British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.02117.x
www.brjpharmacol.org

RESEARCH PAPER

Regulation of apoptosis in HL-1 cardiomyocytes by phosphorylation of the receptor tyrosine kinase EphA2 and protection by lithocholic acid

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Keywords
apoptosis; atrial fibrillation; EphA2; cardiac myocyte; heart failure

Received
27 April 2012
Revised
20 July 2012
Accepted
20 July 2012

BACKGROUND AND PURPOSE

Heart failure and atrial fibrillation are associated with apoptosis of cardiomyocytes, suggesting common abnormalities in pro-apoptotic cardiac molecules. Activation of the receptor tyrosine kinase EphA2 causes apoptosis *in vitro*, and dysregulation of EphA2-dependent signalling is implicated in LEOPARD and Noonan syndromes associated with cardiomyopathy. Molecular pathways and regulation of EphA2 signalling in the heart are poorly understood. Here we elucidated the pathways of EphA2-dependent apoptosis and evaluated a therapeutic strategy to prevent EphA2 activation and cardiac cell death.

EXPERIMENTAL APPROACH

EphA2 signalling was studied in an established model of doxazosin-induced apoptosis in HL-1 cells. Apoptosis was measured with TUNEL assays and as cell viability using a formazan method. Western blotting and siRNA for EphA2 were also used.

KEY RESULTS

Apoptosis induced by doxazosin (EC_{50} = 17.3 μM) was associated with EphA2 activation through enhanced phosphorylation (2.2-fold). Activation of pro-apoptotic downstream factors, phospho-SHP-2 (3.9-fold), phospho-p38 MAPK (2.3-fold) and GADD153 (1.6-fold) resulted in cleavage of caspase 3. Furthermore, two anti-apoptotic enzymes were suppressed (focal adhesion kinase, by 41%; phospho-Akt, by 78%). Inactivation of EphA2 with appropriate siRNA mimicked pro-apoptotic effects of doxazosin. Finally, administration of lithocholic acid (LCA) protected against apoptosis by increasing EphA2 protein levels and decreasing EphA2 phosphorylation.

CONCLUSIONS AND IMPLICATIONS

EphA2 phosphorylation and activation of SHP-2 are critical steps in apoptosis. Reduction of EphA2 phosphorylation by LCA may represent a novel approach for future anti-apoptotic treatment of heart failure and atrial fibrillation.

Abbreviations

AF, atrial fibrillation; EphA2, erythropoietin-producing human hepatocellular carcinoma receptor tyrosine kinase A2; ER, endoplasmic reticulum; FAK, focal adhesion kinase; GADD153, growth arrest and DNA damage inducible gene 153; hERG, human ether-a-go-go-related gene; HF, heart failure; LCA, lithocholic acid; LS, LEOPARD syndrome; NS, Noonan syndrome; SHP-2, Src homology domain-containing protein tyrosine phosphatase 2; siRNA, small interfering RNA; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt

Introduction

Heart failure (HF) has become an epidemic cardiovascular disorder with high morbidity and mortality. Atrial fibrillation (AF) is the most common sustained arrhythmia and is in itself associated with serious deleterious effects such as reduced ventricular function and thromboembolic events. Despite their clinical and socioeconomic impact, effective treatment of HF and AF still remains an unmet medical need. At the molecular level, increased apoptosis, which is observed in both conditions, leads to cardiomyocyte loss, contractile dysfunction, electrical remodelling and disease progression (Aimé-Sempé *et al.*, 1999; Kang and Izumo, 2000; Cardin *et al.*, 2003; Shih *et al.*, 2011). Recently, attenuation of apoptosis has emerged as innovative approach for the treatment of HF and AF (Hilfiker-Kleiner *et al.*, 2006; Trappe *et al.*, 2012). However, a better understanding of underlying pathways is required to identify targets for specific therapeutic strategies. Novel anti-apoptotic approaches may be generated by transferring mechanistic information from tumour biology to cardiovascular research. In particular, pro-apoptotic pathways that contribute to cancer growth when suppressed are expected to promote HF and AF. The analysis of analogous mechanisms between heart and cancer biology could improve our understanding of myocardial cell death and indentify novel compounds targeting cardiac apoptosis.

The erythropoietin-producing human hepatocellular carcinoma receptor tyrosine kinase A2 (EphA2) and associated downstream signalling were reported to regulate cell death and differentiation in development and cancer (Wykosky and Debinski, 2008). Phosphorylation and down-regulation of EphA2 result in tumour cell death, whereas increased EphA2 expression and receptor dephosphorylation functionally contribute to the malignant phenotype of many invasive cancers (Kinch and Carles-Kinch, 2003; Wykosky and Debinski, 2008). Lithocholic acid (LCA), a secondary bile acid, prevents EphA2 phosphorylation and suppresses apoptosis in prostate and colon adenocarcinoma cells (Kozoni *et al.*, 2000; Giorgio *et al.*, 2011). The significance of EphA2 signalling in normal and diseased myocardial cells is less well understood, and cardiac EphA2 signal transduction has not yet been investigated in detail. EphA2 expression was only recently detected in murine cardiac myocytes and human cardiac stem cells, where it was implicated in the regulation of cell migration, regeneration and cardioprotection (Dries *et al.*, 2011; Goichberg *et al.*, 2011). EphA2 activates the Src homology domain-containing protein tyrosine phosphatase 2 (SHP-2) associated with autosomal-dominant LEOPARD syndrome (LS) and Noonan syndrome (NS) (Gelb and Tartaglia, 2006). Mutations in SHP-2 account for the majority of LS and NS cases, exhibiting pulmonary valve stenosis, atrial/ventricular septal defects and primary hypertrophic cardiomyopathy among their multisystemic features. At the cellular level, SHP-2 activation is associated with cardiomyocyte apoptosis (Rafiq *et al.*, 2006).

Based on these findings, we hypothesized that EphA2 and SHP-2 would be critical factors in cardiomyocyte apoptosis, and that LCA would prevent cardiac cell death. The first aim of this study was to elucidate EphA2-dependent pathways in cardiac myocytes. An LCA-based anti-apoptotic therapeutic approach was then evaluated *in vitro*. EphA2 signalling was assessed using a cellular model of drug-induced apoptosis in HL-1 cardiac myocytes (González-Juanatey *et al.*, 2003; Eiras *et al.*, 2006; Thomas *et al.*, 2008).

