Arrhythmogenic Delayed Afterdepolarizations Are Promoted by Severe Hypothermia But Not Therapeutic Hypothermia

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Background: Severe hypothermia (SH) is known to be arrhythmogenic, but the effect of therapeutic hypothermia (TH) on arrhythmias is unclear. It is hypothesized that susceptibility to Ca-mediated arrhythmia triggers would be increased only by SH.

Methods and Results: Spontaneous Ca release (SCR) and resultant delayed afterdepolarizations (DADs) were evaluated by optical mapping in canine wedge preparations during normothermia (N, 36°C), TH (32°C) or SH (28°C; n=8 each). The slope (amplitude/risetime) of multicellular SCR (mSCR) events, a determinant of triggered activity, was suppressed in TH (24.4±3.4%/s vs. N: 41.5±6.0%/s), but significantly higher in SH (96.3±8.1%/s) producing higher amplitude DADs in SH (35.7±1.6%) and smaller in TH (5.3±1.0% vs. N: 10.0±1.1%, all P<0.05). Triggered activity was only observed in SH. In isolated myocytes, sarcoplasmic reticulum (SR) Ca release kinetics slowed in a temperature-dependent manner, prolonging Ca transient rise time [33±3 (N) vs. 50±6 (TH) vs. 88±12 ms (SH)], which can explain the decreased mSCR slope and DAD amplitude in TH. Although the SR Ca content was similar in TH and SH, Ca spark frequency was markedly increased only in SH, suggesting that increased ryanodine receptor open probability could explain the increased triggered activity during SH.

Conclusions: Temperature dependence of Ca release can explain susceptibility to Ca-mediated arrhythmia triggers in SH. This may therefore explain the increased risk of lethal arrhythmia in SH, but not during TH.

Key Words: Arrhythmia; Optical mapping; Therapeutic hypothermia; Triggered activity

Therapeutic hypothermia (TH) improves neurologic outcomes and survival in patients with return of spontaneous circulation (ROSC) after cardiac arrest. Current cardiopulmonary resuscitation guidelines recommend that all comatose patients with ROSC should be cooled to have temperature maintained between 32 and 36°C for 24 h. In addition to improving neurological outcomes, several recent experimental and clinical studies suggest that TH decreases infarct size in acute myocardial infarction. Importantly, the incidence of lethal arrhythmias in pre-resuscitated patients treated with TH is relatively low, suggesting that temperatures used in clinical TH may be arrhythmogenic, although this is controversial.

Unlike TH, severe hypothermia (SH, less than 30°C), is well known to be pro-arrhythmic. Arrhythmias in patients with accidental SH are common and include ventricular fibrillation (VF), which can be refractory to standard therapy. Experimental studies have shown that hypothermia causes dysregulation of cellular calcium (Ca), which has been associated with intracellular Ca overload and ryanodine receptor (RyR) dysfunction. Given clinical observations of increased ventricular ectopy and irritability during SH, frequent triggered arrhythmias would be expected. Moreover, although repolarization abnormalities induced by hypothermia are known to promote substrates for re-entrant VF, the effect of hypothermia on VF triggers is poorly understood.

Despite the contrast in arrhythmia susceptibility associated with TH vs. SH, there have been very few systematic studies that have investigated the underlying arrhythmia mechanisms. Existing data on the effect of hypothermia on Ca dysregulation and triggered activity is difficult to interpret because many studies have been performed at temperatures even colder than those observed during SH (e.g., room temperature) and in varied species. Moreover, the effect of hypothermia on cellular Ca regulation is complex, such that the net impact on susceptibility to arrhythmias is difficult to predict. Therefore, we aimed to investigate the effects of clinically relevant temperatures on Ca dysregulation and triggered arrhythmias in a single animal model at the tissue, cellular and subcellular level. We hypothesized...
that susceptibility to Ca-mediated arrhythmia triggers would only be increased by SH, due to the dominant effect of colder temperature on RyR open probability (RyR-P).

