Ultrastructure and Regulation of Lateralized Connexin43 in the Failing Heart

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Rationale: **Gap junctions mediate cell-to-cell electric coupling of cardiomyocytes. The primary gap junction protein in the working myocardium, connexin43 (Cx43), exhibits increased localization at the lateral membranes of cardiomyocytes in a variety of heart diseases, although the precise location and function of this population is unknown.**

Objective: **To define the subcellular location of lateralized gap junctions at the light and electron microscopic level, and further characterize the biochemical regulation of gap junction turnover.**

Methods and Results: **By electron microscopy, we characterized gap junctions formed between cardiomyocyte lateral membranes in failing canine ventricular myocardium. These gap junctions were varied in structure and appeared to be extensively internalizing. Internalized gap junctions were incorporated into multilamellar membrane structures, with features characteristic of autophagosomes. Intracellular Cx43 extensively colocalized with the autophagosome marker GFP-LC3 when both proteins were exogenously expressed in HeLa cells, and endogenous Cx43 colocalized with GFP-LC3 in neonatal rat ventricular myocytes. Furthermore, a distinct phosphorylated form of Cx43, as well as the autophagosome-targeted form of LC3 (microtubule-associated protein light chain 3) targeted to lipid rafts in cardiac tissue, and both were increased in heart failure.**

Conclusions: **Our data demonstrate a previously unrecognized pathway of gap junction internalization and degradation in the heart and identify a cellular pathway with potential therapeutic implications. (*Circ Res*. 2010; 106:1153-1163.)**

Key Words: gap junctions ■ connexin43 ■ heart failure ■ autophagy ■ lipid rafts

Gap junctions (GJs) allow ions and small molecules to be exchanged between the cytoplasm of adjacent cells. Intercellular communication through GJs mediates coordinated cell behavior in nearly every mammalian tissue, contributing to diverse physiological processes. Efficient electric activation and action potential propagation in the heart requires current passage between cardiomyocytes, a function carried out by GJs. Thus, accurate targeting and maintenance of GJs in cardiomyocytes is essential for normal heart function. Consistent with this concept are studies that demonstrate impaired heart function on genetic disruption of connexin (Cx)43, the primary GJ channel protein in mammalian ventricular muscle.[1–3] Furthermore, a variety of structural heart diseases in both humans and animal models are associated with remodeling of GJ proteins, including decreased expression and altered subcellular distribution of Cx43.[4–10] Despite the critical role GJs play in the heart, and extensive evidence for GJ remodeling in cardiac disease, a mechanistic understanding of the GJ life cycle in cardiomyocytes is limited.

GJ channels are assembled from 2 hexameric hemichannels, or connexons, (formed by connexins) which dock together at contacts between cells. GJ channels cluster into tightly packed 2D arrays consisting of a few to thousands of channels, known as GJ plaques. In cardiac tissue GJs are formed primarily at the intercalated disk (ID), which is the site of contact between the ends of cardiomyocytes. This arrangement accounts for anisotropic current flow, with conduction progressing rapidly in the direction of tissue fiber orientation. GJ proteins have been shown to have a remarkably rapid rate of turnover, with half lives on the order of 1.5 to 5 hours both in vitro and in vivo.[11–13] Such rapid turnover kinetics necessitates well coordinated and regulated trafficking. GJ plaques are internalized by a cellular process in which

Original received June 24, 2008; revision received January 28, 2010; accepted February 8, 2010.

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This manuscript was sent to Hans Michael Piper, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

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Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.108.182147**

Non-standard Abbreviations and Acronyms	
AGJ	annular gap junction
AP	alkaline phosphatase
Cx43	connexin43
GFP	green fluorescent protein
GJ	gap junction
HF	heart failure
ID	intercalated disk
LC3	microtubule-associated protein light chain 3
LR	lipid raft
NRVM	neonatal rat ventricular myocyte
TEM	transmission electron microscopy
ZO-1	zonula occludens-1

the plasma membranes of both coupled cells are internalized into one of the 2 cells, producing double membrane intracellular inclusions.[14] Intracellular circular GJ membranes, termed annular GJs (AGJs), have been morphologically characterized in isolated cells and tissues by electron microscopy.[15,16] Dynamic studies of GJs in cell systems have demonstrated internalization of entire plaques forming AGJs consisting of membranes from both coupled cells.[17,18] Since their earliest description, annular GJs have been implicated in degradative pathways involving lysosomes[14,15,19–22] or the proteasome.[23] More recent studies have sought to characterize the cellular processes by which GJs are internalized and degraded,[18,24] although many details remain unknown, especially in the physiological context.

Cx43 phosphorylation is widely implicated in the assembly and turnover of GJs. Cx43 can be phosphorylated on multiple amino acids on its carboxyl-terminal tail, with different phosphorylation sites having distinct effects on GJ function. It has been reported that the phosphorylation state of Cx43 is altered with cardiac disease and on pharmacological treatment of cardiac cells[6,7,25–29]; however, the precise phosphorylation changes that occur, and the effect they have on GJ function, are unresolved.

In this article, we provide an ultrastructural characterization of GJs formed between cardiomyocyte lateral cell borders in failing canine cardiac tissue. Internalized GJs are incorporated into heterogeneous multilamellar membranes, reminiscent of autophagosomes. These data suggest that an autophagic pathway is enhanced in the failing heart, and involves a distinct phosphorylated form of Cx43 that targets to lipid rafts (LRs).

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and describes antibodies, plasmids, cell culture methods, biochemical fractionation and analysis of tissue, and both light and electron microscopic methods used in the study.

Canine Heart Failure Model

Dogs were rapidly paced into heart failure (HF) as previously described.[6]

Neonatal Rat Ventricular Myocyte Isolation and Culture

Neonatal rat ventricular myocytes (NRVMs) were enzymatically dissociated from the ventricles of 2-day-old rats as previously described.[30]

Statistical Analysis

For the frequency of lateralized GJs (normal versus failing hearts), statistical significance was determined by a proportion test of the mean frequencies. Statistical significance of Western blot data were determined using unpaired, 2-tailed Student's *t* tests. Error bars represent SD of the mean.

All procedures involving animals were approved by the Johns Hopkins Animal Care and Use Committee.

Results

Lateralized Cx43 Does Not Colocalize With Zonula Occludens-1 or Cadherin

Lateralized Cx43 was first characterized in canine ventricular tissue by immunofluorescent confocal microscopy. Figure 1A and 1B shows representative failing cardiac tissue sections stained with antibodies to Cx43, zonula occludens (ZO)-1 or a pan-specific cadherin antibody, revealing extensive targeting of both Cx43 and mechanical junction proteins to the ID (boxed regions). Extensive Cx43 staining was also observed along cardiomyocyte lateral borders, a pattern previously shown to be exaggerated in a variety of structural heart diseases (arrowheads). The lateralized population of Cx43 exhibited diminished colocalization with ZO-1 and cadherin. ZO-1 staining was also present in spaces between cardiomyocytes, representing staining of noncardiomyocyte cell types including vascular endothelial cells within capillaries and fibroblasts.

