Stimulation of “Stress-regulated” Mitogen-activated Protein Kinases (Stress-activated Protein Kinases/c-Jun N-terminal Kinases and p38-Mitogen-activated Protein Kinases) in Perfused Rat Hearts by Oxidative and Other Stresses*

(Received for publication, July 28, 1997, and in revised form, December 22, 1997)

Angela Clerk‡§, Stephen J. Fuller‡¶, Ashour Michael‡, and Peter H. Sugden¶

From the National Heart and Lung Institute Division, Imperial College School of Medicine, Royal Brompton Campus, London SW3 6LY, United Kingdom and the Division of Biomedical Sciences, Imperial College School of Medicine, Charing Cross Campus, London W6 8RF, United Kingdom

“Stress-regulated” mitogen-activated protein kinases (SR-MAPKs) comprise the stress-activated protein kinases (SAPKs)/c-Jun N-terminal kinases (JNKs) and the p38-MAPKs. In the perfused heart, ischemia/reperfusion activates SR-MAPKs. Although the agent(s) directly responsible is unclear, reactive oxygen species are generated during ischemia/reperfusion. We have assessed the ability of oxidative stress (as exemplified by H2O2) to activate SR-MAPKs in the perfused heart and compared it with the effect of ischemia/reperfusion. H2O2 activated both SAPKs/JNKs and p38-MAPK. Maximal activation by H2O2 in both cases was observed at 0.5 mM. Whereas activation of p38-MAPK by H2O2 was comparable to that of ischemia and ischemia/reperfusion, activation of the SAPKs/JNKs was less than that of ischemia/reperfusion. As with ischemia/reperfusion, there was minimal activation of the ERK MAPK subfamily by H2O2. MAPK-activated protein kinase 2 (MAPKAPK2), a downstream substrate of p38-MAPKs, was activated by H2O2 to a similar extent as with ischemia or ischemia/reperfusion. In all instances, activation of MAPKAPK2 in perfused hearts was inhibited by SB203580, an inhibitor of p38-MAPKs. Perfusion of hearts at high aortic pressure (20 kilopascals) also activated the SR-MAPKs and MAPKAPK2. Free radical trapping agents (dimethyl sulfoxide and N-t-butyl-α-phenyl nitrone) inhibited the activation of SR-MAPKs and MAPKAPK2 by ischemia/reperfusion. These data are consistent with a role for reactive oxygen species in the activation of SR-MAPKs during ischemia/reperfusion.

Ischemic and hypertensive myocardial disease is currently a major cause of mortality and morbidity. In these conditions, the heart is exposed to numerous cell stresses including increased production of reactive oxygen species (ROS),† ionic imbalances, osmotic stress, mechanical stress, and metabolic deprivation (reviewed in Refs. 1–4). In numerous cell lines, primary cultures, and tissues, cellular stresses activate “stress-regulated” mitogen-activated protein kinases (SR-MAPKs) (reviewed in Refs. 5 and 6). There are two relatively well characterized SR-MAPK families, the stress-activated protein kinases (SAPKs) and the p38-MAPKs. SAPKs are alternatively known as the c-Jun N-terminal kinases (JNKs), although, strictly speaking, the JNK terminology applies to the human enzymes, whereas the SAPK terminology applies to the rat enzymes. Substrates for SAPKs/JNKs include the transcription factors c-Jun (7, 8), ATF-2 (9–12), and Elk-1 (13, 14). Phosphorylation of these transcription factors in their trans-activation domains leads to an increase in their ability to trans-activate transcription. p38-MAPK (15, 16) (alternatively known as cytokine-suppressive antiinflammatory drug-binding protein (17), reactivating kinase (18), Mxi2 (19), or stress-activated protein kinase-2 (20)) is a mammalian homolog of the yeast osmosensing protein kinase HOG-1. Like SAPKs/JNKs, p38-MAPKs also phosphorylate transcription factors (ATF2 (11), CHOP/GADD153 (21), and MEF2C (22)), increasing their trans-activating activity or altering their specificity. In addition, p38-MAPK phosphorolysates and activates protein kinase proteins (MAPKAPKs) 2 and 3 (18, 23), which in turn phosphorylate the small heat shock proteins (Hsp25/27) (18, 23–25). This may modulate the cytoprotective activity of Hsp25/27 (26). The third subfamily of MAPKs (the extracellularly responsive kinases, ERKS) is involved in the regulation of cell growth and differentiation (reviewed in Refs. 27 and 28). In the heart (as in other tissues), these MAPKs are more strongly activated by growth-promoting stimuli (phorbol esters, G protein-coupled receptor agonists, and peptide growth factors) than by cell stresses, with the reverse situation applying to the SR-MAPKs (29–31).

