Abnormal response of costal chondrocytes to acidosis in patients with chest wall deformity

A. Asmar[a], I. Semenov[a], R. Kelly Jr[b], M. Stacey[a],*

[a] *Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, VA, USA*
[b] *Department of Surgery, Eastern Virginia Medical School, Pediatric Surgery Division, Children's Hospital of the King's Daughters, Norfolk, VA, USA*

A B S T R A C T

Costal cartilage is much understudied compared to the load bearing cartilages. Abnormally grown costal cartilages are associated with the inherited chest wall deformities pectus excavatum and pectus carinatum resulting in sunken or pigeon chest respectively. A lack of understanding of the ultrastructural and molecular biology properties of costal cartilage is a major confounder in predicting causes and outcomes of these disorders. Due to the avascular nature of cartilage, chondrocytes metabolize glycolytically, producing an acidic environment. During physical activity hydrogen ions move within cartilage driven by compressive forces, thus at any one time, chondrocytes experience transient changes in pH. A variety of ion channels on chondrocytes plasma membrane equip them to function in the rapidly changing conditions they experience. In this paper we describe reduced expression of the *ASIC2* gene encoding the acid sensing ion channel isoform 2 (previously referred to as ACCN1 or ACCN) in patients with chest wall deformities. We hypothesized that chondrocytes from these patients cannot respond normally to changes in pH that are an integral part of the biology of this tissue. Activation of ASICs indirectly creates a cascade ultimately dependent on intracellular calcium transients. The objective of this paper was to compare internal calcium signaling in response to external pH changes in costal chondrocytes from patients with chest wall deformities and healthy individuals. Although the molecular mechanism through which chondrocytes are regulated by acidosis remains unknown, we observed reduced amplitudes of calcium rise in patient chondrocytes exposed to low pH that become further impaired upon repeat exposure.

1. Introduction

Chest wall deformities (CWD) are significant disorders of costal cartilage and a leading health problem affecting young adults. CWD patients are often described as having 'weak' cartilage and typically face exercise intolerance due to associated heart and lung limitations, in addition to substantial psychosocial problems in a vulnerable age group (Kelly Jr. et al., 2013). In severe cases where access to proper reparative surgery is unavailable, marked functional consequences may be encountered. CWD are a complex inherited disorder affecting 1 in 400 to 1 in 1000 individuals, primarily males (~4M:1F) (Creswick et al., 2006; Horth et al., 2012) and can be divided into those with sunken chests (pectus excavatum, PE) and those with pigeon chests (pectus carinatum, PC). Repair of PE is now routinely performed following the minimally invasive Nuss procedure (Nuss, 2008, Pilegaard and Licht, 2017) where one or two substernal support titanium bars are inserted and kept in place for approximately 2–3 years. Outcomes are reported as excellent (Goretsky et al., 2004). With the introduction of stabilizers,

bar displacements dropped significantly. Safety concerns regarding the absorption of electro-magnetic (EM) energy (specific energy absorption rate; SAR) by the implanted bar arose due to the length of time the bars are in place. This was addressed by Miaskowski et al. (2016) whose results clearly indicated that a conductive object, such as a concave implant may cause possible local enhancement of power absorption and produce characteristic SAR peaks around the bar-implant. However, the obtained maximum SAR values did not exceed the recommended reference levels for environmental and occupational exposures.

Chondrocytes, the cartilage forming cells, reside under an environment that is toxic to most cell types due to a lack of vasculature, persistent hypoxia, and related acid production through glycolytic metabolism. In other cell types low pH activates a unique family of membrane ion channels; the acid-sensing ion channels (ASICs) (Chu et al., 2011). In pathological conditions of articular cartilage such as osteoarthritis and rheumatoid arthritis, the tissue pH become acidic, falling to approximately pH 5.5 (Hu et al., 2012). In this paper we describe reduced expression of the *ASIC2* gene in patients with chest wall

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deformities. We hypothesized that chondrocytes from these patients exhibit an abnormal response to changes in external pH that are experienced as an integral part of the biology of this tissue. Activation of ASICs typically creates a cascade ultimately dependent on the formation of intracellular calcium transients. Changes in intracellular calcium signaling cause downstream changes in gene expression. An excessive amount of intracellular calcium induces cell death through apoptosis in chondrocytes (Rong et al., 2012; Li et al., 2014; Zhou et al., 2015). Low pH is known to cause a decrease in extra cellular matrix (ECM) production (Yuan et al., 2010). The objective of this study was to measure intracellular calcium changes in response to reducing environmental pH around costal chondrocytes from patients with CWD.

