Contribution of Macrophage Migration Inhibitory Factor to Extracellular Signal-regulated Kinase Activation by Oxidative Stress in Cardiomyocytes

Jun Fukuzawa†‡, Jun Nishihira§, Naoyuki Hasebe†, Takashi Haneda‡, Junzo Osaki‡, Tetsuya Saito‡, Tomoaki Nomura‡, Takayuki Fujino**, Nobutaka Wakamiya‡‡, and Kenjiro Kikuchi‡

From the †First Department of Medicine, ‡Department of Pharmacology, and ‡‡Department of Microbiology, Asahikawa Medical College, 2-1-1 Midorigaoka-Higashi, Asahikawa 078-8510, Japan; the ¶Central Research Institute, Hokkaido University School of Medicine, Kita 15 Nishi 7 Kita-ku Sapporo 060-8638, Japan, and the §§Nemuro Municipal Hospital, 1-2 Ariso, Nemuro 087-8686, Japan

In response to oxidative stress, the pathogenesis of a number of cardiovascular events and several genes are stimulated by extracellular signal-regulated kinases (ERK1/2). Biphasic (early, 10 min; and delayed, 120 min) ERK1/2 activation by H₂O₂, a reactive oxygen species, was observed in cultured neonatal rat cardiomyocytes. We investigated the hypothesis that the delayed activation of ERK1/2 depends on a factor secreted by oxidative stress (FSO). The delayed activation was inhibited by calphostin C, a protein kinase C inhibitor. Conditioned medium (CM) obtained from cells stimulated with H₂O₂ induced rapid and monophasic ERK1/2 activation, which was not inhibited by calphostin C. In contrast, calphostin C-pretreated CM did not activate ERK1/2. Macrophage migration inhibitory factor (MIF) was one of the candidate FSOs activating ERK1/2. The existence of MIF in CM, the recombinant MIF-stimulated ERK1/2 rapid activation, and anti-MIF neutralizing antibody-induced inhibition of the delayed activation implied that MIF could be the FSO. Pretreatment of cardiomyocytes with a mitogen-activated protein kinase/ERK kinase (MEK) inhibitor did not suppress the MIF secretion, although it prevented the ERK1/2 activation by H₂O₂. These results indicate that MIF is secreted from cardiomyocytes as a result of oxidative stress and activates ERK1/2 through a MEK1/2-dependent mechanism, although the secretion is not regulated by ERK1/2 but by protein kinase C.

Oxidative stress is important in the pathogenesis of ischemic/reperfusion injury (1, 2), apoptosis (3, 4), and hypertrophy in cardiomyocytes (5, 6). In response to reactive oxygen species (ROS), 1 transcription of several genes is activated through some mechanisms in which association with stimulated protein kinases is included (7). Mitogen-activated protein kinases (MAPKs), which are serine/threonine protein kinases, regulate gene promoter activity and play an important role in apoptosis and cell growth (8, 9). The targets for extracellular signal-regulated kinase (ERK1/2), a classical molecule in the MAPK family (10), are nuclear transcription factors, metabolic enzymes, cytoskeletal proteins, and other signaling components (11). ERK1/2 acts as a modulator of many aspects of cellular function and is acutely stimulated by growth and differentiation factors, including oxidative stress in pathways mediated by receptor tyrosine kinase, G protein-coupled receptors, or cytokine receptors (11). However, the number and nature of the mechanisms leading to activation of ERK1/2 evoked by oxidative stress, that is, redox-sensitive regulation in cardiomyocytes, are poorly understood at present. Some reports have shown that ERK1/2 is activated bidirectionally not only by basic fibroblast growth factor and nerve growth factor but also by hydrogen peroxide (H₂O₂) and ROS generators such as LY83583 in vascular smooth muscle cells (12–14). The mechanism of late phase activation of ERK1/2 by oxidative stress has not been well examined, although early phase activation of ERK1/2 by oxidative stress has been well studied (15). One of the possible mechanisms of the late-phase activation of ERK1/2 by H₂O₂ is autocrine and paracrine secretion of the factors activating ERK1/2 (14).

