Increased Protein Kinase C Activity and Expression of Ca\textsuperscript{2+}-Sensitive Isoforms in the Failing Human Heart

Nancy Bowling, BA; Richard A. Walsh, MD; Guojie Song, MD, PhD; Thomas Estridge, MS; George E. Sandusky, PhD, DVM; Rebecca L. Fouts, BA; Karen Mintze; Todd Pickard, BS; Robert Roden, MS; Michael R. Bristow, MD, PhD; Hani N. Sabbah, PhD; Jacques L. Mizrahi, PhD; Gianni Gromo, MD; George L. King, MD; Chris J. Vlahos, PhD

Background—Increased expression of Ca\textsuperscript{2+}-sensitive protein kinase C (PKC) isoforms may be important markers of heart failure. Our aim was to determine the relative expression of PKC-\(\beta_1\), -\(\beta_2\), and -\(\alpha\) in failed and nonfailed myocardium.

Methods and Results—Explanted hearts of patients in whom dilated cardiomyopathy or ischemic cardiomyopathy was diagnosed were examined for PKC isoform content by Western blot, immunohistochemistry, enzymatic activity, and in situ hybridization and compared with nonfailed left ventricle. Quantitative immunoblotting revealed significant increases of >40% in PKC-\(\beta_1\) (\(P<0.05\)) and -\(\beta_2\) (\(P<0.04\)) membrane expression in failed hearts compared with nonfailed; PKC-\(\alpha\) expression was significantly elevated by 70% in membrane fractions (\(P<0.03\)). PKC-\(\epsilon\) expression was not significantly changed. In failed left ventricle, PKC-\(\beta_1\) and -\(\beta_2\) immunostaining was intense throughout myocytes, compared with slight, scattered staining in nonfailed myocytes. PKC-\(\alpha\) immunostaining was also more evident in cardiomyocytes from failed hearts with staining primarily localized to intercalated disks. In situ hybridization revealed increased PKC-\(\beta_1\) and -\(\beta_2\) mRNA expression in cardiomyocytes of failed heart tissue. PKC activity was significantly increased in membrane fractions from failed hearts compared with nonfailed (1021 ± 189 versus 261 ± 89 pmol \(\cdot\) min\textsuperscript{-1} \(\cdot\) mg\textsuperscript{-1}, \(P<0.01\)). LY333531, a selective PKC-\(\beta\) inhibitor, significantly decreased PKC activity in membrane fractions from failed hearts by 209 pmol \(\cdot\) min\textsuperscript{-1} \(\cdot\) mg\textsuperscript{-1} (versus 42.5 pmol \(\cdot\) min\textsuperscript{-1} \(\cdot\) mg\textsuperscript{-1} in nonfailed, \(P<0.04\)), indicating a greater contribution of PKC-\(\beta\) to total PKC activity in failed hearts.

Conclusions—In failed human heart, PKC-\(\beta_1\) and -\(\beta_2\) expression and contribution to total PKC activity are significantly increased. This may signal a role for Ca\textsuperscript{2+}-sensitive PKC isoforms in cardiac mechanisms involved in heart failure. (Circulation. 1999;99:384-391.)

Key Words: cardiomyopathy ■ heart failure ■ hypertrophy ■ myocytes ■ signal transduction

Heart failure is a disease of multiple etiologies that lead to progressive cardiac dysfunction. Whereas initial cardiac and vascular adaptations preserve cardiac output and peripheral tissue perfusion, these changes ultimately lead to increased vascular resistance, with elevations in both preload and afterload adversely affecting the heart. On a cellular level, adaptive responses involve cardiac hypertrophy, contraction, ion transport, gene expression, endothelial function, and vasoconstriction.\(^1\) Protein kinase C (PKC) has been identified with these responses through multiple signal transduction pathways.\(^2\textsuperscript{-}4\) PKC is composed of a family of serine/threonine kinases. Classical PKCs (isoforms \(\alpha\), \(\beta_1\), \(\beta_2\), and \(\gamma\)) are activated by phosophatidylyserine, Ca\textsuperscript{2+}, and diacylglycerol (or PMA). Novel PKCs (\(\delta\), \(\epsilon\), \(\eta\), \(\theta\), and \(\mu\)) are not activated by Ca\textsuperscript{2+} but are activated by PMA and diacylglycerol. The atypical PKCs (\(\zeta\), \(\iota\), and \(\lambda\)) are not activated by Ca\textsuperscript{2+}, PMA, or diacylglycerol.\(^5\)

