Effect of pressure overload-induced hypertrophy on the expression and localization of p38 MAP kinase isoforms in the mouse heart

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Abstract

p38 mitogen-activated protein kinases (MAPKs) are serine/threonine specific protein kinases that respond to cellular stress and regulate a broad range of cellular activities. There are four major isoforms of p38MAPK: α , β , γ , and δ . To date, the prominent isoform in heart has been thought to be p38 α . We examined the expression of each p38 isoform at both the mRNA and protein level in murine heart. mRNA for all four p38 isoforms was detected. p38 γ and p38 δ were expressed at protein levels comparable to p38 α and p38 β , respectively. In the early phase of pressure-overload hypertrophy (1–7 days after constriction of the transverse aorta), the abundance of p38 β , p38 γ and p38 δ mRNA increased; however, no corresponding changes were detected at the protein level. Confocal immunofluorescence studies revealed p38 α and p38 γ in both the cytoplasm and nucleus. In the established phase of hypertrophy induced by chronic pressure overload (7–28 days after constriction of the transverse aorta), p38 γ immunoreactivity accumulated in the nucleus whereas the distribution of p38 α remained unaffected. Hence, both p38 α and p38 γ are prominent p38 isoforms in heart and p38 γ may play a role in mediating the changes in gene expression associated with cardiac remodeling during pressure-overload hypertrophy.

Keywords

p38 MAPK; Isoforms; mRNA; Protein; Heart; Hypertrophy; Chronic pressure overload

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1. Introduction

Five distinct mitogen-activated protein kinase (MAPK) pathways have been classified in mammalian cells: c-jun amino-terminal kinases (JNKs) 1, 2, and 3; p38 MAP kinases α , β , γ , and δ ; extracellular signal-regulated kinases (ERKs) 1, 2; ERK3/4; and ERK5 [1]. The principle MAPKs investigated in cardiac myocytes are ERK1/2, which are activated by hypertrophic stimuli, and JNK and p38 MAPK, which are activated by cellular stress [2]. p38 α and p38 β are ubiquitously expressed, while p38 γ is expressed in striated muscle and p38 δ is expressed in lung, kidney, testis, pancreas, and small intestine [3,4]. To date p38 α , p38 β , and p38 γ have been detected in heart [3,5].

p38 MAPK is activated by phosphorylation of the TGY motif within the activation loop by upstream kinases MKK3, MKK6, and MKK4. In addition, there exists at least 3 means whereby p38 activation is independent of MKK activity. Interactions with TAB1 or ZAP-70 [6,7] induce autophosphorylation of p38 within the activation loop whereas interactions with Hsp90-Cdc37 suppressed this autophosphorylation [8]. Although the exact role of each p38 isoform remains to be determined, each p38 isoform has been independently knocked out in mice: p38 α -deficient mice show embryonic lethality due to placental defects, whereas p38 β -, p38 γ -, and p38 δ -null mice appear to have normal phenotypes [9]. Transgenic mice expressing dominant negative forms of p38 α and p38 β are resistance to cardiac fibrosis during pressure overload [10].

Due to the availability of pharmacological inhibitors, p38 α and p38 β have been far more extensively studied than p38 γ and p38 δ . Inhibition of p38 α / β activity reduces apoptotic cell death in cultured myocytes and intact heart upon pressure overload or ischemia/reperfusion [11,12]. Additionally, improved cardiac function and reduced remodeling is observed upon inhibition of p38 α / β during ischemia/reperfusion injury or myocardial infarction [13,14]. p38 γ is the only MAPK containing a PDZ binding domain sequence (KETXL) within its carboxyl-terminus [15], which makes it unique in the MAPK family. In addition, p38 γ expression is induced during muscle differentiation [16] and it localizes in cytoplasm whereas p38 α and p38 β are present in the cytoplasm and nucleus in isolated cardiac myocytes [3].

p38 isoforms are currently thought to serve non-redundant functions. We have studied the expression of each p38 isoform in mouse heart to determine their presence and relative abundance. In addition, we examined the effect of pressure overload-induced cardiac hypertrophy on their expression and subcellular localization.

2. Materials and methods

2.1. Materials

SDS-polyacrylamide gel electrophoresis reagents, nitrocellulose, and Bradford protein assay reagents were from Bio-Rad Laboratories. Membrane grade (reduced) Triton X-100 (TX-100), leupeptin, and PMSF were from Roche Molecular Biochemicals. Anti-HSP27 was gift from Dr. William Gerthoffer (Reno, NV). Anti-p38 α was from Santa Cruz Biotechnology (sc-535). Anti-p38 γ was from Upstate Biotechnology (#07-474), anti-p38 β

was a gift from Dr. Jacques Landry, anti-p38 δ (#2308), anti-phospho p38 MAPK (#2911S) and anti-phospho HSP27 (S-82) were from Cell Signaling Technology. Anti-phospho HSP27 (S-15) was from StressGen Biotechnologies Co. (SPA-525). Mouse anti-GAPDH was from Ambion Inc. (#4300). ALEXA fluor 488-conjugated donkey anti-rabbit secondary antibody was from Invitrogen. HRP-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. All other reagents were of analytical grade or best grade available. Primers for PCR and qPCR were from Invitrogen. GST fusion proteins were purified by affinity chromatography on glutathione Sepharose (GE Healthcare).

2.2. Preparation of murine cardiac lysates

Mice were sacrificed after sedation with pentobarbital. Hearts were rapidly removed, snap frozen in, and pulverized under, liquid nitrogen. Heart powders were divided into two and used for both RNA extraction and protein extraction. To prepare lysates, powdered tissue was resuspended using a 2 ml Potter-Elvehjem tissue grinder (25–30 passes) in 1.2 ml of ice-cold lysis buffer comprising 50 mM Tris (pH 7.5 at 4 °C), 20 mM β -glycerophosphate, 20 mM NaF, 5 mM EDTA, 10 mM EGTA, 1.0% TX-100, 1 mM Na₃VO₄, 1 μ M microcystin LR, 5 mM DTT, 10 μ g/ml leupeptin, 0.5 mM PMSF, and 10 mM benzamidine. Homogenates were then cleared of cellular debris by centrifugation for 30 min at 100,000 $\times g$ (48,000 rpm) and 4 °C in a Beckman TLA-100.3 rotor. Finally, supernatants were collected, aliquoted, snap-frozen using liquid nitrogen, and stored at –80 °C.

