Evolution of the atrial fibrillation substrate in experimental congestive heart failure: angiotensin-dependent and -independent pathways

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Abstract

Objective: Augmented atrial apoptosis, angiotensin II expression, and related signalling pathway activation have been shown in clinical atrial fibrillation (AF), but their significance is poorly understood. This study evaluated temporal relationships between changes in atrial histopathology, selected signalling mediators, and AF promotion, as well as effects of angiotensin-converting enzyme (ACE) inhibition, in a canine model of congestive heart failure (CHF).

Methods: Dogs were subjected to ventricular tachypacing (VTP) for varying periods up to 5 weeks. Apoptosis was assessed by terminal dUTP nick-end labelling (TUNEL) and DNA fragmentation. Protein expression was determined by Western blot, angiotensin II concentration by ELISA (tissue) and radioimmunoassay (plasma), and caspase-3 activity by enzymatic assay. Histopathological analyses were used to quantify fibrosis, inflammation, and cell death.

Results: Significant apoptosis developed 24 h after VTP onset and persisted for 1 week, returning to baseline thereafter. Apoptosis was preceded by increases in tissue (but not plasma) angiotensin II concentration; enhanced expression of phosphorylated mitogen-activated protein (MAP) kinases p38, JNK, and ERK; and augmented ratios of the proapoptotic protein Bax to the antiapoptotic protein Bcl-2. Increased cell death, leukocyte infiltration, and caspase-3 activity occurred at the time of peak apoptosis. Apoptosis was followed by interstitial fibrosis, which peaked at 5 weeks. ACE inhibition (enalapril) prevented increases in tissue angiotensin II concentration, phosphorylated ERK expression, Bax/Bcl-2 ratio, and cellular apoptosis, but did not affect total cell death, leukocyte infiltration, JNK or p38 activation, and reduced but did not eliminate tissue fibrosis.

Conclusions: AF-promoting atrial structural remodeling in experimental CHF involves angiotensin II-dependent and angiotensin II-independent pathways. Significant apoptosis occurs, but prevention of apoptosis by ACE inhibition only partially prevents atrial structural remodeling.

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1. Introduction

Increased apoptosis has been detected in atrial tissues of patients with atrial fibrillation (AF), along with decreased expression of the antiapoptotic protein Bcl-2 and increased expression of the proapoptotic protein caspase-3 [1]. However, it is not clear whether these changes are due to AF per se, or to the underlying cardiac conditions that cause AF. Apoptosis (programmed cell death) causes cell loss in congestive heart failure (CHF) and may be a significant determinant of prognosis [2,3]. Although apoptosis is a candidate to underlie AF-promoting pathology [4], atria from goats kept in AF for 19–23 weeks show no evidence of apoptosis [5].

Ventricular tachypacing (VTP)-induced CHF produces atrial interstitial fibrosis in dogs comparable to atrial
pathology in clinical AF, and strongly promotes AF maintenance [6,7]. Angiotensin II is an important mediator in CHF-induced structural remodelling [8] and induces apoptosis in isolated cardiomyocytes [9]. Angiotensin II also activates mitogen-activated protein (MAP) kinases [10], which are capable of inducing apoptosis and fibrosis [11]. We previously found that inhibition of angiotensin-convertase enzyme (ACE) attenuates MAP kinase activation and fibrosis in experimental CHF [8]. The possibility that apoptosis plays a role in angiotensin II-related atrial structural remodeling has not, to our knowledge, been evaluated. Furthermore, although 5 weeks of ventricular tachypacing have been shown to cause atrial fibrosis, the time course of fibrosis development in relationship to changes in MAP kinases and other signalling changes has not been established. We therefore designed the present study to evaluate the relative time course during the development of ventricular tachypacing-induced CHF of changes in: (1) atrial apoptosis; (2) atrial and plasma angiotensin II concentration; (3) expression of the apoptotic determinants Bax, Bcl-2, and caspase-3; (4) total and phosphorylated (activated) MAP kinase expression; (5) atrial histopathology; and (6) the AF substrate as estimated by the duration of AF induced by atrial burst pacing. Because angiotensin II appeared to be important in inducing apoptosis, we also evaluated the effects of ACE inhibition with enalapril on apoptosis and related changes.

