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p21$^{CIP1/WAF1}$-dependent inhibition of cardiac hypertrophy in response to Angiotensin II involves Akt/Myc and pRb signaling

Ludger Hauck[a], Daniela Grothe[a], Filio Billia[a,b,c,d,*]

[a] Toronto General Research Institute, 100 College St., Toronto, Ontario, M5G 1L7, Canada
[b] Division of Cardiology, University Health Network (UHN), 200 Elizabeth St., Toronto, Ontario, M5G 2C4, Canada
[c] Heart and Stroke Richard Lewar Centre of Excellence, University of Toronto, Canada
[d] Institute of Medical Science, University of Toronto, 1 King's College Circle, Toronto, Ontario, M5G 1A8, Canada

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ABSTRACT

The cyclin-dependent kinase inhibitor p21$^{CIP1/WAF1}$ (p21) is highly expressed in the adult heart. However, in response to stress, its expression is downregulated. Therefore, we investigated the role of p21 in the regulation of cardiac hypertrophic growth. At 2 months of age, p21 knockout mice (*p21*KO) lack an overt cardiac phenotype. In contrast, by 10 months of age, *p21*KO developed age-dependent cardiac hypertrophy and heart failure. After 3 weeks of trans-aortic banding (TAB), the heart/body weight ratio in 11 week old *p21*KO mice increased by 57%, as compared to 42% in wild type mice indicating that *p21*KO have a higher susceptibility to pressure overload-induced cardiac hypertrophy. We then chronically infused 8 week old wild type mice with Angiotensin II (2.0 mg/kg/min) or saline subcutaneously by osmotic pumps for 14 days. Recombinant TAT conjugated p21 protein variants (10 mg/kg body weight) or saline were intraperitoneally injected once daily for 14 days into Angiotensin II and saline-infused animals. Angiotensin II treated mice developed pathological cardiac hypertrophy with an average increase of 38% in heart/body weight ratios, as compared to saline-treated controls. Reconstitution of p21 function by TAT.p21 protein transduction prevented Angiotensin II-dependent development of cardiac hypertrophy and failure. Taken together, our genetic and biochemical data show an important function of p21 in the regulation of growth-related processes in the heart.

1. Introduction

p21 (*Cdkn1a*) belongs to the Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitors (Cdki) that includes p27 (*Cdkn1b*) and p57 (*Cdkn1c*) [1,2]. p21 is a potent inhibitor of proliferation and apoptosis that is regulated through p53-dependent (for example, oxidative stress) [3–5] and p53-independent (for example, c-Myc) [6] mechanisms. Transition through the cell cycle is tightly controlled by periodic alterations in the activity of Cdks [1]. Mechanistically, p21 functions as a nuclear protein that antagonizes directly the phosphotransferase activities of Cyclin/Cdk2 and Cdk4 complexes, thereby inhibiting cell proliferation [7,8]. Cells with mutated p53 proliferate aberrantly and generate outgrowths of genetically unstable cells, leading to tumorigenesis as demonstrated by the early cancer predisposition of p53 knockout mice [9]. In contrast, p21 knockout (*p21*KO) mice are not cancer prone and develop normally without displaying any gross physiological abnormalities [10]. However, on a subcellular level, p21 loss confers a higher sensitivity to genotoxic insult caused by perturbations in cell cycle control.

Shortly after birth, mammalian cardiomyocytes exit the cell cycle and downregulate cell cycle promoting genes, such as E2f1, cyclin D1, or the E3 ubiquitin ligase Skp2 [11–17]. Adult cardiomyocytes are differentiated post-mitotic cells that lack significant proliferative potential caused by high levels of cell cycle inhibitors, such as Cdkis p21/p27 and retinoblastoma family proteins pRb (*Rb1*) and p130 (*Rbl2*) [18–22]. The limited mitotic capacity of mature cardiomyocytes renders the adult mammalian heart functionally unable to repair itself after ischemic injury [23]. Instead, surviving cardiomyocytes undergo hypertrophy to compensate for the ensuing hemodynamic stress manifested as cell enlargement, myofibrillar disarray and re-expression of fetal genes [24–26]. This

* Corresponding author at: Toronto General Research Institute, 100 College St., Toronto, Ontario, M5G 1L7, Canada.
E-mail addresses: lhauck@uhnres.utoronto.ca (L. Hauck), dgrothe@uhnres.utoronto.ca (D. Grothe), phyllis.billia@uhn.ca (F. Billia).

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process becomes maladaptive with time leading to the development of heart failure with significant morbidity and mortality. The molecular mechanisms underlying heart failure remain poorly understood. As such, identifying the factors which effectively maintain cardiac tissue homeostasis is of great scientific and clinical import [27,28].

Cyclin D2-mediated regulation of cardiac growth by the c-Myc (Myc) and pRb pathways has been well established [29–32]. Moreover, FoxO3a-dependent activation of p21 is important for the anti-hypertrophic impact of statins in the heart [33]. However, a direct mechanistic role of p21 in cardiac growth control has not been previously investigated. One distinguishing feature of adult cardiomyocytes is the mechanistic dissociation of cell growth from proliferation which is the cardinal feature of hypertrophy [19]. Both events are intrinsically linked in mitotic cells, indicating common regulatory pathways for these two processes [34–36]. Therefore, we investigated the effect of p21 on cardiac hypertrophy in established murine models of heart failure. Our study shows that reconstitution of p21 protein abundance in the myocardium of adult mice prevents Ang II-induced hypertrophy and heart failure.

