Clinical Effects of Phosphodiesterase 3A Mutations in Inherited Hypertension With Brachydactyly


Abstract—Autosomal-dominant hypertension with brachydactyly is a salt-independent Mendelian syndrome caused by activating mutations in the gene encoding phosphodiesterase 3A. These mutations increase the protein kinase A-mediated phosphorylation of phosphodiesterase 3A resulting in enhanced cAMP-hydrolytic affinity and accelerated cell proliferation. The phosphorylated vasodilator-stimulated phosphoprotein is diminished, and parathyroid hormone-related peptide is dysregulated, potentially accounting for all phenotypic features. Untreated patients die prematurely of stroke; however, hypertension-induced target-organ damage is otherwise hardly apparent. We conducted clinical studies of vascular function, cardiac functional imaging, platelet function in affected and nonaffected persons, and cell-based assays. Large-vessel and cardiac functions indeed seem to be preserved. The platelet studies showed normal platelet function. Cell-based studies demonstrated that available phosphodiesterase 3A inhibitors suppress the mutant isoforms. However, increasing cGMP to indirectly inhibit the enzyme seemed to have particular use. Our results shed more light on phosphodiesterase 3A activation and could be relevant to the treatment of severe hypertension in the general population. (Hypertension. 2015;66:800-808. DOI: 10.1161/HYPERTENSIONAHA.115.06000.)

Key Words: blood platelets • brachydactyly • cyclic nucleotide phosphodiesterases, type 3 • genetics • HTNB • hypertension

Considerable strides have been made elucidating the molecular genetics of hypertension.1 Mendelian syndromes have defined pathways for blood pressure regulation. The common underlying mechanisms seem to involve perturbations that may increase distal nephron sodium reabsorption and also impair normal vasodilator responses to increases in salt intake.2,3 We reported on the sole Mendelian exception to date, autosomal-dominant hypertension with brachydactyly type E, first described in a Turkish kindred (hypertension with brachydactyly [HTNB], OMIM #112410).4,5 Per protocol, we determined that affected persons had salt-resistant hypertension.6 Blood pressure remained elevated during ganglionic blockade, consistent with an intrinsic vascular abnormality.6 Furthermore, affected persons exhibited dramatic decreases in blood pressure when exposed to nitroprusside.6 Dissected arterioles from HTNB7 showed robust medial hyperplasia.8 The increased blood pressure is strongly age-dependent.9 At age 50 years, the mean arterial blood pressure differed ≈50 mm Hg between affected and nonaffected family members and stroke commonly occurred. The penetrance of the syndrome is 100%.

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HTNB is linked to chromosome 12p, as shown in 6 unrelated families. The locus seems to be relevant to essential hypertension supported by a linkage study in Chinese hypertensive families without brachydactyly type E. Furthermore, a recent genome-wide association study from Framingham also identified the location we report.

The molecular causes for HTNB are heterozygous missense, gain-of-function mutations in the gene encoding the cGMP-inhibited cAMP phosphodiesterase 3A (PDE3A). PDE3A belongs to the PDE3 family, which catalyzes the hydrolysis of the intracellular second messengers, cAMP and cGMP. PDE3A plays a prominent role in heart, vascular smooth muscle cells (VSMCs), oocytes, and platelets. In HTNB, the mutational PDE3A enhances VSMC proliferation that together with increased constriction is likely causative for the increased peripheral vascular resistance. Hypertension arises in childhood, and treatment modalities are flexible. Nevertheless, conventional hypertension-induced target organ damage in the form of cardiac hypertrophy, hypertensive retinopathy, and kidney damage is remarkably mild. PDE3A is abundant in platelets. We investigated ventriculoarterial coupling and platelet function in HTNB patients. We used in vitro pharmacological testing to study the mutated enzymes and suggest that increasing cGMP in VSMC could be of therapeutic use in patients with HTNB.

Methods

After internal review-board (IRB, Charité University Medicine Berlin) approval, we invited 4 affected and 4 non-affected members from our Turkish kindred with the T445N mutation to participate in clinical research center–based studies. Written informed consent was obtained from all participants. Detailed methods are available in the online-only Data Supplement.

Central Blood Pressure

Our technique is based on the wave reflection method using a common oscillometric cuff (ARCSolver) and was validated against the standard tonometric method, as well as compared with invasive aortic blood pressure measurements. During cardiovascular magnetic resonance imaging (CMR), a compatible blood pressure measurement device was used. Blood pressure was measured 3× at baseline and during handgrip testing.

