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REVIEW

MAPK signalling in cardiovascular health and disease: molecular mechanisms and therapeutic targets

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ABSTRACT

Intracellular MAPK (mitogen-activated protein kinase) signalling cascades probably play an important role in the pathogenesis of cardiac and vascular disease. A substantial amount of basic science research has defined many of the details of MAPK pathway organization and activation, but the role of individual signalling proteins in the pathogenesis of various cardiovascular diseases is still being elucidated. In the present review, the role of the MAPKs ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 MAPK in cardiac hypertrophy, cardiac remodelling after myocardial infarction, atherosclerosis and vascular restenosis will be examined, with attention paid to genetically modified murine model systems and to the use of pharmacological inhibitors of protein kinases. Despite the complexities of this field of research, attractive targets for pharmacological therapy are emerging.

INTRODUCTION: INTRACELLULAR SIGNAL TRANSDUCTION PATHWAYS

The growth and survival of adult cardiomyocytes, SMCs (smooth muscle cells) and macrophages are regulated by extracellular ligands, growth factors and cytokines that bind to cell-surface receptors and activate intracellular signal transduction cascades. These signalling pathways control essential processes in all eukaryotic cells, including gene transcription, protein translation, cytoskeletal remodelling, endocytosis, cell metabolism, cell proliferation and survival. The analysis of the function of specific signalling proteins in cardiovascular pathophysiology is a major goal for biomedical researchers.

Key words: atherosclerosis, hypertrophy, intracellular signalling, mitogen-activated protein kinase (MAPK), myocardial infarction, remodelling.

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ANF, atrial natriuretic factor; apoE, apolipoprotein E; ASK1, apoptosis signal-regulating kinase 1; ATM, ataxia telangectasia mutated; CKO, cardiac-specific knockout; DN, dominant-negative; DUSP6, dual-specificity phosphatase 6; eIF4E, eukaryotic initiation factor 4E; ERK, extracellular-signal-regulated kinase; ET1, endothelin 1; FGF1, fibroblast growth factor 1; GPCR, G-protein-coupled receptor; Grb2, growth-factor-receptor-bound protein 2; JNK, c-Jun N-terminal kinase; LAD, left anterior descending; LDL low-density lipoprotein; acLDL, acetylated LDL; L-NAME, N^G-nitro-L-arginine methyl ester; LV, left ventricular; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MHC, myosin heavy chain; MI, myocardial infarction; MKK, MAPK kinase; MKKK, MAPK kinase kinase; MLC, myosin light-chain; Mnk, MAPK-interacting kinase; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T-cells; oxLDL, oxidized LDL; $p90^{RSK}$, 90 kDa ribosomal protein S6 kinase; PDGF, platelet-derived growth factor; PI3Kα, phosphoinositide 3-kinase α; Rb, retinoblastoma protein; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; SH2, Src homology 2; SMC, smooth muscle cell; SOS, Son of sevenless; SR-A, scavenger receptor A; TAC, transverse aortic constriction; TAK1, transforming growth factor-β-activated kinase 1; VSMC, vascular SMC.

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When extracellular growth factors, ligands or cytokines bind to cell-surface receptors, conformational changes occur in these receptors altering the intracellular signalling potential of these proteins. In one of the best-understood examples, the extracellular dimeric PDGF (platelet-derived growth factor) ligand binds simultaneously to two single-membrane-pass PDGF RTKs (receptor tyrosine kinases) and this facilitates the clustering of the two receptors, so that they are able to phosphorylate one another on tyrosine residues on their intracellular portions [1]. These phosphorylated intracellular tyrosine residues, in turn, are docking sites for proteins that are able to bind to phosphorylated motifs, such as the SH2 (Src homology 2)-domain-containing protein Grb2 (growth-factor-receptor-bound protein 2) [2]. Grb2 is constitutively bound to the guanine nucleotide-exchange factor SOS (Son of sevenless) [3,4]. When Grb2 binds to phosphotyrosine motifs on the intracellular portion of RTKs, it brings SOS in close proximity to the plasma-membrane-tethered small GTPase Ras and facilitates Ras activation [5–7]. Ras is a master regulator of intracellular signalling cascades and it promotes the activation of MAPK (mitogen-activated protein kinase) cascades as well as other signalling pathways.

In addition to the direct activation of RTKs by growth-factor-mediated receptor clustering, there are several alternative mechanisms for transmembrane receptor activation that result in Ras activation. For example, direct activation of GPCRs (G-protein-coupled receptors) by extracellular ligands can result in the transactivation of RTKs, via the metalloproteinase-mediated extracellular release of tethered growth factors, such as heparin-binding EGF (epidermal growth factor), or by the action of intracellular signalling proteins, such as Src family tyrosine kinases, on the intracellular portions of RTKs [8]. Furthermore, RTK activation by growth factor binding can result in the transactivation of GPCRs, which promote the activation of G_{α} and $G_{\beta\gamma}$ subunits. Transactivation of GPCRs can either occur as a result of GPCR ligand production downstream of RTK activation or because of complex formation between RTKs and GPCRs [9].

MAPK cascades are triple kinase pathways that include a MKKK (MAPK kinase kinase), a MKK (MAPK kinase) and a terminal MAPK. MAPK cascades may be organized in this fashion in order to promote signal amplification and fidelity [10]. Furthermore, scaffolding proteins help to cluster particular components of MAPK cascades in specific subcellular localizations [11]. The first MKKK to be well characterized was Raf-1, a proto-oncogene that is a serine/threonine protein kinase [12]. Raf-1, and its highly related family members A-Raf and B-Raf, binds directly to activated GTP-bound Ras. Raf-1 binding to Ras results in several complicated post-translational modifications of Raf-1, ultimately leading to full kinase activation [13]. Once fully activated, Raf-1 phosphorylates and activates MKK1 or MKK2 {also called MEK1/2 [MAPK/ERK (extracellular-signal-regulated kinase) kinase 1/2]}. MKK1/2, in turn, phosphorylates ERK1 or ERK2 (ERK1/2) on a threonine and a tyrosine residue in its activation loop, leading to kinase activation. Fully activated ERK1/2 has a variety of substrates at the plasma membrane, in the cytosol and in the nucleus that regulate important aspects of cell physiology.

In addition to the Raf–MKK–ERK cascade, at least three other major MAPK cascades, the JNK (c-Jun N-terminal kinase), the p38 MAPK and the big MAPK (ERK5) cascades, play an important role in the regulation of cell physiology [10]. The ERK5 cascade will not be discussed further in the present review because it has been characterized in fewer models of cardiovascular disease than the other three MAPK cascades. Activated GTP-bound Ras indirectly promotes the activation of the JNK and p38 MAPK cascades, perhaps via the ability of Ras to directly bind to and activate PI3Kα (phosphoinositide 3-kinase α), which facilitates the activation of the Rac GTPase in some cell types [14,15]. Activated Rac, in turn, is able to bind to MEKK (MEK kinase) family members that are MKKKs for the JNK and p38 MAPK cascades [16]. Although the Grb2–SOS–Ras–PI3Kα–Rac pathway plays an important role in the activation of the JNK and p38 MAPK cascades in some situations, there are other important stimuli that activate these cascades independent of Ras, such as cell 'stress' due to the accumulation of ROS (reactive oxygen species), hyperosmolarity, genotoxicity or dysfunction of the endoplasmic reticulum [17–20]. In the case of the JNK and p38 MAPK cascades, there are several MKKKs, such as MEKK1–4, ASK1 (apoptosis signal-regulating kinase 1), TAK1 (transforming growth factor-β-activated kinase 1) and MLK3 (mixed-lineage kinase 3) [21–27]. The specific MKKK that regulates activation of the JNK or the p38 MAPK cascade is not known in many physiological contexts. There are two major MKKs in the JNK cascade, named MKK4 and MKK7. There are three genes that encode the JNK family members JNK1 (*MAPK8*), JNK2 (*MAPK9*) and JNK3 (*MAPK10*). There are four splice variants of JNK1, four splice variants of JNK2 and two splice variants of JNK3 that lead to the production of ten isoforms of either 46 or 54 kDa [28]. For the p38 MAPK cascade, there are several MKKKs, such as ASK1 and TAK1, and two major MKKs, named MKK3 and MKK6. There are four genes encoding members of the p38 MAPK family, called p38α (*MAPK14*), p38β (*MAPK11*), p38γ (*MAPK12*) and p38δ (*MAPK13*) [29]. It is apparent that upstream activators of the JNK and p38 MAPK pathways are overlapping, especially at the level of MKKKs, and this has led to confusion about the independent regulation of these pathways. A leading model is that scaffolding proteins establish specificity by binding to unique combinations of MAPK pathway kinases and substrates in particular subcellular localizations [11,30,31].