Methods

HL-1 cell culture

HL-1 cells, a cardiac cell line derived from the AT-1 mouse atrial myocyte tumour lineage, were kindly provided by Dr William Claycomb (New Orleans, LA) (Claycomb *et al.*, 1998). Cells were cultured and maintained as described previously (Claycomb *et al.*, 1998; Thomas *et al.*, 2008; Trappe *et al.*, 2012). Drug treatment was performed in norepinephrine-free Claycomb medium (Sigma-Aldrich, St. Louis, MO) when cells were 80% confluent, unless indicated otherwise.

TUNEL staining

Apoptosis was detected by TUNEL. Following exposure to doxazosin, cells grown in 24-well tissue culture dishes were fixed, and TUNEL reaction mixture (Roche Applied Science, Mannheim, Germany) was added to the sections according to the manufacturer's instructions, followed by incubation at 37°C for 60 min. After removal of the TUNEL reagent, slides were rinsed with PBS, and TUNEL-positive cells were evaluated using a fluorescence microscope (IX 50; Olympus, Hamburg, Germany).

2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) cell viability assay

Cell viability was assessed using an assay that utilizes the ability of live cells to reduce XTT to produce a colored formazan compound. Cells grown in 96-well tissue culture dishes were transferred into doxazosin-free media after 24 h incubation with the drug. XTT (AppliChem, Darmstadt, Germany) was then added, and cells were maintained at 37°C and 5% CO_2 for 2–4 h in accordance with the manufacturer's instructions. Optical density was determined at 450 nm using a spectrophotometer (PHOmo, Anthos Mikrosysteme, Krefeld, Germany) and was normalized to control measurements obtained from corresponding cells cultured in drug-free medium.

Small interfering RNA (siRNA) knockdown

Drug and molecular target nomenclature follows Alexander *et al.*, (2011). Anti-EphA2 siRNA (sc-35320; Santa Cruz Biotechnology, Heidelberg, Germany) and anti-human ether-a-go-go-related gene (hERG; $K_v11.1$) siRNA (sc-42498; Santa Cruz Biotechnology) were used to knock down protein expression. Scrambled siRNA (sc-37007; Santa Cruz Biotechnology) served as control. HL-1 cells were allowed to settle in six-well tissue culture dishes and antibiotic- and norepinephrine-free Claycomb medium for 24 h to achieve 50% confluence. siRNAs (10 μM stock solutions) were then transfected according to the manufacturer's recommendation. After 13 h, norepinephrine-free Claycomb medium supplemented with 20% FBS and 2% penicillin/streptomycin was

added, and cells were incubated for 24 h in the absence or presence of doxazosin, as indicated. Following siRNA transfection procedures, HL-1 cell viability was quantified.

Western blot analysis

Protein immunodetection was performed by SDS gel electrophoresis and Western blotting as reported (Bikou *et al.*, 2011; Soucek *et al.*, 2012; Trappe *et al.*, 2012). HL-1 cells were solubilized for 20 min at 4°C in RIPA lysis buffer containing SDS and sodium deoxycholate supplemented with 'Complete' protease inhibitors and 'PhosSTOP' phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany). Nitrocellulose membranes were developed by sequential exposure to blocking reagent (5% dry milk); primary antibodies directed against EphA2 (1:100; sc-924, Santa Cruz Biotechnology); phospho-EphA2/Tyr^{594} (1:1000; CB4368, Cell Applications, San Diego, CA); SHP-2 (1:1000; 3752, Cell Signaling Technology, Danvers, MA); phospho-SHP-2/Tyr^{542} (1:1000; 3751, Cell Signaling); focal adhesion kinase (FAK; 1:1000; ab40794, Abcam, Cambridge, MA); growth arrest and DNA damage inducible gene 153 (GADD153; 1:500; ab11419, Abcam); p-Akt/Ser^{473} (1:1000; 9271, Cell Signaling); phospho-p38 MAPK/Thr^{180}/Tyr^{182} (1:1000; 9211, Cell Signaling); caspase 3 (1:1000; 9665, Cell Signaling), cleaved caspase 3 (1:1000; 9661, Cell Signaling), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:40 000; G8140-11, US Biological, Swampscott, MA); and appropriate HRP-conjugated secondary antibodies (Abcam). Detailed antibody information is provided in Table S1. Signals were developed using the enhanced chemiluminescence assay (GE Healthcare, ECL Western Blotting Reagents, Buckinghamshire, UK) and quantified using ImageJ 1.41 Software (National Institute of Health, Bethesda, MD). Protein content was normalized to GAPDH for quantification of optical density.

Statistics

Data are presented as mean ± SEM. Statistical differences of continuous variables were determined with Origin 6 software (OriginLab, Northampton, MA) using unpaired Student's *t*-tests (two-sided tests). Multiple comparisons were performed using one-way ANOVA. If the hypothesis of equal means could be rejected at the 0.05-level, pair-wise comparisons of groups were made and the probability values were adjusted for multiple comparisons using the Bonferroni correction. Statistical analyses were carried out before normalization of data. $P < 0.05$ was considered statistically significant.

Materials

Doxazosin and LCA (Sigma-Aldrich) were prepared as 10 mM or 50 mM stock solutions in DMSO respectively. LCA and doxazosin were applied simultaneously when anti-apoptotic effects of LCA were studied. Murine ephrin A1/Fc chimera (Sigma-Aldrich) was dissolved in PBS to a 0.4 μg μL^{-1} stock solution. All chemicals were stored at –20°C.

