Targeted Antioxidant Treatment Decreases Cardiac Alternans Associated With Chronic Myocardial Infarction

Bradley N. Plummer, PhD; Haiyan Liu, MD; Xiaoping Wan, MD, PhD; Isabelle Deschênes, PhD; Kenneth R. Laurita, PhD

Background—In myocardial infarction (MI), repolarization alternans is a potent arrhythmia substrate that has been linked to Ca\(^{2+}\) cycling proteins, such as sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA2a), located in the sarcoplasmic reticulum. MI is also associated with oxidative stress and increased xanthine oxidase (XO) activity, an important source of reactive oxygen species (ROS) in the sarcoplasmic reticulum that may reduce SERCA2a function. We hypothesize that in chronic MI, XO-mediated oxidation of SERCA2a is a mechanism of cardiac alternans.

Methods and Results—Male Lewis rats underwent ligation of the left anterior descending coronary artery (n=54) or sham procedure (n=24). At 4 weeks, optical mapping of intracellular Ca\(^{2+}\) and ROS was performed. ECG T-wave alternans (ECG ALT) and Ca\(^{2+}\) transient alternans (Ca\(^{2+}\)ALT) were induced by rapid pacing (300–120 ms) before and after the XO inhibitor allopurinol (ALLO, 50 \(\mu\)mol/L). In MI, ECG ALT (2.32±0.41%) and Ca\(^{2+}\) ALT (22.3±4.5%) were significantly greater compared with sham (0.18±0.08%; \(P<0.001\); 0.79±0.32%; \(P<0.01\)). Additionally, ROS was increased by 137% (\(P<0.01\)) and oxidation of SERCA2a by 30% (\(P<0.05\)) in MI compared with sham. Treatment with ALLO significantly decreased ECG ALT (−77±9%; \(P<0.05\)) and Ca\(^{2+}\) ALT (−56±7%; \(P<0.05\)) and, importantly, reduced ROS (−65%; \(P<0.01\)) and oxidation of SERCA2a (−38%, \(P<0.05\)). CaMKII inhibition and general antioxidant treatment had no effect on ECG ALT and Ca\(^{2+}\) ALT.

Conclusions—These results demonstrate, for the first time, that in MI, increased ROS from XO is a significant cause of repolarization alternans. This suggests that targeting XO ROS production may be effective at preventing arrhythmia substrates in chronic MI. (Circ Arrhythm Electrophysiol. 2015;8:165-173. DOI: 10.1161/CIRCEP.114.001789.)

Key Words: allopurinol ■ arrhythmia (mechanisms) ■ myocardial infarction ■ oxidative stress ■ SERCA2a

Sudden cardiac death following myocardial infarction (MI) is the most common cause of mortality from heart disease. F"{"u}rthermore, declining death rates from acute MI have increased the population of patients with chronic MI that are at risk for developing ventricular arrhythmias. In patients with chronic MI, cardiac repolarization alternans is a potent arrhythmia substrate. As we and others have shown, abnormal intracellular Ca\(^{2+}\) cycling is a well-established mechanism of alternans. Specifically, we have recently shown that the sarcoplasmic reticulum (SR) Ca\(^{2+}\) ATPase (SERCA2a), an important regulator of SR Ca\(^{2+}\) reuptake, can significantly modulate the magnitude of alternans.

Reactive oxygen species (ROS) production is increased in many diseases, including ischemic heart disease. Acute ROS exposure in isolated myocytes and whole hearts has been shown to significantly disrupt intracellular Ca\(^{2+}\) regulation and promote a broad range of arrhythmia substrates. Interestingly, however, heart disease patients do not see a reduction in cardiac events with general antioxidant therapy, which raises 2 interesting possibilities: either increased oxidative stress is not a determinant of cardiac events, or targeting a specific source of ROS is more important. Support of the latter is evidenced by Sovari et al, who have recently shown that targeting mitochondrial ROS is effective at suppressing in vivo arrhythmias associated with connexin proteins in a nonischemic heart failure model.

