Review

Mitogen-Activated Protein Kinases and Their Role in Regulation of Cellular Processes

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Abstract. Mitogen-activated protein kinases (MAPKs) are evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells. The three major MAPK cascades are known, the extracellular signal-regulated protein kinase (ERK) cascade, c-Jun amino-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) cascade and p38-MAPK cascade. This paper is focused on characterization of these MAPK cascades in terms of their distribution and biological role in some pathological processes (apoptosis, hypertrophy) with a special orientation on the role of MAPKs in cardiovascular system during ischemia/reperfusion.

Key words: Mitogen-activated protein kinase — Ischemia — Kinase inhibitors

Introduction and characterization of MAPK cascades

Eukaryotic cells respond to extracellular stimuli by transmitting intracellular signals to coordinate appropriate responses. Protein phosphorylation is a major regulatory mechanism utilized by second messenger systems that are coupled to cell surface receptors or induced by cellular disturbances, such as stress stimuli and oncogenic transformation. Among the pathways that use protein phosphorylation to transduce these signals from the plasma membrane to nuclear and other cellular targets there are highly conserved mitogen-activated protein kinase (MAPK) cascades. These cascades are involved in the regulation of a wide variety of cellular processes such as proliferation, differentiation, development, cell cycle, cell death (Robinson and Cobb 1997; Chang and Karin 2001). These protein kinase cascades consist of a three-kinase module (Cobb and Goldsmith 1995) that includes:

- an activator of MEK (MEK kinase [MEKK] or MAPK kinase kinase),
- an activator of MAPK (MEK, MKK or MAPK kinase),
- the MAPK.

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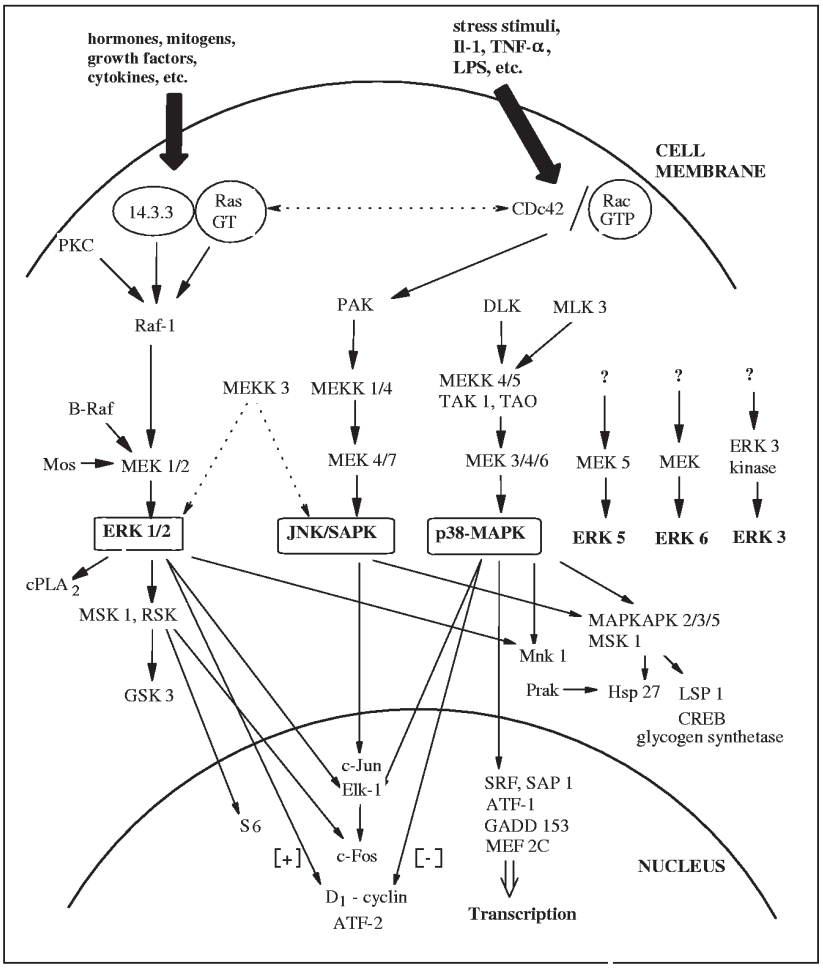

Figure 1. Schematic diagram of the currently known MAPK pathways. **ATF**, activating transcription factor; **CDc42**, cell division cycle protein 42; **cPLA2**, phospholipase A2; **CREB**, cAMP response element binding protein; **DLK**, dual leucine zipper-bearing kinase; **ERK**, extracellular signal-regulated protein kinase; **GADD 153**, growth arrest and DNA damage inducible gene 153; **GSK 3**, glycogen synthase kinase 3; **GTP**, guanosin triphosphate; **Hsp 27**, heat shock protein; **Il-1**, interleukin-1; **JNK/SAPK**, c-Jun amino – terminal kinase/stress-activated protein kinase; **LPS**, lipopolysaccharide; **LSP 1**, lymphocyte-specific protein 1; **MAPK**, mitogen-activated protein kinase; **MAPKAPK**, MAPK-activated protein kinase; **MEK**, MAPK kinase; **MEKK**, MAPK kinase kinase; **MEF 2C**, myocyte enhancer factor 2C; **MLK 3**, mixed-lineage kinase 3; **Mnk and Prak**, MAPKAPK homologues; **MSK 1**, mitogen- and stress-activated protein kinase 1; **PAK**, p21-activated protein kinase; **PKC**, protein kinase C; **RSK**, ribosomal S6 kinase; **S6**, ribosomal protein; **SAP 1**, accessory protein 1; **SRF**, serum response factor; **TAK 1**, TGF-β activated kinase 1; **TAO**, 1000 and 1 amino acid kinase; **TNF-α**, tumor necrosis factor-α.