Results

Cellular model: doxazosin induces apoptosis of HL-1 cells

Apoptosis was studied in an established model of mouse atrial cardiac tumour cells (HL-1 cells) exposed to doxazosin (González-Juanatey *et al.*, 2003; Eiras *et al.*, 2006). The antihypertensive α_1-adrenoceptor antagonist doxazosin is implicated in HF and triggers apoptosis in cardiomyocytes and in experimental cells, independent of its antagonism of adrenoceptors (ALLHAT Trial Investigators, 2000; González-Juanatey *et al.*, 2003; Thomas *et al.*, 2008; Kyprianou *et al.*, 2009; Staudacher *et al.*, 2011). Compared with baseline conditions (Figure 1A), increased apoptosis rates were detected by TUNEL following exposure to doxazosin (10–50 μM) for 24 h (Figure 1B–D). Quantification of HL-1 cell death using a XTT-based cell viability assay revealed a half-maximal effective doxazosin concentration of 17.3 ± 1.8 μM ($n = 3$ independent assays; Figure 1E). The incubation time required to achieve half-maximal pro-apoptotic effects of 30 μM doxazosin was 19.5 ± 3.6 h ($n = 5$; Figure 1F).

Apoptosis of HL-1 cells is associated with increased EphA2 phosphorylation

To evaluate the significance of EphA2 signalling in cardiac apoptosis, EphA2 expression was determined by Western blot analysis in apoptotic cells following doxazosin treatment for 24 h (Figure 2A, B, Figure S1). Figure 2A illustrates total EphA2 protein content under control conditions and after application of increasing concentrations of doxazosin. EphA2 protein levels were not significantly affected by doxazosin treatment ($n = 3$; $P = 0.99$) (Figure 2A). In contrast, EphA2 phosphorylation at the intracellular amino acid Tyr^{594} was enhanced in concentration-dependent manner (Figure 2B), consistent with previous reports on cell differentiation and motility in cancer research (Fang *et al.*, 2008). After exposure to 30 μM doxazosin, the phospho-EphA2 content was significantly increased, compared with untreated controls ($n = 4$; $P = 0.031$).

EphA2 phosphorylation triggers pro-apoptotic signalling in HL-1 cells

Signalling pathways associated with EphA2 hyperphosphorylation were elucidated in detail. The non-receptor tyrosine phosphatase SHP-2 is a well-recognized substrate of EphA2 phosphorylation at Tyr^{542}, resulting in SHP-2 activation. SHP-2 phosphorylation increased significantly in apoptotic cells following doxazosin treatment (30 μM) ($n = 3$; $P = 0.007$), whereas total SHP-2 protein was not affected ($n = 3$; $P = 0.64$) (Figure 2C, D, Figure S1). Pro-apoptotic signalling involved cleavage and non-significant reduction of FAK ($n = 3$; $P = 0.22$) after doxazosin application (30 μM) (Figure 2E, Figure S1). FAK cleavage marks a critical step in cardiomyocyte apoptosis that modulates established pro- and anti-apoptotic factors. HL-1 cell apoptosis was associated with increased phosphorylation (i.e. activation) of p38 MAPK ($n = 3$; $P = 0.007$), leading to increased protein expression of the pro-apoptotic nuclear transcription factor GADD153 ($n = 3$; $P = 0.35$) that did not reach statistical significance (Figure 2F, G, Figure S1). In contrast, expression of anti-apoptotic, phosphorylated protein kinase B (Akt) was clearly suppressed ($n = 4$; $P = 0.002$), relative to control cells (Figure 2H, Figure S1).

Finally, caspase 3 executes the EphA2-dependent apoptotic pathway in cardiac myocytes (Figure 2I, J, Figure S1). Increased GADD153 expression and reduced Akt phosphorylation were associated with activation of caspase 3 ($n = 3$;