The tremendous progress made in the identification and characterization of the components of the MAPK cascades has led to an explosion in translational research attempting to link these signalling pathways to cardiovascular disease. In the present review, the role of the ERK, JNK and p38 MAPK cascades in the pathophysiology of cardiac hypertrophy, pathological remodelling after MI (myocardial infarction), development of atherosclerosis and vascular neointima formation will be discussed.

SIGNALLING MECHANISMS IN CARDIAC HYPERTROPHY: BACKGROUND AND MAPK-OVEREXPRESSION STUDIES

Most adult cardiomyocytes are unable to proliferate, and they respond to stress by enlarging due to increased protein synthesis or decreased protein degradation. If cardiomyocytes are stressed because of pressure overload, then the growth of individual cardiomyocytes is typically associated with abnormal gene expression and the increased deposition of extracellular matrix materials [32]. Extracellular matrix is primarily deposited in the heart by cardiac fibroblasts. When many cardiomyocytes get larger and extracellular matrix is deposited, the entire heart enlarges, resulting in the development of cardiac hypertrophy. Pressure-overload-induced cardiac hypertrophy, also called 'pathological' cardiac hypertrophy, is associated with a very poor prognosis in humans and often contributes to the development of cardiac arrhythmias, diastolic dysfunction and congestive heart failure [33]. The growth of individual cardiomyocytes in response to pressure overload is characterized by the increased width of cells. Interestingly, in response to exercise or volume overload, cardiomyocytes enlarge but do not exhibit altered gene expression, and this cardiomyocyte growth is not associated with extracellular matrix deposition [32]. The growth of individual cardiomyocytes in response to exercise is characterized by the increased length of cells. The enlargement of the heart that occurs in response to exercise or volume overload is called 'physiological' cardiac hypertrophy and is not associated with a poor prognosis in humans.

In most cases, pathological cardiac hypertrophy develops as a consequence of pressure overload due to hypertension or valvular heart disease. To model cardiac hypertrophy in animals, surgical procedures were developed that mimic coarctation of the aorta and result in pressure overload [34,35]. The development of these animal models of pressure overload, combined with the ability to genetically manipulate mice, has resulted in a series of important basic science discoveries that have implications towards the clinical management of patients.

When TAC (transverse aortic constriction) is performed on mice, all three major MAPK pathways are activated in cardiac tissue [36]. In one study, JNK activation appeared to occur earliest and was fully activated within 3 h of surgery, whereas ERK and p38 MAPK activation occurred within 7 days of surgery [36]. In another study, ERK1/2 and p38 MAPK activation occurred within 10 min of TAC [37]. Activation of ERK, JNK and p38 MAPK has been demonstrated in other animal model systems and also in humans with heart failure [38].

An important study in 1995 showed that cardiac-specific overexpression of an activated form of H-Ras (Harvey-Ras) in transgenic mice leads to cardiac hypertrophy and diastolic dysfunction [39]. In that study, an MLC (myosin light-chain) 2v promoter fragment was linked to a cDNA encoding the oncogenic Val^{12} mutant form of H-Ras to create a construct that was used to generate several lines of transgenic mice. Mice that were homozygous for the MLC-Ras transgene had a marked 57.5 % increase in LV (left ventricular) mass and in the LV weight/body weight ratio when compared with non-transgenic control mice. Increased LV weight was associated with an increased average area of cardiomyocytes. No increase in cardiac fibrosis was observed in transgenic mice; however, myofibrillar disarray was observed on histological analysis and ANF (atrial natriuretic factor) mRNA levels were markedly increased. In addition, cardiac catheterization showed that diastolic function was depressed in MLC-Ras transgenic mice [39].

In related work on the role of the Ras–Raf–MKK–ERK signalling cascade, overexpression of an activated form of MKK1 (MEK1) was shown to lead to profound cardiac hypertrophy without fibrosis [40]. In that study, the cardiac-specific α-MHC (myosin heavy chain) promoter was linked to a cDNA encoding an activated form of MKK1 (Ser^{217}/Ser^{221} to glutamic acid) to create a construct that was used to generate several lines of transgenic mice. All lines of mice developed concentric LV hypertrophy with increased cardiomyocyte cell size but without interstitial fibrosis. Indeed, cardiac hypertrophy was observed beginning at 3 weeks of age. ERK1/2 activation was increased in cardiac tissue obtained from MHC-MKK1 transgenic mice. ANF, BNP (brain natriuretic peptide), α-skeletal actin and β-MHC gene expression were all elevated in 8-week-old transgenic MHC-MKK1 mice. Isolated working heart preparations were used to evaluate cardiac function and this showed that MHC-MKK1 transgenic mice had increased systolic function, determined by dP/dt_{max}, and reduced diastolic function, determined by dP/dt_{min} [40].

The molecular mechanisms linking activated MKK1 to the development of cardiac hypertrophy in intact animals are not firmly established; however, there are several potential mechanisms that have been demonstrated in cultured cells. Activated MKK1 may promote cardiac hypertrophy by the phosphorylation and activation of the transcription factors Elk1 and GATA4 by ERK [41,42]. Furthermore, activated MKK1 may promote hypertrophy via the activation of the cellular translational

machinery. ERK can directly phosphorylate the *TSC2* gene product tuberin on Ser^{664}, and this leads to the activation of mTOR (mammalian target of rapamycin), a master regulator of protein synthesis [43]. Furthermore, additional ERK substrates, including $p90^{RSK}$ (90 kDa ribosomal protein S6 kinase) and Mnk1/2 (MAPK-interacting kinase 1/2), are known to promote increased translation [44]. Activated $p90^{RSK}$ can also phosphorylate and inactivate tuberin, leading to mTOR activation [45]. Mnk1 can phosphorylate and activate eIF4E (eukaryotic initiation factor 4E) [46]. Therefore activated MKK1 may promote cardiac hypertrophy via ERK-mediated effects on cardiomyocyte transcription and translation.

Although activation of the Ras–Raf–MKK–ERK cascade in the heart promotes cardiac hypertrophy, activation of the JNK cascade does not. Transgenic mice were generated with cardiac-specific overexpression of an activated form of MKK7 (Ser^{281} and Thr^{275} to aspartic acid, called MKK7D) [47]. Transgenic MKK7D mice had increased activation of both JNK1 and JNK2 in cardiac tissue, without activation of ERK1/2 or p38α MAPK. Transgenic MKK7D mice died at approx. 7 weeks of age with signs of congestive heart failure, including oedematous lungs and ascites. Although there was biatrial enlargement in transgenic MKK7D mice, there was no LV hypertrophy and cardiomyocyte cell size was not increased. However, ANF and α-skeletal actin mRNA levels were increased in cardiac tissue from MKK7D transgenic mice. Diastolic LV filling was impaired in MKK7D transgenic mice. Interestingly, MKK7D mice also had reduced connexin 43 protein levels and gap junction formation between cardiomyocytes in ventricular sections [47]. In a related study, transgenic mice with cardiac-specific overexpression of an MKK7–JNK1 fusion protein were generated and were found to have normal ventricular weight at baseline, but were resistant to cardiac hypertrophy induced by the overexpression of calcineurin A [48]. The ability of JNK1 activation to antagonize calcineurin A-induced cardiac hypertrophy may be explained by the ability of JNK1 to phosphorylate members of the NFAT (nuclear factor of activated T-cells) transcription factor family, thereby preventing their nuclear translocation. Indeed, overexpression of JNK1 and MKK7 in cultured cardiomyocytes blocked calcineurin A-induced nuclear translocation of an NFATc3–GFP (green fluorescent protein) fusion protein [48].