See p 334

Studies using rat ventricular cardiomyocyte preparations have shown that Ca\textsuperscript{2+}-dependent PKC isoforms \(\alpha\), and possibly \(\beta\), are expressed in fetal and neonatal heart,\(^5\textsuperscript{-}7\) but they are not expressed or are only sparsely detected in adult cardiac tissue.\(^8\) Increased expression of these PKC isoforms in adult heart is associated with conditions such as diabetes and heart failure.\(^9\textsuperscript{-}10\) Transgenic mice with targeted cardiac overexpression of PKC-\(\beta_2\) exhibit gross hypertrophy and diminished ventricular function.\(^10\textsuperscript{-}11\) Preliminary data\(^12\) suggest that PKC-\(\beta\) isoforms are detectable in cardiomyopathic human heart tissue, but the cellular
origin of the kinases was not reported. Erdbrugger did not detect PKC- in nonfailed human heart. To define the role of Ca\textsuperscript{2+}-sensitive PKC isoforms in human heart failure, we began a systematic investigation of PKC- \( a \), -b1, and -b2 in human left ventricle tissue by means of Western blot analysis and immunohistochemistry. PKC- was further characterized by in situ mRNA expression and isoform specific PKC activity, using the PKC- selective inhibitor LY333531. Results confirmed that PKC- and -b2 were expressed in human cardiomyocytes, and both protein and mRNA expression were greatly increased in hearts from end stage heart failure patients. PKC- immunostaining, while negligible in nonfailed cardiomyocytes, was distinctly evident in intercalated disks from failed hearts. Also, total PKC activity was significantly increased in membrane fractions from failed hearts. The extent of PKC activity inhibited by LY333531 demonstrated that PKC- was responsible for approximately 21\% of total enzyme activity.

### Methods

#### Subjects

Failing hearts were obtained from patients undergoing cardiac transplant \((n=7)\) or in whom NYHA Class IV heart failure was diagnosed at death \((n=5)\). The group consisted of 11 men and 1 woman with a mean age of 52.0 years and a mean ejection fraction of 15.8. When patients with dilated cardiomyopathy were compared with patients with ischemic cardiomyopathy, no significant differences were found in age, ejection fraction, pulmonary wedge pressure, or cardiac index. Nonfailing hearts rejected for transplant were obtained from a group consisting of 6 men and 5 women with a mean age of 37.2 years (Table 1). Whereas difference in mean age between failed and nonfailed patients was significant \((P<0.05)\), there was no significant correlation between age and expression of any PKC isoform.

#### Tissue Source and Sample Preparation

Transmural sections of left ventricular free wall were obtained from failed hearts excised at death or time of transplant or from nonfailed donor hearts unmatched for transplant. Sections were frozen in liquid...
N2 and stored at −70°C until used. Sample preparation was done at 4°C. Tissue samples (>0.2 g) were homogenized using the procedure of Paul et al.15 Samples ≤0.2 g were prepared as described by Pucaet et al.16 All protein determinations used the method of Bradford.17

**Western Blot Analysis**

Samples were separated by SDS-PAGE using 10% or 4% to 20% Tris-glycine gels. Samples were normalized to equal protein concentrations; equal amounts of cytosol and membrane protein were used in all experiments. Experiments to assure antibody specificity were performed using control peptides for each isoform. Recombinant PKC-β1, -β2, -α, or -ε (CalBiochem), or rat brain extract in some experiments, were included as standards. Antibody cross-reactivity between PKC isoforms was assessed using recombinant PKC enzymes. No cross-reactivity was observed at amounts at which samples were loaded onto the gel (5 ng), although slight cross-reactivity between antiPKC-β and recombinant PKC-α was observed at amounts ≥20 ng. Proteins were transferred onto Immobilon-P membranes (Millipore). Membranes were blocked using 5% dry milk. Immunoblots using polyclonal rabbit antibodies to PKC-β1, -β2, -α, or -ε (1:1000 dilution), were incubated for 1 hour at room temperature or overnight at 4°C. Goat anti-rabbit IgG horseradish peroxidase conjugate (1:3000 dilution) was used as secondary antibody. Chemiluminescent detection (Amersham) and autoradiography were used to identify bands comigrating with the PKC standards. Control experiments without primary antibody or in the presence of appropriate blocking peptide were negative. The relative abundance of individual proteins identified was quantified using a Coomassie blue-stained gel coupled to an image capture board and NIH 1.61 image analyzer software. Scanning units were normalized to recombinant standard in individual autoradiographs to correct for experiment-to-experiment variations. Densitometry scanning of Ponceau-stained membranes was used to correct for differences in sample loading and protein transfer as described by Ping et al.18