2. Materials and methods

2.1. General

Animal handling was in accordance with the guidelines of the Canadian Council on Animal Care and was in agreement with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Seven groups of mongrel dogs (n = 10–13/group) weighing 24–37 kg were subjected to right ven-
tricular tachypacing for: 0 (controls), 6, 12, and 24 h, and 1, 2, and 5 weeks. Under sterile technique, a unipolar tined pacing lead (Medtronic) was inserted into the right ventricular apex via the jugular vein in (1.5%) halothane-
anesthetized dogs. A pacemaker implanted in the neck was programmed to capture the ventricles at 240 beats/ min, with the rate decreased to 220 beats/min after 3 weeks to limit mortality. The presence of CHF was confirmed by clinical signs (lethargy, oedema, dyspnea, and loss of appetite) as in previous studies [6,8]. On the study day, an ECG was recorded to confirm continued ventricular capture and the pacemaker was deactivated. Dogs were then anaesthetized (2 mg/kg morphine, s.c.; 120 mg/kg α-chloralose, i.v. load; 29.25 mg/kg/h maintenance infusion) and ventilated. Femoral veins and arteries were cannulated and body temperature was maintained at 37 °C with a heating pad. A median sternotomy was performed and Teflon-coated stainless steel electrodes were hooked into left and right atrial (RA) appendages. AF was induced with four times threshold intensity burst pacing (10 Hz, 5–10 s) to measure mean AF duration (DAF) in each dog. For DAF <5 min, 15 measurements were performed; for DAF of 5–10 min, 10 measurements were performed; for DAF of 10–20 min, five measurements were performed; and for DAF >20 min, three measurements were performed.

Blood samples were taken for angiotensin II plasma concentration measurements. Dogs were sacrificed by α-chloralose overdose and hearts removed for subsequent analysis. For terminal dUTP nick-end labelling (TUNEL) and histopathological studies, hearts were excised, rinsed, and immersed into 10% neutral-buffered formalin. Tis-

In order to evaluate the effects of ACE inhibition on apoptosis and on fibrosis, four additional groups of five dogs each were subjected to ventricular tachypacing for 24 h or 5 weeks: two were treated with oral enalapril at 2 mg/kg/day, and the two others served as a placebo-
treated control group. The investigators were blinded to treatment allocation to prevent possible bias. Enalapril treatment began 3 days prior to tachypacing onset, to ensure steady-state conditions throughout the ventricular tachypacing period. After 24 h of tachypacing, hearts were removed and portions of the atria placed in either formalin for subsequent histopathological study, or liquid N2 for biochemical assays. Tissues from control dogs were analyzed at the same time as tissues from enalapril-
treated dogs for each determination, to control for inter-
experiment variability in assays. Similarly, in the time course analysis, tissues from each group of dogs were analyzed simultaneously (e.g., on each gel, each assay, etc.).

2.2. Apoptosis measurements

2.2.1. TUNEL

Paraffin-embedded sections were deparaffinized, rehy-
drated, saponified, and marked with Biotin-dUTP via re-
combinant terminal deoxynucleotidyltransferase (TdT). For the measurements of apoptotic myocytes, slides were then incubated sequentially with 1:50 ExtrAvidin–fluores-
cein isothiocyanate (FITC), which binds to biotinylated dUTP, 1:40 α-sarcosomic actin antibody (raised in mice), 1:100 tetramethylrhodamine (TRITC)-coupled antimouse IgG, and propidium iodide (PI). For the fibroblasts, slides were incubated sequentially with 1:50 ExtrAvidin–FITC, 1:25 antivimentin, 1:200 Cy5-coupled antimouse IgG, and PI. Each step was separated by three 3-min washes in phosphate-buffered saline (PBS). Cell counting was per-
formed with a fluorescence microscope equipped with green and red filters.

2.2.2. DNA laddering (fragmentation)

DNA was extracted from pulverized tissues, submitted to proteinase-K and RNAse-A degradation, followed by three passing cycles into a chloroform:isoamyl (24:1)/phenol solution and by two washing cycles with ethanol (70%). DNA was marked with [32P]dCTP via rTdt and then subjected to electrophoresis on a 1.5% agarose gel. The gel content was transferred to a Hybond-N+ membrane with a Vacugene apparatus, then radioactivity was detected and imaged with a PhosphorImager.