Echocardiography

Transthoracic echocardiography (Vivid E9, GE Healthcare) was performed using a 4V-D active matrix 4D volume phased array transducer in steep left lateral decubitus position. A standardized ultrasound examination protocol was developed for this clinical study and applied in each patient. Electrocardiographic-guided loops using standard acoustic windows were stored for off-line image analysis. To assess left-ventricular (LV) size, volume, and contractility, a complete set of standardized views encompassing LV and atria was obtained at rest in 2-dimensional (2D) and color Doppler mode. A volume mode was used for 4D data sets. A pulsed Doppler at the tips of the mitral valve leaflets and pulsed tissue Doppler in the mitral valve plane were obtained to assess diastolic function and time intervals.

Cardiovascular Magnetic Resonance Imaging

All CMR scans were performed on a clinical 1.5 Tesla MR scanner (Avanto, Siemens Medical Solutions AG, Erlangen, Germany) using a 12-channel cardiac array coil. We performed cine imaging with a standard steady-state free precession sequence to assess cardiac structure and function. We acquired 3 long axes and a complete short-axis package covering the left ventricle from base to apex during repetitive breath holds in end-expiration. To assess aortic stiffness, we measured aortic distensibility and pulse wave velocity according to the transit time method by validated CMR techniques.

Handgrip Testing

For the handgrip test, the subject squeezed a rubber ball with 30% of his maximal voluntary force for 3 minutes. Handgrip testing was also performed during CMR imaging. A CMR compatible prototype was used (Sensory-Motor Systems Laboratory, Zurich, Switzerland) consisting of the plastic handgrip, a plastic fiber cable, an electronic switch box, and corresponding software measuring force by an optical principle.

Platelet Aggregation

For the preparation of platelet-rich plasma, citrate anticoagulated blood samples were centrifuged at 100g for 10 minutes. Supernatant was carefully transferred to polypropylene tubes (Falcon tubes, BD Bioscience, Bedford). Platelet count was adjusted to 2×10^5 platelets/µL (accepted range, 1.9–2.1×10^5 platelets/µL) with blood group AB plasma and stored at 37°C until use. Platelets were activated with thrombin receptor activator peptide (TRAP) and collagen. Transmission of light through a platelet suspension was continuously measured over time with conventional automated data acquisition and analysis (Labview). Nine different concentrations of TRAP-6 (H-Ser-Phe-Leu-Leu-Arg-ASN-OH; Bachem Inc, Switzerland) and Collagen reagent Horm (Takeda Austria GmbH, Austria) were examined in replicates for each patient and control. Measurements of the area under the aggregation curve were plotted against the concentrations of each stimulus with DeltaGraph v7.0.1 software (Red Rock software, Inc and Equation Editor Design Sciences, Inc).

Milorafine (1,6-dihydro-2-methyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile) was used to inhibit platelet aggregation (Sigma-Aldrich). Platelets were preincubated with the inhibitor for 5 minutes. Milorafine concentrations between 0.03 and 100 µmol/L were tested for the inhibition of TRAP-6 and collagen-induced aggregation (100 and 33.3 µmol/L TRAP; 20 and 6.6 mg/mL collagen).

Immunoblotting of Transfected HeLa, Platelets, and Human Heart Lysates

A commonly used, cancer-derived cell line named after Henrietta Lacks (HeLa) cells were transiently transfected with an empty vector (sc300-w/o) or a vector encoding full-length wild-type PDE3A cDNA (Origene, SC300151, NM_000921), and 48 hours after transfection, cells were stimulated for 1 hour.
with 20 µmol/L Forskolin and 100 ng/mL phorbol 12-myristate 13-acetate (PMA). HeLa cells were scraped in PBS and subsequently centrifuged (5 minutes, 4°C, 275g). Pelletted cells were lysed in RIPA buffer (50 mmol/L Tris, pH 7.8, 10% glycerol, 150 mmol/L NaCl, 1% Triton X, 0.025% sodium deoxycholate, and 1 mmol/L EDTA) supplemented with protease and phosphatase inhibitors (Complete and PhosSTOP; Roche), incubated for 10 minutes on ice, and subsequently centrifuged (15 minutes, 4°C, 21,250g). Platelet-rich plasma was stimulated with 200 µmol/L TRAP-6 and 40 µg/mL collagen to induce platelet aggregation. Human cardiac tissue was kindly provided by Dr med. Sabine Klaassen, MDC, Berlin.