Xanthine oxidase (XO) is a superoxide-producing enzyme that is localized to the SR. We have recently shown that inhibition of XO activity can normalize the oxidative status of key SR Ca\(^{2+}\) regulatory proteins. Furthermore, in MI, XO activity is increased. Based on this and the prominent role SERCA2a plays in Ca\(^{2+}\) cycling, we hypothesize that in chronic MI, XO-mediated oxidation of SERCA2a is a mechanism of cardiac alternans.

Methods

Chronic MI Model

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85–23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of Case Western Reserve University. Male Lewis rats underwent ligation of the left anterior descending coronary artery (n=54) or sham procedure (n=24). At 4 weeks, optical mapping of intracellular Ca\(^{2+}\) and ROS was performed. ECG T-wave alternans (ECG ALT) and Ca\(^{2+}\) transient alternans (Ca\(^{2+}\)ALT) were induced by rapid pacing (300–120 ms) before and after the XO inhibitor allopurinol (ALLO, 50 \(\mu\)mol/L). In MI, ECG ALT (2.32±0.41%) and Ca\(^{2+}\) ALT (22.3±4.5%) were significantly greater compared with sham (0.18±0.08%; \(P<0.001\); 0.79±0.32%; \(P<0.01\)). Additionally, ROS was increased by 137% (\(P<0.01\)) and oxidation of SERCA2a by 30% (\(P<0.05\)) in MI compared with sham. Treatment with ALLO significantly decreased ECG ALT (−77±9%; \(P<0.05\)) and Ca\(^{2+}\) ALT (−56±7%; \(P<0.05\)) and, importantly, reduced ROS (−65%; \(P<0.01\)) and oxidation of SERCA2a (−38%, \(P<0.05\)). CaMKII inhibition and general antioxidant treatment had no effect on ECG ALT and Ca\(^{2+}\) ALT.

Conclusions—These results demonstrate, for the first time, that in MI, increased ROS from XO is a significant cause of repolarization alternans. This suggests that targeting XO ROS production may be effective at preventing arrhythmia substrates in chronic MI. (Circ Arrhythm Electrophysiol. 2015;8:165-173. DOI: 10.1161/CIRCEP.114.001789.)

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WHAT IS KNOWN

- In myocardial infarction (MI), cardiac alternans is an important arrhythmia substrate that has been linked to abnormal calcium cycling.
- MI is also associated with oxidative stress and increased xanthine oxidase activity, an important source of reactive oxygen species.

WHAT THE STUDY ADDS

- Cardiac alternans observed in chronic MI is associated with increased reactive oxygen species in parallel with reduced intracellular calcium cycling.
- In chronic MI, decreasing oxidation of sarcoplasmic reticulum Ca\(^{2+}\) ATPase enhances calcium cycling and reduces cardiac alternans.
- Xanthine oxidase–mediated oxidation of sarcoplasmic reticulum Ca\(^{2+}\) ATPase is an important mechanism of cardiac alternans in chronic MI.

the Institutional Animal Care and Use Committee of Case Western Reserve University. To create a model of chronic MI, we performed permanent ligation of the left anterior descending coronary artery in male Lewis rats weighing 300 to 350 g (n=54), as described previously. Sham (n=24) surgeries were performed as controls, and animals with no procedure (n=4) were used to assess nonspecific drug effects. After 4 weeks, animals were euthanized for optical mapping and tissue sample analysis.

Optical Mapping

Rats were anesthetized (0.3 cc Ketamine/0.1 cc Xylazine, IP), and their hearts were rapidly removed via medial thoracotomy and Langendorff perfused with oxygenated (95% O\(_2\)–5% CO\(_2\)) Tyrode’s solution containing (mmol/L): 137 NaCl, 5.4 KCl, 3.0 CaCl\(_2\), 1 MgSO\(_4\), 5.0 dextrose, and 10 HEPES (pH 7.45, 34°C). Perfusion pressure was maintained between 50 and 70 mm Hg by regulating coronary flow using a pulsatile flow system. To measure intracellular Ca\(^{2+}\), hearts were stained with the Ca\(^{2+}\)-sensitive indicator Indo-1 or untreated fluorescence measurements were normalized to the baseline. All samples were then treated with mBB (5 mmol/L) or its inactive analogue KN-92 (0.5 mmol/L) or its inactive analogue KN-92 (0.5 mmol/L) and reduced intracellular Ca\(^{2+}\) was measured using the free-thiol-specific fluorescence ratio (SERCA2a-SOx) was measured using the free-thiol-specific fluorescence ratio (SERCA2a-SOx). SERCA2a oxidation (SERCA2a-SOx) was measured using the free-thiol-specific fluorescence ratio (SERCA2a-SOx). Monobromobimane Assay

SERCA2a oxidation (SERCA2a-SOx) was measured using the free-thiol-specific fluorescence ratio (SERCA2a-SOx).