2.3. Caspase-3 activity measurement

Proteins were extracted from pulverized tissues submitted to three freeze/thaw cycles into a lysis buffer (Triton X-100, 1%; sucrose, 0.32 mol/l; Tris–HCl, 10 mol/l; EDTA, 5 mmol/l; DTT 2 mmol/l; PMSF 1 mmol/l; leupeptin, 10 μg/ml; Pepstatin-A, 10 μg/ml; aprotinin, 10 μg/ml), followed by 10 min of 10,000 rpm centrifugation. The supernatant was kept and protein concentration was quantified by the Bradford method. For each sample, two enzymatic reactions were performed in 390 μl of buffer (Tris–HCl, pH 7.0, 50 mmol/l; MgCl2, 5 mmol/l; EGTA 1 mmol/l; CHAPS, 0.1%; DTT, 1 mmol/l) to which was added 10 mmol/l N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) and 50 μg of extracted proteins, one with and one without 0.8 mmol/l N-acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO). Nonspecific activity was evaluated with the tubes, which included the Ac-DEVD-CHO, and the specific activity was evaluated by subtraction of the nonspecific activity from the total activity measured in the tubes without Ac-DEVD-CHO. All reactions were performed at 37 °C for 3 h. Fluorescence was measured by spectrofluorometer at 365 nm excitation and 465 nm emission.

2.4. Histopathology and immunohistochemistry

Histopathological analyses were performed on five hearts from control dogs and four hearts for each ventricular tachypacing group. It was also performed on the 10 dogs submitted to 5 weeks of ventricular tachypacing and treated with placebo or enalapril. Samples were obtained from Bachmann’s bundle, right atrial and left atrial (LA) appendages, LA posterior and inferior walls, crista terminalis, and RA-free wall. Tissue blocks were collected following longitudinal and transverse planes for each region and 5-μm sections cut at room temperature. Since changes were qualitatively homogeneous across regions, results shown are based on averages for all regions in each dog.

Microscopic images of Masson’s trichrome-stained sections at 400 × magnification were digitized (Scion Image Software) and analyzed with Sigmascan 4.0 (Jandel). Connective tissue content was quantified as a percentage of surface area, excluding blood vessels. To analyze cell death, sections were stained with hematoxylin–phloxin–safran (HPS). Dead (acidophilic) and viable cells were counted in 5–10 transverse section fields at 400 ×. HPS-stained longitudinal sections were used to quantify white cell...
infiltration at high magnification (1000 ×). Mononuclear and polymorphonuclear cells per field were counted for 12 fields of each slide.

2.5. Western blots

Protein extracts (200 μg) were denatured and electrophoresed on 15% sodium dodecyl sulfate (SDS) polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (0.45 μm), blocked for 90 min with 5% nonfat dry milk (NFDM) in Tris-buffered saline (TBS), and incubated overnight in primary antibody solutions. After three washes in 0.1% Tween 80–TBS (TTBS), membranes were incubated with horseradish peroxidase-conjugated primary antibodies in 5% NFDM in TTBS for 60 min, followed by four washes in TTBS. Antibodies were detected with Chemiluminescence Reagent Plus and quantified by densitometry (Quantity One software). Each gel contained samples from control and each ventricular tachypacing group.

Primary antibodies were: rabbit anti-ERK polyclonal IgG, rabbit anti-p38 polyclonal IgG, rabbit anti-JNK polyclonal IgG, mouse anti-Bcl-2 monoclonal IgG, and rabbit anti-Bax polyclonal IgG. Although we have previously assessed changes in MAP kinases at 24 h to 5 weeks after tachypacing onset [8], we felt it important to repeat these measurements in the present series of dogs, both to obtain results at earlier time points and, because results in different series of animals can vary, to be able to relate MAP kinase changes to other signalling events in the same animals. MAP kinases were probed with antibodies raised specifically against phosphorylated forms, which detect only MAP kinases catalytically activated by threonine or tyrosine.

Fig. 3. Atrial expression of phosphorylated (phospho) and total ERK1/2 (A), P38 (B), and JNK (C). Representative gels are shown on the top of each panel, and mean ± S.E.M. data (n = 5 hearts for CTL, four for each ventricular tachypacing group) are shown at the bottom. *P<0.05, **P<0.01 vs. CTL.