We and others have recently shown that p38-MAPK and MAPKAPK2 are strongly activated by ischemia in the perfused rat heart (32, 33). On reperfusion, activation of these kinases is maintained, and in addition SAPKs/JNKs are activated (32–34). Consistent with this, SAPKs/JNKs are activated in neonatal cardiac myocytes subjected to hypoxia/reoxygenation but not hypoxia alone (35). The roles of these SR-MAPKs remain obscure. We are interested in signals that may potentially activate the SR-MAPKs in ischemic/reperfusion stress. During ischemia and on reperfusion of the ischemic myocardium, there is release of ROS as well as other factors (reviewed in Refs. 1, 29–31) dependent protein kinase inhibitory peptide; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; SR-MAPKs, stress-regulated MAPKs (SAPKs/JNKs and p38-MAPKs).
Stimulation of “Stress-regulated” MAPKs in Heart

2, and 4). Here, we have examined the potential of oxidative stress (as exemplified by perfusion with H$_2$O$_2$) to activate SR-MAPKs and MAPKAPK2 in the isolated rat heart and have compared the effects of H$_2$O$_2$ with those of ischemia and ischemia/reperfusion. Using antioxidants, we investigated the role of ROS in the activation of SR-MAPKs in ischemia/reperfusion.

In addition, we have examined the effects of another pathophysiologically important stress (hypertensive stress) on the activation of SR-MAPKs.

EXPERIMENTAL PROCEDURES

Materials—Prestained molecular mass markers, biotinylated anti-rabbit IgG, ECL blotting reagents, and Hyperfilm MP were from Amersham International. [γ-³²P]ATP was from NEN Life Science Products. Protein A-Sepharose, N-t-butyl-$\beta$-phenyl nitrore (BNP), and other biochemicals were from Sigma. SDS-polyacrylamide gel electrophoresis reagents and Bradford (36) protein assay reagent were from Bio-Rad. SB203580 was a gift from Dr. John C. Lee (SmithKline Beecham, King of Prussia, PA). Nicotrocellose was from Schleicher & Schuell. cAMP-dependent protein kinase inhibitory peptide (PKI, amino acid sequence TTYADFIASGRTGRRNAIHD) was from Bachem. The MAPKAPK2 substrate peptide (KKLRLTSLVFA (18, 37)) was synthesized by Severn Biotech. General laboratory chemicals were from Merck. Antibodies to JNK1, JNK2, and p38-MAPK were from Santa Cruz Biotechnology Inc. The JNK1 antibody (sc-474) was a rabbit antibody raised to a C-terminal region of human JNK1 (amino acids 368–384). Antibodies to JNK2 (sc-572 and sc-827) were raised to the full-length 424-residue protein and amino acids 5–22 from human JNK2, respectively. The p38-MAPK antibody (sc-355) was a rabbit antibody raised to amino acids 341–360 at the C terminus of the mouse sequence. The N-terminal trans-activation domain of human c-Jun (amino acid residues 1–135) and the catalytic domain of murine MAPKAPK2 (amino acid residues 46–400) were expressed as glutathione S-transferase (GST) fusion proteins in Escherichia coli and were purified by glutathione-Sepharose (Pharmacia) chromatography (38). They were used without cleavage from the GST moiety. The c-Jun(1–135) region encompasses the following residues that can be phosphorylated by SAPKs/JNKs: Ser-63, Ser-73, Thr-91, and Thr-93 (39). The MAPKAPK2(46–400) region encompasses the following residues that can be phosphorylated by p38-MAPK. Thr-222, Thr-272, and Thr-334 (40).

Heart Perfusions—Adult male (250–300 g) Sprague-Dawley rat hearts were perfused retrogradely at a pressure of 10 kilopascals (70 mm Hg) with Krebs-Henseleit bicarbonate buffer. NaHCO$_3$, 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$ (pH 7.6) at 37 °C supplemented with 10 mM glucose and equilibrated with 95% O$_2$/5% CO$_2$. The temperature of the perfusates and hearts was maintained at 37 °C by the use of a water-jacketed apparatus. Coronary flows were determined at 10 min after cannulation and were also measured during and at the end of the experiments. All other times given refer to times following the equilibration period. The c-Jun(1–135) region was used as an in vitro phosphorylation substrate.

