl of lysis buffer (10% glycerol, 1% NP-40, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES, 20 mM β-glycerol phosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate; pH 7.5 with protease and phosphatase inhibitors). Homogenates were sonicated for 20 min at 4 °C and further centrifuged (16,000 g) for 20 min at 4 °C. Total protein content in the supernatant was determined via Lowry´s method. A total of 80 μg of protein were loaded onto 4–15% polyacrylamide gels, electrotransferred to a polyvinyl membrane at 18 V for 45 min using a semidry transfer system. Membranes were incubated for 1 h in blocking solution (5% nonfat dry milk in Tris-buffered saline [TBS] plus 0.1% Tween 20 [TBS-T]), followed by overnight incubation at 4 °C with primary antibodies against Ar 1, Ar 2, nitrotyrosine, neuronal (n) NOS, inducible (i) NOS, and eNOS as well as phosphorylated eNOS. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal subunit 6 (SRP6) were used as loading controls. All antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Membranes were washed (3× for 5 min) in TBS-T and incubated for 1 h at 4 °C in the presence of horseradish peroxidase-conjugated secondary antibodies diluted 1:3000 in TBS-T. Membranes were again washed in TBS-T and the immunoblots were developed using an enhanced chemiluminescence detection kit (ImmunoCruz™ Western Blotting Luminol Reagent). The densities of the bands were digitally quantified via scanning densitometry.