Fig. 4. (A) Top: examples of Western blots showing bands corresponding to Bcl-2 (left) and Bax (right), with samples obtained from atria of dogs subjected to VTP for the times indicated. Bottom: atrial expression of Bax and Bcl-2 (mean ± S.E.M., n = 5 hearts for CTL, four for each VTP group). (B) Mean (± S.E.M.) Bax/Bcl-2 ratio as a function of VTP duration. (C) Mean ± S.E.M. caspase-3 activity as a function of VTP duration (n = 5 hearts for CTL, four for each VTP group). *P<0.05, **P<0.01 vs. CTL.
phosphorylation, as well as antibodies that detect total MAP kinase, whether phosphorylated or nonphosphorylated. Changes in the two species of ERK (ERK1 at 44 kDa and ERK2 at 42 kDa) are presented as average band densities of the two moieties, after verification that both moieties responded similarly to the interventions studied.

2.6. Tissue and plasma angiotensin II concentration assays

Tissue angiotensin II concentration was measured in 500-μg protein samples by ELISA (Peninsula Laboratories). Blood for angiotensin II assay was obtained in EDTA-containing tubes containing 100 μl of bestatin solution to prevent angiotensin II breakdown. Plasma angiotensin II concentration was measured in protein extracts by radioimmunoassay (ALPCO-RIA kits), with a standard curve generated for each experiment.

2.7. Statistics

Group variance homogeneity was tested and means were compared by ANOVA. Scheffé or Mann–Whitney U tests were performed when the variance was homocedastic or heterocedastic, respectively. Results are expressed as mean ± S.E.M., and two-tailed P < 0.05 was considered statistically significant.

3. Results

3.1. Fibrosis and AF promotion

The duration of burst pacing-induced AF (DAF) increased progressively during ventricular tachypacing (Fig. 1A). Fibrous tissue content increased by approximately

![Fig. 5](https://example.com/fig5.jpg)

**Fig. 5.** (A) Examples of atrial tissue from control (I) and 24-h VTP (II) dogs. Column (a) represents TRITC (cardiomyocytes) and propidium iodide (nuclei) staining. Column (b) shows FITC staining to detect TUNEL-positive nuclei. Column (c) shows superimposed images. (B) Percentage of TUNEL-positive atrial cardiomyocytes (scale at left) and extent of DNA fragmentation (scale at right) as a function of VTP duration (mean ± S.E.M., n = 6 hearts for control, CTL, and n = 4 hearts for each VTP group). *P < 0.05, **P < 0.01 vs. CTL.
threefold after 1 week, and larger increases were seen after 2 and 5 weeks of tachypacing (Fig. 1B).

3.2. Angiotensin II and MAP kinase expression

Atrial angiotensin II concentrations increased significantly within 6 h of tachypacing onset, reached ~2.5 times control at 24 h, and remained elevated thereafter (Fig. 2A). Plasma angiotensin II concentrations failed to increase significantly until after 1 week of tachypacing (Fig. 2B), suggesting that the early rise in tissue angiotensin II concentration may have been due to local production. Phosphorylated ERK increased sevenfold within 6 h and remained elevated thereafter (Fig. 3A). Phosphorylated p38 almost doubled within the first 6 h and remained elevated throughout the study period (Fig. 3B).

Fig. 6. (A) Examples of atrial histopathology in a control (left), a 24-h (middle), and a 5-week (right) VTP dog (HPS stain, 400 \texttimes magnification). (B) Mononuclear (MN), polymorphonuclear (PMN), and total white cells per high-power field (1000 \texttimes). (C) Percentage of nonviable (acidophilic) and TUNEL-positive cardiomyocytes (mean \pm S.E.M., \(n\)= 6 hearts for control, CTL, and \(n\)= 4 hearts for each VTP group).
ylated JNK increased after 6 h, remained elevated for 24 h, and then returned towards control values (Fig. 3C). Total ERK, p38, and JNK expression did not change significantly with the development of CHF.

