Impaired Myofibrillar Energetics and Oxidative Injury During Human Atrial Fibrillation

Michael J. Mihm, PhD; Fushun Yu, PhD; Cynthia A. Carnes, PharmD, PhD; Peter J. Reiser, PhD; Patrick M. McCarthy, MD; David R. Van Wagoner, PhD; John Anthony Bauer, PhD

Background—Atrial fibrillation (AF) is associated with severe contractile dysfunction and structural and electrophysiological remodeling. Mechanisms responsible for impaired contractility are undefined, and current therapies do not address this dysfunction. We have found that myofibrillar creatine kinase (MM-CK), an important controller of myocyte contractility, is highly sensitive to oxidative injury, and we hypothesized that increased oxidative stress and energetic impairment during AF could contribute to contractile dysfunction.

Methods and Results—Right atrial appendages were obtained from AF patients undergoing the Maze procedure and from control patients who were in normal sinus rhythm and undergoing cardiac surgery. MM-CK activity was reduced in AF patients compared with controls (25.4±3.4 versus 18.2±3.8 μmol/mg of myofibrillar protein per minute; control versus AF; P<0.05). No reduction in total CK activity or myosin ATPase activity was detected. This selective reduction in MM-CK activity was associated with increased relative expression of the β-myosin isoform (25±6 versus 63±5%β, CTRL versus AF; P<0.05). Western blotting of AF myofibrillar isolates demonstrated no changes in protein composition but showed increased prevalence of protein oxidation as detected by Western blotting for 3-nitrotyrosine (peroxynitrite biomarker) and protein carbonyls (hydroxyl radical biomarker; P<0.05). Patterns of these oxidative markers were distinct, which suggests discrete chemical events and differential protein vulnerabilities in vivo. MM-CK inhibition was statistically correlated to extent of nitration (P<0.01) but not to carbonyl presence.

Conclusions—The present results provide novel evidence of oxidative damage in human AF that altered myofibrillar energetics may contribute to atrial contractile dysfunction and that protein nitration may be an important participant in this condition. (Circulation. 2001;104:174-180.)

Key Words: arrhythmia ▪ creatine kinase ▪ nitric oxide ▪ 3-nitrotyrosine ▪ contractility

Atrial fibrillation (AF), the most prevalent sustained arrhythmia, affects over 2 million Americans.1 AF is associated with structural remodeling and impaired atrial contractility, which results from persistent loss of atrial electrical synchrony and subsequent high rate activation.2 AF patients have marked atrial dilatation and atrial myocyte hypertrophy with increased interstitial fibrosis and fatty deposition. Although electrophysiological and structural remodeling are well-established consequences of sustained AF, underlying mechanisms and relationships between phenom- ena remain ill defined.2,3 and molecular mechanisms that contribute to impaired contractility have not been studied extensively. Impaired atrial contractility probably contributes to blood stasis and promotes clot formation, embolism, and stroke in this patient population.4 In addition, decreased ventricular filling during AF can have deleterious effects on patients with impaired ventricular function.5 Current therapeutic strategies do not address this important aspect of AF.

The myocyte contractile apparatus, the myofibril, has complex and tightly regulated high-energy phosphate production and utilization capabilities.6 Myofibrillar energetic controllers (primarily creatine kinase and myosin ATPase) are altered in multiple settings of ventricular dysfunction, but the mechanisms involved are undefined.7 In the present study, we tested the novel hypothesis that myofibrillar energetics are impaired during human AF.

Nitric oxide (NO) modulates cardiac myocyte contractility and blood flow.8 During periods of high oxidative stress or tissue injury, loss of NO control can occur and formation of NO-derived reactive species is favored.9 Of particular biological relevance is peroxynitrite, formed by the reaction of NO with superoxide anion. Peroxynitrite formation is an established event in multiple settings of ventricular dysfunction that results in selective nitration of protein tyrosine residues and disruption of cellular energetic control.9-11 We evaluated roles of altered NO-related signaling and NO-derived reactive species and a possible mechanistic role for myofibrillar oxidation in the energetic impairment observed during human chronic AF.