In mammalian cells, several MAPK signaling pathways were found (Fig. 1): extracellular signal-regulated protein kinase cascade (ERK cascade), c-Jun amino-terminal kinase/stress-activated protein kinase cascade (JNK/SAPK cascade) and p38-MAPK cascade. Sequential phosphorylation and activation of specific components of MAPK cascades initiated by several extracellular signals (growth factors, cytokines, physical and chemical stress) mediates cell signal transmission. The members of MAPK family are activated by phosphorylation on threonine and tyrosine residues by upstream located specific kinases (MEKK, MKK). MAPKs are Ser/Thr protein kinases that transmit the signals by phosphorylating downstream substrates on serine or threonine that are adjacent to proline residues (Avruch et al. 1994; Marshall 1994). The aim of this review is to provide some information about MAPKs starting with their distribution, activation and inactivation by various extracellular and intracellular stimuli up to their final biological actions mediated through different substrates, such as transcription factors, downstream kinases or other signal molecules. In addition, also the role of MAPKs in cardiovascular system during ischemic preconditioning and ischemia/reperfusion injury is described.

MEKK

Up to now, five MEKKs (MEKK 1–5) are known to activate three best known MAPK cascades (ERK, p38-MAPK, JNK/SAPK) with different selectivity. The gene encoding MEKK 1 was cloned on the basis of the similarity of MEKK 1 to the MEKK of the yeast pheromone-response pathway (Lange-Carter et al. 1993). MEKK 1 is a large protein of 195 kDa. When over-expressed in Swiss 3T3 and REF 52 fibroblasts, MEKK 1 can induce apoptosis (Johnson et al. 1996). Cloned cDNAs encoding MEKK 1 homologues include also cDNAs for the close relatives MEKK 2 and MEKK 3 and the more distantly related transforming growth factor-β (TGF-β)-activated kinase (TAK 1) (Yamaguchi et al. 1995; Blank et al. 1996). TAK 1 is stimulated in response to TGF-β and bone morphogenetic protein and activates MEKs of the stress-responsive cascades. Two novel TAK 1-binding proteins (TABs) were identified in mammalian cells, that may stimulate TAK 1 activity (Shibuya et al. 1996).

In transfected fibroblasts, MEKK 1 most potently activates JNK/SAPK; however, in primary cardiac myocytes both ERK and JNK/SAPK pathways are activated (Robinson and Cobb 1997), consistent with the ability of MEKK 1 to phosphorylate MEKs 1–4 *in vitro*. These data suggest that the effects of MEKK 1 may depend on the cell type. MEKKs 2 and 3 are able to transduce signal to both JNK/SAPK and ERK; however, MEKK 2 preferentially activates JNK/SAPKs and MEKK 3 preferentially activates ERKs (Blank et al. 1996). MEKK 5 may serve as an upstream kinase of p38-MAPK pathway (Wang et al. 1996).

MEK

MEK family consists of at least seven kinases MEK 1–7. Although *in vivo* specificities of MEKs have not been fully defined, their *in vitro* specificities suggest that each acts in a single, or maximally in two MAPK pathways. MEK 1/2 act on ERK 1/2, MEK 3 acts on p38-MAPK, MEK 4 stimulates JNK/SAPKs and also p38-MAPK, MEK 5 is specific mainly for ERK 5 (Waskiewicz and Cooper 1995; Zhou et al. 1995; Deacon and Blank 1997) and MEK 6 was shown to activate p38-MAPK (Han et al. 1996). MEK 7 is specific for the JNK/SAPK isoforms (Foltz et al. 1998).

MEK 1 and MEK 2 contain proline-rich inserts in their carboxy-terminal domains that are absent in other MEK family members. Deletion of the insert from MEK 1 impairs its activation by Raf-1 in transfected cells, suggesting that this insert may be involved in the coupling of MEK 1 to components of the signal transduction cascade (Catling et al. 1995). Phosphorylation sites for other kinases are located in this insert; thus, MEK-Raf coupling may be regulated by protein kinases from other signaling systems (Robinson and Cobb 1997).

MAPK

Mitogen-activated protein kinases are grouped into subfamilies on the basis of sequence similarity, mechanisms of upstream stimulation, and sensitivity to activation by different MEKs. Three groups of MAPKs are best known and defined; ERK, p38-MAPK and JNK/SAPK. The ERKs are strongly activated by mitogenic and growth factors (Sadoshima et al. 1995; Sugden and Bogoyevitch 1995), JNK/SAPK and p38-MAPK can be activated by various cell stresses, such as hyperosmotic shock, metabolic stress or protein synthesis inhibitors, UV light, heat shock, cytokines (Rouse et al. 1994; Raingeaud et al. 1995; Iordanov et al. 1998; Yuasa et al. 1998). In several studies it was found that ischemia and ischemia/reperfusion (Knight and Buxton 1996; Htun et al. 1998; Sugden and Clerk 1998; Marais et al. 2001) also activated JNK/SAPK and p38-MAPK.

Many other MAPKs have been discovered, including ERK 3 (Boulton et al. 1991), ERK 5 (Zhou et al. 1995) and Ste20p-related kinases, such as p21-activated protein kinases (PAKs), the mixed-lineage kinases (MLKs) (Manser et al. 1994), Ste20-like oxidant stress response kinase (SOK 1), 1 and 2 kinases responsive to stress (Krs), thousand and one amino acid kinase (TAO 1) and MAPK-upstream kinase (MUK) (Pombo et al. 1996; Taylor et al. 1996).

MAPKs act in parallel with other cell signaling systems. It was found that stress activated p38-MAPK negatively regulates low density lipoprotein (LDL)-receptor expression in an isoform-specific manner *via* modulation of p42/p44-MAPK cascade and this represented one-way communication between p38-MAPK and p42/p44-MAPK (Mehta and Miller 1999). Therefore, cross talking between pathways is highly probable and seems to be crucial for the coordinated responses of the cells. Integration of signals may take place at many levels, e.g. upstream of the cascades, within the cascades, by regulated inactivation of the cascades,

and within the substrates. Accordingly, MAPKs may cooperate with each other as was demonstrated also in steroid-dependent transcription *via* phosphorylation of a receptor (Kato et al. 1995).