Macrophage migration inhibitory factor (MIF), initially identified as a soluble factor derived from activated T lymphocytes, is a cytokine that plays a critical role in several inflammatory conditions by regulating the activation of macrophages and T cells (16–19), although its precise biological function remains unclear (20). However, after cloning of the MIF cDNA, previously unrecognized endocrine and enzymatic functions of MIF were revealed. It also acts as a proinflammatory cytokine produced by macrophages in response to a variety of inflammatory stimuli and as a factor stimulating cell growth in many cell types (18, 21). The responses of cardiovascular systems to MIF have not been reported except for mRNA and protein expression of MIF in the atherosclerotic region in a hypercholesterolemic model of rabbit vessels (22). Recently, increased serum concentrations of MIF have been reported in patients with acute myocardial infarction in which reperfusion injury may be involved (23).

We hypothesized that cardiomyocytes could secrete factors increasing ERK1/2 activity in response to ROS and that MIF could be an important secreted ROS-induced factor in cardiomyocytes. To explore this hypothesis, we used cultured cardiomyocytes from neonatal rat to examine whether H₂O₂-induced MIF secretion in cardiomyocytes and to investigate the
mechanism and the relationship between MIF and H$_2$O$_2$-induced ERK1/2 activation.

**MATERIALS AND METHODS**

Reagents and Antibodies—Reagents for tissue culture were obtained from Invitrogen. Polyclonal antibodies against phosphospecific and total ERK1/2 and phosphospecific and total MEK1 or 1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059, geldanamycin, and calphostin C were purchased from Calbiochem. Rat recombinant MIF was expressed in *Escherichia coli* and purified to homogeneity as described previously (24). A polyclonal anti-rat MIF antibody was generated by immunizing New Zealand White rabbits with recombinant rat MIF as reported previously (25).

Isolation and Culture of Neonatal Rat Ventricular Cardiomyocytes—Cardiomyocytes were isolated from 1–2-day-old Sprague-Dawley rat ventricles by an enzymatic method as described previously (26–28). Twenty-four hours after isolation, serum-containing medium was changed to Dulbecco's modified Eagle's medium/F-12 with a serum substitute as described previously (29). Twenty-four hours before experiments, cells were given the same medium without the serum substitute. Using these methods, cultures that contained 90–95% myocytes were obtained (26).

Immunoblot Analysis—Cardiomyocytes were lysed after each stimulation with a buffer as described previously (30). The lysed samples were subjected to Tris-glycine-SDS-PAGE (5–10%). After separation by electrophoresis, samples were transferred to nitrocellulose membranes (Amersham Biosciences) and subjected to immunoblot analysis using each indicated antibody (30). Signals were visualized with enhanced chemiluminescence (PerkinElmer Life Sciences). For detection of MIF, the samples were subjected to Tri-Tricine SDS-PAGE as described previously (31). In each experiment, three independent analyses were performed to confirm the reproducibility.

Preparation of Conditioned Medium—Conditioned medium was collected and concentrated according to a method described elsewhere (14). Briefly, cells were washed three times with Hank's balanced salt solution (NaCl 130 mM, KCl 5 mM, CaCl$_2$ 1.5 mM, MgCl$_2$ 1 mM, HEPES 20 mM, pH 7.4) and were equilibrated for 24 h. H$_2$O$_2$ (1 mM) was added to the Hank's balanced salt solution pretreated with or without calphostin C (1 μM) or PD98059 (50 μM) or control medium from the cells unstimulated with H$_2$O$_2$, were collected and centrifuged to remove the debris at 800 × g at 4 °C for 10 min. The conditioned medium pretreated with or without calphostin C or PD98059 and the control medium were concentrated 100-fold by using a Centricon™ centrifugal filter device (Millipore, Bedford, MA).

