Application of kinomic array analysis to screen for altered kinases in atrial fibrillation remodeling

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BACKGROUND Dysregulation of protein kinase–mediated signaling is an early event in many diseases, including the most common clinical cardiac arrhythmia, atrial fibrillation (AF). Kinomic profiling represents a promising technique to identify candidate kinases.

OBJECTIVE In this study we used kinomic profiling to identify kinases altered in AF remodeling using atrial tissue from a canine model of AF (atrial tachypacing).

METHODS Left atrial tissue obtained in a previous canine study was used for kinomic array (containing 1024 kinase pseudosubstrates) analysis. Three groups of dogs were included: nonpaced controls and atrial tachypaced dogs, which were contrasted with geranylgeranylacetone-treated dogs with AF, which are protected from AF promotion, to enhance specificity of detection of putative kinases.

RESULTS While tachypacing changed activity of 50 kinases, 40 of these were prevented by geranylgeranylacetone and involved in differentiation and proliferation (SRC), contraction, metabolism, immunity, development, cell cycle (CDK4), and survival (Akt). Inhibitors of Akt (MK2206) and CDK4 (PD0332991) and overexpression of a dominant-negative CDK4 phosphorylation mutant protected against tachypacing-induced contractile dysfunction in HL-1 cardiomyocytes. Moreover, patients with AF show down- and upregulation of SRC and Akt phosphorylation, respectively, similar to findings of the kinome array.

CONCLUSION Contrasting kinomic array analyses of controls and treated subjects offer a versatile tool to identify kinases altered in atrial remodeling owing to tachypacing, which include Akt, CDK4, and SRC. Ultimately, pharmacological targeting of altered kinases may offer novel therapeutic possibilities to treat clinical AF.

KEYWORDS Akt; Atrial fibrillation; Cardiomyocytes; CDK; Kinases; Kinome array; SRC

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Introduction

Atrial fibrillation (AF) is the most prevalent and persistent clinical tachyarrhythmia.[1] Its maintenance and progression is driven by AF-induced structural, contractile, and electrical remodeling of cardiomyocytes, commonly referred to as *electropathology*.[1,2] Atrial remodeling creates a substrate for AF, which limits the efficacy of current electrical cardioversion and pharmacological therapies.[1] Therefore,

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identification of the molecular mechanisms underlying AF-induced atrial remodeling will provide insight into AF maintenance and progression and may identify novel therapeutic targets.

At the molecular level, dysregulation of kinase activity contributes to AF-induced remodeling. In experimental models of AF as well as in patients, activity of several kinases is changed.[3,4] Their altered activity is thought to contribute to AF-induced remodeling through (de)phosphorylation of diverse downstream effector proteins, including diverse ion channels,[4,5] calcium-handling proteins,[5] gap-junction proteins,[6] contractile proteins,[7] cytoskeletal proteins,[3] and transcription factors.[8] Changes in phosphorylation status of these proteins during AF in turn affects electrical properties, calcium homeostasis, contractility, and gene transcription.

Although several kinases have been implicated in AF-induced remodeling, an integrated overview of AF-induced kinases and their targets is lacking. The goals of this study were (1) to examine the putative kinase activity profile in an in vivo dog model of AF,[9] with and without treatment with geranylgeranylacetone (GGA),[9] by analyzing overall kinase activity using a kinome array containing 1024 pseudosubstrates; (2) to evaluate the potential role of target kinases in AF progression by assessing the results of inhibiting 2 candidate kinases on tachypacing-induced remodeling changes in an in vitro assay; and (3) to extrapolate the selected findings of the kinome array in human patients with AF. GGA was previously shown to induce the expression of heat shock proteins (HSPs), resulting in the prevention of AF promotion in the dog model of AF.[9] Given that HSPs interact with kinases,[10,11] results from dogs with AF were contrasted with GGA-treated dogs with AF, which are protected from atrial remodeling, to test the potential relevance to AF-induced remodeling of putative kinases.

Methods

In vivo canine model of AF

Dog left atrial tissue obtained in a previously performed study at the Montreal Heart Institute[9] was used for the kinase profiling. Dog experiments were according to the guidelines for animal handling of the National Institutes of Health and approved by the Animal Research Ethics Committee of the Montreal Heart Institute (see the Supplemental Information for more details).

Kinome array

Kinome arrays were performed according to the manufacturer's instructions (see the Supplemental Information for more details).