Subcellular Fractionation

HeLa cells from two 10-cm cell culture dishes transiently expressing PDE3A (Origene, SC300151; or PDE3A with 1 of the 6 HTNB mutations) were scraped in 1 mL of PBS. Short centrifugation (5 minutes, 4°C, 275g) pelleted the cells for resuspension in 100 µL of fractionation buffer (250 mmol/L sucrose, 20 mmol/L HEPES, pH 7.4, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L dithiothreitol) supplemented with protease and phosphatase inhibitors (see above).

Cell-Based Studies

We performed functional cAMP responsive element luciferase assays in HeLa cells transiently expressing the 6 HTNB PDE3A mutations in comparison with wild-type PDE3A. HeLa cells were transfected with an empty vector (sc300-w/o), a wild-type PDE3A (Origene, SC300151) expression construct, and the 6 full-length mutant PDE3A expression plasmids. HeLa cells were cotransfected with a CAMP responsive element regulating luciferase. The measured luciferase activity was dependent on the cellular cAMP level. A renilla plasmid, and the 6 full-length mutant PDE3A expression plasmids. HeLa cells were cotransfected with a CAMP responsive element regulating luciferase. The measured luciferase activity was dependent on the cellular cAMP level.

Statistics

We used parametric statistics (repeated measures ANOVA) on normally distributed data. We relied on nonparametric statistics where indicated. For cell-based experiments, the functional in vitro experiments were reproduced 2 to 6 times. Numbers (n) of experiments are mentioned in the figure legends. Significance was determined by nonparametrical Wilcoxon rank-sum and Mann–Whitney tests (***P<0.001, **P<0.01, and *P<0.05). Scatter plots show mean, and T-bars indicate SEM.

Results

We were able to recruit 4 affected persons (AFF) harboring the PDE3A T445N mutation and 4 nonaffected relatives (NON) for this study (Table). Heart rate was similar in affected (1 man and 3 women; age, 16–60 years) and nonaffected family members (2 men and 2 women; age, 23–58 years). Central systolic blood pressure and central diastolic blood pressure tended to be higher in affected family members (Figure 1A). One older male nonaffected family member (046) was obese, hypertensive (treated with valsartan and amlodipine), and had known atherosclerosis. Affected family members showed no profound changes in left ventricular mass, cardiac output, distensibility of the ascending aorta, or in aortic pulse wave velocity measured by CMR. The highest values for aortic pulse wave velocity associated with low aortic distensibility were obtained in AFF 035 and in NON 046 (Figure 1B). Effective arterial elastance (cEa), calculated as central aortic end-systolic blood pressure divided by stroke volume, was higher in affected family members (Figure 1C). Left ventricular end-systolic elastance, calculated by a modified single-beat method but factoring in central systolic blood pressure and central diastolic blood pressure, was also higher in affected family members. The ratio between Ea and Ees-sb, which reflects the degree of ventriculoarterial coupling, was normal in both groups. Measurements during handgrip testing showed similar results.

Cardiac visualization results were consistent with the clinical data. For instance, LV function of AFF 035 as assessed by multislice imaging with 4D echocardiography (Figure S1 in the online-only Data Supplement), including quantification of the LV global and regional function by longitudinal strain bull’s eye with averaged values for the 17 LV segments and corresponding curves. Affected persons showed no profound changes in left ventricular mass, cardiac output, or distensibility of the ascending aorta measured by CMR. We performed cardiac MRI and covered completely the left ventricle to quantify volume and function. These images from AFF 035

Table. Demographic Data

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Listed are the status as affected (AFF) and nonaffected (NON); age in years; sex classified as male and female; height in cm; weight in kg; body mass index (BMI) in kg/m²; systolic (SBP), diastolic (DBP), and mean (Mean) arterial blood pressure in millimeters of mercury, sitting position; and SBP and DBP percentile normalized for age, sex, and height.

*Blood pressure values under treatment.
attest to the normal cardiac function in these subjects determined by CMR (Figure 2).

In cardiac myocytes, PDE3A1 interacts with a multiprotein complex, which contains A-kinase anchoring protein (AKAP18) and sarcoplasmic reticulum Ca\(^{2+}\) ATPase 2 (SERCA2A), and controls Ca\(^{2+}\) reuptake into the sarcoplasmatic reticulum. This localization at the sarcoplasmatic reticulum involves Ser\(^{292}/\)Ser\(^{293}\) phosphorylation of PDE3A1.\(^{28,29}\) We

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**Figure 1.** A, Individual values of heart rate (HR, left), central systolic blood pressure (cSBP, middle), and central diastolic blood pressure (cDBP, right) in affected (aff) and nonaffected (nonaff) family members are shown. B, Individual values of left ventricular mass (LVmass, top left), cardiac output (CO, top right), aortic distensibility (Ao-distens, bottom left), and aortic pulse wave velocity (PWV-CMR, bottom right) in aff and nonaff family members measured by cardiac magnetic resonance imaging (CMR). C, Individual values of effective arterial elastance (cEa, left), single-beat LV end-systolic elastance (Ees-sb, middle), and the cEa/Ees-sb ratio (cEa/Ees-sb, right) in aff and nonaff family members measured by echocardiography (Echo). All affected are symbolized using gray, and the nonaffected are symbolized using black color. Nonaff 046 was hypertensive, obese, and had atherosclerosis (large diamond-shaped symbol).