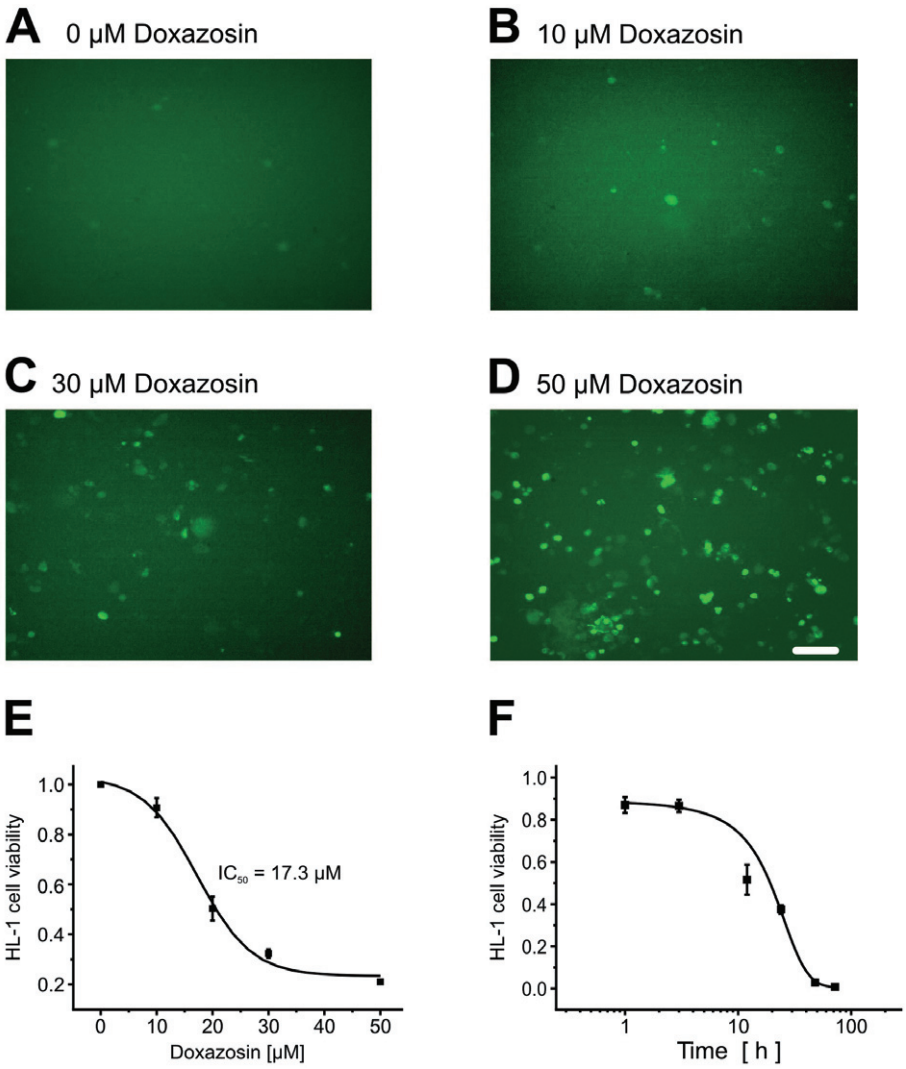

Figure 1

Doxazosin causes apoptosis of HL-1 cells, a cardiac cell line derived from a mouse atrial myocyte tumour lineage. (A–D) Fluorescence microphotographs corresponding to TUNEL assays. A total of 250 000 cells were employed per assay. Increased green nuclear fluorescence reflects endonucleolytic DNA degradation and apoptosis of cells treated with doxazosin. Scale bar, 100 µm. (E) Concentration–response relationship obtained from three independent XTT cell viability assays. (F) Time course of doxazosin-associated apoptosis. Half-maximal pro-apoptotic effects of 30 µM doxazosin were observed after 19.5 h (n = 5). Data are given as mean ± SEM.

P = 0.002) through cleavage of the respective inactive procaspase (Figure 2I, J). Caspase 3 serves as key downstream enzyme in the apoptotic process and directly cleaves apoptotic substrates. The corresponding reduction of inactive procaspases 3 (n = 3; P = 0.43) was not statistically significant (Figure 2I).

Ephrin A1 does not modulate apoptosis of HL-1 cardiac cells

The Eph receptor interacting protein, ephrin A1, is a physiological, membrane-bound ligand of EphA2 receptors (Wykosky and Debinski, 2008). Under experimental conditions, soluble ephrin A1 requires artificial clustering by fusion to the Fc portion of IgG immunoglobulins (ephrin A1/Fc). EphA2 stimulation by ephrin A1 may then induce receptor phosphorylation. Ephrin A1 has been shown to alter cell viability and to trigger apoptosis in malignant cells (Noblitt *et al.*, 2004; 2005; Tandon *et al.*, 2012). To assess the significance of ephrin A1 in HL-1 cells, ephrin A1/Fc was co-administered with 30 µM doxazosin, and apoptosis was quantified using the XTT assay. Cell viability was not affected by ephrin A1/Fc treatment in the absence of doxazosin, and ephrin A1/Fc did not modulate HL-1 cell apoptosis induced by doxazosin (data not shown). Similar results were obtained using different ephrin A1/Fc concentrations (10 ng mL^{-1}; 1 µg mL^{-1}; 5 µg mL^{-1}), incubation temperatures (4°C; 37°C) or drug administration periods (24 h; 48 h), respectively, indicating that ephrin A1 did not affect EphA2-dependent apoptosis in HL-1 cells.