2. Methods

2.1. Patients

Human costal cartilage was obtained from three patients with pectus carinatum and three with pectus excavatum, all severe enough to warrant surgical repair. Informed consent was obtained following IRB approval of the protocol at Eastern Virginia Medical School and Old Dominion University. The IRB protocol currently prevents disclosure of many clinical features, and thus close correlation of clinical phenotype with gene expression is not possible. Costal cartilage samples were collected from ribs 6–8 at surgery. Experiments were performed on the round, rod-like, midsections of cartilage. All patients were male, with an age range of early teen to early 20's. Apparently normal costal cartilage was obtained from three age-matched-controls, one 15 year old and two 17-year-old males, and processed within 24 h. Cells were cultured in Chondrocyte growth medium (PromoCell) at 37 °C with 5% CO_2 in humidified air. Chondrocytes were maintained in suspension culture (Bosnakovski et al., 2004; Wa et al., 2015) to maintain their differentiated phenotype, and briefly expanded in tissue culture flasks before plating onto 10 mm diameter glass coverslips. All experiments used cells that had been passaged < 4 times.

2.2. RNA isolation and real time qPCR

RNA was directly isolated from cells in tissue culture dishes, and genomic DNA eliminated using a Direct-zol™ RNA MiniPrep (Zymo Research, Irvine, CA, USA). Complimentary DNA (cDNA) was generated using an RT-First Strand Kit (Qiagen, Valencia, CA, USA). RNA and cDNA concentrations were measured by NanoDrop (Thermo Fisher). Polymerase chain reactions (PCRs) were performed using SYBR green detection (Qiagen) and customized ion channel array plates (Qiagen) in a BioRad CFX96 system (BioRad, Hercules, CA, USA). Manufacturer guidelines were used for PCR reaction volumes and cycle parameters. The cycling parameters were 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Reaction specificities were assessed with a melt curve of 65 °C to 95 °C in 0.2 °C increments. Data was standardized to five reference genes defined in Fig. 1 (*ACTB, B2M, GAPDH, HPRT1* and *RPLP0*) using the ΔCq method. All experiments were in triplicate and performed with positive and negative controls. At least two independent extractions were included. Gene expression was calculated as $2^{-(Cq_{GOI}-Cq_{Ref})}$ where Cq_{GOI} is the Cq value of the gene of interest, and Cq_{Ref}, is the Cq value for the averaged reference genes. The assay range using the RT^2 profiler array (Qiagen) is 6.8–35 Cq. In order to minimize the possibility of false-positives and account for variability, a constant concentration of total cDNA was used in all reactions and a raw Cq cutoff of 30 was used (Canales et al., 2006; Arikawa et al., 2008). Expression of the acid sensitive G-protein coupled receptor gene *GPR68* (G Protein-Coupled Receptor 68) was also assessed using commercially available primers from Qiagen. Chondrocyte phenotype was confirmed by aggrecan (*ACAN*), biglycan (*BGN*), decorin (*DCN*), and cartilage oligomeric matrix protein (*COMP*), gene expression by RT-PCR using commercially available primers (Qiagen).
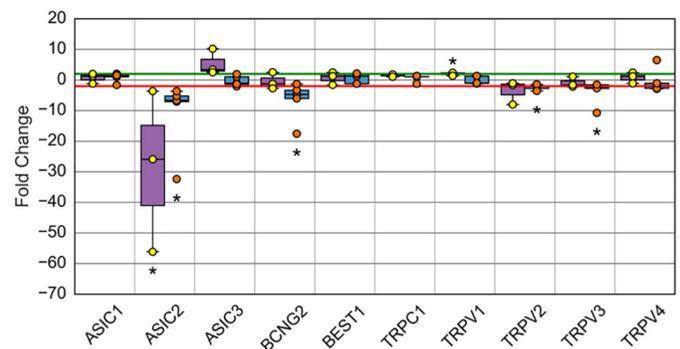