2. Materials and methods

2.1. p21*KO mice*

Mice with targeted homozygous disruption of the gene encoding p21 were purchased from Jackson (B6.129S6(Cg)-*Cdkn1a*^{tm1Led}/J; Bar Harbor, ME 04609 USA). Primer sequences for PCR-based genotyping: 12427 5′-GTTGTCCTCGCCCTCATCTA-3′; 12428 5′-GCCTATGTTGGG AA ACCAGA-3′; primer 5′-CTGTCCATCTGCACGAGACTA-3′. All animal usage in this study was in accordance with approved institutional animal care guidelines of the UHN (AUP 11381, Canadian Council in Animal Care). *p21*KO mice were previously backcrossed into a C57BL/6 background for 7 generations. We used isogenic age-matched male C57BL/6 wild type and *p21*KO mice in this study. We subjected 8 week old *p21*KO and wild type mice to hemodynamic stress induced by trans-aortic banding (TAB). At 3 weeks post TAB, animals were sacrified for determination of HBW, M-mode echocardiography and biochemical measurements. We chronically infused Angiotensin II (A9525, Sigma) subcutaneously by osmotic mini pumps (model 2000, Alzet) at a dose of 2.0 mg/kg/day for 14d. We injected TAT proteins (10 mg/kg; 200 μl/injection) intraperitoneally once daily for 14d. Systolic arterial blood pressure was monitored by tail cuff plethysmography under anesthesia of 2% isoflurane (Panlab; Harvard Apparatus, UK).

2.2. Cell fractionation and heart tissue extracts

Total cell extracts were prepared with Cell Lysis Buffer (Cell Signaling) composed of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, protease inhibitors. Protein content was determined with the Bradford protein assay kit (Pierce) and BSA as standard.

2.3. Antibodies

Anti-$His_{(6)}$G (R940-25, Invitrogen), Akt (9272), Akt.Pi-S473 (4051), actin (4986, Cell Signaling),

Cdk2 (sc-163), Cdk4 (sc-260), Cyclin A (sc-751), Cyclin D2 (sc-593), Cyclin E (sc-481), c-Myc (sc-40), pRb (sc-50), pRb.Pi-Ser795 (sc-7086), p21 (sc-6246), p21.Pi-Thr145 (sc-20220-R), normal rabbit IgG (sc-2027, Santa Cruz), α-actinin, sarcomeric (A7811, Sigma), Alexa Fluor 488-conjugated goat anti-rabbit IgG (A-11034), Alexa Fluor 750 conjugated goat anti-mouse IgG (A-11029; Molecular Probes).

2.4. Echocardiography

Echocardiography in anesthetized mice (2% isoflurane, 98% oxygen) was performed using a 15-MHz linear ultrasound transducer (Vivid7; GE). Body temperature was maintained at 37 °C. M-mode measurements of the left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were made from short-axis views at the level of the papillary muscle. Left ventricular fractional shortening (FS) was calculated as follows: FS = (LVEDD − LVESD)/LVEDD × 100%.

2.5. TUNEL assay and immunofluorescence microscopy of fixed hearts

Fluorescence tiling of whole longitudinal specimens was performed on an Olympus BX50 fluorescent microscope and Metamorph software (AOMF—Advanced Optical Microcopy Facility, Ontario Cancer Institute, Toronto, ON Canada). Hearts were fixed in 4% PBS-buffered formalin for 50–60 min at room temperature. Specimen were permeabilized in 1% Triton X-100 for 60 min at room temperature. Alexa Fluor 488-WGA was from Molecular Probes (W11261). Detection of fragmented genomic DNA was performed by *in situ* TUNEL assay according to the manufacturer's instructions (Roche).

2.6. Immunocomplex kinase assays

For immunocomplex kinase assays, cell extracts were incubated with anti-Cdk2 or anti-Cdk4 and protein A-agarose beads for 3 h at 4 °C. Pelleted beads were washed twice in ice-cold lysis buffer. Negative control reactions contained either control anti-rabbit IgG (sc-2027, Santa Cruz), no antibodies, or sample extracts were omitted (data not shown).The reactions were initiated by addition of kinase buffer containing histone H3 substrate: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 30 μM ATP (Roche), 3 μM histone H1 (Roche). Reactions were stopped by addition of 2× SDS sample buffer. Aliquots of the reaction mixtures were analyzed by immunoblotting.

2.7. Quantitative PCR coupled with reverse transcription, hypertrophy assays, and immunocytochemistry

We carried out RT-PCR (Superscript III; Invitrogen) and real time PCR (SYBR Green; Qiagen) on an Mastercycler EP Realplex (Eppendorf) for mRNA analysis with total RNA (1.0 μg/reaction) isolated from LV apexes from three mice of each genotype and condition using TRIzol reagent (Invitrogen): ANP forward 5′-CATCACCCTGGGCTTCTTCCT-3′, reverse 5′-TGGGCTCCAAT CCTGTCAATC-3′; BNP forward 5′-GCGGCATGGATCTCCTGAAGG-3′, reverse 5′-CCCAG GCAGAGTCAGAAACTG-3′; α-MHC forward 5′-CCAATGAGTACCGCGTGAA-3′, reverse 5′-ACAGTCATGCCG-GGATGAT-3′, β-MHC forward 5′-ATGTGCCGGACCTTGGAA-3′, reverse 5′-CCTCGGGTTAGCTGAGAGATCA-3′. GAPDH forward 5′-ATGTTCCAGTATG ACTCCACTCA CG-3′, reverse 5′-GAAGACACCAGTAGACTCCACGACA-3′.

2.8. Plasmids and production of TAT p21 fusion proteins

TAT-conjugated wt.p21 (Genbank L25610) and p21ΔC (lacking amino acid residues 91-164) were in the pRSET based pTAT vector (kind gift from S. Dowdy, Howard Hughes Medical Institute, La Jolla, USA) providing the N-terminal HIV-1 TAT protein transduction domain and a His-tag.