The mechanism of activation of ERK subfamily of MAPKs

One of the most studied MAPK signaling cascades is the ERK pathway, which begins usually at the plasma membrane level where stimulus-induced binding with guanosin triphosphate (GTP) activates the small G-protein (Ras). This activation is followed by translocation of a 74 kDa protein Ser/Thr kinase, the Raf-1 kinase (a member of MEKK family), to the plasma membrane (Warne et al. 1993), allowing its interaction with another protein termed 14.3 3 (Fantl et al. 1994; Freed et al. 1994). The 14.3.3 proteins are small acidic scaffold and adapter proteins with a molecular mass ranging from 27 to 32 kDa (Fu et al. 2000; Muslin and Xing 2000; Shaw 2000). These proteins are naturally dimeric and can form homo- or heterodimers with the other 14.3.3 isoforms (Jones et al. 1995). 14.3.3-Raf association requires phosphorylation of Raf on the Ser 259 and Ser 621 which substitution by Ala inhibits this association (Michaud et al. 1995). This interaction with 14.3.3 proteins stabilizes Raf-1 and enables its activation by some membrane kinases, which might belong to the protein kinase C (PKC) family (Kolch et al. 1993; Van Der Hoeven et al. 2000). Activation of Raf by 14.3.3 protein was also observed (Fantl et al. 1994; Freed et al. 1994). Jaumot and Hancock (2001) found that PP1 and PP2A, serine-threonine phosphatases, have a positive role in this activation, and that inhibition of PP1 and PP2A causes plasma membrane accumulation of 14.3.3-Raf-1 complexes which cannot be activated. The profound role of 14.3.3 dimerization has been demonstrated in the Raf regulation where both monomeric and dimeric forms of 14.3.3 bind with Raf, but only the dimeric form supports Raf kinase activity (Tzivion et al. 1998; Tzivion et al. 2000).

After Ras/14.3.3-Raf stimulation the signal is transmitted to the next kinases in this cascade, MEKs (MEK 1 and 2), which can be activated primarily by Raf-1 but also by B-Raf (Raf-1 isoform) and Mos (a member of MEKK family) (Ahn et al. 1991). Mechanism of MEK activation involves phosphorylation on two Ser residues within MEK subdomains VII and VIII. Activated MEKs demonstrate a high degree of specificity for the native form of their downstream substrates, the ERK 1 and ERK 2. ERKs are activated by dual phosphorylation of tyrosine (Tyr-phosphorylation by tyrosine kinases) and threonine residues (Thr-phosphorylation by upstream Ser/Thr kinases, such as Raf-1, MEK). This type of activation is common to all known MAPK isoforms based on presence of Thr-X-Tyr motif within subdomain VII and VIII. The only difference is in the residue X between the Thr and Tyr, Glu for ERK (TEY), Gly for p38-MAPK (TGY) and Pro for JNK (TPY) (Payne et al. 1991; Cano and Mahadevan 1995).

Biological consequences of ERK pathway activation

Phosphorylation of Thr and Tyr residues is essential not only for ERK activation but also for translocation of the ERKs to the nucleus. Structural analysis indicates

that some MAPKs dimerize on activation (Khokhlatchev et al. 1998). Though the role of MAPKs dimers is not yet known, they can be involved in the mechanism of action of the MAP kinase family. In the case of ERK 2 it may be involved in stimulus-dependent nuclear accumulation (Khokhlatchev et al. 1998).

A large number of nuclear, cytosolic and structural regulatory proteins can be phosphorylated by ERKs (Widmann et al. 1999). ERKs can directly control several cellular processes including transcription, translation and cytoskeletal rearrangement (Reszka et al. 1995). Even though the major group of ERK substrates includes transcription factors (TF), the ERKs translocation is not essential for their activity, because the phosphorylation of transcription factors can occur *via* three distinct pathways that are not necessarily associated with translocation of ERKs to the nucleus (Widmann et al. 1999; Chang and Karin 2001). Firstly, TF can be phosphorylated by ERKs directly in the nucleus after translocation of activated ERKs. Secondly, ERK can phosphorylate TF in the cytosol and then translocate the phosphorylated TF to the nucleus. Thirdly, ERKs can activate other protein kinases which then act directly or indirectly on TF, either in the cytosol or in the nucleus. In addition, when ERK 1/2 is prevented experimentally from entering the nucleus in mammalian cells, phosphorylation of the transcription factor Elk-1 in the nucleus is disrupted, whereas activation of cytosolic substrates is unperturbed (Brunet et al. 1999).

ERKs can transmit the signal to downstream kinases such as the ribosomal S6 kinase (RSK, also known as p90rsk or MAP kinase-activated protein kinase 1 (MAPKAP kinase 1) (Erikson 1991). RSK can phosphorylate and activate regulatory molecules such as the transcription factor c-Fos, cyclic adenosin monophosphate (cAMP) response element-binding protein (CREB), estrogen receptor, nuclear factor-kappa B (NFκB/IκBα), the ribosomal S6 protein and can also transmit the signal to a downstream kinase, glycogen synthase kinase 3 (GSK 3) which participates in the down-regulation of transcription. RSK also phosphorylates the Ras GTP/GDP-exchange factor Sos leading to feedback inhibition of the Ras-ERK pathway (Eldar-Finkelman et al. 1995; Frödin and Gammeltoft 1999). It has been found that prolonged activation and nuclear retention of ERKs is required for transcription of cyclin D1 in the cell cycle (Lavoie et al. 1996). The contribution of ERKs to the regulation of cell apoptosis is supposed. In human B cells activation of ERKs by surface IgM cross-linking triggered apoptosis (Sakata et al. 1995). On the other hand, when H_2O_2-induced activation of ERK cascade was selectively inhibited in cultured neonatal cardiomyocytes, there was an increased number of apoptotic cells (Aikawa et al. 1997). This suggests a positive anti-apoptotic role of ERK in cardiac cells. This is supported also by the study of Adderley and Fitzgerald (1999) who found that cell death of cardiomyocytes due to exposure to H_2O_2 and doxorubicin (inducers of oxidative stress) was limited by an increase of prostacyclin formation. This increase reflected the induction of cyclooxygenase-2 (COX-2) expression mediated by ERK 1/2. Interesting is also the role of ERKs in cardiac hypertrophy. Hypertrophic G protein-coupled receptors (GPCR) agonists such as endothelin-1 (ET-1) and phenylephrine stimulate ERK pathway in the heart (Clerk

et al. 1994; Markou and Lazou 2001). MEK 1/2 and ERK 1/2 are also strongly activated by phorbol 12-myristate 13-acetate (PMA) in cardiac myocytes (Bogoyevitch et al. 1993). In the left ventricle in rats, when hypertrophy was induced by volume overload, an increase in the ERK 1/2 activity was observed (Sentex et al. 2001).