GJ Ultrastructure

We used transmission electron microscopy (TEM) of canine cardiac tissue to examine the ultrastructure of GJs. By TEM, GJs appear as pentalaminar membranes. Immunogold labeling of Cx43 revealed extensive staining on pentalaminar membranes within the ID, representing GJs formed between cells (not shown). In both normal and failing hearts, Cx43-labeled pentalaminar membranes were also observed in circular structures emanating from the ID region, representing internalizing GJs destined to become AGJs (Figure 2A).

Conventional TEM was used to study the ultrastructure of GJs in greater detail. Figure 2B shows an electron micrograph of normal cardiac tissue demonstrating the orientation of GJs within the ID. The mechanical junctions formed between cardiomyocytes are formed by adherens junctions and desmosomes and run perpendicular to the direction of tissue fibers. GJs are located between intervening portions of mechanical junctions and run parallel to the fiber orientation (Figure 2B, boxed region). GJs located at the outer edge of the ID are not typically flanked by mechanical junctions at the edge which is continuous with lateral membranes (Figure 2B1).

Figure 2C and 2D shows electron micrographs of failing cardiac tissue. GJs are extensively formed between the lateral membranes of adjacent myocytes, in regions distant from the ID (Figure 2C [1 through 3] and 2D [1 and 2]). These

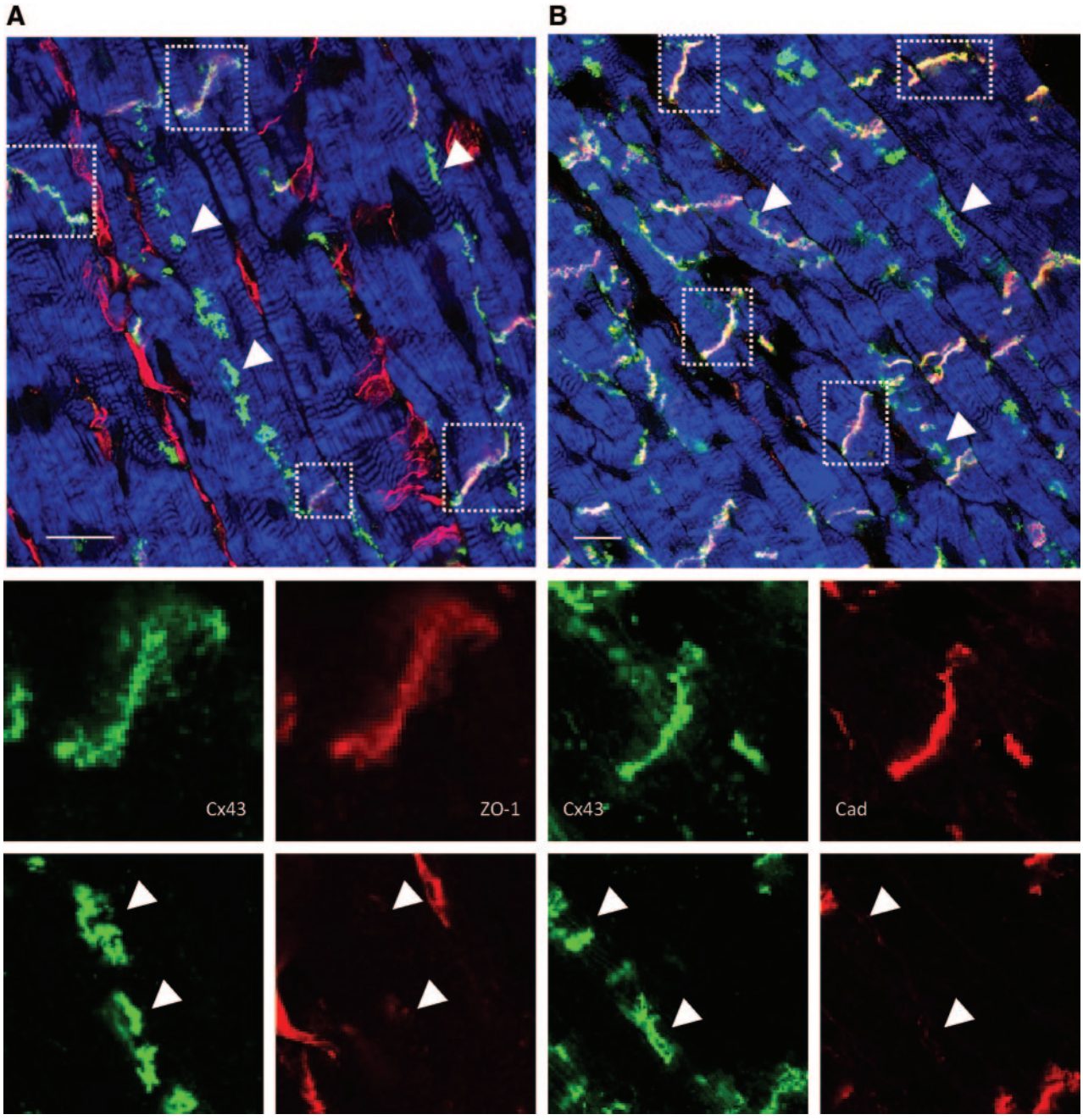

Figure 1. Immunofluorescent localization of Cx43, ZO-1, and cadherin in canine cardiac myocardium. Failing canine left ventricular tissue sections were stained for Cx43 **(green)** and ZO-1 **(red) (A)** or cadherin **(red) (B)** and imaged by confocal microscopy. Actin was stained using phalloidin to reveal tissue architecture **(blue)**. **A and B** are optical sections showing IDs **(boxed regions)** and lateralized GJs **(arrowheads)**. The IDs exhibit staining for Cx43 and ZO-1 and Cx43 and cadherin, whereas lateralized Cx43 signal exhibits diminished signal for ZO-1 and cadherin.

lateralized GJs are not flanked by typical mechanical junctions. Lateralized GJs are atypical of GJs formed at IDs in that they exhibit complex membrane bending and are often in close association with mitochondria, and other indistinct cellular material. Lateralized GJs were more frequently observed in failing compared with normal tissue. A series of images of similar tissue orientations were acquired from 4 normal and 3 failing dog hearts, and the number of cell–cell pairs possessing lateralized GJs was counted (Figure 3A). In tissue sections from 4 normal dogs, 1 of 21, 1 of 22, 4 of 25, and 11 of 21 cell–cell pairs possessed lateralized GJs (average frequency=0.19), whereas in tissue sections from 3 failing dogs, 7 of 14, 14 of 21, and 9 of 12 cell–cell pairs possessed lateralized GJs (average frequency=0.64) (P=0.013) (Figure 3B).