Activation of the p38 MAPK cascade in cardiac tissue does not promote cardiac hypertrophy, as demonstrated by transgenic mice with cardiac-specific overexpression of activated mutant forms of MKK3 (MKK3bE) and MKK6 (MKK6bE) [49]. Both MKK3bE and MKK6bE transgenic mice had increased p38 MAPK activity in cardiac tissue. Both types of transgenic mice died between 5 and 7 weeks of age with signs of congestive heart failure. Neither MKK3bE nor MKK6bE transgenic mice developed LV hypertrophy or cardiomyocyte enlargement; however, they both developed biatrial enlargement. Prominent interstitial cardiac fibrosis was observed in both types of transgenic mice, and ANF, β-MHC and α-skeletal actin mRNA levels were increased. LV systolic function was decreased in MKK3bE mice with reduced LV wall thickness, but systolic function was not reduced in MKK6bE mice. Both types of transgenic mice had increased diastolic chamber stiffness [49].

Taken together, overexpression studies lead to the model that the simultaneous activation of ERK, JNK and p38 MAPK in the heart after pressure overload contributes to the development of pathological cardiac hypertrophy. In this model, ERK activation promotes the growth of cardiomyocytes, JNK activation leads to reduced gap junction formation, p38 MAPK activation promotes cardiac fibrosis, and activation of all three pathways promotes reduced diastolic compliance. Pressure overload also leads to the activation of the calcineurin A/NFAT pathway in the heart that clearly plays a major role in promoting the growth of cardiomyocytes [32].

SIGNALLING MECHANISMS IN CARDIAC HYPERTROPHY: MAPK LOSS-OF-FUNCTION STUDIES

Overexpression studies in mice suggest that Ras-mediated ERK activation plays an important role in the growth of individual cardiomyocytes, whereas p38 MAPK and JNK activation does not promote cardiac hypertrophy but instead promotes cardiac dysfunction. Loss-of-function studies *in vivo* correlate, in part, with overexpression studies.

Several studies have investigated the role of the Raf–MKK–ERK cascade in the development of cardiomyocyte hypertrophy by use of pharmacological inhibitors. In one study, rat neonatal cardiomyocytes were treated with ET1 (endothelin 1) or phenylephrine in the presence of the selective and highly potent MKK1/2 inhibitor U0126, or the selective Raf family member inhibitor SB386023 [50]. Ligand-induced protein synthesis, cardiomyocyte growth, sarcomeric organization and expression of β-MHC were all potently inhibited by U0126 or SB386023. In another study, phenylephrine- and ET1-induced protein synthesis, measured by both radioactive methionine and radioactive leucine incorporation, were dramatically inhibited by treatment of cardiomyocytes with the MKK1/2 inhibitors U0126 or PD184352 [51]. In an *in vivo* study, cardiac hypertrophy was induced in rats after administration of the NOS (NO synthase) inhibitor L-NAME (N^G-nitro-L-arginine methyl ester) that resulted in the development of systemic hypertension [52]. L-NAME-induced cardiac hypertrophy was blocked in animals that were treated with the MKK1/2 inhibitor PD98059. Therefore experiments with pharmacological

inhibitors support a role of the Raf–MKK–ERK pathway in the development of cardiac hypertrophy.

Activation of MAPK cascades is often dependent on the action of the Grb2–SOS complex. To determine the role of Grb2 in the development of pathological cardiac hypertrophy, experiments were performed with Grb2 haplo-insufficient mice [53]. $Grb2^{-/-}$ mice do not survive early embryonic development, but $Grb2^{+/-}$ mice appear normal at birth and have normal cardiac structure and function at baseline. Grb2 haplo-insufficient mice were resistant to pressure-overload-induced cardiac hypertrophy induced by TAC. Furthermore, $Grb2^{+/-}$ mice developed less interstitial cardiac fibrosis and had reduced levels of ANF and β-MHC mRNA in response to TAC when compared with wild-type mice. In response to TAC, cardiac JNK and p38 MAPK activation were reduced in $Grb2^{+/-}$ mice, but ERK1/2 activation was normal. It is important to note that Grb2 may affect the activation of several non-MAPK signalling pathways, including the PI3Kα–Akt1 pathway and the PLCγ (phospholipase Cγ)/Ca^{2+} pathway, through complex interactions with other scaffolding proteins such as GAB1 (Grb2-associated binder 1) [53].

To specifically investigate the role of Raf-1 in the development of cardiac hypertrophy, transgenic mice were generated with cardiac-specific overexpression of DN-Raf (a dominant-negative form of Raf-1) [54]. Cardiomyocytes express Raf-1, B-Raf and A-Raf, and DN-Raf-1 probably inhibits all three family members [55]. Transgenic DN-Raf mice were resistant to TAC-induced cardiac hypertrophy and cardiomyocyte enlargement [54]. Furthermore, ERK1/2 activation was reduced in DN-Raf cardiac tissue 1 week after TAC, but p38 MAPK and JNK activation was unaffected. Pressure-overload-induced ANF and β-MHC gene induction was decreased in cardiac tissue from DN-Raf mice. Interestingly, DN-Raf mice had significantly increased mortality after TAC that was associated with increased cardiomyocyte apoptosis when compared with wild-type mice [54].

To specifically analyse the role of Raf-1 in cardiac hypertrophy, mice with cardiac-specific targeted gene disruption of Raf-1 [Raf CKO (cardiac-specific knockout)] were produced [56]. Although Raf CKO mice had a normal life expectancy, they developed abnormal cardiac function by 10 weeks of age with markedly reduced LV systolic function, LV dilation and reduced LV posterior wall thickness. Furthermore, Raf CKO mice had increased cardiomyocyte apoptosis at 3–5 weeks of age. Cardiac signal transduction was abnormal at baseline in Raf CKO mice with increased ASK1, JNK and p38 MAPK activities. However, ET1-induced MKK and ERK1/2 activation was not affected in cardiac tissue from Raf CKO mice. The cardiac phenotype of Raf CKO mice was ameliorated by breeding with ASK1-knockout mice [56]. Taken together, these results suggest that Raf-1 inhibits ASK1 in an MKK/ERK-independent manner and also suggest that B-Raf and A-Raf function in parallel with Raf-1 to promote ERK1/2 activation in heart.

To specifically examine the role of ERK1/2 in the development of cardiac hypertrophy, the cardiac phenotypes of $ERK1^{-/-}$ and $ERK2^{+/-}$ mice were analysed [37]. ERK2 haplo-insufficient mice were examined because ERK2 comprises approx. 70 % of total ERK protein in the heart. $ERK2^{-/-}$ mice are non-viable, and $ERK1^{-/-}$ $ERK2^{+/-}$ mice have increased embryonic lethality. Both $ERK1^{-/-}$ and $ERK2^{+/-}$ mice have a normal cardiac hypertrophic response to pressure overload by TAC. In addition, a transgenic mouse model was generated with cardiac-specific overexpression of DUSP6 (dual-specificity phosphatase 6) that is a relatively specific phosphatase for ERK1/2. DUSP6 transgenic mice had reduced ERK1/2 activation in response to TAC, but developed a normal degree of LV hypertrophy. However, DUSP6 transgenic mice had more interstitial fibrosis and increased cardiomyocyte apoptosis after TAC when compared with non-transgenic mice [37]. Although these findings suggest that ERK1/2 is not required for the growth of cardiomyocytes, cardiac-specific targeted disruption of ERK2 may provide more definitive information about this question.

To address the role of the JNK pathway in cardiac hypertrophy, mice with altered expression and activity of JNK proteins were investigated [48]. $JNK1^{-/-}$, $JNK2^{-/-}$, $JNK1^{+/-}JNK2^{-/-}$ and transgenic mice with cardiac-specific overexpression of MHC–DN-JNK1/2 (DN forms of JNK1 and JNK2) were subjected to pressure overload by TAC. Although $JNK1^{-/-}$ and $JNK2^{-/-}$ mice developed cardiac hypertrophy to a similar extent as wild-type mice, $JNK1^{+/-}JNK2^{-/-}$ mice and MHC-DN-JNK1/2 transgenic mice developed an exaggerated form of cardiac hypertrophy in response to TAC. Furthermore, 7-month-old $JNK1^{+/-}JNK2^{-/-}$ mice and MHC–DN-JNK1/2 transgenic mice developed spontaneous cardiac hypertrophy with cardiomyocyte enlargement in the absence of pressure overload [48]. MHC-DN-JNK1/2 mice also had increased cardiac NFAT activity measured by use of an NFAT-binding element–luciferase reporter construct. That study suggests that JNK1/2 inhibits cardiac hypertrophy through phosphorylation and inactivation of NFAT transcription factors. Another group more recently examined the response of $JNK1^{-/-}$, $JNK2^{-/-}$ and $JNK3^{-/-}$ mice to pressure overload by TAC [57]. Although all three knockout mice developed cardiac hypertrophy to an extent indistinguishable from wild-type mice, $JNK1^{-/-}$ mice had an abnormal response to TAC manifested by reduced LV systolic function for several weeks after the surgery, which eventually returned to normal. These findings suggest that JNK1 plays a protective role in maintaining LV systolic function in the acute phase after pressure overload.