### 3.3. Apoptosis

Both Bcl-2 and Bax expression increased with ventricular tachypacing, albeit with a slightly different time course (Fig. 4A). Since Bax promotes and Bcl-2 prevents apoptosis, the Bax/Bcl-2 ratio (Fig. 4B) is an index of net proapoptotic protein expression. The Bax/Bcl-2 ratio increased significantly after 12 h, remained elevated for 1 week, and then returned to control values. Fig. 4C shows the activity of caspase-3, an apoptotic cysteine protease. Caspase activity was unchanged by 6 and 12 h of tachypacing, but was strongly increased at 24 h and returned towards control subsequently. Thus, angiotensin II and MAP kinase changes preceded increases in the Bax/Bcl-2 ratio, which in turn preceded the rise in tissue caspase activity.

Fig. 5A (top) shows atrial tissue sections stained (left) with propidium iodide (for nuclei) and TRITC attached to α-sarcomeric actin (to identify cardiomyocytes, seen as red in sections), corresponding sections with FITC staining for TUNEL-positive nuclei (middle, green) and superimposed images (right). In control, TUNEL-positive nuclei were rare (panel I). After 24 h of ventricular tachypacing, TUNEL-positive cardiomyocyte nuclei (panel II) became much more common. The mean percentage of TUNEL-positive cardiomyocytes rose to a maximum, ~10 times control, at 24 h (Fig. 5B), and decreased thereafter. TUNEL-positive fibroblasts were rarely seen (<1/10^5 cells) and no clear changes were seen with ventricular tachypacing. DNA fragmentation was not altered after 6 or 12 h of tachypacing, but increased substantially within 24 h and returned towards control thereafter (Fig. 5B), paralleling changes in TUNEL-positive cardiac myocytes.

### 3.4. Histopathological studies

Fig. 6A shows HPS-stained atrial tissue sections from a control (left), 24 h-tachypaced (middle), and 5 week-tachypaced (right) dog. After 24 h of tachypacing, the number of leukocytes within atrial tissue increased (white arrows show zones of white cell infiltration), as did the number of acidophilic, nonviable cells (black arrows). The number of both mononuclear and polymorphonuclear cells increased significantly (Fig. 6B), accompanied by perceptible tissue edema. The white cell count returned to normal within 1 week. The percentage of dead cells increased 10-fold after 24 h of ventricular tachypacing, coinciding with maximal TUNEL positivity (Fig. 6C). Cell death rate returned to control values within 1 week. Signs of necrosis, including contraction bands and pyknotic nuclei, were seen after ventricular tachypacing, particularly at 24 h, but were not sufficiently frequent for accurate quantification.

### 3.5. Effects of ACE inhibition on CHF-induced apoptosis and related changes

The earliest changes we noted following the onset of ventricular tachypacing were increases in tissue angiotensin II concentration and MAP kinase expression, which were statistically significant within 6 h. Angiotensin II is capable of enhancing expression of phosphorylated ERK, JNK, and p38 [10,11], and of inducing cardiomyocyte apoptosis [9]. We hypothesized that atrial angiotensin II elaborated in response to the hemodynamic stress associated with ventricular tachypacing may be a principal factor inducing atrial MAP kinase activation and apoptosis. Since apoptosis peaked after 24 h of tachypacing, we compared apoptosis and associated changes in atria of additional groups of dogs subjected to 24-h ventricular tachypacing during therapy with the converting enzyme inhibitor enalapril vs. dogs similarly handled but without enalapril therapy. Ventricular tachypacing-induced increases in phosphorylated ERK expression were significantly reduced by enalapril (Fig. 7A), but increases in phosphorylated p38 (Fig. 7B) and JNK (Fig. 7C) were unaffected. Enalapril prevented tachypacing-induced increases in angiotensin II concentration (Fig. 8A, left), Bax/Bcl-2 ratio (Fig. 8A, middle), and caspase-3 activity (Fig. 8A, right). Apoptosis was completely suppressed by enalapril (Fig. 8B, left), but cell death rates (Fig. 8B, middle) and leukocyte infiltration...
Although enalapril prevented atrial angiotensin II accumulation and apoptosis, it did not significantly change total cellular death rate, suggesting the presence of discrete angiotensin II-dependent and -independent events. To evaluate potential changes in structural remodeling, we quantified tissue fibrosis in dogs subjected to 5-week ventricular tachypacing in the presence or absence of enalapril therapy (five dogs per group). Fibrous tissue content in enalapril-treated dog atria averaged 5.7 ± 1.3%, lower than in placebo-treated dogs (9.8 ± 1.1%, \( P = 0.03 \) vs. enalapril-treated), but nevertheless greater than in control dogs (0.8 ± 0.2%, \( P < 0.001 \) vs. enalapril-treated). DAF averaged 700 ± 151 s in placebo-treated dogs, vs. 132 ± 36 s (\( P < 0.05 \)) in enalapril-treated dogs.

