Mitogen-activated protein kinase translocates to the nucleus during ischaemia and is activated during reperfusion

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Growth factors and various cellular stresses are known to activate mitogen-activated protein (MAP) kinase, which plays a role in conveying signals from the cytosol to the nucleus. The phosphorylation of MAP kinase is thought to be a prerequisite for translocation. Here, we investigate the translocation and activation of MAP kinase during ischaemia and reperfusion in perfused rat heart. Ischaemia (0–40 min) induces the translocation of MAP kinase from the cytosol fraction to the nuclear fraction. Immunohistochemical observation shows that MAP kinase staining in the nucleus is enhanced after ischaemia for 40 min. Unexpectedly, tyrosine phosphorylation of MAP kinase is unchanged in the nuclear fraction during ischaemia, indicating that unphosphorylated MAP kinase translocates from the cytosol to the nucleus. During reperfusion (0–30 min), after ischaemia for 20 min, tyrosine phosphorylation of MAP kinase in the

nuclear fraction is increased with a peak at 10 min of reperfusion. The activation is confirmed by MAP kinase activity with similar kinetics to the tyrosine phosphorylation. However, the amount of MAP kinase in the fraction is almost constant during reperfusion for 10 min. Although an upstream kinase for MAP kinase, MAP kinase/extracellular signal-regulated kinase kinase (MEK)-1, remains in the cytosol throughout ischaemia and reperfusion, MEK-2, another upstream kinase for MAP kinase, is constantly present in the nucleus as well as in the cytoplasm, based on analyses by fractionation and immunohistochemistry. Furthermore, MEK-2 activity in the nuclear fraction is rapidly increased during post-ischaemic reperfusion. These findings demonstrate that nuclear MAP kinase is activated by tyrosine phosphorylation during reperfusion, probably by MEK-2.

INTRODUCTION

In response to ischaemia and reperfusion, mammalian cells induce various pathways that lead to cell death and organ dysfunction. In the heart, prolonged ischaemia causes necrosis and contractile dysfunction, but following brief ischaemia the heart can recover from injury. Many genes are expressed in the heart during its recovery from ischaemia. In particular, the expression of immediate early genes (e.g. *c-fos*, *c-jun*, *Egr-1*) is rapidly up-regulated [1–3], and genes considered to be markers of the hypertrophic response (e.g. atrial natriuretic factor, transforming growth factor- β) are subsequently expressed during post-ischaemic reperfusion [4,5]. In clinical studies, subjects with ischaemic heart disease often experience hypertrophy [6]. Reperfusion after brief ischaemia may induce the gene expression that leads to myocardial hypertrophy. It was reported that this hypertrophy occurs through mitogen-activated protein (MAP) kinase activation caused by mitogenic stimuli such as angiotensin II, endothelin-1 or mechanical stretch [7–10]. These findings suggest that MAP kinase may be involved in the genetic responses in ischaemic heart disease. Pombo et al. reported that post-ischaemic reperfusion in rat kidney induces MAP kinase activation and stress-activated protein kinase (SAPK) activation [11]. A more recent report demonstrated that MAP kinase is activated in cardiomyocytes during the recovery from metabolic inhibition [12]. However, the mechanism of MAP kinase activation during ischaemia and reperfusion remains largely unknown.

MAP kinase is a serine/threonine protein kinase whose activity is up-regulated through phosphorylation on tyrosine and threonine residues by MAP kinase/extracellular signal-regulated kinase kinases (MEKs) [13–16]. MEKs are substrates for Raf-1

[17,18], which has been reported to be activated through either receptors involved in Ras or a protein kinase C (PKC)-dependent pathway [19,20]. These MAP kinase activators cause its translocation from the cytosol to the nucleus, where transcription factors such as c-Jun [21], Elk-1 [22] and c-Myc [23] are substrates for MAP kinase. This indicates that MAP kinase serves as an important regulator of nuclear transcriptional activity. Although increasing interest has been paid to the role of MAP kinase in the nucleus, the nuclear translocation of MAP kinase remains poorly elucidated.

In the present study, we investigated the mechanism of MAP kinase activation during ischaemia and reperfusion with special interest in the relationship between nuclear translocation and the activation of MAP kinase.