To address the role of p38 MAPK in cardiac hypertrophy, transgenic mice with cardiac-specific overexpression

of DN-p38α MAPK and DN-p38β MAPK were examined [53,58]. Both DN-p38α MAPK and DN-p38β MAPK transgenic mice developed cardiac hypertrophy to a similar extent as non-transgenic mice, although there was a trend towards greater hypertrophy in DN-p38β MAPK mice. Furthermore, both DN-p38α MAPK and DN-p38β MAPK transgenic mice had reduced interstitial fibrosis after TAC when compared with non-transgenic mice [53]. In another study, transgenic mice were examined with cardiac-specific overexpression of DN-p38α MAPK, DN-MKK3 or DN-MKK6 [59]. In that study, DN-p38α MAPK, DN-MKK3 and DN-MKK6 transgenic mice all developed enhanced cardiac hypertrophy in response to pressure overload induced by TAC with increased cardiomyocyte size. Furthermore, all three types of transgenic mice developed spontaneous cardiac hypertrophy in the absence of pressure overload. Finally, cardiac NFAT activity was increased in DN-p38α MAPK cardiac tissue, as measured by an NFAT-dependent luciferase reporter transgenic mouse [59]. That study suggested that p38α MAPK , similar to JNK1/2, inhibits cardiac hypertrophy through inactivation of NFAT transcription factors.

p38α CKO mice had normal cardiac structure and function in the absence of experimental manipulation [60]. When subjected to pressure overload by TAC, p38α MAPK CKO mice developed cardiac hypertrophy to a similar but slightly greater extent than control p38α MAPK$^{loxp/loxp}$ α-MHC-*Cre*(−) mice. In addition, p38α MAPK CKO mice developed markedly increased cardiomyocyte apoptosis and fibrosis after TAC. One way to resolve the apparent inconsistencies between the various p38α MAPK loss-of-function studies is that there may be a biphasic dose–response curve to p38α MAPK activation in cardiomyocytes. In this model, low-level and/or transient p38α MAPK activation has an important anti-apoptotic function in cardiomyocytes, but high-level and/or sustained p38α MAPK activation promotes cell death. Presumably all cardiac p38α MAPK activity would be eliminated in the p38α MAPK CKO mouse, but only the high-level and/or sustained activity would be eliminated in the various DN transgenic models. An alternative theory is that p38α MAPK has an anti-apoptotic function that is not dependent on kinase activity, similar to the ability of Raf-1 to bind to and inhibit ASK1.

One current model for the role of intracellular signalling pathways in the development of pathological cardiac hypertrophy is that the Raf–MKK1/2–ERK1/2 cascade probably contributes to the growth of cardiomyocytes, but the calcineurin/NFAT pathway is dominant in this process (Figure 1) [32,61]. Furthermore, the MKKK–MKK3/6–p38α MAPK and MKKK–MKK4/7–JNK1/2 pathways both inhibit the growth of cardiomyocytes, and p38α/β MAPK promotes cardiomyocyte dysfunction. This model implies that inhibition of p38 MAPK *in vivo* may lead to increased cardiac hypertrophy with increased cardiac function and, therefore, may be useful for patients with dilated cardiomyopathy, who have depressed cardiac contractility and ventricular wall thinning. On the other hand, inhibition of ERK1/2 *in vivo* may lead to regression of cardiac hypertrophy and may promote cardiomyocyte apoptosis and, therefore, would be useful in patients with hypertrophic cardiomyopathy with preserved systolic function.

ROLE OF MAPKs IN CARDIAC REMODELLING AFTER MI

MI usually occurs when there is acute thrombosis in a coronary artery at the site of an atherosclerotic lesion, resulting in termination of blood flow to myocardium supplied by the affected artery. In the days and weeks after an acute MI, pathological cardiac remodelling can occur that is characterized by cardiomyocyte death in the infarct border zone with resultant infarct extension, fibrosis at the site of infarct and in the unaffected myocardium, dilation of the left ventricle and hypertrophy of the unaffected myocardium [62]. Cardiac remodelling is thought to occur as a result of inflammatory mechanisms that are triggered by the necrotic myocardium, generation of ROS by necrotic myocardium and also because of increased wall stress in the infarct border zone. Although remodelling has acute adaptive features, such as the deposition of extracellular matrix in the infarct zone to prevent cardiac rupture, it eventually promotes functional decompensation and the development of heart failure.

The role of MAPKs in pathological cardiac remodelling has been investigated in several studies. In particular, p38 MAPK has been widely studied in this context. In the minutes after experimental MI, ERK1/2, JNK1/2 and p38α MAPK are all activated in both the ischaemic myocardium and unaffected portions of the left ventricle of mice and rats [63,64]. Although the activation of various MAPKs in the unaffected myocardium is variable in the days following MI, it appears that p38α MAPK is most consistently activated for several weeks after the initial insult [65–67]. In one study, DN-p38α MAPK transgenic mice were evaluated for their ability to remodel after experimental MI [63]. DN-p38α MAPK mice and non-transgenic controls were subjected to LAD (left anterior descending) coronary artery ligation and two-dimensional transthoracic echocardiography performed 2 h after surgery revealed nearly identical initial infarct sizes. Cardiac remodelling was evaluated 7 days after MI and DN-p38α MAPK mice had a decreased area of scarring at the site of infarction with markedly reduced cardiomyocyte apoptosis in the infarct border zone. Furthermore, the LV end-systolic volume was significantly reduced in DN-p38α MAPK mice when compared with non-transgenic mice 7 days after MI. One potential

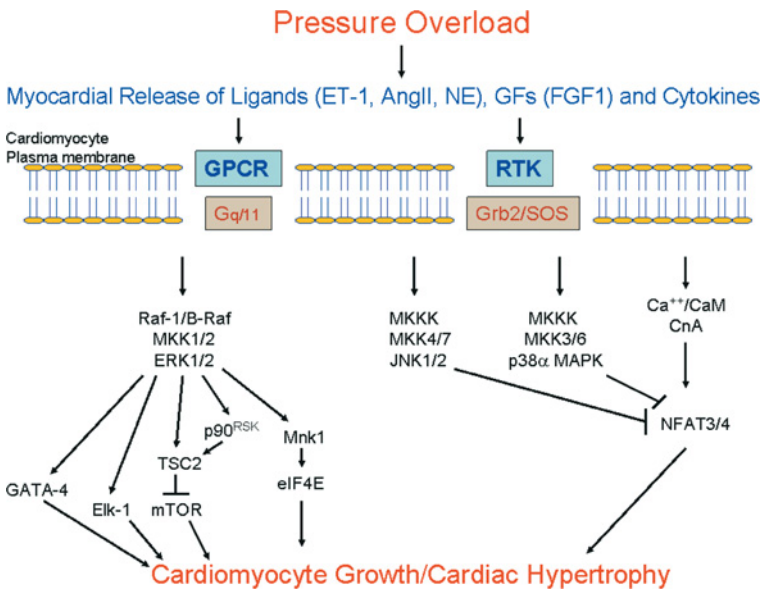

Figure 1 Model of the role of the MAPK cascades in cardiomyocyte hypertrophy

In response to pressure overload, there is an intramyocardial release of ligands, such as ET1, angiotensin II (AngII) and noradrenaline (norepinephrine; NE), growth factors (GFs), including FGF1, and cytokines. These extracellular factors bind to and activate transmembrane GPCRs, RTKs and cytokine receptors (not depicted). Activated transmembrane receptors, in turn, directly activate intracellular signalling proteins, including G-proteins ($G_{q/11}$) and the Grb2–SOS complex, which promote activation of the MAPK cascades and the CaM (Ca^{2+}/calmodulin)–CnA (calcineurin A)–NFAT3/4 cascade. Activation of these signalling cascades modulates the growth of cardiomyocytes in the manner depicted. Specifically, ERK1/2 phosphorylates a variety of targets that may contribute to cardiomyocyte growth, including the transcription factors Elk-1 and GATA4, and several proteins that regulate the translational machinery, including tuberin (*TSC2* gene product), Mnk1 and $p90^{RSK}$. On the other hand, JNK1/2 and p38 MAPK phosphorylate NFAT family members, resulting in inhibition of the calcineurin–NFAT hypertrophic pathway.

molecular mechanism for the reduction in pathological cardiac remodelling is that cardiac tissue from DN-p38α MAPK transgenic mice obtained 2 h after LAD coronary artery ligation had reduced deamidation of the pivotal anti-apoptotic protein Bcl-x_L [63]. Deamidation of Bcl-x_L inhibits its anti-apoptotic function and may result in its degradation. In a related study, overexpression of DN-p38α MAPK in transgenic mouse heart tissue resulted in an increased expression of Bcl-2 at baseline, which was increased after ischaemia/reperfusion injury [68]. Taken together, these results suggest that activation of p38α MAPK promotes pathological remodelling by reducing the activity or expression of the anti-apoptotic family members Bcl-x_L and Bcl-2.