**Immunohistochemistry**

Zn-formalin–fixed, paraffin-processed tissues were sectioned at 5 μm and baked overnight at 60°C. Antigen retrieval was performed in Accutaf tissue unmasking solution (Accurate Scientific) for 10 minutes at 90°C. After cooling, all subsequent steps were performed at room temperature on the Dako Immunostainer. Rabbit anti-PKC antibodies specific to PKC-α, -β1, or -β2 (Santa Cruz Biotechnology) were used as primary antibodies and incubated for 30 minutes prior to detection using the LSAB2 (Dako) peroxidase staining kit with diaminobenzidine/peroxidase substrate to produce a brown color. After a brief counterstain with hematoxylin, slides were coverslipped and reviewed histologically. As controls, coincubation of 10 μg/mL of the appropriate PKC-α, -β1, or -β2 peptide (Santa Cruz Biotechnology) with 5 μg/mL of anti-PKC-α, -β1 or -β2 (Santa Cruz Biotechnology) was performed prior to tissue treatment.

**In Situ Hybridization Protocol**

Five-micrometer tissue sections were prepared as described above. Pretreatment and hybridization were performed as described in the Biozol RNA Super Sensitive mRNA Detection kit. PKC-β1 and -β2 differ only in a 50- to 52- amino acid sequence at their respective C-termini, selective polymerase chain reaction (PCR) primers were designed to amplify 300-bp fragments containing C-terminal regions of PKC-β1 and -β2, as well as some of the 3′-untranslated regions from human spleen cDNA. PCR fragments were directionally cloned into pBlueScript II SK+ (Stratagene) which served as template for T3 and T7 RNA polymerase to generate fluorescein-labeled sense and antisense probes (Boehringer Mannheim). Fluorescein labeled probes were hybridized to the tissue in a Hybaid Omnislide thermal cycler for 10 minutes at 95°C and 2 hours at 37°C. Hybridized probes were detected using an anti-fluorescein primary antibody (Biogenex) and the LSAB2 detection system with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate substrate and nuclear red counterstain.

**PKC Activity**

Total PKC activity was measured in cytosol, and membrane fractions were quantified by radioenzymatic assay involving PKC-catalyzed transfer of 32P from [γ-32P] ATP (DuPont-New England Nuclear) into the PKC-specific substrate peptide RKRTLRRL using a PKC enzyme assay system (Amersham) optimized for Ca2+-dependent PKC isoforms.19 Duplicate protein samples (1 μg) from cytosol or membrane fractions were assayed in the presence of 1.4 mmol/L calcium acetate, 34 μg/mL phosphatidylycerine, and 2.3 μg/mL PMA. Enzymatic activity of human recombinant PKC-β2 (Calbiochem, 0.22 to 56 ng/sample) was used as standard curve. Rat brain extract (2 μg) was assayed as positive control. Nonspecific PKC activity was assayed by replacing the buffer-containing substrate with 50 mmol/L Tris-HCl, pH 7.5. Net PKC activity was calculated by the difference between total and nonspecific PKC activity. To determine the amount of PKC activity contributed by the β isoforms, fractions were assayed in the absence and presence of LY333531. Results were expressed as picomoles phosphate transferred per minute per milligram of sample proteins.

**Statistical Analysis**

Results are expressed as mean±SEM. Statistical analysis consisted of Student’s unpaired t-test and Dunnett’s test using JMP (SAS Institute). The level of significance was assumed at P<0.05.

