Calpains and cytokines in fibrillating human atria

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Goette, Andreas, Marco Arndt, Christoph Röcken, Thorsten Staack, Roland Bechtloff, Dirk Reinhold, Christof Huth, Siegfried Ansorge, Helmut U. Klein, and Uwe Lendeckel. Calpains and cytokines in fibrillating human atria. Am J Physiol Heart Circ Physiol 283:H264–H272, 2002; 10.1152/ajpheart.00505.2001.—Atrial fibrillation (AF) is accompanied by intracellular calcium overload. The purpose of this study was to assess the role of calcium-dependent calpains and cytokines during AF. Atrial tissue samples from 32 patients [16 with chronic AF and 16 in sinus rhythm (SR)] undergoing open heart surgery were studied. Atrial expression of calpain I and II, calpastatin, troponin T (TnT), troponin C (TnC), and cytokines [interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, transforming growth factor (TGF)-β1, and tumor necrosis factor (TNF)-α] were determined. Expression of calpain I was increased during AF (461 ± 201% vs. 100 ± 34%, *P < 0.05). Amounts of calpain II and calpastatin were unchanged. Total calpain enzymatic activity was more than doubled during AF (35.2 ± 17.7 vs. 12.4 ± 9.2 units, *P < 0.05). In contrast to TnC, TnT levels were reduced in fibrillating atria by 26% (*P < 0.05), corresponding to the myofilament disintegration seen by electron microscopy. Small amounts of only IL-2 and TGF-β1 mRNA and protein were detected regardless of the underlying cardiac rhythm. In conclusion, atria of patients with permanent AF show evidence of calpain I activation that might contribute to structural remodeling and contractile dysfunction, whereas there is no evidence of activation of tissue cytokines.

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TnC were examined by Western blotting. Calpain I expression was localized by immunohistochemistry. Expression of calpain I and II, calpastatin, IL-2, and TGF-β1 was analyzed quantitatively at the mRNA level. The presence of TNF-α, IL-1β, IL-6, IL-8, and IL-10 mRNA was assessed by qualitative RT-PCR. Amounts of IL-2 and TGF-β1 protein were determined by enzyme immunoassay. Calpain enzymatic activity was analyzed using the fluorescent substrate Suc-Leu-Tyr-7-amido-4-methyl-coumarin (Suc-Leu-Tyr-AMC). Selected tissue samples from each group were examined by light and electron microscopy. All patients gave written consent to participate in the study, and their baseline characteristics are shown in Tables 1 and 2.

**Western blot.** About 200 mg of tissue were homogenized directly in 2× RotiLoad (Roth; Karlsruhe, Germany) using the dispersing system UltraTurrax (Sigma; Deisenhofen, Germany). The homogenate was cleared by ultracentrifugation at 4°C, 100,000 g, for 30 min. Protein contents were determined using the micro-Lowry-based Protein Assay kit provided by Sigma (Heidelberg, Germany). After samples (3 μg each) were heated for 5 min at 100°C, they were applied onto precast 4–12% gradient SDS-polyacrylamide gels (NuPAGE, Novex Electrophoresis; Frankfurt, Germany) and separated at 300 V, 15 mA constant, using MOPS-SDS running buffer (Novex). Proteins were transferred onto nitrocellulose membranes (BAS-85, Schleicher & Schuell; Goslar, Germany) by means of a semidry blotter (Bio-Rad; Munich, Germany) (25 V, 2 h, 50 mmol/l Tris-borate buffer, pH 9.0). Membranes were blocked by overnight incubation in 7.5% (wt/vol) milk powder in PBS (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4).

**Table 1. Clinical characteristics of patients**

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**Means ± SD**

- **SR patients**
  - 62 ± 11
  - 2.5
  - 55 ± 18
  - 3.7 ± 0.62

- **AF patients**
  - 65 ± 5
  - 47 ± 53
  - 2.7
  - 49 ± 13
  - 4.8 ± 0.35

M, male; F, female. Age was measured in years, and atrial fibrillation (AF) duration was measured in months. CAD, number of diseased coronary arteries; OMI, old myocardial infarction present (1) or absent (0); VD, valve disease; EF, left ventricular ejection fraction (in %); LA, left atrial diameter (in cm); SR, sinus rhythm; MR, mitral regurgitation; AS, aortic stenosis; AR, aortic regurgitation; NA, data not available.

