Mitochondrial ROS initiate phosphorylation of p38 MAP kinase during hypoxia in cardiomyocytes

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Kulisz, Andre, Ningfang Chen, Navdeep S. Chandel, Zuohui Shao, and Paul T. Schumacker. Mitochondrial ROS initiate phosphorylation of p38 MAP kinase during hypoxia in cardiomyocytes. Am J Physiol Lung Cell Mol Physiol 282: L1324–L1329, 2002; 10.1152/ajplung.00326.2001.—The p38 mitogen-activated protein kinase (MAPK) is phosphorylated in response to oxidative stress. Mitochondria in cardiomyocytes increase their generation of reactive oxygen species (ROS) during hypoxia (1–5% O2). These ROS participate in signal transduction pathways involved in adaptive responses, including ischemic preconditioning and gene transcription. The present study therefore tested the hypothesis that hypoxia induces p38 MAPK phosphorylation by augmenting mitochondrial ROS generation. In cardiomyocytes, phosphorylation of p38 was observed in a PO2-dependent manner during hypoxia. This response was inhibited by rotenone, thenoyltrifluoroacetone, and myxothiazol, inhibitors of mitochondrial complexes I, II, and III, respectively. A similar inhibition was observed in the cells pretreated with anion channel inhibitor DIDS, which may block ROS release from mitochondria. During normoxia, increases in mitochondrial ROS elicited by azide (1–2 mM) or by the mitochondrial inhibitor antimycin A caused increased phosphorylation of p38. Brief treatment with exogenous H2O2 during normoxia also induced phosphorylation of p38 as hypoxia, but this effect was not abolished by myxothiazol or DIDS. The antioxidant N-acetyl-cysteine abolished the p38 response to hypoxia, presumably by scavenging H2O2, but the mitochondrial extracellular receptor kinase inhibitor PD-98059 did not inhibit p38 phosphorylation during hypoxia. Thus physiological hypoxia leads to p38 phosphorylation through a mechanism that requires electron flux in the proximal region of the mitochondrial electron transport chain, which suggests that either H2O2 or superoxide participates in activating that process.

hydrogen peroxide; superoxide; respiration; protein kinases; oxidant stress

MITOGEN-ACTIVATED PROTEIN KINASES (MAPK) comprise a superfamily of serine/threonine protein kinases that are critical for various cellular functions in different cell types. MAPK themselves are activated by phosphorylation on threonine and tyrosine residues in a Thr-X-Tyr motif residing in the activation loop proximal to the ATP- and substrate-binding sites of the protein (20). This phosphorylation is carried out by a dual-specificity MAPK kinase, which itself is activated by a phosphorylation event in response to an either intracellular or extracellular stimulus (13, 24). MAPK can be categorized further into three subfamilies based on the size of the activation loop and the identity of the amino acid present between the Thr and Tyr in the activation motif. One of these is p38 MAPK, which contains a Gly in the motif (12, 16, 25). Activation of p38 MAPK occurs in response to ultraviolet light, increased extracellular osmolarity, proinflammatory cytokines, and chemical stress (10, 17, 23).

Oxidative signaling has been implicated in a variety of experimental interventions that lead to the initiation of gene transcription or other adaptive responses (19, 26, 28). Several groups have shown that p38 MAPK is activated by reactive oxygen species (ROS) generated intracellularly, as well as by hydrogen peroxide (H2O2) administered exogenously (8, 14, 18, 23, 27). Other studies have shown that hypoxia leads to the activation of p38 MAPK (9, 27, 32), although neither the mechanism underlying this activation nor its relationship to ROS signals has been described. We previously found that hypoxia causes an increase in ROS generation in cardiomyocytes, as detected using the oxidant-sensitive probe 2',7'-dichlorofluorescein (11). Inhibitors of complexes I and II of the mitochondrial electron transport chain attenuated the oxidant signal during hypoxia, whereas inhibitors of more distal regions augmented ROS production and failed to block the response to hypoxia. This suggested that the increased ROS generation originated from the proximal region of the electron transport chain, most likely complex III. These observations suggested that ROS generated by mitochondria may be responsible for triggering p38 phosphorylation (activation) during hypoxia. The present study therefore sought to test the hypothesis that ROS generated by mitochondria during physiological hypoxia is sufficient to initiate p38 MAPK activation in cardiomyocytes.