**Results**

**Determination of PKC-β Expression by Western Blot; Comparison with PKC-α and -ε**

Hearts from patients with heart failure had elevated expression of the PKC-β. Transmural sections from left ventricles...
were separated into membrane and cytosolic fractions and subjected to SDS-PAGE and Western blot analysis. Representative autoradiograms of cytosol and membrane fractions from a failed and a nonfailed heart are shown in Figure 1. Expression of PKC-β was predominantly localized in membrane fractions of both failed and nonfailed hearts. The identity of PKC-β1 or -β2 was confirmed by using both migration with respective recombinant standard (Figure 1) as well as by obliteration of the respective PKC-β isoform signal with appropriate PKC-β-blocking peptide (Figure 1). Quantitation of isoform expression from autoradiography confirmed that both PKC-β1 and -β2 (Figure 2) were increased by 40% in membrane fractions from failed hearts (P<0.05 and P<0.04, PKC-β1 and -β2, respectively). Comparison of hearts diagnosed with ischemic cardiomyopathy (n=6) or dilated cardiomyopathy (n=6) revealed no significant differences in PKC-β1 or -β2 expression in either cytosol or membrane preparations (data not shown).

PKC-α expression was elevated in failed heart cytosol and was significantly increased by 70% (P<0.03) in failed heart membrane fractions (Figure 3). The identity of PKC-α was also confirmed by using both migration with recombinant standard (Figure 1) as well as by obliteration of signal with PKC-α blocking peptide (Figure 1). By contrast, PKC-ε expression in cytosol and membrane was not significantly different between failed and nonfailed hearts (Figure 4).

**Determination of PKC-α Expression by Immunohistochemistry**

Immunostaining with PKC-α antibody in failed hearts revealed intense localization of PKC-α expression in intercalated disks (Figure 5A), with moderate, diffuse cytoplasmic staining. Slight staining was observed in some intercalated disks in nonfailed hearts, whereas cytoplasmic staining was not present (Figure 5B). PKC-α signal was obliterated by PKC-α antibody-blocking peptide (Figure 5C and 5D).

**Determination of PKC-β Expression by Immunohistochemistry**

In a like manner, left ventricular tissue from explanted hearts of patients with and without heart failure was examined using specific antibodies against PKC-β1 (Figure 6A and 6B) and PKC-β2 (Figure 6E and 6F). Diseased hearts (Figure 6A and 6E) showed strong staining in cardiomyocytes and some intercalated disks (for PKC-β2, Figure 6E), whereas nonfailed hearts (Figure 6B and 6F) showed reduced staining. Immunostaining of individual cases (4 nonfailed and 9 failed) was qualitatively similar (data not shown). PKC-β1 and -β2 immunostaining was obliterated by the respective antiPKC-β blocking peptides (Figure 6C and 6D, and 6G and 6H).

**In Situ Hybridization.**

mRNA expression of PKC-β1 (Figure 7A and 7B) and -β2
Thus, the component of PKC activity attributable to PKC-\(\alpha\) was observed using sense strand of PKC-\(\beta\). Slight scattered positive staining in cardiomyocytes. No signal was observed in nonfailed heart tissue, whereas nonfailed heart tissue had intense staining. Diffuse staining was observed in cardiomyocytes in nonfailed and failed human heart. PKC-\(\alpha\) fractions obtained from failed heart (n = 6) compared with nonfailed (n = 4) membrane fractions (Figure 8; 1021 ± 189 versus 261 ± 89 pmol·mg\(^{-1}\)·min\(^{-1}\), failed and nonfailed, respectively; \(P < 0.04\)). PKC activity was predominantly associated with membrane fractions, which is consistent with PKC protein expression by Western blots. Total PKC activity was greater in cytosol fractions from failed hearts (n = 6) compared with nonfailed (n = 4) (Figure 8; 612 ± 189 versus 248 ± 75 pmol·mg\(^{-1}\)·min\(^{-1}\), failed and nonfailed, respectively) but did not achieve significance. In order to ascertain the contribution of PKC-\(\beta\) to total PKC activity, membrane and cytosolic fractions from nonfailed and failed human hearts were assayed in the presence of LY333531 (10\( \mu \)mol/L). As shown in Figure 8, LY333531 (50 nmol/L) reduced total PKC activity in cytosol fractions by 126 ± 83 and 41 ± 20 pmol·mg\(^{-1}\)·min\(^{-1}\), failed and nonfailed, respectively. In membrane fractions, LY333531 reduced total PKC activity by 209 ± 59 pmol·min\(^{-1}\)·mg\(^{-1}\), whereas PKC activity in nonfailed membrane fractions was decreased by only 42.5 ± 21 pmol·min\(^{-1}\)·mg\(^{-1}\). PKC activity measured in the absence of Ca\(^{2+}\) and lipid was <5% of the activity measured in the presence of Ca\(^{2+}\). Thus, the component of PKC activity attributable to PKC-\(\beta\) was significantly greater in failed heart membrane preparations than nonfailed (\(P < 0.04\)).