*P < 0.01 compared with AF.

**Table 2. Medical therapy in patients with AF and SR**

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<td>ACE inhibitors, %</td>
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n = No. of patients; numbers in parentheses represent the absolute numbers. ACE, angiotensin-converting enzyme. P = not significant.
mM Na2HPO4, and 1.4 mM NaH2PO4; pH 7.4). Monoclonal mouse anti-TnT (clone 1C11), anti-TnC (clone 4T21, HyTest; Turku, Finland), anti-calpain I, and anti-calpastatin antibodies (Chemicon International, Hofheim, Germany) as well as goat anti-mouse horseradish peroxidase (POD) (New England Biolabs; Schwabach, Germany) and “SuperSignal West Dura Extended Duration Substrate” (Pierce, Rockford, USA) were used for immunochromeluminescence detection. Rabbit anti-calpain II polyclonal antibody (Chemicon) together with goat anti-rabbit-POD (New England Biolabs) were used for determination of calpain II protein amounts. The resulting images were densitometrically quantified using two-dimensional image analysis software (RFIP-Scan, MWG Biotech; Ebersberg, Germany). The mean relative absorption units of the group with SR were set as controls and compared with the corresponding means of the AF group.

To avoid artificial variations of individual blots, protein determination, SDS-PAGE, blotting onto nitrocellulose membrane, immunostaining, and chemiluminescence detection were performed simultaneously in the same batch of reagents. Densitometric quantification for comparison of the different groups was done only on blots processed equally and exposed on the same X-ray film.

RNA isolation. Samples of human atrial appendages obtained during open heart surgery were immediately frozen in liquid nitrogen and stored at −192°C for further analysis. Total RNA was prepared as described by Chomczynski and Sacchi (7). About 200 mg of tissue were homogenized on ice in 2 ml TriZOL (GIBCO-BRL; Karlsruhe, Germany) using the dispersing system Ultra Turrax (Sigma). After the addition of 400 μl chloroform, the samples were vortexed and centrifuged at 4°C and 14,000 g for 15 min. Total RNA was precipitated from the aqueous phase by the addition of 1 volume of ice-cold isopropanol. The RNA was dissolved in 0.1% SDS and reprecipitated by the addition of 1/10 volume of 5 mol/l ammonium acetate (pH 4.8) and 2.5 volumes of ice-cold ethanol. Contaminating DNA was removed by Dnase I digestion (Boehringer Mannheim; Mannheim, Germany; 20 U/50-μl reaction, 30 min at 37°C). Subsequently, RNA was subjected to a second round of purification by means of a RNeasy Mini Kit (Qiagen; Hilden, Germany), and the resulting RNA was quantified spectrophotometrically using a GeneQuant (Pharmacia; Freiburg, Germany). RNA was aliquoted and stored ethanol precipitated at −80°C until further use.

RT-PCR. In each case, 1 μg total RNA was transcribed in a final volume of 20 μl by 20 units of AMV reverse transcriptase (Promega; Mannheim, Germany) in the supplied buffer with the addition of 0.5 mmol/l dNTP, 10 mmol/l random hexanucleotides (Boehringer Mannheim), and 50 units placent RNase inhibitor (Ambion; Austin, TX) during a 1-h incubation at 37°C. The enzymes were inactivated by a 10-min incubation at 65°C, and the reaction mixture was kept frozen at −70°C until enzymatic amplification.

To assess atrial cytokine expression, quantitative RT-PCR was performed using 1/10 of the RT reaction per 50-μl reaction in an Autogene II thermocycler (CLF Laboratory; Emsmersacker, Germany). The reaction mixture consisted of 1× reaction buffer, 3 mmol/l MgCl2, 200 μmol/l dNTP, 1 unit InViTaq polymerase (InViTeK; Berlin, Germany), 2 μl cDNA, and one of the commercially available RT primer pairs for IL-1β (Biosource; Solingen, Germany), IL-2, IL-6, IL-10, TNF-α, or TGF-β1 (Stratagene; Heidelberg, Germany).