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From the Cardiothoracic Surgery/Kaufman Center for Heart Failure (P.M.M.); Department of Cardiology (D.V.W.), Cleveland Clinic Foundation, Cleveland, Ohio; and the Division of Pharmacology/College of Pharmacy (M.J.M., P.Y., C.A.C., D.V.W., J.A.B.), OSU Heart and Lung Institute (J.A.B.), and College of Dentistry (P.J.R.): Department of Cardiology (D.V.W.), Cleveland Clinic Foundation, Cleveland, Ohio; and the Division of Pharmacology/College of Pharmacy (M.J.M., P.Y., C.A.C., D.V.W., J.A.B.), OSU Heart and Lung Institute (J.A.B.), and College of Dentistry (P.J.R.); Ohio State University, Columbus, Ohio.
Correspondence to John Anthony Bauer, Ohio State University, 500 W 12th Ave, Columbus, OH 43210. E-mail bauer.140@osu.edu
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174
Myofibrillar fractions were prepared using a previously described isolation protocol. Enriched myofibrillar fractions were suspended in buffers appropriate for determination of creatine kinase or myosin ATPase activity. CK activity was determined by the methods of Oliver. Briefly, phosphocreatine (0.1 to 100 mmol/L) was added to atrial homogenates or myofibrillar isolates. ATP formation was indirectly measured through NADPH formation in the presence of high KCl for its well-established correlation to functional measures of cardiac contractility (shortening velocity and ejection fraction), myofibrillar ATP turnover, and myosin isoform ratio (high- versus low-velocity isoform). Each experiment was calibrated by use of an inorganic phosphate standard curve.

Western Blot Analysis
In parallel experiments, myofibrillar extracts were studied by Western blot, in which myosin isofrom expression, M-CK content, fibrillar protein profiles, and evidence of protein oxidation were assessed. Myosin isofrom separation was performed as previously described. M-CK content (monomeric form of myofibrillar CK [MM-CK]) under reducing gel conditions) was assessed by polyclonal antibody (rabbit anti-human M-CK, Fitzgerald Industries; 1:400 dilution) or 3-nitrotyrosine (3NT; a biological marker of peroxynitrite; polyclonal rabbit anti-3NT, Upstate Biotechnologies; 1:400 dilution). Relative prevalence of myofibrillar protein bands was assessed with a reversible, nonspecific protein stain (Fast-blot, Geno Technologies). Myofibrillar protein migration patterns were determined for each sample. Images were scanned and digitally recorded, and each identified protein band area was delineated and recorded for later use during immunoblot analysis (see below). Preliminary experiments demonstrated a linear response of this staining method within the protein loads investigated.

Protein oxidation was investigated by use of immunochcmical techniques. After general protein identification, blots were thoroughly washed and probed with primary antibodies for detection of 3-nitrotyrosine (3NT; a biological marker of peroxynitrite; polyclonal rabbit anti-3NT, Upstate Biotechnologies; 1:400 dilution) or protein carbonyls (primarily derived from hydroxyl radical; Oxyblot protein carbonyl kit, Intergen), by use of techniques previously described. Diaminobenzidine (0.06% w/v) was used to visualize immunoreactivity. In all cases, antibody specificity was demonstrated either with preimmune serum (isotypic staining control) or antibody preexposed to excess free antigen (preadsorbed staining controls).

**TABLE 1. Clinical Characteristics of AF and Control Patients**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age, y</th>
<th>Sex</th>
<th>Surgery</th>
<th>AF Status, Duration in y</th>
<th>Presurgical Medications</th>
<th>Atrial Enlargement (RDE)</th>
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<td>67</td>
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<td>Maze III</td>
<td>AF, 20</td>
<td>Dig, BB, C</td>
<td>++ + + + +</td>
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<tr>
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<td>AI, D, T, C</td>
<td>++ + + + +</td>
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<td>NSR</td>
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<td>63</td>
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<tr>
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<td>M</td>
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<td>NTG, CC, BB</td>
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<td>F</td>
<td>Donor</td>
<td>NSR</td>
<td>CC</td>
<td>NA NA</td>
</tr>
</tbody>
</table>

**Histology**
Tissues were paraffin-embedded and blocked according to standard procedures. Sections (5 μm each) were evaluated with standard protocols for staining with hematoxylin-eosin and Masson’s trichrome (for fibrosis).

**Methods**

**Patients**
Atrial appendages were obtained as surgical specimens from patients undergoing cardiac surgery by use of procedures approved by the Institutional Review Board of the Cleveland Clinic Foundation. All patients gave informed consent.

