Surgery for Aortic Disease

Differential Protein Kinase C Isoform Abundance in Ascending Aortic Aneurysms From Patients With Bicuspid Versus Tricuspid Aortic Valves

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Background—It is recognized that different events contribute to the initiation of ascending thoracic aortic aneurysms (ATAAs) in patients with bicuspid aortic valves (BAV) versus patients with tricuspid aortic valves (TAV), but the molecular signaling pathways driving aneurysm formation remain unclear. Protein kinase C (PKC) is a superfamily of kinases which differentially mediate signaling events that lead to altered gene expression and cellular function, and may regulate downstream mediators of vascular remodeling. The present study tested the hypothesis that ATAA development in patients with BAV versus TAV proceeds by independent signaling pathways involving differential PKC signaling.

Methods and Results—ATAA samples were collected from BAV (n=57) and TAV (n=55) patients and assessed for 10 different PKC isoforms by immunoblotting. Results were expressed as a percent change in abundance (mean±SEM) from a nonaneurysmal control group (100%, n=21). Correlation analysis was performed, and relationships between PKC and matrix metalloproteinase abundance were identified. In the BAV group, classic and novel PKC isoforms (PKC-α, β1, γ, ε, θ) were increased, whereas PKC-η and atypical PKC-ζ were decreased. In the TAV group, classic and novel isoforms were decreased and atypical PKC-ζ was elevated. Positive correlations between PKC and matrix metalloproteinase abundance were identified.

Conclusions—Differential PKC isoform abundance was observed in ATAA samples from patients with BAV versus TAV, suggesting independent molecular signaling pathways may be operative. Induction of independent transcriptional programs may result and may provide a mechanistic foundation for developing selective diagnostic/therapeutic strategies for patients with ATAA secondary to BAV or TAV. (Circulation. 2007;116[suppl I]:I-144–I-149.)

Key Words: valves ■ aneurysm ■ aorta ■ PKC ■ metalloproteinases

The congenital bicuspid aortic valve (BAV) is the most common cardiac malformation, occurring in 1% to 2% of the population.5,6 Approximately 33% of patients with a BAV will develop serious complications that require treatment. A common consequence of BAV is aortic dilatation leading to the formation of ascending thoracic aortic aneurysms (ATAAs).2,4,5 There have been 2 predominant theories put forth to explain the increased incidence of ATAA in patients with BAV: (1) genetic abnormalities which may include defects in the neural crest-origin cells and alterations in fibrillin-1 function, and (2) enhanced hemodynamic stress on the ascending aortic wall as a result of turbulent blood flow over the malformed valve. ATAA formation is a multifactorial process that involves both cellular and extracellular mechanisms that converge on multiple signaling pathways and result in the maladaptive remodeling of the vascular extracellular matrix. Studies from this laboratory and others have demonstrated differential matrix metalloproteinase (MMP) profiles in ATAA samples from patients with a BAV versus patients with idiopathic medial degenerative disease and a tricuspid aortic valve (TAV).6–10 These differential profiles suggest that aneurysm formation in each valve group may proceed by unique mechanisms that require discrete signaling pathways and different intermediate effectors. However, although there are several studies comparing the deregulation of MMP expression secondary to BAV versus TAV, little information is known regarding the upstream signaling events that orchestrate these changes in each valve group.

Protein kinase C (PKC) is a family of lipid regulated serine/threonine kinases that mediate cellular responses and cellular function.11–13 There are at least 10 different isoenzymes, encoded by 9 different genes, classified into 3 groups that are differentiated by their activation requirements and
downstream targets. The classic isoforms (cPKC; α, βI, βII, and γ) require both diacylglycerol and calcium for activation, whereas the novel isoforms (nPKC; δ, ε, η, and θ) require only diacylglycerol for activation and are calcium independent. The atypical isoforms (aPKC; ξ and ζ) are both calcium and diacylglycerol-independent but are regulated by other glycerol- and sphingo-lipids.\(^\text{11}\)

Because the activation of various PKC isoforms has been implicated in the regulation of downstream effectors of vascular remodeling,\(^\text{14-19}\) we hypothesized that independent signaling pathways involving differential PKC signaling contribute to the unique etiologies of ATAA in patients with BAV versus TAV.