MATERIALS AND METHODS

Materials

Sodium orthovanadate, microcystin, PMA and PMSF were obtained from Sigma. Anti-(MAP kinase) antibody, anti-MEK-2 antibody, Protein A/G-agarose and p42^{MAPK} (the 42 kDa isotype of MAP kinase) were bought from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), anti-phosphotyrosine antibody from Biomakor, anti-(histone H1) antibody from Leinco Technologies, anti-(phospho-MAP kinase) antibody, anti-phospho-Elk-1 antibody, phospho-MAP kinase as control protein and MAP kinase assay kit from New England Biolabs (Beverly, MA, U.S.A.) and anti-MEK-1 antibody from Upstate Biotechnology (Lake Placid, NY, U.S.A.). An enhanced chemiluminescence reaction kit assay and anti-mouse and anti-rabbit IgG antibodies coupled to peroxidase were obtained from

Abbreviations used: MAP, mitogen-activated protein; MEK, MAP kinase/extracellular signal-regulated kinase kinase; p44^{MAPK} and p42^{MAPK}, the 44 kDa and 42 kDa isotypes of MAP kinase; SAPK/JNK, stress-activated protein kinase/Jun N-terminal kinase; PKC, protein kinase C.

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Amersham, the Vectastain kit was from Vector Laboratories (Burlingame, CA, U.S.A.) and the biotin blocking system was from Dako (Carpenteria, CA, U.S.A.). All other chemicals were commercially available.

Perfusion protocol

Male Wistar rats weighing approx. 200 g were anaesthetized with sodium pentobarbital. The aortas were cannulated and attached to a Langendorff apparatus. The hearts were perfused and then subjected to global ischaemia for 0–40 min and reperfusion for 0–30 min after ischaemia for 20 min at 37 °C, as previously described [24,25]. The hearts were quickly frozen in liquid nitrogen and stored at –70 °C for later biochemical analyses.

Subcellular fractionation

Subcellular fractionation was performed essentially as previously described [25,26] with minor modifications. The frozen hearts were minced and homogenized in 2 volumes of STE buffer [0.32 M sucrose/10 mM Tris/HCl (pH 7.4)/1 mM EGTA/2 mM EDTA/5 mM Na₂S₂O₃/10 mM β -mercaptoethanol/20 μ M leupeptin/0.15 μ M pepstatin A/0.2 mM PMSF/50 mM NaF/1 mM sodium orthovanadate/0.4 nM microcystin] with a Polytron homogenizer. The homogenates were mixed with 2 volumes of STE buffer and centrifuged (1000 g, 10 min) to obtain the pellets. The pellet was washed once and suspended in STE buffer (nuclear fraction). The supernatant was centrifuged (100 000 g, 60 min) to obtain the cytosol fraction and the pellet, which was then suspended in STE buffer (membrane fraction). The nuclear fraction was solubilized in Triton buffer [1 % (v/v) Triton X-100/150 mM NaCl/10 mM Tris/HCl (pH 7.4)/1 mM EGTA/1 mM EDTA/0.2 mM sodium orthovanadate/20 μ M leupeptin A/0.2 mM PMSF/50 mM NaF/0.4 nM microcystin LR]. The fraction was centrifuged (15 000 g, 30 min, 4 °C), and the supernatant (nuclear extract) was stored at –70 °C.

Electrophoresis and immunoblotting

The subcellular fractions and molecular-mass standards were electrophoresed on 12.5 % (w/v) polyacrylamide gels in the presence of SDS and transferred to nitrocellulose membranes (0.45 μ m). The blots were blocked with 5 % non-fat dry milk (for MAP kinase, MEK-1, MEK-2, phospho-MAP kinase, phospho-Elk-1 and histone) or 10 % (w/v) BSA (for phosphotyrosine) in Tris-buffered saline containing 0.05 % (w/v) Tween-20, and incubated with antibody. After washing the blots, the antigens were visualized by enhanced chemiluminescent detection reagents.