HL-1 atrial cardiomyocyte cell culture, calcium transients, and Western blot analysis

HL-1 atrial cardiomyocytes were normal paced or tachypaced (TP) for 10 hours with or without pretreatment with the Akt inhibitor MK2206 (Sigma, The Netherlands), the CDK4 inhibitor PD0332991 (Sigma), or the HSP-inducer GGA (Eisai Pharmaceutical, Japan) or transfected with the wild-type or mutant CDK4 or pcDNA3.1+ plasmids. Calcium transients (CaTs) were measured with the Ca^{2+}-sensitive dye Fluo-4 AM (Invitrogen, The Netherlands), and Western blot analysis was performed as described previously[9] (see the Supplemental Information for more details).

Patient material

Before surgery, investigators assessed patient characteristics (Supplemental Table S1) as described previously.[12] Right atrial appendages were obtained from patients with AF and control patients in sinus rhythm (SR) undergoing cardiothoracic surgery, for underlying heart disease, as described in the HALT & REVERSE study (MEC 2014-393).[13] After excision, atrial appendages were immediately snap frozen in liquid nitrogen and stored at −80°C.

Statistical analysis

The results of statistics analyses performed on the kinome array, HL-1 cardiomyocytes, and patient experiments are described in the Supplemental Information.

Results

Identification of key kinases involved in tachypacing-induced atrial remodeling

The principal electrophysiological findings in various groups of dogs in the previous study[9] are summarized in Supplemental Table S2. To identify putative kinases altered in tachypacing-induced atrial remodeling, we contrasted their respective kinase profiles. All kinome arrays showed substantial radioactivity and allowed detailed analysis. Representative phosphor images are shown in Figure 1A, and technical quality of the analysis was good (see the Supplemental information for more details). The substrate profile of left atrial tissue of nonpaced controls was then contrasted with those of tachypacing (TP) and TP+GGA groups. A heat map generated from the profiles showed TP to change the phosphorylation intensity patterns compared to nonpaced controls, which was partially attenuated by GGA treatment (Figure 1B). Hierarchical clustering using the Euclidean distance and average linkage showed that the TP+GGA group clustered with the nonpaced control group, both distinct from the TP group (Figure 1C), supporting that GGA treatment partially attenuates TP-induced alterations in kinase activity.

To identify changes in putative kinase activity, substrates were grouped per upstream kinase, as indicated by the manufacturer, and overall kinase activity was determined per group. When contrasting the TP group with the control group, a total of 50 kinases differed significantly in activity. Of these kinases, TP decreased activity of 21 kinases and increased activity of 29 kinases (Supplemental Tables S3 and S4, respectively). Several kinases with decreased activity are related to proliferation, differentiation, and cell survival