4. Discussion

Although the presence of atrial apoptosis has been demonstrated in tissue samples from patients with AF, its importance has been uncertain and the role of apoptosis in experimental models of AF has not been documented. We found that apoptosis, leukocyte infiltration, and an increased cell death rate occur prior to arrhythmogenic atrial structural remodeling associated with experimental CHF. These changes are preceded by increased tissue angiotensin II concentration; activation of p38, JNK, and ERK1/2 MAP kinases; and an increased Bax/Bcl-2 ratio. ACE inhibition prevents increases in angiotensin II concentration, phosphorylated ERK expression, caspase-3 activity, and apoptosis, but does not alter JNK or p38 activation, total cell death, or leukocyte infiltration, and only partly prevents tissue fibrosis, in this model.

4.1. Apoptosis and AF

Aimé-Sempé et al. [1] reported a high proportion (~29%) of TUNEL-positive cells, increased caspase-3 expression, and an increased Bax/Bcl-2 ratio in atrial samples from patients with chronic AF. Apoptotic changes are absent in atria of goats with electrically induced chronic AF, suggesting that AF per se does not induce apoptosis [5]. We found that ventricular tachypacing-induced CHF results in atrial apoptosis prior to the development of AF-promoting interstitial fibrosis and that prevention of apoptosis is associated with reduced CHF-related atrial structural remodeling.

The number of TUNEL-positive nuclei we observed (0.8% of total) was much less than that (29%) reported by Aimé-Sempé et al. [1]. However, these authors noted a much smaller number of cells with shrunken nuclei and strong TUNEL staining, and did not detect DNA fragmentation, suggesting that the true apoptosis rate in their samples was likely much smaller than 29%. Since apoptosis leads inevitably to cell death within days, it would seem unlikely that an apoptosis rate of almost 30% could be maintained for substantial periods. Nevertheless, by any measure, the presence of apoptotic changes was certainly much greater in AF patients than in controls [1]. Cesselli et al. [12] observed progressive increases in ventricular apoptosis over 5 weeks of ventricular tachypacing, contrasting with the transient changes we saw in the atria and suggesting differences between the atrial and ventricular responses to tachypacing-induced CHF. Heinke et al. [13] noted...
The most prominent structural alteration associated with AF is tissue fibrosis [1,6,14,15]. Apoptotic cell removal is classically considered to be silent, and not to lead to tissue fibrosis [16]. However, caspase-3-induced apoptosis leads to renal cell death that causes renal fibrosis [17] and apoptosis can induce the production of transforming growth factor-β (TGF-β), a known profibrotic cytokine [16]. Administration of an antibody against TGF-β to rats with unilateral ureteral obstruction prevents apoptosis and associated renal tubular fibrosis [18]. Thus, atrial apoptosis may contribute to CHF-related atrial fibrosis, and enalapril-induced prevention of apoptosis may underlie the reduced atrial structural remodeling in CHF seen with the drug. The atrial cell loss caused by apoptosis may also contribute to AF-promoting atrial contractile abnormalities and dilation associated with CHF due to ventricular tachypacing [19].

The mechanisms by which CHF leads to atrial apoptosis are unknown. Mechanical stretch can increase the production of TGF-β and induce apoptosis [18]. Enalapril completely prevented apoptosis in the present study, suggesting a key role for angiotensin II. The angiotensin II causing atrial apoptosis was likely due to tissue production, since atrial angiotensin II concentrations increased within 6 h of the onset of ventricular tachypacing, before the development of apoptosis, whereas plasma angiotensin II concentrations failed to increase significantly until a week later. Cardiomyocytes possess an intrinsic renin–angiotensin system that is upregulated in CHF [20], and there is considerable evidence that locally produced angiotensin II is the principle form involved in remodelling [21].