Immunoprecipitation/kinase assay

MAP kinase activity was determined according to the instruction manual. Briefly, the fractions (600 μ g of protein) were lysed in 200 μ l of lysis buffer [1 % Triton X-100/20 mM Tris (pH 7.5)/150 mM NaCl/1 mM EDTA/1 mM EGTA/2.5 mM sodium pyrophosphate/1 mM β -glycerol phosphate/1 mM Na₃VO₄/leupeptin (1 μ g/ml)] and clarified by centrifugation at 15 000 g for 30 min at 4 °C. After normalization of protein content, the lysates were incubated with anti-(phospho-MAP kinase) antibody (for MAP kinase activity) or anti-MEK-2 antibody (for MEK-2 activity) followed by Protein A/G-agarose. The complexes were washed twice in lysis buffer and twice in kinase buffer [25 mM Tris (pH 7.5)/5 mM β -glycerol phos-

phate/2 mM dithiothreitol/0.1 mM Na₃VO₄/10 mM MgCl₂]. Kinase reactions were carried out by resuspending the complexes in 50 μ l of kinase buffer and 100 μ M ATP, and 1 μ g of Elk-1 (for MAP kinase activity) or 1.5 μ g of MAP kinase (for MEK-2 activity), and incubating for 30 min at 30 °C. The reaction products were electrophoresed on SDS/polyacrylamide (12.5 %) gels, transferred to PVDF membrane, and probed with anti-phospho-Elk-1 antibody (for MAP kinase activity) or anti-(phospho-MAP kinase) antibody (for MEK-2 activity).

Immunohistochemistry

Rat hearts were immersed in OCT compound (embedding medium; Miles) and rapidly frozen in liquid nitrogen. The specimens were fixed on glasses with PLP solution [0.02 M NaIO₄/PBS (pH 7.4)/2 % (w/v) paraformaldehyde] for 10 min at 4 °C and then incubated with 0.3 % (v/v) H₂O₂ in PBS for 30 min to quench the endogenous peroxidase activity. The specimens were blocked with 1.5 % (v/v) normal serum in PBS, then incubated with anti-(MAP kinase) antibody, anti-MEK-1 antibody, anti-MEK-2 antibody or anti-histone antibody in 1 % BSA in PBS for 1 h, and immunostained by the avidin–biotin peroxidase complex method using a Vectastain kit. The peroxidase label was visualized by exposing the sections to diaminobenzidine.

RESULTS

Nuclear translocation and tyrosine phosphorylation of MAP kinase during ischaemia

To investigate the subcellular localization of MAP kinase during ischaemia, we carried out the fractionation of perfused rat hearts. Electron microscopic observation showed that the 1000 g pellet (nuclear fraction) contained the nuclei and myofibrils, and that the 100 000 g pellet (membrane fraction) included membrane vesicles and mitochondria (results not shown). Histone H1, a nuclear protein, was mainly localized in the nuclear fraction [86.1 ($\pm$ 2.0) %, mean $\pm$ S.E., n = 8; Figure 1B], and the recovery remained almost constant throughout ischaemia and reperfusion (results not shown). The fractions were analysed by immunoblotting using a specific antibody against MAP kinase isoforms [p44^{MAPK} (the 44 kDa isotype of MAP kinase) and p42^{MAPK}]. In untreated hearts, MAP kinase predominated in the cytosol fraction [p44^{MAPK}, 82 ($\pm$ 7) % and p42^{MAPK}, 75 ($\pm$ 5) %], and the nuclear fraction contained only 3.5 ($\pm$ 0.9) % of p44^{MAPK} and 12 ($\pm$ 2.5) % of p42^{MAPK}. The amount of MAP kinase in the cytosol fraction was significantly lower after 40 min of ischaemia, with a concomitant increase in p44^{MAPK} [25 ($\pm$ 3.7) %] and p42^{MAPK} [34 ($\pm$ 2.4) %] in the nuclear fraction. MAP kinase in the membrane fraction remained at control levels during ischaemia (0–40 min). The sum of MAP kinase in the three fractions was almost unchanged during ischaemia (Figures 1A, 1D and 1E). These observations indicate that MAP kinase translocates from the cytosol fraction to the nuclear fraction in a time-dependent manner. Immunohistochemical observation using anti-(MAP kinase) antibody showed that MAP kinase was mainly localized in the cytosol and partially in the nucleus, and that nuclear staining was enhanced after 40 min of ischaemia (Figures 2A and 2B). The staining of MAP kinase was blocked by preincubation with the immunizing peptide, and by only secondary antibody (results not shown). This, taken together with the immunoblotting and immunohistochemical analyses, shows that ischaemia induces the translocation of MAP kinase from the cytosol to the nucleus. An upstream kinase for MAP kinase, MEK-1, remains strictly in the cytosol fraction during ischaemia