Subsequently, IL-2 and TGF-β1 mRNA amounts as well as those of calpains I and II and calpastatin were determined quantitatively. Quantitative PCR was performed using the iCycler (real-time PCR device, Bio-Rad). All samples were analyzed in triplicate. A 30-μl reaction mixture consisted of 15 μl HotStarTaq Master Mix (Qiagen; Hilden, Germany), 1.2 μl RT reaction, 0.3 μl SYBR green I (1:10,000) (Molecular Probes; Eugene, OR), and 0.5 μmol/l specific primers for IL-2 and TGF-β1 (Stratagene), calpain I (forward: 5′-GCCAGAGCATCTCTGCAGGGAG-3′; reverse: 5′-GCTTGTGAAGTCTCCTC-TAACGCCC-3′), calpain II (forward: 5′-CCGAGCCTACTTCTG-GATGAA-3′; reverse: 5′-AGGTTGATGAAGTCTCCTGAG-3′), and calpastatin (forward: 5′-GACAGTCAAGTCTGCTC; reverse: 5′-TCAGGTCTCTGGCAATAGTG-3′). Primers for calpains and calpastatin were synthesized by BioTez (Berlin, Germany). Initial denaturation and activation of Taq polymerase at 95°C for 15 min was followed by 40 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. 18S mRNA amounts were determined using the RT primer pair available from Ambion and used to normalize cDNA contents. The fluorescence intensity of the double-strand-specific SYBR green I, reflecting the amount of actually formed PCR product, was read real time at the end of each elongation step. The specific initial temperature at which the linear increase of sample PCR product started relative to the corresponding points of a standard curve obtained by serial dilution of known copy numbers of the corresponding cloned PCR fragments. Data are given as copy numbers normalized to 18S mRNA amounts. Phytohemagglutinin-activated T cells served as positive controls for cytokine expression. For comparison, copy numbers of IL-2 and TGF-β1 mRNAs were determined in resting (IL-2: 300 copies/μg RNA; TGF-β1: 12,000 copies/μg RNA) and activated T cells (IL-2: 1.2 × 106 copies/μg RNA; TGF-β1: 4,000 copies/μg RNA).

Calpain enzymatic activity. Calpain enzymatic activity was determined exactly as described by others (35) using Suc-Leu-Tyr-AMC (Bachem, Heidelberg, Germany) as the substrate. Briefly, 30 μg protein extract were incubated for 10 min at 37°C in a buffered solution (pH 7.4) containing 20 mM Tris-HCl, 5 mM Ca2+, and EDTA-free protease inhibitor cocktail (recommended dilution, Boehringer Mannheim). After the addition of 5 μl of a 50 mM substrate stock solution, buffer was added to adjust the volume of the assay to 2 ml. AMC release was measured by fluorometry (380-nm excitation and 430-nm emission, spectrometer LS50, Perkin-Elmer) immediately after substrate addition (substrate blank) and after incubation for 30 min at 37°C. Control assays were performed in the absence of CaCl2 with 10 mM EDTA and 10 mM EGTA. Total calpain activity was determined as the Ca2+-dependent cleavage of Suc-Leu-Tyr-AMC and given as arbitrary units per milligram of protein per minute.

Light microscopy. For light microscopy, tissue samples were fixed in formalin and embedded in paraffin. Six specimens were obtained from patients in SR, and six specimens were obtained from patients with chronic AF. Deparaffinized sections were stained with hematoxylin and eosin and Masson’s trichrome stain, respectively.

Immunohistochemistry. Immunostaining was performed with a monoclonal anti-calpain I antibody (dilution 1:200). Immunoreactions were visualized with the avidin-biotin complex method, applying a Vectastain ABC-alkaline phosphatase kit (Alexis Deutschland; Grünberg, Germany). Neufuchsin served as chromogen. Specimens were counterstained with hematoxylin. The specificity of immunostaining was controlled by omitting the primary antibody.