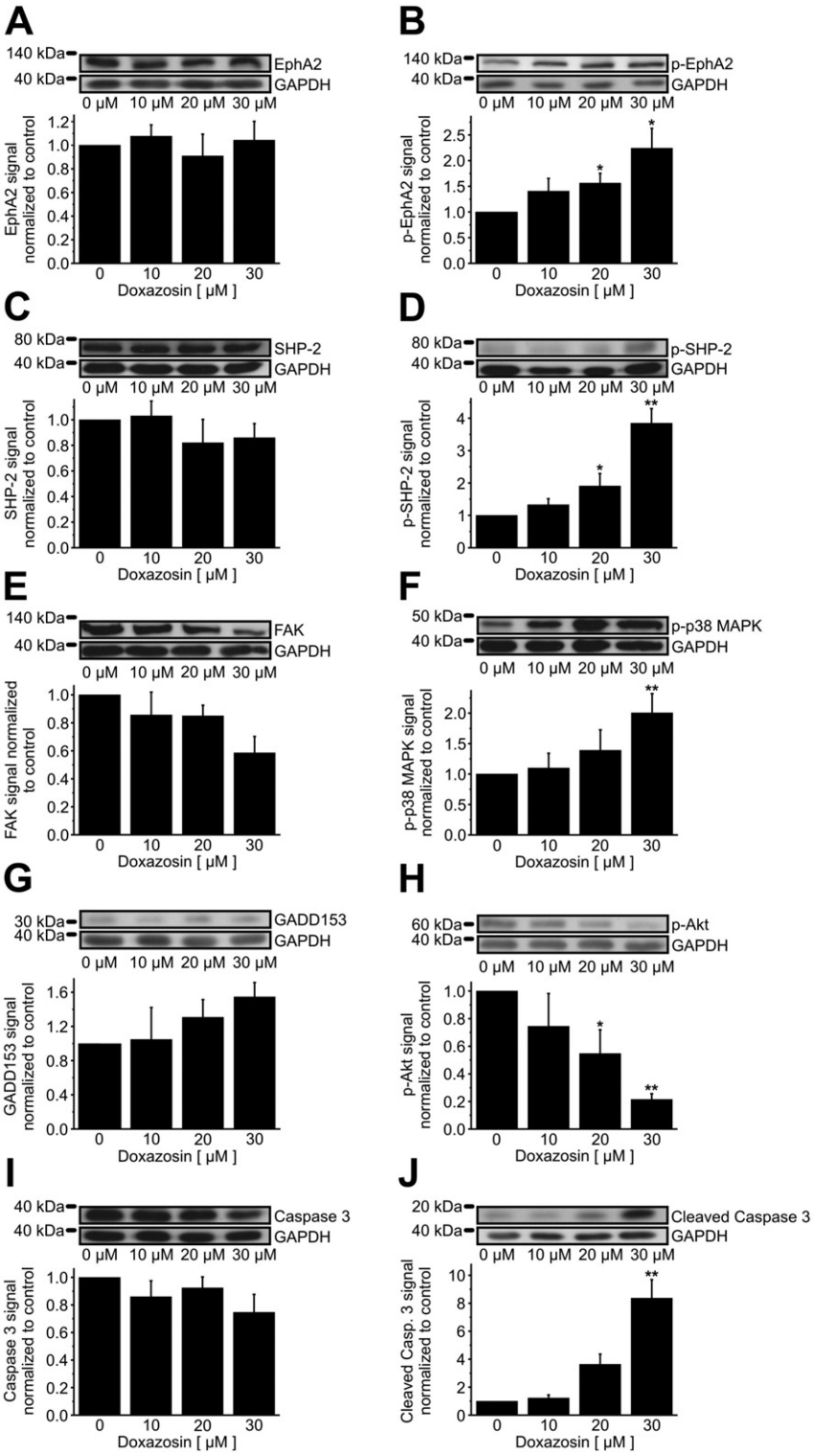

Figure 2

Analysis of proteins involved in pro-apoptotic signalling. (A, C) Total protein levels of EphA2 (A) and SHP-2 (C) were not significantly affected by doxazosin. In contrast, apoptosis was associated with increased phosphorylation (i.e. activation) of EphA2 (B), SHP-2 (D) and p38 MAPK (F). FAK exhibited reduced expression owing to cleavage (E), and growth arrest and DNA damage inducible gene 153 (GADD153) protein levels were elevated (G). (H) The anti-apoptotic protein kinase B (p-Akt) was suppressed. (I, J) Activation of caspase 3 *en route* to apoptosis. Doxazosin treatment induced cleavage of caspase 3 (I) associated with decreased expression of pre-processed caspase 3 levels (J) that did not reach statistical significance. Representative Western blots and mean (± SEM) optical densities normalized to doxazosin-free conditions are presented for HL-1 cells exposed to increasing concentrations of doxazosin (n = 3–5 independent assays; *$P < 0.05$; **$P < 0.01$). See text and Figure 5 for mechanistic details.

LCA protects from doxazosin-induced apoptosis

LCA suppresses apoptosis and prevents EphA2 phosphorylation in tumour-derived cells (Kozoni *et al.*, 2000; Giorgio *et al.*, 2011). To analyse anti-apoptotic effects of LCA, the compound was administered in the presence of 30 μM doxazosin, and cell viability was quantified after 24 h using the XTT method. Compared with control cells treated with the solvent, DMSO (Figure 3A, E), doxazosin reduced cell viability (n = 3; P = 0.0008) (Figure 3B, E). LCA concentration-dependently reduced (50 μM LCA; n = 3; P = 0.006) or prevented cardiomyocyte apoptosis (100 μM LCA; n = 3; P = 0.42), (Figure 3C–E). In this study, enhanced EphA2 phosphorylation was identified as a critical pro-apoptotic mechanism. Consistent with this key finding, LCA (50 and 100 μM) prevented hyperphosphorylation of EphA2 in the presence of the pro-apoptotic stimulus, doxazosin (Figure 3F). Of note, application of LCA in the absence of doxazosin did not significantly reduce baseline EphA2 phosphorylation levels compared with untreated control cells (Figure 3F), emphasizing that this effect of LCA required prior activation of pro-apoptotic signalling. In addition to reduced phosphorylation, expression of total EphA2 was increased at both concentrations of LCA (50 μM LCA; n = 4; P = 0.005: 100 μM LCA; n = 4; P = 0.003) in the presence of doxazosin (30 μM) (Figure 3G). This unexpected result was not obtained following treatment with either LCA or doxazosin alone (Figure 3G).

Reduction of cell viability through siRNA inactivation of EphA2

To further explore the significance of total EphA2 protein content in HL-1 cell apoptosis, EphA2 siRNA was applied to knock down EphA2 expression before assessing viability. Compared with control cells treated with scrambled siRNA (n = 6), EphA2 siRNA reduced cell viability (n = 6; P = 0.0008) (Figure 4A–C). Unfortunately, there was no combination of experimental siRNA conditions that allowed us to measure cell viability (requiring a significant amount of remaining cells despite apoptosis) and significant EphA2 protein reduction (requiring a relatively high siRNA concentration and/or long siRNA application/cell culture time that leads to extreme cell loss) at the same time. High siRNA concentrations and long application or cell culture periods resulted in rapid death and loss of virtually all cells, preventing reasonable Western blot analyses (data not shown). This limitation should be considered when interpreting siRNA-mediated effects shown in the present work.