Perhaps two best-characterized ERK substrates are cytoplasmic phospholipase A_2 ($cPLA_2$) and the transcription factor Elk-1. Phosphorylation of $cPLA_2$by ERKs causes an increase in the enzymatic activity of $cPLA_2$, resulting in an increased arachidonic acid release and formation of lysophospholipids from membrane phospholipids (Lin et al. 1993). ERKs can therefore trigger the formation of multiple secondary signaling molecules. Elk-1 is also a direct target of the ERK cascade. This transcription factor binds to the promoters of many genes and it is possible that an increased phosphorylation and stimulation of its transcriptional activity can mediate the effects of the MAPK signal transduction pathway on gene expression (Davis 1993).

p38-subfamily of MAPKs

Activation and inactivation of p38-MAPK

p38-MAPK was first isolated in the course of a study designed to identify proteins that were phosphorylated on tyrosine in macrophages stimulated with bacterial endotoxin lipopolysaccharide (LPS) (Han et al. 1993). There are at least four members of p38 group of MAPK that have been cloned and characterized: p38-α (SAPK 2) (Han et al. 1994), p38-β (SAPK 2b) (Jiang et al. 1996), p38-γ (ERK 6/SAPK 3) (Lechner et al. 1996) and p38-δ (SAPK 4) (Kumar et al. 1997). Comparison of peptide sequences of p38-MAPKs and other MAP kinases have shown that p38 isoforms have more than a 60% amino acid sequence identity within this group and that each member of p38-MAPK has 40–45% identity with the members of other groups of MAPKs (Jiang et al. 1997). All four p38 isoforms can be activated by a group of extracellular stimulus including proinflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin-1 (Il-1), the protein synthesis inhibitors, and chemically stressful stimuli such as arsenite and H_2O_2 (Rouse et al. 1994; Raingeaud et al. 1995; Iordanov et al. 1998; Yuasa et al. 1998). Although these four isoforms display similar activation profiles, differences were observed in the kinetics and in the level of activation (Jiang 1996). Moreover, ischemic stress was found to reveal a different effect on activation of distinct p38 isoforms (Saurin et al. 1999; Conrad et al. 1999). It is therefore unclear whether these isoforms are specifically regulated through different activators or coactivated by the same upstream regulators *in vivo*. The most extensive data concerning activation of p38-MAPKs in different cells under different conditions regard the activation of p38-α. Increased p38-α activation related to inflammation has been observed in many cellular types, such as LPS-treated macrophages (Han et al. 1994), TNF-stimulated endothelial cells (Pietersma et al. 1997), human platelets stimulated with thrombin, collagen fibers or thromboxan analogues (Saklatvala et al. 1996). Some growth factors, including

granulocyte macrophage-colony stimulating factor (GM-CSF), fibroblast growth factor (FGF), erythropoietin, and interleukin-3 (Il-3) can also trigger the activation of p38-α in certain cell types (Tan et al. 1996). Osmotic stress is also a very strong activator of p38-α in many different cell types including epithelial cells, endothelial cells, and fibroblasts (Moriguchi et al. 1996). On the other hand, down-regulation of p38-α activity by oxidative stress was observed in liver (Mendelson et al. 1996). In neurons, down-regulation of p38-α activity by insulin was found (Heidenreich and Kummer 1996). Thus, the activation of p38-α is both stimulus-dependent and cell-type dependent.

The direct activators of p38-MAPK are upstream-located kinases MEK 3, 4 and MEK 6 (Han et al. 1996; Yuasa 1998). A recently cloned MEKK 5 or TAK 1 may serve as a kinase upstream of MEK 3 and 6. Thus, the p38-MAPK cascade may consist of the sequence:

$$\text{TAO/TAK 1/MEKK 5/4} \rightarrow \text{MEK 3/4/6} \rightarrow \text{p38} - \alpha/\beta/\gamma/\delta$$

With the use of cotransfection techniques, several other potential regulators of the p38-MAPK pathway have been defined. They include GTP-binding protein Rac, PAK (Bagrodia et al. 1995; Zhang et al. 1995) and two new kinases named MLK 3, and DLK (dual leucine zipper-bearing kinase) (Fan et al. 1996; Tibbles et al. 1996). It is unknown, how these signaling molecules are coupled to receptor signals or how they mediate different extracellular signals that ultimately lead to p38-MAPK activation.

Biological consequences of p38-MAPK pathway activation

Extensive studies have been undertaken in search of proteins targeted for phosphorylation by p38-MAPK. It was found that some protein kinases and certain transcription factors are included (New and Han 1998). Protein kinases that can serve as substrates for p38-MAPK are two structurally and functionally related protein kinases, MAP kinase-activated protein kinase 2 and 3 (MAPKAPK 2 and 3). These kinases were found to phosphorylate small heat shock protein (Hsp 27) which is involved in regulation of actin dynamics that may occur in TNF, interleukin-1 and heat shock or phorbol ester treated cells (Freshney et al. 1994; Rouse et al. 1994; Huot et al. 1997). Activated MAPKAPK 2 has also been reported to phosphorylate and activate glycogen synthase (Stokoe et al. 1992), CREB transcription factor (Tan et al. 1996) and lymphocyte-specific protein 1 (LSP 1) (Huang et al. 1997). In addition, data from the *in vivo* and *in vitro* assays indicate that MAPKAPK 3 is a potential substrate of ERK and JNK (Ludwig et al. 1996).