For experiments in which H$_2$O$_2$ was the only agent added, hearts perfused in 20 mM H$_2$O$_2$ were equilibrated for 15 min. The perfusate was then changed to buffer A (20 mM β-glycerophosphate (pH 7.5), 50 mM NaF, 2 mM EDTA, 0.2 mM Na$_2$VO$_4$, 10 mM benzimidazole, 200 μM leupeptin, 10 μM trans-epoxy succinyl-t-leucylamido(4-guanidino)butane, 5 mM dithiothreitol, 300 μM phenylmethylsulfonyl fluoride, 1% (v/v) Triton X-100) and exposed to ice (5 min). The samples were centrifuged (10,000 g, 5 min, 4 °C), and the supernatants were boiled with 0.33 volume of SDS sample buffer (0.33 M Tris-HCl (pH 6.8), 10% (v/v) SDS, 13% (v/v) glycerol, 133 mM dithiothreitol, 0.2 mg/ml bromophenol blue). The pellet was washed in immunoprecipitation buffer (3 × 150 μl, 4 °C), resuspended in 150 μl of sample buffer (30 μl of 1.33 times), and then separated by SDS-polyacrylamide gel electrophoresis on 10% (v/v) polyacrylamide gels that had been formed in the presence of 0.5 mg/ml myelin basic protein (MBP) for the assay of ERKs, 0.5 mg/ml GST-c-Jun(1–135) for the assay of SAPKs/JNKs, or 0.5 mg/ml GST-MAPKAPK2(46–400) for the assay of p38-MAPKs, with a 6% (v/v) stacking gel. Following electrophoresis, SDS was removed from the gels by washing in 20% (v/v) propyl-2-ol in 50 mM Tris-HCl (pH 8.0) (3 × 20 min). The propan-2-ol was removed by washing in 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol (3 × 20 min). Proteins were denatured in 6 M guanidine-HCl, 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol (2 × 30 min) and then renatured in 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.04% (v/v) Tween 40 (1 × 30 min, 2 × 1 h, 1 × 18 h, 1 × 30 min, 4 °C). The gels were equilibrated to room temperature with 30 μg of Heps (pH 8.0), 2 mM dithiothreitol, 10 mM MgCl$_2$ (2 × 30 min) and then incubated for 3 h with 12.5 μCi/gel [³²P]ATP in 5 ml of 40 mM Heps (pH 8.0), 0.5 mM EGTA, 10 mM MgCl$_2$, 50 μM ATP, 0.1 μM PKI. The reaction was stopped, and gels were washed with 1% (w/v) disodium pyrophosphate, 5% (v/v) trichloroacetic acid. The gels were dried onto 3MM Whatman chromatography paper and autoradiographed. In gel bands were identified by laser scanning densitometry.

Western Blot Analysis—Proteins (30 μl) were separated by SDS-polyacrylamide gel electrophoresis on 10% (v/v) polyacrylamide gels and transferred electrophoretically to nitrocellulose (42). Nonspecific binding sites were blocked with 5% (w/v) nonfat milk powder in 20 mM NaHPO$_4$, 80 mM Na$_2$HPO$_4$, 100 mM NaCl, 0.05% (v/v) Tween-20 (pH 7.5) (PBST) for 30 min, and the blots were incubated with JNK1 or p38-MAPK antibodies (1:100 dilution in blocking solution, overnight, 4 °C). Blots were then incubated for 1 h with 1:5000 dilution of horseradish peroxidase-linked anti-rabbit IgG antibodies (1:5000 dilution in blocking solution, overnight, 4 °C). After washing in PBST (3 × 5 min), the blots were incubated with horseradish peroxidase-linked anti-rabbit IgG antibodies (1:5000 dilution in PBST containing 1% (v/v) nonfat milk powder, 1 h, room temperature). The blots were washed again in PBST (3 × 5 min), and the bands were detected using ECL with exposure to Hyperfilm MP. Blots were quantified by laser scanning densitometry.