Measurement of Hydrogen Peroxide Concentration—The concentration (degradation) of the exogenously added H$_2$O$_2$ in the medium with or without cells was measured with a Bioxytech H$_2$O$_2$-560™ (Oxis International Inc., Portland, OR) according to the manufacturer's protocol.

**RESULTS**

H$_2$O$_2$-induced ERK1/2 Activation—Exposure of cardiomyocytes to H$_2$O$_2$ (1 mM) stimulated ERK1/2 phosphorylation (activation) with a peak at 10 min. The ERK1/2 activity returned to the baseline level at 45–60 min. After returning to the base line, a second peak of ERK1/2 activation appeared at 120 min (Fig. 1A). The concentration of H$_2$O$_2$ that was added exogenously to the medium decreased exponentially and returned to the basal (zero) level within 90 min after the exogenous administration (Fig. 1B). Concentration dependences of H$_2$O$_2$ in the early and late phases of ERK1/2 activation occurred in different manners (Fig. 1C). To evaluate whether H$_2$O$_2$-induced ERK1/2 activation was mediated via an ROS-dependent...
or -independent mechanism, cells were treated with the anti-oxidant reagent, catalase (3000 units/ml) before exposure to H2O2 (1 mM). Catalase inhibited both the early and late phases of ERK1/2 activation in response to H2O2 (Fig. 1D).

Roles of MEK, Raf-1, and PKC in H2O2-induced ERK Activation—To assess the upstream mechanism of H2O2-induced ERK1/2 activation, cardiomyocytes were pretreated with agents that inhibit MAPK/ERK kinase (MEK), Raf-1, or protein kinase C (PKC) before exposure to H2O2. The addition of PD98059 (50 \mu M), an inhibitor of MEK, decreased both the early and late phases of ERK1/2 activation in response to H2O2 (Fig. 2A). The activity of MEK1/2 was also up-regulated biphasically by H2O2 administration into cultured medium as was that of ERK1/2 (Fig. 2B). Geldanamycin (2 \mu M), which can bind to HSP-90 and disrupts the Raf-1-HSP90 multimolecular complex leading to destabilization of Raf-1 (32), also inhibited both peaks (Fig. 2C). However, calphostin C (1 \mu M), a specific inhibitor of protein kinase C, diminished only the delayed activation of ERK1/2 by H2O2 (Fig. 2D).

Effect of Conditioned Medium Prepared by Exposing Cardiomyocytes to H2O2 on ERK Activation—Conditioned medium obtained from the cells exposed to 1 mM H2O2 (CM−) stimulated ERK1/2 activation rapidly and monophonically (Fig. 3A), whereas H2O2-unstimulated control medium (CM+) did not stimulate ERK1/2 activation (Fig. 3B). The conditioned medium was fractionated on a molecular weight basis using a commercially available filter device. Adding each fraction to the medium stimulated ERK1/2 activation in cardiomyocytes. CM10–30, CM50, and CM30–50, to a lesser extent, could stimulate ERK1/2 activation (Fig. 3C). In contrast to the H2O2-stimulated cells, catalase did not inhibit ERK1/2 activation in cells stimulated with CM− (Fig. 3D). Calphostin C-pretreated conditioned medium obtained from the cells stimulated with H2O2 (1 mM) showed suppressed ERK1/2 activation (Fig. 4A). In contrast, the conditioned medium-stimulated rapid and monophonical activation of ERK1/2 was not inhibited by pretreatment with calphostin C (1 \mu M) (Fig. 4B).