2.3. Cloning and purification of fusion proteins

Human cDNA for p38 α was purchased in the pOTB7 vector (Open Biosystems), p38 β was in pCMV-SPORT6 (Open Biosystems), p38 γ was in pET 14b (a gift from Dr. Jiahuai Han), and p38 δ was in pOTB7 (ATCC). Full length inserts for each p38 isoform was then subcloned into pGEX-6P (GE Healthcare) in phase 3' to a GST-6His coding sequence. Insertion of cDNA in to pGEX-6P was confirmed by electrophoresis on agarose gels and sequencing. Each pGEX-6P p38 construct was transformed into *E. coli* competent strain BL21 (DE3) (Novagen). Transformed *E. coli* were then selected on DYT agar media containing 100 μ g/ml ampicillin. The transformed bacteria were inoculated in DYT media contained 100 μ g/ml ampicillin, grown to an optical density of 0.6 at 600 nm, and then expression of the GST-fusion protein induced by addition of 1 mM isopropyl- β -D-thiogalactoside for 6–7 h at 37 °C with constant shaking. p38-fusion proteins were purified by affinity chromatography on glutathione Sepharose (GE Healthcare). The integrity and purity of each fusion protein was determined by SDS-PAGE.

2.4. RNA analysis

Mouse heart total RNA was isolated using RNeasy® Mini kits (Qiagen Inc.) with minor modifications. Briefly, total RNA was extracted by homogenizing tissue powders in 2 ml of TRIzol reagent (Sigma) using a Polytron at 10,000 rpm (2 $\times$ 15 s). Chloroform (0.4 ml) was added (for phase separation) and then samples were mixed and centrifuged for 15 min at 18,300 $\times g$ and 4 °C. The upper aqueous phase was collected, diluted with an equal volume of 70% ethanol, loaded onto Qiagen columns, and purified according to the manufacturer's instructions. Total RNA concentrations were determined by measuring the optical density and only samples having an 260/280 ratio between 1.8–2.1 were used. cDNA synthesis was

performed in a 20 μ l reaction volume containing 1 μ g of total RNA, 100 ng of random primers, 1 $\times$ First Strand buffer (50 mM Tris-HCl pH8.3, 75 mM KCl, 3 mM MgCl₂), 0.5 mM dNTP, 10mM DTT, 40 U RNaseOUT recombinant ribonuclease inhibitor, and 200 U of M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's protocol. Real-time qPCR was performed using a MX3000P QPCR system (Stratagene). Each amplification reaction mixture (25 μ l) contained 12.5 ng cDNA equivalent to reverse transcribed RNA, 300 nM forward and reverse primers, 30 nM ROX passive reference, 12.5 μ l platinum SYBR green mix (2 $\times$) (Invitrogen). PCR parameters were 95 °C for 10 min, and 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. SYBR green fluorescence was measured at the end of the annealing and extension phases of each cycle. p38 isoform-specific primers were designed using Clone Manager 6 (Sci Ed Software, USA) and were based on the cDNA sequences in the NCBI database (p38 α , NM_011951; p38 β , NM_011161; p38 γ , NM_013871; p38 δ , NM_011950). The forward and reverse primers used are shown in Table 1. The ability of each primer pair to selectively amplify a single target sequence was verified by analyzing the dissociation curve. In addition, amplicons were gel purified and sequenced to assure amplification of the correct target sequence. The amplification efficiency for each primer pair, determined from a standard curve of 50–3.25 ng reverse transcribed RNA isolated from the murine heart after two-fold serial dilution, was found to be between 90–110%. All samples were normalized to GAPDH, which was amplified in parallel.

2.5. Subcellular localization of p38 α and p38 γ

Following surgical removal, mouse hearts were frozen in 2-methyl butane chilled with liquid nitrogen. Tissue was embedded in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Inc.). After trimming, 14 μ m transverse ventricular cryosections were prepared and stored at –80 °C prior to further use. Three sections per heart were fixed for 20 min in ice-cold 4% paraformaldehyde in PBS (pH 7.2). After fixation, sections were washed three times with ice-cold PBS (5min), blocked and permeabilized in a PBS solution containing 2% donkey serum and 0.5% TX-100 for 60 min. Slides were rinsed once with ice-cold PBS and incubated with primary antibody diluted (1:100 for p38 α and phospho p38; 1:200 for p38 γ) in PBS containing 1% donkey serum and 0.1% TX-100 overnight at 4 °C in a closed humidified chamber. The next day, slides were rinsed three times with ice-cold PBS and incubated for 1 h with ALEXA fluor 488-labeled donkey anti-rabbit IgG antibody (1:600) and 1.5 μ M TO-PRO 3 iodide in PBS containing 1% donkey serum and 0.1% TX-100. Finally, slides were rinsed three times with ice-cold PBS, drained and mounted using 15 μ l of DABCO/glycerol medium. p38 α , p38 γ , and TO-PRO-3 (binds dsDNA) were visualized using an LSM-510 confocal fluorescence microscope (Carl Zeiss, Oberkochen, Germany) as described previously [17–19]. Control experiments were performed omitting the primary antibody.

