The role of altered intercellular coupling in arrhythmias induced by acute myocardial ischemia

Deborah L. Lerner\(^{a,d}\), Michael A. Beardslee\(^b\), Jeffrey E. Saffitz\(^{b,c,d,\ast}\)

\(^a\)Department of Pediatrics, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA
\(^b\)Department of Medicine, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA
\(^c\)Department of Pathology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA
\(^d\)Center for Cardiovascular Research, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA

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1. Introduction

Sudden cardiac death occurs with unacceptably high incidence in patients with ischemic heart disease and cardiomyopathy. As Zipes and Wellens [1] have emphasized, sudden death arises from highly variable interactions between anatomic and/or functional myocardial substrates, transient initiating events and cellular/tissue arrhythmia mechanisms. In our view, a key strategy for developing mechanistic insights into sudden death is to first define the role of individual factors (including specific gene products) that contribute to arrhythmias, and to then understand how these factors interact to cause sudden death.

One of the most common disease settings leading to sudden cardiac death is the acute coronary syndromes. Acute ischemia is marked by alterations in cell metabolism, cell signaling, intercellular communication and electrical impulse propagation [2]. These changes produce a cascade of events that are adaptive in the sense that mechanisms are activated to mitigate injury, forestall cell death and isolate irreversibly injured myocytes from their viable neighbors, but also maladaptive in that they can create a substrate that supports the initiation and maintenance of malignant ventricular arrhythmias. Among the electrophysiologically relevant changes that occur rapidly after the onset of ischemia are reductions in tissue pH, increases in interstitial K\(^+\) and intracellular Ca\(^{2+}\) concentrations and changes in active and passive membrane properties, all of which interact in a complex milieu to slow conduction, alter excitability and refractoriness, promote electrical uncoupling, and generate spontaneous electrical activity [1–4].

In this review, we focus on the specific role of diminished intercellular electrical coupling in the pathogenesis of lethal arrhythmias induced by acute ischemia. Until recently, it has been difficult to isolate the contribution of diminished coupling per se to arrhythmogenesis in the complex setting of acute ischemia. However, the advent of techniques to manipulate gene expression and characterize cardiac electrophysiology in mice has provided a way to isolate and define the role of specific gene products in the pathogenesis of complex disease processes such as sudden cardiac death.

2. Connexins and electrical coupling

Normal electrical function of the heart requires current transfer from one cell to another at gap junctions. Gap junction channels are formed by a family of proteins called connexins (Cx) [5]. Three individual connexins, Cx43, Cx40 and Cx45, are expressed in different amounts and distributions in different cardiac tissues [5]. Cx43, the most abundant connexin, is expressed in atrial and ventricular muscle [6]. Cx40 expression is restricted to atrial muscle and the conduction system; little or no Cx40 is detected immunohistochemically in working adult ventricular myocytes [7,8]. Cx45 expression in the ventricle is concentrated in the Purkinje system with only low levels expressed in working ventricular myocytes [9]. Thus, Cx43 appears to be the principal gap junction protein responsible for electrically coupling working ventricular myocytes.
Although the evidence is still largely circumstantial, it is likely that the number, size and spatial distribution of gap junctions are important determinants of impulse propagation in a functional syncytium. Functionally distinct cardiac tissues exhibit markedly different patterns of intercellular connections that can account for the different anisotropic conduction properties of specific cardiac tissues [10,11]. For example, a typical canine left ventricular myocyte is physically connected by gap junctions to 11 or 12 other myocytes in overlapping side-to-side and end-to-end orientations [10]. In contrast, myocytes of the canine crista terminalis are interconnected almost entirely in end-to-end fashion [10], a pattern that undoubtedly contributes to the greater anisotropy of conduction in the crista compared with the ventricle. Data such as these support the hypothesis that spatial differences in the expression of gap junction channel proteins have functional importance but the specific contributions of altered cellular coupling to conduction disturbances have been difficult to define rigorously because pathophysiological processes or experimental interventions that diminish coupling also affect active membrane properties or cause remodeling of the extracellular matrix and/or conduction pathways.