Right atrial appendages were obtained from 7 patients in permanent AF (>1 month at the time of surgery) undergoing the Maze procedure and mitral valve repair (mean age, 64±5 years). Control data were obtained from right appendages of 6 patients in normal sinus rhythm with no history of AF (mean age 62±8 years). This population included 5 patients undergoing routine cardiac bypass graft surgery and 1 nonfailing heart-transplant donor rejected for transplantation. Surgeries were performed between January and July 1999. Table 1 describes detailed clinical characteristics.

**Enzyme Kinetics**
Myofibrillar fractions were prepared using a previously described isolation protocol. Enriched myofibrillar fractions were suspended in buffers appropriate for determination of creatine kinase or myosin ATPase activity. CK activity was determined by the methods of Oliver. Briefly, phosphocreatine (0.1 to 100 mmol/L) was added to atrial homogenates or myofibrillar isolates. ATP formation was indirectly measured through NADPH formation in the presence of hexokinase and glucose-6-phosphate dehydrogenase. NADPH formation was monitored spectrophotometrically (340 nm; 25°C). Rate of absorbance change (monitored for 6 minutes) was proportional to CK activity. ATP formation was rate-limiting at all CK concentrations analyzed (CK concentrations, 2.5 mg/mL to 5 g/mL). Each sample was investigated in triplicate.

Calcium-dependent myosin ATPase activity was assayed from myofibrillar fractions by monitoring inorganic phosphate generation. Reaction mixture contained 0.3 mol/L KCl and 0.01 mol/L CaCl₂ in 50 mmol/L Tris-maleate buffer, pH 7.6, 25°C. Na₂ATP addition initiated the reaction (5 mmol/L, producing maximal ATPase velocity). The reaction was terminated 70 minutes later by addition of 30% trichloroacetic acid. Myosin ATPase activity was studied in the presence of high KCl for its well-established correlations to functional measures of cardiac contractility (shortening velocity and ejection fraction), myofibrillar ATP turnover, and myosin isoform ratio (high- versus low-velocity isoform). Each experiment was calibrated by use of an inorganic phosphate standard curve.

**Western Blot Analysis**
In parallel experiments, myofibrillar extracts were studied by Western blot, in which myosin isoform expression, M-CK content, fibrillar protein profiles, and evidence of protein oxidation were assessed. Myosin isoform separation was performed as previously described. M-CK content (monomeric form of myofibrillar CK [MM-CK]) under reducing gel conditions) was assessed by polyclonal antibody (rabbit anti-human M-CK, Fitzgerald Industries; 1:200 dilution). Relative prevalence of myofibrillar protein bands was assessed with a reversible, nonspecific protein stain (Fast-blot, Geno Technologies). Myofibrillar protein migration patterns were determined for each sample. Images were scanned and digitally recorded, and each identified protein band area was delineated and recorded for later use during immunoblot analysis (see below). Preliminary experiments demonstrated a linear response of this staining method within the protein loads investigated.

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Immunoblots were scanned with an HP Scanjet 6200c (Hewlett Packard; 1290×960 resolution) and analyzed with digital image-analysis software (Image Pro Plus, Media Cybernetics). Protein-band areas delineated previously from the Fast-blot protein stain were recalled for protein-band identification of each immunoblot; images were then background corrected and average band intensities were determined. In nitration studies, average intensities were corrected for blot-to-blot variability by dividing 3NT signal by nitrated BSA internal standard for the respective blot. Untreated and tetranitromethane-treated M-CK was used to develop standard curves of diaminobenzidine signal versus micrograms of M-CK or moles of 3NT. Comparison of triplicate standard curves developed with tetranitromethane-treated M-CK yielded a detection limit of \( \sim 10 \) pmol of 3NT, whereas detection limit for the M-CK standard curve was \( \sim 100 \) ng. Coefficients of variation for this method were 2% for intrablot variability and 16% for interblot variability.

Data Handling

All data described as mean\( \pm \)SEM. Enzyme-activity data were fit to the Michaelis-Menton model (GraphPad Prizm software). Differences between treatment groups were assessed by use of 2-tailed Student’s \( t \) tests with post hoc Student-Newman-Keuls tests. Correlation analyses were performed using Spearman’s nonparametric correlation. \( P<0.05 \) described statistical significance.

Results

Clinical characteristics of the AF and control patients are presented in Table 1. No age- or sex-related differences were detected in any functional parameter measured (Spearman’s correlation analyses). Atrial appendages were analyzed for collagen deposition using Masson’s trichrome staining. Increased interstitial fibrosis and myocyte hypertrophy were apparent in appendages from AF patients compared with controls (Figure 1A, \( \times 400 \) magnification). Analysis of protein and DNA content from atrial homogenates provided further evidence of remodeling during AF; protein and DNA content was increased 24% in right atrial tissue from AF patients over right atrial controls (Figure 1B). These parameters were used to normalize enzymatic activities to account for morphological changes.

