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to prevent Heart Attack Trial (ALLHAT)\textsuperscript{21} and a trend toward increased mortality in the Vasodilator Heart Failure Trial (V-HeFT).\textsuperscript{22} Nonselective activation of all α1 subtypes in vitro has a robust positive inotropic effect in failing human myocardium.\textsuperscript{23,24} Nonselective α1-activation in vitro also protects against ischemia.\textsuperscript{25–27} These beneficial effects of α1-AR activation suggest a novel interpretation of the harmful results of excessive norepinephrine reduction in clinical trials (Moxonidine Safety and Efficacy (MOXSE), Moxonidine Congestive Heart Failure (MOXCON), and Beta-Blocker Evaluation of Survival Trial (BEST)).\textsuperscript{28–30} Specifically suggesting that some degree of α1-activation is essential in HF. Further consistent with a beneficial or compensatory role for human myocardial α1-ARs, chronic therapy with the cardioprotective β-blocker carvediol potentiates α1-AR effects,\textsuperscript{31} and total α1-ARs are not downregulated in HF, in contrast with β-ARs that are downregulated.\textsuperscript{32–34}

The distinct and important roles of the α1A, α1B, and α1D in mouse heart and the contrasting harmful effects of nonselective α1-blockade and beneficial effects of nonselective α1-stimulation in human heart emphasize the need to define expression of the α1 subtypes in the human heart. Recently, we found that the α1D is the predominant subtype in human coronary arteries.\textsuperscript{35} However, little is known about the α1 subtypes in human myocardium. In fact, previous limited mRNA studies have concluded that the human heart expresses only the α1A\textsuperscript{36–39} and that mouse models of α1-AR biology might not be relevant to human heart disease.\textsuperscript{40,41} The α1-subtype proteins in human myocardium have never been measured.

Here, we test the hypothesis that the α1A and α1B subtypes are both present in human myocardium, similar to the mouse, and are not downregulated in HF. This is the first report of α1-AR subtype proteins in human heart and is the first characterization of α1-subtypes in nonfailing (NF) and failing heart.

Methods

Patients

With the approval of the University of California, San Francisco (UCSF) Committee for Human Research, we obtained tissue from hearts removed at the time of transplant at UCSF or from organ donors whose hearts were not transplanted for technical reasons. Full informed consent was obtained from all UCSF transplant recipients before surgery. The California Transplant Donor Network provided the unused donor hearts and obtained informed consent from the donor’s next of kin.

Tissue Collection

Cold cardioplegia was perfused antegrade before cardiectomy, and the explanted heart was placed immediately in ice-cold physiological solution. Full-thickness samples from multiple regions of the left ventricle (LV) and right ventricle (RV) were cleaned rapidly of all epicardial fat, flash frozen in liquid nitrogen, and stored at −80°C.

RNA Preparation

Tissue was homogenized in TRIzol reagent (Invitrogen, Gibco BRL), using a rotor-stator homogenizer (Polytron) at speed 7 of 10. RNA was extracted in chloroform and isopropyl alcohol, purified on QiaGen Mini-Prep columns, treated with DNase (Turbo DNasefree, Ambion), and quantified using spectrophotometry (BioRad SmartSpec 3000). Selected RNA samples were analyzed to confirm the absence of significant degradation (Agilent 2100 BioAnalyzer).

Quantitative Real-Time Reverse Transcription PCR

Primer3 (version 0.4.0) and BLAST were used to design multiple potential primer pairs for each target and reference gene. α1-AR subtype primers spanned the 25-kb intron at the end of the 6th transmembrane domain, and final primer pairs (supplemental Figure I) were chosen for comparable reaction efficiencies as measured by serial dilution. Specificity of amplification was confirmed by (1) sequencing; (2) polymerase chain reaction (PCR) with human α1-AR cDNAs; and (3) a dissociation step in all quantitative real-time reverse transcription PCR (qRT-PCR) reactions. Amplification of genomic DNA was excluded by (1) use of intron-spanning primers; (2) DNase treatment of RNA; and (3) PCR run on agarose gels using no-RT templates as negative controls.

For qRT-PCR, 1 µg of RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) with both random hexamers (Invitrogen) and oligo-dT (Roche). qRT-PCR reactions contained 5% of the cDNA product, primers at 1.25 nM per reaction, and SYBR Green Master (Roche) with ROX reference dye. All reactions were performed in triplicate in an ABI PRISM 7900HT Sequence Detection System. Data were analyzed with SDS software version 2.3 (Applied Biosystems).