### Methods

#### Study Population

The study population consisted of aortic tissue samples collected from ATAA patients at time of surgical resection that had either a BAV (\(n = 57\)) or a TAV (\(n = 55\)). All patients underwent aortic root replacement, and all tissue specimens were collected from the ascending aorta as opposed to the sinus segment or proximal arch. Results were compared with nonaneurysmal aortic samples collected from the ascending aorta of 21 reference control patients (8 heart donors, 12 heart transplant recipients, and 1 coronary artery bypass graft patient). Thus, in all cases, reference control and aneurysmal tissue specimens were collected from the same region of the ascending aorta for biochemical comparison. There were no gender differences between groups (control: 76±4% male; BAV: 75±7% male; TAV: 62±6% male; \(P = 0.231\ \chi^2\)); however, the mean ages between groups were different (control: 48±3 years; BAV: 57±2 years, \(P = 0.002\) from control; and TAV 66±2 years, \(P < 0.001\) from control, and \(P = 0.002\) from BAV). Aortic diameters were not different between ATAA subgroups, but both were different from the reference control group (control: 2.8±0.1 cm; BAV: 5.3±0.1 cm, \(P < 0.001\); TAV: 5.4±0.2 cm, \(P < 0.001\)). Significant proportions of the BAV (47.9%) and TAV (45.8%) patients had documented aortic insufficiency or regurgitation, and 25% of the BAV patients had documented aortic stenosis that was moderate to critical. Approximately half of the patients in all 3 groups had hypertension (Control 42.9%, BAV 56.3%, and TAV 54.2%). All patient demographics are summarized in the Table. No patient experienced giant cell arteritis, had an aortic dissection, or had an established connective tissue disorder. This study was approved by the Institutional Review Boards of both the Medical University of South Carolina and the University of Pennsylvania, and informed consent was obtained from all patients.

#### Sample Preparation

Resected aortic tissue specimens were homogenized in cold acidic extraction buffer to prevent protease activation during the extraction process.\(^\text{20}\) The aortic homogenates were then centrifuged (4°C, 10 minutes, 12000g) and the final protein concentration was determined (BCA Protein Assay).

### Immunoblotting

The relative abundance of 10 different PKC isoforms were determined using quantitative immunoblotting techniques.\(^\text{20}\) Briefly, 10 μg of each aortic homogenate was fractionated on a 4% to 12% Bis-Tris gradient polyacrylamide gel (Invitrogen Corp). The proteins were then transferred to nitrocellulose membrane (0.45 μm; Bio-Rad) and blocked with 5% nonfat dry milk in phosphate buffered saline (PBS) for 1 hour at room temperature. The membrane was incubated with antisera (0.4 μg/mL in 5% nonfat dry milk/PBS) containing specific antibodies for each of the PKC isoforms tested (\(\alpha =\)alpha, \(\beta I =\)beta-I, \(\beta II =\)beta-II, \(\gamma =\)gamma, \(\delta =\)delta, \(\epsilon =\)epsilon, \(\theta =\)theta, \(\eta =\)eta, \(\iota =\)iota, and \(\zeta =\)zeta; Santa Cruz Biotechnology). Following incubation with primary antibody, the membrane was washed extensively (3×10 minutes, 25 mL PBS) to reduce nonspecific antibody interactions. A secondary peroxidase-conjugated antibody (species dependent on the primary antibody used) was applied (1:5000 dilution in 5% nonfat dry milk/PBS) and allowed to incubate for 1 hour at room temperature. The membrane was again washed extensively (4×15 minutes, 25 mL PBS). Positive immunoreactivity was identified by briefly incubating the membranes with a chemiluminescent substrate (Western Lighting Chemiluminescence Reagent Plus; Perkin Elmer) and exposing the blot to film (Hyperfilm; GE Healthcare).

### Data Analysis

Immunoblots were digitized and quantitative densitometric image analysis was performed (Gel Pro Analyzer; Media Cybernetics). PKC abundance in ATAA specimens was expressed as a percentage increase or decrease as compared with reference control values, which were set at 100%.

All statistical procedures were carried out using the Stata statistical package (Intercooled Stata v8.2; StataCorp LP). Patient demographics were compared by \(\chi^2\) analysis or 1-way ANOVA using Tukey wsd post hoc analysis. One-sample mean comparison tests were performed to determine statistical differences in PKC abundance. Pairwise correlation analysis was performed to reveal relationships between PKC abundance and MMP/tissue inhibitor of metalloproteinase (TIMP) expression (previously reported\(^\text{3}^\)) or ascending aortic diameter. Significant correlations were reported with regression values and probability values. Statistical analyses were also performed to compare PKC isoform abundance to age, presence of hypertension, aortic valve pathology (insufficiency/regurgitation versus stenosis), and aortic diameter indexed to body surface area. All data are presented as a mean±SEM, and values of \(P < 0.05\) were considered statistically significant.