Significant decreases in MM-CMK maximum velocity (\( V_{\text{max}} \)) were observed in myofibrillar fractions from AF patients compared with controls, when normalized to total myofibrillar protein or DNA content or as a percent of total CK activity (Figure 2A through 2C). No significant change in \( K_m \) was observed (data not shown). The selective reduction in MM-CMK activity was not due to decreased M-CMK content, given that myofibrillar M-CMK content was unchanged in AF versus control tissue in terms of both M-CMK mass and as a percentage of total myofibrillar protein (Figures 2D and 2E).

In contrast to significant reductions in M-CMK activity, no change in myosin ATPase activity was observed in AF versus control myofibrillar fractions (Table 2). Total CK activity, measured from whole homogenates before myofibrillar fractionation, was not different between groups, which suggests selective MM-CMK impairment in this setting.

Isoform switching from the high- (\( \alpha \)-myosin) to the low-velocity (\( \beta \)-myosin) isoform of myosin was observed in AF atria (representative gel, Figure 3A). Figure 3B plots summary data for the \( \beta \)-myosin fraction in control and AF patients. Changes in myosin isoform gene expression were significantly correlated to the myosin ATPase \( V_{\text{max}} \) (Figure 3C, \( \circ \), Spearman’s nonparametric correlation, \( P<0.01 \)). Interestingly, MM-CMK \( V_{\text{max}} \) was also predictive of isoform switching in these tissues (\( P<0.01 \)). Slope of the relationship for M-CMK \( V_{\text{max}} \) versus percentage of \( \beta \)-myosin was 4-fold steeper than that of ATPase \( V_{\text{max}} \).

Relative protein compositions of myofibrillar extracts from AF and control atria are shown in Figure 4A. Western blotting methods and nonspecific protein staining showed that the primary protein constituents of the myofibrillar compartment were identical to those of previously published reports (identities shown at top according to Yates and Greaser17). No between-group differences in relative protein quantities were detected for any band identified (Figure 4B).

Figure 5A shows evidence of oxidative protein modifications in AF versus control myofibrillar extracts. Tyrosine nitration and carbonyl formation were evaluated by use of immunoblotting methods in parallel studies of the same myofibrillar fractions. In both cases, low levels of these oxidative markers were detected in control tissues, whereas significant increases were observed in AF tissues. Digital quantification of protein bands was used to assess relative intensities of nitration (Figure 5B) or carbonyl signals (Figure 5C). Relative distribution of these oxidative markers was distinct, which suggests differential sensitivities of myofibrillar proteins to these oxidative events.

Figure 6 illustrates the relationships between functional energetic parameters and myofibrillar oxidation.

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**Figure 1.** Atrial myocyte hypertrophy and fibrosis deposition was observed during AF. A, Trichrome-stained AF and control right atrial appendages (\( \times 400 \)). AF atria demonstrated interstitial fibrosis (blue staining), myocyte elongation, and hypertrophy. B, Protein/DNA ratios were increased in AF atria. CTRL indicates control (normal sinus rhythm). \( P<0.05 \).
significant increases in carbonyl formation in AF tissue, the extent of carbonyl formation in myofibrillar creatine kinase or myosin did not correlate with any of the energetic changes observed. In contrast, extent of 3NT formation in MM-CK was inversely correlated with $V_{\text{max}}$ (Figure 6A; $P<0.02$). Furthermore, extent of nitration in myosin heavy chain was correlated to percentage myosin isoform switching (Figure 6B; $P=0.03$). No similar correlation was observed for carbonyl events in relation to any parameter measured.

**Discussion**

We tested the hypothesis that myofibrillar energetics are impaired during, and may participate in, chronic human AF. Because we previously demonstrated that myofibrillar energetic controllers (particularly MM-CK) are highly vulnerable to oxidative inhibition, an additional component of our investigations was to define the contribution of oxidative injury in the observed energy impairments during AF. The major findings of our investigations are (1) that striking evidence exists of oxidative injury during AF, (2) that myofibrillar energetics are altered in this setting, and (3) that protein oxidative events (particularly nitration) may participate in the impairment of MM-CK and myosin isoform switching observed in AF.