MAPKAPK homologues, MAP kinase-interacting kinase (Mnk) and p38-regulated/activated protein kinase (Prak), were identified recently (Waskiewicz 1997). Prak is specifically regulated by p38-α and p38-β and Hsp 27 is a substrate of Prak. Mnk 1 binds to p38-MAPK and ERK and was shown to be phosphorylated by both ERK 1 and p38-α (Fukunaga et al. 1997; Waskiewicz 1997) *in vitro*. Activation of Mnk 1 *in vivo* is initiated by growth factors, phorbol esters or physiological stress and can be blocked by specific inhibitors of MEK and p38-MAPK.

This suggests that Mnk 1 can also transmit the p38-MAPK signal (Waskiewicz 1997).

Many transcription factors have been suggested as potential substrates for p38-MAPK. These include activating transcription factor-1 (ATF-1), ATF-2, Elk-1, serum response factor (SRF), growth arrest and DNA damage inducible gene 153 (CHOP 10 or GADD 153) and myocyte enhance factor 2C (MEF 2C) (Raingeaud et al. 1995; Hazzalin et al. 1996; Price et al. 1996; Tan et al. 1996; Wang and Ron 1996; Han et al. 1997; Janknecht 1997; Whitmarsh et al. 1997).

p38-MAPK is activated mainly by numerous inflammatory stimuli (Han et al. 1994; Raingeaud et al. 1995) and thereafter affects directly or indirectly the transcription of genes encoding inflammatory molecules. In biological terms this indicates an important role of p38-MAPK in inflammatory responses. Moreover, involvement of p38 pathway in cardiac hypertrophy (Clerk and Sugden 1999) and apoptosis (Cross et al. 2000; Feuerstein and Young 2000) is suggested. In cardiac myocytes infected with recombinant adenoviruses encoding up-stream activators of p38-MAPK (MEK 3b and MEK 6b) typical hypertrophy response was observed and it was enhanced by co-transfection of p38-β (wild type) and suppressed by a dominant negative p38-β mutant (Wang et al. 1998a). Clerk and Sugden (1999) observed that ET-1 and phenylephrine (powerful hypertrophic agonists) stimulate p38-MAPK pathway in the heart. In another study it was also observed that stimulation of cardiac myocytes with phenylephrine resulted in a strong activation of p38-MAPK whereby phosphorylation was maximal at 5–10 minutes (Markou and Lazou 2001). With respect to apoptosis, it was found that inhibition of p38-MAPK by SB 203580 before the ischemic insult markedly diminished the consequences of the ischemia/reperfusion injury, including apoptosis (>50 %) in a Langendorff-perfused rabbit heart (Ma et al. 1999). In the cardiovascular system activation of two different isoforms of p38-MAPK has been found to play divergent roles in cell death (Wang et al. 1998a). It suggests that the involvement of p38 isoforms in apoptosis may be cell-type and stimulus-type dependent. The influence of p38-MAPK on cell growth first became apparent following the observation that overexpression of mammalian p38-MAPK in yeast leads to significant slowing of proliferation (Han et al. 1994). Yeast with a mutation of Spc-1, the yeast homologue of p38-MAPK, exhibits a delay in the G2 phase of the cell cycle (Shiozaki and Russel 1995). Microinjection of p38-MAPK into NIH-3T3 cells can cause G1 arrest (Molnar et al. 1997). Lavoie et al. (1996) found that activation of p38-MAPK has a negative effect on D1-cyclin expression. All these facts suggest a relevant link between p38-MAPK activity and control of cell cycle.

JNK/SAPK – subfamily of MAPKs

JNKs (c-Jun-NH_2-terminal kinases) were first identified as protein kinases that phosphorylate the transcription factor c-Jun within its amino-terminal activation domain at Ser 63 and Ser 73 in cells exposed to UV radiation (Pulverer et al. 1991). Assays using the c-Jun activation domain as a substrate demonstrated the presence of 46 kDa (JNK 1) and 55 kDa (JNK 2) forms of JNKs (Derijard et al. 1994).

Simultaneously, three genes encoding MAPK-related kinases were cloned from a rat brain cDNA library and these kinases turned out to have all the properties of JNKs. Because these kinases were more strongly activated by stress-inducing stimuli, they were named SAPK 1α, β, γ (Kyriakis et al. 1994). The genes for SAPK 1α and SAPK β encode kinases of approximately 55 kDa, similar in size to JNK 2, whereas SAPK γ and JNK 1 have relative molecular masses of approximately 45 kDa (Derijard et al. 1994; Kyriakis et al. 1994). It is suggested that both groups of kinases are the same and therefore they were named as JNK/SAPKs.

JNK/SAPKs are activated by UV radiation, inhibitors of translation, epidermal growth factor (EGF), TNF, chemical stress and heat shock (Kyriakis et al. 1994; Rouse et al. 1994; Raingeaud et al. 1995; Iordanov et al. 1998; Yuasa et al. 1998). The exact mechanism of JNK/SAPK cascade activation is not fully elucidated, but it is possible that it starts with the activation of Rac (the small GTP-binding protein) or CDc42 cell division cycle protein 42 followed by PAK activation. PAK binds to MEKK 1, 2 or 4 and this mediates phosphorylation of MEK 4, an upstream activator of JNK/SAPK.

$$\text{Rac/CDc42} \rightarrow \text{PAK} \rightarrow \text{MEKK1, 2, 4} \rightarrow \text{MEK4/7} \rightarrow \text{JNK/SAPK}$$

A lower level of JNK/SAPK activation was also observed with other GTP-binding proteins, through Ras activation. It was found that Ras activation amplifies the effects of some stimuli on JNK/SAPKs activity (Derijard et al. 1994).