In failing cardiac tissue, internalized GJs were often observed as concentric rings, suggesting extensive involution of GJ membranes during internalization. (Figure 2D2, arrowhead; Figure 4A through 4D). Internalized GJs are typically large (≈0.1 to 1 μm in diameter), highly heterogeneous structures with undefined cellular debris in their lumens (Figure 4A). Internalized GJ membranes also

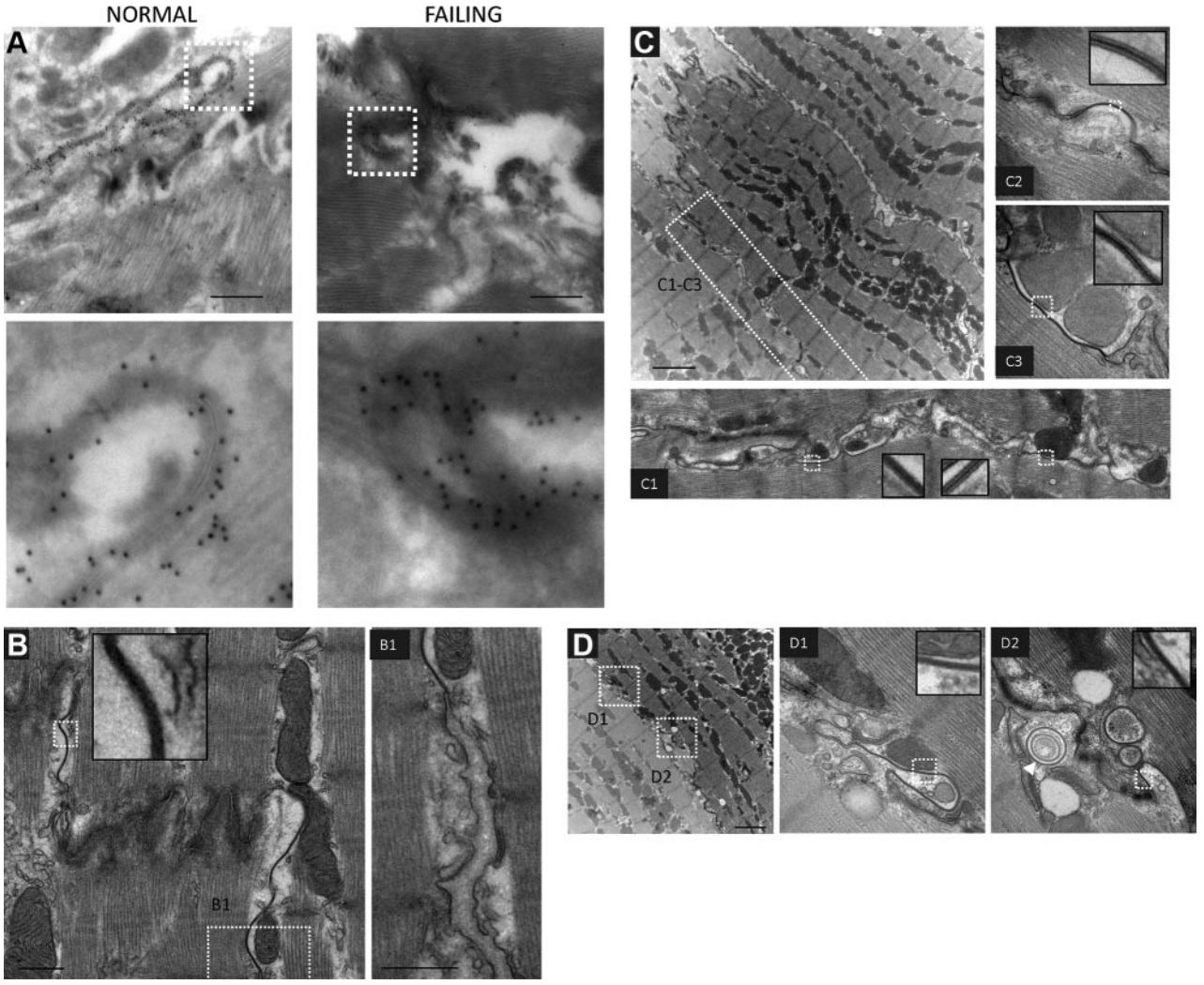

Figure 2. TEM of cardiac tissue sections. A, Micrographs of normal and failing canine ventricular tissue sections labeled with anti-Cx43 primary antibody and a secondary antibody conjugated to 12-nm colloidal gold and then imaged by TEM. The **bottom images** show magnified regions of the **boxed areas**. **B,** Conventional TEM of nonfailing canine left ventricular tissue sections. The ID is magnified in the inset. **B1,** Magnified and extended view of the boxed region in **B**. **C and D,** Micrographs of failing canine ventricular tissue sections, highlighting contacts between cardiomyocyte lateral cell borders. **C (1 through 3) and D (1 and 2),** Magnified images from within the **boxed regions** in **C** and **D**, respectively. The **insets** are from **boxed regions** highlighting pentalaminar membranes. A concentric internalized GJ is indicated by a **white arrowhead** in **D, 2**. Scale bars A, B,=500 nm C, D=2 μm.

formed multilamellar membrane structures (Figure 4B and 4C) reminiscent of autophagosomes. Further morphological support for autophagic sequestration of internalized GJs were crescent-shaped, putative isolation membranes that appeared to envelope internalized GJs (Figure 4D1, arrowheads), as well as multiple putative isolation membranes in close proximity to the ID (Figure 4E).

To test the hypothesis that internalized GJs associate with autophagosomes, we transiently transfected HeLa cells with both Cx43 and a specific marker of autophagosomes, LC3 (microtubule-associated protein light chain 3) fused to enhanced green fluorescent protein (GFP-LC3). Figure 5A shows 2 HeLa cells, each expressing Cx43 and GFP-LC3. GJs are formed at sites of contact between the 2 cells (arrowhead in Figure 5A, merge) and exhibit strong Cx43 staining. A significant fraction of Cx43 staining is intracellular and punctate, representing trafficking intermediates of Cx43. GFP-LC3 is expressed as a cytoplasmic protein, resulting in diffuse green signal throughout the cell. However, GFP-LC3 is also concentrated on maturing autophagosomes by a covalent lipid modification, resulting in punctate green staining of autophagosomes. A proportion of intracellular Cx43 signal colocalized with GFP-LC3 (Figure 5A, merge), suggesting an association of internalized GJs with autophagosomes. To determine whether endogenous cardiac Cx43 associates with autophagosomes we cultured primary NRVMs that were transiently transfected with GFP-LC3. Under baseline culture conditions cytoplasmic Cx43 and GFP-LC3 only sporadically colocalized (data not shown). We hypothesized that internalized GJs may be rapidly cleared from the cytoplasm through lysosomal degradation. We therefore treated cells with a lysosomal inhibitor (10 μmol/L chloroquine) for 2 hours before fixing, immunostaining and