Several studies have employed pharmacological inhibitors of p38 MAPK to evaluate the role of this protein kinase in cardiac remodelling after experimental MI. In one study, rats were subjected to experimental MI by ligation of the LAD coronary artery [69]. On day 6 after surgery, mice were evaluated by echocardiography, and animals with anterior wall infarction affecting >40 % of the left ventricle were randomized to receive RWJ-67657, a p38α and p38β MAPK inhibitor, or vehicle for 3 weeks. On day 27 after MI, animals were evaluated for the presence of cardiac remodelling. Echocardiography demonstrated that fractional shortening was significantly higher in animals that received RWJ-67657 when compared with animals treated with vehicle. Echocardiography also showed that infarct expansion was reduced in RWJ-67657-treated animals. Cardiac catheterization showed that systolic function, measured by determining the dP/dt_{max}, and diastolic function, measured by determining the LV end-diastolic pressure, were both better preserved in animals that received RWJ-67657 after MI compared with those that received vehicle [69]. Finally, cardiac fibrosis was reduced in the unaffected myocardium of animals treated with RWJ-67657 after MI. In another recent study in rats, animals were subjected to experimental MI and then immediately started on treatment with SB203580, a p38α and p38β MAPK inhibitor, or vehicle for 1 or 6 weeks [70]. At both 1 and 6 weeks after MI, treatment with SB203580 resulted in reduced myocardial fibrosis, reduced TNFα (tumour necrosis factor α) levels and collagen I levels, and increased LV contractile function. In a mouse study, animals were subjected to experimental MI and p38-MAPK-inhibition therapy was begun 2 weeks later, after early cardiac remodelling was completed [71]. Mice were treated with SC-409, a p38 MAPK inhibitor, the ACEI (angiotensin-converting enzyme inhibitor) enalapril, enalapril + SC-409 or vehicle for 12 weeks. At the end of the therapy period, mice treated with SC-409 had an improved

ejection fraction, increased cardiac output, decreased LV chamber dilation and reduced myocardial collagen deposition. Inhibition of p38 MAPK activity had a similar benefit as ACEI therapy in this mouse model system.

Analysis of mice haplo-insufficient for 14-3-3τ also demonstrated the role of p38α MAPK in post-infarction cardiac remodelling [72]. 14-3-3 proteins are intracellular dimeric phosphoserine-binding proteins that inhibit the activation of p38α MAPK and JNK1/2 by binding to ASK1 and other MKKKs, but they promote activation of the ERK cascade via complex interactions with Raf family members [73]. 14-3-3$\tau^{-/-}$ mice did not survive embryonic development, but haplo-insufficient mice appeared normal at birth. Cardiac tissue from 14-3-3$\tau^{+/-}$ mice had increased basal activation of ASK1, p38α MAPK and JNK1/2, but reduced ERK1/2 activation [72]. Surgical ligation of the LAD coronary artery was performed in 14-3-3$\tau^{-/-}$ and wild-type mice and both groups had similar initial infarct sizes by echocardiography 1 day after surgery. However, 7 days after surgery, 14-3-3$\tau^{-/-}$ mice had increased LV chamber dilation, increased infarct size and increased cardiomyocyte apoptosis in the infarct border zone when compared with wild-type mice. Furthermore, 14-3-3$\tau^{-/-}$ mice had significantly increased mortality in the days after MI, chiefly as a result of ventricular rupture at the site of MI. Treatment of 14-3-3$\tau^{-/-}$ mice with SB202190 improved the survival of these animals after MI [72].

The mechanisms by which p38α MAPK promotes pathological cardiac remodelling may include the induction of apoptosis, via modulation of Bcl-x_L and Bcl-2 activity, and the production of inflammatory cytokines. Another possible explanation was recently elaborated in a study that showed that p38α MAPK can block cardiomyocyte mitosis [74]. In that study, microarray analysis of neonatal rat cardiomyocytes treated with SB203580 showed that p38 MAPK inhibition resulted in the up-regulation of many cell-cycle proteins, including cyclin A2, cyclin B, cdc2 and aurora B. Treatment of cultured adult rat cardiomyocytes with FGF1 (fibroblast growth factor 1) and SB203580 induced mitosis. Analysis of mice with cardiac-specific disruption of p38α MAPK (MLC-2v-*Cre* p38α MAPK$^{loxp/loxp}$) showed that these mice had a 92.3 % increase in neonatal cardiomyocyte mitoses. In a second study, rats were subjected to experimental MI, and animals were treated with SB203580 by intraperitoneal injection every 3 days for 1 month, a single injection of FGF1 + self-assembling peptides into the infarct border zone immediately after coronary artery ligation, SB203580 + FGF1 or vehicle alone [75]. Evidence of cardiomyocyte mitosis, measured by cyclin A and H3P (phosphorylated histone H3) staining, was increased within the infarct and infarct border zones of rats treated with SB203580 or SB203580 + FGF1 for 2 weeks. Evidence of pathological cardiac remodelling was reduced in rats treated with SB203580 or SB203580 + FGF1 at 2 weeks after MI, with treated animals having increased fractional shortening, reduced LV scar volume, reduced ventricular muscle loss and reduced thinning at the site of MI [75]. When rats were analysed 3 months after MI and 2 months after SB203580 injection was discontinued, the salutary effects of combined SB203580 + FGF1 treatment on cardiac structure and function persisted, although the effects of SB203580 treatment alone were diminished, and this difference may be due to increased myocardial capillary density seen in rats treated with FGF1 [75].

An important MKKK in the heart that can activate both the JNK1/2 and p38α MAPK pathways is ASK1. This stress-inducible MKKK is interesting because of its ability to be regulated by ROS via thioredoxin [76]. Thioredoxin binds to and inhibits the activity of ASK1 in a reduction/oxygenation-sensitive matter. The concentration of ROS is increased in unaffected myocardium following MI in mice [77]. To evaluate the role of ASK1 in cardiac remodelling, one group subjected ASK1$^{-/-}$ and wild-type mice to experimental MI [78]. Although the initial infarct sizes were the same in both groups, ASK1$^{-/-}$ mice had reduced cardiac remodelling, with reduced fibrosis in the border zone and remote myocardium, reduced diastolic LV dimension, improved fractional shortening and reduced cardiomyocyte apoptosis in the border zone. Activation of JNK1/2 in the infarct border zone was reduced in ASK1$^{-/-}$ mice at 2 and 7 days after MI, but p38α MAPK activation was not affected in the knockout animals [78]. These results suggest that ASK1–JNK1/2 signalling promotes pathological cardiac remodelling after MI.

A current model of cardiac remodelling after MI is that the activation of the p38α MAPK and JNK1/2 cascades promotes fibrosis in the infarct area and unaffected myocardium, apoptosis in the infarct border zone with resultant infarct expansion and LV chamber dilation (Figure 2). However, not all studies support this model of cardiac remodelling, and the role of the ERK1/2 cascade in this process is not well known [66,79]. The use of pharmacological agents to inhibit ASK1, p38α MAPK or JNK1/2 may reduce pathological cardiac remodelling after MI in humans.