ROS Measurement in Frozen Sections

Fresh heart tissue was frozen in Tissue-Tek OCT, sectioned into 20 µm slices along the short axis, washed with PBS, and stained with 10 µmol/L DCF for 30 minutes at 37°C. DCF fluorescence was then imaged using an epifluorescence microscope (Olympus).

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Experimental Protocol

Decremental rapid pacing (300–120 ms) was used to elicit Ca\(^{2+}\) transients alternans under baseline conditions in sham and MI hearts. To inhibit XO, allopurinol (ALLO, 50 µmol/L; Sigma-Aldrich) was perfused for 15 minutes. To test for CaMKII activity, KN-93 (0.5 µmol/L) or its inactive analogue KN-92 (0.5 µmol/L) was perfused for 15 minutes. The nonspecific antioxidant N-acetylcysteine (10 µmol/L) was perfused for 15 minutes to scavenge ROS. After each treatment (performed in separate animals), the alternans induction protocol was repeated. Finally, arrhythmia incidence was assessed by quantifying the occurrence of extra beats or ventricular tachycardia/ventricular fibrillation after termination of rapid pacing (120 ms) with one-to-one capture.

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Data Analysis

ECG T-wave alternans (ECG ALT) was measured by signal averaging consecutive beat pairs over a 5 s window. The beat averages were time aligned by the pacing artifact, and the peak-to-peak amplitude was normalized to 100%. Then, the average percent difference between beat pairs from the end of the QRS to the end of the T wave was calculated. Ca²⁺ transient alternans (Ca²⁺ ALT) were measured as the difference in amplitude between 2 consecutive beats, expressed as a percentage of the largest beat and averaged across all 256 channels within the mapping field. For regional analysis, any site within 2 mm of the visible scar was considered border zone and all other sites were considered remote. Ca²⁺ transient duration was calculated from the time of release to 85% of diastolic values. For the optical mapping measurements of ROS, DCF fluorescence (F) at each site was normalized to the mean background fluorescence (F₀) at each site was normalized to the mean background fluorescence (F₀) after staining from an area of viable tissue representing 5% of the mapping field. For each heart, DCF fluorescence was quantified by averaging over all recording sites. In a couple of experiments, signal quality near the scar was not suitable for analysis or 1-to-1 pacing capture was lost. Such experiments were not included in the analysis. For unpaired comparisons with small sample size, Wilcoxon rank-sum test was used. Otherwise, student’s paired and unpaired t-tests were performed where appropriate. In situations with unequal data variance, we used the Welch t test with unpooled variance and Satterthwaite equation. Fisher exact test was used for statistical analysis of categorical data. Comparisons were considered significant for P values <0.05. Values reported are mean±SE.

Results

Cardiac alternans is significantly increased in MI compared with sham-operated animals. Shown in Figure 1A are representative ECG recordings from isolated sham and MI hearts measured during steady state pacing at a cycle length (CL) of 120 ms. In the sham heart, 2 consecutive beats (a and b) are superimposed and demonstrate the absence of ECG ALT (beats a and b are identical). In contrast, ECG ALT is evident in the MI heart. Over all animals tested, ECG ALT measured at the same CL is significantly higher in MI (2.3±0.41%, n=20) compared with shams (0.18±0.08%, n=8, P<0.001).

Similarly, Ca²⁺ ALT is larger in MI hearts compared with shams. This can be seen in the Ca²⁺ recordings shown in Panel B that demonstrate the absence of beat-to-beat Ca²⁺ ALT in a sham heart and large Ca²⁺ ALT in a heart with MI paced at the same CL (120 ms). Similar to ECG ALT, Ca²⁺ ALT is significantly higher in MI (22.3±4.5%, n=7) compared with shams (0.79±0.32%, n=8, P<0.01). Shortening fraction is significantly less in MI 25±4% compared with shams 55±2% (P<0.01). These data demonstrate that Ca²⁺ ALT and global repolarization alternans (ECG ALT) are higher in MI compared with shams.