Figure 2 Immunohistochemical localization of MAP kinase in ischaemic heart

Hearts were subjected to ischaemia for 0 min (**A**) or 40 min (**B**), and then cryosectioned, fixed with PLP solution, and stained with anti-(MAP kinase) antibody (**A**) and (**B**), anti-histone antibody (**C**), or haematoxylin and eosin (**D**) as described in the Materials and methods section. The Figure shows representative photographs from three independent experiments. Bar represents 2 μ m.

Figure 3 Tyrosine phosphorylation of MAP kinase in the nuclear fraction of rat heart during ischaemia

Nuclear fractions (40 μ g of protein) were prepared from hearts after ischaemia for 0–40 min and subjected to immunoblotting with anti-(phospho-MAP kinase) antibody. Purified p42^{MAPK} was used as the negative control and phosphorylated p42^{MAPK} by MEK-2 was used as the positive control. The Figure shows a representative immunoblot from three independent experiments.

(0–40 min, Figure 1C), indicating that the ischaemia-induced translocation of MAP kinase is not due to a non-specific translocation of protein kinases. MAP kinase has previously been shown to require tyrosine phosphorylation for its activation [28]. To determine whether the nuclear MAP kinase is phosphorylated, we carried out immunoblotting with anti-(phospho-MAP kinase) antibody in the nuclear fraction. The tyrosine phosphorylation of MAP kinase was unchanged in the fraction during ischaemia (Figure 3). It has also been reported that the electrophoretic mobility of MAP kinase is decreased by its phosphorylation and activation [29]. Consistent with the results on the tyrosine phosphorylation, no mobility shift of

Figure 1 Subcellular localization of MAP kinase in ischaemic heart

Subcellular fractions were prepared from hearts after ischaemia for 0–40 min, and subjected to immunoblotting with anti-(MAP kinase) antibody (**A**), anti-(histone H1) antibody (**B**), and anti-MEK-1 antibody (**C**). N, nuclear; M, membrane; C, cytosol. (**A**)–(**C**) show representative immunoblots of three independent experiments and (**D**) and (**E**) show the amounts of p44^{MAPK} (**D**) and p42^{MAPK} (**E**) [(%) sum of nuclear (○), membrane (●), and cytosol (△) fractions of control heart, $n = 3$] densitometrically determined from the immunoblots with anti-(MAP kinase) antibody (* $P < 0.05$ versus control, mean $\pm$ S.E.).

Figure 4 Phosphorylation of MAP kinase in the nuclear fraction of rat heart during post-ischaemic reperfusion

Nuclear fractions (40 μ g of protein) were prepared from hearts undergoing reperfusion for 0–30 min after ischaemia for 20 min, and subjected to immunoblotting with anti-(phospho-MAP kinase) antibody. The Figure shows a representative immunoblot from three independent experiments.

nuclear MAP kinase was detectable during ischaemia (0–40 min), which was in contrast with the shift observed after treatment with 1 μ M PMA (results not shown). These findings indicate that phosphorylation of MAP kinase is not required for its nuclear translocation during ischaemia.