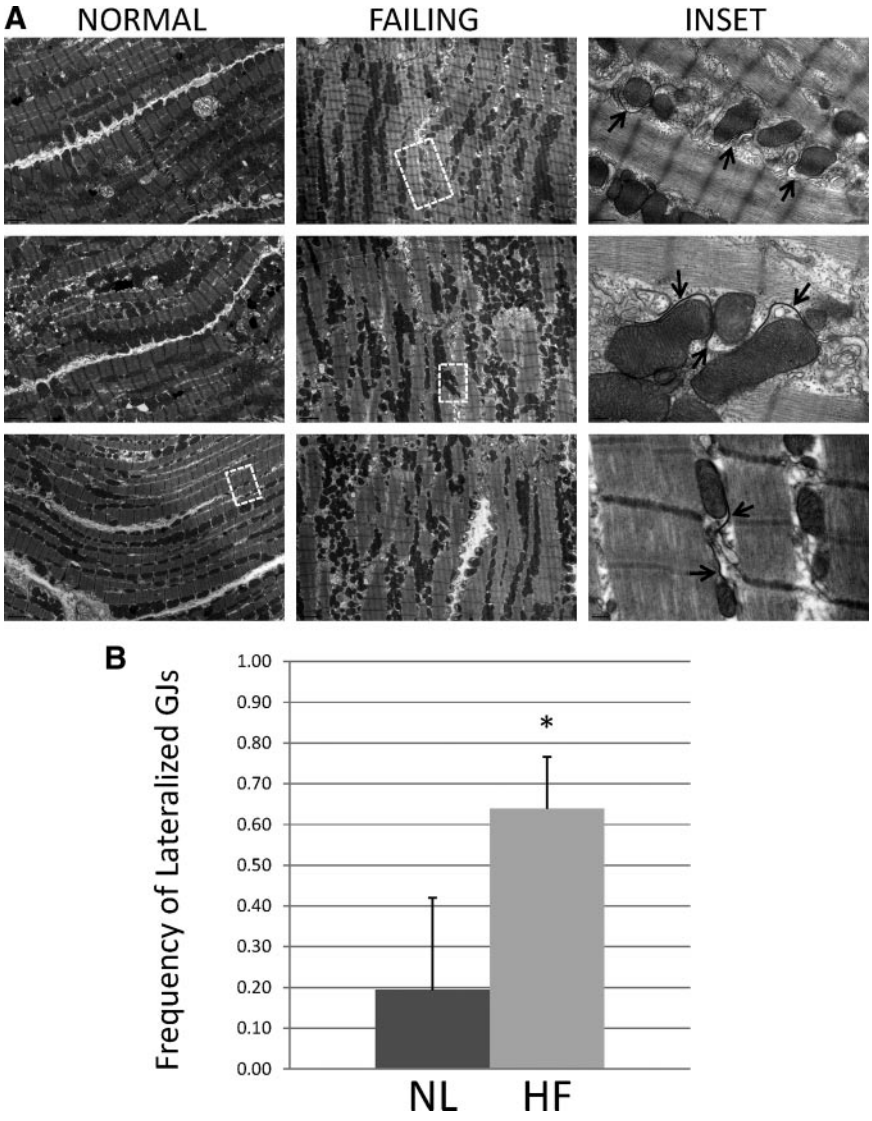

Figure 3. Frequency of lateralized GJs in normal and failing myocardium. A, Representative micrographs of apposing lateral cell borders in normal and failing canine myocardium. The **inset images** are from the **boxed regions**, highlighting GJs formed between myocyte lateral cell membranes. Pentalaminar GJ membranes are indicated by **arrows**. **B,** The frequency with which apposing lateral cell borders exhibited GJ formation was quantified in normal and failing tissue sections. In normal hearts (n=4), 1/22, 1/22, 4/25, and 11/21 cell–cell borders possessed lateralized GJs, whereas in failing hearts (n=3), 7/14, 14/21, and 9/12 cell–cell borders possessed lateralized GJs. *P=0.013.

imaging the cells. Inhibiting lysosomal activity resulted in significant intracellular colocalization between endogenous Cx43 and expressed GFP-LC3 (Figure 5B).

LR Targeting of Cx43 and LC3-II

Cx43 has been reported to target to buoyant, cholesterol and sphingolipid-rich membrane domains termed LRs,[31–33] although the significance of LR targeting is not known. Our observation that internalized GJs associate with multilamellar membranes led us to hypothesize these structures might represent detergent resistant, buoyant populations of Cx43. We prepared LRs from cardiac tissue by cold Triton X-100 extraction and sucrose density centrifugation. Western blotting revealed 2 populations of Cx43 (Figure 6A): a buoyant population (fraction 5) that cofractionated (but is not necessarily associated) with the muscle-specific caveolar protein caveolin-3 and the glycosylated sphingolipid G_M1 (markers of LR domains) and a dense population (fractions 9 to 11). Cx43 is well known to separate into multiple bands by SDS-PAGE, representing distinct phosphorylated species of the protein.[34] To better separate Cx43 into its distinct phosphorylated forms, we analyzed the sucrose fractions by large format SDS-PAGE (Figure 6B, top). The slowly migrating form of Cx43 (designated P2) was uniquely targeted to the LR fraction, whereas intermediate and fast migrating bands (designated P1 and P0 respectively) remained predominantly in the dense non-LR fractions. To confirm that the slow migration of LR targeted Cx43 was attributable to phosphorylation and not an artifact of the LR isolation procedure, we treated proteins isolated from fraction 5 with alkaline phosphatase (AP) (Figure 6C). AP treatment increased the mobility of Cx43, indicating that LR-targeted Cx43 constitutes a highly phosphorylated form of the protein.

We examined the distribution of the autophagosome marker LC3 in LR fractions (Figure 6B, bottom). LC3 migrates as 2 forms, LC3-I and LC3-II. LC3-I is cytoplasmic and migrates at ≈18kDa. During autophagy LC3-I is partially cleaved and covalently linked to maturing autophagosomes, resulting in the LC3-II form that migrates at ≈15 kDa. The dense, non-LR fractions contained both LC3-I and LC3-II. The buoyant fraction 5 contained LC3-II, indicating that a subpopulation of autophagosomes

