

Recovery in *ERG* gene expression with biventricular pacing in a rabbit model of myocardial infarction

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Background: Improved clinical and echocardiographic parameters have been documented with biventricular (BIV) pacing in patients after myocardial infarction (MI). We investigated the changes in gene expression in cardiac tissue with BIV pacing, using a rabbit model of MI.

Method: New Zealand White rabbits were divided into four groups: sham-operated controls, MI with no pacing, MI with right ventricle (RV) pacing (MI + RV), and MI with BIV pacing (MI + BIV). Pacing was initiated 1–2 weeks after the coronary ligation. At 5 weeks, the hearts were excised. The tissue extracted from the left ventricle (LV) and RV underwent analysis for protein and messenger ribonucleic acid (mRNA) levels.

Results: The ether-a-go-go-related gene (*ERG*) protein levels recovered from the base of the LV away from the MI area were two- to threefold lower in the MI and the MI + RV compared with the MI + BIV groups ($P = 0.07$). The *ERG* protein levels were similar between the MI + BIV and the control groups. However, the RNA levels were comparable between the four study groups, suggesting that a posttranscriptional mechanism accounted for the difference in protein levels.

Conclusion: In this rabbit model of MI, we demonstrated a recovery in *ERG* protein levels with BIV pacing, after MI. This recovery may underlie some of the benefits seen with BIV pacing in ischemic cardiomyopathy.

Keywords: heart failure, biventricular pacing, cardiac reverse remodeling, ether-a-go-go-related gene

Cardiac resynchronization therapy, also known as biventricular (BIV) pacing, is a modality used in the treatment of advanced heart failure.^{1–3} In patients with ventricular conduction abnormalities who continue to suffer from severe heart failure symptoms (New York Heart Association Class III–IV)^{1–3} despite optimal pharmacological therapy, BIV pacing can improve left ventricle (LV) muscle function and induce reverse mechanical remodeling, manifested as smaller LV end-systolic and end-diastolic dimensions. Clinical trials have also demonstrated the benefits of BIV pacing in improving the endpoints of death and hospitalization.^{2,3}

Despite this, little is known about the regional, cellular, and molecular mechanisms responsible for the benefits seen with BIV pacing. In a previous study,⁴ we demonstrated the prevention of remodeling with BIV pacing initiated at the time of myocardial infarction (MI), manifested as the preservation of LV function and dimensions as well as preservation of the protein levels of the ether-a-go-go-related gene (*ERG*) protein. In this current study, we investigated whether changes in the myocardial *ERG* protein levels can be demonstrated when the BIV pacing is initiated 1–2 weeks after the MI, which better simulates the clinical scenario observed with heart failure patients.

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Methods

Study design

The study protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee. New Zealand White rabbits were housed at the animal facility in individual cages and allowed free access to food pellets, hay, and water. The rabbits ($n = 12$; weight = 3.0–5.0 kg) were divided into four study groups: (1) sham-operated controls (C) ($n = 3$), in which the rabbits underwent pericardial stripping only; (2) MI with no pacing (MI group) ($n = 3$), in which the rabbits underwent pericardial stripping and coronary ligation but no pacing; (3) MI with right ventricle (RV) pacing (MI + RV group) ($n = 3$), in which the rabbits underwent pericardial stripping, coronary ligation, and RV pacing with the intent of creating ventricular dyssynchrony; and (4) MI with BIV pacing (MI + BV group) ($n = 3$), in which the rabbits underwent pericardial stripping, coronary ligation, and BIV pacing. Rabbits in the pacing groups were continuously paced at a rate of 270 beats per minute for 3–4 weeks beginning 1–2 weeks after coronary ligation and pacemaker implantation, depending on the rate of animal recovery after surgery. At the end of this period, they were sacrificed, and their hearts were excised. Cardiac tissue was collected from the base of the LV away from the MI site as well as from the RV. Tissue analysis was performed as described below.

Surgical preparation of the rabbits

The rabbits were anesthetized using an intramuscular injection of a mixture of ketamine (35 mg/kg) and xylazine (5 mg/kg). A 22 gauge intravenous catheter was inserted into the marginal ear vein for venous access. An arterial line was inserted into the middle ear artery, for continuous hemodynamic monitoring. The rabbits were intubated with an endotracheal tube (3.0 mm ID) and mechanically ventilated (rate 40/min, tidal volume 15 mL) using room air enriched with oxygen. Isoflurane anesthesia (2.0%–2.5%) was delivered, to maintain general anesthesia during surgery. The rabbits were placed on a water blanket adjusted to 38°C. A pulse oxymeter was placed on the rabbit's tongue for continuous monitoring of oxygen saturation.

