Journal of Molecular and Cellular Cardiology 67 (2014) 60–68

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

The signaling pathway for aldosterone-induced mitochondrial production of superoxide anion in the myocardium

M.B. Nolly [1], C.I. Caldiz [1], A.M. Yeves, M.C. Villa-Abrille, P.E. Morgan, Nicolas Amado Mondaca, Enrique L. Portiansky, G.E. Chiappe de Cingolani, H.E. Cingolani, I.L. Ennis *

Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, UNLP-CONICET, Argentina

ARTICLE INFO

Article history:
Received 13 June 2013
Received in revised form 4 December 2013
Accepted 8 December 2013
Available online 16 December 2013

Keywords:
Mineralocorticoid receptor
Oxidative stress
siRNA
Transactivation

ABSTRACT

Mineralocorticoid receptor (MR) antagonists decrease morbidity and mortality in heart failure patients for whom oxidative stress is usual; however, the underlying mechanism for this protection is unclear. Since aldosterone stimulates reactive oxygen species (ROS) production in several tissues, we explored its effect and the intracellular pathway involved in the rat myocardium. Aldosterone dose-dependently increased O_2^- production in myocardial slices. At 10 nmol/L, aldosterone increased O_2^- to 165 ± 8.8% of control, an effect prevented not only by the MR antagonists eplerenone and spironolactone (107 ± 7.8 and 103 ± 5.3%, respectively) but also by AG1478 (105 ± 8.0%), antagonist of the EGF receptor (EGFR). Similar results were obtained by silencing MR expression through the direct intramyocardial injection of a lentivirus coding for a siRNA against the MR. The aldosterone effect on O_2^- production was mimicked by the mK_{ATP} channel opener diazoxide and blocked by preventing its opening with 5-HD and glibenclamide, implicating the mitochondria as the source of O_2^-. Inhibiting the respiratory chain with rotenone or mitochondrial permeability transition (MPT) with cyclosporine A or bongkrekic acid also canceled aldosterone-induced O_2^- production. In addition, aldosterone effect depended on NADPH oxidase and phosphoinositide 3-kinase activation, as apocynin and wortmannin, respectively, inhibited it. EGF (0.1 μg/mL) similarly increased O_2^-, although in this case MR antagonists had no effect, suggesting that EGFR transactivation occurred downstream from MR activation. Inhibition of mK_{ATP} channels, the respiratory chain, or MPT did not prevent Akt phosphorylation, supporting that it happened upstream of the mitochondria. Importantly, cardiomyocytes were confirmed as a source of aldosterone induced mitochondrial ROS production in experiments performed in isolated cardiac myocytes.
These results allow us to speculate that the beneficial effects of MR antagonists in heart failure may be related to a decrease in oxidative stress.

1. Introduction

The renin–angiotensin–aldosterone system is activated during heart failure (HF). Mineralocorticoid receptor (MR) antagonists have been shown to decrease morbidity and mortality not only in patients with severe HF [1] and after myocardial infarction [2] but also in patients with HF class II of the NYHA functional class [3]. The mechanism by which this inhibition induces beneficial effects, however, has not yet been completely clarified [4,5]. On the other hand, a deleterious increase in oxidative stress has been recognized in HF [6–8], and endothelial dysfunction is linked to this abnormality [9].

A role for aldosterone in increasing reactive oxygen species (ROS) production in smooth muscle cells, cardiac myocytes, and other tissues has been reported recently, but the intracellular signaling pathway involved in this phenomenon in the myocardium remains unknown [10–13]. In the current study, experiments were performed in rat myocardium and isolated cardiac myocytes to analyze in detail the mechanism underlying aldosterone-induced myocardial O_2^- production. Our results indicate that aldosterone induces the opening of mitochondrial ATP-dependent potassium (mK_{ATP}) channels via a non-genomic mechanism that relies on MR-dependent epidermal growth factor receptor (EGFR) transactivation, increasing mitochondrial ROS production.

2. Methods

2.1. Animals

All the procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996)

Abbreviations: HF, heart failure; MR, mineralocorticoid receptor; ROS, reactive oxygen species; mK_{ATP}, mitochondrial ATP-dependent potassium; EGFR, epidermal growth factor receptor; MPT, permeability transition pore; 5-HD, 5-hydroxydecanoate; CsA, cyclosporine A; DHE, dihydroethidium.

* Corresponding author at: Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, UNLP, Calle 60 y 120, 1900 La Plata. Argentina. Tel./fax: +54 221 483 4833.
E-mail address: iennis@med.unlp.edu.ar (I.L. Ennis).

[1] Both authors equally contributed to this manuscript.

0022-2828/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.yjmcc.2013.12.004

and the experimental protocol was approved by the Animal Welfare Committee of La Plata School of Medicine. Rats (body weight 300–400 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/kg body weight) and hearts rapidly excised when plane three of phase III of anesthesia was reached.

2.2. Construction and production of lentiviral vectors

DNA encoding for siRNA against the MR ($siRNA_{MR}$) or the scrambled sequence ($siRNA_{SCR}$) was inserted in the lentiviral vector backbone PPT.CDsRed2.H1 as previously described [14]. Sequence for $siRNA_{MR}$ was obtained from the work of Wang et al. [15] and subcloned, at the BamHI cloning site (5′) and PacI cloning site (3′), following the H1 RNA Polymerase promoter, to generate PPT.CDsRed2.H1.$siRNA_{MR}$.

2.3. Injection of lentiviral vector

Four-month-old male Wistar rats were injected with lentivirus at two sites in the anterolateral wall of the left ventricle as described before [14]. Four weeks after injection animals were sacrificed, heart removed and sliced. Some slices were used to measure O_2^- production and others were immediately processed to obtain a protein homogenate and stored at −70 °C for immunoblot analysis.