**Figure 2.** Image shows the full coverage of the left ventricle of AFF 035 for quantification of volumes and function using cardiovascular magnetic resonance as an accepted gold standard for volumetry.
aimed to elucidate whether the 6 HTNB-causing PDE3A mutations that affect its phosphorylation at Ser\textsuperscript{428} and Ser\textsuperscript{438} also had an effect on compartmentalization. In PMA/forskolin-stimulated cells, we observed a slight increase of the mutational PDE3A isoforms phosphorylated at Ser\textsuperscript{428} in the microsomal fraction, supporting the idea that mutation-dependent increased Ser\textsuperscript{428} phosphorylation leads to an enhanced association of the mutational enzymes to the membrane (Figure S2).

We found no differences in platelet aggregation by activation using TRAP or collagen (Table S1). Although the data suggest a minor but consistent shift in the platelets responsiveness to milrinone, as an inhibitor of platelet aggregation in affected individuals, the high interindividual variability does not allow demonstrating a statistical significance of this effect. We compared the PDE3A isoforms of human cardiac tissue, HeLa cells, and transiently transfected HeLa cells expressing full-length wild-type PDE3A versus platelet PDE3A by Western blotting. We illustrate the identified mutation sites, the known PDE3A isoforms, their relationship to the catalytic domain, and the protein domains recognized by the antibodies (Figure 3A). As expected, the 3 known isoforms, PDE3A1 (microsomal), PDE3A2, and PDE3A3 (both microsomal and cytosolic), were detected in the cardiac tissue and in transfected HeLa cells. Platelets expressed predominantly the PDE3A2 isoform (Figure 3B).\textsuperscript{30} Intense signals of the antibody to PDE3A phosphoSer\textsuperscript{438} were observed, suggesting a highly phosphorylated PDE3A2 isoform on platelet activation with TRAP-6 or collagen.

To test for possible pharmacological intervention aiming at increasing cAMP levels, HeLa cells were transfected with full-length wild-type or 1 of the 6 full-length PDE3A mutational expression constructs and with a cAMP responsive element regulating luciferase transcriptional activity. Increasing forskolin concentrations enhanced the endogenous cAMP levels. Previously, we have shown that the PDE3 mutations in the sole presence of forskolin or l-arginine showed a significant reduction of the cAMP responsive element–mediated luciferase activity as a result of the higher cAMP hydrolysis when compared with wild-type PDE3A (http://www.nature.com/ng/journal/v47/n6/fig_tab/ng.3302_SF6.html).\textsuperscript{14} The mutated PDE3A proteins hydrolyzed more cAMP in the presence of cilostazol (Figure 4A) compared with controls (\(P<0.002\)). Milrinone decreased (\(P<0.002\)) the enhanced cAMP hydrolysis more effectively than cilostazol with increasing cAMP levels (Figure 4B). The effect on the mutants was less than on wild-type PDE3A cAMP hydrolysis. Moreover, PDE3A was competitively inhibited by cGMP.\textsuperscript{31} cGMP stimulation using cilostazol showed that cGMP competitively inhibited cAMP hydrolysis with a significant (\(P<0.002\)) difference between mutants and wild-type PDE3A (Figure 4C). The data indicate that the more cGMP is present, the less was the cAMP hydrolysis, and the greater was the measured luciferase activity. Cilostazol abrogated the inhibitory effect of the increasing l-arginine concentrations on the mutants more (\(P<0.002\)) than on the wild-type PDE3A (Figure 4C). The cGMP inhibition and milrinone inhibition were synergistic and equalized the mutant and the wild-type PDE3A hydrolytic activities (Figure 4D). The soluble guanylate cyclase stimulator, BAY 41 to 8543, reduced the enhanced cAMP hydrolysis of the PDE3A mutations to the levels of the wild-type PDE3A (Figure 4E).