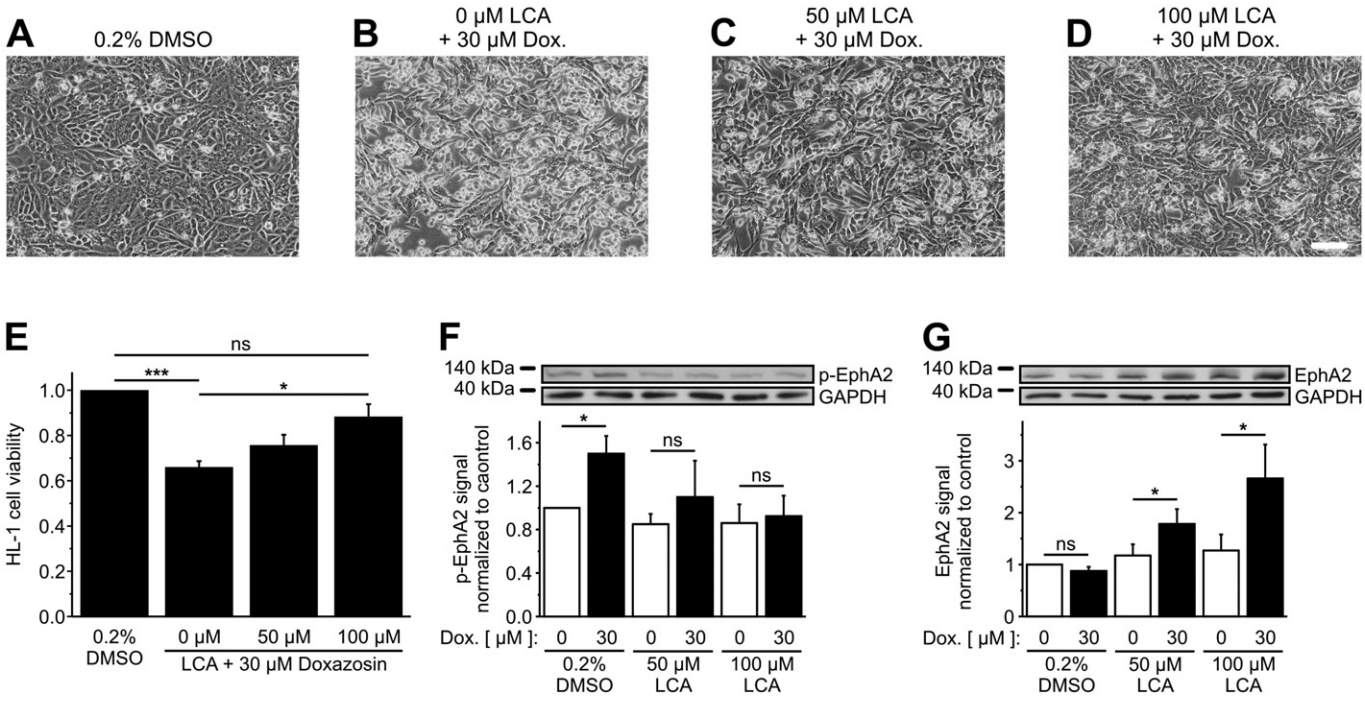

Figure 3

LCA prevents apoptosis. (A, B) Microscopic findings after treatment of HL-1 cells with vehicle (A) or 30 μM doxazosin (B) illustrate doxazosin-associated cell death. (C, D) LCA reduced (C) or prevented (D) apoptosis of cardiac myocytes (scale bar, 100 μm). (E) Quantification of mean cell survival normalized to controls using the XTT assay revealed dose-dependent apoptosis protection by LCA (n = 3 assays). (F, G) Activation of anti-apoptotic EphA2 signalling by LCA is illustrated by Western blot analyses of phosphorylated (F; n = 3–4 assays) and total EphA2 protein (G; n = 4 assays), respectively, revealing reduced phosphorylation and increased expression of EphA2 during doxazosin administration. LCA did not affect cells grown in the absence of the pro-apoptotic stimulus, doxazosin. Protein samples obtained from n = 4 assays was analysed per group. Data are provided as mean ± SEM [*P < 0.05; ***P < 0.001; ns, not significant vs. control medium (E) or doxazosin-free conditions (F, G) respectively].

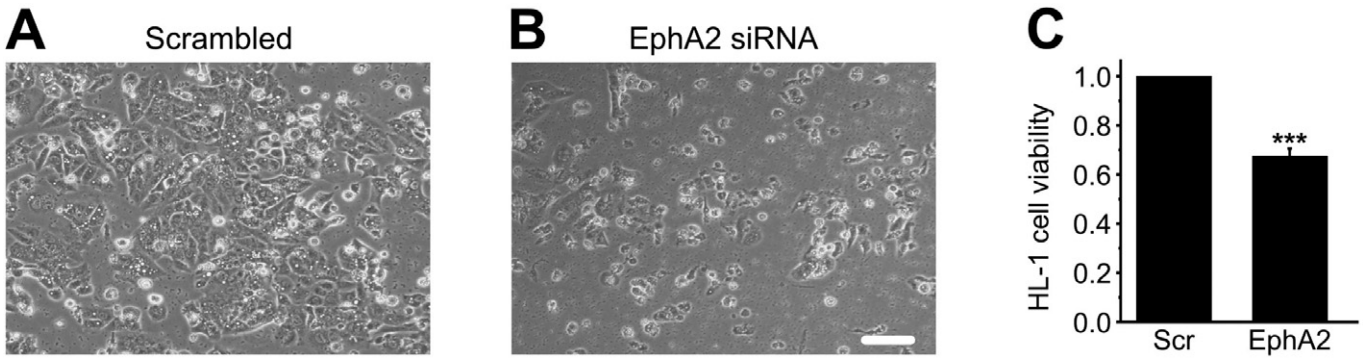

Figure 4

Knock-down of EphA2 impairs cell viability. (A, B) Representative microphotographs illustrate reduced viability of HL-1 cells treated with siRNA to suppress EphA2 expression compared with controls exposed to scrambled siRNA (scale bar, 100 μm). (C) Quantification of viable cells normalized to controls. Cell survival was determined using an XTT-based assay ($n = 6$ assays; $^{***}P < 0.001$). Data are given as mean ± SEM. Scr, srambled siRNA.

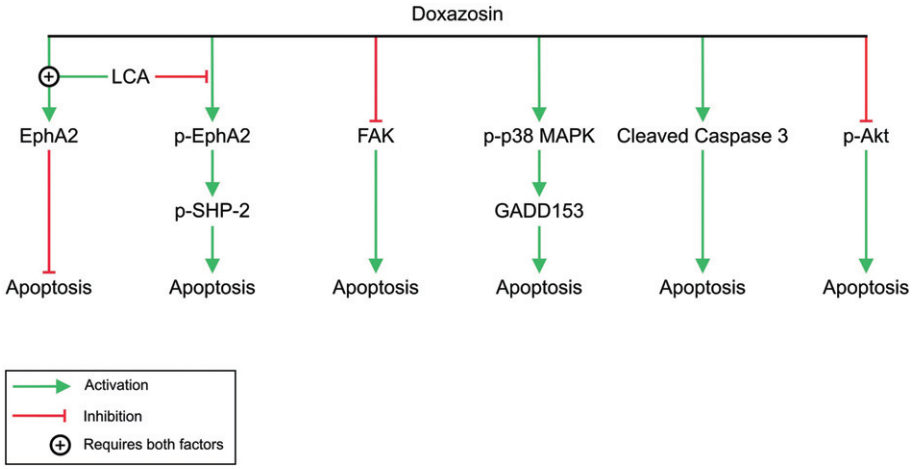

Figure 5

EphA2 signalling in cardiac myocytes. Doxazosin triggers EphA2 phosphorylation, resulting in SHP-2 phosphorylation. FAK was inactivated. In addition, dephosphorylation of protein kinase B (Akt), phosphorylation of p38 MAPK, stimulation of growth arrest and DNA damage inducible gene 153 (GADD153) and activation (i.e. cleavage) of caspase 3 occurred. LCA inhibits apoptosis in the presence of doxazosin through prevention of EphA2 phosphorylation and *via* increased EphA2 protein expression. Note that, for clarity, intermediate signalling steps are not shown.