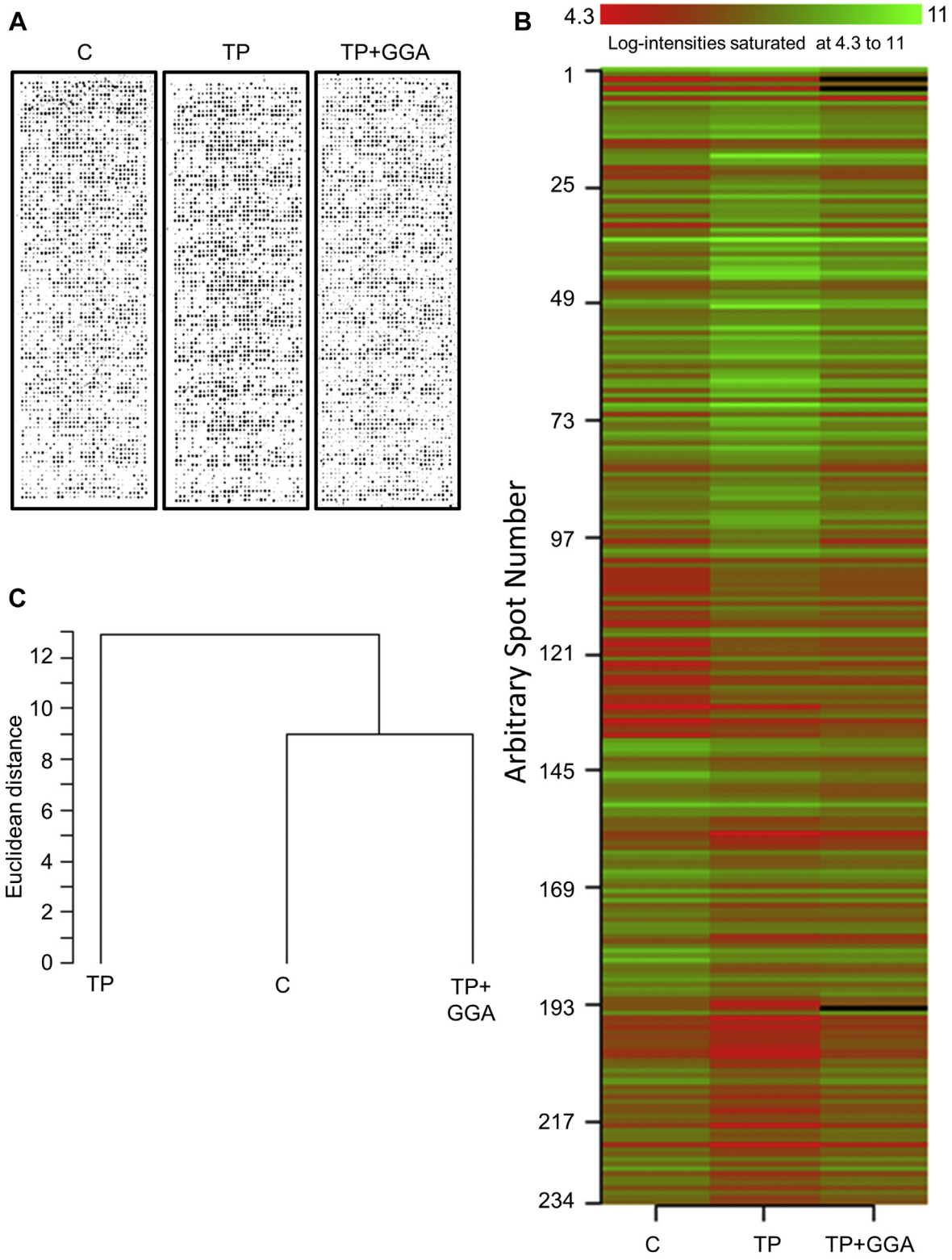

Figure 1 GGA treatment partially protects against tachypacing-induced changes in substrate phosphorylation. **A:** A representative phosphor image of the kinase array per group: C, TP group, and TP+GGA group. **B:** Heat map of the overall substrate phosphorylation changes in response to TP and TP+GGA in comparison to C. Tachypacing caused a significant change in the spot intensity of 232 substrates, of which 95 decreased and 137 increased, which is partially normalized by GGA treatment. **C:** Dendogram showing the hierarchical clustering using the Euclidean distance with average linkage for all 3 groups. C = non-paced controls; GGA = geranylgeranylacetone; TP = tachypaced; TP+GGA = tachypaced and geranylgeranylacetone treated.

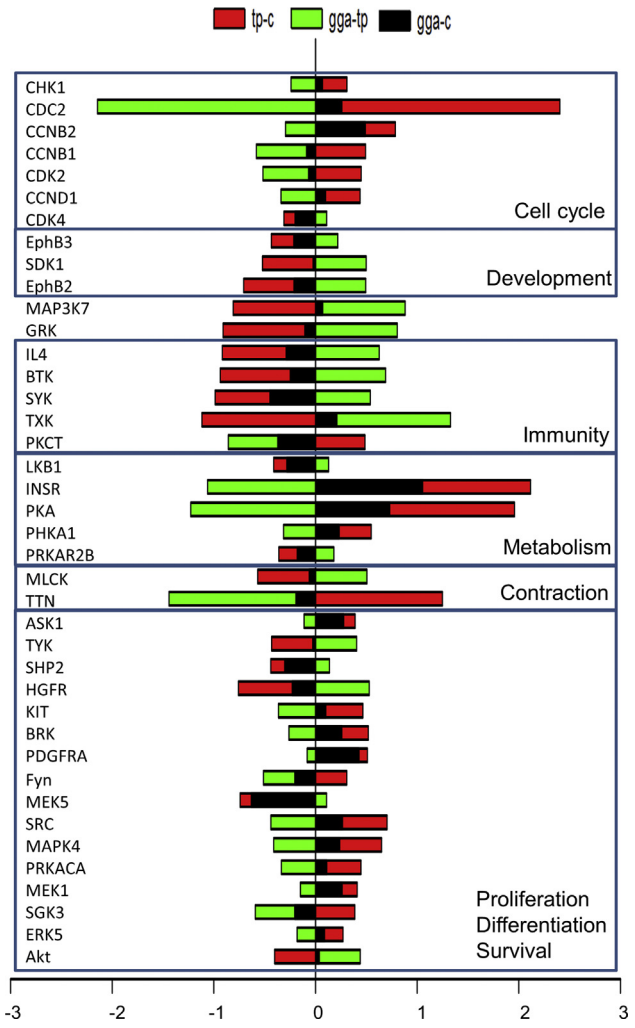

Figure 2 GGA treatment partially protects against tachypacing-induced changes in overall kinase activity. Kinases with changed activity were identified by determining changes in substrate phosphorylation. Differences between the indicated groups of log2(normalized overall kinase activity) are displayed. Kinases are grouped according to their main function. The only kinases displayed are those whose activity is (partially) attenuated by GGA treatment. *Red bars* indicate changes of TP vs C; *green bars* indicate changes of GGA vs TP; and *black bars* indicate changes of GGA vs C. C = nonpaced controls; GGA = tachypacing and geranylgeranylacetone treatment; TP = tachypacing.