H2O2-induced MIF Secretion—The MIF in the conditioned medium was detected by Tris-Tricine SDS-PAGE and immunoblotting, whereas no MIF was detectable in the control medium or mock-stimulated medium (Fig. 5A). To confirm the effect of ERK1/2 on MIF secretion induced by H2O2, PD98059 (50 \mu M)-pretreated conditioned medium was analyzed. PD98059 did not inhibit H2O2-induced MIF secretion from cardiomyocytes. However, protein kinase C inhibition by calphostin C (1 \mu M) inhibited H2O2-induced MIF secretion. MIF concentration in the conditioned medium increased from 90 min after H2O2 stimulation (Fig. 5B). By contrast, MIF was not secreted in the calphostin C-pretreated conditioned medium. To determine whether the H2O2-induced MIF secretion was controlled via a regulatory or constitutive mechanism (33), the changes in the MIF concentration in cells stimulated with H2O2 (1 mM) were determined by using whole cell lysate. Intracellular MIF contents were decreased at 90 and 120 min after stimulation (Fig. 5C).

Effect of MIF and Anti-MIF Antibody on ERK1/2 Activation—Cells stimulated with a high concentration of recombinant MIF showed rapid and monophonical activation of ERK1/2-like conditioned medium-stimulated cells (Fig. 6A). We also determined the effect of an anti-MIF antibody on H2O2-induced ERK activation. Anti-MIF antibody (1:1000) pretreatment inhibited the late phase activation of ERK1/2, whereas pretreatment of the cells with preimmune serum did not decrease the H2O2-induced late phase activation (Fig. 6B). In each ERK1/2 activation stimulated with the conditioned medium fractionated by the centrifugal filtration system, CM30–50-induced...
ERK1/2 activation was completely inhibited by the anti-MIF antibody, and the one stimulated with CM10–30 was partially inhibited (Fig. 3C).

**DISCUSSION**

The MAPK signaling pathway plays a pivotal role in the mediation of cellular responses to a variety of signaling molecules. ERK1/2 regulates cell growth, embryonic development, cell survival (9), and cell differentiation in different cell types through the stimulation of specific gene expression. The signaling pathway for ERK1/2 activation includes intracellular calcium, heterotrimeric G proteins, protein kinase C, JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathways, and phosphatidylinositol 3-kinase. In cardiomyocytes, the Src/Ras/Raf-1 cascade has also been reported as a pathway for the redox-sensitive regulation of ERK1/2 activation.
the early phase (15). In the present study, we observed that exogenously supplied H$_2$O$_2$ (1 mM) caused biphasic activation of ERK1/2, which was inhibited by catalase, an antioxidant reagent, in cultured cardiomyocytes from neonatal rats. The mechanism of this phenomenon was inferred to be ROS-dependent. Pharmacological blockade of the Raf-1/MEK cascade suppressed the activation in both phases. On the other hand, inhibition of the PKC pathway by calphostin C activation in various cells has already been reported. Like other PKC, cardiomyocytes were stimulated with H$_2$O$_2$ for the indicated times, separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the anti-MIF antibody as described under “Materials and Methods.”

It was obvious that stimulation by regeneration of H$_2$O$_2$ could be excluded as a mechanism for the late phase activation of ERK1/2 by H$_2$O$_2$. The concentration of the exogenously applied H$_2$O$_2$ in the medium decreased exponentially in the presence of the cells (Fig. 1B). The phenomenon of biphasic ERK1/2 activation in various cells has already been reported. Like H$_2$O$_2$, basic fibroblast growth factor, nerve growth factor, and ROS generators such as LY83583 were reported to stimulate the biphasic ERK1/2 activation (12–14). The Ras/Raf/MEK cascade is known as an ERK1/2 activation system. In the present study, Raf-1 and MEK were shown to be upper signaling cascades for both phases of the ERK1/2 activation. Because geldanamycin, which was used as an inhibitor of Raf-1 in the present experiments, could function as an inhibitor of HSP-90 (32), biphasic activation of ERK1/2 with H$_2$O$_2$ could also be explained by an HSP-90-related mechanism. Further examination will be needed to confirm whether the phenomenon is HSP-90-dependent. We have demonstrated that the late phase activation of ERK1/2 is PKC-dependent. Although PKC is also known as a Raf-1 stimulator followed by ERK1/2 activation, the early phase activation of ERK1/2 by H$_2$O$_2$ was not mediated through this cascade in the present experiments. This indicated that the mechanisms of H$_2$O$_2$-induced ERK1/2 activation were different in the early and late phases. This finding was supported by the differences in H$_2$O$_2$ concentration dependence of ERK1/2 activation between the early and late phases.