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

#### Analysis of PKC Abundance

The aortic specimens were assayed by immunoblotting for 10 different PKC isoforms that were classified into 3 different
activation groups (Figure 1). Of the classic PKC isoforms (presented as integrated optical density ratio, percent change from reference control, probability value) PKC-α (479.1/293.1, 163.5±19.3%, P=0.002), -βI (230.0/165.9, 138.7±16.2%, P=0.020), and -γ (269.1/165.9, 162.2±21.7%, P=0.006) were elevated in the BAV specimens, whereas PKC-βII remained unchanged. In the TAV samples, PKC-βII (617.9/947.9, 65.2±8.1%, P<0.001) and -γ (80.6/165.9, 48.6±7.7%, P<0.001) were decreased from control values, whereas PKC-α and -βI remained unchanged. Of the novel PKC isoforms PKC-ε (168.3/105.1, 160.0±24.9%, P=0.020) and -θ (308.8/194.7, 158.6±20.3%, P=0.006) were elevated in the BAV specimens, whereas PKC-η (3973.9/5397.8, 73.6±9.9%, P=0.010) was decreased and PKC-δ remained unchanged. In the TAV specimens, PKC-ε (80.0/105.1, 76.1±11.5%, P=0.043) and -η (2766.0/5397.8, 51.2±8.1%, P<0.001) were decreased from control values and PKC-δ and -θ remained unchanged. Of the atypical PKC isoforms, PKC-ζ (968.0/1180.1, 82.0±5.2%, P=0.001) was decreased in the BAV specimens, whereas PKC-τ remained unchanged. In the TAV specimens, PKC-ζ (1443.7/1180.1, 122.3±7.0%, P=0.002) was elevated from control values, whereas PKC-τ remained unchanged. The abundance of the following PKC isoforms was significantly different between BAV and TAV specimens: PKC-α (P=0.034), -βII (P=0.001), -γ (P<0.001), -ε (P=0.006), -θ (P=0.011), and -ζ (P<0.001). These results are summarized in Figure 2.

Pairwise Correlation Analysis

To examine the relationships between PKC abundance and potential downstream regulators of vascular remodeling, pairwise correlation analysis was performed. The percent change from reference control of each PKC isoform was compared with the abundance of each MMP and TIMP species, as previously reported, for each patient sample. Significant correlations were identified between PKC-θ (170±23%, P<0.05 versus reference control) and MMP-2 (138±7%, P<0.05 versus reference control) in the BAV samples (r=0.4995, P=0.0002; Figure 3A), and PKC-ζ (122±7%, P<0.05 versus reference control) and MMP-7 (300±82%, P<0.05 versus reference control) in the TAV samples (r=0.4076, P=0.0025; Figure 3B). No significant correlations were identified between PKC isoform abundance and ascending aortic diameter in either BAV or TAV patients. Additional analyses examining the affect of age, aortic valve pathology (aortic insufficiency/regurgitation, stenosis), and aortic size indexed to body surface area on PKC isoform abundance failed to reveal any significant relationships. Comparisons of PKC isoform abundance in ATAA specimens from BAV and TAV patients with and without hypertension, revealed the PKC-α (P=0.007), -ε (P=0.014), -θ

*Figure 1. Comparison of classic, novel, and atypical PKC isoform abundance in BAV and TAV aneurysm samples (BAV [dark gray], TAV [light gray]; representing the mean±SEM). A representative immunoblot of each PKC isoform is shown on the right. The number of observations are shown in parentheses on each bar; *P<0.05 versus reference control, #P<0.05 versus BAV.

Figure 2. Summary of the significant changes in PKC isoform abundance in ATAA specimens from BAV versus TAV patients.

Figure 3. Correlation analysis between PKC and MMP abundance. A, PKC-θ versus MMP-2 in BAV samples; B, PKC-ζ versus MMP-7 in TAV samples. The r value and P value for the correlation are shown. Gray lines indicate the 95% CI.
(P=0.018), and \(-\) (P=0.034) were elevated in ATAA specimens from BAV patients with hypertension as compared with those without.