Fast Protein Liquid Chromatography of ERKs and MAPKAPK2—Supernatants of heart powders homogenized with 3 volumes of buffer A were diluted 4-fold with buffer B and centrifuged (10,000 g, 5 min, 4 °C). Proteins in samples (0.5 ml) were separated by fast protein liquid chromatography (FPLC). ERKs were separated on a Mono Q H5/5 column equilibrated with 50 mM Tris/HCl (pH 7.3), 2 mM EDTA, 2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, 0.03% (v/v) Brij-35, 0.3 mM Na$_2$VO$_4$, 1 mM benzimidazole, and 4 μg/ml leupeptin. Following a 5-ml isocratic wash, ERKs were separated using a linear NaCl gradient (20 ml, 0–0.33 M NaCl) at flow rate of 1 ml/min with collection of 0.5-ml fractions. They were assayed by the incorporation of [³²P]ATP into MBP by the direct method as described previously (43). Samples of fractions were also taken for in gel kinase assay. All of the samples were boiled with 0.33 volume of SDS sample buffer for 5 min.

For MAPKAPK2, heart extracts were applied to a Mono S HR5/5 column equilibrated with 25 mM β-glycerophosphate (pH 7.3), 2 mM EDTA, 5% (v/v) glycerol, 0.1% (v/v) Triton X-100, 50 μM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol. Following a 5-ml isocratic wash, MAPKAPK2 was eluted using a linear NaCl gradient (15 ml, 0–0.3 M NaCl) at flow rate of 1 ml/min with collection of 1-ml fractions.
MAPKAPK2 was assayed by the incorporation of $^{32}$P from $[^{32}P]ATP$ into KKLINTLNSVA peptide substrate (37) as described previously (32), except that the incubation time was decreased to 10 min. For the determination of total MAPKAPK2 activity, the areas under the FPLC peaks were integrated.

**RESULTS**

**Activation of “Stress-regulated” MAPKs by $H_2O_2$**—In gel kinase assays of hearts perfused for 30 min showed maximal activation of both p46 and p54 SAPKs/JNKs with 0.5 mM $H_2O_2$ (Fig. 1A). Activation was reduced at higher concentrations. This activation was never as great as that observed after ischemia (20 min) followed by reperfusion (10 min) (Fig. 1A). We also confirmed that ischemia alone (20 min) did not activate the SAPKs/JNKs (Fig. 1A). JNK1 antibodies immunoprecipitated all of the p46 SAPK/JNK activity (Fig. 1B, top panel) and protein (Fig. 1B, bottom panel), and approximately 25% of the p54 SAPK/JNK activity (Fig. 1B, top panel). It is not possible to determine the proportion of p54 SAPK/JNK protein immunoprecipitated by the JNK1 antibody (Fig. 1B, bottom panel) because of the interference by immunoglobulins in this region of the gel. These data confirm that the activities principally responsible for phosphorylation of c-Jun(1–135) in the in gel kinase assays (Fig. 1A) were SAPKs/JNKs.

Analogous experiments showed that p38-MAPK was also maximally activated by perfusion with 0.5 mM $H_2O_2$ for 30 min (Fig. 2A). In this case, activation was comparable with that induced by ischemia (20 min) alone or ischemia/reperfusion (10 min) (Fig. 2A). In gel phosphorylation of GST-MAPKAPK2(46–400) was essentially completely inhibited by 10 $\mu$M SB203580 confirming that the activity responsible was p38-MAPK (Fig. 2B). p38-MAPK antibodies immunoprecipitated only approximately 50% of the p38-MAPK activity (Fig. 2C, top panel) and p38-MAPK protein detected on immunoblots (Fig. 2C, bottom panel). This proportion was not altered by increasing the p38-MAPK antibody concentration (results not shown), demonstrating that the incomplete immunoprecipitation did not result from insufficient antibody. Furthermore, the apparent molecular mass of the immunoprecipitated p38-MAPK activity in whole extracts was determined by in gel kinase assays with GST-c-Jun(1–135) as substrate as described under “Experimental Procedures.” The experiments were repeated two further times with similar results.