Relative quantitation of PCR products used the ΔΔCT method.\textsuperscript{42} Values for each mRNA are arbitrary units (AU) relative to two reference genes, β-actin and TATA-binding protein, for improved accuracy,\textsuperscript{43} as AU = 2\textsuperscript{−ΔΔCT} × 1000, where ΔΔCT = [(mean target gene CT) − (mean reference genes CT)], and CT is cycles to threshold.

Radioligand Binding

Approximately 120 mg wet weight of tissue was homogenized (5 mM Tris-HCl, 5 mM EDTA, 250 mM sucrose pH 7.4 plus 0.1 mM phenylmethylsulfonyl fluoride) and centrifuged at 100 000g for 1 hour. The pellet was resuspended in homogenization buffer and centrifuged as before. The resulting final membrane pellet was resuspended in assay buffer (α1 binding: 50 mM Tris pH 7.4, 1 mM EDTA; β-AR binding: 154 mM NaCl, 5 mM MgCl\textsubscript{2}, 20 mM Tris pH 7.4) and used for saturation and competition radioligand binding.

α1-AR saturation binding was at 30°C for 60 minutes with 200 µg membrane protein per tube (~2.5 mg tissue), 6 concentrations (0.04 to 1.2 nM) to define nonspecific binding.\textsuperscript{44} β-AR binding was at 25°C for 90 minutes with 50 µg membrane protein per tube, 6 concentrations (0.04 to 1.0 nM) in triplicate of [\textsuperscript{125}I]-cyanopindolol (CYP), and L-propranolol (1 µM) to define nonspecific binding.

The subtype proteins were quantified by competition binding. For α1-ARs, [\textsuperscript{3}H]-prazosin binding (0.5 nM) was competed with 22 concentrations (0.05 nM to 500 µM) in duplicate of BMY-7378, an α1D-selective antagonist,\textsuperscript{8,45} or 5-methylurapidil, an α1A-selective antagonist.\textsuperscript{7} For β-ARs, [\textsuperscript{125}I]-CYP binding (50 pM) was competed with 22 concentrations (50 pM to 500 µM) in duplicate of the β1-selective antagonist, CGP 20712A, or the β2-selective antagonist, ICI-118,551. Binding data were analyzed using GraphPad Prism 4.0 (GraphPad Software Inc.). Subtype percent calculations were calculated from fitting competition curves, and subtype levels in femtomoles per milligram were calculated from total binding in saturation analysis multiplied by percent from competition in the same preparation.

Data Analysis

Results are presented as mean±SEM. Significant differences (P<0.05) were tested using 1-way ANOVA and Tukey’s multiple comparison for more than two groups or Student’s unpaired t test for two groups, and a normal distribution was assumed for all continuous variables. Linear regression tested for the association between mRNA abundance and clinical variables. The F test compared goodness-of-fit to 1- or 2-site models for competition-binding analyses (GraphPad Prism version 4.0).
Statement of Responsibility
The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Patients
We tested α1-AR subtype expression in human myocardium from 21 explanted hearts of 14 transplant recipients and 7 unused donors (Table 1). As shown in Table 1, there were 9 NF and 12 failing patients, one third of whom had ischemic cardiomyopathy. NF and failing were similar in age and sex, but the mean ejection fraction (EF) was 59±3% in the NF group and 24±2% in the failing group (P<0.0001).

Characterization of NF and Failing LV Myocardium
To characterize NF and failing LV myocardium, we assayed select myocyte and nonmyocyte mRNAs by qRT-PCR and correlated these with EF (Figure 1). As EF decreased, LV β-myosin heavy chain (MyHC) levels did not change (P=0.71), but α-MyHC decreased (P=0.01) and type I collagen increased (P=0.02; Figure 1A through 1C). α-MyHC mRNA in the NF group was 19% of total MyHC, but only 1% of total in the failing group. Importantly, the levels of the reference genes, α-actin and TATA-binding protein, were comparable in the NF and failing groups (Figure 1D).

Competition binding for α2-AR subtypes using ICI-118,551, a selective antagonist for the α2-subtype, showed that α1-subtype levels decreased markedly in failing, whereas...
the β2-subtype was not changed (Figure 1E). Competition binding with CGP 20712A, a β1-selective antagonist, confirmed these values (data not shown).