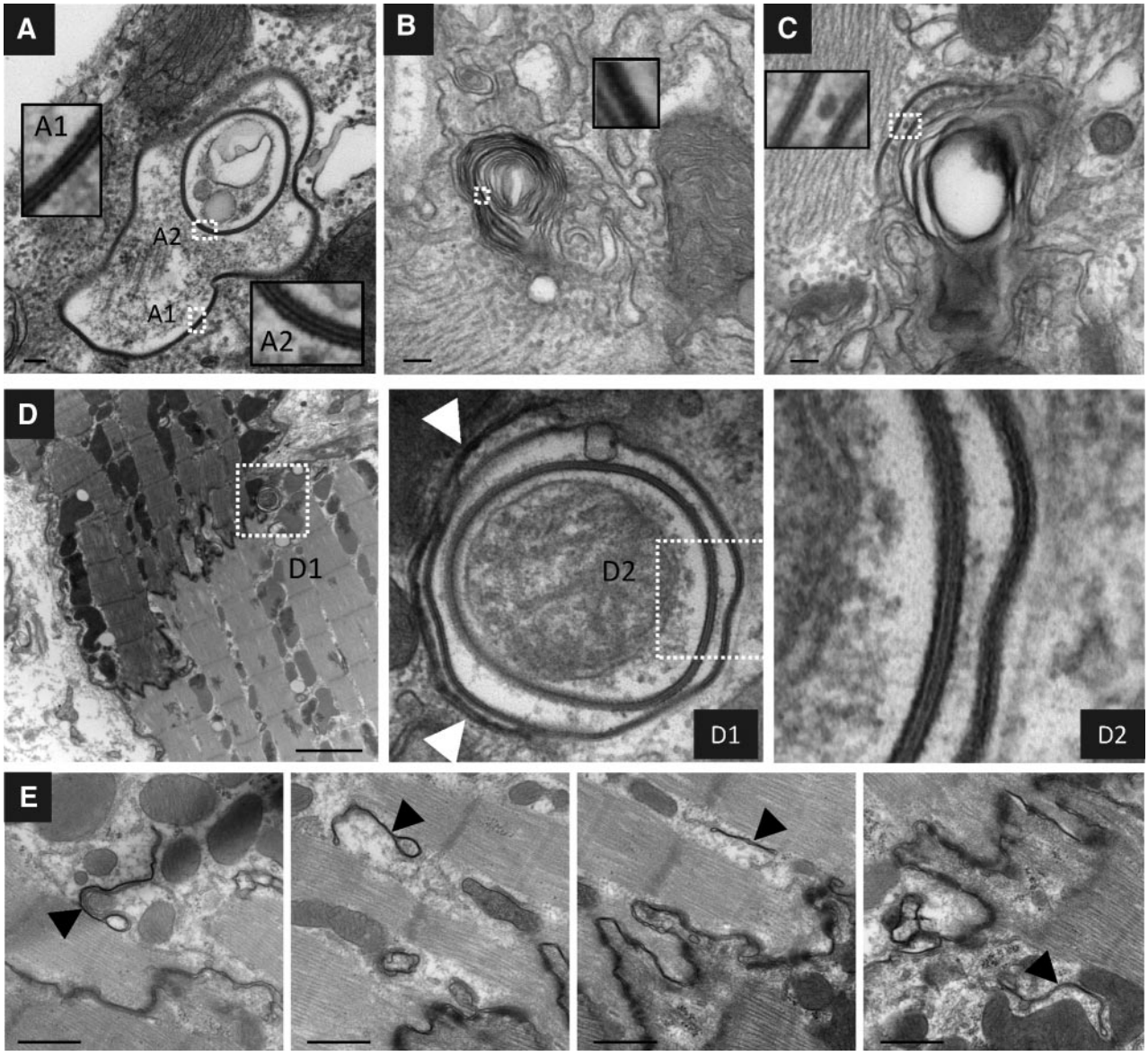

Figure 4. Ultrastructure of internalized GJs from failing myocardium. Representative micrographs of failing myocardium showing internalized GJs with concentric pentalaminar membranes and cellular debris in their lumens **(A),** as well as pentalaminar membranes incorporated into multilamellar membrane structures **(B and C)**. **D,** Internalized and/or internalizing concentric GJ in close association with a putative isolation membrane shown at higher resolution in **D1 (arrowheads)**. **E,** Multiple double membrane structures with the appearance of isolation membranes in close proximity to the ID. The **insets** show the detail of pentalaminar membranes from boxed regions. **Scale bars:** 100 nm **(A through C)**; 2 μm **(D)**; 500 nm **(E)**.

possess the physicochemical properties of LRs. The LR fraction, as expected, is devoid of ZO-1, which was confined to the pellet (Figure 6D).

We then examined the ultrastructure of membranous material from fraction 5 (Figure 6E). By TEM, fraction 5 consists primarily of multilamellar membrane structures, with no discernable structure or morphology. However, interspersed in this material we observed pentalaminar membranes. The edges of these pentalaminar membranes were not associated with structures consistent with mechanical junctions (fascia adherens and desmosomes). This observation is consistent with a population of Cx43 possessing LR properties after incorporation into GJs, but not associated with mechanical junctions, which may include the internalized, multilamellar membranes associated with GJs we observed in cardiac tissue.

Cx43 and LC3 Expression in HF

We examined LR fractions from both normal and pacing-induced HF dog ventricles (Figure 7A).[6,7] LR domains isolated from failing hearts exhibited an approximately 3.5-fold greater Cx43 signal than LR domains from normal hearts ($P=0.011$). In these same samples, the total Cx43 levels decreased by approximately 2-fold ($P=0.01$). We examined the expression levels of LC3-I and LC3-II in normal and HF hearts (Figure 7B). The levels of LC3-I were not statistically different, whereas the levels of LC3-II increased by approximately 2-fold in HF hearts as