**Activation of MAPKs by 0.5 mM $H_2O_2$**—Activation of ERKs by $H_2O_2$ was also assessed in hearts perfused with 0.5 mM $H_2O_2$ for 30 min (results not shown). However, because ERKs are generally activated at earlier times than the SR-MAPKs in heart (29–31), activation of ERKs by $H_2O_2$ was also assessed in hearts perfused for 5 min using both in gel kinase assays and following FPLC on Mono Q columns. As a positive control, the effects of phorbol 12-myristate 13-acetate (PMA, which powerfully activates ERKs in hearts (29, 43, 44)) were also studied, p42 and p44 ERKs eluted from the Mono Q column at 0.22 M NaCl, respectively, consistent with published data (29, 43, 44) and were activated in hearts perfused with PMA (1 $\mu$M, 5 min) (Fig. 3, A and B). Relatively little activation of p42 and p44 ERKs (ERK2 and ERK1 respectively) was observed after perfusion with $H_2O_2$, indicating that more than one isoform of p38-MAPK may be activated in perfused rat heart. It is apparent that although this antibody will detect more than one denatured form of p38-MAPK, it may be activated in perfused rat heart. It is apparent that although this antibody will detect more than one denatured form of p38-MAPK, it may be activated in perfused rat heart.

**Activation of ERKs—Activation of MAPKs by 0.5 mM $H_2O_2$**—Activation of MAPKs by 0.5 mM $H_2O_2$ was mainly confined to the stress-regulated forms. In gel kinase assays showed minimal phosphorylation of the ERK substrate, MBP, by extracts of hearts perfused with $H_2O_2$ for 30 min (results not shown). However, because ERKs are generally activated at earlier times than the SR-MAPKs in heart (29–31), activation of ERKs by $H_2O_2$ was also assessed in hearts perfused for 5 min using both in gel kinase assays and following FPLC on Mono Q columns. As a positive control, the effects of phorbol 12-myristate 13-acetate (PMA, which powerfully activates ERKs in hearts (29, 43, 44)) were also studied, p42 and p44 ERKs eluted from the Mono Q column at 0.22 M NaCl, respectively, consistent with published data (29, 43, 44) and were activated in hearts perfused with PMA (1 $\mu$M, 5 min) (Fig. 3, A and B). Relatively little activation of p42 and p44 ERKs (ERK2 and ERK1 respectively) was observed after perfusion with $H_2O_2$, indicating that more than one isoform of p38-MAPK may be activated in perfused rat heart. It is apparent that although this antibody will detect more than one denatured form of p38-MAPK, it may be activated in perfused rat heart.
ERKs by in gel kinase assays with MBP as substrate (Fig. 3B).

**Activation of MAPKAPK2**—MAPKAPK2 is an established substrate of p38-MAPK (18). The activation of MAPKAPK2 was studied following FPLC on Mono S columns. Ischemia (Fig. 4A), ischemia/reperfusion (Fig. 4B), and H2O2 (Fig. 4C) all induced activation of MAPKAPK2. The effects of ischemia and H2O2 were comparable, but the effects of ischemia/reperfusion were consistently greater. SB203580 is a selective inhibitor for MAPKAPK2 by ischemia or ischemia/reperfusion as compared with perfusions in the absence of Me2SO (results not shown).

**Activation of SR-MAPKs and MAPKAPK2 by High Pressure Perfusion**—Hearts were perfused at 20 kilopascals (140 mm Hg) for 20 min to simulate aortic hypertension. p46 and p54 SAPKs/JNKs (Fig. 5A, top panel) and p38-MAPK (Fig. 5A, bottom panel) were activated. Activation of MAPKAPK2 was also detected (Fig. 5B).

**Effects of Free Radical Scavengers on the Activation of SR-MAPKs and MAPKAPK2 by Ischemia and Ischemia/Reperfusion**—To demonstrate that ROS and other free radicals are involved in the activation of SR-MAPKs and MAPKAPK2 by ischemia and/or ischemia/reperfusion, hearts were perfused with the OH scavenger, MeSO (0.4% (v/v), 56 mM), or with the lipophilic spin trap radical scavenger, BPN (10 mM, added in MeSO (56 mM final concentration)). Perfusion of hearts with MeSO or BPN/MeSO under control conditions (no interruption in coronary flow) did not affect the activity of either p38-MAPK or SAPKs/JNKs. The activation of p38-MAPK by ischemia was unaffected by the presence of MeSO but was greatly reduced by BPN/MeSO (Fig. 6A). Consistent with this, the activation of MAPKAPK2 by ischemia was not reduced (and may even be increased) in the presence of MeSO, whereas BPN essentially abolished the activation of MAPKAPK2 by ischemia (Fig. 7). In contrast, the activation of SAPKs/JNKs (Fig. 6B, upper panel), p38-MAPK (Fig. 6B, lower panel) and MAPKAPK2 (Fig. 7) by ischemia/reperfusion was essentially completely inhibited by 56 mM MeSO.