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MATERIALS AND METHODS

Primary cell isolation. Embryonic chick cardiomyocytes were isolated using a method (4) modified from Barry et al. (2). Briefly, hearts were removed from 10–11-day-old chick embryos and washed in Hank’s balanced salt solution lacking magnesium and calcium (Life Technologies). The ventricular tissue was minced and then dissociated using 4–6 cycles of trypsin (0.025%, Life Technologies) digestion with gentle shaking at 37°C. After 8 min, the trypsin digestion was stopped by transferring the cells to a trypsin inhibitor solution. The cells were then filtered (100-μm mesh), centrifuged for 5 min at 1,200 rpm at 4°C, and resuspended in nutritive medium [54% Barry’s solution (116 mM NaCl, 1.3 mM KCl, 22 mM NaHCO3, 0.8 mM MgSO4, 1.0 mM NaH2PO4, 0.87 mM CaCl2, and 5.6 mM glucose)], 4% medium 199 with Earle’s salts (Life Technologies), 6% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were preplated for 45 min on a petri dish in a humidified incubator (5% CO2–95% air at 37°C) to promote early adherence of fibroblasts. The nonadherent cells were counted with a hemacytometer, and their viability was assessed using trypan blue (0.4%). Approximately 1 × 10⁶ cells in nutritive medium were plated onto glass coverslips (25 mm) in 35 × 10 mm dishes. Cells were maintained in a humidified incubator for 2–3 days, at which time synchronous contractions of the monolayer of cells were noted. Experiments were carried out on spontaneously contracting cells on day 4 or 5 after the isolation.

Hypoxia chambers. To expose cells to hypoxic conditions, petri dishes containing cells on coverslips were placed into sealed glass jars (Naigene) fitted with fluid transfer tubes to permit gas flushing. The jars were continuously flushed with humidified gas at known [O2], 5% CO2, balance nitrogen, supplied by a precision mass flow controller. The chambers were maintained at 37°C in an incubator throughout the exposure period. Control normoxic cells were maintained in a humidified incubator with 5% CO2 in air.

Cell lysis and protein extraction. The nutritive medium was quickly aspirated from the dishes and replaced with 500 μl of ice-cold radioimmunoprecipitation assay buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate, 200 mM phenylmethylsulfonyl fluoride, 200 mM Na3VO4, and 50 mM NaF). Cells were then scraped off of the coverslips and drawn into a 3-ml syringe fitted with a 27.5-gauge needle to further lyse the cells. The lysate solution was then centrifuged at 14,000 × g for 10 min at 4°C, and the supernatant (soluble fraction) was transferred into new tubes and stored at −70°C until needed.

Cell electrophoresis and Western blotting. Samples were mixed with equal volumes of loading buffer and were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) as per Laemmli (15). All lanes were loaded with equal quantities of protein. In addition to the samples, Kaleidoscope prestained standards (Bio-Rad) and/or p38 MAPK control proteins (New England Biolabs) were also run out as per manufacturers' specifications to verify the specificity of the antibodies used. Proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Life Science) using Towbin’s buffer (30) and the Trans-Blot SD Semi-Dry system (Bio-Rad), and effective transfer was verified by staining the membrane with Ponceau S solution (Sigma). The membrane was incubated at room temperature in Tris-buffered saline with Tween 20 (TBS-T) plus 5% milk to block nonspecific binding, then washed 3 × with TBS-T and incubated overnight at 4°C with the primary antibody [1:1,000 anti-phospho-specific p38 (New England Biolabs) or 1:5,000 anti-p38 (Santa Cruz Biotechnology) in TBS-T plus 5% BSA. The next day, the membrane was washed 3 × with TBS-T, incubated at room temperature with the secondary antibody [1:2,000 anti-rabbit-horseradish peroxidase (New England Biolabs)] in TBS-T plus 5% milk, and washed 3 × with TBS-T. Antibody-bound proteins were detected utilizing ECL Western blotting detection reagents (Amersham Life Science) and visualized on Hyperfilm-ECL (Amersham Life Science). Verification of equal loading of proteins was confirmed once again by staining the membrane with 0.1% India ink. Each experiment was repeated several times, with a representative blot shown for each experiment.