pathways, whereas kinases with increased activity are related to proliferation, differentiation, survival pathways, and, in addition, cell cycle, muscle contraction, and metabolism. As GGA treatment protects against tachypacing-induced atrial remodeling,[9] kinase normalization by GGA treatment points to a contribution in atrial remodeling. Of the 50 kinases changed by tachypacing, GGA treatment prevented 34 completely and 6 partially (Figure 2 and Supplemental Table S5). The 6 partially restored kinases were protein kinase A (PKA), myosin light chain kinase (MLCK), group of genes that encode for cytoplasmic nonreceptor protein kinases (SRC), tyrosine-protein kinase (TXK), cyclin B1 (CCNB1), and ephrin type-B receptor (EphB2). We next grouped a total of 40 putative kinases changed by GGA according to their function (Figure 2) and observed that they are involved in the regulation of cell cycle, development, immunity, metabolism, and muscle contraction or have a role in proliferation, differentiation, and survival pathways.

Identification of a modulating role of Akt and CDK4 in TP HL-1 cardiomyocytes

The kinome array identified various kinases altered in AF-induced remodeling (Supplemental Table S5). To expand their role in AF progression, we selected 2 kinases from Supplemental Table S5 that were activated upon tachypacing and for which inhibitors are currently available.

Akt is an interesting candidate because of its role in cellular processes, including survival pathways, metabolism, and cell cycle.[14] Consistent with the array analysis, TP dogs showed a significant increase in the Thr308 phosphorylation of Akt and a trend toward an increase in phosphorylation of S473 while phosphorylation was normalized in the TP+GGA group to control levels (Figures 3A and 3B). To obtain further information on the potential role of Akt in tachypacing-induced contractile dysfunction, HL-1 cardiomyocytes were pretreated with the Akt activation inhibitor 100 nM MK2206 before and during tachypacing. MK2206 both reduced Akt-Ser473 and Akt-Thr308 phosphorylation and attenuated tachypacing-induced loss of contractile function by preserving CaT (Figures 3C and 3D and Supplemental Figure S1). However, MK2206 treatment also significantly reduced the CaT amplitude in nonpaced cardiomyocytes, indicating that Akt inhibition may have undesirable effects.

The other interesting target is CDK4, as it is part of the larger group of cell cycle–related kinases, which are involved in cardiac hypertrophy,[15] as also found in AF. Palbociclib (PD0332991) is a US Food and Drug Administration–approved selective CDK4 inhibitor used for the treatment of breast cancer.[16] To test whether CDK4 is involved in AF remodeling, TP HL-1 cardiomyocytes were pretreated with PD0332991. PD0332991 (500 nM) attenuated tachypacing-induced loss of CaT (Figures 3E and 3F). In addition, we transfected HL-1 cardiomyocytes with CDK4 (wild type) and a nonphosphorylatable mutant, CDK4-T172A. Control and wild-type transfected HL-1 cardiomyocytes showed a reduction in CaT after tachypacing. In contrast, transfection with the nonphosphorylatable mutant attenuated tachypacing-induced loss of CaT (Figures 3G and 3H and Supplemental Figure S2).

Finally, we show that treatment with GGA (10 μM) attenuated tachypacing-induced loss of CaT in HL-1 atrial cardiomyocytes (Supplemental Figure S3), as also shown previously.[9]

Together, these findings indicate that the kinases Akt and CDK4 might contribute to tachypacing-induced loss of CaT in HL-1 atrial cardiomyocytes and suggest that understanding the role of CDK4 and, depending on potential undesirable