2.6. Activation of p38

Purified GST-p38 isoforms (0.5 mg/ml) were activated by incubating in the presence of constitutively active His6-MKK6(EE) (0.16 mg/ml) in the presence of 1 mM ATP, 10 mM MgCl₂, 0.62 mM EGTA, 0.25mM DTT, 1 mM Na₃VO₄, 10 μ g/ml leupeptin, and 1 μ M microcystin in PBS overnight at room temperature without mixing [20]. This procedure has been shown previously to give complete dual (TGY) phosphorylation of p38 α [20]. To

separate phosphorylated GST-p38 from His6-MKK6(EE), reaction mixtures were incubated with glutathione Sepharose beads for 2–3 h at 4 °C on a clinical rotor, centrifuged for 2 min at 5000 rpm and 4 °C, washed thrice with TBS containing 1 mM EDTA (TBSE), and bound GST-p38 eluted with reduced glutathione. Purified, activated p38 was then dialyzed against TBSE buffer followed by a storage buffer comprising 50% glycerol, 50mM Tris (pH 7.5 at 5 °C), 150 mM NaCl, 0.1 mM EGTA, 0.03% Brij 35, 1 mM benzamidine, 0.2 mM PMSF, and 0.1% β -mercaptoethanol. Protein concentrations for each isoform were determined on 10–20% SDS-PAGE using BSA as a standard. Proteins were separated on gels and stained with Coomassie Brilliant Blue R250. Gels were scanned using a VersaDoc 4000 imaging system and p38 and BSA band densities determined using Quantity One software (Bio-Rad Laboratories, Inc). The specific activity of each phosphorylated p38 isoform was determined by incubating activated p38 (20 ng) with 0.33mg/ml MBP, 100 μ M [γ ³²P]ATP (10 μ Ci), 1 mM EGTA, 10 mM MgCl₂, 10mM DTT, 10 μ g/ml leupeptin, 1 mM Na₃VO₄, and 1 μ M microcystin LR at 30 °C for 2, 5, 8, and 10 min. Reactions were stopped with SDS-PAGE sample buffer and separated on 10–20% SDS-PAGE, stained with Coomassie blue, the MBP band excised from the gel, and ³²P incorporation in MBP measured by liquid scintillation counting.

2.7. Immunoblotting

SDS-PAGE and immunoblotting were performed as described previously [17]. Following the transfer, nitrocellulose membranes were rinsed in PBS and fixed by with glutaraldehyde [21].

2.8. Pressure-overload hypertrophy

Chronic pressure-overload was induced in male C57BL/6 mice (10 weeks old) by transverse aortic constriction (TAC) for 1, 3, 7, 14, and 28 days as described [21]. Sham mice underwent the same surgical procedure except the aortae were not constricted.

Animal handling followed the guidelines of the Canadian Council of Animal Care.

2.9. Miscellaneous methods

Protein concentrations were determined by the method of Bradford [22] using bovine serum albumin (BSA) as a standard.

3. Results

3.1. Expression of p38 isoforms in heart

Although the expression of p38 α , p38 β , and p38 γ in heart has been shown previously [5], nothing is known about the expression of p38 δ or the relative abundance of each isoform. To further our understanding of p38 signaling in heart we quantified the relative mRNA abundance for each p38 isoform using real time quantitative PCR. As the p38 isoforms show high degree of DNA sequence homology (>60%), primers were directed towards regions of lower homology and on different exons. In addition, primers were designed to specifically amplify amplicons of similar size (p38 α 271 bases, p38 β 264 bases, p38 γ 269 bases, and p38 δ 202 bases). Equal amplicon size allowed us to better compare the relative expression

levels. The specificity of each primer pair was verified both by melting curve and sequencing. Using these isoform specific primers (Table 1), the mRNA abundance was measured in mouse heart and expressed relative to the abundance of p38 α mRNA. We detected mRNA for all four p38 isoforms with p38 α and p38 γ mRNAs being the most abundant followed by p38 β (10.6%), and p38 δ (0.08%) (Fig. 1A).

As it's the level of protein that is important with respect to function, we examined the expression of each p38 isoform by immunoblotting. At the amino acid level p38 isoform have more than 60% sequence homology [23], hence there is possibility that antibodies may cross react. We first used purified recombinant p38 isoforms (Fig. 1B) to assess the isoform-selectivity of each antibody. Different commercially available antibodies were screened against pure p38 isoforms and only those that were specific for the intended isoform were employed in subsequent studies (Fig. 1C). Using these isoform-specific antibodies, expression of each p38 isoform in hearts lysate was assessed by immunoblotting. All four p38 isoforms were detected (Fig. 1D). We then quantified the expression of p38 isoform expression by immunoblotting using purified GST-p38 isoforms as standards. Similar to mRNAs, p38 α and p38 γ were the most abundant isoforms at the protein level, followed by p38 β (0.018%) and p38 δ (0.016%) (Fig. 1E). In conclusion p38 δ is expressed in heart and at a level comparable to p38 β . Furthermore, p38 α and p38 γ are expressed at similar levels, indicating that p38 γ might also play an important role in regulating cardiac function.

3.2. p38 expression during chronic pressure overload

p38 α/β dominant negative transgenic mice are resistant to cardiac fibrosis during pressure overload [10]. In addition, inhibition of p38 α/β activity reduces apoptotic cell death in cultured myocytes and intact heart during pressure overload or ischemia/reperfusion [11,12]. However, although they are activated by similar upstream kinases, there is very little known about the function of p38 γ and p38 δ . Our results (Fig. 1A and E) showed p38 γ is expressed at levels similar to p38 α . Therefore, we examined the effect of chronic pressure overload on the expression of each p38 isoform to see if any particular isoform shows alterations in expression, as this would suggest potentially a more prominent role in cardiac pathophysiology. Chronic pressure overload was induced by transverse aorta constriction and mice were sacrificed after 1, 3, or 7 days. Total RNA was subsequently isolated and the relative mRNA abundance of each p38 isoform was quantified by qPCR. There was no change in the quantity of p38 α mRNA. In contrast, p38 β and p38 δ mRNA increased within 1 day of pressure overload and remained elevated whereas p38 γ mRNA showed an initial increase but had returned to basal levels by day 7 (Fig. 2A). However, although increases in mRNA abundance were observed for p38 β , p38 γ , and p38 δ , the protein level of each isoform was unaltered within this time-frame (Fig. 2B).