**DISCUSSION**

Global ischemia stimulates p38-MAPK and MAPKAPK2 activities in the perfused heart (32, 33). On reperfusion of ischemic hearts, the stimulation of p38-MAPK and MAPKAPK2 are maintained or increased (32, 33). In addition, activities of SAPKs/JNKs are stimulated by ischemia/reperfusion (32–34). A variety of potentially cytotoxic activators of the SR-MAPKs are produced by the heart in response to ischemia and reperfusion. However, it is not clear which agent(s) is responsible for the activation of these kinases. It was recognized some years ago that there was a significant release of ROS under these conditions (reviewed in Refs. 1, 2, and 4). Thus increases in HzO2 (45), OH (46, 47), and O2 (48, 49) have been detected in ischemia and in reperfusion following ischemia. These species then induce the production of other radicals (e.g., alkoxy and alkyl radicals) by reaction with membrane lipids (50). Here, we
have assessed the potential of ROS, as exemplified by H$_2$O$_2$, to activate the SR-MAPKs in perfused heart and tested whether the production of ROS and free radicals may be responsible for the activation of these kinases by ischemia and ischemia/reperfusion.

**Activation of SR-MAPKs and MAPKAPK2 by H$_2$O$_2$**—Perfusion of rat hearts with H$_2$O$_2$ stimulated SAPKs/JNKs (Fig. 1, A and B) and p38-MAPK (Fig. 2, A and C). These results contrast with those of Knight and Buxton (34), who failed to detect activation of SAPKs/JNKs in hearts perfused with 0.5 mM H$_2$O$_2$ (34). Activation of both SAPKs/JNKs and p38-MAPK appeared to be critically dependent on the concentration of H$_2$O$_2$ because there was minimal activation at 0.1–0.2 mM, but with 0.5 mM H$_2$O$_2$ activation was maximal (Figs. 1A and 2A). At higher concentrations (1 mM H$_2$O$_2$), SAPK/JNK activity declined (Fig. 1A), but p38-MAPK activity was maintained. A similar activation pattern of the SAPKs/JNKs has been noted in astrocytes exposed to H$_2$O$_2$ (51). The activation of p38-MAPK by 0.5–1 mM H$_2$O$_2$ was comparable with the activation seen after ischemia and ischemia/reperfusion (Fig. 2A). These data suggest that increases in H$_2$O$_2$ concentrations (or other ROS) in the heart during ischemia and ischemia/reperfusion (45) could play a role in activation of p38-MAPK. In contrast to the activation of p38-MAPK, the activation of SAPKs/JNKs by 0.5 mM H$_2$O$_2$ was greater than that seen after ischemia but less than that after ischemia/reperfusion (Fig. 1A). This suggests that factors other than ROS may be involved in the activation of SAPKs/JNKs during ischemia/reperfusion.

The species of ROS responsible for the activation of the SR-MAPKs by H$_2$O$_2$ is not clear. Exposure of isolated hearts or ventricular myocytes to H$_2$O$_2$ leads to an iron-dependent formation of OH$^-$ (52–54). Although OH$^-$ may be partly responsible for some of the cardiotoxic effects of H$_2$O$_2$, OH$^-$-independent effects of H$_2$O$_2$ have also been detected (55). At low concentrations (14 mM), the OH$^-$ scavenger Me$_2$SO did not significantly inhibit the activation of p38-MAPK or MAPKAPK2 (an index of p38-MAPK activation) by 0.5 mM H$_2$O$_2$ (results not shown). Further experiments examining the effects of higher concentrations of Me$_2$SO on the activation of SR-MAPKs by H$_2$O$_2$ are indicated.

Several isoforms of the SAPKs/JNKs have been identified by molecular cloning (56). At least three genes produce alternatively spliced transcripts encoding proteins of approximately 46 and 54 kDa (56). An antibody to human JNK1 immunoprecipitated all of the p46 SAPK/JNK activity and approximately 25% of the p54 SAPK/JNK activity stimulated by ischemia, ischemia/reperfusion, or H$_2$O$_2$ in perfused heart (Fig. 1B, top panel). This suggests that there is activation of at least one other isoform of p54 SAPK/JNK. Although antibodies to JNK2 detect...
a protein in neonatal rat ventricular myocytes, we found that these antibodies were not suitable for immunoprecipitation (results not shown).