**Discussion**

We determined earlier that HTNB featured increased peripheral vascular resistance without salt sensitivity.\textsuperscript{5} In that study, we also found that fibroblasts from the patients proliferated at a slightly increased rate, compared controls.\textsuperscript{5}
Analogous observations have been made earlier in VSMC in spontaneously hypertensive rats. Results from our recent study confirmed our earlier observations. We also observed altered phosphorylation of vasodilator-stimulated phosphoprotein that could influence VSMC proliferation and function. The findings bring to mind the arguments raised by Folkow, who taught that vascular hypertrophy, remodeling, and reactivity could be a driver of peripheral vascular resistance in hypertension. Also contributing to an increase in peripheral vascular resistance is an increase in myosin light-chain phosphorylation via myosin light-chain kinase. MYH9 mutation-related disturbances originating in the vasculature itself or in sites outside the vasculature, such as brain or adrenal glands, that have the capacity to affect vascular function. We would support such an interpretation.

We aimed to study ventriculoarterial coupling in patients with HTNB during rest and isometric handgrip challenge. Under normal conditions, the heart and the vasculature are fine tuned in structure and function to maintain the oxygen supply for peripheral organs and demand at a minimum of energy consumption. The relationship between the heart and the vasculature is well maintained in animals at different levels of volume load and during exercise. Several groups have proposed a new approach to evaluate the interaction between the ventricle and the vasculature based on the relation between LV end-systolic elastance (Ees) and Ea in health and disease. According to Chen et al, left ventricular end-systolic elastance can be calculated by a modified single-beat method using systolic (P(s)) and diastolic (P(d)) arm-cuff pressures, echo-Doppler stroke volume, and echo-derived ejection. An Ea/Ees coupling ratio of 0.6 to 1.2 is close to optimal efficiency. This range is normally maintained under various physiological stresses. However, the coupling ratio can become high in systolic heart failure, where depressed systolic function (low Ees) is coupled to high arterial impedance (high Ea). In the observations reported here, we could detect no differences between

Figure 4. A, Hela cells were transfected with empty vector (sc300-w/o, blue line), wild-type (red line), and mutated PDE3A expression vectors (gray and black lines). Hydrolysis of cAMP was monitored as cAMP responsive element (CRE) luciferase activity. With incremental forskolin concentrations, the PDE3A mutations showed a significant reduction of the CRE-mediated luciferase activity as a result of the higher cAMP hydrolysis compared with the wild-type PDE3A. Cilostazol showed no effect abrogating the PDE3A mutations gain-of-function effects. B, Milrinone decreased the enhanced cAMP hydrolysis more effectively than cilostazol with increasing cAMP levels. C, cGMP stimulation with increasing L-arginine concentrations and cilostazol had light effects blocking the enhanced cAMP hydrolysis. D, cGMP in combination with milrinone determined that cGMP competitively inhibited cAMP hydrolysis. E, The soluble guanylate cyclase stimulator BAY 41–8453 reduced the enhanced cAMP hydrolysis of the PDE3A mutations compared with the wild-type PDE3A. **P<0.002, mean±SD; n=3; Wilcoxon and Mann–Whitney test).
affected and nonaffected persons. Cardiac function and morphology were remarkably normal in affected persons, similar to our earlier observations. The findings raise the hypothesis that perhaps the PDE3A overactivity somehow protects the heart from injury.

Earlier observations support the idea that phosphoinositide 3-kinase (PI3K) coordinates the coincident signaling of the major cardiac PDE3 and PDE4 isoforms, thus orchestrating a feedback loop that prevents calcium-dependent ventricular arrhythmia. Other investigators examined the role of PDE3A in regulating myocardial function and survival in vivo using transgenic mice with myocardial PDE3A1 overexpression. These mice were protected against ischemia–reperfusion injury presumably because of reduced β-adrenergic receptor–mediated signaling. Still other investigators used the same transgenic overexpression model to determine PDE3A-related effects on angiotensin-II–induced cardiac hypertrophy. The findings suggested that PDE3A protects the heart from angiotensin-II–induced cardiac remodeling through its modulation of the functional connection between angiotensin-II and transforming growth factor-β. Although PDE3A2 is the prominent isoform in VSMC, PDE3A1 associates with SERCA2, phospholamban, and AKAP18 in a multiprotein signalosome in human cardiac sarcoplasmatic reticulum, where it regulates a discrete cAMP pool that controls contractility by modulating Ca²⁺ reuptake into the sarcoplasmatic reticulum during diastole. Our preliminary experiments using a HeLa cell transfection model suggest that increased phosphorylation at Ser428 and Ser 438 phosphorylation. We found that platelets derived from a control person showed brisk phosphorylation of PDE3A2 at Ser438 on stimulation with TRAP-6 or collagen. Hunter et al observed that platelet activation stimulates protein kinase C–dependent phosphorylation of PDE3A on various serine residues, among others Ser428 and Ser438, leading to a subsequent increase in cAMP hydrolysis. We postulate that platelet activation induces nearly saturated phosphorylation of residue Ser438 that would impede and compensate the effects of the PDE3A mutations on the phosphorylation status, as well as the hydrolytic activity of the enzyme in our HTNB patients. Furthermore, the T445N mutation could support the interaction of the enzyme with milrinone in platelets.