Chronic pressure overload induces a hypertrophic response that is associated with both the re-expression of fetal genes and activation of the p38 MAPK pathway. To verify the establishment of a hypertrophic response, we assessed the levels of atrial natriuretic peptide (ANP) and β -myosin heavy chain (β -MHC) [24] mRNA in sham and banded hearts by qPCR. ANP and β -MHC mRNA was increased in TAC hearts on day 1 and gradually increased further through day 7 (Fig. 2D). The time-course for activation of p38 signaling

was also examined. We examined the effect of pressure overload on the phosphorylation of p38 MAPK and its downstream effector hsp25 using phospho-specific antibodies to the T-G-Y motif in p38 and Ser-15 and Ser-82 in hsp25 (Fig. 2C). p38 phosphorylation was increased on day 1, peaked on day 3, and then declined but remained elevated, relative to sham animals, on day 7. The phospho-p38 antibody revealed a doublet on day 3, suggested at least 2 isoforms were activated. A similar time course was observed for phosphorylation of hsp25 (Fig. 2C). Hence, although chronic pressure overload include a sustained increase in ANP and β -MHC mRNA, high levels of p38 activation were not sustained.

Similar to ANP and β -MHC, the abundance of p38 β and p38 δ mRNA increased in response to pressure overload. ANP and β -MHC are fetally expressed genes that become re-expressed during pathological cardiac hypertrophy. We sought to determine the temporal expression of each p38 isoform in the ventricular myocardium during post-natal development. mRNA was isolated from neonatal, 7 day, 20 day, and adult (8 week) hearts. Analysis by qPCR showed that (relative to neonatal hearts) p38 α mRNA was reduced by 2-fold at 7 and 20 days (Fig. 3A), p38 β mRNA dropped 2-fold at 20 days (Fig. 3B), p38 γ mRNA was reduced at 7 and 20 days (Fig. 3C), and p38 δ mRNA dropped more than 3-fold within 7 days of birth (Fig. 3D). However, whereas the mRNA content for p38 α , p38 β , and p38 γ had returned to levels comparable to those in the neonatal heart by 8 weeks, p38 δ mRNA remained low in the adult heart.

3.3. Specific activity

The level of expression is only one determinant of the impact an enzyme may have on cellular function. Other factors include its subcellular localization, substrate specificity, activation, and turnover number. Therefore we examined the specific activity of each p38 isoform, following phosphorylation *in vitro* by His6-MKK6(EF) and repurification, towards MBP. The specific activity of p38 α was 75.70 U/mg (Fig. 4A), p38 β was 76.05 U/mg (Fig. 4B), p38 γ was 96.13 U/mg (Fig. 4C), and p38 δ was 190.9 U/mg (Fig. 4D). Hence, the specific activity of p38 δ towards MBP was more than two fold higher than the other isoforms. However, this cannot compensate for the comparatively low levels of p38 δ protein relative to p38 α and p38 γ , and suggesting p38 α and p38 γ may play more prominent roles in the heart.

3.4. Subcellular distribution of p38 γ and p38 α during pressure overload induced hypertrophy

Although each p38 isoforms are activated, with variable efficiency, by similar upstream kinases MKK3, MKK4, and MKK6 [25], no study to date has described the effect of chronic pressure overload on the cellular localization of p38. Hence, we studied TAC induced pressure-overload in mice. Mice were sacrificed 7, 14, and 28 days after surgery, time-points where molecular and morphological changes associated with pathological hypertrophy were clearly apparent. These time points were chosen to examine localization once hypertrophy had been established rather than during the acute or early phases of the response to pressure overload [26]. Cryosections of the ventricles were prepared and immunostained with anti-p38 α , anti-p38 γ , or anti-phospho-p38 antibodies. As immunoblotting showed p38 β and p38 δ 1) were in far lower abundance and 2) their antibodies reveal non-specific bands, we

did not determine the subcellular localization of these isoforms. p38 α and p38 γ localized to the cytoplasm and nucleus in sham hearts. However, increased nuclear localization of p38 γ was observed in sections from TAC hearts at 7, 14, and 28 days (Left panel of Fig. 5A, B and C). In contrast, no nuclear accumulation of p38 α was observed (Right panel of Fig. 5A, B and C). Reexpression of BNP and β -MHC confirmed a persistent hypertrophic response in these hearts (Fig. 5D). No differences between TAC and sham were observed with the anti-phospho-p38 antibody (results not shown), which is consistent with the low levels of phospho-p38 detected by immunoblot after day 3 (Fig. 2C). These results suggest that p38 α and p38 γ respond differently to chronic pressure overload in terms of their subcellular localization. These differences in localization would result in access to different substrates and hence distinct functional effects. Nuclear accumulation for p38 γ and not p38 α , as well as differential regulation at transcriptional level during heart development and hypertrophy strongly suggests non-redundant roles of p38 α and p38 γ in the heart as well as a potential role for p38 γ in the maintenance and/or progression of pathological ventricular hypertrophy.

To understand the consequences of the nuclear localization of p38 γ , we sought to identify substrates in cytosolic and nuclear fractions from rat heart specific to phosphorylated, activated GST-p38 α / β / γ / δ upon incubation in the presence of [γ - 32 P]ATP. However, no isoform-specific phosphorylation was observed in either subcellular fraction (results not shown). The failure to detect added incorporation of 32 P into proteins in the cytosolic and nuclear fractions might reflect low copy number or phosphorylation status of target proteins in these fractions or, perhaps, the absence of a suitable scaffolding protein.