SB203580 is a selective inhibitor of p38-MAPK (17, 57). The 38-kDa MAPK activity detected using in gel assays with GST-MAPKAPK2(46–400) as a substrate was completely inhibited by 10 μM SB203580 (Fig. 2B), indicating that this activity is indeed attributable to p38-MAPK. However, an antibody to the C terminus of murine p38-MAPK immunoprecipitated only approximately 50% of the activity (Fig. 2C, top panel). The immunoprecipitated form of p38-MAPK migrated slightly more slowly than the residual activity in the supernatant (Fig. 2C, bottom panel), suggesting that more than one isoform of p38-MAPK is activated by ischemia, ischemia/reperfusion, or H₂O₂ in perfused heart. Using the same antibody for Western blots, bands of approximately 38 kDa were detected in both the immunoprecipitates and the residual supernatants (Fig. 2C, bottom panel), although, consistent with the p38-MAPK activity data (Fig. 2C, top panel), the band in the immunoprecipitates migrated more slowly than the band in the supernatants (Fig. 2C, bottom panel). This suggests that although the antibody is more selective for a particular isoform(s) of p38-MAPK in the native form, it also detects other forms after denaturation. At least five isoforms of p38-MAPKs have been identified: p38-MAPK (15, 16, 18) of which there are two alternatively spliced isoforms (17), p38-MAPKβ (58), p38-MAPKγ (20, 59), and p38-MAPKδ (60, 61). However, p38-MAPKγ and p38-MAPKδ transcripts are minimally expressed in heart and neither phosphorylates MAPKAPK2 effectively in an in gel kinase assay (59, 61). Furthermore, p38-MAPKγ and p38-MAPKδ are resistant to inhibition by SB203580 (20, 60). It is thus likely that ischemia and ischemia/reperfusion activate the alternatively spliced isoforms of p38-MAPK and/or p38-MAPKδ in the heart.

One downstream substrate of p38-MAPK is MAPKAPK2 (18). Perfusion of hearts with 0.5 mM H₂O₂ for 30 min powerfully activated MAPKAPK2 (Fig. 4C). The activation was similar to that seen after ischemia (Fig. 4A) but less than that after ischemia/reperfusion (Fig. 4B). In all cases, inclusion of 10 μM SB203580 in the perfusion medium abolished MAPKAPK2 activation (Fig. 4, A–C). These data are consistent with activation of p38-MAPKs stimulating MAPKAPK2 activities.

**Activation of ERKs by H₂O₂**—H₂O₂ activates the ERKs in HeLa, Rat1, NIH 3T3, and PC12 cells line (62) and in primary cultures of rat astrocytes (51). There is disagreement about whether H₂O₂ does (62) or does not (63) produce a significant activation of ERKs in primary cultures of vascular smooth muscle cells. In the study in which no ERK activation was detected (63), there was significant activation of ERKs by another ROS, namely O₂. We detected only minimal ERK1 and ERK2 activation in hearts perfused with 0.5 mM H₂O₂ for 5 min, although there was significant activation in hearts perfused with 1 μM PMA (Fig. 3A and B). There was no detectable activation of ERKs after 30 min of perfusion with 0.5 mM H₂O₂ (results not shown). These data contrast with a previous study that showed activation of ERKs rather than SAPKs/JNKs in hearts perfused with H₂O₂ (34). However, in this study (34), ERKs were partially purified by batch elution from DEAE-Sepharose with 0.5 M NaCl and assayed with a tetrapeptide derived from MBP. Such an elution protocol would elute all MAPK species and is therefore less specific than separation on Mono Q FPLC using a NaCl gradient, which was used here.