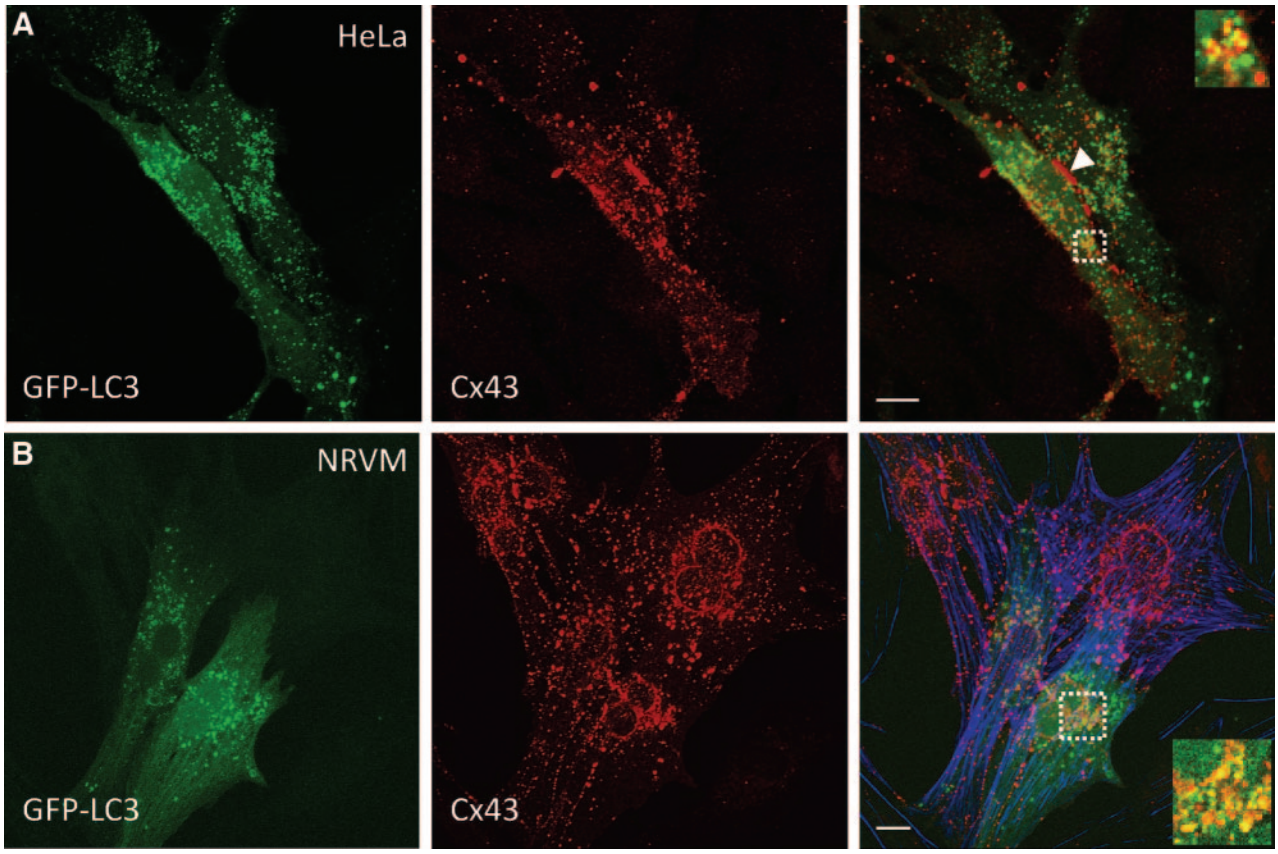

Figure 5. Association of internalized GJs with autophagosomes. A, HeLa cells were transfected with Cx43 and GFP-LC3. Fixed cells were immunostained and imaged by confocal microscopy (GFP-LC3 [**green, left**], Cx43 [**red, middle**], merge [**right**]). **B,** Cultured NRVMs were transfected with GFP-LC3 and treated with a lysosomal inhibitor (10 μmol/L chloroquine for 2 hours before fixation). Fixed cells were then immunostained for endogenous Cx43 and imaged as in **A**. NRVMs were stained for actin using phalloidin **(blue)**. All **images** are projections of 17 optical sections. **Insets** in **merged images** are single optical sections from within **boxed regions**. **Scale bars:** 2 μm.

compared to normal hearts ($P=0.016$), indicative of increased autophagosome formation in failing hearts.

Discussion

In cardiac tissue, Cx43 forms GJs primarily at the ID. A prominent feature of structural heart disease is a redistribution of Cx43 to the lateral cell borders and a decrease in Cx43 expression levels.[6,7,29] Despite the consistency of altered Cx43 expression and distribution in structural heart disease, the functional status and structural nature of lateralized Cx43 is incompletely understood. In this study, we demonstrate that GJs are formed between the lateral membranes of cardiomyocytes with increased frequency in a canine model of pacing induced HF. However, the lack of colocalization with ZO-1 and cadherin, and the heterogeneous structure morphology of lateralized GJs demonstrate that they are structurally and likely functionally distinct from those formed at the ID.

Cx43 has been shown to be directly bound by ZO-1[35] and it is thought that ZO-1 in part regulates the assembly of Cx43 into the periphery of GJ plaques.[36] Changes in the relationship between ZO-1 and Cx43 have been reported in cardiac disease[37,38] and is emerging as a potentially critical interaction for the maintenance of proper GJ-mediated intercellular communication. Furthermore, Cx43-containing vesicles have been shown to be directly delivered to cadherin-containing adherens junctions via microtubules.[39] The formation of apparently atypical GJs at cardiomyocyte lateral membranes, devoid of ZO-1 and cadherin colocalization, suggests impairment in the mechanisms responsible for direct delivery of Cx43 to sites of mechanical junction formation.

AGJs have been described in a number of cells and tissues[14,17,18] including isolated cardiomyocytes[15,40] and cardiac tissue subjected to stress.[29,41] AGJs have been suggested to be endocytosed GJ plaques destined for lysosomal or proteasomal degradation.[14,15,19,21,23] Direct evidence for the origin of AGJs comes from the work of Jordan et al[17] using time lapse studies of fluorescently tagged Cx43, and Piehl et al[18] using dye injection studies to demonstrate internalization of intercellular GJ plaques into 1 of 2 coupled cells. We suggest that intracellular uni- and multilamellar GJ-containing membrane rings observed in cardiac tissue represent AGJs in progressively advanced stages of processing for degradation.

The incorporation of internalized GJs into multilamellar membranes, their close association with cellular debris, and the colocalization of Cx43 with LC3 is suggestive of autophagy playing a role in GJ clearance. The association of internalized GJs with autophagosomes has been suggested in the literature[15,42] but is not an accepted mechanism of GJ degradation. Autophagosomes are dynamic organelles which