Electron microscopy. For electron microscopy, specimens were fixed in a mixture of 2% formalin-2.5% glutaraldehyde (pH 7.2, overnight at 4°C) and then in 3.125% glutaraldehyde.
Following standard procedures of tissue processing for electron microscopy, the specimens were finally embedded in Epon supplemented with 2% 2,4,6-tris(dimethylaminomethyl)phenol (DMP-30, Sigma). Polymerization took place over 24 h at 60°C. Semithin sections (1–2 μm) were stained with Toluidine blue. Ultrathin sections (80–120 nm) were mounted on copper grids and counterstained with 3% aqueous uranyl acetate (30 min at room temperature) and contrasted with 1% aqueous lead citrate (15 min at room temperature).

**Statistical analysis.** All values are expressed as means ± SD. Differences between the groups were evaluated using an unpaired Student's t-test. Pearson's correlation coefficient (r) was used to determine the relation between AF duration and calpain I and TnT expression. A value of P < 0.05 was considered to be statistically significant.

**RESULTS**

**Patient characteristics.** Tables 1 and 2 summarize the baseline characteristics of all patients. The underlying heart diseases (coronary artery or valve diseases) as well as the medical therapy were equally distributed between the two groups. The incidence of noncardiac diseases like hypertension (AF: 11 of 16, 69%, and SR: 9 of 16, 56%) and diabetes (AF: 9 of 16, 56%, and SR: 6 of 16, 38%) was not significantly different. The left atrial diameter was increased in patients with chronic AF compared with patients in SR (Table 1).

**Expression of calpains and calpastatin.** At the mRNA level, there were no significant differences in the expression of calpain I (AF: 159.6 ± 137.9%; SR: 100 ± 68.3%, P = not significant [NS]), calpain II (AF: 87.0 ± 41.3%; SR: 100 ± 45.2%, P = NS), and calpastatin (AF: 104.1 ± 165.3%; SR: 100 ± 92.7%, P = NS) between AF and SR patients (Fig. 1).

Amounts of calpain I protein were significantly increased in patients with AF (461 ± 201%) compared with patients in SR (100 ± 34%, P < 0.05; Fig. 2). In contrast, calpain II (AF: 92 ± 30.6%; SR: 100 ± 33.8%, P = NS) and calpastatin expression (AF: 126.2 ± 98.6%; SR: 100 ± 83.2%, P = NS) was not different in the two groups (Fig. 2). The duration of AF did not correlate with the amounts of calpain I expression (r = 0.20, P = NS). The underlying cardiac disease and the intake of digoxin or calcium channel blockers did not affect calpain I expression. Calpain I expression was localized in atrial myocytes by immunohistochemistry regardless of the underlying atrial rhythm (Fig. 3).

**Calpain enzymatic activity.** Calpain enzymatic activity was more than doubled in fibrillating atrial tissue samples (35.2 ± 17.7 units) compared with SR samples (12.4 ± 9.2 units; Fig. 2). Calpain activity was not related to the medical therapy or clinical parameters (data not shown).

**TnT and TnC content.** Consistent with previous reports, two TnT isoforms were detected in all samples. The total amount of TnT was significantly reduced in
patients with AF (74.3 ± 8%) compared with patients in SR (100 ± 9%, P < 0.05; Fig. 4). There was no significant correlation between TnT protein levels and AF duration (r = 0.18, P = NS). Small TnT subfragments could not be detected by the used blotting technique. In contrast, the amounts of TnC were not significantly different in patients with and without AF (AF: 128 ± 78% vs. SR: 100 ± 61%, P = NS; Fig. 4).

Cytokine expression. Expression of TNF-α, IL-1β, IL-6, and IL-10 mRNA could not be detected in any of the tissue samples. IL-8 mRNA was found in one patient with chronic AF only (patient 27).

IL-2 mRNA was found in all patients with and without AF. However, the relative amount of IL-2 mRNA was not different between the groups (SR: 75 ± 185 vs. AF: 64.7 ± 79 copies/μg RNA, P = NS; Fig. 5). At the
protein level, IL-2 was found in minute amounts (<1.6 ng/mg protein) in four patients only (2 AF and 2 SR).