PKC plays important roles in many cellular responses in various types of cells. These responses include contraction, migration, hypertrophy, proliferation, apoptosis, and secretion (34). In addition, lung surfactant phospholipid from alveolar type II cells is secreted via a conventional type PKC (PKC-α and -β) dependent mechanism (35). Considering these reports, we hypothesized that the mechanism of the PKC-dependent late phase activation of ERK1/2 by H$_2$O$_2$ could be explained by a factor secreted by oxidative stress (FSO). To examine this hypothesis, we examined the effect of conditioned medium derived from the cells stimulated with H$_2$O$_2$ (1 mM) for 120 min on ERK1/2 activation in cardiomyocytes. As demonstrated in Fig. 3, concentrated control medium obtained from the cells unstimulated with H$_2$O$_2$ did not activate ERK1/2 in cardiomyocytes. In contrast, the concentrated conditioned medium rapidly and monophasically activated the ERK1/2. These facts confirmed the hypothesis that a secreted factor plays a key role in the H$_2$O$_2$-induced late phase activation of ERK1/2 in cardiomyocytes. Catalase, which abrogated H$_2$O$_2$-induced ERK activation, did not inhibit the rapid and monophasic activation of ERK1/2 induced by conditioned medium. This suggested that the mechanism of the conditioned medium-induced ERK1/2 activation was independent of ROS. The decay of H$_2$O$_2$ shown in Fig. 1 indicated that the mechanism of the residual H$_2$O$_2$ in...
the medium was excluded for the rapid and monophasic activation of ERK1/2 by conditioned medium. Conditioned medium pretreated with calphostin C did not activate ERK1/2 in cardiomyocytes, as demonstrated in Fig. 4. This, along with the result that the late phase activation of ERK1/2 by H$_2$O$_2$ was PKC-dependent, indicated that the mechanism of the oxidative stress-induced secretion was PKC-dependent.

In the present study, we demonstrated that the ERK1/2 activation in conditioned medium could be fractionated into various molecular weight ranges and that each fraction induced ERK1/2 activation to some extent. This indicated that some secreted factors were responsible for the late phase activation. By means of a literature search (36), we examined which growth factors or cytokines were activated by oxidative stress, including ischemia and hypoxia, and stimulated ERK1/2 activation. Considering the molecular weight, MIF could be one of the candidate molecules that activate ERK1/2. The molecular weight of MIF is 13,000; it forms oligomers, especially trimers, as confirmed by crystal structure analysis (37–39) and cross-linking studies (40, 41). It has been reported to stimulate ERK1/2 in fibroblasts via a protein kinase A-dependent pathway (42). MIF was originally described as a T-cell-derived cytokine that inhibits macrophage migration in vitro and promotes macrophage accumulation in the delayed-type hypersensitivity reaction (16, 17). As shown in Fig. 6, we observed that neutralizing antibodies against MIF partially inhibited the late phase activation of ERK1/2 by H$_2$O$_2$ and that recombinant MIF induced ERK1/2 activation in a manner similar to the ERK1/2 activation induced by conditioned medium. The medium fraction of CM30–50 (containing major parts of the MIF trimer)-induced ERK1/2 activation was completely inhibited and that of CM10–30 (containing a possible MIF monomer or dimer) was partially inhibited by the anti-MIF antibody. These results suggested that MIF can act as a monomer or oligomer and that some other factors, induced by H$_2$O$_2$ and increased ERK1/2 activation, existed in the CM10–30. These results indicated that MIF, either alone or with one or more other factors, could be the FSO.