Methods

Dual Calcium-Voltage Optical Mapping in Canine Wedge Preparation

All experiments were approved by the Animal Care and Use Committee of our institution and carried out in accordance with Public Health Service guidelines for the care and use of laboratory animals. Hearts from 24 mongrel dogs (15–20 kg) were rapidly excised by right lateral thoracotomy under pentobarbital (50 ng/mL) anesthesia. Intracellular Ca transients and action potentials were simultaneously recorded from identical 256 sites with high spatial (0.89 mm), temporal (0.5 ms), and voltage (0.5 mV) resolution from cells spanning the entire left ventricular wall. Details of the experimental procedure for dual optical mapping of Ca and membrane voltage (Vm) in the canine wedge preparation have been previously described.

Briefly, wedges (~40 mm height x30 mm width x12 mm depth) harvested near the base of the left ventricle were isolated with coronary arteries, and perfused (50–70 mmHg) with oxygenated (95% O2, 5% CO2) Tyrode’s solution containing (in mmol/L) 135 NaCl, 0.9 NaH2PO4, 0.492 MgSO4, 4.03 KCl, 5.5 dextrose, 1.8 CaCl2 and 10 HEPES (pH 7.4). Wedges were loaded with indo-1AM, a Ca-sensitive indicator (10 μmol/L), for 40 min, and also with 4-[2-[6-(dibutylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl) hydroxide inner salt (di-4-ANEPPS), a voltage-sensitive dye (15 μmol/L; Sigma-Aldrich) was used to prevent motion artifact in the optical recordings.

Experimental Protocols

Baseline intracellular Ca and action potential recordings were obtained during steady-state endocardial pacing at twice the diastolic threshold. As transmural heterogeneity of Ca cycling and cellular electrophysiology may be important in susceptibility to triggered activity, measurements were evaluated at epicardial, midmyocardial, and endocardial regions. Spontaneous Ca release (SCRs) and subsequent delayed afterdepolarizations (DADs) were induced by rapid pacing over a range of HRs (240–333 beats/min), with one-to-one capture for 10–15 s followed by a halt in pacing, in a stepwise fashion. Data are reported at 240 and 300 beats/min. Isoproterenol (0.2 μmol/L) was used in an additional set of experiments to induce triggered arrhythmias (n=8, 5, 3 in N, TH, SH, respectively).

Temperature Control

A Lexan insulated imaging chamber contained a heat exchanger, which allowed for precise and rapid (<5 min) temperature control. Temperature was measured using a digital temperature probe (Omega®) in the chamber, allowing for temperature precision of ±0.1°C. We have previously reported negligible heterogeneities in temperature across the transmural imaged surface of the canine wedge preparation or within the imaging chamber. The perfusion temperature was set to match the chamber temperature, so it is unlikely there were any intramyocardial temperature gradients. To study the effect of temperature on Ca-mediated arrhythmias, we performed studies during normothermia (N, 36°C) in 8 preparations, at moderate hypothermia, using temperatures more typical for clinical TH (32°C, n=8), and at temperatures considered clinically as SH (28°C, n=8).

Data Analysis

For optical recordings, the Ca transient decay rate was measured by the time constant (τ, s) of single exponential fit (e-τs). The Ca transient duration was measured as the time from the beginning of Ca transient to 90% recovery of peak amplitude. As previously described, action potential activation time was defined as the point of maximum positive first derivative for the action potential upstroke, and repolarization time was defined as the point of maximum positive second derivative of the repolarization phase. Action potential duration (APD) was measured as the time between activation time and repolarization time.

The SCR measured from multiple cells (multicellular SCR events: mSCRs, roughly 25,000 to 30,000 per imaging site) and DADs were measured as a percent of the fluorescence intensity of the average of the last 2 paced beats at each site. Time-to-peak of mSCRs was measured as the time between the maximum dCa/dt of the last paced beat and the peak of the first mSCR. The mSCR rise time was defined as the time between the minimal fluorescence after the last paced beat, to the peak fluorescence of the mSCRs. The slope of mSCRs, a determinant of triggered activity, was calculated by dividing mSCR amplitude by mSCR rise time. In any given experiment, under every condition, the 10 sites with the largest mSCR amplitudes were analyzed for mSCR and DAD parameters; average data of these sites is presented. Ca-mediated triggered activity was defined as one or more ectopic beats immediately following cessation of rapid pacing, which was preceded by a mSCR and induced by a DAD.