Discussion

EphA2, a regulator of apoptosis

HF and AF account for significant cardiovascular morbidity and mortality. Standard treatment of HF and AF is based on medical therapy that is limited by reduced efficacy, side effects and safety issues in a significant number of patients. In search for novel therapeutic strategies and targets, suppression of cardiomyocyte apoptosis has come into focus (Hilfiker-Kleiner *et al.*, 2006; Trappe *et al.*, 2012). The present study reveals a previously unrecognized, pro-apoptotic molecular mechanism. Apoptosis of HL-1 cardiac cells was triggered by phosphorylation of the receptor tyrosine kinase, EphA2. Based on these data, a therapeutic strategy to prevent apoptosis was developed. LCA reduced EphA2 phosphorylation and protected cardiomyocytes against apoptosis.

EphA2-dependent pro-apoptotic signalling

Pro-apoptotic signalling was elucidated using a cellular model. Doxazosin time- and concentration-dependently triggered cardiomyocyte apoptosis (Figure 1). EphA2 receptor phosphorylation was identified as an integral event, initiating apoptosis independent of the physiological EphA2 receptor ligand, ephrin A1 (Figures 2, 5). EphA2-induced phosphorylation and activation of the tyrosine phosphatase SHP-2 were associated with cleavage of FAK (Figures 2, 5). In addition, doxazosin-induced apoptosis was linked to attenuated FAK activity. Furthermore we detected dephosphorylation of protein kinase B (Akt), representing the withdrawal of a survival signal. Stimulation of the endoplasmic reticulum (ER) stress-related apoptotic pathway (Eiras *et al.*, 2006) was indicated by increased phosphorylation of the pro-apoptotic factor p38 MAPK and enhanced protein expression of the nuclear transcription factor growth arrest and DNA damage

inducible gene 153 (GADD153) respectively. Finally, the effector caspase 3 was activated (by cleavage) in response to doxazosin treatment (Figures 2, 5).

SHP-2 activation and FAK cleavage have been observed earlier in apoptosis of neonatal rat cardiomyocytes induced by cathepsin G (Rafiq *et al.*, 2006). Furthermore, the EphA2-dependent pathway identified here resembles EphA2 regulation of integrin signalling detected in PC-3 prostate epithelial cells (Miao *et al.*, 2000), suggesting a broader role of EphA2/SHP-2 signalling in the heart and in extracardiac tissue. Previous work reported beneficial effects of EphA2 activation by its ligand, ephrin A1, in the heart (Dries *et al.*, 2011; Goichberg *et al.*, 2011). In a mouse model of myocardial infarction, Ephrin A1/Fc reduced infarct size and increased cell survival (Dries *et al.*, 2011). This effect was associated with increased gene expression of EphA2 and other EphA subtypes. The anti-apoptotic effect of increased EphA2 expression corresponds well to our observation that siRNA-mediated suppression of EphA2 resulted in reduced cell viability (Figure 4) and is in mechanistic accordance with previous work (Noblitt *et al.*, 2004; Tandon *et al.*, 2012). In hCSC, Ephrin A1 caused EphA2 internalization and enhanced cellular motility, resulting in improved cardiac repair (Goichberg *et al.*, 2011), whereas this protein reduced motility in pancreatic adenocarcinoma cells (Duxbury *et al.*, 2004a). Although different experimental procedures were followed in these studies, EphA2-mediated effects appeared to be cell-specific and should be interpreted within their respective cellular environment. Furthermore, the individual cellular EphA2 response depends on the mode of EphA2 activation (this study: doxazosin; Dries *et al.*, 2011; Goichberg *et al.*, 2011: ephrin A1). The latter conclusion is further supported by our finding in the present study that ephrin A1/Fc did not affect EphA2 signalling at baseline or after stimulation with doxazosin in HL-1 cells.

Different effects of EphA2 protein levels as distinct from EphA2 phosphorylation on apoptotic signalling highlight the complex role of EphA2 in apoptosis. Anti-apoptotic effects of enhanced EphA2 protein levels are well established in the heart and in tumour cells and cell lines (Carles-Kinch *et al.*, 2002; Duxbury *et al.*, 2004a,b; Noblitt *et al.*, 2004; Nasreen *et al.*, 2006; Brantley-Sieders *et al.*, 2008; Zhou *et al.*, 2008; Shahzad *et al.*, 2009; Dries *et al.*, 2011; Goichberg *et al.*, 2011; Mohammed *et al.*, 2011; Tandon *et al.*, 2012). Increased total EphA2 protein levels could result in a relative increase of phosphorylated EphA2, which may exert opposing effects on apoptosis. Unfortunately, the EphA2 phosphorylation status was not investigated in most studies conducted to date, preventing further evaluation of this potential mechanistic contradiction. In addition, the relative content of phosphorylated EphA2 compared with total EphA2 may be important in cells with regulation of both EphA2 protein and EphA2 phosphorylation. Furthermore, increased EphA2 phosphorylation and apoptosis were previously associated with either enhanced (Dohn *et al.*, 2001) or suppressed (Noblitt *et al.*, 2004; 2005; Tandon *et al.*, 2012) EphA2 protein content in cancer cell lines (Wykosky and Debinski, 2008; Tandon *et al.*, 2011). In these studies, EphA2 phosphorylation appeared to be the critical trigger of apoptosis, whereas EphA2 protein levels were not consistently associated with cell death. The present work advances this concept and provides the first direct evidence for identifying EphA2 phosphorylation as a critical pro-apoptotic event.

Two previous studies have reported doxazosin-induced apoptosis in human and neonatal rat cardiomyocytes and in HL-1 cells (González-Juanatey *et al.*, 2003; Eiras *et al.*, 2006). The α_1–adrenoceptor antagonist prazosin was pro-apoptotic as well, whereas terazosin and 5-methylurapidil did not induce apoptosis in HL-1 cells (González-Juanatey *et al.*, 2003). Furthermore, terazosin did not enhance atrial natriuretic peptide-induced apoptosis in neonatal rat ventricular myocytes (Wu *et al.*, 1997). The significance of the cellular context is highlighted by data obtained from prostate cells. In contrast to cardiac tissue, apoptosis was caused by *both* doxazosin and terazosin in prostate epithelium (Kyprianou, 2003). While pro-apoptotic signalling in cardiac myocytes has been explored in the present study and in an earlier report (Eiras *et al.*, 2006), the mechanism by which doxazosin triggers apoptosis in the prostate remains to be elucidated.