3. The Cx43 knockout mouse

In 1995, Reaume et al. [12] produced a Cx43 knockout mouse. Cx43 −/− fetuses develop to term but die soon after birth due to obstruction of pulmonary blood flow caused by a conotruncal malformation that arises from altered intercellular coupling in cardiac neural crest cells. Because Cx43 −/− mice have a cardiac malformation and survive for only a limited time, we have focused our attention primarily on heterozygotes (Cx43 +/−) which show no overt abnormalities and live a normal life span. Cx43 immunoreactive signal was attributable entirely to a reduction in Cx43 protein content in ventricular myocardium compared with the ventricle. Data such as these support the expectation that spatial differences in the expression of gap junction channel proteins have functional importance but the specific contributions of altered cellular coupling to conduction disturbances have been difficult to define rigorously because pathophysiological processes or experimental interventions that diminish coupling also affect active membrane properties or cause remodeling of the extracellular matrix and/or conduction pathways.

4. Effects of diminished Cx43 expression on gap junction structure

Myocytes of different cardiac tissues are interconnected by gap junctions that vary widely not only in terms of spatial distribution but also in total amount [10,11]. For example, aggregate gap junction profile length per unit cell area measured in electron micrographs of myocytes of the left ventricle and sinus node differ by more than 20-fold [11]. Despite the apparent importance of gap junction distribution as a determinant of conduction, little is known about how specific patterns of intercellular connections are established or how the number and size of gap junctions are determined in different tissues of the heart. To gain insights into the relationship between the amount of a connexin expressed and the structure of gap junctions, we analyzed Cx43 +/− and +/+ mice and asked whether the 50% reduction in Cx43 protein content in ventricular myocardium would result in fewer gap junctions, smaller gap junctions, both fewer and smaller gap junctions, or no change in gap junction structure which might occur if there were a significant pool of intracellular Cx43. Using quantitative confocal microscopy, we measured the total amount of Cx43 immunoreactive signal in gap junctions (but not in potential intracellular pools) as well as the number and size of discrete clusters of immunoreactive signal which we defined operationally as individual gap junctions. As shown in Table 1, diminished Cx43 expression in Cx43 +/− mice caused a significant reduction in total ventricular immunoreactive signal compared with Cx43 +/+ mice, consistent with the presence of one null allele in the heterozygotes [18]. The difference in total Cx43 immunoreactive signal was attributable entirely to a reduction in the number of gap junctions [18]. Mean gap junction size in ventricular myocytes was identical in Cx43 +/− and +/+ samples [18]. Thus, when faced with a

<table>
<thead>
<tr>
<th>Cx43 expression</th>
<th>% Tissue area</th>
<th>Objects/field</th>
<th>Object size (μm²)</th>
</tr>
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<tbody>
<tr>
<td>Cx43 +/+</td>
<td>0.79±0.24</td>
<td>226±52</td>
<td>0.86±0.12</td>
</tr>
<tr>
<td>Cx43 +/−</td>
<td>0.43±0.13*</td>
<td>150±32*</td>
<td>0.80±0.10</td>
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*Sections of mouse left ventricle were stained with anti-Cx43 antibodies and analyzed by quantitative confocal microscopy. The proportion of total tissue area occupied by high-intensity immunoreactive Cx43 signal (expressed as % tissue area) is reduced significantly in sections of Cx43 +/− ventricular myocardium compared with Cx43 +/+ tissue. This reduction is due to a significant decrease in the number of clusters of high intensity signal (objects/field, operationally defined as the number of individual gap junctions within a test field) but no significant difference in the size of individual clusters of high intensity signal. *p<0.05 vs. Cx43 +/+.
genetic deficiency in Cx43 expression, ventricular myocytes form fewer but not smaller gap junctions.

A general relationship appears to exist between the total amount of Cx43 expressed and the number of gap junctions formed. For example, we have shown previously that a ~2-fold increase in Cx43 expression in cultured neonatal rat ventricular myocytes exposed to cAMP is associated with an increase in gap junction number and a 25–30% increase in conduction velocity [19]. More recently, we observed that application of pulsatile stretch to cultured neonatal rat ventricular myocytes also causes a 2- to 3-fold increase in Cx43 expression associated with a significant increase in both gap junction number and conduction velocity [20]. Taken together, these observations indicate that changes in the total amount of Cx43 expression on the order of a 50% reduction (in Cx43/−/− mice) or a 2-fold increase (in cells exposed to cAMP or stretch) lead to corresponding changes in gap junction number (but no consistent changes in gap junction size) and a gain or loss of function (a change in conduction velocity) of a magnitude that is remarkably well predicted by cable theory [20].