Measurement of Ca Transients and Ca Content in Sarcoplasmic Reticulum (SR) in Isolated Myocytes

Endocardial and mid-myocardial myocytes were isolated from canine wedges using an enzymatic dispersion technique described previously. To measure intracellular Ca, myocytes were incubated in Tyrodes containing indo-1AM (Molecular Probes®) and 0.025% (wt/wt) Pluronic F-127 (Molecular Probes®) for 20 min at room temperature. The intracellular indo-1AM was excited at 355 nm and fluorescence emitted at 405 nm and 485 nm was collected by 2 matched photomultipliers. The ratio of the intensity of fluorescence emitted at 405 nm over that at 485 nm was calculated after subtraction of background fluorescence, and was used to evaluate the SR Ca content between groups of myocytes after caffeine pulse-induced SR Ca release. The rise time of the Ca transient was measured from 10 to 90% of the upstroke phase.

Confocal Imaging

Ca sparks were recorded within 2 h of isolating canine myocytes. Before imaging, cells were incubated in Tyrodes (as described above) plus 5 μmol/L Fluo-4-AM (Sigma-Aldrich) and an equal amount of Pluronic (20% W/V) for 45 min. Myocytes were then washed with normal Tyrodes solution before being placed in a 35-mm culture dish with a glass bottom and field stimulating electrodes. Imaging was performed using a Leica TCS SP8 tandem scanner HyD detec-
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Statistical Analysis
Statistical analysis was performed using JMP statistical software (SAS, Cary, NC, USA). ANOVA was used to determine statistical differences in Ca transient and mSCR characteristics, APD, and DAD amplitudes, between the 3 groups. When statistically significant differences were observed by ANOVA, the Tukey-Kramer post-hoc test was performed. Results are expressed as mean±standard error of the mean, and a value of P<0.05 was considered statistically significant.

Results
Severe But Not Therapeutic Hypothermia Promotes Triggered Activity
Ca mediated-triggered activity, which we defined as one or more ectopic beats immediately following termination of rapid pacing and is associated with a mSCR, was not observed in N (0 of 8) or TH (0 of 8) preparations. In contrast, we observed triggered activity in 4 of 8 preparations and sustained arrhythmia in 2 of 4 preparations in SH but no sustained ventricular arrhythmia in either N or TH. Sustained arrhythmia was observed in the presence of isoproterenol in 2 of 8 N, 1 of 5 TH, and 3 of 3 SH experiments (P=NS). Figure 1A shows representative Ca transients (Ca), action potentials (Vm), and the electrocardiogram (ECG) are shown from canine wedge preparations during normothermia (N), therapeutic hypothermia (TH) and severe hypothermia (SH). Although multicellular SCRs (mSCRs) and DADs are observed during N and TH, only during SH is mSCR-induced triggered activity observed. (B) Ca, Vm and the ECG are shown during SH in the presence of isoproterenol (0.2 μmol/L). During SH, a mSCR (Middle panel) is observed in the optical recordings, which causes DADs (Lower panel), followed by triggered activity and ventricular tachycardia.

Effect of Hypothermia on mSCRs and Subsequent DADs
To determine the mechanism of increased susceptibility to triggered arrhythmias under conditions of SH, we examined the temperature dependence of mSCR activity and resultant DADs. Figure 2A shows representative Ca transients (Upper panels) demonstrating mSCRs recorded after a halt in pacing. Resultant DADs that were recorded simultaneously are observed in the corresponding Vm recordings (Lower panels). When the temperature was decreased (TH), mSCR amplitude was almost identical to that in N (20.1±2.7 μmol/L vs. 21.6±3.0% in N vs. TH, P=NS), but the mSCR slope was decreased (41.5±6.0 vs. 24.4±3.4% in N vs. TH, P<0.05). This was associated with a decrease in DAD amplitude in TH compared to N (10.0±1.0 vs. 5.3±1.0% in N vs. TH, P<0.05). In contrast, when the temperature was further decreased (SH), mSCR amplitude markedly increased (69.4±1.5%), as did the mSCR slope (96.3±8.1%/s), compared to both N and TH. DAD amplitude in SH was also markedly increased (35.7±1.6%, P<0.05 vs. N and vs. TH, respectively), suggesting a close relationship with the mSCR slope. Summary data showing the effect of temperature on mSCR characteristics and
To further demonstrate the relationship between DAD amplitude and mSCR slope, DAD amplitude was plotted as a function of corresponding mSCR slope over all temperatures (Figure 3). Shown is a strong linear relationship between the mSCR slope and DAD amplitude when the mSCR slope and DAD amplitude were smallest during TH (gray boxes), moderate during N (white boxes), and largest during SH (black boxes). This result is consistent with the relationship between the mSCR slope and DAD amplitude previously shown in failing hearts.31,33