4. Discussion

To date p38 α , p38 β , and p38 γ have been reported in heart with p38 α being considered to be the dominant isoform [3–5]. We now show that p38 δ is expressed in the mouse heart at protein levels that are comparable to p38 β . In addition, at both the mRNA and protein levels, expression of p38 α and p38 γ are similar, suggesting that both play important roles in the heart. Finally, p38 γ was shown to accumulate in the nucleus in hearts hypertrophying due to chronic pressure overload.

p38 δ was detected in mouse heart by immunoblotting and found to be expressed at levels similar to p38 β . There are numerous reports suggesting that, although expressed at levels much lower than p38 α , p38 β plays a distinct role in the heart. p38 α and p38 β are activated in the heart in response to ischemia and reperfusion [27] and pressure overload [26], as well as following the exposure of cardiac myocytes to cytokines [28], hypertrophic agonists [29] and mechanical stretch [30]. In simulated ischemia phosphorylation of p38 α increases whereas that of p38 β decreases [31]. Moreover inhibition of p38 α during ischemia has a protective effect [31,32]. Similarly, p38 α ^{+/-} mice are resistant to infarction [33]. In contrast, during preconditioning, activation of p38 α decreases and that of p38 β increases [31], suggesting that p38 β is protective whereas p38 α is detrimental. Expression of constitutively active MKK6bE or MKK3bE along with wild-type or dominant negative p38 α (DN-p38 α) or p38 β (DN-p38 β) in neonatal myocytes indicates p38 α mediates apoptosis whereas p38 β is involved in the hypertrophic response [34]. Mice expressing a dominant-negative form of 14-3-3 η (DN-14-3-3) are unable to compensate for pressure overload resulting in increased

myocyte apoptosis and mortality. DN-14-3-3/DNp38 β mice, and to a lesser extent DN-14-3-3/DN-p38 α mice, show reduced mortality and cardiac myocyte apoptosis in response to pressure overload [35]. Taken together, these data indicate that, although expressed at lower levels than p38 α or p38 γ , p38 β plays a distinct role in the heart. Hence, further study might also reveal a unique role for p38 δ in the heart.

The anti-apoptotic role of p38 α and p38 β has been studied in pressure overload hypertrophy and ischemia/reperfusion [11,12]. However, little is known regarding the roles of p38 γ or p38 δ . We therefore studied the effect of chronic pressure overload on the expression and subcellular localization of p38 isoforms in the mouse heart. No change in expression of p38 α mRNA was observed in response to chronic pressure overload, whereas mRNA levels for p38 β / γ / δ was increased. However, no corresponding change in expression was detected at protein level during the first 7 days following TAC. It has been shown in transfected HEK293T cells overexpressing p38 α and p38 γ that p38 α activation reduces p38 γ protein via activation of the c-jun-dependent ubiquitin–proteasome pathway [36]. However, in heart, although there was a peak of p38 phosphorylation at day 3 of TAC, p38 γ protein levels were unaffected.

p38 γ was detected in the cytoplasm and nucleus. Due to the -KETXL sequence in its C-terminus [15] p38 γ is able to bind PDZ domains. As a result of this, p38 γ binds to a number of PDZ-containing proteins such as α 1-syntrophin, SAP90/PSD95, and SAP97/hDlg [15,37]. These proteins are scaffold proteins that are usually targeted to the cytoskeleton. Upon phosphorylation by p38 γ , hDlg is released from the cytoskeleton [37]. Other substrates include the microtubule-associated protein tau [38] and CapZ-interacting protein [39]. Although p38 γ was previously reported to be exclusively cytosolic in neonatal rat ventricular myocytes [3], in the present study, p38 γ immunoreactivity accumulated in the nuclei during chronic pressure overload. Nuclear substrates for p38 γ identified to date include transcription factors such as ATF2, AP-1, Elk-1, MEF2A, p53, SAP1 and SAP2 [40–42]. MEF2 transcription factors are involved in the regulation of inducible gene expression during hypertrophy and MEF2 activity is increased during pressure or volume overload [43]. Activation of MEF2A and MEF2C is stimulated by p38; however, p38 α may be more efficient than p38 γ at activating MEF2 [44–46]. Expression of an active variant of p38 γ attenuates, whereas active p38 α induces, expression of AP-1-driven reporter genes [42]. Furthermore, in the skeletal muscle cell line C2C12, over expression of p38 γ leads to differentiation from myoblasts to myotubes whereas expression of dominant negative p38 γ prevents this differentiation [16,47]. Given that p38 γ is a prominent p38 isoform in heart and it accumulated in the nucleus during chronic pressure overload, it is quite possible that p38 γ plays a role in the changes in gene expression associated with pathological hypertrophy.

Nuclear translocation of p38 γ but not p38 α 7–28 days after the initiation of pressure overload may result from 1) the activation of TAB-1, which binds to and activates p38 α independently of MKK3/6 but retains the activated p38 α in the cytosol [48], 2) the sustained activation of a p38 γ -specific MKK resulting in its nuclear translocation, 3) the interaction of p38 γ with a PDZ-domain containing protein that shuttles p38 γ into the nucleus, or 4) the interaction of activated p38 γ with a nuclear PDZ-domain containing protein which retains

p38 γ within the nucleus. Similar to p38 γ , YES kinase-associated protein (YAP) contains the PDZ-binding motif-FLTWL in its C-terminus that is essential for both translocation of YAP from the cytosol to the nucleus and its ability to promote apoptosis in response to low serum [49]. It is thought that one or more yet unidentified PDZ-domain containing proteins are responsible for the nucleocytoplasmic shuttling of YAP. In addition, PDZ-containing proteins including the Ca²⁺/calmodulin-associated Ser/Thr kinase (CASK), L-periaxin, ZO-1, MAGI-1c, and LIM-kinase 1 contain nuclear localization signals and shuttle between the cytoplasm and the nucleus [50–55].

The small GTP-binding protein RhoA activates p38 γ , but not p38 α , through MKK3/6 [41]. Similarly, overexpression of the MEK kinase Cot in NIH3T3 cells activates cotransfected p38 γ , but not p38 α or p38 δ [56]. MKK6 activates all p38 isoforms but the efficiency varies between isoforms and with the nature of the stimuli [57]. Remy et al. recently characterized the phosphorylation and activation of each p38 isoform in mouse embryonic fibroblasts (MEF) lacking MKK3 and/or MKK6 in response to hyperosmotic shock, ultraviolet (UV) radiation, anisomycin, and TNF α [25]. These authors showed that MKK3 and MKK6 are both essential for full activation of p38 α , p38 γ and p38 β by environmental stress, whereas MKK6 is the major p38 γ activator in response to TNF α . In contrast, MKK3 mediated p38 δ activation in response to UV radiation, hyperosmotic shock, anisomycin or TNF α . In the absence of MKK3 and MKK6, MKK4 mediates the activation of p38 α [58] but not p38 β / δ / γ to UV radiation [25]. These authors concluded that the ability of MKK3 versus MKK6 to activate an individual p38 isoform is dependent upon the nature and intensity of the stimulus as well cell-specific properties such as the expression level of MKK3, MKK6 and of the p38 isoforms. As the actual effect of activation of a given isoform would, then depend upon its subcellular localization and substrate specificity, p38 signaling may actually be spatially and temporally compartmentalized in physiological systems. Further study is required to understand role of p38 γ in the myocyte nucleus during chronic pressure overload.