5. Diminished intercellular coupling in the pathogenesis of arrhythmias induced by acute ischemia

To gain insights into the role of diminished coupling in the pathogenesis of ventricular arrhythmias induced by acute regional ischemia, we compared arrhythmogenesis in isolated perfused hearts from Cx43+/− and +/+ mice in which acute regional ischemia was created by occlusion of the proximal left anterior descending (LAD) coronary artery [21]. Control hearts of either genotype which did not undergo coronary occlusion remained in sinus rhythm, contracted vigorously and exhibited no arrhythmias during 60 min of normoxic perfusion. Aggressive electrical stimulation protocols involving single and multiple extrastimuli at coupling intervals near the effective refractory period repeatedly failed to induce either ventricular tachycardia (VT) or premature ventricular beats (PVBs) in any Cx43+/− or +/+ hearts during this time, thus demonstrating that the preparations were electrically stable under normoxic conditions [21]. In response to LAD occlusion, however, spontaneous ventricular arrhythmias were observed (Fig. 1).

As shown in Fig. 2, Cx43+/− hearts subjected to LAD occlusion exhibited marked increases in the incidence,
limited to modest slowing of ventricular conduction ve-
creased PVBs in Cx43

Uncoupling and mechanisms of arrhythmias could prolong repolarization and promote the generation of

Cx43

was observed in 11 Cx43

hearts and occurred significantly earlier than in Cx43

PVBs were also significantly more abundant in Cx43

heart did not occur until after 13 min of ischemia. Isolated

course of uncoupling induced by ischemia in the mouse

®ve Cx43

produced VT in 11 Cx43

spontaneous VT compared with only one Cx43

exhibited nearly continuous bursts of VT with minimal wave of arrhythmias (Type Ib) occurs concomitant with

wildtype hearts (

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The time of onset of the ®rst spontaneous VT following temporal patterns of uncoupling in Cx43

early onset (Type Ia) arrhythmias antecede electrical uncoupling, whereas a second wave of arrhythmias (Type Ib) occurs concomitant with uncoupling [23]. Because reentrant circuits in Type Ia arrhythmias have an inner core size of several millimeters, these early arrhythmias may not occur frequently in hearts of small mammals such as mice. We have observed some arrhythmias in mice early after the onset of coronary occlusion but it is unknown whether these events were related to reentry or triggered activity. Most arrhythmias observed in our studies were probably analogous to Type Ib arrhythmias which occur concomitantly with electrical uncoupling in larger animals [23]. Although reentry is most likely, multiple mechanisms could contribute to arrhythmias induced by acute ischemia in Cx43

The time of onset of the ®rst spontaneous VT following LAD occlusion occurred signi®cantly earlier in Cx43

hearts (P<0.05) (Fig. 3). Of nine Cx43

hearts that mechanisms in acute regional ischemia. Among the unre-
solved questions to be addressed are: What is the time course of uncoupling induced by ischemia in the mouse ventricle? What critical level of uncoupling, if any, must be achieved before arrhythmias can arise? What are the speci®c contributions of diminished coupling to reentry and triggered activity in the setting of acute ischemia?

The greater number and earlier onset of PVBs in Cx43

mice following coronary artery occlusion suggest potential links between changes in coupling and development and propagation of PVBs. Saiz et al. [24] showed in a modeling study that a speci®c degree of uncoupling could prolong repolarization and promote the generation of early afterdepolarizations and, thereby, allow for a critical rise in membrane potential to achieve transfer of the impulse to the surrounding tissue. Our ®ndings of increased PVBs in Cx43

frequency and duration of VT compared with Cx43

hearts [21]. Of 16 hearts studied in each group, 12 Cx43

+/+ and Cx43

+/− hearts exhibited at least one run of either spontaneous or pacing-induced VT. Eleven Cx43

+/− hearts developed multiple runs of VT compared with only five wildtype hearts (P<0.05). Four Cx43

+/− hearts exhibited nearly continuous bursts of VT with minimal wave of arrhythmias (Type Ib) occurs concomitant with uncoupling [23]. Because reentrant circuits in Type Ia arrhythmias have an inner core size of several millimeters, these early arrhythmias may not occur frequently in hearts of small mammals such as mice. We have observed some arrhythmias in mice early after the onset of coronary occlusion but it is unknown whether these events were related to reentry or triggered activity. Most arrhythmias observed in our studies were probably analogous to Type Ib arrhythmias which occur concomitantly with electrical uncoupling in larger animals [23]. Although reentry is most likely, multiple mechanisms could contribute to arrhythmias induced by acute ischemia in Cx43

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Fig. 3. Time of onset of the ®rst spontaneous VT after coronary artery occlusion in Cx43

+/+ and Cx43

+/− hearts (n=16 for each group). Reprinted with permission from Ref. [21].