We investigated the pharmacological effects of milrinone, cilostazol, and a soluble guanylate cyclase stimulator in cells expressing each of the 6 PDE3A mutational variants. These studies indicated that mutated PDE3A was associated with increased cAMP-hydrolytic activity, even when exposed to the stimulator of adenylyl cyclase, forskolin. We found that milrinone and cilostazol diminished the increased PDE3A activity. Adding arginine helped, presumably by generating cGMP, which inhibits binding and hydrolysis of cAMP. The cGMP also activates cGMP-dependent protein kinase G1. Eliminating the binding of PKG1 to myosin light-chain phosphatase leads to VSMC abnormalities of contraction, relaxation, and systemic blood pressure in mice, independently of the renal function, a situation similar to that in our patients. Increasing cGMP by stimulating soluble guanylate cyclase has developed into a sound, promising, therapeutic concept and has shown utility in pulmonary hypertension. Augmenting natriuretic peptide–mediated cGMP production, physical exercise, or nephrilysin inhibition could all ameliorate cAMP deficiency in affected persons.

**Perspectives**

HTNB is a novel Mendelian model of increased peripheral vascular resistance. The current clinical data of patients carrying the PDE3A T445N mutation suggest that function of platelets is not affected and that the effects of the hyperactive enzyme may actually be cardioprotective. The mechanism of hypertension is increased peripheral resistance from vasoconstriction, vascular remodeling, or both. Our preliminary data suggest that soluble guanylate-cyclase stimulation could be of clinical benefit in this syndrome and perhaps in difficult-to-control hypertension in general.

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The project runs over 20 years. O. Toka, A. Aydin, H.R. Toka, S. Bähring, and F.C. Luft were initiators. O. Toka and A. Aydin organized the current study. J. Tank, O. Toka, A. Aydin, S. Elitok, M. Boschmann, and G. Rahn were responsible for local clinical research center (CRC) organization and conduct. J. Tank, J. Jordan, M. Boschmann, A. Mühl, and K. Mai were responsible for the vascular function and CRC protocol including MSC harvesting, imaging was performed by A. Töpper, W. Utz, and J. Schulz-Menger, platelet studies were performed by T. Müller, A. Döescher, and S. Gnoth, and cell-based studies were performed by C. Schichterle, P.G. Maass, E. Bartels-Klein, I. Hollfinger, A. Mühl, C. Lindschau, and Y. Wefeld-Neuenfeld with input from M.A. Movsesian, S. Bähring, and E. Klassmann.

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References


12. Toca et al. PDE3A and Hypertension
What Is New?

- We performed additional phenotyping in patients with Mendelian hypertension and brachydactyly type E and conducted functional studies on the phosphodiesterase 3A mutations responsible for the condition. Affected persons exhibit little in the way of cardiac changes. We can attribute all the phenotypes in hypertension and brachydactyly type E to overactive phosphodiesterase 3A.

What Is Relevant?

- This Mendelian form of hypertension is the first not related to sodium reabsorption in the distal nephron.

Novelty and Significance

Summary

Hypertension and brachydactyly type E is a model genetic disease highly relevant to essential hypertension because a generalized increase in peripheral vascular resistance and not sodium reabsorption is responsible. Linkage studies in families and a genome-wide association study in patients with essential hypertension underscore that interpretation.
Clinical Effects of Phosphodiesterase 3A Mutations in Inherited Hypertension With Brachydactyly


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Clinical effects of phosphodiesterase 3A mutations in inherited hypertension with brachydactyly

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Supplemental methods

**Echocardiography**
Assessment of LVEF took place at rest and during the third minute of isometric handgrip testing using standardized 4- and 2-chamber views. For assessment of systemic flow at rest and during handgrip testing, we obtained a velocity time integral by pulsed Doppler in the left ventricular outflow tract (LVOT). The systolic diameter of the LVOT was obtained from parasternal long axis view.