Previous studies have associated a slower decay of the Ca²⁺ transient with greater cardiac alternans. In the present study, we also found that the duration of the Ca²⁺ transient, which depends on the decay phase of the Ca²⁺ transient, is longer in MI compared with shams. Figure 2A shows representative examples of Ca²⁺ transients recorded from a sham and MI heart at the same pacing CL (300 ms). When measured at 85% of Ca²⁺ transient amplitude, the durations are on average significantly longer in MI (n=6) compared with sham (n=5, P<0.05). The decay of the Ca transient is also slower in MI (119±7 ms) compared with sham (101±6 ms). Interestingly, we found no significant change in the expression of SERCA2a (Panel B) in animals with MI (1.2±0.03 AU) compared with shams (1.1±0.12 AU, P=0.31). These results suggest that the
increase in cardiac alternans observed in MI is because of slower \( Ca^{2+} \) cycling, which may be explained by decreased SERCA2a function rather than reduced expression.

When \( Ca^{2+} \) ALT is compared across the heart surface, the largest levels are observed near the scar. Shown in Figure 3 are representative examples of the spatial distribution of \( Ca^{2+} \) ALT in a sham and MI heart paced at the same CL (120 ms). In the sham heart, no \( Ca^{2+} \) ALT is observed across the mapping field. In contrast, in the MI heart, \( Ca^{2+} \) ALT is maximum near the scar (dashed outline). In the center of the scar, no \( Ca^{2+} \) ALT is observed; however, this is because \( Ca^{2+} \) signals are absent due to poor dye perfusion or the presence of nonviable tissue.

The high level of \( Ca^{2+} \) ALT and prolonged \( Ca^{2+} \) transient duration observed in MI hearts may be related to increased ROS associated with MI. Images of DCF fluorescence measured in an isolated MI heart using optical mapping techniques reveal increased ROS, especially near the scar (Figure 4). In contrast, ROS is much lower from the same region in a sham heart. When ROS levels are normalized for each animal, significantly higher levels \((136.9\pm39\%, P<0.01)\) are observed in MI hearts \((n=8)\) compared with shams \((n=7)\). In separate experiments, several hearts were prepared for in situ ROS detection in tissue slices using DCF (Panel B). This method, which is independent of dye perfusion (unlike measurements in the whole heart), also showed increased levels of ROS associated with MI. Shown at the bottom (Panel C) is \( Ca^{2+} \) ALT plotted against ROS for SHAM hearts and for MI hearts that were divided into remote and border zone regions. Overall, \( Ca^{2+} \) ALT is positively correlated with ROS levels.

It is possible that increased ROS associated with MI is responsible for the increased \( Ca^{2+} \) ALT we observed. In MI hearts treated with ALLO, a specific inhibitor of XO, ROS \((1.3\pm0.1 F/F_0, n=8)\) was significantly lower compared with MI \((3.7\pm2.8 F/F_0, n=8, P<0.005)\), as demonstrated in a representative example (Figure 4A, ALLO+MI). Cardiac alternans was also measured before and after the administration of ALLO. Shown in Figure 5A are ECG ALT and \( Ca^{2+} \) ALT in MI before (MI) and then after ALLO administration (MI+ALLO). The representative traces show that ALLO essentially eliminated beat-to-beat ECG ALT and \( Ca^{2+} \) ALT. On average, ALLO significantly decreased ECG ALT \((-77\pm9\%, P<0.05)\) and \( Ca^{2+} \) ALT \((-56\pm7\%, P<0.05)\) in paired comparisons \((n=5)\). \( Ca^{2+} \) ALT contour maps from an MI heart before and after ALLO demonstrate a significant reduction of \( Ca^{2+} \) ALT (Panel B). Panel C demonstrates the effectiveness of ALLO (open circles) compared with MI alone (filled circles) on \( Ca^{2+} \) ALT and ROS for border zone and remote regions. Time controls in MI animals showed no change in alternans over a similar time period \((0.1\pm4\%, P=ns)\). Finally, arrhythmia incidence was reduced in MI+ALLO \((11\%)\) compared with MI \((44\%)\), but this did not reach statistical significance.