**Effect of Hypothermia on Ca Cycling and APD**

Figure 4A shows representative simultaneously recorded endocardial Ca and action potentials from all groups. Hypothermia produced an expected temperature-dependent prolongation of APD (which is expected to increase SR Ca content) and Ca transient duration, which was greatest in SH. APD in N, TH, and SH was 236.5±13.3, 284.8±15.3, and 347.4±18.7 ms, respectively, while duration of Ca transient in N, TH, and SH was 354.3±9.6, 425.3±12.0, and 515.5±9.3 ms, respectively. Ca transient decay, an indicator of SR Ca uptake, was significantly slowed by both TH and SH vs. N (tau of Ca transient in N, TH, and SH was 169.2±13.3, 283.9±18.7 ms, respectively, Figure 4B), which might reduce SR Ca con-

**Figure 2.** SH increases the slope of mSCR and resultant DADs. (A) Representative mSCRs and DADs from all groups are shown. The slope of mSCRs and amplitude of subsequent DAD were increased by SH (Right panel). Paradoxically, the slope of mSCRs and DAD amplitude were attenuated by TH (Middle panel). (B) Summary data of mSCR and DAD characteristics are shown (each n=8). Although there was no significant difference in the amplitude of mSCRs between N and TH, TH decreased the slope of mSCRs, resulting in subsequent attenuation of DAD amplitude. However, in SH, because the mSCR amplitude was significantly greater, the slope of mSCRs was increased, resulting in greater DAD amplitude. These data suggest that SH can promote triggered activity by increasing DADs, whereas TH suppresses the DAD response to mSCRs. Abbreviations as in Figure 1.

**Figure 3.** Slope of mSCR events determines susceptibility to resultant DADs. The amplitude of DADs was plotted as a function of mSCR slope across experiments (each n=8). A close linear relationship between mSCR slope and DAD amplitude is observed, demonstrating that mSCR slope is a primary determinant of the magnitude of resultant DADs. Abbreviations as in Figure 1.
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Increased mSCR slope, which corresponds to the condition when DADs and triggered activity were greatest (Figures 1, 2). This suggests that in SH increased RyR- 

**Figure 4.** Therapeutic and severe hypothermia significantly slow cellular Ca cycling and prolong action potential duration (APD). (A) Representative endocardial optically recorded Ca and action potentials (Vm) from the canine wedge preparation in N, TH, and SH measured during pacing at 100 beats/min are shown. TH prolonged the duration and decay of the Ca transient, and prolonged APD compared to N. SH further slowed Ca cycling and prolonged APD. (B) Summary data of these parameters are shown (each n=8). These data demonstrated that cellular Ca cycling was significantly slowed in a temperature-dependent manner. Similar alterations in Ca cycling and APD were observed in epicardial and mid-myocardial sites (data not shown), indicating there were no spatial heterogeneities in the effect of hypothermia on Ca cycling and APD. Abbreviations as in Figure 1.