TGF-β1 mRNA was present in all samples. The relative amounts of mRNA, however, were comparable in the two groups (SR: 62.5 ± 74 vs. AF: 58.6 ± 52.1 copies/μg RNA, P = NS; Fig. 5). In addition, TGF-β1 protein amounts were not significantly different (AF: 0.56 ± 0.34 vs. SR: 0.66 ± 0.32 pg/μg protein, P = NS).

Light microscopy/electron microscopy. A cellular inflammatory infiltrate was not found in any tissue specimen investigated histologically. Occasionally, few mononuclear cells were present in the lumen of atrial vessels. Structural changes, such as fibrosis, were common regardless of the underlying atrial rhythm. However, the amount of fibrosis varied and appeared to be more pronounced in specimens from patients suffering from AF compared with in SR (Fig. 3).

On electron microscopy, desintegration of myofilaments was apparent in tissue samples from fibrillating atria only (Fig. 6). No other pathological alteration was found that separated fibrillating from nonfibrillating atria.

DISCUSSION

This study shows, for the first time, an increased atrial expression of calpain I and calpain enzymatic activity in patients with AF. These changes are accompanied by reduced amounts of TnT and morphological degradation of myofilaments in fibrillating human atria. In contrast, structural atrial changes were not associated with an increased expression of proinflammatory (TNF-α, IL-1β, and IL-6) or immunosuppressive (IL-10) cytokines. Only small amounts of IL-2 and TGF-β1 mRNA and protein were found in atrial tissue compared with the expression level detected in human resting or activated T cells. However, their expression was not related to the presence of AF.

Molecular mechanisms for contractile dysfunction. Recent studies suggest that AF is associated with intracellular calcium overload (3, 9, 18, 44). An increase in cytosolic calcium levels is known to cause an activation of calcium-dependent calpains (12). Calpain I-dependent degradation of troponins has been reported in ischemia-reperfusion models causing myocardial stunning (12, 17). The results of the present study imply a similar effect of calpain I in fibrillating atrial muscle. The amounts of TnT protein were reduced and the normal sarcomere structure was partially disturbed in patients with AF. In accordance with previous studies, calpain I had no effect on TnC levels (12, 17). In contrast to previous ischemia-reperfusion models, small fragments of TnT were not detected in this study. One explanation for this finding might be that cleaved TnT subfragments were too small or carried no specific epitope to be detected by the antibody used. However, myofilament destruction was clearly shown by electron microscopy, which implies that, in addition to TnT, other contractile myofilaments (like myosin and actin) were partly degraded as well.

Loss of a regular sarcomere structure would help to explain the prolonged mechanical dysfunction of the atria after successful cardioversion of AF. Mechanical atrial dysfunction can last for several weeks after cardioversion (13, 14, 33). Recently, downregulation of L-type calcium channels and an altered intracellular calcium handling has been described to be involved in the pathogenesis of contractile dysfunction during AF (26, 34, 35, 38, 44). However, Schotten et al. (34) have also demonstrated that there is a strong correlation...
between the maximum force of contraction and sarcomere content in atrial muscle preparations. Although changes in number and/or function of L-type calcium channels seem to contribute predominantly to the reduced contractility during AF, the time course of resolution of these functional changes after restoration of SR has not been studied in detail. A recent study (13) implies that structural abnormalities persist after cardioversion. However, in that particular study, a mitral regurgitation model was used so that the effects of AF per se and its resolution cannot be fully assessed. Nevertheless, structural abnormalities of contractile proteins may help to explain especially the long-lasting dyscontractility of the atria after cardioversion. In addition to these intracellular changes, interstitial collagen accumulation as well as atrial dilatation may further contribute to prolonged alterations of the atrial contractile performance (2, 19, 27, 28, 33).