5. Conclusions

We have shown that p38 α and p38 γ are present at comparable protein levels in the mouse heart. In addition, p38 δ is expressed at protein levels similar to p38 β . Although their RNA abundance for p38 β , p38 δ , and p38 γ changed in response to chronic pressure overload, the corresponding protein levels remained unchanged. Furthermore, p38 γ accumulated in nucleus in hypertrophying hearts whereas p38 α did not, which might suggest a distinct role for p38 γ during the maintenance or progression of ventricular hypertrophy in response to chronic pressure overload.

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The authors declare no conflicts of interest.

Abbreviations

DTT	dithiothreitol
ERK	extracellular signal-regulated kinase
GST	glutathione <i>S</i> -transferase
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
NES	nuclear export signal
PKI	cyclic AMP-dependent protein kinase inhibitory peptide
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
TAC	transverse aortic constriction
TX-100	Triton X-100

References

1. Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, Wright A, Vanderbilt C, Cobb MH. *Chem Rev.* 2001; 101(8):2449. [PubMed: 11749383]
2. Clerk A, Sugden PH. *Circ Res.* 2006; 99(5):455. [PubMed: 16946139]
3. Court NW, dos Remedios CG, Cordell J, Bogoyevitch MA. *J Mol Cell Cardiol.* 2002; 3:413.
4. Ono K, Han J. *Cell Signal.* 2000; 12(1):1. [PubMed: 10676842]
5. Seta K, Sadoshima J. *J Mol Cell Cardiol.* 2002; 34(6):597. [PubMed: 12054845]
6. Ge B, Gram H, di Padova F, Huang B, New L, Ulevitch RJ, Luo Y, Han J. *Science.* 2002; 295:1291. [PubMed: 11847341]
7. Salvador JM, Mittelstadt PR, Guszczynski T, Copeland TD, Yamaguchi H, Appella E, Fornace AJ Jr, Ashwell JD. *Nat Immunol.* 2005; 6:390. [PubMed: 15735648]
8. Ota A, Zhang J, Ping P, Han J, Wang Y. *Circ Res.* 2010; 106:1404. [PubMed: 20299663]
9. Gerits N, Kostenko S, Moens U. *Transgenic Res.* 2007; 16(3):281. [PubMed: 17219248]
10. Zhang S, Weinheimer C, Courtois M, Kovacs A, Zhang CE, Cheng AM, Wang Y, Muslin AJ. *J Clin Invest.* 2003; 111:833. [PubMed: 12639989]
11. Kerkela R, Force T. *J Am Coll Cardiol.* 2006; 48(3):556. [PubMed: 16875983]
12. Baines CP, Molkentin JD. *J Mol Cell Cardiol.* 2005; 38(1):47. [PubMed: 15623421]
13. Ma XL, Kumar S, Gao F, Loudon CS, Lopez BL, Christopher TA, Wang C, Lee JC, Feuerstein GZ, Yue TL. *Circulation.* 1999; 99(13):1685. [PubMed: 10190877]
14. Ren J, Zhang S, Kovacs A, Wang Y, Muslin AJ. *J Mol Cell Cardiol.* 2005; 38(4):617. [PubMed: 15808838]
15. Hasegawa M, Cuenda A, Spillantini MG, Thomas GM, Buee-Scherrer V, Cohen P, Goedert M. *J Biol Chem.* 1999; 274(18):12626. [PubMed: 10212242]
16. Lechner C, Zahalka MA, Giot JF, Moller NP, Ullrich A. *Proc Natl Acad Sci U S A.* 1996; 93(9):4355. [PubMed: 8633070]
17. Boivin B, Chevalier D, Villeneuve LR, Rousseau E, Allen BG. *J Biol Chem.* 2003; 278:29153. [PubMed: 12756260]