**CK Kinetics**

Severe dysregulation of ventricular myofibrillar energetics has been demonstrated during experimental and human cardiac failure, in addition to changes in myosin isoform expression, altered ATP usage, and decreased creatine kinase activities. These changes even have been observed in the absence of impaired oxidative phosphorylation. We now report selective impairment of myofibrillar creatine kinase activities in atria of AF patients in the absence of alterations in atrial MM-CK content or myosin ATPase activity. This result is consistent with previous studies that demonstrated that impaired MM-CK activity may participate in the initiation and progression of cardiac myocyte contractile dysfunction and failure. Reduced MM-CK activity can lead to contractile deficits, particularly during periods of high rate activity (ie, AF). Interestingly, the present deficit was not accompanied by a decrease in total CK activity, which suggests that MM-CK may be uniquely impaired during AF and that compensatory expression of other CK isoforms may occur during AF. The latter conclusion may be supported by the observed trend toward increased nonmyofibrillar CK activity. These data are the first evidence of atrial myofibrillar energetic impairment during AF and may represent a novel mechanistic basis for contractile impairment observed in these patients.

**Myosin Isoform Switching**

In rodent models, adaptive changes in myosin isoform expression frequently accompany cardiac dysfunction. This response may be of particular relevance in human atrial (versus ventricular) tissue, as atrial expression of the high-velocity myosin isoform dominates ($\alpha$-myosin, $\approx 80\%$). We observed significant myosin isoform shifts from the $\alpha$- to the $\beta$-myosin isoform in chronic human AF tissues. We hypothesize that atrial myosin isoform switching may be a compensatory response to decreased myofibrillar ATP production or accumulation of ADP (as evidenced by MM-CK impairment). Ratio of MM-CK and ATPase activities was unchanged in AF tissues, and both MM-CK and ATPase activities were highly correlated to percentage of $\beta$-myosin isoform expression, with MM-CK showing a 4-fold steeper slope (Figure 3). We hypothesize that reduced MM-CK

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**TABLE 2. Myosin ATPase and Total CK Activities Were Unchanged During Chronic Human AF**

<table>
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<th>Treatment</th>
<th>n</th>
<th>Protein, $\mu$mol $\cdot$ min $^{-1} \cdot$ mg $^{-1}$</th>
<th>DNA, nmol $\cdot$ min $^{-1} \cdot$ mg $^{-1}$</th>
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<td></td>
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<td>CTRL</td>
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<td>6.26±0.98</td>
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<td>AF</td>
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<td>3.77±0.47</td>
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<td><strong>Total CK</strong></td>
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<tr>
<td>CTRL</td>
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<td>AF</td>
<td>7</td>
<td>69.4±1.2</td>
<td>167±14</td>
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activity (which resulted in significant accumulation of ADP at the myofibril) may initiate a sequence that leads to altered myosin isoform expression and ATP use. Some physiological rationale supports matched ATP production and use at the myofibrillar level, because accumulation of ADP at the site of contraction can adversely affect contractile performance. Further studies into regulation of myofibrillar high-energy phosphate production and use during AF are clearly warranted.

Evidence of Oxidative Events in AF

Despite striking evidence of structural remodeling, we found that AF caused no significant changes in the relative protein composition of the myofibril. CK can be inactivated due to oxidative posttranslational modification. Increased metabolic burden developed in the fibrillating myocyte suggests that increased production of reactive oxygen species is likely in AF. Myofibrillar proteins were probed for evidence of 2 distinct oxidative posttranslational modifications: tyrosine nitration (marker of peroxynitrite-protein interactions) and carbonyl formation (hydroxyl radical mediated event). Both modifications have been associated with important structural and functional alterations in a variety of protein and enzyme systems.

Although we found evidence that both nitration and carbonyl formation were increased in myofibrillar proteins in AF patients, the relative distribution of these events was distinct. Prevalence of nitration in control tissues was much higher than basal carbonyl events, consistent with the emerging perspective that low levels of tyrosine nitration may be a physiological regulatory or signaling pathway. Significant elevations in nitration events were dispersed throughout the myofibrillar compartment, whereas carbonyl events appeared to predominate in high-molecular-weight proteins, particularly C-protein. Important structural proteins (myosin heavy chain, C-protein, α-actinin, and desmin) and functional proteins (myosin heavy chain, actin, and M-CK) were modified during AF. Our data provide the first experimental evidence that protein oxidative events occur in atrial myocytes during AF, and may represent an important pathological mechanism in this arrhythmia. Several sources of reactive oxygen species may be involved, including perturbation of mitochondrial electron transport or intracellular oxygenase activities and/or decreased antioxidant capacities. Relative contributions of these oxidative events to the electrophysiological and structural remodeling and contractile deficits in AF warrant further investigation.