**Figure 5.** SR Ca content is increased by both therapeutic and severe hypothermia. Summary data of SR Ca content are shown. SR Ca content in TH (0.85±0.12) and SH (0.88±0.07) are similarly increased as compared to N conditions (0.63±0.15 AU, both P<0.05, each n=7). Abbreviations as in Figure 1.
increase in spark frequency (2.66±0.28/s) compared to that during N (1.1±0.24/s, P<0.01) and TH (1.77±0.27/s, P<0.05). In addition, SH significantly slowed (i.e., increased) the time to peak (TTP) of Ca sparks [13.90±6.22 (SH) vs. 9.79±2.96 (N, P<0.05), 8.57±2.01 ms (TH, P<0.01)]. However, there was no difference in Ca spark amplitude at any temperature [0.33±0.11 (N), 0.30±0.04 (TH), 0.32±0.13 (SH), P=NS]. Finally, a colder temperature slightly prolonged the duration and decay of Ca sparks, but had no effect on their full width at half maximum (data not shown). Thus, Ca spark frequency was impacted the most by decreasing the temperature to that of SH.

**Discussion**

In this study, we demonstrate that SH (28°C) promoted (leak) may underlie the marked increase in the mSCR slope and susceptibility to triggered arrhythmias observed, rather than increased Ca release kinetics. This is consistent with observations of increased RyR-P$_s$ with more significant cooling.$^{17,35}$

**Effect of Hypothermia on Ca Spark Activity**

To further determine the mechanisms of mSCR activity, we assessed Ca spark characteristics in isolated canine myocytes. Shown in Figure 7 (Upper image) are examples of line scan images recorded upon termination of pacing (1 Hz) for myocytes at N, TH, SH temperatures. As can be seen in these examples, spark frequency increased with decreasing temperature. Quantification of Ca spark characteristics exhibited similar results (Lower graphs). Decreasing the temperature to SH caused a significant increase in spark frequency (2.66±0.28/s) compared to that during N (1.1±0.24/s, P<0.01) and TH (1.77±0.27/s, P<0.05). In addition, SH significantly slowed (i.e., increased) the time to peak (TTP) of Ca sparks [13.90±6.22 (SH) vs. 9.79±2.96 (N, P<0.05), 8.57±2.01 ms (TH, P<0.01)]. However, there was no difference in Ca spark amplitude at any temperature [0.33±0.11 (N), 0.30±0.04 (TH), 0.32±0.13 (SH), P=NS]. Finally, a colder temperature slightly prolonged the duration and decay of Ca sparks, but had no effect on their full width at half maximum (data not shown). Thus, Ca spark frequency was impacted the most by decreasing the temperature to that of SH.

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(Figures and tables are not included in this text. The full article should be consulted for detailed visual representations and data.)
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There are even case reports that TH promotes VF, which is linked to hypothermic modulation of the transient outward potassium and L-type Ca currents, producing an inward shift in the balance of early repolarization currents, and therefore an increase in early repolarization promoting phase-2 re-entry. So, although TH may not promote DOR or DADs, it may under some circumstances enhance other arrhythmia substrates. These data emphasize the need for additional translational and clinical studies to determine the combined effects of arrhythmia substrates on clinical outcomes during TH.

In contrast to TH, the association between SH and arrhythmogenesis is well known. The incidence of refractory ventricular arrhythmias significantly increases when body temperature decreases to less than 30°C. We have previously reported in a canine model of SH, that increased DOR caused re-entrant arrhythmias. In this same model during bradycardia, transmural DOR was markedly enhanced in SH, but not TH, and created a substrate for inducible re-entry. Thus, SH promotes arrhythmogenesis by additional mechanisms, which are separate, but potentially complimentary to triggered activity described in the current study. The effects of SH on triggered activity has not been previously demonstrated, and the current study is the first to demonstrate a relationship between Ca dysregulation and DAD-induced triggered activity. Clinical observations of increased ventricular ectopy and arrhythmias induced by cardiac manipulation during SH, suggest that triggered activity is a mechanism of arrhythmogenesis in SH, which is consistent with our findings.

Therefore, increased Ca-mediated triggers and re-entrant substrates (DOR, alternans) during SH is a potent combination for arrhythmias. Although increases in early afterdepolarizations (EADs) due to excessive prolongation of APD by hypothermia have been recognized, we did not observe EADs or EAD-induced triggered activity at the temperatures tested, despite significant APD prolongation.