The chest was opened through the fourth left intercostal space. The heart was exposed through an incision of the pericardium and explored. The MI was induced by ligating the posterolateral branch of the left coronary system, using a 5-0 Prolene™ suture (Ethicon Inc, Somerville, NJ, USA) at a level midway between the atrioventricular groove and the apex. Successful ligation was confirmed by the presence of

myocardial cyanosis, with bulging and ST-segment changes in the amplified electrocardiogram (ECG) signal. These changes in the surface ECG were previously described in detail by our group.⁴ Lidocaine (1–4 mg/kg) was administered pre- and postligation as an antiarrhythmic agent, and a prophylactic antibiotic (Ancef 100 mg intravenous [IV]) was administered before and after surgery.

Pacemaker implantation

Pacemaker leads were sutured to the epicardial surface of both the RV and LV, or on the RV only, depending on the study group of the rabbit. For RV pacing, the lead was sutured to the RV free wall, close to the apex. For LV pacing, the lead was sutured to the LV free wall, close to the base of the heart. The leads (model 4965 and model 4968; Medtronic, Inc, Minneapolis, MN, USA) were connected to a permanent pacemaker configured to pace the RV alone or both the RV and LV simultaneously. In case of BIV pacing, a Y-connector (model 2872; Medtronic) was used to pace the RV and LV simultaneously from a single-chamber pacemaker (Kappa KSR403; Medtronic), programmed through research software (Medtronic Inc) to pace at a fast rate of 270 beats per minute (slightly above the encaged rabbits' ambulatory heart rate). The pacemaker and leads were placed in a subcutaneous pocket in the abdominal area of the rabbit.

Cellular and molecular analysis of cardiac tissue

The hearts were excised from the rabbits, and 200 mg samples from the base of the LV free wall and the RV free wall were collected. We performed immunoblots (Western blots) using anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Bethyl Laboratories, Inc., Montgomery, TX, USA) anti-Erg (Life Technologies Corp, Carlsbad, CA, USA), anti-KVLQT1 (Alomone Labs, Jerusalem, Israel), and anti-Kv4.3 (Alomone Labs). As previously described,⁵ crude membrane preparations were isolated, by differential centrifugation, from the same regions of the hearts described above. Channels were solubilized with 1% sodium dodecyl sulfate (SDS), quantitated (Bio-Rad Laboratories, Hercules, CA, USA), and ~100 µg of protein per lane was run on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel, transferred by semidry apparatus, blocked with phosphate-buffered saline (PBS [Cellgro, Manassas, VA, USA])-5% milk, incubated overnight at 4°C with the primary antibody, washed with PBS-Tween® (Bio-Rad Laboratories, Inc., Hercules, CA, USA), incubated with horseradish peroxidase (HRP)-conjugated

chicken anti-rabbit 2° antibody (Amersham Biosciences, Piscataway, NJ, USA),[®] and quantitated by chemiluminescence (Perkin-Elmer, Waltham, MA, USA). Coomassie Blue (Life Technologies, Grand Island, NY, USA) staining of the SDS-PAGE blots was performed to confirm equal loading. Custom-made polyclonal antibodies to rabbit ERG, directed against the peptide RQRKRKLSFRRRTD (AA 885-898), were generated in the chicken (affinity-purified IgY; Life Technologies). Preimmune IgY, from egg yolk, was used to determine the SERCA2a- and ERG-specific bands.

The autoradiographs were scanned using a Visioneer OneTouch 9220 scanner (Visioneer Inc, Pleasanton, CA, USA) and Scanner and Camera Wizard software (Microsoft Corp, Redmond, WA, USA). The images were digitized for analysis using Quantity One™ quantitation software (Bio-Rad). Bands of interest were delineated and densitometries were calculated, based on the number of pixels. We performed Western blot analyses on tissue from all rabbits of each of the C, MI, MI + RV, and MI + BIV groups.