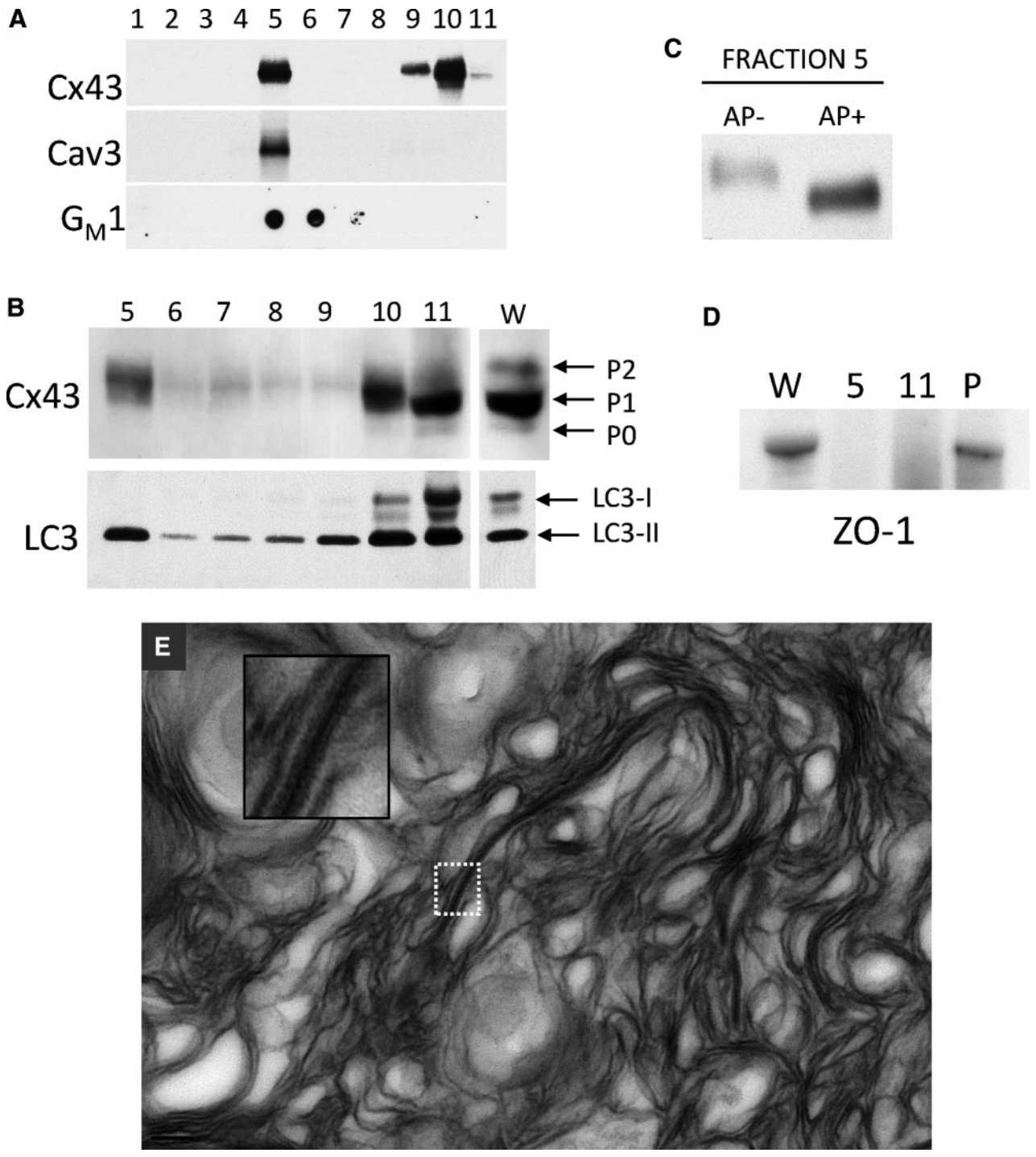

Figure 6. LR targeting of Cx43 and LC3 in cardiac tissue. A, Sucrose gradient fractionation of canine ventricular myocardium Western blotted for Cx43, caveolin-3 (Cav-3), and G_M1 (dot blot probed with horseradish peroxidase–conjugated cholera toxin B subunit). Fractions 1 to 4 correspond to 5% sucrose, 5 to 10 correspond to 38% sucrose, and 11 corresponds to 40% sucrose. **B,** Fractions 5 to 11 probed for Cx43 (separated by large format [18 cm^2] 10% acrylamide gel) and LC3. The **lane** marked **W** is a whole tissue lysate. Distinct phosphorylated species of Cx43 (P0, P1, P2) and LC3-I and LC3-II are indicated with arrows. **C,** Cx43 Western blot of fraction 5 that was untreated (AP-) or treated (AP+) with AP and separated on a 10% acrylamide gel. **D,** ZO-1 is absent from fraction 5 and 11 but was present in the pellet (P). **E,** Material in fraction 5 was pelleted and prepared for TEM. A representative TEM of fraction 5 exhibits primarily multilamellar membranous material with an incorporated pentalaminar membrane (the **inset** is a higher-power view of the **boxed region**).

sequester cell contents for delivery to lysosomes.[43] In postmitotic cells, such as cardiomyocytes, autophagy is constitutively active to help maintain cell size and integrity. Under stressful conditions, the rate of autophagy is increased, where it serves an adaptive function by providing a source of nutrients through catabolism of "unnecessary" cellular contents, as well as clearing damaged organelles.[44] Autophagy has been implicated in the pathogenesis of many diseases,

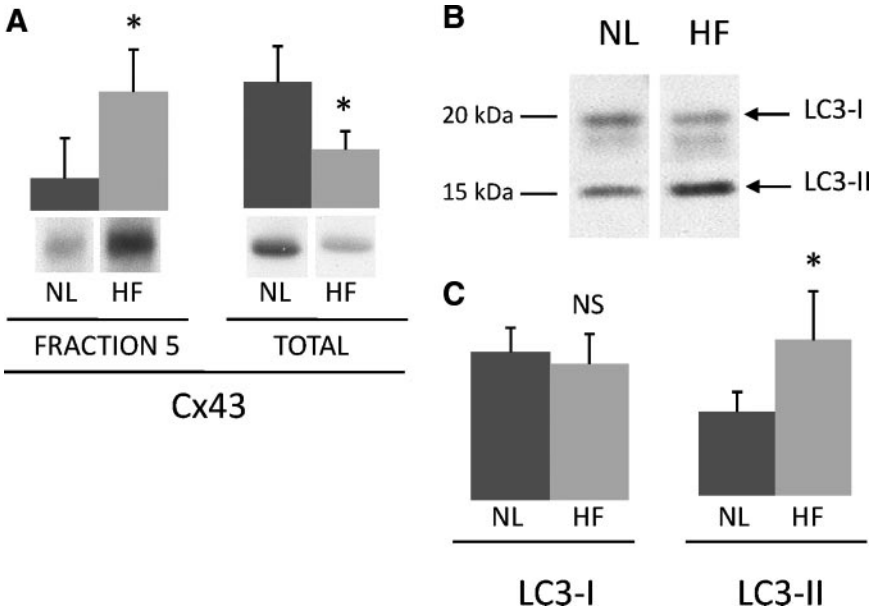

Figure 7. LR-targeted Cx43 and LC3-II are increased in HF. A, Bar graphs and representative Western blots of fraction 5 **(left)** from normal (NL) and failing (HF) dog hearts (n=5 each) and whole tissue lysates **(right)** from the same hearts (separated by 4% to 12% acrylamide minigels to collapse Cx43 bands) probed for Cx43 (arbitrary units, $^*P<0.02$). **B,** The whole tissue lysates used in **A** were also probed for LC3. The migration of LC3-I and LC3-II are indicated by **arrows**. **C,** Bar graphs of LC3-I and LC3-II levels in normal and failing hearts (n=5 each, arbitrary units, NS indicates not significant, $^*P<0.02$).

including ischemia/reperfusion injury and HF,[45,46] as a mechanism of cell survival. Internalized GJs are quite large and structurally distinct relative to typical endocytic organelles, and connexins are relatively nonstandard endocytic cargo. The cellular machinery involved in GJ internalization and degradation is therefore likely to be distinct from that involved in more conventional endocytic processes. Thus, it is plausible that GJs would be sequestered by autophagic machinery for delivery to lysosomes.