Mechanisms Underlying the Temperature-Dependent Effects of Hypothermia and Ca-Mediated Triggered Activity

The clinical data suggest that SH is more arrhythmogenic than TH or N temperatures. Our results suggest a mechanism for this observation based on, in part, temperature-dependent Ca dysregulation. Prior experimental studies have demonstrated important temperature-dependent effects on Ca cycling. For example, increased SR Ca content is observed with decreasing temperature, which is consistent with our data, as we observed increased SR Ca content between control and TH. However, we did not observe a significant increase in SR Ca content between TH and SH. This, and the fact that Ca reuptake always decreased with temperature, rules out SR Ca overload as a mechanism of our observations.

Our results suggest that changes in SR Ca release determine the relationship between decreasing temperature and triggered activity. We found that RyR release kinetics (conductance) decreases with temperature, as shown by the prolongation of Ca transient rise time (Figure 6B) and Ca spark TtP (Figure 7 Lower, Middle). This is consistent with a known decrease in conductance and kinetics of RyR Ca release with temperature. Alternatively, decreasing temperature also increases RyR-P, as well as fractional and net Ca release from RyR. We contend that these 2 processes combined determine the occurrence of triggered activity. This is manifest in the biphasic nature of the mSCR slope and DAD amplitude (Figure 2B), both of which are directly related to triggered activity occurrence.

In other words, slow RyR release kinetics is the predominant effect during TH, resulting in decreased mSCR slope and DAD amplitude. However, RyR-P increases exponentially with decreasing temperature, which would make it much more predominant at SH, relative to RyR conductance. This promoted a “leaky RyR,” as evidenced by increased spark frequency at SH, which markedly increases diastolic RyR Ca release. It is important to note that this increase in Ca spark frequency was despite no significant increase in SR Ca content at SH compared to TH. Therefore, the significant increase in susceptibility to DADs and triggered activity in SH we observed is consistent with RyR-P increasing and becoming dominant at severely hypothermic temperatures.

Study Limitations

The canine wedge preparation may not be the best model to test susceptibility to sustained arrhythmias, and in vivo studies may be necessary to fully evaluate arrhythmia susceptibility. In these studies, we did not evaluate the hypothermia-induced effects of additional arrhythmia substrates, although we have previously shown that in this model, that only SH promotes DOR and re-entrant arrhythmias. We did not directly examine outward sodium-Ca exchanger (NCX) current, which is the electrogenic current most responsible for DADs in response to SCR. NCX is decreased in hypothermia. However, we observed the most significant triggered activity during SH, suggesting the effect
of hypothermia on NCX was not a primary determinant of hypothermia on triggered arrhythmias. In addition, we did not determine whether there were temperature-dependent differences in resting membrane potential (RMP). Although we recently reported no difference in RMP between normal temperature and TH, we cannot rule out that in SH, there might be a difference in RMP. In the present study, Ca dysregulation and triggered activity was assessed by rapid pacing. Even though SH is typically associated with bradycardia, our data may help explain frequent ventricular ectopy during SH.  

We evaluated the data at 32°C as TH, as clinical data and AHA guidelines have recommended 32–34°C as target temperatures for TH. This temperature was also chosen based on previous data and a recent randomized clinical trial suggesting that patients treated at 32°C showed better prognosis and cardioprotection compared to those treated at 34°C. However, a recent clinical trial suggests no differences in outcomes of cardiac arrest survivors treated with a temperature controlled at 33°C vs. 36°C, and the optimal temperature for TH remains controversial. We did not examine the effect of dynamic temperature changes or rewarming, as is implemented during TH or occurs during accidental SH, on triggered activity. During clinical TH, additional factors may be important in determining susceptibility to triggered arrhythmias, such as ongoing ischemia or reperfusion during resuscitation. Additional experiments are needed to elucidate the interactions between dynamic changes in temperature or ischemia/reperfusion on triggered arrhythmias.

Conclusions

We reveal a novel mechanism of Ca-mediated triggered arrhythmias that occur in SH but not in TH. Increased susceptibility to Ca-mediated triggered arrhythmias during SH was associated with a marked increase in Ca spark frequency, without a significant increase in SR Ca content, suggesting that increased RyR-P is the underlying mechanism.

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