**Discussion**

Although it is recognized that different natural histories and pathophysiological events contribute to the initiation of ATAA formation in patients with a BAV versus patients with a TAV, the molecular signaling pathways that drive aneurysm formation in these two valve groups remain unclear. Because PKC has been directly implicated in regulating downstream effectors of extracellular matrix remodeling in other disease states, the present study measured the abundance of 10 different PKC isoenzymes in ATAA samples resected from BAV and TAV patients. When compared with nonaneurysmal aortic specimens collected from a reference control group, the unique findings of this study demonstrate that differential PKC isoenzyme expression profiles exist in ATAA from BAV versus TAV patients (Figures 1 and 2). In the BAV samples, increased abundance of the diacylglycerol-sensitive classic and novel PKC isoforms was observed along with decreased novel PKC-\(\eta\) and atypical PKC-\(\zeta\). Alternatively, in the TAV samples, the classic and novel isoforms were decreased from control, whereas atypical PKC-\(\zeta\) was increased. Moreover, when pairwise comparisons were performed between the abundance of PKC isoenzyme species and the MMPs and their endogenous inhibitors (as previously reported), significant correlations were identified (Figure 3). Together, these data suggest that independent transcriptional programs mediated by differential PKC signaling may account for the disparate proteolytic processes driving ATAA formation in BAV versus TAV patients. Accordingly, this differential expression of PKC isoforms may hold future diagnostic and prognostic implications.

PKC functions as a critical signaling mediator, transducing intracellular and extracellular signals, by regulating kinase cascades that ultimately influence a diverse number of cellular mechanisms including mitogenic and apoptotic pathways, vesicular transport, lipid metabolism, and gene expression. Importantly, PKC has been shown to be operative in pathways regulating the expression of MMPs. The MMPs are a family of at least 27 distinct zinc-dependent proteases capable of degrading all extracellular matrix constituents, and thus have been established as critical mediators of extracellular matrix remodeling. Recently, this laboratory has reported that the MMPs and their endogenous inhibitors are differentially expressed in aortic tissue from patients with BAV versus TAV. Because regulation of MMP expression has been suggested to occur primarily at the transcriptional level, the differential MMP profiles identified in these valve groups likely arose from the activation of different transcriptional programs during aneurysm development. The present study results suggest that PKC signaling, affected through differential isoenzyme expression, may contribute to the disparate transcriptional programs regulating the distinct MMP expression profiles demonstrated in each ATAA valve group.

Previous studies have implicated PKC as an upstream mediator of MMP production and release. Park and colleagues demonstrated in glioblastoma cells that phorbol ester treatment could induce MMP-9 secretion, MMP-2 activation, and the translocation of MT1-MMP to the plasma membrane. These observations were shown to be dependent on PKC-mediated p38MAP kinase activation, and were accompanied by the down regulation of TIMP-1 and TIMP-2. PKC-mediated MMP production was also confirmed by Chu and coworkers who demonstrated that inflammatory cytokines could induce the production and secretion of MMP-2 and MMP-9 by a PKC-dependent mechanism in osteoarthritis cell cultures. Furthermore, MMP release from neutrophils was shown to be dependent on PKC activity by Chakrabarti et al, who demonstrated that 85% of MMP-9 specific granule release could be blocked by a pan-specific PKC inhibitor. Together these data strongly support the hypothesis that the expression and release of MMPs is at least in part dependent on a common fundamental signaling pathway involving PKC.

In the present study, the increased abundance of the diacylglycerol-sensitive classic and novel PKC isoforms \(-\alpha\), \(-\beta1\), \(-\gamma\), \(-\varepsilon\), and \(-\theta\) in BAV samples suggests that aneurysm formation may be driven by a process that results in the sustained activation of upstream mediators of diacylglycerol production; such as phospholipase C (PLC). PLC is a family of lipid metabolic enzymes that function to remove the phospho-head group of several specific phospholipid species, resulting in the formation of diacylglycerol. Schütze and coworkers demonstrated that tumor necrosis factor-\(\alpha\) could stimulate diacylglycerol levels in U937 cells by activating a phosphatidylycholine-specific PLC. The increase in diacylglycerol was linked to the potent activation of PKC, as PKC activation could be inhibited with a specific PLC inhibitor. Although there is a paucity of information regarding the active signaling pathways in aortic aneurysm formation, previous studies of intracranial aneurysms have identified increased PLC activity in both experimental models and patient samples, and associated the increase in PLC activity with increased PKC activation. Thus, the persistent activation of lipid metabolic enzymes such as PLC could contribute to aneurysm formation through the activation of the diacylglycerol-sensitive PKC isoforms and the subsequent induction of PKC-mediated MMP expression.