The signaling mechanisms regulating the internalization and degradation of Cx43 GJs are poorly understood, but likely involve post-translational modification of Cx43. Cx43 is phosphorylated extensively on its carboxyl terminus, regulating both the trafficking and permeability of Cx43 GJ channels.[47,48] By SDS-PAGE, the 3 major bands observed for Cx43 are typically referred to as P0 (fastest migrating, least phosphorylated), P1, and P2 (slowest migrating, most phosphorylated), and the P2 form only appears after Cx43 has reached the plasma membrane and formed GJs.[34] Changes in the phosphorylation state of cardiac Cx43 have been reported in heart disease[7,29]; however, the precise changes that occur and the functional consequences of these changes are unclear. In cell-based models, pharmacological interventions that promote the phosphorylation of Cx43 to the P2 state have been shown to enhance internalization and degradation of Cx43 GJs, suggesting that hyperphosphorylation of Cx43 may be involved in GJ degradation.[24,49–52] Other studies have reported enhanced expression and GJ formation on pharmacological treatments also likely to result in Cx43 phosphorylation.[26,28] It is likely that the specific amino acids that are phosphorylated dictate the effects that Cx43 phosphorylation has on GJs. Thus, increased Cx43 phosphorylation, or perhaps differences in the pattern of phosphorylation, may result in more rapid kinetics at multiple stages of the GJ life cycle, enhancing the formation of GJs, as well as their internalization and degradation, increasing the overall rate of GJ assembly and turnover. Further studies will be required to determine the effect that specific phosphorylation events have on GJ function and to determine which of these events are altered in disease.

Cx43 has been reported to target to buoyant, cholesterol- and sphingolipid-rich membrane domains termed lipid rafts,[31–33] and there is evidence that the phosphorylation state may differ between LR- and non–LR-targeted populations.[32–34,53] Our observation that the P2 form of Cx43 is uniquely targeted to LRs suggests that LR targeting of Cx43 is a late event in the GJ life cycle and may be involved in GJ degradation. Consistent with this hypothesis are studies which demonstrate that connexins can target to LRs, but that intercellular GJs themselves are not LRs.[31] Musil and Goodenough[34] have described the relationship between Cx43 Triton solubility, phosphorylation state, and assembly into GJs in vitro. The authors suggest that Cx43 begins as a Triton soluble, hypophosphorylated form during its progression toward the plasma membrane. On arrival at the plasma membrane Cx43 acquires Triton resistance and matures into a hyperphosphorylated form. The authors further suggest that the acquisition of Triton resistance and maturation to the P2 phosphorylated form corresponds to the formation of mature GJ plaques. Based on our ultrastructural characterization of fraction 5 (Figure 6E), which contains P2 phosphorylated Cx43, we do not believe these buoyant pentalaminar membranes represent GJ plaques coupling 2 cells at IDs, but rather internalized GJs incorporated into multilamellar membranes. We therefore hypothesize that maturation to the P2 form in fact corresponds to the degradation of GJs through autophagic processing, although further experiments will be required to confirm this hypothesis.

Our data demonstrate that GJs with highly variable morphology are formed between cardiomyocyte lateral membranes with increased frequency in HF and that GJ turnover likely occurs via an autophagic pathway. The mechanisms by which Cx43 GJs are targeted for internalization and degradation by autophagy appears to involve changes in phosphorylation, as well as processing through membranes with physicochemical properties of LRs. The regulated process of

GJ turnover via autophagy suggests a novel pathway for the regulation of cardiac GJs and therefore conduction in the mammalian ventricle. These findings offer insight into the mechanisms regulating electric conduction in mammalian myocardium, and may in part contribute to the arrhythmogenic electric remodeling associated with HF.

There are limitations to the present study. We did not perform serial sectioning and/or 3D reconstruction of TEM sections. It is therefore possible that apparently internalized GJs are in reality extensively convoluted GJs still attached to the plasma membrane. Regardless of this possibility, the presence of GJs at lateral membranes is nonetheless more frequent in failing hearts, and lateral GJs are more convoluted, if not more extensively internalized, than GJs at the ID.

These data were obtained in a canine model of HF that in many respects mimics human HF but is clearly different from the most common forms of HF occurring in humans. The detailed regulation of Cx43 and GJ localization may not be identical with human disease. Reassuringly, many of the features of GJ remodeling characterized in this model are similar to those previously observed in diseased human hearts.[5,9]

Although we have provided ultrastructural, microscopic, and biochemical evidence in support of GJ association with autophagosomes, we have not demonstrated connexin degradation via autophagy per se. The precise contribution of autophagy to connexin turnover will require a more quantitative examination.

Acknowledgments

We acknowledge Dr David R. Graham for assistance with lipid raft isolation.

Sources of Funding

This work was supported by NIH grant P01 HL077180. G.F.T. holds the Michel Mirowski, MD, Professorship in Cardiology.

Disclosures

None.

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Novelty and Significance

What Is Known?

- Gap junctions (GJs), formed by connexins, allow electric current to flow from one cardiomyocyte to another and are required for synchronous electric activation and contraction of cardiac muscle.
- In healthy hearts, GJs are formed primarily between the ends of cardiomyocytes (intercalated disks); however, in a variety of heart diseases, the major ventricular GJ protein (connexin [Cx]43) increases its localization at cardiomyocyte lateral membranes, as well as decreases in overall expression.
- The precise location, the functional status, and mechanisms of regulation of lateralized Cx43 are not known.

What New Information Does This Article Contribute?

- This article demonstrates increased GJ formation between the lateral membranes of adjacent cardiomyocytes, which are not flanked by typical mechanical junctions, have highly variable morphology and appear to undergo extensive internalization.
- Internalized GJs are incorporated into multilamellar membrane structures characteristic of autophagosomes, and Cx43 colocalizes with an autophagosome marker (LC3-II) in both HeLa cells and primary cultured neonatal rat ventricular myocytes.
- This article also shows that a distinct phosphorylated form of Cx43 targets to lipid rafts, and it is this population that we hypothesize is involved in the autophagic degradation of GJs.

We and others hypothesize that remodeling of GJ expression and localization contributes, at least in part, to the high incidence of lethal arrhythmias associated with cardiovascular disease. In this study, we have demonstrated that GJs are formed between adjacent cardiomyocyte lateral membranes with increased frequency in failing hearts. Lateralized GJs have highly variable morphology and appear to be extensively internalized, and internalized GJs associate with autophagosomes. Furthermore, a distinct phosphorylated form of Cx43 targets to lipid rafts, and we hypothesize that it is this population that undergoes autophagic degradation. Of particular novelty in this study is the ultrastructural characterization of lateralized GJs. Until now, the precise location of lateralized Cx43 was unknown. Our observation suggests that Cx43 may be aberrantly targeted to lateral membranes with increased frequency in a disease setting, highlighting the importance of further study into the mechanisms of Cx43 targeting to the plasma membrane. Finally, our study suggests that GJs are degraded through autophagy, which until now has not been incorporated into models of GJ degradation. From this observation should stem further study into the role of autophagy in GJ turnover, both in the heart and in general.