**Cytokines and AF.** Intracellular calcium overload induced by myocardial reperfusion is a potent stimulus to cause invasion of inflammatory cells and increased expression of cytokines (20, 42). Some studies suggest that the pathophysiological atrial changes during AF are caused by ischemia as well (4, 21). However, these results have been questioned by other groups (5, 16, 18). In addition, it has been speculated that AF is promoted by an atrial myocarditis or autoimmune process (15, 16, 29). Frustaci et al. (15, 16) have found atrial infiltration of inflammatory cells in about two-third of patients with lone AF. In contrast to ischemia-reperfusion models or patients with lone AF, expression of inflammatory cytokines was not observed in fibrillating atria during this study. The present study included a heterogenous group of patients with severe valve and/or coronary artery disease. In all of the patients, neither cellular inflammatory infiltrates nor expression of proinflammatory cytokines could be demonstrated. In addition, the lack of significant amounts of the immunosuppressive cytokine IL-10 demonstrates that the absence of proinflammatory cytokines during AF was not induced by a predominant synthesis of anti-inflammatory cytokines. Small amounts of IL-2 mRNA, however, were found in all tissue samples. Despite the absence of inflammatory infiltrates, activated T lymphocytes within the atrial capillaries might have been a source for its expression. Previous studies have shown that plasma IL-2 levels are increased in patients with coronary artery disease (31). In addition, soluble IL-2 receptor levels are inversely related to left ventricular function (40). Thus the demonstrated IL-2 expression in the present study is most likely due to the underlying ventricular disease and not related to atrial alterations. A recent study by Chung et al. (8) has demonstrated elevated systemic CRP levels in patients with persistent AF (defined as AF > 30 days). In that study, the detection limit for CRP (~0.18 mg/l) was significantly lower compared with the routine CRP assay used in our study. However, elevated systemic CRP levels may not necessarily correspond to inflammatory changes in atrial tissue. In addition, differences in the patient population with longlasting episodes of AF (average 47 mo) in this study may have contributed to the conflicting findings, because the impact of inflammatory effects may decrease with time.

Nevertheless, degenerative and fibrotic changes of human fibrillating atria have consistently been reported (15, 16, 19, 36). TGF-β1 contributes to the phenotypic conversion of fibroblasts to myofibroblasts and regulates myofibroblast turnover of collagen (6, 38). An increased expression of TGF-β1 is found at sites of increased tissue repair and is associated with fibrous tissue formation (39). The presence of small amounts of TGF-β1 within all tissue samples in the present study may help to explain that there were some fibrotic changes even in patients with SR. However, due to our findings, increased amounts of interstitial fibrosis in fibrillating atria cannot be explained by TGF-β1. Instead, recent data imply that activation of the atrial angiotensin system and altered bradykinin metabolism contribute to progressive atrial fibrosis during AF (19, 27, 28). In addition to these molecular pathways, increased apoptotic death of myocytes can also contribute to the development of fibrosis. Aime-Sempe et al. (1) provided evidence of an increased rate of apoptosis in fibrillating human atria. Importantly, inflammatory infiltrates were not observed in that study either. TNF-α is one potential proapoptotic stimulus. The absence of TNF-α expression in the present study suggests, however, that other stimuli than TNF-α are responsible for the described apoptotic cell death in patients with AF. Due to the observed cytokine expression pattern and in contrast to previous results in patients with lone AF, it seems unlikely that locally expressed inflammatory cytokines are responsible for specific alterations of atrial tissue during AF in patients with underlying cardiovascular diseases.

**Study limitations.** As with all studies using human atrial biopsies, there are some limitations that may have influenced the results of the present study. Right atrial appendages were analyzed only. Therefore, no comment can be made regarding the distribution of histological changes or possible interatrial differences in cytokine expression. Although pathological alterations might be more pronounced in left atria, there are no data supporting the principally different employment of molecular signaling cascades within left and right atria. All patients (AF and SR) had significant coronary artery or valve disease. Thus, in contrast to animal experiments, no healthy controls were used for comparison. Several calcium-dependent proteases exist in cardiac myocytes. Therefore, it cannot be fully excluded that, besides calpain I, other proteases have also contributed to some of the observed structural changes. Light microscopy was used to detect inflammatory infiltrates. Structural alterations (amount of fibrosis, etc.) observed on microscopy were qualitatively assessed only. However, previous studies have already shown quantitative differences in the amount of atrial fibrosis in patients with and without AF (10).

The present study demonstrates an increased atrial expression and activity of calpain I during AF. In-
creased activity of this calcium-dependent protease might contribute to structural remodeling and contractile dysfunction, whereas there is no evidence of activation of tissue cytokines.

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