**Mechanisms of Activation of SR-MAPKs and MAPKAPK2 During Ischemia and Ischemia/Reperfusion**—As discussed above, H₂O₂ generates free radical ROS in the heart and activates SR-MAPKs (Figs. 1 and 2) and MAPKAPK2 (Fig. 4). Equally, ROS are generated in the heart during ischemia and ischemia/reperfusion. We therefore sought evidence that ROS and other free radicals mediate the activation of SR-MAPKs in ischemia and ischemia/reperfusion. We used two different free radical scavengers, the OH⁻ radical scavenger Me₆SO and the lipophilic spin trap BPN. Both have been found to be cardioprotective under situations of increased oxidative stress (54, 64). At a concentration of 10 mM BPN/56 mM Me₆SO (solvent carry-over), activation of p38-MAPK and MAPKAPK2 by ischemia was inhibited (Figs. 6A and 7). In contrast, 56 mM Me₆SO alone inhibited activation of SR-MAPKs and MAPKAPK2 after ischemia/reperfusion (Fig. 6B and 7). These data implicated ROS and free radicals in the activation of SR-MAPKs during ischemia and ischemia/reperfusion but suggest that different radicals may be involved during the ischemic and reperfusion phases. It has recently been suggested that O₂ and H₂O₂ are generated during simulated ischemia in isolated cardiac myocytes, whereas OH⁻ and further H₂O₂ are generated during simulated reperfusion (65). This is entirely consistent with our data (Figs. 6 and 7).

**Activation of SR-MAPKs and MAPKAPK2 by High Pressure Perfusion**—Using in gel kinase assays with MBP as a substrate, we have previously shown that short term perfusion (5 min) of hearts at high aortic pressures activates ERKs (44). Furthermore, the upstream activators of the ERKs, the MAPK (or ERK) kinase group of MAPK kinases, were also activated (44). This work was completed prior to the identification of the SR-MAPKs. Here, we show that perfusion of hearts at high aortic pressure for longer times (20 min) additionally stimulated the activities of SAPKs/JNKs, p38-MAPK, and MAPKAPK2 (c.f. ischemia, ischemia/reperfusion, and H₂O₂). Although the mechanism of activation of the SR-MAPKs by high pressure perfusion is unclear, it could represent ischemia/reperfusion effects, because hypertension in vivo is known to induce subendomyocardial ischemia (reviewed in Ref. 3).

**Significance of SR-MAPKs in the Heart**—The biological consequences of activation of SR-MAPKs in the heart are poorly understood. Activation of the SAPKs/JNKs would be expected to result in phosphorylation of the c-Jun and ATF2 transactivation factors, increasing their trans-activating activity (reviewed in Ref. 6). In this regard, we have shown that hyperoxic stress activates SAPK/JNKs in ventricular myocytes (31), and c-Jun and ATF2 become phosphorylated (66). Transcription of c-Jun is regulated by a number of cis-acting regulatory sequences in the c-Jun promoter region, including two sites (junA and junB) that bind c-Jun/ATF2 heterodimers. Thus, activation of SAPKs/JNKs is potentially able to up-regulate c-Jun expression. Increased expression of c-Jun occurs during ischemia/reperfusion in isolated hearts (67) and during hypoxia in cultured myocytes (68). Furthermore, H₂O₂ induces c-Jun expression in NIH 3T3 cells (69). Thus SAPK/JNK-dependent activation of c-Jun might be expected to increase gene expression and anabolism. Indeed, SAPKs/JNKs have been proposed to be mediators of the α₁-adrenergic stimulation of hypertrophic growth in the ventricular myocyte (70). However, activation of SAPKs/JNKs and c-Jun (71) induces apoptosis in a number of cell lines, which may be particularly pertinent in the ischemic heart. The p38-MAPKs have also been implicated in α₁-adrenergic stimulation of hypertrophic growth in the ventricular myocyte (72). One of their substrates (MAPKAPK2) phosphorylates the small heat shock proteins Hsp25/27, which may be cytoprotective (26). However, activation of p38-MAPK may be apoptotic (73). The ultimate biological effects of activation of SR-MAPKs may depend on the duration and extent of their activation (74).
Stimulation of "Stress-regulated" Mitogen-activated Protein Kinases (Stress-activated Protein Kinases/c-Jun N-terminal Kinases and p38-Mitogen-activated Protein Kinases) in Perfused Rat Hearts by Oxidative and Other Stresses

Angela Clerk, Stephen J. Fuller, Ashour Michael and Peter H. Sugden

J. Biol. Chem. 1998, 273:7228-7234.
doi: 10.1074/jbc.273.13.7228

Access the most updated version of this article at http://www.jbc.org/content/273/13/7228

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 73 references, 34 of which can be accessed free at http://www.jbc.org/content/273/13/7228.full.html#ref-list-1