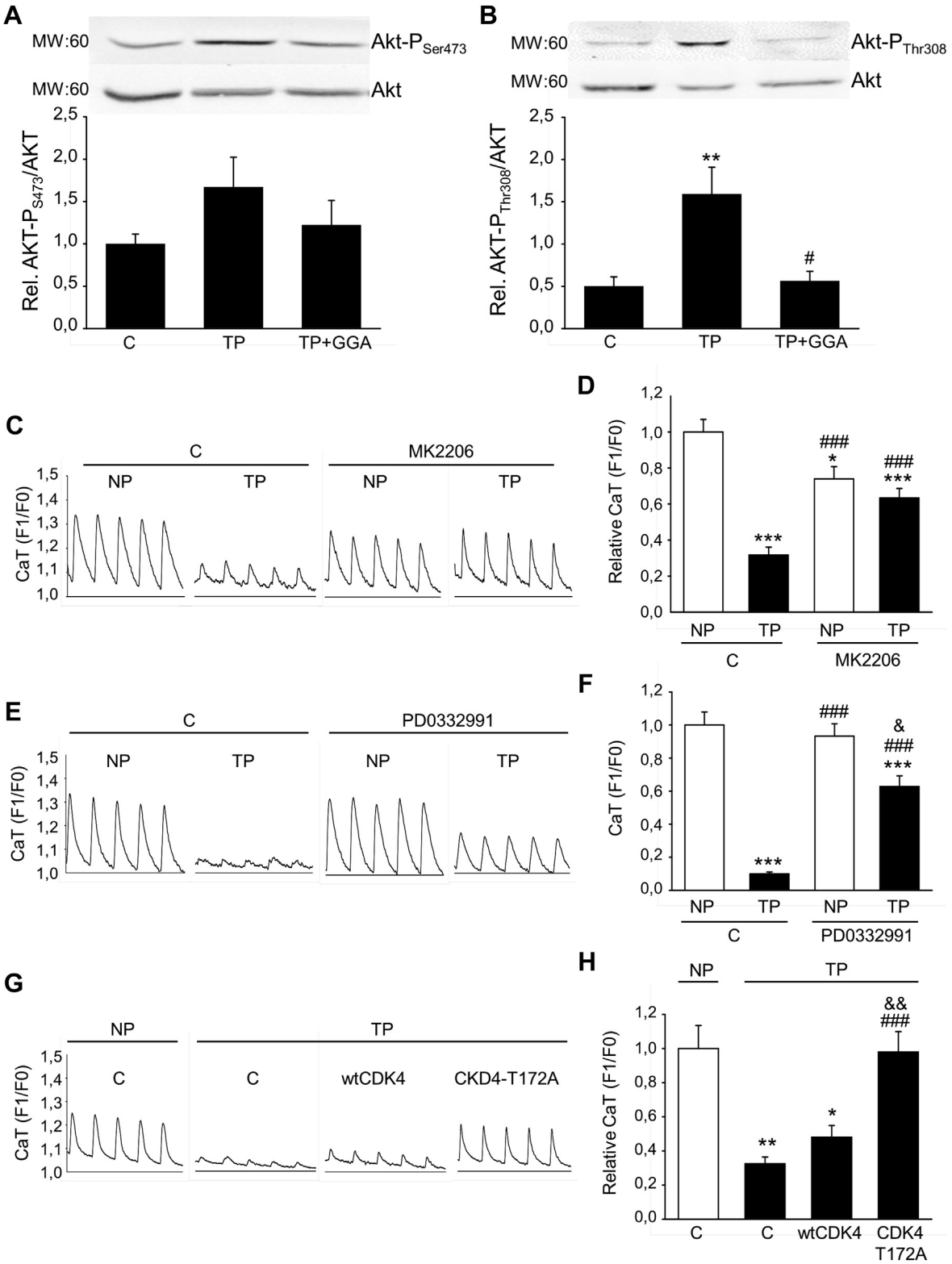

Figure 3 Inhibition of Akt and CDK4 phosphorylation protects against TP-induced contractile dysfunction in HL-1 cardiomyocytes. **A** and **B:** The *top panel* presents Western blots of phospho-Akt Ser473 and Thr308, respectively, and Akt, and the *lower panel* presents quantified data of the ratio phosphorylated protein normalized to basal protein levels. $^{**}P \leq .01$ vs C; $^{\#}P \leq .05$ vs TP. **C:** Representative CaT of HL-1 cardiomyocytes after NP or TP, pretreated with the Akt inhibitor MK2206. **D:** Quantified relative CaT amplitude of NP and TP HL-1 cardiomyocytes, each from groups as indicated. $^{*}P \leq .05$; $^{***}P \leq .001$ vs C NP; $^{\#\#\#}P \leq .001$ vs C TP. **E:** Representative CaT of HL-1 cardiomyocytes after NP or TP, pretreated with the CDK4 inhibitor PD0332991. **F:** Quantified CaT amplitude of NP and TP HL-1 cardiomyocytes, each from groups as indicated. $^{***}P \leq .001$ vs C NP; $^{\#\#\#}P \leq .001$ vs C TP; $^{\&}P \leq .05$ vs NP PD0332991. **G:** Representative CaT of HL-1 cardiomyocytes transfected with wtCDK4 or CDK4-T172A, followed by NP (Supplemental Figure S2) or TP. **H:** Quantified relative CaT amplitude of NP and TP HL-1 cardiomyocytes transiently transfected with wtCDK4 or CDK4-T172A. $^{*}P \leq .05$; $^{**}P \leq .01$ vs C NP; $^{\#\#\#}P \leq .001$ vs C TP; $^{\&\&}P \leq .01$ vs wtCDK4 TP. C = nonpaced controls; CaT = calcium transient; NP = normal pacing; TP = tachypacing; wt = wild type.