Fig. 1. Median fold changes in non-selective ion channels in chondrocytes of CWD patients using qPCR. Pectus excavatum (PE; purple box plot) and pectus carinatum (PC; blue box plot) are shown relative to normal costal cartilage. Each sample was analyzed with an $n = 3$ and significant changes ($p < .05$) relative to normal costal cartilage are indicated (*). The green and red horizontal lines indicated a +2 or −2 fold change, respectively. Outlying data points in PC patients are indicated by unconnected orange dots. Genes analyzed are ASIC1, 2, and 3 Acid Sensing Ion Channel unit 1, 2, and 3; BCNG2 (HCN2) Hyperpolarization Activated Cyclic Nucleotide Gated Potassium and Sodium Channel 2; BEST1 Bestrophin; TRPC1 Transient Receptor Potential Cation Channel Subfamily C Member 1; TRPV1–4 Transient Receptor Potential Cation Channel Subfamily V Members 1–4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Protein extraction and ELISA

Protein was isolated from chondrocytes grown in 3D as cell pellets by rinsing the cells in cold PBS three times, centrifuged at 300 × g for 10 min at 4 °C. RIPA buffer (Sigma) with protease inhibitors (Roche) was added for 10 min on ice to lyse cells and extract protein. The RIPA-cell mixture was transferred to a conical tube with a 0.1–0.6 mm bead, loaded in a TissueLyser bullet blender (Qiagen) and run at 20–30 Hz for 2 min. The homogenate was centrifuged at 14,000 × g for 15 min at 4 °C and the supernatant used for protein analysis. Total protein extract was quantified using a Bradford assay and ASIC2 determined by ELISA (a human ASIC2 monoclonal antibody detected by a biotin labeled polyclonal secondary antibody and measured colorimetrically via avidin/peroxidase conjugates using a Molecular Devices Spectra-Max i3 plate reader) by comparing samples to an ASIC2 standard curve following manufacturer's guidelines (MyBioSource San Diego CA).

2.4. Calcium imaging

Intracellular calcium imaging was performed on control and a PC patient chondrocytes using procedures previously described (Semenov et al., 2013). Cells were cultured on glass coverslips, loaded with Fura-2/AM dye (Sigma-Aldrich, St. Louis, MO, USA), and placed into a vacuum perfusion chamber mounted on an IX71 microscope (Olympus, Center Valley, PA, USA) while being maintained using a physiological solution consisting of 5.4 mM KCl, 140 mM NaCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES. Normal pH (pH 7.4) and low pH (pH 5.5) physiological solutions were used. Cells were recorded in normal pH solution for 60 s and then perfused with low pH solution. After 3 min low pH solution was replaced with normal pH solution. Alternating excitation at 340 and 380 nm was provided with a xenon lamp using a Lambda DG4 switcher (Sutter, Novato, CA, USA), emission at 510 nm was collected via a UApoN340 40×/1.35 objective (Olympus America, Center Valley, PA) and recorded with a iXon Ultra 897 electron multiplication CCD digital camera (Andor Technology, Belfast, UK). The intracellular calcium concentration was calculated using a calibration kit (Life Technologies, Carlsbad, CA, USA) and the equation:

$$[Ca^{2+}]_i = \left(\frac{R - R_{\min}}{R_{\max} - R} \right) \times K_D \times \beta \qquad (1)$$

where $[Ca^{2+}]_i$ refers to internal calcium concentration and the recorded ratios are R, zero calcium ratio is $R_{\min}$, ratio at calcium saturation is $R_{\max}$, the effective dissociation constant is K_D, and the ratio of free to bound dye is β (Grynkiewicz et al., 1985).

2.5. Metabolic activity

Metabolic activity of cells was evaluated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Proliferation Assay Kit (ATCC) following manufacturer guidelines. The reduction of tetrazolium by metabolically active cells was quantified spectrophotometrically at 570–650 nm. Cells from two controls (C-08 and C-10), two PC patients (PC16 and PC34), and two PE patients (PE01 and PE02) were cultured in 96-well plates, then treated with growth mediums at pH 5.5, 6.0, and 6.5 and evaluated at 0, 5, 10, 30, 60 min, and 24 h prior to spectrophotometric analysis. Each replicate was measured three different times and data pooled for each control and patient sample.

2.6. Statistical analysis

Statistical analysis was performed using Student t-test to determine significance between sample and control means. For all tests, $p < .05$ indicated the difference as significant. The number of repeat experiments performed is n.