6. Uncoupling and mechanisms of arrhythmias during acute ischemia

The cardiac phenotype in Cx43

mice appears to be limited to modest slowing of ventricular conduction ve-
lucity. Otherwise, cardiac structure and function are normal. For example, echocardiographic measurement of left ventricular mass, wall thickness, internal chamber dimensions and percent fractional shortening are the same in Cx43

+/+ and Cx43

+/− hearts (unpublished observations).

Histologically, Cx43

+/− hearts are indistinguishable and there is no evidence of fibrosis or other changes in the extracellular matrix in Cx43

+/− hearts [13,14]. Furthermore, action potential morphology, peak Na+ current and activation and inactivation kinetics, and Na+ channel protein expression and distribution are the same in neonatal Cx43

+/− mice (unpublished observations). These observations suggest that the marked increase in arrhythmias in Cx43

+/− mice following coronary artery occlusion is not related to changes in myocardial structure or active membrane properties of myocytes. Rather, our observations indicate that diminished coupling per se is a powerful independent determinant of arrhyth-
mias induced by acute ischemia.

Two distinct phases of arrhythmias have been observed during acute ischemia in larger animal models although the same may not hold true in smaller animals. In pig hearts subjected to acute ischemia, early onset (Type Ia) arrhyth-
mias antecede electrical uncoupling, whereas a second wave of arrhythmias (Type Ib) occurs concomitant with uncoupling [23]. Because reentrant circuits in Type Ia arrhythmias have an inner core size of several millimeters, these early arrhythmias may not occur frequently in hearts of small mammals such as mice. We have observed some arrhythmias in mice early after the onset of coronary occlusion but it is unknown whether these events were related to reentry or triggered activity. Most arrhythmias observed in our studies were probably analogous to Type Ib arrhythmias which occur concomitantly with electrical uncoupling in larger animals [23]. Although reentry is most likely, multiple mechanisms could contribute to arrhythmias induced by acute ischemia in Cx43

+/− mice. A goal of future studies is to characterize spatial and temporal patterns of uncoupling in Cx43

+/− and Cx43

+/+ mice and elucidate their relationship to speci®c arrhythmia mechanisms in acute regional ischemia. Among the unre-
solved questions to be addressed are: What is the time course of uncoupling induced by ischemia in the mouse ventricle? What critical level of uncoupling, if any, must be achieved before arrhythmias can arise? What are the speci®c contributions of diminished coupling to reentry and triggered activity in the setting of acute ischemia?

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+/− mice following coronary artery occlusion suggest potential links between changes in coupling and development and propagation of PVBs. Saiz et al. [24] showed in a modeling study that a speci®c degree of uncoupling could prolong repolarization and promote the generation of early afterdepolarizations and, thereby, allow for a critical rise in membrane potential to achieve transfer of the impulse to the surrounding tissue. Our ®ndings of increased PVBs in Cx43

+/− hearts is consistent with the
results of these computer models and provides experimental evidence potentially linking alterations in coupling with the generation and propagation of triggered beats.

Biochemical mechanisms mediating uncoupling during ischemia are undoubtedly related to multiple pathophysiological processes including progressive increases in intracellular Ca$^{2+}$ and H$^+$ concentrations and accumulation of amphipathic lipid metabolites [25,26]. Other mechanisms could also promote uncoupling, however, and contribute to the development of conduction abnormalities and arrhythmias. Like many of the connexins, Cx43 is a phosphoprotein and changes in its phosphorylation can affect channel properties and connexin turnover dynamics [27–30]. Because acute ischemia may activate or inhibit protein kinases and phosphatases [31], we performed studies to test the hypothesis that electrical uncoupling induced by myocardial ischemia is mediated, at least in part, by alterations in phosphorylation of Cx43.