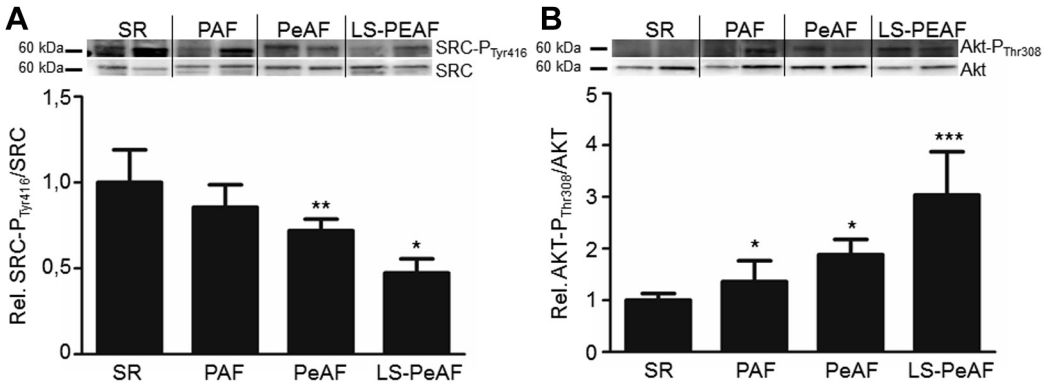

Figure 4 Kinase activity in patients with atrial fibrillation is comparable to findings of the kinome array. **A:** The *top panel* presents Western blots of phospho-SRC Tyr416 and SRC, and the *lower panel* presents quantified data of the ratio phosphorylated protein normalized to basal protein levels. **B:** The *top panel* presents Western blots of phospho-Akt Thr308 and Akt, and the *lower panel* presents quantified data of the ratio phosphorylated protein normalized to basal protein levels. LS-PeAF = long-standing persistent atrial fibrillation; PAF = paroxysmal atrial fibrillation, PeAF = persistent atrial fibrillation; SR = sinus rhythm. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$ vs SR.

effects on healthy cardiomyocytes, Akt inhibitors may provide clues for the development of novel treatment paradigms to counteract AF-induced remodeling.

Identification of kinases altered in tachypacing-induced atrial remodeling in patients with AF

To extrapolate the findings in humans with AF, we studied the expression of 2 kinases, SRC and Akt, which showed the strongest and clearest signals after Western blot analyses. SRC and Akt showed reduced and increased kinase activity, respectively, in the kinome array. The expression of phospho-SRC (Tyr416) and phospho-Akt (Thr308) relative to total SRC and Akt was determined in right atrial appendages of patients with paroxysmal AF, persistent AF (PeAF), and long-standing persistent AF (LS-PeAF) along with control patients in SR. Patients with AF showed, similar to the kinome array, a decrease in SRC phosphorylation at Tyr416 (Figure 4A and Supplemental Figure S4). Moreover, the degree of SRC phosphorylation at Tyr416 is dependent on the stage of AF, as the level of SRC phosphorylation in patients with PeAF and LS-PeAF is significantly reduced compared with that in patients in SR. In addition, the phosphorylation levels of Akt at Thr308 were gradually increased in AF (Figure 4B), which also corresponds with the findings of the kinome array. Similar to SRC, the phosphorylation levels of Akt at Thr308 were significantly increased in patients with PeAF and LS-PeAF compared with those in SR. Expression of CDK4 was inconclusive (Supplemental Figure S5) because of the poor quality of the available antibodies. These findings suggest that kinases identified by the kinome array may be relevant to atrial remodeling changes in patients with AF.