3. Results

3.1. Expression of acid sensing ion channel genes

Chondrocyte phenotype was confirmed through four structural genes of cartilage expressed in the differentiated chondrocyte (Stacey et al., 2012; Asmar et al., 2015). Cq values confirming expression of *ACAN*, *BGN*, *DCN*, and *COMP* are all below our conservative cut off value of Cq30 (Table 1).

Median changes in gene expression of non-selective ion channels in three PE samples and three PC samples versus three normal costal chondrocyte samples showed few statistically significant differences (Fig. 1). No significant down regulation was observed in PE samples except for the acid sensing ion channel gene *ASIC2*. *ASIC2* was the only gene to be down regulated in both PE and PC samples (-25.94-fold, $p < .02$ and -6.71-fold, $p < .01$ respectively). In PC samples, significant down regulation was also observed in *TRPV2* (-2.09, $p < .001$), *TRPV3* (-2.35, $p < .03$), *BCNG2* (-3.36, $p < .04$), and *GPR68* (-3.13, $p < .002$) compared to controls. *ASIC3* up regulation was observed in PE, however levels were not significant ($+5.35$, $p < .22$). No significant up regulation was observed in any patient samples compared to controls.

Table 1
Chondrocyte phenotype confirmation was determined by measuring gene expression Cq values of four genes expressed in the differentiated cell (*ACAN*, *BGN*, *DCN*, and *COMP*) and three housekeeping genes (*GAPDH*, *ACTB* and *B2M*).

Gene	PC19 Cq average	PC16 Cq average	C-08 Cq average	C-10 Cq average
ACAN	26.01	26.94	25.22	22.14
BGN	28.74	27.73	23.19	26.03
DCN	27.21	22.95	18.68	23.10
COMP	24.69	N/A	21.72	20.44
GAPDH	20.01	20.32	18.83	19.15
ACTB	16.76	20.62	18.33	18.15
B2M	18.06	N/A	19.68	20.18

3.2. Protein expression of ASIC2

ASIC2 protein was calculated from an ASIC2 standard curve for C-08, C-10, and PC34 as 2.75, 1.01, and 0.91 ng/mL respectively and expressed as a percentage of total protein for each sample (Table 2). Normalized to each control, protein levels of ASIC2 in PC34 were 67% and 85% of C-08 and C-10 respectively; agreeing with the reduced expression profiles obtained in Fig. 1.

3.3. Calcium transits following increased acidity

The significant down regulation of the *ASIC2* gene in both PE and PC samples led to the question of whether chondrocytes of CWD patients are desensitized to external acidosis. Using live cell ratiometric monitoring of intracellular calcium, measurements from chondrocytes of normal costal and PC patients were performed (Fig. 2). Comparison of transients from PC patient (PC16) and control (C-08) cells (Fig. 3) showed the transient began to form at around 68.09 ± 10.23 s and peaked at 103.03 ± 15.36 s in control cells ($n = 8$); while in PC16 cells, the transient began with a significant delay at 160.81 ± 12.72 s and peaked at 200.54 ± 2.32 s ($n = 3$). Amplitudes of calcium transients also showed significant differences ($p < .05$) in C-08 ($0.892 \pm 0.152\,\mu M$) as compared to PC16 cells ($0.715 \pm 0.0714\,\mu M$). Moreover, PC16 had significantly higher basal levels of intracellular calcium than control cells (PC16: $0.156 \pm 0.00747\,\mu M$, C-08: $0.121 \pm 0.00937\,\mu M$, $p < .01$). Amplitudes of calcium release were consistently lower in patients, however the delayed onset of the calcium transit appeared not to be consistent finding.

3.4. Response of patient chondrocytes following further exposure to acidity

Patient (PC34) and control (C-10) cells were exposed to reduced pH 5.5 followed by restoration to pH 7.4 and then a second exposure to pH 5.5. Similar to PC16, there was a significant difference in amplitude of calcium release between control and patient cells when first exposed to pH 5.5 ($0.133\,\mu M$ compared to $0.0987\,\mu M$, $p < .005$). Control cells showed near identical amplitudes of calcium release upon a second exposure to pH 5.5 (Fig. 4. $0.133\,\mu M$ and $0.121\,\mu M$ respectively, $p < .32$) as compared to PC34, which showed a significant reduction in amplitude ($0.098\,\mu M$ and $0.051\,\mu M$ respectively, $p < .0001$). Differences in amplitude of calcium release between control and patient samples were highly significant upon second exposure ($0.121\,\mu M$ compared to $0.051\,\mu M$ respectively, $p < .0001$).