In summary, we found in failing LV the expected repression of β1-MyHC, induction of collagen indicative of fibrosis, and downregulation of the β1-AR subtype, showing a molecular phenotype similar to that reported in previous studies on human HF.\cite{33,46-48}

α1-AR Subtype mRNAs in the NF Myocardium

To begin to test if all 3 α1-subtypes were present in human myocardium, we did qRT-PCR with validated primers spanning the long intron in each α1-subtype gene. We made RNA from transmural samples of myocardium from 3 regions: LV free wall (LVFW), LV septum (LVS, the LV side of the interventricular septum), and RV free wall (RVFW), all taken at the level of the papillary muscles. Figure 2 (left) shows that the α1A was the predominant α1-subtype mRNA in all NF myocardial regions. The α1B and α1D were present at much lower levels (P<0.05) and did not differ among the regions (Figure 2). In NF myocardial LVFW and LVS, the α1A was 63% of total α1-AR mRNA, α1B was 22%, and α1D was 15% (Table 2).

α1-AR Subtype Proteins in the NF Myocardium

We quantified α1-AR subtype protein levels using radioligand binding. Recently, we found that commercial α1-AR antibodies are not specific for α1-ARs,\cite{49} and thus, we could not use immunoblot or immunohistochemistry to detect or quantify the α1-subtype proteins.

**Figure 1.** Characterization of NF and failing (F) LV myocardium. RNA from LV myocardium was used in qRT-PCR for β-MyHC (A), α-MyHC (B), and type I collagen (C), and mRNA levels were related to EF by linear regression. D, Levels of β-actin and TATA-binding protein, the reference mRNAs used for normalization, are shown as cycles to threshold in real-time PCR (P by t tests). E, The β1- and β2-subtype binding percents were measured by competition for 125I-CYP binding with ICI-118,551, a β2-selective antagonist, and the femtomole per milligram protein for each subtype was calculated as in Methods section (P by t test).

**Figure 2.** α1-AR subtype mRNA Levels in NF and failing myocardium. Levels of α1-subtype mRNAs by qRT-PCR in 3 regions of NF and failing myocardium. Values are mean±SE AU normalized to β-actin and TATA-binding protein, n=8 NF, 10 failing (P by ANOVA and Tukey multiple comparison). LVFW indicates left ventricle free wall; LVS, LV septum; RVFW, right ventricle free wall.
Saturation radioligand binding in myocardial membranes with $^3$H-prazosin, a nonselective $\alpha_1$-AR antagonist, produced typical curves (Figure 3A), with $B_{\text{max}} \approx 4$ fmol/mg protein. Specific binding at the $^3$H-prazosin Kd (0.2 nM) averaged 40% of total $^3$H-prazosin bound. Specific total $\alpha_1$-binding reflecting all $\alpha_1$-subtypes in NF myocardium was about 10% of $\beta$-AR binding (Table 2), similar to that reported in previous studies. Total $\alpha_1$-binding was similar in NF LVFW, LVS, and RVFW (Figure 3B, left).

To detect and quantify the $\alpha_1$-subtype proteins, we used competition for $^3$H-prazosin binding with subtype-selective antagonists. Competition with 5-methylurapidil, an $\alpha_1$A-selective antagonist, produced classic 2-site curves (Figure 4A, left). In NF LVFW myocardium, there was a high-affinity component (Ki 11 ± 4 nM, n = 6), representing the $\alpha_1$A subtype, and a low affinity component (Ki 99 ± 53 μmol/L, n = 6), which could have been the $\alpha_1$B and/or $\alpha_1$D. To distinguish these, we did competition binding in the same preparations with BMY-7378, an $\alpha_1$D-selective antagonist. Competition binding with BMY-7378 yielded 1-site binding curves (Figure 4A, right), with only a low-affinity component (Ki 11 ± 6 μmol/L, n = 6), indicating no detectable $\alpha_1$D binding. Taken together, these results together indicate that $\alpha_1$A and $\alpha_1$B binding are present, but $\alpha_1$D is not. B, $\alpha_1$-subtype proteins as receptor fmol/mg membrane protein, calculated from levels determined by saturation binding and percents measured from competition binding, n = 6 LV, n = 4 RV (P = NS by ANOVA). Failing RV has a significant increase in relative $\alpha_1$A binding (failing 44% versus NF 27%, P = 0.04 by t test).
results suggested that the α1A and α1B subtype proteins were both present in human NF LV myocardium but that the α1D was not present. NF LV had 40±2% α1A and 60±2% α1B (P<0.001, n=6; Figure 4B, left; Table 2). Total α1-binding in NF RV myocardium was similar to LV (Figure 3B), but the NF RV contained relatively less α1A (25±5%, n=3, P=0.02; Figure 4B, left).

In summary, human NF myocardium expressed all 3 α1 subtype mRNAs, with a striking predominance of the α1A. However, binding assays detected only the α1A and the α1B, and the α1B was predominant.