**Discussion**

Detection of Ca\(^{2+}\)-sensitive isoforms PKC-\(\alpha\) and -\(\beta\) in human cardiomyocytes remains controversial.\(^5,6,12,21\) Our objective was to determine whether expression of Ca\(^{2+}\)-sensitive isoforms PKC-\(\alpha\) and -\(\beta\) was detectable in ventricular tissue from failed human heart, specifically in cardiomyocytes. Through Western blot analysis we have defined the presence of both isoforms in explanted human hearts, and localization of PKC-\(\beta\) and -\(\beta\) in cardiomyocytes was verified by immunohistochemistry and in situ mRNA probes. In addition, immunohistochemistry showed intense staining of PKC-\(\alpha\) at intercalated disks between cardiomyocytes in failed hearts. The portion of total PKC activity in heart that could be blocked by LY333531 was significantly increased in preparations from end stage failed hearts compared with nonfailed.

The importance of PKC in regulating cellular processes has been established.\(^9,10\) In addition, changes in PKC expression during fetal development and in certain pathological states have been noted.\(^4,12,21,22\) However, it has been difficult to document the presence of a specific PKC isoform in any particular cell type. We identified expression of PKC-\(\alpha\), -\(\beta\), and -\(\gamma\) staining throughout the tissue sections (arrows). Nonfailed heart shows reduced immunostaining for PKC-\(\beta\) (Figure 5C and 5D) or -\(\beta\) (Figure 5G and 5H), or in controls to which probe was omitted (data not shown).

**Determination of PKC Enzymatic Activity**

Total PKC activity was significantly increased in membrane fractions obtained from failed heart (n = 4) compared with nonfailed (n = 4) membrane fractions (Figure 8; 1021 ± 189 versus 261 ± 89 pmol·mg\(^{-1}\)·min\(^{-1}\), failed and nonfailed, respectively; \(P < 0.04\)). PKC activity was predominantly associated with membrane fractions, which is consistent with PKC protein expression by Western blots. Total PKC activity was greater in cytosol fractions from failed hearts (n = 4) than nonfailed (n = 4) (Figure 8; 612 ± 189 versus 248 ± 75 pmol·mg\(^{-1}\)·min\(^{-1}\), failed and nonfailed, respectively) but did not achieve significance. In order to ascertain the contribution of PKC-\(\beta\) to total PKC activity, membrane and cytosolic fractions from nonfailed and failed human hearts were assayed in the presence of LY333531 (10\( \mu \)mol/L). As shown in Figure 8, LY333531 (50 nmol/L) reduced total PKC activity in cytosol fractions by 126 ± 83 and 41 ± 20 pmol·mg\(^{-1}\)·min\(^{-1}\), failed and nonfailed, respectively. In membrane fractions, LY333531 reduced total PKC activity by 209 ± 59 pmol·min\(^{-1}\)·mg\(^{-1}\), whereas PKC activity in nonfailed membrane fractions was decreased by only 42.5 ± 21 pmol·min\(^{-1}\)·mg\(^{-1}\). PKC activity measured in the absence of Ca\(^{2+}\) and lipid was <5% of the activity measured in the presence of Ca\(^{2+}\). Thus, the component of PKC activity attributable to PKC-\(\beta\) was significantly greater in failed heart membrane preparations than nonfailed (\(P < 0.04\)).