18. Boivin B, Lavoie C, Vaniotis G, Baragli A, Villeneuve LR, Ethier N, Trieu P, Allen BG, Hébert TE. *Cardiovasc Res*. 2006; 71(1):69. [PubMed: 16631628]
19. Boivin B, Villeneuve LR, Farhat N, Chevalier D, Allen BG. *J Mol Cell Cardiol*. 2005; 38:665. [PubMed: 15808843]
20. Keesler GA, Bray J, Hunt J, Johnson DA, Gleason T, Yao Z, Wang SW, Parker C, Yamane H, Cole C, Lichenstein HS. *Protein Expr Purif*. 1998; 14(2):221. [PubMed: 9790884]
21. Connern CP, Halestrap AP. *Biochemistry*. 1996; 35(25):8172. [PubMed: 8679570]
22. Bradford MM. *Anal Biochem*. 1976; 72:248. [PubMed: 942051]
23. New L, Han J. *Trends Cardiovasc Med*. 1998; 8(5):220. [PubMed: 14987568]
24. Du XJ. *Br J Pharmacol*. 2007; 152(2):169. [PubMed: 17592504]
25. Remy G, Risco AM, Inesta-Vaquera FA, Gonzalez-Teran B, Sabio G, Davis RJ, Cuenda A. *Cell Signal*. 2010; 22:660. [PubMed: 20004242]
26. Esposito G, Prasad SVN, Rapacciuolo A, Mao L, Koch WJ, Rockman HA. *Circulation*. 2001; 103:1453. [PubMed: 11245652]
27. Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, Marshall CJ, Sugden PH. *Circ Res*. 1996; 79:162. [PubMed: 8755992]
28. Clerk A, Harrison JG, Long CS, Sugden PH. *J Mol Cell Cardiol*. 1999; 31(12):2087. [PubMed: 10640438]
29. Clerk A, Sugden PH. *Am J Cardiol*. 1999; 83(12A):64H.
30. Kudoh S, Komuro I, Hiroi Y, Zou Y, Harada K, Sugaya T, Takekoshi N, Murakami K, Kadowaki T, Yazaki Y. *J Biol Chem*. 1998; 273(37):24037. [PubMed: 9727021]
31. Saurin AT, Martin JL, Heads RJ, Foley C, Mockridge JW, Wright MJ, Wang Y, Marber MS. *FASEB J*. 2000; 14:2237. [PubMed: 11053245]
32. Kumphune S, Bassi R, Jacquet S, Sicard P, Clark JE, Verma S, Avkiran M, O'Keefe SJ, Marber MS. *J Biol Chem*. 2010; 285(5):2968. [PubMed: 19996096]
33. Otsu K, Yamashita N, Nishida K, Hirotani S, Yamaguchi O, Watanabe T, Hikoso S, Higuchi Y, Matsumura Y, Maruyama M, Sudo T, Osada H, Hori M. *Biochem Biophys Res Commun*. 2003; 302(1):56. [PubMed: 12593847]
34. Wang Y, Huang S, Sah VP, Ross J Jr, Brown JH, Han J, Chien KR. *J Biol Chem*. 1998; 273:2161. [PubMed: 9442057]
35. Zhang S, Ren J, Zhang CE, Treskov I, Wang Y, Muslin AJ. *Circ Res*. 2003; 93(11):1026. [PubMed: 14593000]
36. Qi X, Pohl NM, Loesch M, Hou S, Li R, Qin JZ, Cuenda A, Chen G. *J Biol Chem*. 2007; 282(43):31398. [PubMed: 17724032]
37. Sabio G, Arthur JS, Kuma Y, Pegg M, Carr J, Murray-Tait V, Centeno F, Goedert M, Morrice NA, Cuenda A. *EMBO J*. 2005; 24(6):1134. [PubMed: 15729360]
38. Goedert M, Hasegawa M, Jakes R, Lawler S, Cuenda A, Cohen P. *FEBS Lett*. 1997; 409(1):57. [PubMed: 9199504]
39. Evers CE, McNeill H, Knebel A, Morrice N, Arthur SJ, Cuenda A, Cohen P. *Biochem J*. 2005; 389(Pt 1):127. [PubMed: 15850461]
40. Cuenda A, Cohen P, Buee-Scherrer V, Goedert M. *EMBO J*. 1997; 16(2):295. [PubMed: 9029150]
41. Marinissen MJ, Chiariello M, Gutkind JS. *Genes Dev*. 2001; 15(5):535. [PubMed: 11238375]
42. Askari N, Diskin R, Avitzour M, Capone R, Livnah O, Engelberg D. *J Biol Chem*. 2007; 282(1):91. [PubMed: 17088247]
43. Akazawa H, Komuro I. *Circ Res*. 2003; 92(10):1079. [PubMed: 12775656]
44. Zetser A, Gredinger E, Bengal E. *J Biol Chem*. 1999; 274(8):5193. [PubMed: 9988769]
45. Zhao M, New L, Kravchenko VV, Kato Y, Gram H, di Padova F, Olson EN, Ulevitch RJ, Han J. *Mol Cell Biol*. 1999; 19(1):21. [PubMed: 9858528]
46. Wang BW, Chang H, Kuan P, Shyu KG. *J Endocrinol*. 2008; 197(1):85. [PubMed: 18372235]
47. Perdiguero E, Ruiz-Bonilla V, Gresh L, Hui L, Ballestar E, Sousa-Victor P, Baeza-Raja B, Jardi M, Bosch-Comas A, Esteller M, Caelles C, Serrano AL, Wagner EF, Munoz-Canoves P. *EMBO J*. 2007; 26(5):1245. [PubMed: 17304211]

48. Lu G, Kang YJ, Han J, Herschman HR, Stefani E, Wang Y. J Biol Chem. 2006; 281(9):6087. [PubMed: 16407200]
49. Oka T, Sudol M. Genes Cells. 2009; 14(5):607. [PubMed: 19371381]
50. Gottardi CJ, Arpin M, Fanning AS, Louvard D. Proc Natl Acad Sci U S A. 1996; 93(20):10779. [PubMed: 8855257]
51. Dobrosotskaya I, Guy RK, James GL. J Biol Chem. 1997; 272(50):31589. [PubMed: 9395497]
52. Yang N, Mizuno K. Biochem J. 1999; 338(Pt 3):793. [PubMed: 10051454]
53. Sherman DL, Brophy PJ. J Biol Chem. 2000; 275(7):4537. [PubMed: 10671475]
54. Traweger A, Lehner C, Farkas A, Krizbai IA, Tempfer H, Klement E, Guenther B, Bauer HC, Bauer H. Differentiation. 2008; 76(1):99. [PubMed: 17973926]
55. Ojeh N, Pekovic V, Jahoda C, Maatta A. J Cell Sci. 2008; 121(Pt 16):2705. [PubMed: 18664494]
56. Chiariello M, Marinissen MJ, Gutkind JS. Mol Cell Biol. 2000; 20(5):1747. [PubMed: 10669751]
57. Alonso G, Ambrosino C, Jones M, Nebreda AR. J Biol Chem. 2000; 275(51):40641. [PubMed: 11010976]
58. Brancho D, Tanaka N, Jaeschke A, Ventura JJ, Kelkar N, Tanaka Y, Kyuuma M, Takeshita T, Flavell RA, Davis RJ. Genes Dev. 2003; 17(16):1969. [PubMed: 12893778]

Fig. 1.