Biological consequences of JNK/SAPK pathway activation

The best characterized substrate phosphorylated by JNK/SAPKs is the transcription factor c-Jun. Detailed analysis of c-Jun activation by these kinases demonstrated that JNK/SAPKs bind to small regions (the δ-subdomains) of the amino-terminal activation domains of c-Jun (Hibi et al. 1993; Derijard et al. 1994). These binding sites are physically separated from the sites of c-Jun phosphorylation. Interestingly, addition of a peptide based on the sequence of the δ-subdomain blocks the ability of JNK/SAPK to phosphorylate c-Jun (Adler et al. 1994). Together, these data indicate that the strong binding interaction between JNK/SAPK and the δ-subdomain of c-Jun is required for the phosphorylation of c-Jun by JNK/SAPK. So far, additional substrates, such as ATF-2, Elk-1, insulin receptor substrate 1 and Bcl-2 having a function regulated by JNK/SAPK-mediated phosphorylation were identified (Gupta et al. 1995; Whitmarsh et al. 1995; Gross et al. 1999; Aguirre et al. 2000).

JNK/SAPKs may have an important role in cell signaling, cardiac cell hypertrophy and in regulating of apoptosis in various cell types (Sakata et al. 1995; Bogoyevitch et al. 1996; Wang et al. 1998b). Specific activation of the JNK pathway by over-expression of MEK 7 in cardiac myocytes induced characteristic features of cardiomyocytes hypertrophy along with increased expression of atrial natriuretic factor and sarcomeric organization (Wang et al. 1998b). Similarly, activation of JNKs through transfection of MEKK 1 and MEK 4 also led to hypertrophy (Bogoyevitch et al. 1996). Stimulation of both p46 and p54 JNK/SAPK by ET-1 was

observed in cardiac myocytes and in isolated perfused rat hearts (Bogoyevitch et al. 1995; Lazou et al. 1998). In human B-cells JNK/SAPKs are selectively activated by cluster of differentiation (CD)-40 (a cell surface receptor) (Sakata et al. 1995), and its activation salvages cells from apoptosis. JNK/SAPK stimulation has also been associated with hepatic regeneration and with T-cell activation (Hsueh and Lai 1995; Westwick et al. 1995). Andreka et al. (2001) observed a cytoprotection by JNK during NO-induced apoptosis in neonatal rat cardiac myocytes. In other study, inhibition of JNK 1, but not JNK 2, suppressed apoptosis induced by ischemia/reoxygenation in rat cardiac myocytes (Hreniuk et al. 2001). In a rabbit model of *in vivo* ischemia and reperfusion robust apoptosis and activation of JNK/SAPK has been demonstrated. This increase of JNK was significantly diminished by carvedilol (multiple action β-blocker) (Yue et al. 1998). Inhibition of JNK/SAPK by bioactive cell-permeable peptide inhibitors protected pancreatic βTC-3 cells against apoptosis induced by Il-1β (Bonny et al. 2001). All these data suggest that consequential effects of JNK/SAPK activation are dependent on cell type, stimulus of apoptosis induction, intensity and duration of their activation and these effects could be also isoenzyme specific (Hreniuk et al. 2001).

The inhibitors of MAPKs

Identification and use of the specific inhibitors of MAPKs represents an efficacious strategy for the study of physiological substrates and the roles of MAPKs in mammalian cells. Nowadays, the inhibitors PD 98059, UO 126, SB 203580 and SB 202190 are most used (Fig. 2).

PD 98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one] is a flavone compound that binds to the inactive form of MEK 1/2, preventing its activation by c-Raf and other upstream activators (Alessi et al. 1995). It is effective in low, i.e. micromolar concentrations and does not inhibit the phosphorylated (fully activated) form of MEK. PD 98059 indirectly acts on ERK 1/2 by inhibiting the activation of MEK 1/2 (Alessi et al. 1995; Dudley et al. 1995). Although the amino acid sequence of MEK 2 is 90% identical to that of MEK 1, this inhibitor is at least tenfold less effective in preventing the activation of MEK 2 than MEK 1 *in vitro*. The strong suppression of MEK 1 activation by PD 98059 suggests that MEK 1 is the dominant ERK 1/2 activator in nearly all cells examined so far (Cohen 1997).

UO 126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenyl-thio]butadiene) represents a novel specific inhibitor of ERK cascade and acts directly on both MEK 1/2 and ERK 1/2 (Duncia et al. 1998). It has been also found that UO 126 is characterized by 100-fold higher affinity for MEKs than PD (Favata et al. 1998).

SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulphiphenyl)-5-(4-pyridinyl)-1H-imidazole] and SB 202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridinyl)-1H-imidazole] are representatives of a novel class of pyridinyl imidazole derivatives developed as inhibitors of the LPS-induced synthesis of Il-1 and TNF in monocytes. It was found that SB 203580 inhibits p38-α and SAPK 2b/p38-β in submicromolar concentrations *in vitro* (Lee et al. 1994; Cuenda et al. 1997). When added to the