Factors secreted in response to oxidative stress mediate production of the oxidative stress-induced growth factor in several cell types (43). Although different growth factors are involved depending on the type of tissue as well as the type of oxidative stress, it is obvious that responses in an autocrine/paracrine manner could be a common mechanism utilized in oxidative stress-induced signal transduction. MIF mRNA expression in cardiomyocytes was up-regulated 6 h after H$_2$O$_2$ stimulation (44), and the concentration of MIF in the cardiomyocytes decreased after H$_2$O$_2$ stimulation. It is likely that myocytes utilize the regulated type (preformed MIF within cells) but not the constitutive type of secretion for oxidative stress-induced MIF secretion, like atrial natriuretic factor secretion in response to endothelin (45). This suggests that oxidative stress causes secretion of preformed MIF, although an oxidative stress-induced increase in MIF production is likely to occur at a later stage. Several possibilities could be considered for the mechanism of MIF secretion induced by oxidative stress. First, oxidative stress activates signaling pathways such as that of PKC, Ca$^{2+}$, and small G protein (Rab), which may in turn stimulate growth factor secretion. The present work suggests this PKC-dependent explanation. Second, oxidative stress causes an alteration in myocyte sarclemma permeability, which indicates release of intracellular growth factors. Pacing-induced basic fibroblast growth factor release from adult rat ventricular cardiomyocytes accounts for this mechanism (46). Although the plasma MIF level increases in patients with acute myocardial infarction, it does not increase in patients with other ischemic conditions such as unstable angina pectoris (23). These data suggest that MIF is secreted from necrotic cardiomyocytes. Thus, these possibilities will have to be examined.

Next, the question arises as to whether linkage between the
early phase and late phase activation of ERK1/2 exists. PKC is known to stimulate Raf-1 followed by ERK1/2 in some types of cells (47), indicating that PKC signaling could be the upper signaling cascade for each phase of ERK1/2 activation. As shown in Fig. 4, a PKC inhibitor, calphostin C, inhibited neither conditioned medium-induced (that is, FSO-induced) ERK1/2 activation nor H2O2-induced early phase activation of ERK1/2, indicating that PKC was not the upper signaling cascade for either early or late phase ERK activation evoked by H2O2. A similar result for the early phase activation of ERK1/2 was reported previously (15). Because we used calphostin C, known as a pan-specific PKC inhibitor, in the present study as well as in a previous one (15), additional experiments using isotype-selective PKC inhibitor such as Go-6983 will be needed to determine which PKC isoforms contribute to the H2O2-induced MIF secretion. PD98059, an inhibitor of MEK, and geldanamycin, an inhibitor of Raf-1, had inhibitory effects on H2O2-induced ERK1/2 activation in both phases as demonstrated in Fig. 2, indicating that the Raf-1/MEK pathway was the upper signaling cascade for ERK1/2 activation in both phases. On the other hand, ERK1/2 did not play a major role in MIF secretion, which had been suggested by the finding that H2O2-stimulated conditioned medium pretreated with PD98059 did not affect the secretion of MIF from cardiomyocytes, as shown in Fig. 5. These data indicate that the early phase activation of ERK1/2 by oxidative stress is not required for the late phase activation of ERK1/2. For the secretion of MIF, PKC plays a key role. The present study indicated a novel role for MIF in the mediation of the oxidative stress-induced signaling cascade up-regulation in cardiomyocytes.

In summary, we have demonstrated that oxidative stress activates ERK1/2 biphasically in cardiomyocytes. A PKC-dependent mechanism contributes to the late phase activation of ERK1/2 by oxidative stress. Secretion of MIF from cardiomyocytes themselves, induced by oxidative stress, plays a key role in late phase activation of ERK1/2 through a MEK-dependent mechanism, although its secretion is not regulated by the MEK-ERK1/2 signaling cascade but by PKC. These phenomena provide important insights into the cellular response to oxidative stress.

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