Cardiac expression of p38 in murine heart. (A) The abundance of them RNA for each p38 isoform was measured in murine heart total RNA by qPCR. Shown are the mean $\pm$ S.E. (n=5). (B) Purified GST-p38 isoforms (2 μ g) were separated on 10–20% acrylamide-gradient SDS-PAGE and visualized using Coomassie Brilliant Blue R250. (C) Isoform selectivity of p38 antibodies. Purified GST-p38 isoforms (5 ng) were separated on 10–20% acrylamide-gradient SDS-PAGE, transferred to nitrocellulose, and probed with the indicated p38 isoform-specific antibodies. (D) Detection of p38 isoforms in mouse heart lysates. Lysates (50 μ g) from 2 separate hearts (a, b) were separated on 10–20% acrylamide-gradient SDS-PAGE, transferred to nitrocellulose and probed with the indicated p38 isoform-specific antibody. Detection conditions were separately optimized for each isoform. (E) Quantification of p38 isoforms in mouse heart lysates. Standard curves were constructed for each isoform using the purified recombinant p38 isoforms shown in Fig. 1B. Heart lysates (5 and 50 μ g) and were resolved, together with a standard curve of the appropriate p38 isoform, on 10–20% acrylamide-gradient SDS-PAGE, transferred to nitrocellulose, and probed with an appropriate isoform specific antibody. The absolute amount of each isoform was calculated (in fmol), using the predicted molecular mass of the GST-p38 isoform, and expressed per microgram of heart lysate. Numbers at the left indicate the positions of the molecular mass marker proteins (kDa).

Fig. 2.

Effect of chronic pressure overload on p38 expression. Mice were sacrificed 1, 3, and 7 days after constriction of the transverse aorta (TAC) and the hearts isolated. (A) mRNA for each p38 was quantified by qPCR and normalized to GAPDH expression. Shown are the relative abundance of p38 α , p38 β , p38 γ , and p38 δ mRNA. (B) To assess p38 expression at the protein level, 50 μ g heart lysate were separated on 10–20% acrylamide-gradient SDS-PAGE, transferred to nitrocellulose and probed the indicated antibodies. Detection conditions were separately optimized for each isoform. (C) Phosphorylation of p38 and HSP25 was studied using phosphospecific antibodies. (D) Cardiac fetal gene expression. To confirm that TAC induced a hypertrophic response, in each heart atrial natriuretic peptide (ANP) and β -myosin

heavy chain (β -MHC) mRNA was quantified by qPCR. Shown are the mean $\pm$ S.E. (n=6).
***, $p<0.001$: **, $p<0.01$: *, $p<0.05$: one-way ANOVA with Newman–Keuls post-hoc analysis.

Fig. 3.

Changes of p38 mRNA during post-natal heart development. The abundance mRNA each p38 isoform was determined during post-natal heart development. Total RNA was isolated from neonatal, 7 day, 20 day, and adult (8 weeks) hearts and the level of mRNA quantified by qPCR. ((A) p38 α , (B) p38 β , (C) p38 γ , and (D) p38 δ). Samples were normalized to GAPDH. Shown are the mean $\pm$ S.E. (n=3). ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$: one-way ANOVA with Newman-Keuls post-hoc analysis.

Fig. 4.

Specific activity of p38 isoforms. An aliquot (20 ng) of each activated p38 isoform was incubated with MBP (0.33 mg/ml) and [γ - 32 P]ATP. Reactions were terminated after 2, 5, 8, or 10 min by addition of SDS-PAGE sample buffer. Proteins were separated on 10–20% SDS-PAGE, stained with Coomassie blue, the MBP band excised and 32 P incorporation in MBP determined by scintillation counting. 32 P incorporation was plotted against time for (A) p38 α , (B) p38 β , (C) p38 γ , and (D) p38 δ . Shown are the mean $\pm$ S.E. (n=2).

Fig. 5.

Subcellular localization of p38 α and p38 γ during chronic pressure overload. Mice were sacrificed after 1, 2, or 4 weeks of TAC and 14 μ m sections prepared. After fixation and blocking, sections were probed with anti-p38 α , anti-p38 γ or anti-phospho-p38 (not shown) antibodies and visualized using an ALEXA fluor 448-coupled secondary (anti-rabbit) antibody followed by confocal microscopy. Immunolocalization of p38 α and p38 γ after (A) 7 days, (B) 14 days, and (C) 28 days of TAC. Nuclei were visualized by staining with TO-PRO3 iodide. In 14-d samples, actin distribution was also revealed using phalloidin. Shown are the representative images of $n=3$ and 3 sections per heart. (D) To confirm that TAC induced a hypertrophic response, sections from each heart were also assessed for expression of B-type natriuretic peptide (BNP) and β -myosin heavy chain (β -MHC). Shown are the mean $\pm$ S.E. ($n=3$). ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$: one-way ANOVA with Newman-Keuls post-hoc analysis.

Table 1

Primers used during real-time qPCR.

Target	Primers
p38 α S	5'-AAT CTC CTC AGA GTC TGC AAG-3'
p38 α AS	5'-GCT CTT CCA CTC ATC TAT GAG AAG G-3'
p38 β S	5'-GAC CTC AGC AGT GTC TTC CAT-3'
p38 β AS	5'-GAG CTG TGA GGG TTC CAG G-3'
p38 γ S	5'-TTT GCC TCT GTC CTG ACC AAC-3'
p38 γ AS	5'-GGA ACT CTG GCT CCT AGC TGC-3'
p38 δ S	5'-GAT TTC ACA CAG CTC TTT CCA CGC-3'
p38 δ AS	5'-CCA CAC TGA GTT TCT CAT GTT CT-3'
ANP S	5'-GTG CGG TGT CCA ACA CAG A-3'
ANP AS	5'-TTC TAC CGG CAT CTT CTC CTC-3'
BNP S	5'-GTT TGG GCT GTA ACG CAC TGA-3'
BNP AS	5'-GAA AGA GAC CCA GGC AGA GTC A-3'
β -MHC S	5'-AGG GTG GCA AAG TCA CTG CT-3'
β -MHC AS	5'-CAT CAC CTG GTC CTC CTT CA-3'
GAPDH S	5'-CTG CAC CAC CAA CTG CTT AGC-3'
GAPDH AS	5'-ACT GTG GTC ATG AGC CCT TCC A-3'

S, sense; AS, anti-sense; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; qPCR, quantitative polymer chain reaction; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; β -MHC, β -myosin heavy chain.