Previous studies have shown that ROS decreases SERCA2a function,\(^1^2\) which may explain why \( Ca^{2+} \) transients are longer in MI. If so, inhibiting XO ROS with ALLO should shorten \( Ca^{2+} \) transient duration. Shown in Figure 6 are \( Ca^{2+} \) transients measured before and after ALLO treatment. The representative traces show that ALLO decreases \( Ca^{2+} \) transient duration
However, as shown in Figure 8, when hearts were treated with KN-93 or its inactive analog KN-92, no significant change in Ca\(^{2+}\) ALT was observed. In comparison, treatment with ALLO (\(\Delta\text{Ca}^{2+}\) ALT calculated from Figure 5) caused a significant decrease in Ca\(^{2+}\) ALT. Similarly, acute treatment with the general antioxidant, N-acetylcysteine, had no effect on alternans. In addition, we have previously shown that ALLO has no effect on \(I_{\text{Ca,L}}\) and in the present study, we found no change in peak \(I_{\text{Ca,L}}\) or \(I_{\text{Ca,L}}\) inactivation (inset) from MI hearts compared with shams (\(P=\text{ns}\)). However, the reversal potential was increased in MI (64±2 mV) compared with sham (55±1 mV, \(P<0.01\)). Nevertheless, changes in \(I_{\text{Ca,L}}\) cannot explain the occurrence of repolarization alternans in our model of MI or the effects of ALLO. It is also possible that ALLO decreased APD, which could also decrease Ca\(^{2+}\) ALT independent of any changes in Ca\(^{2+}\)regulation. However, QT interval, if anything, was slightly prolonged by 16±7 ms with ALLO; however, this did not reach statistical significance. Finally, in control animals (n=4), ALLO had no effect on ECG QT interval (1±1%, \(P=\text{ns}\)), ECG QRS width (0±4%, \(P=\text{ns}\)), CaD (1±1% \(P=\text{ns}\), or the decay of the Ca\(^{2+}\) transient (1±1%, \(P=\text{ns}\)). Taken together, these data demonstrate that cardiac alternans associated with MI is due to, in large part, XO-mediated oxidation of SERCA2a.

**Discussion**

The primary findings of this report are (1) ECG T-wave and Ca\(^{2+}\) transient alternans in chronic MI are associated with elevated ROS and reduced SR Ca\(^{2+}\) cycling, (2) in chronic MI, decreased SERCA2a-SOx enhanced SR Ca\(^{2+}\)cycling and reduced ECG T-wave and Ca\(^{2+}\) transient alternans, and (3) increased ROS from XO is an important mechanism of SERCA2a-SOx and, thus, cardiac alternans in chronic MI. Importantly, this study is the first to report that XO-mediated oxidation of SERCA2a plays a significant role in repolarization alternans associated with MI. These results increase the growing body of evidence that suggests arrhythmic ROS production is ubiquitous in cardiovascular disease and that...
targeting specific oxidant sources may be effective at preventing sudden cardiac death.

Alternans Mediated by Oxidative Stress in Chronic MI

Like acute ischemia, chronic MI has been previously associated with cardiac alternans in experimental models and in humans. However, much less is known about the mechanisms of alternans in the setting of chronic MI. Previous studies have shown that oxidative stress is increased in MI and that increased oxidative stress can disrupt Ca2+ cycling. For example, Belevytch et al. have shown that oxidative stress is increased in MI and that increased oxidative stress can disrupt Ca2+ cycling. This is consistent with previous reports showing that reduced SERCA2a is a well-known mechanism of Ca2+ transient and repolarization alternans. In the present study, we show that alternans associated with chronic MI is secondary to increased ROS and resultant SERCA2a-SOx. Our results could have also been explained by decreased SERCA2a expression; however, this is not what we found, and decreased SERCA2a expression is not a universal finding in chronic MI.