**Discussion**

Detection of Ca\(^{2+}\)-sensitive isoforms PKC-\(\alpha\) and -\(\beta\) in human cardiomyocytes remains controversial.\(^5,6,12,21\) Our objective was to determine whether expression of Ca\(^{2+}\)-sensitive isoforms PKC-\(\alpha\) and -\(\beta\) was detectable in ventricular tissue from failed human heart, specifically in cardiomyocytes. Through Western blot analysis we have defined the presence of both isoforms in explanted human hearts, and localization of PKC-\(\beta\) and -\(\beta\) in cardiomyocytes was verified by immunohistochemistry and in situ mRNA probes. In addition, immunohistochemistry showed intense staining of PKC-\(\alpha\) at intercalated disks between cardiomyocytes in failed hearts. The portion of total PKC activity in heart that could be blocked by LY333531 was significantly increased in preparations from end stage failed hearts compared with nonfailed.

The importance of PKC in regulating cellular processes has been established.\(^9,10\) In addition, changes in PKC expression during fetal development and in certain pathological states have been noted.\(^4,12,21,22\) However, it has been difficult to document the presence of a specific PKC isoform in any particular cell type. We identified expression of PKC-\(\alpha\), -\(\beta\), and -\(\gamma\) staining throughout the tissue sections (arrows). Nonfailed heart shows reduced immunostaining for PKC-\(\beta\) (Figure 5C and 5D) or -\(\beta\) (Figure 5G and 5H), or in controls to which probe was omitted (data not shown).

**Determination of PKC Enzymatic Activity**

Total PKC activity was significantly increased in membrane fractions obtained from failed heart (n = 4) compared with nonfailed (n = 4) membrane fractions (Figure 8; 1021 ± 189 versus 261 ± 89 pmol·mg\(^{-1}\)·min\(^{-1}\), failed and nonfailed, respectively; \(P < 0.04\)). PKC activity was predominantly associated with membrane fractions, which is consistent with PKC protein expression by Western blots. Total PKC activity was greater in cytosol fractions from failed hearts (n = 4) than nonfailed (n = 4) (Figure 8; 612 ± 189 versus 248 ± 75 pmol·mg\(^{-1}\)·min\(^{-1}\), failed and nonfailed, respectively) but did not achieve significance. In order to ascertain the contribution of PKC-\(\beta\) to total PKC activity, membrane and cytosolic fractions from nonfailed and failed human hearts were assayed in the presence of LY333531 (10\( \mu \)mol/L). As shown in Figure 8, LY333531 (50 nmol/L) reduced total PKC activity in cytosol fractions by 126 ± 83 and 41 ± 20 pmol·mg\(^{-1}\)·min\(^{-1}\), failed and nonfailed, respectively. In membrane fractions, LY333531 reduced total PKC activity by 209 ± 59 pmol·min\(^{-1}\)·mg\(^{-1}\), whereas PKC activity in nonfailed membrane fractions was decreased by only 42.5 ± 21 pmol·min\(^{-1}\)·mg\(^{-1}\). PKC activity measured in the absence of Ca\(^{2+}\) and lipid was <5% of the activity measured in the presence of Ca\(^{2+}\). Thus, the component of PKC activity attributable to PKC-\(\beta\) was significantly greater in failed heart membrane preparations than nonfailed (\(P < 0.04\)).

**Discussion**

Detection of Ca\(^{2+}\)-sensitive isoforms PKC-\(\alpha\) and -\(\beta\) in human cardiomyocytes remains controversial.\(^5,6,12,21\) Our objective was to determine whether expression of Ca\(^{2+}\)-sensitive isoforms PKC-\(\alpha\) and -\(\beta\) was detectable in ventricular tissue from failed human heart, specifically in cardiomyocytes. Through Western blot analysis we have defined the presence of both isoforms in explanted human hearts, and localization of PKC-\(\beta\) and -\(\beta\) in cardiomyocytes was verified by immunohistochemistry and in situ mRNA probes. In addition, immunohistochemistry showed intense staining of PKC-\(\alpha\) at intercalated disks between cardiomyocytes in failed hearts. The portion of total PKC activity in heart that could be blocked by LY333531 was significantly increased in preparations from end stage failed hearts compared with nonfailed.