With regard to the TAV samples, a general decrease was observed in the diacylglycerol-sensitive isoforms coconcurrent with an increase in atypical PKC-\(\zeta\). PKC-\(\zeta\), although insensitive to calcium and diacylglycerol, requires specific interactions with phospholipids or sphingolipids for activation. Among these lipids, the generation of phosphatidylinositol-3,4,5-trisphosphate by phosphatidylinositol-3 kinase has been identified as a potent regulator of PKC-\(\zeta\) activation. Interestingly, PKC-\(\zeta\) has been shown to be a potent activator of nuclear factor \(\kappa\)-B, a transcription factor with target binding sites in several MMP promoter regions. Esteve et al demonstrated that interleukin-1 and tumor necrosis factor-\(\alpha\) could induce MMP-9 expression by an nuclear factor \(\kappa\)-B-dependent mechanism that required the activation of PKC-\(\zeta\). This work confirmed the observations of Hussain and coworkers who demonstrated that PKC-\(\zeta\) activity was essential for cytokine-dependent induction of MMP-1, -3, and -9 through a mechanism that involved the activation of nuclear factor \(\kappa\)-B. Thus, activation of atypical PKC in ATAA samples from TAV
patients may also result in the production of MMPs through the stimulation of a potentially distinct transcriptional program. In an effort to establish a relationship between the MMP/TIMP profiles and PKC isoenzyme expression in BAV versus TAV aneurysms, pairwise correlation analysis was performed comparing MMP or TIMP abundance with PKC isoform abundance. Several statistically significant interactions were uncovered. Of greatest interest was a strong positive relationship ($r = 0.4995$, $P = 0.0002$) between MMP-2 abundance and PKC-θ in the BAV samples. Both MMP-2 and PKC-θ were elevated as compared with reference control values. Previous studies from several investigators have described elevated MMP-2 in valvular and aneurysmal aortic tissues from BAV patients, representing the most consistent finding in BAV specimens across laboratories.$^6$–$^{10}$ Interestingly, Xie and co-workers, through a series of in vitro studies in adult rat cardiac fibroblasts, have demonstrated that activation of PKC-θ plays a critical role in interleukin-1β-mediated induction of MMP-2 expression and activity.$^39$ Thus, these data support the present study findings and may suggest that PKC-θ activation is required for MMP-2 induction during ATAA formation in patients with BAV. In the TAV samples, MMP-7 and PKC-ζ were both elevated compared with reference control values and likewise displayed a positive relationship ($r = 0.4076$, $P = 0.0025$) by correlation analysis. Although no literature precedence has been established directly linking PKC-ζ activation with MMP-7 expression, indirect evidence suggests they may be linked. Activator protein-1 (AP-1) is a heterodimeric leucine zipper transcription factor comprised of the Fos and Jun family of transcriptional regulators. MMP-7, as well as several other MMPs, have AP-1 consensus binding sites within their promoter regions.$^{40}$ In a study by Ways et al, overexpression of PKC-ζ in U937 cells induced differentiation which was characterized by increased levels of the c-jun proto-oncogene and increased AP-1 DNA binding activity; implicating PKC-ζ as an upstream activator of AP-1.$^{41}$ More recently, lipopolysaccharide induced activation of AP-1 was likewise shown to require PKC-ζ activation.$^{42}$ Thus, PKC-ζ could potentially activate AP-1-mediated transcription of MMP-7 during ATAA formation in patients with TAV. Because this study was performed on resected aortic specimens collected at the time of surgical intervention, there are some inherent limitations to this data. First, it is unclear whether the differential PKC profiles identified in each valve group are representative of specific phases of aneurysm progression or summary of all phases. Furthermore, other confounding factors such as patient exposure to cyclo-oxygenase inhibitors, steroids, or statins may also affect aneurysm formation and progression. Until noninvasive methods of specific activity will be required to further establish the role of PKC activation in aneurysm formation. Last, it is also important to note that potential upstream activators of PKC (diacylglycerol, phospholipase C, phosphatidylinositol-3 kinase) were not measured in this study. Identification of the operative signaling intermediates that function upstream of PKC in each valve group will need to be addressed in future experimentation. Nevertheless, the results of the present study imply that differential PKC signaling may be operative during aneurysm formation in ATAA samples from BAV versus TAV patients. This differential signaling may result in the activation of distinct transcriptional programs that could explain the differential proteolytic profiles previously described in these ATAA subgroups. Furthermore, the altered abundance of the different classes of PKC isoforms in BAV (classic and novel) versus TAV (atypical) suggest that the upstream stimuli are different and add to the data further describing the divergent operational pathobiology in ATAA formation secondary to BAV versus TAV. Continued definition of the roles played by upstream signaling pathways in these pathological conditions may eventually allow identification of the critical stimuli which initiate aneurysm formation with obvious therapeutic implications.

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Disclosures
None.

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