Determinants of CREB degradation and KChIP2 gene transcription in cardiac memory

Nazira Özgen, MD, PhD,* David H. Lau, MD, PhD,† Iryna N. Shlapakova, MD,* Warren Sherman, MD,† Steven J. Feinmark, PhD,* Peter Danilo, Jr., PhD,* Michael R. Rosen, MD*‡

*From the Departments of *Pharmacology, †Medicine, and ‡Pediatrics, Columbia University, New York, New York.*

BACKGROUND Left ventricular pacing (LVP) to induce cardiac memory (CM) in dogs results in a decreased transient outward K current (I_{to}) and reduced mRNA and protein of the I_{to} channel accessory subunit, KChIP2. The KChIP2 decrease is attributed to a decrease in its transcription factor, cyclic adenosine monophosphate response element binding protein (CREB).

OBJECTIVE This study sought to determine the mechanisms responsible for the CREB decrease that is initiated by LVP.

METHODS CM was quantified as T-wave vector displacement in 18 LVP dogs. In 5 dogs, angiotensin II receptor blocker, saralasin, was infused before and during pacing. In 3 dogs, proteasomal inhibitor, lactacystin, was injected into the left anterior descending artery before LVP. Epicardial biopsy samples were taken before and after LVP. Neonatal rat cardiomyocytes (NRCM) were incubated with H_2O_2 (50 μmol/l) for 1 hour with or without lactacystin.

RESULTS LVP significantly displaced the T-wave vector and was associated with increased lipid peroxidation and increased tissue angiotensin II levels. Saralasin prevented T-vector displacement and lipid peroxidation. CREB was significantly decreased after 2 hours of LVP and was comparably decreased in H_2O_2–treated NRCM. Lactacystin inhibited the CREB decrease in LVP dogs and H_2O_2-treated NRCM. LVP and H_2O_2 both induced CREB ubiquitination, and the H_2O_2-induced CREB decrease was prevented by knocking down ubiquitin.

CONCLUSION LVP initiates myocardial angiotensin II production and reactive oxygen species synthesis, leading to CREB ubiquitination and its proteasomal degradation. This sequence of events would explain the pacing-induced reduction in KChIP2, and contribute to altered repolarization and the T-wave changes of cardiac memory.

KEYWORDS Cardiac memory; cAMP response element binding protein; Angiotensin II; Reactive oxygen species; Ubiquitination; Proteasomal degradation

ABBREVIATIONS **CREB** = cyclic adenosine monophosphate response element binding protein; **LA** = left atrium/atrial; **LAD** = left anterior descending; **LV** = left ventricle/ventricular; **NRCM** = neonatal rat ventricular cardiomyocytes; **ROS** = reactive oxygen species; **TFA** = trifluoroacetic acid.

(Heart Rhythm 2010;7:964–970)

Introduction

The T-wave changes of cardiac memory are a long-recognized phenomenon.[1] The persistent memory after long periods of ventricular pacing depends on a transcriptional process, the outcome of which is reduction of the transient outward current, I_{to},[2] and an altered ventricular repolarization gradient.[3,4] I_{to} is carried via a channel whose pore-forming unit in dogs and humans is Kv4.3.[5] The accessory protein KChIP2 influences both the magnitude and kinetics of I_{to}.[6]

The cyclic adenosine monophosphate response element binding protein (CREB) binds in the KChIP2 promoter region and seems necessary for baseline I_{to} expression.[7] For example, in regions of hearts treated with CREB antisense,[7] nuclear CREB is reduced and neither an action potential notch nor I_{to} is seen. During ventricular pacing of normal hearts for 1 to 2 hours or longer, nuclear CREB is also reduced and this reduction accompanies the T-wave changes of long-term memory. However, the mechanism for CREB reduction has not been determined in the setting of cardiac pacing.

In this study, we hypothesized that ubiquitination and proteasomal degradation are mechanisms by which CREB reduction occurs, and that these processes are initiated by pacing-induced angiotensin II production and synthesis of reactive oxygen species (ROS). Because the CREB reduction commences within 2 hours of onset of pacing, we used a 2-hour pacing protocol to test our hypothesis.

Methods

Experiments were performed using protocols approved by Columbia University's Institutional Animal Care and Use Committee and conform to the Guide for Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, revised 1996).

Supported by U.S. Public Health Service/National Heart, Lung, and Blood Institute grant HL-67101. **Address reprint requests and correspondence:** Dr. Nazira Özgen, College of Physicians and Surgeons, Center for Molecular Therapeutics, Department of Pharmacology, Columbia University, 630 West 168 Street, P&S 7-509, New York, New York 10032. E-mail address: no2127@columbia.edu. (Received February 5, 2010; accepted March 19, 2010.)

1547-5271/$ -see front matter doi:10.1016/j.hrthm.2010.03.024

Intact animal studies

We used previously described procedures[8] to isoflurane-anesthetize 10 adult male mongrel dogs (22 to 25 kg, Chestnut Ridge Kennels, Chippensburg, Pennsylvania) and implant bipolar pacing electrodes to the left atrium (LA) and the anterior wall of the left ventricle (LV). After 45-minute LA pacing, an epicardial reference biopsy sample was obtained 1 to 2 cm from the LV pacing electrode. We used a programmable stimulator (Bloom Associates, Reading, Pennsylvania) to AV sequentially pace (basic cycle length 400 ms, AV delay 50 ms), ensuring 100% ventricular capture for 2 hours. Additional LV epicardial biopsy samples were then taken 1 to 2 cm from the electrode. All biopsy samples were immediately frozen in liquid nitrogen.

