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Differential phosphorylation of LZ+/LZ− MYPT1 isoforms regulates MLC phosphatase activity

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ABSTRACT

The vascular response to NO is due, in part, to a Ca^{2+} independent activation of myosin light chain (MLC) phosphatase, a trimeric enzyme of 20 kDa, 38 kDa catalytic and 110–130 kDa myosin targeting (MYPT1) subunits. Alternative mRNA splicing produces MYPT1 isoforms that differ by the presence or absence of a central insert (CI) and a leucine zipper (LZ), and the presence of a LZ+ MYPT1 isoform is important for protein kinase G (PKG) mediated activation of MLC phosphatase. This study was designed to determine the molecular basis for the differential sensitivity of the vasculature to NO. Our results demonstrate that the presence of the MYPT1 LZ domain is required for PKG to both phosphorylate MYPT1 at S668 and activate MLC phosphatase. Further for LZ+ MYPT1 isoforms, an S668A MYPT1 mutation prevents the PKG mediated, Ca^{2+} independent activation of MLC phosphatase. These data demonstrate that differential PKG mediated S668 phosphorylation of LZ+/LZ− MYPT1 isoforms could be important for determining the diversity in the sensitivity of the vasculature to NO mediated vasodilatation. Thus, the relative expression of LZ+/LZ− MYPT1 isoforms, in part, defines the vascular response to NO and NO based vasodilators, and therefore, plays a role in the regulation of vascular tone in both health and disease.

Introduction

Changes in vascular tone and function are associated with a number of diseases including systemic hypertension [1,2], heart failure [3] and pulmonary hypertension [4,5]. And although blood pressure would be expected to be regulated by changes in vascular resistance or function, until recently, there was little direct evidence supporting this hypothesis. Crowley et al. [6] demonstrated that mice lacking AT1 receptors in both the peripheral vasculature and kidney (AT1 KO) were hypotensive compared to WT mice, and the blood pressure of mice lacking only peripheral or only renal AT1 receptors was intermediate between WT and AT1 KO animals. Michael et al. [7] reported that mice with a smooth muscle specific mutation in the LZ domain of PKGIα, which disrupts the interaction of PKGIα and MYPT1, were hypertensive. Further the smooth muscle from these transgenic mice, compared to WT, were less sensitive to both NO and cGMP mediated relaxation [7], suggesting that the decrease in the vasodilatory response to NO produces hypertension. These data demonstrate that hypertension is not only the result of abnormalities in renal Na^+ excretion [8], but that changes in the regulation of vascular smooth muscle, in isolation, produce both a change peripheral vascular resistance and blood pressure. Thus, the regulation of vascular tone and function is important in both health and disease.

The regulation of vascular tone is dependent on the level of phosphorylation of the 20 kDa smooth muscle myosin regulatory light chain (LC),[1] which is determined by relative activities of myosin light chain kinase (MLCK) and MLC phosphatase [9]. MLCK is regulated by Ca^{2+}-calmodulin [10], while MLC phosphatase is regulated by a number of signaling pathways [11,12]. MLC phosphatase is a holoenzyme consisting of a catalytic, 20 kDa and myosin targeting (MYPT1) subunits [11]. Alternative mRNA splicing produces 4 MYPT1 isoforms [11], which differ by the presence and absence of a central insert (CI) and leucine zipper (LZ).

Nitric oxide (NO) mediated vasodilatation is a fundamental response of the vasculature [13], and the signaling pathway for NO mediated vasodilatation is known to involve both Ca^{2+} dependent and Ca^{2+} independent pathways [14]. During NO mediated vasodilatation, NO activates cGMP dependent protein kinase (PKG), which phosphorylates a number of target proteins to reduce intracellular Ca^{2+}, and also produces a Ca^{2+} independent activation of MLC phosphatase [14]. The expression of a LZ+ MYPT1 isoform

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[1] *Abbreviations used:* LC, light chain; MLCK, myosin light chain kinase; CI, central insert; LZ, leucine zipper; DMEM, Dulbecco's Modified Eagle's Media; FBS, Fetal Bovine Serum.

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has been demonstrated to be important for a PKG mediated, Ca^{2+} independent activation of MLC phosphatase [15,16], and PKG signaling results in a rapid MYPT1 phosphorylation at S668 [17] and a slower phosphorylation at S695 [17–19]. However, the mechanism for the PKG mediated, Ca^{2+} independent activation of MLC phosphatase has yet to be elucidated. The relative expression of LZ+/LZ− isoforms as well as sensitivity to NO mediated vasodilatation has been demonstrated to be modulated during development [20], heart failure [21,22], pulmonary hypertension [23], portal hypertension [24], preeclampsia [25] and nitrate tolerance [24], which could suggest that relative LZ+ MYPT1 expression is a key determinant of the vascular response to NO.

This study was designed to determine if PKG mediated MYPT1 phosphorylation at S668 results in an activation of MLC phosphatase activity, and whether differential S668 phosphorylation of LZ+/LZ− MYPT1 isoforms is the basis for the heterogenous response of the vasculature to Ca^{2+} desensitization produced by NO and NO based vasodilators.