Activation of nuclear MAP kinase during post-ischaemic reperfusion

Rats were subjected to reperfusion for 0–30 min after global ischaemia for 20 min. MAP kinase activation in the nuclear fraction during post-ischaemic reperfusion was initially examined by immunoblotting using anti-(phospho-MAP kinase) antibody. Post-ischaemic reperfusion induced the tyrosine phosphorylation of p42^{MAPK}. The level of tyrosine phosphorylation reached a maximum at 10 min reperfusion (Figure 4). A mobility shift of the MAP kinase in the nuclear fraction was observed at 10 min reperfusion, and correlated with the tyrosine phosphorylation (results not shown). To confirm the tyrosine phosphorylation of MAP kinase, we carried out immunoprecipitation of the nuclear fraction using anti-(MAP kinase) antibody. The immunoprecipitates obtained showed that p42^{MAPK} is tyrosine phosphorylated at 10 min reperfusion (results not shown). MAP kinase activity in each fraction was estimated as described in the Materials and methods section. The magnitude and kinetics of the activity in the nuclear fraction were consistent with those for MAP kinase phosphorylation during ischaemia and reperfusion (Figures 5A and 5B). However, the amount of MAP kinase in the nuclear fraction remained almost constant during 10 min of reperfusion (results not shown). Although MAP kinase activity in the membrane fraction was increased during reperfusion, the activity in the cytosol fraction was not detectable throughout ischaemia and reperfusion under the conditions used in this study (results not shown). These findings indicate that nuclear MAP kinase is activated by tyrosine phosphorylation during reperfusion. During ischaemia and reperfusion, MEK-1 was localized in the cytosol fraction and was not detectable in the nuclear fraction (Figure 6A). However, another upstream kinase for MAP kinase, MEK-2, was constantly present not only in the cytosol fraction but also in the nuclear fraction, based on immunoblotting analysis (Figure 6B). Immunohistochemical observations also show that MEK-2 was found primarily in the nucleus as well as in the cytoplasm, although MEK-1 was predominantly in the cytoplasm (Figures 7A and 7B). The staining of MEK-2 was blocked by preincubation with the immunizing peptide, and no stains were observed by incubating with only secondary antibody (results not shown). The findings indicate that MEK-2 is partially present in the nucleus as well as the cytoplasm. MEK-2 activity in the nuclear fraction was rapidly

Figure 5 MAP kinase activity in the nuclear fraction of rat heart during post-ischaemic reperfusion

Nuclear fractions (600 μ g) were prepared from hearts undergoing reperfusion for 0–30 min after ischaemia for 20 min, and subjected to immunoprecipitation with anti-(phospho-MAP kinase) antibody followed by Protein A/G-agarose. The complexes were incubated with 100 μ M ATP and 1.0 μ g of Elk-1, and subjected to immunoblotting with anti-phospho-Elk-1 antibody as described in the Materials and methods section (A). (B) p42^{MAPK} activity densitometrically determined from the immunoblots with anti-phospho-Elk-1 antibody (control %, mean $\pm$ S.E., $n = 3$; * $P < 0.05$ versus control) as described in the Materials and methods section.

increased during post-ischaemic reperfusion (Figure 8), suggesting that MEK-2 may be involved in nuclear MAP kinase activation during reperfusion.

DISCUSSION

In this report, we investigated the nuclear translocation and activation of MAP kinase during ischaemia and reperfusion. During the study we demonstrated that ischaemia induces the nuclear translocation of unphosphorylated MAP kinase, which is then activated during reperfusion by tyrosine phosphorylation, probably by MEK-2, an upstream kinase for MAP kinase.

It is generally thought that growth factors induce the phosphorylation of MAP kinase by upstream kinases, MEK-1 and MEK-2, which leads to the translocation of MAP kinase to the nucleus and membrane [30]. However, as shown in this study, the nuclear translocation of MAP kinase is independent of its phosphorylation under pathogenic conditions. It has previously been reported that MAP kinase mutants (T-192A and Y-194F) that lack MAP kinase activity undergo nuclear translocation like wild-type MAP kinase [31,32]; these data are consistent with our findings. The nuclear translocation of unphosphorylated MAP kinase implies that the nuclear membrane, like the plasma membrane, may have a signalling pathway for MAP kinase activation. MEK-2 is constitutively present in the nuclear fraction, where MAP kinase translocates during ischaemia. Several studies have shown the nuclear transfer of MAP kinase cascades (e.g. Raf-1, PKC isoenzymes) other than

Figure 6 Subcellular localization of MEK-1 and MEK-2 in rat heart during post-ischaemic reperfusion

Subcellular fractions were prepared from hearts undergoing reperfusion for 0–30 min following ischaemia for 20 min, and subjected to immunoblotting with anti-MEK-1 antibody (**A**) or anti-MEK-2 antibody (**B**). N, nuclear; M, membrane; C, cytosol. The Figure shows representative immunoblots from three independent experiments.