Messenger ribonucleic acid (mRNA) levels

ERG gene expression levels of mRNA levels were determined by fluorescence-based kinetic real-time polymerase chain reaction (RT-PCR) using an Applied Biosystems ABI PRISM[®] model 7000 (Life Technologies) sequence detection system. Total RNA was isolated using TRIzol[®] reagent (Life Technologies), as described elsewhere.^{6,7} First, strand complementary deoxyribonucleic acid (cDNA) synthesis was performed with 1 µg of RNA and SuperScript[®] III RNase H⁻ Reverse Transcriptase (200 u/µL) (Life Technologies), and the RT-PCR reaction was performed using 1 µg of cDNA, Absolute QPCR SYBR Green ROX Mix (Thermo Fisher Scientific, Waltham, MA, USA), and rabbit-specific ERG forward: 5'-CAGGCACCACGCATCCA-3' and reverse: 5'-CAGTCCCACACAGCCTTGAA-3' primers.⁶ The quantification of the ERG PCR product was performed with ABI PRISM 7000 SDS software v 1.1, and the expression of *ERG* was compared with the gene expression of β-actin using rabbit specific forward: 5'-CTGGCTGGC-CGCGACCT-3' and reverse: 5'-GAACCGCTCATTGC-CAATGGG-3' β-actin primer pairs with a similar RT-PCR condition as that of *ERG*. The threshold cycle (C_T) number at which the detectable fluorescence for the ERG product exceeded the background was compared with the β-actin product, for each sample.

Data analysis

Data is presented as mean ± standard error unless otherwise indicated. Continuous variables were compared by the Fischer's exact test between the various study groups. Bar graphs were constructed for all analyses and visually inspected. A one-sided *P*-value less than ≤0.05 was considered statistically significant and ≤0.1 was considered to indicate a strong trend towards significance. All statistical analyses and graphs were performed on SPSS software v 13 (IBM, Armonk, NY, USA).

Results

Myocardial infarction

As previously described,⁴ all myocardial infarctions were ascertained at the time of the coronary ligation of the posterolateral coronary artery, by myocardial blanching and bulging, as well as by ST elevations on the surface ECG (Figure 1). As previously shown,⁴ when the coronary artery is ligated halfway between the LV base and apex, the sizes of the MIs are predictable and comparable between study groups.

Tissue analysis

Tissues were collected from rabbits in the C, MI, MI + RV, and MI + BIV groups from prespecified regions of the LV and RV, with focus on the normal myocardial zone, away from the infarcted area. Testing the expression of the K⁺ channels responsible for repolarization showed no differences in the expression of Kv4.3 or KVLQT1, between the groups (data not shown).

Compared with the C group, ERG protein levels, adjusted for nicotinamide adenine dinucleotide phosphate (NAPDH) loading, obtained from tissues collected from the LV base were two to threefold reduced in the MI group and the MI + RV groups compared with both the MI + BIV and C groups (*P* = 0.07) (Figure 2). In fact, ERG levels in the MI + BIV group were comparable to those of the C group (*P* = 0.49). These difference were not seen in the RV free wall tissue, where the mere presence of pacing (MI + RV and MI + BIV groups) resulted in a significant increase (greater than threefold) in the ERG protein expression (*P* = 0.01 and *P* = 0.08, respectively).

mRNA levels

The levels of mRNA for the *ERG* gene were not different among the four groups of rabbits (Figure 3), suggesting a posttranscription mechanism was responsible for the reduction in the LV ERG protein levels in the MI and MI + RV groups compared with the C and MI + BIV groups.

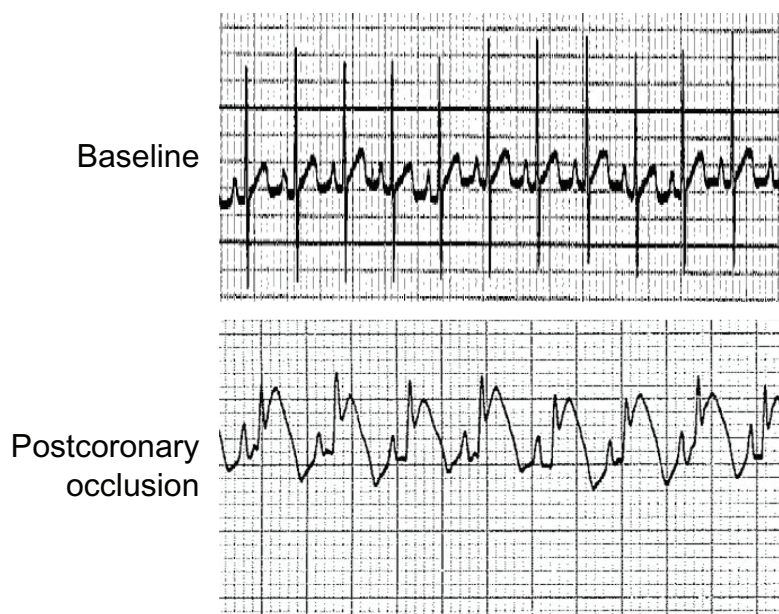


Figure 1 Surface electrocardiogram lead II demonstrating baseline and postcoronary occlusion tracings in a rabbit included in the MI + BIV study group.
Notes: Paper speed 25 mm/second; resolution 20 mm/mV. Note the significant ST elevation after coronary occlusion and the loss of R wave amplitude.
Abbreviation: MI + BIV, myocardial infarction plus biventricular pacing.