CaMKII activity is increased in heart disease and has been previously shown to significantly modulate Ca2+ regulatory proteins and cause arrhythmias. In addition, modeling studies have shown that increased CaMKII activity is linked to increased cardiac alternans. Because ROS can activate CaMKII, it is possible that the increase in alternans we observed is caused by increased CaMKII activity. Surprisingly, we saw no significant effect of the CaMKII inhibitor KN-93 or its inactive analogue KN-92 on alternans activity. This does not necessarily mean that CaMKII is unrelated to alternans. Rather, it is possible that CaMKII is not significantly increased in the rat model of chronic MI, as previously shown.

Role of XO in Alternans

Cardiac alternans is highly dependent on SR Ca2+ regulatory proteins and their redox status. XO, an important source of ROS, is expressed in the SR where it is inhibited by neuronal nitric oxide synthase. In ischemic heart failure, neuronal nitric oxide synthase can translocate to the sarcolemma, relieving its inhibition of XO, which may explain increased ROS local to the SR. We have previously shown that ALLO, a specific blocker of XO, can normalize RyR oxidation (RyR-SOx) secondary to neuronal nitric oxide synthase inhibition. Similarly, Gonzalez et al. have shown that ALLO reduces myocardial ROS and improves RyR function in a nonischemic rat heart failure model. In addition to the effect ALLO has on RyR, we found in the present study that ALLO reduced alternans secondary to increased rate of Ca2+ cycling and reduced SERCA2a-SOx. Previous studies have shown that oxidation of SERCA2a is associated with reduced function, which is consistent with our findings. ALLO can also reduce sodium–calcium exchanger, but this would slow Ca2+ cycling and promote Ca2+ alternans, which is not what we observed. Thus, our results suggest that SERCA2a-SOx, along with RyR oxidation shown by others, are important mechanisms of arrhythmia and cardiac alternans associated with chronic MI.

Interestingly, we found that acute treatment with the general antioxidant, N-acetylcysteine, had no effect on alternans activity (Figure 8). This has 2 important implications. First, this result suggests limited ROS-induced ROS release between mitochondria and XO in our model because previous studies have shown that N-acetylcysteine can suppress ROS-induced ROS release. Second, this result suggests that targeted inhibition of XO rather than a general antioxidant can reduce SERCA2a-SOx and, thus, alternans. This idea is consistent with a recent study by Sovari et al., who showed that targeted mitochondrial antioxidant therapy, as opposed to nonspecific antioxidants, can decrease reentrant arrhythmias in a sudden cardiac death mouse model by preventing ROS-mediated connexin 43 destabilization. The present study and that by Sovari et al. may explain why clinical trials have shown that nonspecific antioxidants, such as Vitamin C and E, are ineffective at improving cardiac outcomes. Importantly, the present study adds further evidence in
support of the idea that targeted antioxidant therapies may be an effective approach to reduce cardiac events associated with heart disease.

Clinical Implications
Our results suggest that treatment with ALLO may be effective at ameliorating cardiac alternans in patients with chronic MI. Recent clinical trials have shown that ALLO improves myocardial relaxation in patients with ischemic heart disease, as evidenced by a trend toward reduced left ventricular end-diastolic volume. Moreover, overexpression of SERCA2a in humans may also decrease left ventricular end-diastolic volume. Thus, our result demonstrating that ALLO treatment increases SERCA2a function is consistent with these findings in patients with heart disease. However, such hemodynamic parameters must be compared cautiously with SERCA2a function. It is important to note that oxypurinol (active ALLO metabolite) has been shown to be ineffective at improving outcomes in a moderate to severe heart failure population, but there was a trend for reduced mortality in patients with high uric acid levels (XO activity). The EXACT-HF trial is currently underway to determine the benefit of ALLO for heart failure patients with high serum uric acid levels (ClinicalTrials.gov, NCT00987415).

Limitations
We did not measure alternans throughout the whole heart; however, we observed significant ECG T-wave ALT, suggesting alternans over large portions of the heart. ALLO treatment did not completely ameliorate alternans, suggesting the involvement of other molecular mechanisms. For example, RyR can influence alternans in chronic MI independent of SERCA2a activity, and ATP depletion associated with chronic MI can have similar action. In addition, we cannot exclude the influence of other ROS sources beyond XO, such as the mitochondria or NADPH oxidases. Although we cannot exclude these possibilities, they are independent of and, thus, not mutually exclusive with SERCA2a-SOx by XO as a cause of repolarization alternans in chronic MI.
References


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