The importance of PKC in regulating cellular processes has been established.\(^9,10\) In addition, changes in PKC expression during fetal development and in certain pathological states have been noted.\(^4,12,21,22\) However, it has been difficult to document the presence of a specific PKC isoform in any particular cell type. We identified expression of PKC-\(\alpha\), -\(\beta\), and -\(\gamma\) staining throughout the tissue sections (arrows). Nonfailed heart shows reduced immunostaining for PKC-\(\beta\) (Figure 5C and 5D) or -\(\beta\) (Figure 5G and 5H), or in controls to which probe was omitted (data not shown).
hybridization. mRNA expression of PKC-β in failed human heart tissue was also demonstrated by in situ staining localized in cardiomyocytes (Figure 7).

PKC-β sensitive PKC-isoforms (Figures 1 through 3), which contrast with the finding that PKC-ε was most intense in intercalated disks. Because of slight cross-reactivity between antiPKC-β and recombinant PKC-α, we cannot rule out that localization of PKC-β in the intercalated disks represents staining of PKC-α. PKC-β1 and -β2 expression in failed human heart tissue was also demonstrated by in situ hybridization. mRNA expression of PKC-β1 and -β2 was elevated in failed heart tissue compared with nonfailed, with staining localized in cardiomyocytes (Figure 7).

PKC-ε was also confirmed by immunostaining in cardiomyocytes and was highly expressed in failing heart. PKC-ε staining was most intense in intercalated disks (Figure 6). PKC-α was shown to induce immediate-early genes,2,25 and direct activation of PKC with PMA or agonists that signal through PKC (eg, endothelin-1, α1-adrenergic agonists, angiotensin II) induced hypertrophy.2,26–28 In rat neonatal cardiomyocytes, PKC was shown to induce early stages of cardiac hypertrophy.29,30

Immunostaining of both PKC-β1 and -β2 in failed heart tissue was increased, predominantly in cardiomyocytes, with some PKC-β2 staining observed in intercalated disks (Figure 6). PKC-α was also confirmed by immunostaining in cardiomyocytes and was highly expressed in failing heart. PKC-α staining was most intense in intercalated disks. Because of slight cross-reactivity between antiPKC-β and recombinant PKC-α, we cannot rule out that localization of PKC-β in the intercalated disks represents staining of PKC-α. PKC-β1 and -β2 expression in failed human heart tissue was also demonstrated by in situ hybridization. mRNA expression of PKC-β1 and -β2 was elevated in failed heart tissue compared with nonfailed, with staining localized in cardiomyocytes (Figure 7).

Total PKC activity was significantly higher in membrane fractions from failed human heart than from nonfailed (Figure 8), which was consistent with protein expression. In nonfailed hearts, PKC activity was approximately equal in cytosol and membrane. PKC activity assay conditions were such that both Ca++-sensitive and -insensitive isoenzyme activity were measured. The high specificity of LY333531 for PKC-β allowed us to determine the involvement of PKC-β in cardiac preparations. LY333531 reduced total PKC activity in failed membrane fractions by 209 pmol·min⁻¹·mg⁻¹, nearly 5-fold the β-specific activity reduced by LY333531 in nonfailed membrane (Figure 8). Although 10-fold higher than the IC₅₀ for PKC-β inhibition, the concentration of LY333531 (50 nmol/L) used in these experiments does not appreciably inhibit other PKC isoenzymes14 in vitro. These data suggest that increased PKC-β expression in failed human hearts is associated with a substantial increase in PKC enzymatic activity.

Reports suggesting involvement of PKC in early stages of heart failure are inconclusive. Induction of immediate early genes (c-fos, c-myc) and induction of late-responsive genes such as β-myosin heavy chain and skeletal actin occur during early phases of murine heart failure.23,24 In rat neonatal cardiomyocytes, PKC was shown to induce immediate-early genes,2,25 and direct activation of PKC with PMA or agonists that signal through PKC (eg, endothelin-1, α1-adrenergic agonists, angiotensin II) induced hypertrophy.2,26–28 In addition, transfection of myocytes with constitutively active PKC were reported to transcriptionally transactivate genes for atrial natriuretic factor and β-MHC, which are characteristically activated in cardiac hypertrophy.29,30