3.5. Calcium is released from internal stores

In a single control sample (C-08) external Ca^{2+} was chelated with EGTA and cells exposed to a reduction of pH to 5.5. Fig. 5 shows an immediate increase in calcium concentration indicating release from internal stores. Interestingly, when external calcium was depleted the shape of the curves was not uniform, but rather showed two-step dynamic with fast initial rising followed by a slower increase towards the peak (Fig. 6). Results indicate the compound nature of Ca^{2+} increase as compared to one-step Ca^{2+} release from the endoplasmic reticulum (ER) (via RyR or IP3 receptors). This was not observed when external calcium was present.

3.6. Metabolic activity of chondrocytes

Cell metabolism was analyzed by the MTT assay in costal chondrocytes (C-08, C-10, and four different samples from CWD patients; PC16, PC34, PE01, and PE02) following acid challenge. Data was pooled for each sample type (Fig. 7). At pH 6.5 and 6.0, similar levels of cell metabolism were observed at almost all time points (0, 5, 10, 30, 60 min and 24 h). Unexpectedly, challenge with media at pH 5.5 lead to consistent decreases in metabolism in both PC and PE cells, while

Table 2
Reduced protein expression of ASIC2 in PC34 cells compared to controls.

Sample	Total protein Conc mg/mL	ASIC2 conc ng/mL	ASIC2 expression % total protein	ASIC2 expression (PC) as a % of each control
C-08	18.88	2.75	0.014	100 (67%)
C-10	13.74	1.01	0.007	100 (83%)
PC34	15.76	0.91	0.0058	
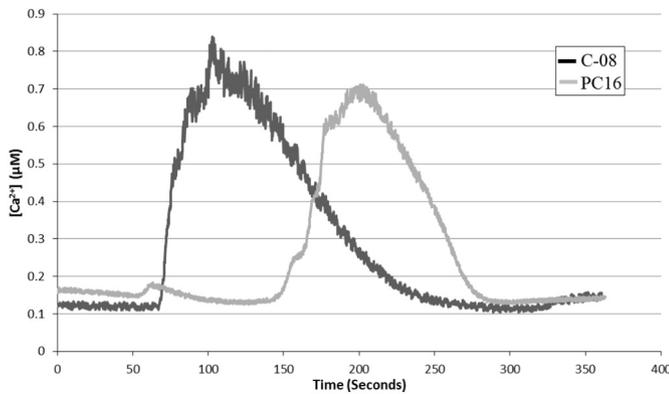
Fig. 2. Costal chondrocytes from a control (C-08) and a PC patient (PC16) were exposed to change of pH and ratiometric measurements of calcium release made. pH was changed from 7.4 to 5.5 at 60 s and calcium release plotted against time.
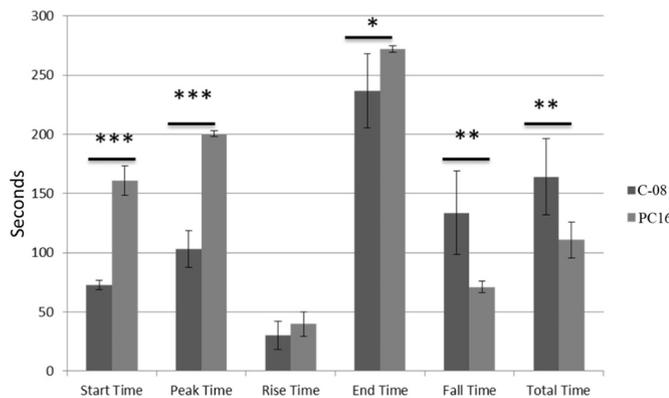

Fig. 5. Calcium is released from internal stores upon exposure to low pH. Calcium release was measured in C-08 cells when the pH was reduced to 5.5 at 30 s. External calcium was chelated with EGTA.

control samples had a mixed response. The results show that the metabolic response by PC and PE cells may only be significant at pH < 6.0.

4. Discussion

Costal cartilage is a much-understudied type of hyaline cartilage where deformities have significant clinical consequences. The spatial and temporal mechanical and electrochemical events in cartilage (Mow and Guo, 2002) led us to investigate the response of chondrocytes to their continually changing environment. The plasma membrane of a cell is the point where the cell directly interacts with its environment, and ion channels provide a conduit for cells to rapidly respond and maintain homeostasis specifically related to changes in pH, osmolarity, and ionic concentrations (Stacey et al., 2014).