Cardiac output at rest and during handgrip test was calculated using the post-processing platform EchoPAC (GE Healthcare) for one representative R-R interval with two methods. First, we used two-dimensional volume measurements with the biplane method of disks summation (modified Simpson’s rule) based on the manual tracing of endocardial border. Papillary muscles were excluded from the cavity. Second, we relied on the product of the velocity-time integral (VTI) and the cross-sectional area (CSA) of the LVOT.

**Cardiac magnetic resonance (CMR)**
Furthermore, we acquired a double-oblique parasagittal image of the aorta to estimate the path length of the pulse wave along the aorta. All CMR imaging was done under resting conditions, but pulse wave velocity was estimated also following standardized stress. Subjects performed isometric handgrip exercise over a 3-minute stress period adjusting the pressure force at 30% of their individual maximum. Imaging and semiautomatic blood pressure measurement on the contralateral upper arm was started synchronously after 2 min handgrip exercise completing all acquisitions within 3 min.

Parameters of cardiac volumes and stroke volume were received from manual endo-contouring of end-systolic and end-diastolic short-axis images using cvi 42 (circle cvi, Calgary, Canada). For estimation of distensibility at the different aortic levels maximum and minimum areas of the aorta were determined from manual tracing of the vessel lumen. To estimate pulse wave velocity, the temporal delay of the pulse wave between ascending aorta and abdominal aorta was calculated from flow velocity curves and finally divided by the distance between these points. For this purpose, we acquired cine and phase-contrast images perpendicular to the flow direction at three aortic levels: ascending aorta and descending aorta at the level of the pulmonary artery bifurcation and abdominal aorta 5 cm below the diaphragm.

**Immunoblotting of transiently transfected HeLa, platelet, and human heart tissue lysate**
PBS washed platelet aggregates were resuspended in RIPA buffer supplemented with protease and phosphatase inhibitors, homogenized ultrasonically (Bandelin SONOPULS) and subsequently centrifuged (15 min, 4°C, 21,250 x g). Human cardiac tissue was homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors using a speed mill (SpeedMill P12; Analytik Jena AG). The cell homogenate was incubated for 30 min on ice followed by centrifugation (15 min, 4°C, 21,250 x g). The cleared lysates (transfected HeLa cells, platelet and human cardiac tissue) were quantified with Coomassie PlusTM Protein Assay Reagent (Thermo Scientific) and 20 µg of total protein of HeLa lysate and 50µg of total protein of platelet and human cardiac lysate were used for immunoblotting according to standard protocols. Detection of PDE3A isoforms was performed with antibodies to PDE3A (A302-740A; Bethyl Laboratories Inc. and SC-11830; Santa Cruz Biotechnology) and antibodies to PDE3A phospho-serine 428 and 438 (S446B
and S442B; University of Dundee). The A302-740A polyclonal antibody (Bethyl Laboratories Inc.; YA) was raised against an epitope of residues 450-500 and SC-11830 was raised against a peptide mapping near the C-terminus of PDE3A. Antibody to β-Actin (CP01; Calbiochem) was used for loading control. Blots were overlaid with peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat secondary antibodies (711-036-153, 715-035-151, 705-035-147; Jackson Immuno Research) and peroxidase-conjugated anti-sheep secondary antibody (P016302-2; DAKO), and signals were visualized using Immobilon™ Western (Merck Millipore) and the Odyssey® Fc Dual Mode Imaging System (Li-Cor®).

Subcellular fractionation
Cells were homogenized using a plastic pistil (Destroy-S-15, Biozym Scientific), incubated for 10 min on ice and centrifuged (10 min, 4°C, 700 x g). The nuclear pellet was discarded and the supernatant was ultra-centrifuged at 100,000 x g for 60 min at 4°C (Beckman Coulter). The supernatant was considered as the cytosolic fraction, and the pellet as membrane fraction; the latter was washed five times with fractionation buffer and finally resuspended in RIPA buffer supplemented with protease and phosphatase inhibitors. Proteins were detected by Western blotting using 20 µg of total protein of each fraction. Immunoblotting was performed for detection of PDE3A (A302-740A; Bethyl Laboratories Inc.), PDE3A phospho serine 428 and 438 (S446B and S442B; University of Dundee), Hsp90 (SPA-830; Stressgen), PanCadherin (ab6528; Abcam) and Renilla luciferase (MAB4400; Millipore). Peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies (711-036-153 and 715-035-151; Jackson Immuno Research) and peroxidase-conjugated anti-sheep secondary antibodies (P016302-2; DAKO) were used, and signals were visualized using Immobilon™ Western (Merck Millipore) and the Odyssey® Fc Dual Mode Imaging System (Li-Cor®).