In an aortic-banded rat model of heart failure, increases in cardiac PKC activity and expression of PKC isoforms β and
PKC-\(\alpha\), \(-\zeta\), \(-\xi\), and \(-\zeta\) were not elevated. In a coronary artery ligation model of heart failure in rats, \(^{32}\) cardiac PKC activity and expression of PKC isoforms \(\beta_1, \beta_2\), and \(\alpha\) increased with progression of failure (N. Bowling, BA, and C.J. Vlahos, PhD, unpublished data, 1998). Thus, findings from these studies are in limited agreement with our data showing increased PKC activity and PKC-\(\alpha\) and \(-\beta\) expression in failing human heart.

Differential elevation of PKC isoforms in cardiac hypertrophy may result from stretch. \(^{27}\) Neonatal myocytes and adult isolated isovolumic left ventricle stretched to pathophysiologically relevant levels show activation of the phospholipase C pathway with resultant translocation of PKC. \(^{15}\)

Furthermore, cardiac-specific postnatal overexpression of PKC-\(\beta_2\) or the GTP-binding protein for phospholipase C \(\beta_1\) (Goq) recapitulates the fetal gene program and produces cardiac hypertrophy and failure in transgenic mice. \(^{10,32}\)

PKC-\(\beta\) is an integral part of the \(\alpha_1\)-adrenergic receptor signaling pathway, which regulates transcription of \(\beta\)-MHC during cardiac myocyte hypertrophy. \(^{26}\) Transcription of \(\beta\)-MHC is upregulated by PKC-\(\beta\) during \(\alpha_1\)-adrenergic induced hypertrophy. \(^{33,34}\) \(\alpha_1\)-Adrenergic agonists and PKC-\(\beta\) act on the same element in the \(\beta\)-MHC promoter to induce expression, suggesting that PKC-\(\beta\) activation may be involved in modulating MHC isoform expression in failed heart. Furthermore, transgenic mice overexpressing PKC-\(\beta\) show signs of overt left ventricular failure, hypertrophy, and impaired calcium handling. \(^{10,11}\) Treatment of these mice with LY333531 reduces left ventricular hypertrophy and improves cardiac function. \(^{10}\) LY333531 also ameliorated diabetic complications in streptozocin-treated rats by restoring endothelial function, which resulted in improved kidney performance and decreased retinal blood flow. \(^{14}\) Many clinical symptoms resulting from endothelial dysfunction in diabetics (eg, increased albumin clearance, loss of vascular tone, and decreased glomerular filtration rate) are similar to peripheral vascular complications observed in patients with heart failure. Therefore, PKC-\(\beta\) may be a therapeutic target for treating heart failure because its inhibition restores cardiac function and also alleviates microvascular complications.

In conclusion, elevation of Ca\(^{2+}\)-sensitive PKC isoforms \(\beta\) and \(\alpha\) has been demonstrated in failed human heart tissue. Increased expression of these PKC isoforms was demonstrated by Western blot and immunohistochemistry, and total PKC activity was elevated. LY333531 showed that 21% of this PKC activity was due to PKC-\(\beta\). Finally, the presence of PKC-\(\beta\) in failed human heart tissue is confirmed by in situ hybridization. These data suggest that PKC isoform expression may play an important role in the pathophysiology of heart failure and that inhibitors of PKC-\(\beta\) may be useful in its treatment.

Acknowledgments

We thank Kirk Ways for helpful insights and discussions and Eric Powell for in situ studies. This work was supported in part by NIH Specialized Center for Heart Research grant HL52318 (R.A.W.), and National Eye Institute grant EY5110 (G.L.K.).
Increased Protein Kinase C Activity and Expression of Ca\textsuperscript{2+}-Sensitive Isoforms in the Failing Human Heart

Nancy Bowling, Richard A. Walsh, Guojie Song, Thomas Estridge, George E. Sandusky, Rebecca L. Fouts, Karen Mintze, Todd Pickard, Robert Roden, Michael R. Bristow, Hani N. Sabbah, Jacques L. Mizrahi, Gianni Gromo, George L. King and Chris J. Vlahos

*Circulation.* 1999;99:384-391
doi: 10.1161/01.CIR.99.3.384

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/99/3/384

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/