Channelopathies, pathologies with underlying defects in ion channel/transport, have been widely described in cell types known to possess a large number and diversity of ion channels, specifically in nerve and muscle. Chondrocytes also possess a large number and diversity of ion channels (Barrett-Jolley et al., 2010; Asmar et al., 2016). Although their functions are largely unknown in the environment in which this cell type resides, we posed the question as to whether this

Fig. 3. Graphical display of intracellular calcium transient time properties following pH challenge in C-08 and PC16 chondrocytes. Significant differences ($p < .05$, < 0.01, and < 0.001) are indicated by asterisks (*, **, and ***, respectively).
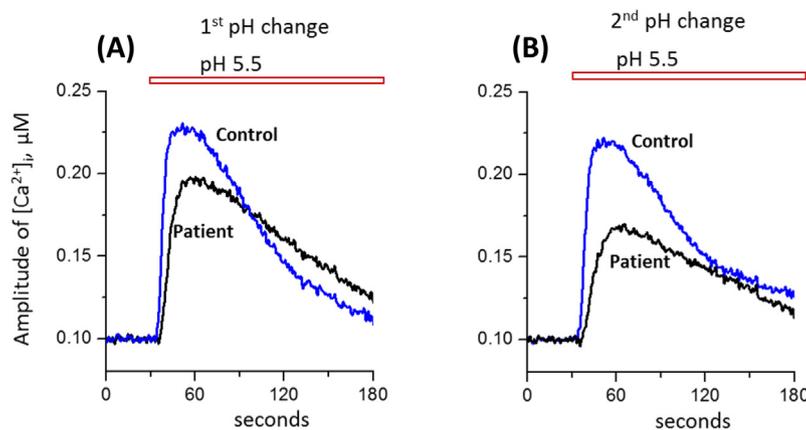

Fig. 4. Calcium release from patient and control cells following a second exposure to acidic pH. (A) The pH was reduced to pH 5.5 at 30 s and calcium release measured in PC34 and C-10 cells. (B) Cells were restored to pH 7.4 and then changed for the second time to pH 5.5. Calcium release from patient cells were significantly lower than the first exposure ($p < 0001$).
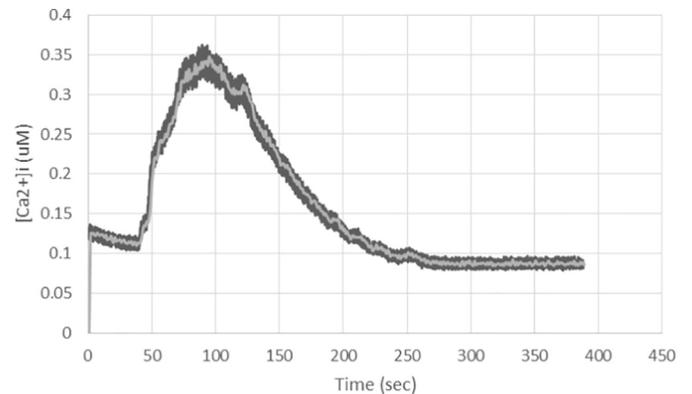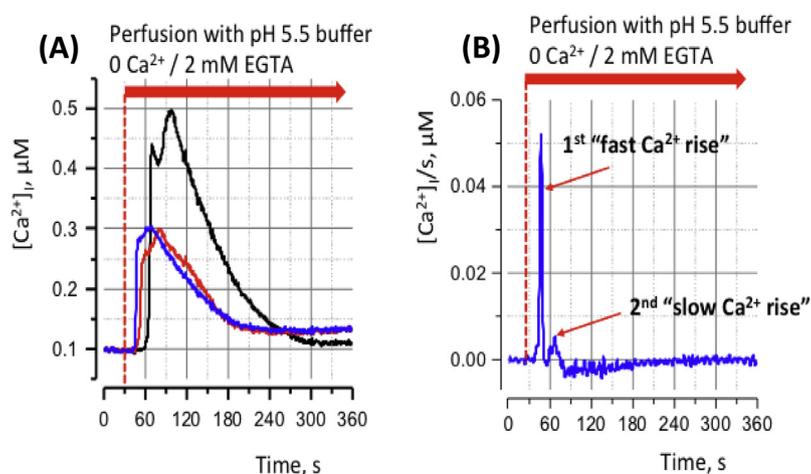
Fig. 6. (A) Ca^{2+}-release from intracellular stores of three individual C-08 cells (black, red, and blue traces) upon perfusion with low pH (5.5) in zero Ca^{2+} solution (B) Dynamic of Ca^{2+} release from internal stores in zero Ca^{2+} solution. To distinguish components of compound Ca^{2+} release traces from (A) were shifted to the same point in the moment of initial Ca^{2+}-rise differentiated and averaged. (B) show two speed components (fast and slow) in the dynamic release of Ca^{2+}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tissue, hyaline cartilage, may also be susceptible to channel related pathologies. In this study we identified consistent down regulation of the acid sensing ion channel gene *ASIC2* in patients with chest wall deformities.

