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Myosin phosphatase isoforms and related transcripts in the pig coronary circulation and effects of exercise and chronic occlusion

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ABSTRACT

Myosin phosphatase (MP) is a key target of signaling pathways that regulate smooth muscle tone and blood flow. Alternative splicing of MP targeting subunit (MYPT1) exon 24 (E24) generates isoforms with variable presence of a C-terminal leucine zipper (LZ) required for activation of MP by NO/cGMP. Here we examined the expression of MP and associated genes in a disease model in the coronary circulation. Female Yucatan miniature swine remained sedentary or were exercise-trained beginning eight weeks after placement of an ameroid constrictor around the left circumflex (LCX) artery. Fourteen weeks later epicardial arteries (~1 mm) and resistance arterioles (~125 μm) were harvested and assayed for gene expression. MYPT1 isoforms were distinct in the epicardial arteries (E24−/LZ+) and resistance arterioles (E24+/LZ−) and unchanged by exercise training or coronary occlusion. MYPT1, CPI-17 and PDE5 mRNA levels were not different between arteries and arterioles while Kir2.1 and eNOS were 6.6-fold and 3.9-fold higher in the arterioles. There were no significant changes in transcript abundance in epicardial arteries of the collateralized (LCX) vs. non-occluded left anterior descending (LAD) territories, or in exercise-trained vs. sedentary pigs. There was a significant 1.2 fold increase in CPI-17 in collateral-dependent arterioles, independent of exercise, and a significant 1.7 fold increase in PDE5 in arterioles from exercise-trained pigs, independent of occlusion. We conclude that differences in MYPT1 E24 (LZ) isoforms, eNOS, and Kir2.1 distinguish epicardial arteries and resistance coronary arterioles. Up-regulation of coronary arteriolar PDE5 by exercise and CPI-17 by chronic occlusion could contribute to altered vasomotor responses and requires further study.

Introduction

De-phosphorylation of myosin by the myosin phosphatase (MP) is the primary mechanism for vascular smooth muscle relaxation. Vasoconstrictor and vasodilator signals regulate vascular smooth muscle tone in part through inhibition or activation, respectively, of MP (reviewed in Grassie et al., 2011; Hartshorne et al., 2004). Based on the pattern of expression of MP subunits and their isoforms in different vascular and smooth muscle tissues, it has been proposed that their regulated expression may determine vessel-specific responses to these signals (reviewed in Fisher, 2010). The myosin phosphatase targeting (regulatory) subunit (MYPT1) Exon 24 (E24) is alternatively spliced, generating isoforms that contain or lack a C-terminal leucine zipper motif required for NO/cGMP-mediated activation of MP (Huang et al., 2004; Khatri et al., 2001; Surks et al., 1999). Protein Kinase C (and Rho Kinase)-potentiated myosin phosphatase inhibitor of 17 kDa (CPI-17) mediates contractile agonist-triggered inhibition of MP (reviewed in Eto, 2009; Eto et al., 1997). Variable levels of expression of CPI-17 correlate with sensitivity to agonist-mediated inhibition of MP (Kitazawa and Kitazawa, 2012; Su et al., 2013; Woodsome et al., 2001).

The contractile function and gene programs of the resistance arterioles that regulate blood flow are distinct from that of larger conduit arteries (reviewed in Chilian, 1997; Tanko and Matrougui, 2002). In the splanchnic circulation the expression of myosin phosphatase subunits is vessel-specific, developmentally regulated and modulates in disease (Payne et al., 2004, 2006; Zhang and Fisher, 2007) (reviewed in Fisher, 2010). In the coronary circulation functional differences between conduit and resistance vessels have been described (reviewed in Camici and Crea, 2007; Duncker and Bache, 2008), yet there is little understanding of the molecular basis of these functional differences particularly with regard to smooth muscle contractile function. In disease models of reduced coronary blood flow, such as chronic coronary occlusion induced by placement of an ameroid constrictor, a number of changes in the contractile function of the collateral-dependent arterioles have been described in sedentary or exercise-trained pigs (reviewed in Heaps and Parker, 2011). The molecular bases of these functional changes are mostly unknown. Given the critical role of MP in setting calcium sensitivity and vascular smooth muscle responses to constrictor and dilator signals, we hypothesized that the increased basal tone and increased responses to vasoconstrictors and vasodilators

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in this model is due to altered expression of MP subunits and related gene products that determine vascular function. In addition to the MP subunits, we measured eNOS and PDE5 given their critical role in determining vasodilator function, and Kir2.1 as a mediator of Endothelium-Derived Hyperpolarizing Factor (EDHF)-triggered vasorelaxation predominant in the smaller arteries (Quayle et al., 1996). These were measured in pig epicardial arteries and resistance arterioles from the chronically occluded collateral-dependent left circumflex territory and reference non-occluded left anterior descending artery in sedentary and exercise-trained pigs.