Discussion

In this study, we present a rabbit model of MI and pacing, in which we demonstrated the recovery of ERG protein levels with BIV pacing compared with RV pacing or no pacing, to levels comparable with those of C group rabbits. This was shown in the normal areas of myocardium away from the

infarcted LV zone. The mechanism underlying the differences in ERG protein levels must have been posttranscriptional given the identical mRNA levels in the four study groups, possibly related to the translation of mRNA to protein or to other mechanisms, such as protein trafficking to the cell membrane. Our data shows that BIV pacing prevents changes in ERG

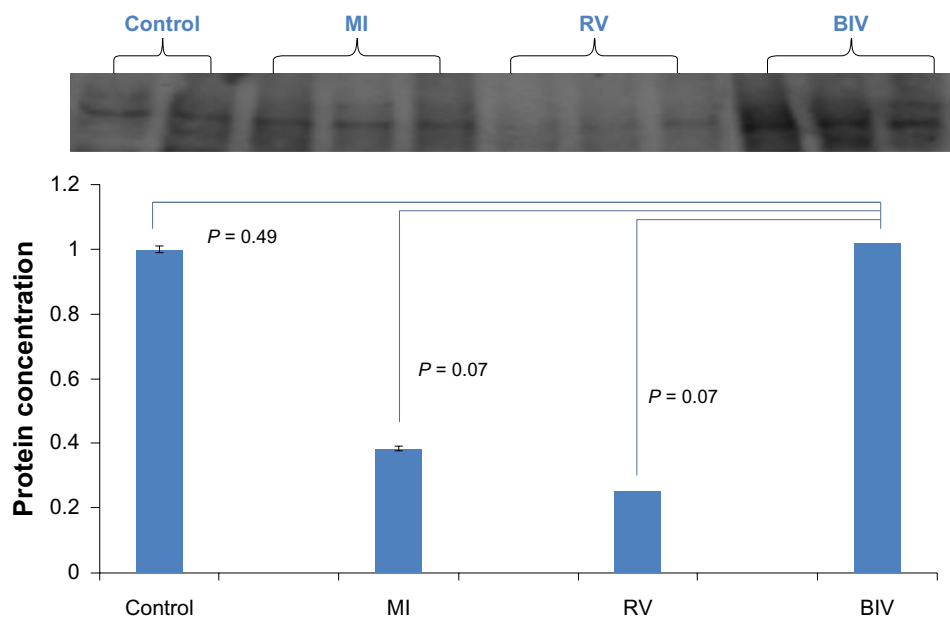


Figure 2 Western blot showing ERG protein levels in two rabbits from the C group and three rabbits from each of the MI, MI + RV, and MI + BIV study groups.
Notes: Underneath the Western blot gel, a bar graph represents the levels of ERG protein, corrected for the loading of the gel, with the 45 kD band used as a reference. Note that ERG protein levels are reduced by a factor of two- to threefold in the MI ($P < 0.07$) and MI + RV ($P < 0.07$) groups compared with the control and MI + BIV groups.
Abbreviations: BIV, biventricular pacing; C, control group; ERG, ether-a-go-go-related gene; MI, myocardial infarction; MI + BIV, myocardial infarction plus biventricular pacing; MI + RV, myocardial infarction plus right ventricular pacing; RV, right ventricular pacing.

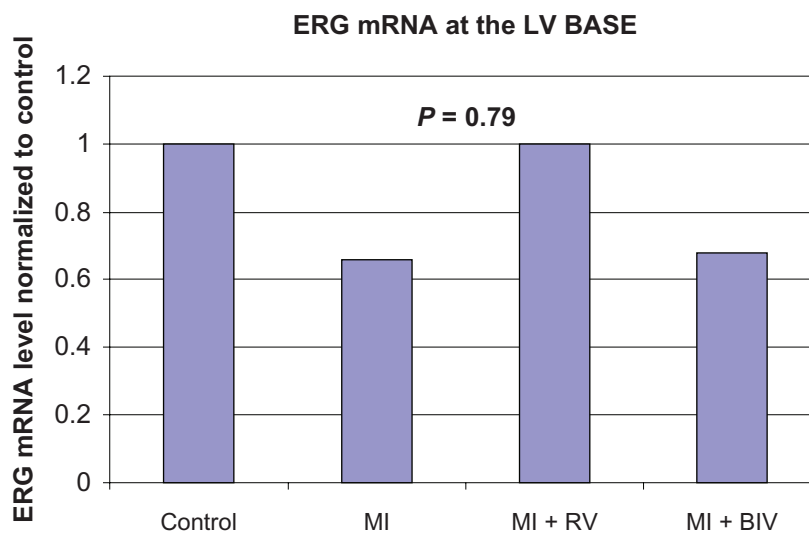


Figure 3 mRNA levels of the *ERG* gene demonstrating no significant differences among these four groups of rabbits.