Cell-based studies
The data describe the relative increase of luciferase activity normalized to the DMSO control of empty-vector transfected cells. 5x104 HeLa cells were seeded 12–24 h prior to transfection. 75 fmol each of the CRE-luciferase construct (pGL4.29, Promega) and the full-length wildtype PDE3A or full-length mutation constructs were transfected with Fugene Extreme Gene HP (Roche), according to the manufacturer’s recommendations. 12.5 ng of pRL-TK (Promega) were added to control transfection efficiency. After 48 h of transfection cell lysates were analyzed using the Dual-Glo Assay (Promega) in a Berthold luminometer. 4 h prior to cell lysis, forskolin (0.3; 1; 10 µM) was added. L-Arginine (1; 30; 100 µM) was added for 10-15 min before cell lysis. The media of transfected HeLa cells was supplemented with BAY 41-8543 (1; 30; 100 µM; kindly provided by Dr. Damian Brockschnieder, Bayer AG), 30 min before harvesting. The compound (0.5 mg/ml) was solved in dimethyl formamide (DMF):PBS (pH 7.2), 1:1. DMSO or DMF served as controls. The results are means of three independent transfections (mean±SD; n = 3; Wilcoxon-Mann-Whitney test).
Supplemental references


Supplemental results

Table S1. Platelet aggregation and inhibition by milrinone. Values (mean ± SD [min – max]) represent half maximal effective concentrations (EC50) for the platelet aggregation in response to either thrombin receptor agonist peptide-6 (TRAP) or collagen (Col) and the milrinone-induced inhibition of platelet aggregation in response to the different TRAP or collagen concentrations.

<table>
<thead>
<tr>
<th>TRAP (EC50; µM)</th>
<th>TRAP (100 µM) Milrinone (EC50; µM)</th>
<th>TRAP (33.3 µM) Milrinone (EC50; µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.1±2.9 [12.3 - 20.0]</td>
<td>6.7±1.8 [4.4 - 8.5]</td>
<td>0.9±0.6 [0.2 - 1.7]</td>
</tr>
<tr>
<td>17.3±3.8 [11.5 - 22.1]</td>
<td>3.6±3.3 [0.7 - 7.0]</td>
<td>0.4±0.5 [0.1 - 1.2]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Col (EC50; µg/ml)</th>
<th>Col (20 µg/ml) Milrinone (EC50; µM)</th>
<th>Col (6.7 µg/ml) Milrinone (EC50; µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2±1.4 [0.2 - 3.6]</td>
<td>1.3±0.8 [0.1 - 2.1]</td>
<td>0.41±0.27 [0.10 - 0.69]</td>
</tr>
<tr>
<td>1.2±0.4 [0.8 - 1.9]</td>
<td>0.8±0.4 [0.2 - 1.0]</td>
<td>0.14±0.12 [0.02 - 0.33]</td>
</tr>
</tbody>
</table>

Platelet responsiveness to TRAP or collagen in affected persons (grey) did not differ from nonaffected relatives. Similar values have been observed for the general population. The mean values for the half-maximal inhibitory effect of milrinone seem to be consistently lower for affected in comparison to non-affected individuals.
(A) Image shows Multi-slice imaging by 4D echocardiography exhibiting normal LV-Function of AFF 035. A movie (not shown) exhibits a normal LV-function: (B) Image shows assessment of the LV global and regional function by longitudinal strain bull eye with averaged values for the 17 LV-segments and corresponding curves.
(A) The fractionation of transfected and non-stimulated HeLa cells showed no differences in compartmentalization between endogenous or wildtype PDE3A and mutational PDE3A. As previously described, the A1 isoform was predominantly detected in the membrane fraction, the A2 isoform mainly in the cytosol. Hsp90 was positive marker for cytosolic fraction, whereas PanCad (Pan Cadherin) was in the membrane fraction. (B) In PMA- and forskolin-stimulated transfected HeLa cells, a slight increase of mutational PDE3A1 was detected in microsomal fractions using the polyclonal anti-PDE3A antibody A302-740A (Bethyl Laboratories Inc.). The mutational PDE3A1 shows a modest increased Ser428 phosphorylation that could lead to an enhanced association of the enzyme to the membrane.
The schematic shows overactive PDE3A hydrolyzing cAMP, impacting on protein kinase A (PKA), myosin light-chain kinase (MLCK) and the phosphorylation of myosin light chain (MLC). We speculate that altered vasodilator-stimulated phosphoprotein phosphorylation (pVASP) could influence VSMC proliferation.