Material and methods

Animals

Coronary arteries and arterioles were obtained from a well-established porcine model of chronic coronary artery occlusion and collateral-dependent perfusion (Heaps et al., 2000). Female Yucatan miniature pigs (Sinclair Research Center, Auxvasse, MO) were used according to the National Institutes of Health (NIH) Guide of "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training" and approved by the Institutional Animal Care and Use Committee at Texas A&M University. Pigs were subjected to proximal left circumflex coronary (LCX) artery ameroid occlusion and allowed to recover for eight weeks. Animals were randomly assigned to sedentary ($n = 4$) or exercise-training ($n = 4$) groups, in which pigs underwent a progressive treadmill exercise training program for 14 weeks, 5 days/week or remained pen-confined, as described previously (Heaps et al., 2000; Xie et al., 2012, 2013). With the aid of a dissection microscope, epicardial arteries (~1 mm) and subepicardial arterioles (~125 μm) were dissected from the non-occluded (LAD) and collateral-dependent (LCX) myocardial regions, respectively. Vessels were cleaned of myocardium and trimmed of fat and connective tissue.

Immunoblots

The coronary epicardial arteries (~1 mm internal diameter; 1 cm length) and arterioles (~100–150 μm diameter; ~15–20 mm total length) were isolated from pig hearts obtained from a local abattoir, quick-frozen in liquid N_2 and stored at −80 °C for later immunoblot analysis as described previously (Fogarty et al., 2009). Arterial and arteriole lysates (20 μg of total protein) were subjected to SDS-polyacrylamide gel electrophoresis (4–20% gradient gel), transferred to polyvinylidene difluoride (PVDF) membrane, and probed overnight with primary antibody. Primary antibodies included rabbit polyclonal antibodies that specifically recognize the MYPT1 LZ-isoform (1:1000; rabbit polyclonal IgG (Bhetwal et al., 2011)), β-actin (1:1000; Novus Biologicals NB600-501), and GAPDH (1:1000; Advanced Immunochemical RGM2). Membranes were incubated with HRP conjugated secondary antibodies followed by SuperSignal West Dura Substrate (Thermo Scientific Pierce) detection. Scanning densitometry was used to quantify signal intensities which were normalized against the on-blot signals for β-actin or GAPDH.

Conventional PCR and real-time PCR

Total RNA from pig vessels was isolated using the RNeasy mini or micro kits (QIAGEN) as per instructions. The yield of total RNA was ~50 ng and ~500 ng from the arterioles and arteries, respectively. Pig bladder RNA was purchased from ZYAGEN (PR902). Total RNA was reverse transcribed by standard methods and splice variants of MYPT1 amplified by conventional PCR: sense primer 5′-gaaagcccagctccatgata-3′; anti-sense primer 5′-tcaaggctccattttcatc-3′ for pig and sense primer 5′-cgaagcggagacagataaga-3′,anti-sense primer 5′-gttgtcacagcggcagga-3′ for rat, then quantified as previously described (Khatri et al., 2001) with minor modifications. cDNAs derived from purified RNA pools from endothelial cells (EC) and smooth muscle cells (SMC) from adult Wistar rat mesenteric arteries were provided by Dr. An Huang (Sun et al., 2011). MYPT1, CPI-17, PDE5, eNOS, Kir2.1, and smooth muscle α-actin message were quantified by real-time PCR using Taqman probes (Table 1) and Taqman Fast Advanced Master mix (Life Technologies) using 1 ng input cDNA. Relative expression was calculated as $2^{-\Delta\Delta Ct}$ normalized to smooth muscle α-actin.

Sequencing and bioinformatical analysis of pig MYPT1

Pig MYPT1 PCR products were gel purified, sequenced and aligned with pig genomic sequence on the UCSC and Ensembl genome browsers (http://genome.ucsc.edu; http://useast.ensembl.org). Sequences were aligned using Clustal Omega from EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Statistical analysis

Data are expressed as mean ± SEM or as fold change. Non-parametric Wilcoxon signed ranks test was used for comparing the differences between related samples of non-occluded and collateral-dependent groups and Mann–Whitney test was used for analysis of the independent samples of sedentary and exercise-trained pigs. $P < 0.05$ was considered statistically significant.

Results

Myosin phosphatase isoforms in the pig

Pig (*Sus scrofa*) genomic sequence in the publicly accessible genome browsers identified the MYPT1 gene with high conservation with other mammals (XM_003355700.2). This sequence aligned with other mammal MYPT1 sequences through exon 23. Poor alignment of exons 24–26 suggested error in sequencing or gene annotation. A cDNA of MYPT1 derived from pig aortic endothelial cells also only contained the first 17 exons (Hirano et al., 1999). To identify the 3′ end of the pig MYPT1 transcript containing the alternative exon, PCR primers were designed based on the known pig 5′ sequence and conserved mouse 3′ sequence (Fig. 1 and Method section). Sequencing of PCR products obtained from the bladder and aorta cDNAs identified the E24 alternative exon and flanking sequence (Figs. 1B and C). The E24 sequence is also present in the human genomic sequence though it has yet to be identified as an alternative exon. The pig and mouse MYPT1 E24 sequence differ by 2 nt (Fig. 1C). The nt difference at position 4 of the exon (A vs. G) is

Table 1
Real-time PCR Taqman probes used in this study.

Gene	HGNC name	Taqman assay ID	Assay location	Product length, bp
Smooth muscle α-actin	ACTA2	Ss04245588_m1	165	84
MYPT1	PPP1R12A	Ss03372900_m1	2092	72
CPI-17	PPP1R14A	Ss03384219_u1	348	58
PDE5A	PDE5A	Ss03376347_u1	656	99
eNOS	NOS3	Ss03383840_u1	3452	77
Kir2.1	KCNJ2	Ss03382889_u1	1153	79