Down regulation of *ASIC* genes are attributed to increased cell survival by prevention of acid-induced injury in rat articular chondrocytes. Acid-induced cell death in chondrocytes occurs through a mitochondrial-dependent pathway, and ASIC-induced calcium entry cause induction of caspase 3-mediated apoptosis (Hu et al., 2012). Rat articular chondrocytes show reduced cellular death using ASIC blockers during external acidosis (Rong et al., 2012). Although down regulation of ASICs is expected to increase cell viability under acidic pH stress, it also causes the cell to become more desensitized to extracellular acidosis. Shifts in pH, which occur regularly in cartilage, are crucial to proper maintenance of this tissue, so desensitization could impair or modify its development and growth. Down regulation of *ASIC2* gene in PC and PE samples led to the question of whether chondrocytes of CWD patients are desensitized to external acidosis.

We consistently observed reduced amplitude of response in patient samples to repeated exposure of acidic conditions suggesting normal homeostasis is not achieved. As a result, cell injury may occur, apparent through cell metabolism/cell death analysis. Cell metabolism by MTT assay in normal, PC, and PE costal chondrocytes following prolonged

acid challenge at pH 6.5 and 6.0, showed very similar levels of cell metabolism at almost all time points. Challenge with media at pH 5.5 lead to consistent decreases in metabolic activity in chondrocytes from PC and PE patients, while control samples had a mixed response. The response by chondrocytes from PC and PE patients and gradual response by control cells may be indicative of homeostatic pathways being immediately compromised in pectus-affected cells. This compromised response due to external acidosis at pH 5.5 may trigger downstream signals that would otherwise be blocked by a consistent calcium transient formation.

Mechanistically, ASICs import calcium from the exterior of the cell and pathways do not appear to involve IP3 and RyR receptors, receptors of the ER involved in release of calcium from internal stores and formation of intracellular calcium transients. When external calcium was depleted, calcium transits were still observed, suggesting release from internal stores. However, the calcium release response appeared unusual in that the rise curve appears to have two components, "fast" and "slow", instead of a single fast rise time. This indicates two different mechanistic pathways may be responsible for internal calcium release. Release of calcium from internal stores mechanistically implicates IP3R, there are also three isoforms of the ryanodine receptor, RyR1–3. Our previous studies show that *RyR3* gene was only expressed in fetal costal chondrocytes (Asmar et al., 2016), and we cannot presently assign a
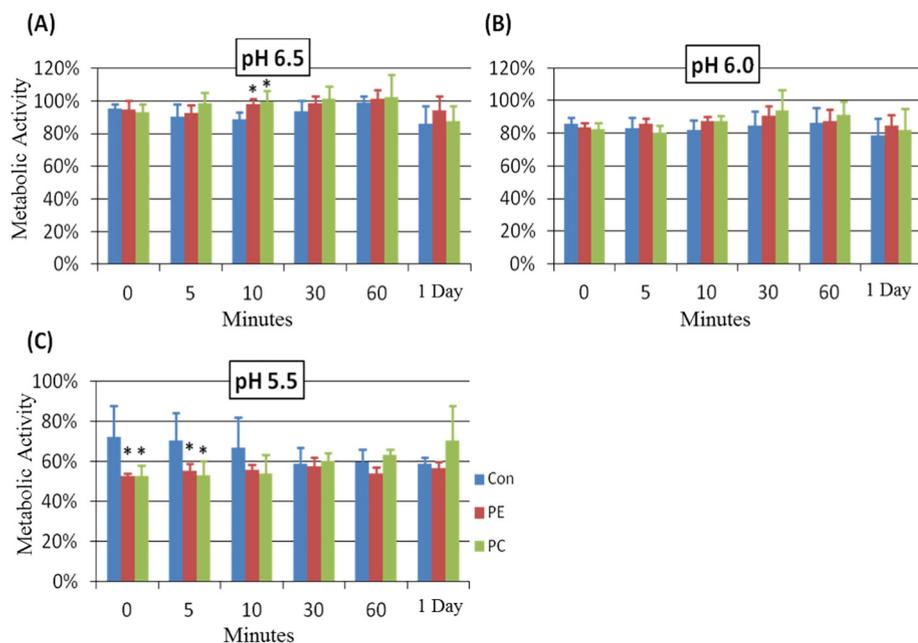