We subjected isolated perfused rat hearts to global ischemia for up to 40 min. Changes in electrical coupling were monitored during this interval by measuring whole tissue resistance and changes in the amount and distribution of phosphorylated and non-phosphorylated isoforms of Cx43 were measured by immunoblotting and confocal microscopy using isoform-specific antibodies [32]. We observed that virtually all Cx43 identified immunohistochemically at intercellular junctions is phosphorylated under control conditions [32]. During ischemia, however, Cx43 underwent progressive dephosphorylation with a time course similar to that of electrical uncoupling (Fig. 4). Although the total amount of Cx43 did not change (Fig. 4), there was a progressive reduction in total Cx43 immunofluorescent signal and concomitant accumulation of nonphosphorylated Cx43 signal at sites of intercellular junctions (Fig. 5). These observations suggest that uncoupling induced by ischemia is associated with dephosphorylation of Cx43, accumulation of nonphosphorylated Cx43 within gap junctions, and translocation of Cx43 from gap junctions into intracellular pools [32]. Further studies will be required to define the pathophysiological relationship between changes in phosphorylation at specific amino acid residues of Cx43 and uncoupling, and the potential effects of modulating Cx43 phosphorylation on the development of arrhythmias during ischemia.

Fig. 4. Upper panel: time course of changes in tissue resistance in a representative rat heart subjected to global ischemia for 40 min. Whole-tissue resistance was plotted relative to the value at the onset of ischemia. Initial changes in tissue resistance after cessation of perfusion are related to vascular collapse and changes in resistance of the extracellular compartment. The later, more marked phase of increased resistance is presumably due to electrical uncoupling. The average time of onset of uncoupling in five experiments was 15.3 ± 3.3 min. Middle panel: a representative immunoblot of samples from rat left ventricles subjected to selected intervals of ischemia and probed with a polyclonal anti-Cx43 antibody that recognizes both phosphorylated and non-phosphorylated isoforms of Cx43. Phosphorylated isoforms migrate at 44–46 kDa whereas nonphosphorylated Cx43 migrates at 41 kDa. These results indicate progressive loss of phosphorylated Cx43 and concomitant accumulation of nonphosphorylated Cx43 during ischemia. Lower panel: quantitative densitometric analysis of total Cx43 signal (phosphorylated plus nonphosphorylated isoforms) measured in four hearts at selected time points after the onset of ischemia. Values were normalized to the 0-min time point and demonstrate no significant change in total tissue content of Cx43 during 40 min of global ischemia. Reprinted with permission from Ref. [32].
Fig. 5. Representative confocal images of sections of rat left ventricles subjected to selected intervals of ischemia and immunostained with either a polyclonal rabbit antibody that recognizes both phosphorylated and nonphosphorylated isoforms of Cx43 or a monoclonal anti-Cx43 antibody that binds selectively to nonphosphorylated Cx43. In control samples (0 min of ischemia), abundant immunoreactive signal is concentrated in discrete spots of intercellular apposition in sections stained with the polyclonal antibody but virtually no signal is present in sections stained with the mouse monoclonal antibody. Thus, most of the immunoreactive signal in gap junctions appears to be phosphorylated Cx43. With increasing intervals of ischemia, there is progressive loss of immunoreactive signal produced by the polyclonal antibody with a corresponding increase in signal observed with the monoclonal antibody. Because the relative titers and binding affinities of the two anti-Cx43 antibodies are not known, it is not possible to directly compare the amounts of phosphorylated and nonphosphorylated Cx43 in gap junctions during ischemia. However, these results indicate that during uncoupling, nonphosphorylated Cx43 accumulates in sites of intercellular apposition while at the same time the amount of total Cx43 (phosphorylated and nonphosphorylated) in gap junctions is progressively reduced. Reprinted with permission from Ref. [32].

7. Conclusions

The pathogenic role of altered connexin expression and function in lethal arrhythmias in patients is still a matter of speculation. Furthermore, the mouse heart differs from the human heart in important ways and mouse models of human disease are imperfect. Nevertheless, the application of modern methods in mouse genetics and ongoing refinements in analytical methods to characterize physiology in the mouse heart will undoubtedly yield insights into the specific contributions of a multitude of gene products in the pathogenesis of complex pathophysiological events such as sudden death. Interbreeding of mice will provide new models in which the effects of multiple defined molecular defects (e.g., defects in coupling and repolarization) on arrhythmogenesis can be elucidated. Development of new research systems involving genetically engineered mouse myocytes grown under defined conditions in vitro will also provide novel information [33]. Analysis of mouse models will play a valuable role in identifying specific genes as appropriate therapeutic targets and in testing principles and concepts to fulfill the promise of human gene therapy.

References