2.4. Myocardial slices

Left ventricular myocardial slices (1 × 5 mm) were obtained from anesthetized 4-month-old male Wistar rats and incubated in Krebs–Hepes assay buffer as previously described [16].

2.5. Cell isolation

Rat ventricular myocytes were isolated from 4-month-old Wistar rats according to the technique described previously [17]. Myocytes were kept in 1 mmol/L CaCl2 K-H solution at room temperature (20–22 °C) until use.

2.6. Measurement of ROS production

2.6.1. In myocardial slices

Myocardial O_2^- production was measured by the lucigenin-enhanced chemiluminescence method as previously described [16]. Since high lucigenin concentrations (>20 μmol/L) may favor redox cycling, we used 5 μmol/L lucigenin, at which the amount of artifacts has been proven insignificant [18]. For each intervention evaluated the lucigenin-chemiluminescence signal was normalized to milligrams of dry weight tissue per minute and expressed relative to the basal production.

2.6.2. In isolated cardiomyocytes

Freshly isolated cardiomyocytes were loaded with 10 μmol/L 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA, Invitrogen) in K-H solution for 30 min at 37 °C in dark. This dye is deacetylated intracellularly by non-specific esterase, which was further oxidized by cellular peroxides to the fluorescent compound 2,7-dichlorofuorescein (DCF), which reported H_2O_2 levels [19]. Cardiomyocytes were washed with K-H solution to remove dye excess, placed in six-well plates (a different well for each treatment) and excited at 495 nm. Emission spectra were acquired trough a 510 nm filter with a Nikon camera attached to an inverted Nikon Eclipse TE2000-S microscope. Photographs were taken immediately in control or stimulated cells (aldosterone 10 nmol/L, or EGF 0.1 μmol/L) every 3 min, during 18 min. The inhibitors and antagonists were placed 10 min before stimulus. *Image J* software was used for image analysis. Results of fluorescence (arbitrary units) vs. time were fitted with lineal function and obtained a slope for each treatment. Results were expressed as % of control slope.

2.7. Immunoanalysis by Western blot

At the end of the experimental protocols cardiac tissue slices were homogenized in lysis buffer (300 mmol/L sacarose; 1 mmol/L DTT; 4 mmol/L EGTA, protease inhibitors cocktail (Complete Mini Roche); 20 mmol/L Tris–HCl, pH 7.4) for determination of EGFR and Akt phosphorylation; and in 50 mM Tris–HCl, pH 7.4 for NADPH oxidase (Nox) and MR expression. Samples were denatured and equal amounts of protein were subjected to PAGE and electrotransferred to PVDF membranes. Membranes were then blocked with non-fat-dry milk and incubated overnight with: anti gp91-phox polyclonal antibody (Santa Cruz Biotechnology, sc-5827); anti-phospho-EGFR (Tyr1173) monoclonal antibody (Cell signaling Technology # 4407); anti-phospho-Akt polyclonal antibody (Cell Signaling Technology #4060) or anti-ratMR (antibody 4G5, kindly provided by Dr. Celso Gomez Sanchez). Antibodies against EGFR (Santa Cruz Biotechnologies sc-03) or Akt (Cell Signaling Technology #9272) respectively were assayed to normalize the amount of the phosphorylated form to the total content of the corresponding protein, while the detection of GAPDH (Millipore MAB374) was used as loading control. Peroxidase-conjugated anti-rabbit (NA934, GE Healthcare Life Sciences) or anti-mouse IgG (NA931, GE Healthcare Life Sciences) was used as secondary antibodies and bands were visualized using the ECL-Plus chemiluminescence detection system (Amersham). Autoradiograms were analyzed by densitometric analysis (Scion Image).

2.8. Chemicals

All drugs used in the present study were of analytical reagent. Aldosterone, EGF, lucigenin (5 μmol/L), Tiron (10 mmol/L), nitro-L-arginine methyl ester (L-NAME 1 mmol/L), 5-hydroxydecanoate (5-HD, 100 μmol/L), rotenone (10 μmol/L), cycloheximide (20 μg/mL), Mifepristone (RU-486; 1 μmol/L), spironolactone (10 μmol/L), KT 5823 (1 μmol/L), wortmannin (10 nmol/L), cyclosporine A (CsA, 0.5 μmol/L) and bongkrekic acid (10 μmol/L) were purchased from Sigma. PP1 (20 μmol/L) was purchased from Biomol. Eplerenone (10 μmol/L) was kindly donated by Gador SA, Argentina. Apocynin (300 μmol/L) was from FLUKA. Glibenclamide (50 μmol/L) was purchased from RBI. AG 1478 (1 μmol/L) and MMP inhibitor (3 μmol/L) were from Calbiochem.

2.9. Statistics

Data are expressed as mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by Student–Newman–Keuls test. $P < 0.05$ was considered significant.

3. Results

We explored the effect of aldosterone on cardiac O_2^- production by the lucigenin-enhanced chemiluminescence method in myocardial slices [16]. Aldosterone induced a dose-dependent increase in the lucigenin-chemiluminescence signal indicating a stimulatory effect of the hormone on myocardial O_2^- production (Fig. 1A). To investigate the underlying signaling pathway involved in this effect as well as the source of O_2^-, we performed experiments to evaluate the influence of 10 nmol/L aldosterone in the presence of different inhibitors. This concentration of aldosterone is one of the most frequently used for in vitro studies [10,11,20,21] and showed submaximal stimulation of O_2^- production. Absolute baseline values among groups were not statistically different (Supplemental Fig. 1). Two different MR antagonists, spironolactone and eplerenone completely blocked the increased O_2^- production induced by aldosterone. Notably, the glucocorticoid receptor antagonist RU-486 (mifepristone) had no effect. Interestingly, the effect