ROLE OF MAPKs IN ATHEROSCLEROTIC LESION DEVELOPMENT

Atherosclerosis is a complex inflammatory condition characterized by the development of fatty deposits in the inner layers of arteries [80,81]. Atherosclerotic lesions contain many cell types, including SMCs, endothelial cells and T-lymphocytes, but always contain a large number of abnormal lipid-laden macrophages that are called foam cells. The development of atherosclerosis in humans is influenced by the presence of important risk factors, such as hypercholesterolaemia, hypertension,

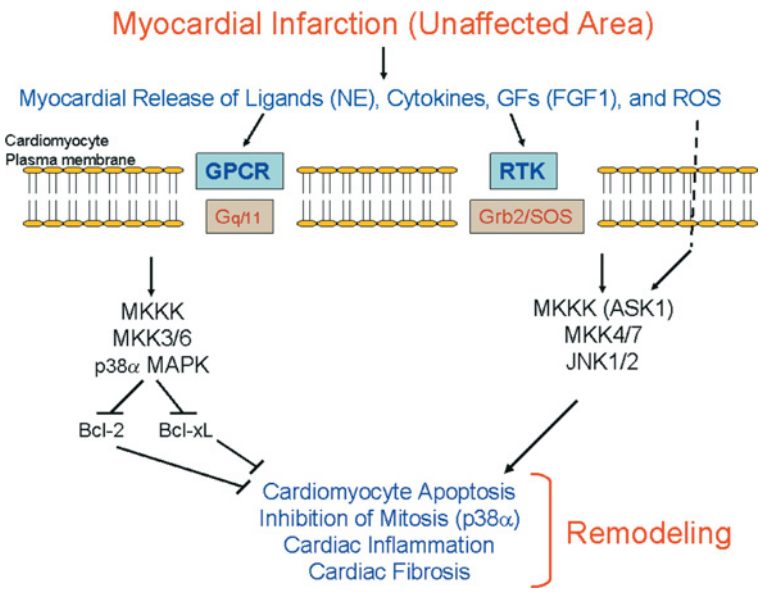

Figure 2 Model of the role of MAPK cascades in pathological cardiac remodelling after MI

After MI, there is a local release of ligands, such as noradrenaline (NE), cytokines, growth factors (GFs), including FGF1, and ROS that lead to the activation of transmembrane receptors and intracellular MAPK cascades. ROS may modify the activity of the MKKK ASK1 by blocking the ability of thioredoxin to inhibit ASK1. The activation of the MKKK–MKK3/6–p38α MAPK and the ASK1–MKK4/7–JNK1/2 cascades promotes pathological cardiac remodelling which includes cardiomyocyte apoptosis, inhibition of cardiomyocyte mitosis, inflammation and fibrosis. Specifically, p38α MAPK promotes cardiomyocyte mitosis by inhibiting the activity or expression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L.

diabetes mellitus, cigarette smoking and a family history of early atherosclerosis.

Although research on atherosclerosis in the past has focused on cholesterol and lipid metabolism, more recently scientists have concentrated on the cell biology of atherosclerosis, including the molecular determinants of foam cell formation. In the response to injury hypothesis, damage to the arterial intima, perhaps because of hypertension, toxins in cigarette smoke or the toxic effects of oxLDL [oxidized LDL (low-density lipoprotein)], leads to the recruitment, adherence and invasion of monocytes into the arterial intima [80,81]. Once monocytes invade the arterial intima, they differentiate into macrophages and begin to take up lipids, especially modified forms of LDL to form foam cells. The molecular mechanisms that regulate foam cell formation are not yet well understood, although many important studies have investigated this issue. Mice are naturally resistant to the development of atherosclerosis despite high-fat feeding, but the development of mice deficient for apoE (apolipoprotein E) or the LDLR (LDL receptor) have provided important model systems for the *in vivo* analysis of atherosclerosis [82,83]. A popular *in vitro* model system of foam cell formation is the treatment of peritoneal or bone marrow macrophages with copper (or myeloperoxidase)-modified oxLDL or acLDL (acetylated LDL) [84].

The role of MAPK cascades in foam cell formation *in vitro* has been investigated recently [85]. Mouse peritoneal macrophages were treated with oxLDL and ERK1/2, p38α MAPK and JNK1/2 were all activated within 15 min, with ERK1/2 activation occurring at the earliest time point. Peritoneal macrophages derived from $CD36^{-/-}$ mice were resistant to oxLDL-induced activation of JNK1/2, but still exhibited ERK1/2 and p38α MAPK activation [85]. CD36 is a transmembrane scavenger receptor which, with SR-A (scavenger receptor A) and other scavenger receptors, facilitates the uptake of oxLDL by macrophages [86]. CD36 binds to the Src family tyrosine kinase Lyn and also to MEKK2 via its short C-terminal cytoplasmic tail. Treatment of macrophages with the Src inhibitor AG1879 or the JNK pathway inhibitor SP600125 blocked oxLDL-induced foam cell formation, but treatment of macrophages with the ERK pathway inhibitor U126 had no effect [85]. In a related study, oxLDL-induced foam cell formation in the J774 macrophage cell line was found to be blocked by administration of the p38 MAPK inhibitor SB203580, but was not blocked by administration of the ERK pathway inhibitor PD98059 [87]. Furthermore, p38α MAPK and MKK6, but not ERK, were found to be required for oxLDL-stimulated CD36 expression in J774 cells.

To determine the specific role of JNK1 and JNK2 in the development of atherosclerosis, apoE-null mice were bred with $JNK1^{-/-}$ or $JNK2^{-/-}$ mice and placed on a high-fat diet [88]. $JNK2^{-/-}apoE^{-/-}$ but not $JNK1^{-/-}apoE^{-/-}$ mice were found to be resistant to

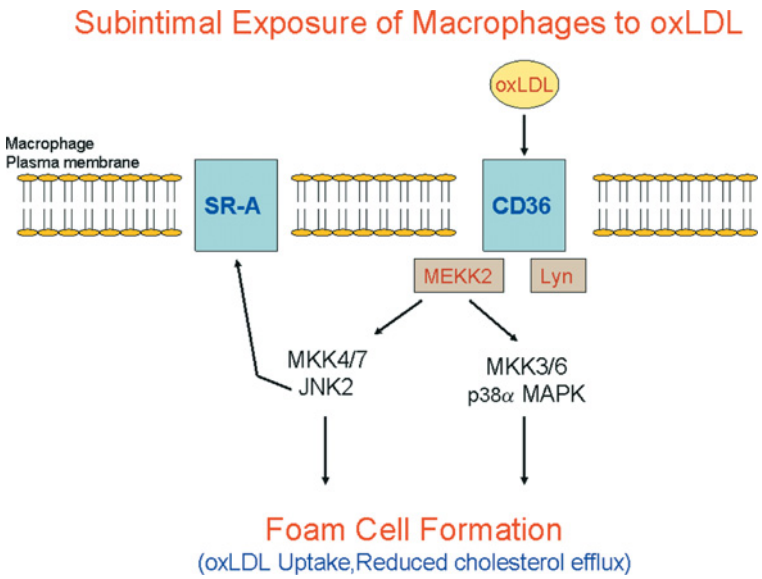

Figure 3 Simplified model of macrophage foam cell formation in the arterial subintimal space

In response to vascular injury, monocytes are recruited to the intimal surface of arteries where they adhere and invade the vessel, differentiating into macrophages. In the subintimal space, macrophages are exposed to oxLDL that binds to the transmembrane protein CD36. Binding of oxLDL to CD36 triggers the activation of Src-family kinases such as Lyn and MEKK2. Activation of Lyn and possibly MEKK2 leads to the activation of JNK2 and also p38α MAPK. Through largely undetermined mechanisms, activation of JNK2 and p38α MAPK promote the internalization of oxLDL via CD36, SR-A or through other scavenger receptors. JNK2 promotes the phosphorylation of SR-A, and this may lead to SR-A internalization while it is associated with modified LDL. The activation of MAPK cascades in macrophages may also modulate cholesterol efflux pathways.