Discussion

In the present study, we used kinomic array analysis to identify potential kinases altered in tachypacing-induced AF promotion in a dog model of AF. We then used a strategy to contrast kinase activity profiles of untreated TP dogs with those of GGA-treated TP dogs, which are protected from atrial remodeling. Our kinomic analysis and diagnostics confirmed excellent reproducibility within and between slides for canine atrial lysates, confirming the applicability of this kinase array method for this in vivo canine model. In addition, selected findings were further tested by studying atrial tissue samples from humans with AF and controls in SR. Tachypacing induced alterations in the activity of 50 putative kinases, of which 29 kinases were increased in activity. GGA treatment suppressed activity changes in 40 kinases (80%), suggesting that GGA partially prevents kinome dysregulation in TP dogs. Two of these kinases, Akt and CDK4, showed enhanced activity due to tachypacing and met criteria for further analysis in dog tissue and HL-1 model systems. Similar to kinomic array results, Western blot analysis of Akt in dog atrial tissue demonstrated that the tachypacing-induced Akt phosphorylation at Thr308 was restored to control values in GGA-treated dogs. In addition, pharmacological inhibition of both Akt and CDK4 protected against tachypacing-induced contractile dysfunction in HL-1 cardiomyocytes. The present study suggests a role for kinomic array analysis as a method in identifying changes in kinase activity in TP dog hearts, which show similar expression in human AF and which might have value in identifying novel therapeutic target approaches for clinical AF treatment.

Identification of kinases involved in tachypacing-induced atrial remodeling

In this study, we identified various kinases that were altered by atrial tachypacing but conserved by tachypacing in combination with a cardioprotective GGA treatment. GGA has been used in Japan as an antiulcer drug since 1984 without serious side effects.[17] Previous studies revealed that the protective effect of GGA occurs via the upregulation of HSPs, which results in the attenuation of contractile dysfunction and electrical and structural remodeling of cardiomyocytes.[9] The upregulation and phosphorylation of heat shock protein

27 is particularly important for the protective effects of GGA.[9] Consistent with these previous findings, the present study shows that GGA partially conserved kinome homeostasis in TP dogs. Consequently, the ability of HSPs to alter kinase activity may play a role in GGA-mediated protection of the kinome as suggested previously.[10,11] Specifically, HSPs may bind to substrates, thereby shielding their phosphorylation sites from kinases. However, the identified kinases cover a broad spectrum of functions and substrates, including pathways of differentiation and proliferation, contraction, metabolism, immunity, development, cell cycle, and survival. Consequently, as an alternative to substrate shielding, a broad induction of HSPs may prevent AF-induced derailment of cellular protein homeostasis (ie, proteostasis) by maintaining protein folding, trafficking, function, and clearance, thus attenuating kinase activation. Such conservation of the proteostasis network by HSPs may represent an upstream event in AF, thereby explaining why GGA treatment precludes activation of the vast majority of kinases in TP dogs.

Among the significantly altered kinases, PKA,[18,19] protein kinase C (PKC),[18] and SRC[4] were previously reported to regulate ion channel function. Increased phosphorylation of the substrates connexin 43,[20] phospholamban,[19] and the ryanodine receptor[21] has been reported in human and experimental models of AF. Phosphorylation of connexin 43 alters gap junction function, changes that are associated with the development of reentrant arrhythmias, including AF.[22] Increased phosphorylation of phospholamban[19] and the ryanodine receptor,[5] both important for cardiac calcium homeostasis, promotes diastolic sarcoplasmic reticulum calcium leak and delayed afterdepolarizations.

Phosphorylation of kinases involved in cardiac contraction appeared differentially regulated. MLCK was reduced in the TP group and (partially) restored in the TP+GGA group. Reduced phosphorylation of MLCK may lead to modulation of cardiac muscle contractility by increasing the sensitivity of the contractile elements to calcium.[23] Conversely, titin phosphorylation was induced, which may contribute to its altered localization or degradation upon tachypacing.[7]

Kinomic array analysis further showed increased extracellular signal regulated kinase 5 (ERK5) phosphorylation upon TP, which may induce activation of its downstream substrate myocyte enhancer factor 2A (MEF2A). MEF2A has been recognized as an important component in the induction of the myocardial hypertrophic genetic program in response to stretch and maladaptive cardiac remodeling[24] and regulates metabolism and sarcomeric organization in the heart.[25]