Fig. 7. Metabolic activity of costal chondrocytes were measured after being subject to low pH: (A) 6.5, (B) 6.0, and (C) 5.5, at several time points between control (blue), PC (green), and PE (red) cells. Metabolic activity levels displayed are compared to cells treated with normal media (y-axis) at each time point (x-axis). Significant differences ($p < .05$) between control and both PC and PE are indicated (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

role for these genes. In our PC samples we identified a small but significant down regulation of the gene *GPR68*, a G-coupled protein receptor that is activated by protons (H^+), resulting in cleavage of PIP2 to DAG and IP3 and release of calcium from internal stores (Hu et al., 2017). Down regulation of the gene *GPR68* may reduce enzymatic kinetics that delays the calcium response when chondrocytes are exposed to decreased pH. ASIC2 may have a separate role, possibly through cell membrane depolarization by Na^+ transport.

Intracellular calcium levels are not well characterized for pH response in chondrocytes with previous recordings of calcium transient levels mainly in response to hypo-osmotic stress showing inconsistent peak levels and total time of intracellular calcium transients (Dascalu et al., 1996; Edlich et al., 2001; Parvizi et al., 2002; Yellowley et al., 2002; Sanchez et al., 2003; Sanchez and Wilkins, 2004; Kurita et al., 2015). To our surprise, we observed a delayed formation of calcium transients in PC16 cells. More samples will need to be analyzed to determine whether there is patient-to-patient variation in time of response to acidic pH.

Critical information missing in the literature is short-term temporal studies on cell responses to external acidosis. Although the hypoxic nature of cartilage creates a slightly acidic environment, it is the mechanical changes of the tissue that produce the low pH environments for short time periods. Analyzing the literature yields only studies monitoring chondrocyte response to external acidosis for time points > 1 h (Yuan et al., 2010; Rong et al., 2012; Collins et al., 2013; Li et al., 2014; Zhang et al., 2016). Many studies monitored changes over days, but none immediately following a pH challenge. Our data show that chondrocytes are able to immediately respond to fluxes in pH, and that these responses are compromised in chondrocytes derived from patients with chest wall deformities.

There are a number of limitations to this study. Patients undergoing repair of CWD tend to be of late teens to early 20's in age and there can be difficulty in obtaining age-matched controls. Our controls derived from accidental deaths (e.g. car/climbing accidents) where there is no record of osteopathology. Additionally the study numbers are small, however statistical significance has been shown. We plan to improve this study with larger numbers that we are able to link directly to full clinical presentation. In conclusion, we have identified reduction in *ASIC2* gene expression in costal chondrocytes from patients with chest wall deformity. We speculated that patient chondrocytes may show abnormal response to acidosis and were able to show that calcium transient amplitudes are reduced in patient chondrocytes. Reduced amplitude of Ca^{2+} response appeared to become exacerbated upon repeat exposure to acidosis. Calcium also appears to be released from internal stores as a two-step process. We believe that we have identified a biomarker for patients with chest wall deformities that may point to the underlying defect of this complex inherited disorder. Once genetic/protein components to these pathways are identified then it is possible to think of a therapy and/or 'cure'. Manipulation of chondrocytes in fully differentiated cartilage will be difficult. Cartilage grown from mesenchymal stem cells can be genetically manipulated prior to differentiation into cartilage and will likely be a more fruitful approach compared to manipulation of a fully differentiated tissue. As a result of this study, the fields of ion channel function and calcium biology with respect to pediatric cartilaginous disorders have been opened that may have beneficial consequences in pathological and regenerative medicine.

Acknowledgements

Authors declare that no conflicts of interest exist.

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