the development of atherosclerosis after 14 weeks of high-cholesterol feeding. Furthermore, apoE-null mice that were treated with the JNK1/2 pathway inhibitor SP600125 were found to develop significantly less atherosclerosis than apoE-null mice treated with vehicle. Lesions from animals with absent JNK2 did not have altered cellular composition, but were simply smaller in absolute size. Bone marrow transplantation from $ApoE^{-/-}$ donors into $JNK2^{-/-}apoE^{-/-}$ recipients resulted in increased atherosclerotic lesion formation, whereas bone marrow transplantation from $JNK2^{-/-}apoE^{-/-}$ donors into $apoE^{-/-}$ recipients resulted in reduced lesion formation; both of these results suggested that the effect of JNK2 deficiency on lesion formation was due to the abnormal function of blood-borne cells, such as macrophages [88]. Indeed, peritoneal macrophages from $JNK2^{-/-}apoE^{-/-}$ mice were found to have decreased oxLDL- and acLDL-induced foam cell formation *in vitro* when compared with macrophages from $apoE^{-/-}$ mice. To determine the mechanism by which JNK2 promotes foam cell formation, the status of SR-A in macrophages from $JNK2^{-/-}$ mice was investigated [88]. Serine phosphorylation of SR-A was markedly reduced in macrophages from $JNK2^{-/-}$ mice, although total SR-A protein levels were increased. One model is that JNK2 phosphorylates SR-A in macrophages, and this promotes foam cell formation by facilitating the internalization of SR-A that is bound to modified LDL (Figure 3) [88].

Additional evidence about the role of MAPKs in atherosclerotic lesion development was provided by the analysis of $Grb2^{+/-}apoE^{-/-}$ mice [89]. When these mice were placed on a high-fat diet for 2 months, they were found to be highly resistant to atherosclerotic lesion formation when compared with $apoE^{-/-}$ mice. Furthermore, bone marrow transplantation from $Grb2^{+/-}apoE^{-/-}$ donors into $apoE^{-/-}$ recipients resulted in reduced lesion formation when compared with $apoE^{-/-}$ mice transplanted with $apoE^{-/-}$ bone marrow. Activation of p38α MAPK and JNK in response to oxLDL treatment was reduced in cultured bone marrow macrophages from $Grb2^{+/-}apoE^{-/-}$ mice when compared with macrophages from $apoE^{-/-}$ mice [89]. The uptake of oxLDL was diminished in bone marrow macrophages from $Grb2^{+/-}$ $apoE^{-/-}$ mice, although the binding was unchanged. It is not apparent how Grb2-mediated signalling is affected by oxLDL binding to scavenger receptors and this is an area of active investigation.

A study evaluating mice deficient for ATM (ataxia telangectasia mutated) also showed that MAPKs regulate atherosclerotic lesion formation [90]. The finding that humans deficient in ATM have an increased risk of death due to ischaemic heart disease led Schneider and co-workers to investigate mice deficient for both ATM and apoE. $ATM^{+/-}apoE^{-/-}$ mice developed the metabolic syndrome on a high-fat diet with hypertension, hyperglycaemia, increased adiposity and increased aortic

atherosclerosis when compared with apoE$^{-/-}$ mice on a similar diet [90]. Furthermore, the aortae of ATM$^{+/-}$apoE$^{-/-}$ mice had increased JNK1/2 activation, but decreased ERK1/2 and Akt1 activation. Transplantation of bone marrow from ATM$^{-/-}$apoE$^{-/-}$ mice into apoE$^{-/-}$ recipients resulted in increased atherosclerotic lesion formation compared with bone marrow transplantation from apoE$^{-/-}$ animals, suggesting that blood-borne cells deficient in ATM conferred the atherosclerosis phenotype [90]. In addition, peritoneal macrophages from ATM$^{-/-}$apoE$^{-/-}$ mice had increased JNK1/2 activation and increased lipoprotein lipase gene expression. These results suggest that ATM deficiency promotes JNK1/2 activation in macrophages, leading to increased foam cell formation.

Taken together, foam cell formation appears to be dependent on the activation JNK2 and also perhaps p38α MAPK (Figure 3). Treatment of cultured macrophages with oxLDL results in the rapid activation of JNK, p38α MAPK and ERK, but treatment of macrophages with an ERK inhibitor does not block foam cell formation [85,87]. Treatment of macrophages with Src, JNK or p38 MAPK inhibitors does block oxLDL-induced foam cell formation [85,87]; however, it is unclear whether oxLDL-mediated activation of JNK2 or p38α MAPK directly regulates the uptake of oxLDL by endocytosis via some other process. It is possible that JNK2 or p38α MAPK activity regulates the expression or activity of proteins required for internalization of oxLDL prior to oxLDL binding to cell-surface receptors. Nevertheless, JNK2 and p38α MAPK remain interesting targets for drug therapy to reduce atherosclerotic lesion formation.

ROLE OF MAPKs IN RESTENOSIS AFTER PERCUTANEOUS ARTERIAL INTERVENTION

Atherosclerotic vascular disease is often treated by percutaneous transluminal balloon angioplasty and/or by arterial stenting. Unfortunately, in many cases, neointima formation in the weeks and months after balloon angioplasty or stenting results in arterial restenosis with resultant morbidity and mortality. Neointima formation is primarily a disorder of SMCs, with SMC migration and proliferation and extracellular matrix deposition in the intima being important features of this disorder [91,92]. Although the incidence of restenosis is markedly reduced when drug-eluting stents are employed that release sirolimus or taxol, the occurrence of delayed in-stent thrombosis due to deficient endothelialization suggests that more complete information about the biology of neointima formation will be useful clinically.

Neointima formation may be triggered by the local release of growth factors, cytokines and ligands in response to balloon angioplasty or stent placement. The release of bioactive factors at the site of vascular intervention is thought to be a consequence of endothelial cell injury and denudation, stretch injury to cells throughout the vessel and local deposition of fibrin and platelets [92]. Growth factors, cytokines and ligands that are released in response to vascular injury bind to receptors on the surface of SMCs promoting the activation of intracellular signalling cascades and leading to cell migration and proliferation. Balloon injury of the rat carotid artery results in the rapid activation of ERK1/2, p38α/β MAPK and JNK1/2 [93–95].

To address the role of MAPK cascades in neointima formation, mice that were haplo-insufficient for Grb2 were subjected to carotid injury by use of a beaded probe method [96]. At 3 weeks after injury, carotid arteries were examined for neointima formation and signalling pathway activation. Grb2$^{+/-}$ mice were resistant to carotid-injury-induced neointima formation with dramatically reduced intimal SMCs. In addition, Grb2 haplo-insufficient mice had reduced ERK, JNK and p38 MAPK activation in carotid artery extracts after probe injury [96].

To more specifically examine the role of Ras activation in neointima formation, mice with SMC-specific targeted disruption of the *Nf1* gene, which encodes neurofibromin, a GTPase that deactivates Ras, were examined [97]. Nf1smKO mice (SMC-specific *Nf1* knockout mice) were subjected to carotid artery injury by external ligation. At 4 weeks after carotid ligation, arteries were examined and Nf1smKO mice were found to develop exaggerated neointima formation with a dramatically increased intima-to-media ratio. Cell proliferation and ERK1/2 activation in the neointima were significantly increased in Nf1smKO mice when compared with control animals [97]. The activation of p38 MAPK and JNK in carotid arteries from Nf1smKO mice after injury was not examined in that study.