4.2. Novel aspects and potential significance

Ours is the first study, to our knowledge, to observe atrial apoptosis in a defined experimental model of AF and to characterize its time course, along with that of related phenomena. Our observations are relevant to understanding the mechanisms of atrial structural remodeling, an important component of the AF substrate [7]. The atrial apoptosis observed in patients with AF [1] could be related to the causes of AF, or a consequence of the arrhythmia. Taken together with the observation that apoptosis is absent in goats with AF sustained in the context of atrial tachycardia-induced remodelling [5], our results suggest that apoptosis is more likely associated with the pathophysiological mechanisms leading to the AF substrate rather than being a result of AF per se.

In patients with AF, tissue ACE is upregulated and angiotensin II type 1 receptors are downregulated, compatible with increased angiotensin II production [22,23]. ACE inhibition attenuates experimental atrial structural remodeling [8] and reduces the prevalence of AF in post-myocardial infarction (MI) patients with left ventricular dysfunction [24]. Our findings provide information about the potential mechanisms and limitations of ACE inhibitor therapy. Angiotensin II activates a variety of MAP kinases and induces fibroblast proliferation [11]. Phosphorylated ERK is increased in atrial tissues of patients with AF [22], and ERK activation promotes fibroblast proliferation [25]. We found that ACE inhibition prevents one constellation of CHF-related atrial changes (ERK activation, apoptosis) while leaving others (JNK and p38 activation, white cell infiltration, cell death) unaltered, and provides only partial protection against atrial structural remodeling. Thus, there must be significant angiotensin-independent atrial remodeling pathways in CHF. These angiotensin-independent pathways account for the incomplete efficacy of ACE inhibition in preventing atrial structural remodeling. The discovery and characterization of these pathways may providealternate and/or additional targets for therapy to prevent atrial remodeling and the development of the AF substrate in CHF.

In addition to apoptosis, we noted substantial cell death associated with an inflammatory response 24 h after the onset of ventricular tachypacing. This observation is interesting in the light of recent evidence for an inflammatory component in AF [26]. The transient nature of atrial apoptosis, cell death, and inflammation suggests that early events may be important in atrial structural remodeling, and that early intervention may be necessary for effective prevention of the development of the AF substrate.

4.3. Potential limitations

The present study evaluated the time course of changes involved in atrial structural remodeling in a well-defined experimental model, but extrapolation to clinical atrial disease should be cautious. Studies in other animal models of atrial structural remodeling and/or CHF would be of interest. The total percentage of dead cells after 24 h of ventricular tachypacing was much greater than the percentage of TUNEL-positive nuclei. Along with evidence for necrosis and the fact that enalapril prevented apoptosis but did not significantly alter the total rate of cell death, this observation suggests that the majority of atrial cell deaths in this model occur by nonapoptotic pathways. It is not possible, based on the results of the present study, to know definitively whether apoptosis mediates the atrial profibrotic effects of angiotensin II, or whether they are associated but causally unrelated events. Further studies in transgenic models with deletions of specific components of potential signalling pathways would be very useful in defining causal relations in atrial structural remodeling.

We found a significant angiotensin-independent component to cell death and structural remodeling. The mechanisms of the angiotensin-independent component remain to be determined. There is evidence for a significant increase in oxidative stress in the atria of patients with AF [27] and in
the ventricular remodeling associated with CHF [12]. Further work assessing the potential role of angiotensin-independent oxidative injury in CHF-induced atrial remodeling would clearly be of interest. Another important potential contributor to CHF-induced atrial remodeling is tissue chymase, a cardiac serine protease that is believed to be an important contributor to angiotensin II formation in both dogs [28] and humans [29]. In addition, chymase can activate cytokines such as transforming growth factor-β [30] and could therefore contribute to atrial remodeling independently of changes in tissue angiotensin II concentration. Further assessment of this issue will be of great interest, particularly since selective chymase inhibitors have begun to be synthesized and have been shown to affect cardiac remodeling [31]. Changes in cytokines may be important in tissue inflammation and fibrosis and were not measured in the present study; however, evaluation of cytokine expression in patients with AF found no significant changes [32].

It is generally believed that p38 is a prominent proapoptotic mediator, but we found that enalapril strongly inhibited apoptosis without affecting p38 activation. Recent evidence indicates that JNK inhibits proapoptotic effects of p38 [33], suggesting that the simultaneous activation of p38 and JNK may explain the lack of enhanced apoptosis in the presence of enalapril.

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