Figure 7 Immunohistochemical localization of MEK-1 and MEK-2 in rat heart

Hearts were cryosectioned, fixed with PLP solution and stained with anti-MEK-1 antibody (**A**) or anti-MEK-2 antibody (**B**) as described in the Materials and methods section. The Figure shows representative photographs from three independent experiments. Bar represents 2 μ m.

Figure 8 MEK-2 activity in the nuclear fraction of rat heart during post-ischaemic reperfusion

Nuclear fractions (600 μ g) were prepared from hearts undergoing reperfusion for 0–30 min after ischaemia for 20 min, and subjected to immunoprecipitation with anti-MEK-2 antibody followed by Protein A/G–agarose. The complexes were incubated with 100 μ M ATP and 1.5 μ g of p42^{MAPK}, and subjected to immunoblotting with anti-(phospho-MAP kinase) antibody as described in the Materials and methods section. The Figure shows a representative immunoblot from two independent experiments.

MEKs in response to stimulation, but their physiological role is presently unknown [33–35].

During reperfusion after ischaemia, nuclear MAP kinase might be activated. First, MAP kinase activation in the nuclear fraction increased significantly with a peak at 10 min of reperfusion, but the amount of MAP kinase in the fraction remained nearly throughout the same time course. Secondly, MEK-2 activity in

the nuclear fraction was increased during reperfusion, which was consistent with MAP kinase activation. Finally, immunohistochemical observation using anti-(MAP kinase) antibody showed nuclear staining, but no staining in the myofibrils. Recently, a MAP kinase superfamily, SAPK/Jun N-terminal kinase (JNK), in the nucleus was reported to be activated without translocation by γ -radiation, which produces oxygen radicals upon reaction with water [36]. During reperfusion, oxygen free radicals are produced by xanthine oxidase in the heart [37], and the radicals can activate MAP kinase [38]. Therefore the radicals generated during reperfusion may be implicated in MAP kinase activation in the nucleus.

It is well known that cytosolic Ca^{2+} levels increase during ischaemia and reperfusion. Cytosolic and nuclear Ca^{2+} concentrations rapidly equilibrate in the cell, since the nuclear envelope is very permeable to Ca^{2+} [39]. The rise in cellular Ca^{2+} triggers cellular responses through the activation of various enzymes such as PKC [40–42], calmodulin-dependent protein kinase II [43], calpain [44] and nitric oxide synthase [45]. MAP kinase is also reported to be activated by an increase in cellular Ca^{2+} in response to angiotensin II [7]. However, reperfusion with a low- Ca^{2+} buffer does not significantly affect MAP kinase phosphorylation, suggesting that Ca^{2+} influx is not involved in MAP kinase activation during ischaemia and reperfusion (Y. Mizukami and K. Yoshida, unpublished work).

There is much evidence that nuclear MAP kinase may be involved in transcriptional regulation. MAP kinase in the nucleus can phosphorylate transcription factor p62^{TCF} at the *c-fos* promoter, leading to *c-fos* induction [46]. Members of the MAP kinase-related family, SAPK/JNK, can phosphorylate c-Jun and enhance its transcriptional activity [47]. During post-ischaemic reperfusion, SAPK/JNK was also activated, and then mRNA expression for *c-fos* and *c-jun* was strongly enhanced (Y. Mizukami, K. Yoshioka and K. Yoshida, unpublished work). Therefore we speculate that c-Fos and c-Jun synergistically activate the function of AP-1 complex [48,49] during reperfusion, and this may induce the gene expression of contractile proteins (e.g. skeletal α -actin, myosin heavy chain) having an AP-1 element in their promoter regions [50,51]. Such gene expression may have relevance to the tissue repair and pathogenic processes that follow ischaemia. Clearly, further investigations are required to clarify the pathophysiological role of MAP kinase in the nucleus.

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