### α1-AR Subtype mRNAs and Binding in the Failing Myocardium

The relative pattern of α1-subtype mRNA expression within the failing myocardium was identical to NF, with a predominance of α1A mRNA and no differences between α1B and α1D in any region (Figure 2, right). Total α1-subtype mRNA levels were also similar in failing and NF myocardium, although α1A mRNA was increased in failing LV (P<0.05; Table 2) and tended to increase in failing RV (Figure 2).

Total α1-binding was not reduced in any region of failing myocardium (Figure 3B, right). Similarly, binding levels of the α1A and α1B subtypes were not reduced in failing myocardium (Figure 4B), and the α1D remained undetectable (data not shown). The relative levels of α1A and α1B binding also were unchanged in failing LV (Table 2), but in RV, there was a relative increase in α1A binding in failing versus NF (44% versus 27%, P=0.04; Figure 4B).

In summary, the α1A and α1B were not repressed or downregulated in the failing myocardium. The α1A tended to increase.

### Comparison of β-AR Subtypes in Failing Myocardium

We measured β-AR subtype mRNAs and binding to test if the 2 AR families had distinct regulation in HF. Myocardium from failing LV had a significant decrease versus NF in β2-subtype mRNA and β1-subtype binding (Table 2; Figure 1E). Stable binding levels in HF of the α1A- and α1B-subtypes and downregulation of the β1-subtype caused a marked increase in the ratio of binding of α1-ARs to β-ARs, from about 10% in NF LV to 20% to 40% in failing LV (Table 2; the 41% α1/β ratio in the failing LV in Table 2 is from 4 samples with α1- and β-binding on the same membranes).

### Demographic and Clinical Variables and Myocardial α1-AR mRNA Levels

The qRT-PCR results were analyzed to determine whether demographic or clinical factors affected the expression of α1-subtype mRNAs. We found that age, EF, sex, β-blocker use, and coronary artery disease (CAD) had no effect on myocardial total or α1-subtype mRNAs (Figure 5A through 5D; data not shown). On the other hand, β-agonist use was
-associated with a decrease in both α1B (P=0.04) and α1D (P<0.01), but did not affect α1A levels (Figure 5E). We compared α1-AR mRNAs in a similar analysis and found that α1- and α2-subtype mRNA levels in myocardium did not change with age, EF, sex, β-blocker use, β-agonist use, or CAD (supplemental Figure II and data not shown).

Discussion

Here, we present the first data on α1-AR subtype proteins in human heart and the first comparison of α1-AR subtypes in NF and failing human hearts. The main findings are that the α1A is the predominant α1-subtype mRNA, the α1A and α1B are both present by binding, with the α1B being predominant, and the α1D is undetectable by binding. In HF, α1-subtype mRNAs are not repressed, and α1-binding is not downregulated, in contrast with β-AR subtypes.

Previous studies of human myocardial α1-AR subtypes used semiquantitative mRNA assays with a very limited number of undefined patients and never measured α1-subtype proteins.36–39 These studies identified the α1A as the most abundant or only α1-subtype mRNA in myocardium, and a separate study concluded that the α1A was the only α1-subtype in human coronary arteries.40 These results prompted the conclusion that the human heart is exclusively α1A and, thus, that mouse models are irrelevant to human cardiac α1-AR biology.40,41 On the contrary, the current and our recent studies show that α1-subtype expression is the same in the human and mouse heart, with the α1A and α1B subtypes in myocardium and the α1D in coronary arteries. This is important because it implies that findings in mouse genetic models, where α1 subtype functions can be studied with precision, are relevant to human cardiac α1-AR biology, in particular the adaptive and protective effects of the α1A- and α1B subtypes.

Technical aspects of this study warrant emphasis. Although our patient population was modest in size, it was significantly larger and more thoroughly characterized than that in previous studies. We quantified α1-subtype mRNAs in DNase-treated RNA, using qRT-PCR with carefully evaluated primer pairs that cross the large intron in all α1-AR genes to eliminate contamination from genomic DNA. We quantified α1-subtype proteins by radioligand binding. Previous studies have used commercial antibodies to measure α1 subtypes in
human noncardiac tissues, but we found that ten different antibodies are not specific for α1-ARs, indicating that binding is currently the only valid method for detecting and quantifying α1-AR proteins. Our membrane preparation for binding was not “purified,” ie, we did not discard any low-speed pellets. This meant that we did not discard the large number of receptors that are found in low-speed pellets and that our denominator of milligram protein was higher than in purified membranes.