Figure 3. Myosin isoform switching from high- to low-velocity isoform during AF. A, Relative myosin isoform expression by SDS-PAGE. B, Shift from α-(high-ATPase-velocity) to β-(low-ATPase-velocity) isoform observed in AF atrial appendages. C, Extent of β-isoform expression statistically correlated with ATPase and M-CK activities (Spearman’s correlation, *P < 0.001). *P < 0.05.

Figure 4. Relative myofibrillar protein composition was unchanged during AF. A, Atrial myofibrillar isolates were assessed for protein content by SDS-PAGE and nonspecific protein stain. Molecular-weight markers and protein-band identifications shown at right. MHC indicates myosin heavy chain; MLC, myosin light chain. B, No differences in relative protein composition were observed. n = 6 samples; blots were run in triplicate.
Relationships of these oxidative events to the observed changes in CK activity and myosin isoform expression were probed using correlation analyses (Figure 6). Despite a statistically significant increase in carbonyl formation on the myosin heavy chain and M-CK, the prevalence of carbonyl formation did not correlate with deficits in MM-CK $V_{\text{max}}$ or extent of isoform switching, respectively. In contrast, the extent of tyrosine nitration in MM-CK was inversely correlated to MM-CK $V_{\text{max}}$. This is consistent with our previous studies regarding interactions of peroxynitrite with MM-CK, and it suggests that peroxynitrite participates in the impairment in CK function in this setting. Similarly, the extent of myosin-heavy-chain nitration was correlated with relative β-isofrm expression. Our results indicate that tyrosine nitration, and, therefore, NO control, may be an important event in the pathology of AF. Further studies regarding regulation of NO pathways during human AF are also warranted.

Limitations

Atrial contractile deficit is a significant cause of morbidity and mortality in this patient population, particularly with regard to atrial stasis, clot formation, and subsequent embolic stroke. Mechanisms by which atrial contractile dysfunction develops are largely unexplored, and current dogma attributes this dysfunction primarily to loss of atrial electrical synchrony and structural remodeling. Given the established role of myofibrillar activities in myocyte energy usage and contractility, we focused on myofibrillar energetic controllers known to be essential to cardiomyocyte contractility and highly sensitive to oxidative influences. Although our present studies provide new evidence of biological oxidation in the setting of chronic AF and mechanistic insight regarding atrial contractile impairment, the time sequence and interrelationships among oxidative events, energetics perturbations, and electrophysiological remodeling remain to be determined.

Summary

Although atrial contractile dysfunction contributes to stroke risk in AF, few studies have addressed the mechanisms underlying this pathology. We have demonstrated for the first time that oxidative modification of myofibrillar proteins is increased in atrial myocytes from AF patients and that this modification contributes to the loss of fibrillar protein function. Further studies to investigate regulation of oxidative processes in atria are warranted and may lead to design of novel therapeutic strategies.

Acknowledgments

The present work was supported by NIH grants HL-57262 (to D.V.W.) and HL-59791, HL-63067, and DK-55053 (to J.A.B.).

Figure 5. AF was associated with significant increases in myofibrillar oxidative events. A, Atrial myofibrillar isolates were assessed for tyrosine nitration and carbonyl formation by immunoblotting. Representative lanes of protein stain and immunoblotting shown. MW STDS indicates molecular weight standards. AF samples showed increased myofibrillar protein oxidative events. B and C, Quantification of relative immunoreactivity for bands identified in Figure 4 demonstrated significant elevations in relative protein nitration (B) and carbonyl events (C) in multiple myofibrillar structural and functional proteins. ND indicates none detected. n=6 samples; blots were run in triplicate. *P<0.05.

Figure 6. Energetic changes were strongly associated with nitration events. A, Correlation analyses demonstrated an inverse correlation between MM-CK function and extent of MM-CK tyrosine nitration but no significant relationship for protein carbonyl events. B, Correlation analyses demonstrated significant relationship between percentage of β-myosin and extent of myosin-heavy-chain tyrosine nitration but no analogous relationship for carbonyl events.
References
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