Abbreviations: BIV, biventricular pacing; ERG, ether-a-go-go-related gene; LV, left ventricle; MI, myocardial infarction; MI + BIV, myocardial infarction plus biventricular pacing; MI + RV, myocardial infarction plus right ventricular pacing; mRNA, messenger ribonucleic acid.

level that are associated with MI, which may underlie some of the mechanisms of benefit of cardiac resynchronization therapy. In keeping with our previous findings,⁴ our present data demonstrate similar changes in the ERG protein levels despite the fact that BIV pacing was not initiated immediately at the time of the MI but rather a week later, simulating real clinical scenarios. ERG is a human protein that enters into the composition of the rapid rectifier potassium channel in myocardial cells, responsible primarily for myocardial repolarization. ERG protein deficiencies have been implicated in inherited and acquired cardiac arrhythmogenesis, leading to sudden cardiac death. In the rabbit model, the ERG protein responds differently to RV pacing under physiological conditions, where its levels increase,⁸ versus disease conditions, such as the aftermath of myocardial infarction, where its levels decrease.⁴ However, the exact mechanism underlying these changes and the role of BIV pacing in restoring ERG levels remain unclear and require further investigation.

The choice of the rabbit model for the present study was driven by numerous considerations, primarily our established experience with this model.^{4,8,9} Our choice of the rabbit in our earlier study of the effect of pacing after MI was dictated by several inherent characteristics of the rabbit heart.^{10–14} The rabbit heart has minimal collateral arteries and no transmural gradient in the collateral blood flow, which leads to MIs that are predictable in size and location. Also, arrhythmic deaths after coronary ligation are less frequent in the rabbit compared with other species, such as dogs.¹³ For these reasons, rabbits have served as a useful animal model for MI provoked by coronary artery ligation. Also, using coronary

artery ligation (on the basis of a bifurcation/trifurcation classification) in the rabbit, a reliable chronic heart failure model was developed,¹⁵ which was the model that we adopted in the current study. The rabbit is also a good model system for electrophysiologic changes. The rabbit cardiac action potential is similar to that of humans, and the underlying channels and currents are also homologous.^{16–20} Moreover, the rabbits' size is large enough to accommodate the implantation of permanent pacemakers designed for humans (but modified to pace at fast rates). For all these reasons, we chose to use the rabbit again, in our present study.

Although human studies have focused on the effect of BIV pacing at the organ level, demonstrating the reduction in LV dyssynchrony²¹ and mitral regurgitation,²² limited knowledge has been accumulated over the past several years as to the cellular and molecular mechanisms involved in the effect of pacing on myocardial function alteration. The present study narrows the gap in knowledge, by documenting changes in the protein expression of genes involved in the ventricular repolarization process. Such an effect may explain the lower incidence of lethal arrhythmias with BIV pacing compared with no pacing, in patients with advanced heart failure.²³

The present study has a number of limitations. First, the pacing in our rabbit model was initiated 1–2 weeks after coronary ligation, unlike the case in humans, where pacing usually commences years after the MI. However, based on our previous studies, many of the electrical and mechanical remodeling changes occur within this short time frame. It is reasonable therefore, to conclude that BIV pacing works by reversing the adverse remodeling of MI and not only by

preventing it. Second, in our model, pacing the RV was done epicardially, which is different from the endocardial pacing typically used in humans, in clinical practice. It is unclear whether this difference significantly affects our findings. Third, in our model, the rabbits did not exhibit overt signs of heart failure. This is different from the original indications for BIV pacing. However, more recent data demonstrate benefits from BIV pacing in even mild forms of heart failure.²⁴ Lastly, the number of animals included in this study was small thus precluding statistical significance despite the high magnitude of recovery in ERG protein levels (about threefold compared with MI + RV group).

In summary, we present a rabbit model of MI and pacing that demonstrates recovery of ERG LV protein expression with BIV pacing. This may underlie some of the benefits of cardiac resynchronization therapy post MI.

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Disclosure

The authors report no conflicts of interest in this work.

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