A potential technical concern was the discordance between α1-subtype mRNA levels and binding levels. The α1A mRNA in human myocardium was by far most abundant, but α1A binding was less than α1B. The myocardial α1D mRNA was as abundant as α1B, but α1D binding was undetectable. Importantly, the same discordance between the levels of α1A and α1B mRNAs and binding is seen also in the mouse and rat heart, and mouse myocardium has α1D mRNA without α1D binding. Furthermore, we were able to detect α1D binding in human coronary arteries in which the α1D is 75% of total binding, ruling out a technical problem. In summary, these results together indicate that the α1-AR subtypes in myocardium have substantial post-transcriptional regulation in man and rodent.

A major finding was that α1-subtype mRNAs and α1-subtype binding were unchanged or increased in HF, in contrast with β-AR subtypes, where the β2 mRNA was repressed, and β1-binding, as shown before, was downregulated. To simplify characterization of the AR phenotype in failing versus NF myocardium, we normalized all subtype binding, given in Table 2, to binding of the α1A in NF LV. Normalization provides the following binding ratios in human LV myocardium of α1A:α1B:β1:β2: in NF LV, 1:2:19:7, and in failing LV, 1:2:5:6. Thus, the failing human myocardium has potentially much greater relative signaling through the α1A, α1B, and β2 subtypes. The failing RV was especially notable, with a significant increase in relative α1A binding (Figure 4).

Clinical Relevance
Loss and gain-of-function studies in the mouse heart show adaptive and protective effects of stimulation of the α1A and/or α1B subtypes (see Introduction section), raising the possibility of using α1-subtype-selective agonists as therapy for HF. Here, we demonstrate that the α1A and α1B are both present in human myocardium and are not downregulated in HF, indicating that this idea might be feasible. Furthermore, the fact that the α1 subtype in human coronary arteries is the α1D suggests that an agonist for the α1A or α1B would not cause coronary constriction. Without the potential harmful side effects of nonselective blockade of myocardial α1A and α1B subtypes, α1B are not downregulated in HF. Thus, α1 subtypes in the human heart seem to be similar to mouse heart, where adaptive and protective effects of α1 subtypes are most convincing. Further studies will help determine whether it might be feasible to treat HF with a drug targeting the α1A and/or α1B.

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Disclosures
None.

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12. Akhter SA, Milano CA, Shotwell BF, Cho MC, Rockman HA, Lefkowitz RJ, Koch WJ. Transgenic mice with cardiac overexpression of alpha1B-adrenergic receptors. In vivo alpha1B-adrenergic receptor-


**CLINICAL PERSPECTIVE**

Increased levels of catecholamines bind two classes of adrenergic receptors (ARs) in the failing heart: α1-ARs and β-ARs. The toxic effect of excessive β-AR stimulation is well studied. Less is known about α1-ARs in the human heart, although evidence ranging from genetically altered mice to large clinical trials suggests that they play adaptive and protective roles. α1-ARs exist as 3 distinct molecular subtypes: A, B, and D. In the rodent heart, the A and B subtypes are on cardiomyocytes, where they mediate beneficial processes, including positive inotropy, physiological hypertrophy, and protection from cell death. The α1D protein is absent in rodent cardiomyocytes but regulates coronary vasoconstriction. In a separate study, we found that the α1D is also the most abundant subtype in human coronary arteries. However, very little is known about the α1-AR subtypes in human myocardium. We used tissue from failing and nonfailing hearts to show that the α1A and α1B are the predominant α1 subtypes in human myocardium and that their abundance is maintained in heart failure. Our findings demonstrate that the distribution of α1-AR subtypes in the human heart is similar to that in the mouse heart, indicating that findings from mouse models can be applicable to human α1-biology. Our findings also suggest the possibility of therapeutically activating a beneficial α1 subtype in the myocardium without causing α1D-mediated coronary vasoconstriction. Similarly, it is possible that the benefits of α1-blockade in the treatment of prostate disease might be best achieved by selective antagonism of the α1D.
α1-Adrenergic Receptor Subtypes in Nonfailing and Failing Human Myocardium
Brian C. Jensen, Philip M. Swigart, Teresa De Marco, Charles Hoopes and Paul C. Simpson

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Online Figure 1. Human qRT-PCR Primers & Validation

A. Primer sequences & efficiencies.

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</table>

B. α1-AR primers flank the single long intron.

C. Any contaminating genomic DNA is not amplified.
Online Figure 2. β-Subtype mRNAs by Clinical and Demographic Variables

A. Age

B. Ejection Fraction

C. Sex

D. β-blocker Use

E. β-agonist Use

p = NS

M. Sex

Male

Female

No β-blocker

β-blocker

p = NS

No β-agonist

β-agonist

p = NS

β1

β2

All β-ARs

β1

β2

All β-ARs

Lin Reg of β-ARs: Line