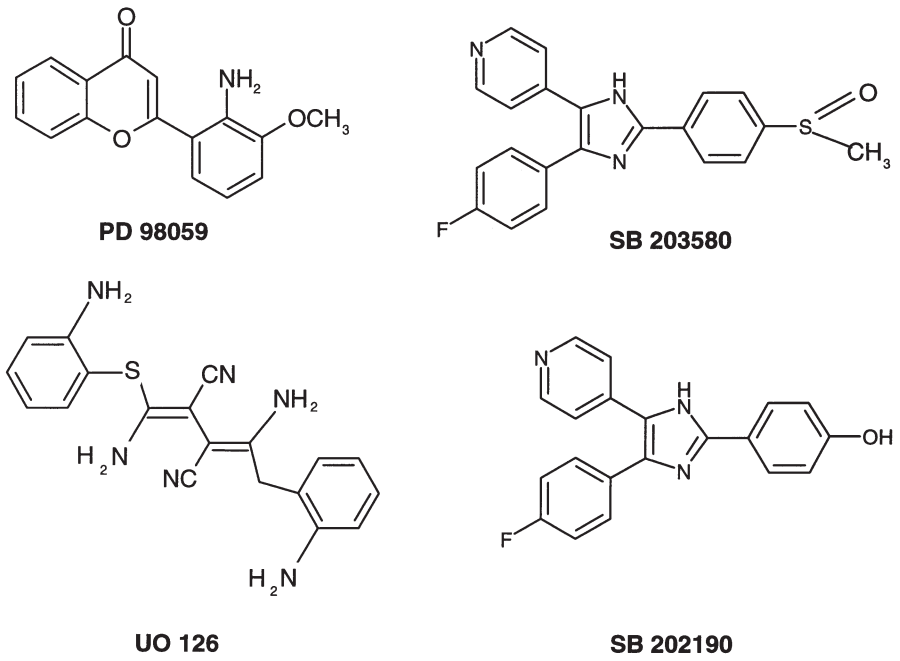

Figure 2. Structures of a flavone and pyridinyl imidazoles synthetic inhibitors of MAPKs.

cells, SB 203580 blocks the activation of MAPKAP kinase 2, a direct substrate of p38-MAPK, in response to various stress and cytokine stimuli (Cuenda et al. 1995). In addition, SB 203580 inhibits two alternatively spliced forms of SAPK 1γ/JNK 2 (JNK 2β_1 and JNK 2β_2), but with 10 to 20-fold lower potency than it inhibits p38-α. SB 203580 inhibits also two further alternatively spliced forms, JNK 2α_1 and JNK 2α_2 with 100-fold lower potency as compared with p38-α (Whitmarsh 1997).

Role of MAPKs in cardiovascular system during ischemia and ischemic preconditioning

Mammalian cells respond to ischemia and ischemia/reperfusion by activation of various pathways that lead to cell death and organ dysfunction. In myocardium, prolonged ischemia causes necrosis and contractile dysfunction, whereas brief episodes of ischemia substantially enhance tolerance of the myocardium to a subsequent long-lasting ischemic insult. This phenomenon is known as ischemic preconditioning (IP) (Murry et al. 1986). Recently, many studies (Aikawa et al. 1997; Weinbrenner et al. 1997, 1998; Barancik et al. 1999, 2000; Mackay and Mochly-Rosen 1999; Omura et al. 1999; Armstrong et al. 2000; Mocanu et al. 2000; Nakano et al.

2000a,b; Sato et al. 2000; Gysembergh et al. 2001; Marais et al. 2001) demonstrated both positive or negative role of MAPKs during ischemia and in the mechanism of IP. It was found that ischemia and reperfusion contributes to the regulation of MAPK cascades, however, some differences in the intensity and duration of their activation, as well as species-related differences (rat, rabbit, pig) were observed.

In the porcine myocardium (*in vivo* model), it was found that the activities of the ERKs increased moderately during brief ischemia and even more markedly during the following reperfusion period (Barancik et al. 1997). Stimulation of the ERKs activity by ischemia/reperfusion was also observed in the isolated perfused rat hearts (Knight and Buxton 1996). In cardiomyocytes, in an *in vitro* model of ischemia, the activities of protein kinases with molecular weights of 42 and 44 kDa were stimulated (Bogoyevitch et al. 1995). These kinases appeared to correspond to the ERKs. An increased activation of the ERKs by hypoxia and hypoxia/reoxygenation has been demonstrated also by Seko et al. (1996). After exposure of the myocardium to ischemic stress, a rapid activation of ERKs was found (Strohm et al. 2000). Shimizu et al. ascertained that ERKs, JNKs, and p38-MAPKs were activated by ischemia after experimentally induced *in vivo* myocardial infarction in rats (Shimizu et al. 1998). In contrast to these findings, other studies showed that global ischemia and ischemia/reperfusion did not activate ERKs (Bogoyevitch et al. 1996; Clerk et al. 1998).

In context with the positive role of ERKs in ischemic preconditioning it was found that the local and systemic infusion of this protein cascade inhibitors (PD 98059 and UO 126) before and during the IP protocol reversed the IP-induced cardioprotection and specifically inhibited the activities of ERKs *in vivo* (Strohm et al. 2000). Another study (Aikawa et al. 1997) showed that when H_2O_2-induced activation of ERKs was selectively inhibited by PD 98059, the number of apoptotic cardiomyocytes increased. A positive role of ERKs in cell protection was confirmed also by the study, in which the authors observed cardiotrophin-1-mediated inhibition of apoptosis in serum-deprived neonatal rat myocytes (Sheng et al. 1997). This effect was associated with an activation of both, ERK 1 and ERK 2 and the application of PD 98059 resulted in the inhibition of cardiotrophin-1-mediated protective effects against cell death. These results suggest that the activation of ERKs can protect cardiomyocytes during ischemia/reperfusion and this activation could play an important positive role in the mechanism of IP as well.

Numerous studies of the regulation of p38-MAPK cascade during ischemia and reperfusion brought unequivocal results. In a study by Barancik et al. (1997), ischemia initially induced activation of p38-MAPK, but during the following reperfusion and subsequent period of ischemia p38-MAPK was deactivated. On the other hand, another study demonstrated that the activity of p38-MAPK was strongly stimulated by ischemia and this activation was further increased during reperfusion (Bogoyevitch et al. 1996). In contrast, ischemia has also been reported not to cause any increased phosphorylation (activation) of p38-α in the rabbit heart, whereas it occurred when the heart was previously preconditioned (Weinbrenner et al. 1997). In isolated rabbit hearts, the stimulation of MAPKAP kinase 2 was

also observed only after IP (Nakano et al. 2000a). However, Maulik et al. (1996) described the stimulation of MAPKAP kinase 2 and of protein kinases that phosphorylate myelin basic proteins (MBP) in isolated rat hearts both, after IP and after ischemia/reperfusion in the absence of IP.