Material and methods

For the present study, we expressed avian MYPT1 in HEK293T cells. There are minor differences in the sequence of avian and mammalian MYPT1; phosphorylation occurs at S668, S695, T696 S852 and T853 of the mammalian (human) sequence, which is equivalent to S667, S694, T695, S849 and T850 of the avian sequence. To avoid confusion and for consistency, we will refer only to the human sequence for both the endogenous and exogenous MYPT1 throughout the manuscript.

Cloning of full length his-tagged MYPT plasmids

Previously obtained pcDNA4 vector and MYPT1 isoform cDNAs containing a his-tag linker were both digested sequentially with PstI and KpnI then ligated and used to transform JM109 cells. The sequence verified MYPT clones used in previous experiments [17] were the template to add the his-tag. Following addition of the his-tag, the clones were sequenced from the his-tag to the join area to ensure that the added sequence was in frame and correct.

Cloning of his-tagged MYPT mutants

His-tagged alanine MYPT mutants (CI−LZ+ and CI+LZ+) were created from the above plasmid templates using the QuikChange site-directed mutagenesis kit (Stratagene). Mutagenesis primers were generated to mutate S668 to an alanine (Ala) to preclude phosphorylation yielding S668A CI−LZ+ MYPT1 and S668A CI+LZ+ MYPT1. Both mutants were sequenced to confirm the desired mutation.

Cell Culture – transient transfections

Human embryonic cells (HEK293T) were grown in P100 plates until ~60–75% confluent. Prior to transfection, the media was changed to 1× Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin and streptomycin at 37 °C with 5% CO_2. Cells were transfected by adding Opti-MEM, 24 μg of Lipofectamine 2000 reagent (Life Technologies), and 24 μg of desired MYPT isoform plasmid per P100 plate. Transfections were allowed to incubate for 24 h before being lysed or subjected to time course assays.

After transient transfections were conducted, low serum media (1× DMEM, 0.01% FBS, and 1% penicillin and streptomycin) was added to P100 plates and cells were incubated for 24 h. The media was removed and replaced with either fresh low serum media (as a control) or low serum media containing 0.1 mM 8-Br-cGMP. Cells exposed to fresh media only underwent a 30 min incubation at 37 °C while cells exposed to media with 8-Br-cGMP underwent incubation times at 37 °C ranging from 1 to 30 min. After the desired incubation time was completed, plates were washed quickly with cold 1× DPBS. Cells were scraped off the plate using cold 1× DPBS with 1× phosphatase inhibitor (Roche) and were sonicated, spun, and placed in fresh tubes. Protein concentrations were calculated, and the cell lysates were stored at −80 °C until ready for use.

For pulldown assays, an appropriate amount of total cell lysate was added to 1× Binding/Wash buffer (600 mM NaCl, 100 mM Phosphatase Buffer pH 7.0, and 0.02% Tween 20) and rotated with Dynabeads (Life Technologies) as well as a phosphatase inhibitor (1× concentration, Roche) for 4 h at 4 °C. The supernatant was removed prior to three washings with 1× Binding/Wash buffer. Bound sample was removed from Dynabeads by incubating beads with 1× LDS for 15 min at room temperature. Samples were stored at −80 °C until ready for use.

Phosphatase assay

The protocol for these experiments has been published [26,27]. Briefly, cell lysate (12.5 μg) was diluted in phosphatase assay buffer (50 mM HCl–Tris, 0.1 mM $CaCl_2$, 0.1% BSA, pH 7.0). Each reaction was pipetted into an individual well of a black 96-well microplate and allowed to incubate at room temperature for 2 min (±1 μM 8Br-cGMP, to phosphorylate MYPT1). To initiate the reaction, a titrated amount of DifMUP substrate (Life Technologies) was added to each well (0–600 μM) yielding a final volume of 100 μL per well. The entire mixture was then briefly mixed, and time resolved fluorescence was measured in a multi-mode plate reader (Biotek Synergy HT) for 10 min at 32 s intervals using emission and excitation wavelengths of 455 nm and 358 nm, respectively. The same total protein input was used for every experiment, and in the presence of a phosphatase inhibitor (PhosStop, Roche), there was no detectable activity, and thus, V_{max} and Km were calculated from the rate of Pi release vs. [DiFMUP]. Data for each experiment was fit to a single exponential equation ($y = yo + Ae^{(x/\tau)}$); where $A = V_{max}$ and τ = Km. Then, the resulting V_{max} and Km for each experiment were averaged to calculate the mean ± SEM for each MYPT1 construct.

Immunoblotting

As we have described [15,17,28], MYPT phosphorylation was determined using phospho-specific antibodies, and data are normalized to total MYPT1. Following pull-down of the His-tagged MYPT1, total MYPT1 was determined using either a polyclonal anti-MYPT1 antibody, a monoclonal MYPT1 antibody (IC2, [21,28]) or a monoclonal anti-LZ+ MYPT1 antibody [21,28].

Statistics

All data are presented as mean ± SEM, and n represents the number of individual experiments. A Student's *t*-test was used to evaluate for the differences between groups. Differences were considered significant at $p < 0.05$, and for multiple comparisons between groups, a Bonferroni correction was performed.

Results

We established HEK293T cells expressing the four avian MYPT1 isoforms; CI+LZ+, CI−LZ+, CI+LZ− and CI−LZ−, which have a predicted Mr and pI of 114.61and 5.11, 109.98 and 5.01, 111.47 and