In 5 additional dogs, we infused saralasin, a competitive angiotensin II inhibitor,[8,9] (0.5 μg/kg/min, intravenously) starting 30 minutes before LV pacing and continuing throughout 2 hours of LV pacing. Biopsy samples were taken as above.

In 3 additional dogs, we catheterized the left femoral artery with a 7-Fr introducer sheath, and engaged the left coronary artery ostium with a 6-Fr Amplatz left 0.75 guiding catheter (Cordis, Warren, New Jersey) under fluoroscopy. We infused the selective proteasomal inhibitor, lactacystin[10] (40 μmol/l, Boston Biochem, Cambridge, Massachusetts), via a transit microcatheter 2.8/2.5-Fr (Cordis) with the tip just distal to first diagonal branch. LA descending (LAD) vessel infusion began after 15 minutes of atrial pacing and continued for 30 minutes (total volume 15 ml). After the infusion, a reference biopsy sample was taken and AV sequential pacing was initiated. Two hours later, epicardial biopsy samples were taken, 1 from the LAD distribution within 2 cm of the ventricular pacing electrode, and the other from remote site.

Western blotting

Western blotting was performed as described previously[11] using CREB, ubiquitin (Cell Signaling Technology, Danvers, Massachusetts), PCNA (Abcam, Cambridge, Massachusetts), and Cyclophilin A (Upstate Biotech, Billerica, Massachusetts) antibodies.

Immunochemistry

Immunochemistry of sections from LV epicardial tissues before and after LV pacing or from neonatal rat ventricular cardiomyocytes (NRCM) treated with or without H_2O_2 was performed as previously described[12] using CREB antibody.

Angiotensin II measurement

Tissue angiotensin II levels were measured by enzyme-linked immunoassay (Peninsula Labs, Torrance, California) according to the manufacturer's instructions using a modified extraction procedure.[13,14] Angiotensin II was extracted from LV tissue that had been homogenized with a Polytron in 4 mol/l guanidine thiocyanate/1% trifluoroacetic acid (TFA) (250 mg tissue/10 ml buffer) containing phenylmethanesulfonylfluoride (0.1 mmol/l), aprotinin (1 ng/ml), leupeptin (1 ng/ml), pepstatin (0.1 μmol/l), and benzamidine (1 μg/ml). The homogenate was clarified (10,000g for 20 minutes) before application by gravity flow to a preconditioned Sep-Pak C18 column (Varian; prewashed with methanol 3 ml followed by 1 % TFA 10 ml). Then the column was washed 1% TFA/1% NaCl (5 ml) $\times$2, and MeOH/H_2O/TFA (30/69/1, v/v; 2 ml) $\times$2. The sample was eluted with MeOH/H_2O/TFA (80/19/1, v/v; in 2 portions 1 ml followed by 0.5 ml), evaporated to dryness, and reconstituted in assay buffer. Recovery of [^{3}H]angiotensin II was 75% $\pm$ 2%.

Lipid peroxidation measurement

Lipid peroxidation was measured in LV tissues using a colorimetric assay kit LPO-586 (Bioxytech, Portland, Oregon) that detects malondialdehyde and 4-hydroxyalkenal.

Intracellular ROS measurements

NRCM were loaded with dihydroethidium (10 μmol/l), a nonfluorescent membrane-permeant probe that interacts with O_2^- to liberate membrane-impermeant ethidium cations that fluoresce upon intercalating with nuclear DNA, in the presence of vehicle, angiotensin II (1 μmol/l), or H_2O_2 (50 μmol/l). Cell images were viewed every 3 to 5 minutes using a Zeiss LSM 510 NLO confocal microscope (excitation 470 to 490 nm, emission 510 to 550 nm).

Cardiomyocyte culture

NRCM were prepared as previously[11] with a density of 5×10^6 cells/100-mm dishes, and used in 4 different experiments: 1) treatment with 50 μmol/l H_2O_2 for different time intervals followed by Western blotting of whole-cell lysates for CREB and cyclophilin A; 2) treatment with 50 μmol/l H_2O_2 for different time intervals followed by Western blotting of cytosolic and nuclear fractions for CREB and PCNA; 3) treatment with angiotensin II for measurement of intracellular ROS; 4) treatment with lactacystin (10 μmol/l) $\times$30 minutes and 50 μmol/l H_2O_2 $\times$1 hour. Whole-cell lysates were then Western blotted for CREB.

Ubiquitin siRNA transfection

Ubiquitin siRNA knockdown was performed per manufacturer's instructions. NRCM were transiently transfected with ubiquitin siRNA (Santa Cruz Biotech, Santa Cruz, California). A scramble siRNA was used as a control. Silencing efficiency was evaluated by Western blotting using ubiquitin antibody (Stratagene, La Jolla, California). At 24 hours after transfection, cells were treated with H_2O_2 $\times$1 hour and whole-cell lysates were Western blotted.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Comparisons between group means were made by Student t-test except for angiotensin II, for which the Mann-Whitney U test was used. $P < .05$ was considered significant.