Although Akt signaling is predominantly viewed as a prosurvival and proliferative pathway, our results imply that tachypacing-induced phosphorylation of Akt has a detrimental action in TP cardiomyocytes. Activation of Akt may induce changes in the cytoskeleton owing to Akt-induced phosphorylation of tumor suppressor tuberous sclerosis complex 2 and paladin involved in F-actin stress bundle formation[14] and actin bundling,[26] respectively. Moreover, Akt signaling is involved in fibrosis formation, which creates a substrate for AF initiation and maintenance. Cardiac hypertrophy, a major risk factor for the development of congestive heart failure, which often occurs concomitantly with AF,[27] may be due to activation of CDK4.[15,28] Interestingly, both Akt and CDK4 activation are linked to Ras homolog gene family member A (RhoA) activation, as observed in experimental models of tachypacing and clinical AF, which leads to the formation of F-actin stress fibers, resulting in changes in structural proteins and gene expression and thereby creating a substrate for AF.[3] RhoA was shown to inhibit expression of HSPs by precluding HSF1 binding to promotor regions.[29] The beneficial action of GGA may thus consist of overriding the RhoA-mediated inhibition of the heat shock response, thus enabling TP cardiomyocytes to mount a proper cell protective response. This mechanism may offer an alternative explanation as to why this treatment normalized the activity of the majority of the kinases activated by tachypacing.

Novel inhibitors of kinases to treat clinical AF

Interestingly, the 2 kinases identified in the present study offer therapeutic options that might be within clinical reach. Various Akt inhibitors are currently being tested in clinical phase II trials for beneficial effects against myelofibrosis and breast cancer.[30] However, agents that inhibit Akt phosphorylation have been shown to increase AF susceptibility.[31] In addition, inhibition of Akt phosphorylation reduced contractility in nonpaced cardiomyocytes (Figure 3C). This may indicate that Akt inhibition in healthy cardiac tissue may increase AF susceptibility and may therefore not be a realistic therapeutic option for clinical AF. Interestingly, recent findings indicate tachypacing-induced endoplasmic reticulum stress to result in downstream Akt activation, activation of autophagy, and, consequently, AF progression. In line with this, pharmacological inhibition of endoplasmic reticulum stress by 4-phenylbutyrate protected from AF promotion.[32] These results indicate that inhibition of molecular mechanisms upstream of Akt activation may represent alternative routes to attenuate the effects of Akt activation. CDKs have been considered promising drug targets for a number of years, but most CDK inhibitors have failed rigorous clinical testing. Recent studies demonstrating clear anticancer efficacy and reduced toxicity of CDK4/6 inhibitors such as palbociclib and multi-CDK inhibitors such as dinaciclib have rejuvenated the field. Favorable results with palbociclib and its recent US Food and Drug Administration approval demonstrate that CDK inhibitors with narrow selectivity profiles can have clinical utility for therapy based on individual tumor genetics.[33]

Our findings identified kinases with a potential role in the pathophysiology of AF. More research is needed to elucidate whether this protective effect occurs in in vivo animal models of AF and, if so, what downstream targets and signaling pathways are involved.

Current limitations of the kinase array technique

The kinome array technique that we applied here, using short pseudosubstrates based on human sequences, has been used successfully in diverse other studies and species.[34] A limitation of the present study is the restricted verification of our kinome array by additional methods due to poor cross-reactivity of antibodies, which prevented us from verifying the phosphorylation status of the majority of proteins in the dog tissue. In addition, it might be possible that the identified kinases are regulated in response to AF, rather than in a primary pathophysiological manner. While a substantial number of dysregulated kinases were identified with the current approach, some may have gone undetected because of technical limitations including cell lysis–induced dilution of cofactors/activators for kinases and nonspecificity of consensus peptides due to interspecies differences.[35] Furthermore, false-positive results likely include consensus peptide sequences on the array representing proteins that are not expressed in cardiac tissue. By using a design essentially contrasting TP group with TP+GGA group, we substantially enhanced specificity of identified substrates altered by TP. Another limitation of this study is the lack of a nonpaced GGA-treated dog group. However, it was previously shown that GGA treatment of isolated nonpaced dog atrial cardiomyocytes did not affect electrical and contractile function.[9] Although the kinome array technique is a valuable tool to provide an overview of altered kinase activity, the targets are putative and the limitations warrant the verification of the phosphorylation status and biological effect of the candidate targets in AF by other molecular biological techniques.

Conclusion

The present study shows that kinomic array analysis of control and treated subjects may offer a versatile tool to identify kinases altered in atrial remodeling in a dog model of AF, which might be translatable to humans with AF. Although the kinome array identified changes in kinases that could lead to further testing of candidate kinases, testing of 1 of the 2 drugs yielded unexpected opposite results, so results of kinome arrays can only be hypothesis generating.

Appendix

Supplementary data

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.hrthm.2018.06.014.

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