Recently, pharmacological inhibition of hERG K^+ channels has been implicated in apoptosis of tumour-derived cells (Thomas *et al.*, 2008; Jehle *et al.*, 2011; Staudacher *et al.*, 2011). However, siRNA-mediated knock-down of hERG did not affect baseline viability of HL-1 myocytes or apoptosis rates upon doxazosin exposure (30 μM; 24 h) compared with controls treated with scrambled siRNA (data not shown), arguing against a hERG-dependent mechanism in cardiac HL-1 cells.

Prevention of apoptosis by LCA

The secondary bile acid LCA prevented doxazosin-induced apoptosis in a dose-dependent manner (Figure 3). At the mechanistic level, LCA antagonized two pro-apoptotic signalling events, EphA2 hyperphosphorylation (Figure 2) and EphA2 down-regulation (Figure 4). Increased EphA2 protein expression and reduced EphA2 phosphorylation were detected following LCA treatment (Figures 3, 5). Of note, a statistically significant increase in EphA2 protein expression was observed when LCA and doxazosin were co-applied, whereas neither of these compounds increased EphA2 protein when applied alone. In an attempt to explain this phenomenon mechanistically, we hypothesize that EphA2 hyperphosphorylation in response to doxazosin represents a strong stimulus for EphA2 receptor internalization and compensatory up-regulation of EphA2 protein synthesis may occur. As a result, the suppression of doxazosin induced EphA2 phosphorylation by LCA treatment could prevent protein internalization, while EphA2 production remains activated and causes a net increase of EphA2 protein levels. In addition, LCA may directly affect protein kinases and/or phosphatases that regulate EphA2 receptor phosphorylation in the presence of a pro-apoptotic stimulus. Whether pro-apoptotic triggers in general could induce a similar molecular LCA response, or whether a direct or indirect drug-specific interaction between LCA and doxazosin is required remains to be investigated.

An LCA-based strategy to prevent apoptosis was previously evaluated in pre-cancerous colon epithelium. LCA reduced the apoptosis rate of colonic epithelial cells in the presence of the carcinogen 1,2-dimethylhydrazine and inhibited EphA2 phosphorylation in adenocarcinoma cell lines (Kozoni *et al.*, 2000; Giorgio *et al.*, 2011). Competitive and reversible inhibition of ephrin A1 binding to EphA2 has been proposed as a mecha-

nism of LCA action in tumour cells. In cardiac myocytes, ephrin A1 did not interfere with EphA2-dependent apoptosis, suggesting a cell-specific molecular interaction.

Clinical implications and potential limitations

The present study indicates that pharmacological intervention to suppress EphA2-dependent signalling reduces cardiac apoptosis. Specifically, inhibition of EphA2 phosphorylation constitutes a potential therapeutic strategy to treat and prevent heart failure. Furthermore, the mechanistic link between doxazosin, EphA2 signalling, and cardiomyocyte apoptosis provides a mechanistic explanation for increased incidence of HF observed with doxazosin in the Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial (ALLHAT; The ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group, 2000). In addition to HF, apoptosis is a critical factor in AF-associated structural remodelling, and reduction of atrial apoptosis through anti-caspase 3 gene therapy has proven effective in suppressing AF in a preclinical model (Aimé-Sempé *et al.*, 1999; Cardin *et al.*, 2003; Trappe *et al.*, 2012). We propose that inhibition of EphA2 may provide an advanced approach to apoptosis suppression in AF. While experimental data obtained from cellular or animal models may yield significant mechanistic data, any transfer to human pathology should be made with caution. In follow-up investigations, human cardiac stem cells or cardiomyocytes may provide a better approximation to human pathology (Goichberg *et al.*, 2011). Preclinical and clinical investigations in HF and AF are then required to determine the relative significance of EphA2 and SHP-2 and to evaluate the viability of EphA2-based medical therapy. Furthermore, the safety of anti-apoptotic treatment strategies needs to be established as neoplastic side effects pose potential limitations. The LCA-based therapeutic approach presented here antagonized the apoptotic potential of doxazosin in HL-1 cells and did not interfere with cell viability in the absence of a pro-apoptotic trigger. Thus, LCA is not expected to exhibit neoplastic properties.

In conclusion, EphA2 receptor tyrosine kinase phosphorylation and activation of the protein tyrosine phosphatase SHP-2 represent crucial steps in apoptosis. Suppression of EphA2 phosphorylation by LCA protected HL-1 cardiac myocytes from cell death. The link between the EphA2 pathway and cardiac apoptosis provides a novel target for the treatment of heart failure and atrial fibrillation.

Acknowledgements

We thank Jennifer Gütermann, Bianca Stadler and Kai Sona for excellent technical assistance; and we are grateful to Dr Eckhard Ficker for helpful discussions and suggestions. This work was supported in part by grants from the German Heart Foundation/German Foundation of Heart Research (Kaltenbach Scholarships to JJ and FW, project F/06/10 to DT) and from the Max-Planck-Society (TANDEM project to PAS).

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Original Western blots corresponding to protein analysis displayed in Figure 2. Apoptosis was associated with increased activation/phosphorylation of EphA2 (B), SHP-2 (D) and p38 MAPK (F); while total EphA2 (A) and SHP-2 (C) levels were not significantly affected. FAK exhibited reduced expression owing to cleavage (E), and growth arrest and DNA damage inducible gene 153 (GADD153) protein levels were elevated (G). The anti-apoptotic protein kinase B (p-Akt) was suppressed (H). (I, J) Doxazosin treatment induced cleavage of caspase 3 (I) associated with decreased expression of pre-processed caspase 3 levels (J).
Table S1 Overview: primary antibodies used in this study.