The role of p38-MAPK cascade in cardioprotection is also controversial. In isolated rabbit cardiomyocytes (Weinbrenner et al. 1997; Armstrong et al. 2000), rabbit and rat hearts (Maulik et al. 1998; Mocanu et al. 2000; Nakano et al. 2000b), it was shown that inhibition of p38-MAPK by SB 203580 completely abolished protection conferred by IP and a positive role of p38-MAPK in preconditioning was suggested. In accord, Nagarkatti and Sha'fi (1998) also found that protective effect of preconditioning was attenuated in the presence of SB 203580, but not in the presence of PD 98059 in rat myoblast cells). On the other hand, negative effect of p38-MAPK activation on the myocardium during ischemia was demonstrated when administration of SB 203580 before ischemia and during reperfusion resulted in inhibition of p38-MAPK pathway, reduction of apoptosis and recovery of myocardial function (Ma et al. 1999). Mackay and Mochly-Rosen (1999) observed a protective effect of SB 203580 against extended ischemia in cultured neonatal rat cardiomyocytes. When p38-MAPK activation was investigated during three ischemic episodes of preconditioning protocol in isolated perfused rat and rabbit hearts, there was a progressive decreasing of p38-MAPK activity during sustained ischemia and reperfusion and it was associated with improved functional recovery (Gysembergh et al. 2001; Marais et al. 2001). Administration of SB 203580 before and during preconditioning protocol had no effect on cardiomyocytes morphology and viability after hypoxia, but when it was applied in non-preconditioned cells before the onset of hypoxia, it caused a significant improvement in both morphology and viability (Marais et al. 2001). The systemic or local application of SB 203580 before and during the IP protocol caused an inhibition of p38-MAPK activity in pig myocardium, but it did not influence the IP-mediated cardioprotection *in vivo* (Barancik et al. 2000). In this study it was also found that application of SB 203580 before ischemia significantly reduced the infarct size suggesting a negative role of the p38-MAPK pathway in myocardium during ischemia.

The changes in the JNK/SAPK activity probably also play an important role in response of myocardial cells to ischemia and reperfusion. Several studies showed that the activities of JNK/SAPK in myocardium are unaffected by ischemia alone, but their activation only occurred during the following reperfusion (Pombo et al. 1994; Bogoyevitch et al. 1996; Knight and Buxton 1996; Barancik et al. 1997). In canine kidney epithelial cells, ATP repletion following ATP depletion induced by chemical anoxia was also associated with activation of JNK/SAPKs with a time course of activation similar to that seen in the kidney after ischemia and reperfusion (Pombo et al. 1994). However, this kinase pathway was also found to be moderately activated during ischemia (Omura et al. 1999). It is known that the MAPKs are phosphoproteins and require increased phosphorylation for their activation. The degree of phosphorylation depends on the balance between phosphorylation mediated by upstream kinases and dephosphorylation mediated by the action of protein

phosphatases. Recently the protein phosphatase inhibitors (okadaic acid, calyculin A, fostriecin) were found to mediate pharmacological protection of myocardium against ischemia and reperfusion, but the mechanisms involved in this protection are unclear (Armstrong et al. 1998; Barancik et al. 1999; Weinbrenner et al. 1998).

It was found that the application of okadaic acid (OA) exerted cardioprotective effects and this was associated with the inhibition of Ser/Thr protein phosphatases as well as with the activation of JNK/SAPK (Barancik et al. 1999). Other phosphatase inhibitors, such as calyculin A and fostriecin also caused the IP-like protective effects in isolated rat cardiomyocytes (Armstrong et al. 1998). Furthermore, cyclosporine A, FK-506 phosphatase-2B (PP-2B)-inhibitors, were shown to protect ischemic myocardium in isolated rabbit and rat hearts (Cumming et al. 1996; Weinbrenner et al. 1998).

Stimulation of JNK/SAPK as well as p38-MAPK was also observed after treatment with anisomycin, a protein synthesis inhibitor (Cano et al. 1996; Barancik et al. 1999; Mackay and Mochly-Rosen 1999; Nakano et al. 2000a; Sato et al. 2000). Application of anisomycin stimulated either solely the activity of JNK/SAPK (Barancik et al. 1999) or activities of both JNK/SAPK and p38-MAPK (Mackay and Mochly-Rosen 1999; Nakano et al. 2000a; Sato et al. 2000). This was connected with cardioprotective effects (reduction of infarct size, imitation of IP-mediated protection) in several animal models. It suggests the involvement of "stress" kinase pathway activation in IP.

The results of many studies suggest that ischemia and reperfusion induce different stimulation of various MAPK cascades and reveal differences in the time course of kinase stimulation and variability in the intensity of MAPK activation. In addition, the studies performed using inhibitors and activators of individual MAPK activities indicate a different role of MAPK cascades in the process of ischemic preconditioning. It appears that ERKs play a positive role in this process, while the role of p38-MAPK and JNK/SAPK pathways is more controversial.

Perspectives

Kinases constituting MAPK pathways have come to be acknowledged as key cellular signal transducers. Numerous studies have been performed to reveal a participation of MAPKs in important cellular processes, such as gene expression, cell proliferation and differentiation, as well as cell survival and death. However, the issue of contribution of these kinase pathways to the cellular response to various stress stimuli including ischemia, reperfusion and ischemic preconditioning still remains unresolved. Some confusion may arise from cross-talking between several MAPK pathways, which complicate a complete understanding of MAPK effects in the cardiovascular and other systems. Therefore, further studies, oriented towards identification of all possible and physiologically relevant MAPK substrates that regulate these processes, employing proteomic analyses and/or development and application of novel small cell-permeable modulators specific for each family

of MAPKs are a major challenge. The recognition of detailed and complex interactions between enzymes and these inhibitors through mechanistic enzymology, the elucidation of kinase structures and the sequencing of human genome, as well as development of dominant negative or constitutively active mutants of several members of MAPK would promote not only the definition of genes regulated in response to individual MAPKs but also potential application of specific kinase inhibitors in human pharmacotherapy.

Acknowledgements. This study was supported, in part, by grant VEGA SR 2/2063/22.

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Final version accepted: June 27, 2002