To specifically examine the role of p38α MAPK in the pathogenesis of neointima formation, compound transgenic mice with SMC-specific inducible expression of DN-p38α MAPK were produced (A. J. Muslin, unpublished work). In these mice, tetracycline administration resulted in the expression of DN-p38α MAPK in the SMCs of the aorta and carotid arteries. Compound transgenic or control mice were treated with tetracycline for 2 weeks and then subjected to carotid injury by the beaded probe method. Mice expressing DN-p38α MAPK in SMCs were resistant to neointima formation after carotid injury when compared with control animals. To address the mechanism by which p38α MAPK promotes the proliferation of SMCs, PDGF stimulation was demonstrated to result in the p38α MAPK-dependent phosphorylation of Rb (retinoblastoma protein), a master regulator of cell-cycle progression. Furthermore, PDGF stimulation of cultured SMCs promoted the p38α MAPK-dependent expression of MCM6 (minichromosome maintenance protein 6), a

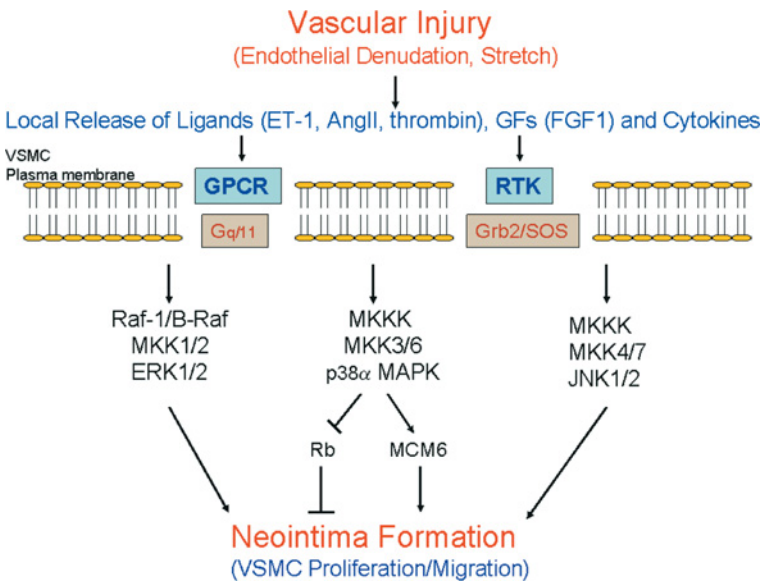

Figure 4 Model of SMC proliferation in neointima formation after vascular injury

In response to arterial injury that includes endothelial denudation and stretch, there is a local release of ligands, such as ET1, angiotensin II (AngII) and thrombin, growth factors (GFs) and cytokines that bind to transmembrane receptors. These receptors include GPCRs, RTKs and cytokine receptors (not depicted) that activate intracellular signalling proteins such as G-proteins ($G_{q/11}$) and the Grb2–SOS complex. In turn, intracellular MAPK cascades are activated that ultimately lead to VSMC migration into the intima and proliferation, resulting in neointima formation. The activation of p38α MAPK in VSMCs promotes the hyperphosphorylation of Rb and the expression of MCM6 (minichromosome maintenance 6) which both contribute to cell proliferation.

protein required for DNA synthesis in the S-phase of the cell cycle (A.J. Muslin, unpublished work).

In one *in vivo* study that employed the HVJ (haemagglutinating virus of Japan) liposome-mediated delivery of DN-ERK1 and DN-JNK1 to rat arteries, both reagents were found to be effective at inhibiting neointima formation after balloon injury of the common carotid artery [98]. Both DN-ERK1 and DN-JNK1 were highly expressed in the carotid arteries, and the carotid intima-to-media ratio was decreased at both the 14 and 28 day time points after injury compared with control animals. Furthermore, liposome-mediated delivery of wild-type forms of ERK1 and JNK1 increased neointima formation after balloon injury [98].

Several animal studies of neointima formation have employed pharmacological inhibitors of MAPKs. In one early study, administration of PD98059, a MKK1 inhibitor, via the external application of a pluronic gel containing the compound, blocked medial smooth cell proliferation after balloon injury of the carotid artery, but did not block neointima formation [91]. However, ERK1/2 activation was not completely blocked in the carotid arteries by administration of the pluronic gel. In another study, administration of a novel oral MKK1 inhibitor, PD0185625, to rats blocked carotid neointima formation at both 14 and 28 days after balloon injury. Administration of PD0185625 completely blocked ERK1/2 activation in balloon-injured carotid arteries and also inhibited BrdU (bromodeoxyuridine) incorporation in VSMCs (vascular SMCs) [99]. Finally, administration of an oral p38α/β MAPK inhibitor FR167653 to rats significantly inhibited carotid neointima formation 14 days after balloon injury [100]. Furthermore, carotid artery production of the cytokine IL1β (interleukin 1β) was induced by balloon injury, consistent with p38α MAPK activation, but was blocked by FR167653 administration.

The results of animal studies investigating neointima formation after vascular injury suggest that ERK1/2, JNK1/2 and p38α MAPK all promote neointima formation and SMC proliferation (Figure 4). The ability of p38α MAPK to promote VSMC proliferation dramatically contrasts with its ability to suppress mitosis in adult cardiomyocytes. This difference emphasizes the fact that MAPKs often have widely disparate biological roles in different cell types and in different physiological contexts.

CONCLUSIONS

Tremendous progress has been achieved in the past decade to characterize the role of MAPK cascades in various forms of cardiovascular disease. Although there are important inconsistencies and controversies within the field that should be resolved, four major themes emerge from this area of biomedical research. First, MAPK cascades modulate the hypertrophic response of the heart to pressure overload. Activation of the Raf–MKK–ERK1/2 cascade promotes the growth of individual

cardiomyocytes, whereas activation of the MKKK–MKK4/7–JNK and MKKK–MKK3/6–p38 MAPK cascade antagonizes the growth of individual cardiomyocytes. Furthermore, activation of ERK1/2 promotes cardiomyocyte survival after pressure overload, whereas activation of p38α MAPK promotes cardiomyocyte contractile dysfunction. Secondly, MAPK cascades regulate cardiac remodelling after MI. Activation of p38α MAPK and JNK1/2 promote pathological cardiac remodelling which includes cardiomyocyte apoptosis in the infarct border zone, infarct expansion and fibrosis at the site of the infarction and in the unaffected myocardium. Thirdly, MAPKs modulate atherosclerotic lesion formation via the regulation of macrophage foam cell formation. JNK2 and p38α MAPK activity are required for the uptake of oxLDL by macrophages in culture, and JNK2 is required for atherosclerotic lesion development *in vivo* because of its ability to facilitate the transformation of macrophages into foam cells. Fourthly, MAPK cascades play a pivotal role in the formation of neointima after vascular injury. Activation of ERK1/2, JNK1/2 and p38α MAPK in VSMCs is required for the formation of neointima after vascular injury.

Although these four themes suggest therapeutic targets for the development of pharmacological agents to treat cardiovascular disease, there are many important limitations of the studies reviewed above that merit discussion. In some cases, these limitations explain the conflicting results obtained by different research groups. First, studies that examined the activation of MAPK cascades in cells were limited by the fact that they all examined the pan-cellular activation of signalling proteins. In other words, variations in the subcellular activation of various pathways, such as whether a MAPK was active in the nucleus compared with the cytosol, were not examined in any of the studies discussed. This may be a significant limitation as activation of a pathway in one location often has a different biological effect than activation in another part of the cell [101]. Secondly, studies that examined the activation of MAPK cascades typically did not consider the integrated quantity of pathway activation. It is apparent that the length of time and quantity of activation of a particular MAPK is critical for determining the biological response [102]. Thirdly, studies that employed overexpression of activated forms of a kinase are limited by the fact that the overexpressed kinase may be targeted to atypical subcellular locations, that the overexpressed kinase may phosphorylate non-ideal or non-specific substrates and that the kinase is continuously active and does not reflect the normal timing of pathway activation. Fourthly, global gene knockout studies are limited by the lack of tissue specificity of the knockout and the possibility that an observed cardiovascular phenotype may be due to disruption of the gene in other tissues. Furthermore, when a gene is disrupted in a continuous fashion from the onset of embryonic development, compensatory pathways are often up-regulated that mitigate or alter the phenotype. Fifthly, tissue-specific knockout studies are limited by the toxicity of the *Cre* recombinase and the variability of gene disruption within cells in a specific tissue [103]. Sixthly, studies that use pharmacological agents are limited by the frequent off-target effects of these agents. Finally, loss-of-function studies with protein kinases that utilize pharmacological agents answer different questions than loss-of-function studies that employ gene disruption technology or loss-of-function studies that involve the expression of a DN mutant. Many protein kinases are multifunctional proteins that have non-kinase activities. For example, Raf-1 is both a protein kinase and an ASK1 inhibitor: if the kinase function of Raf-1 is inhibited with a small molecule inhibitor, the ability of Raf-1 to bind to and inhibit ASK1 remains [56]. In contrast, targeted disruption of the Raf-1 gene disrupts both functions of Raf-1. Overexpression of a DN mutant inhibits the kinase activity of native Raf-1, but also probably potentiates its ability to inhibit ASK1.

Despite all of these caveats about the scientific investigation of MAPK pathway function in cardiovascular disease, promising targets have been identified. It will be exciting to translate some of these discoveries to the clinical realm in order to reduce the terrible burden of cardiovascular disease.

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