Reagents. In different experiments the cells were treated with H2O2, antimony A, myothiazol, sodium azide, 4,4'-dihydroxyanostibene-2, 2'-disulfonic acid (DIDS), rotenone, thenoyltrifluoroacetone (TFFA), and N-acetyl-cysteine (NAC) (Sigma). The MAPK kinase inhibitor 2'-amino-3'-methoxyflavone (PD-98059) was obtained from Biomol Research Laboratories.

RESULTS

Effects of hypoxia on phosphorylation of p38 MAPK.

Cardiomyocytes on glass coverslips were exposed to 100 μM H2O2 for 15 min to determine whether phosphorylation of p38 MAPK occurs in cardiomyocytes in a manner similar to that observed with other cell types. Total protein was extracted from the cells, electrophoresed on an SDS-PAGE, and transferred to a nitrocellulose membrane that was probed with an anti-phospho p38 antibody, which recognizes only the phosphorylated form of the protein (Fig. 1). A single band was visualized which corresponded to a band visualized in the lane loaded with an external positive control. The external positive and negative control lysates loaded in the first two lanes were prepared from C6 cells that had or had not been treated with antisomycin, respectively. Cells on coverslips were also exposed to hypoxia (humidified 1% O2, PO2 ~7 Torr, 5% CO2, balance N2) for 1, 2, or 3 h. Control coverslips were maintained in a humidified 37°C, 5% CO2 incubator (normoxia) to serve as an internal negative control.
Phosphorylation of p38 MAPK was not detected in the normoxic sample but was detected in each of the hypoxic samples and H$_2$O$_2$ treatment sample. Equal loading of protein was confirmed by probing with an anti-p38 antibody (Fig. 1).

**PO$_2$ dependence of the phosphorylation of p38 in cardiomyocytes.** To confirm that the decrease in PO$_2$ was responsible for the phosphorylation of p38 MAPK, coverslips with cardiomyocytes were exposed to decreased levels of O$_2$ for 2 h. Cells incubated at 4, 2, and 1% O$_2$ showed increases in p38 phosphorylation, whereas cells maintained in the incubator (normoxic) or those incubated at 16 or 8% O$_2$ did not exhibit increased levels of p38 phosphorylation (Fig. 2).

**Role of mitochondrial ROS during hypoxia in the phosphorylation of p38 MAPK.** We previously found increased levels of p38 phosphorylation (Fig. 2). By inhibiting electron transfer at complex IV, the noncompetitive inhibitor antimycin A (100 ng/ml) during normoxia to augment the formation of ROS from mitochondria exhibited phosphorylation of p38 (Fig. 3A). Separate coverslips were preincubated for 30 min with either myxothiazol (0.5 μM), to inhibit complex III, or DIDS (50 μM), to inhibit anion channels in the mitochondria inner membrane, and then exposed to 2 h of hypoxia (1% O$_2$). Myxothiazol and DIDS both prevented the phosphorylation of p38 in response to hypoxia (Fig. 3A). If complex III acts as a source of ROS production, this should be attenuated by inhibiting electron flux at more upstream sites. To inhibit electron flux through complexes I and II, cells were pretreated with rotenone (2.5 μM) and TTFA (20 μM) for 15 min. An external positive control lysate was used to verify the specificity of the antibody.

Effect of exogenous H$_2$O$_2$ on phosphorylation of p38 MAPK. Exogenous H$_2$O$_2$ (100 μM) has been reported to induce phosphorylation of p38 MAPK. However, the concentration of H$_2$O$_2$ required to activate p38, which suggests that H$_2$O$_2$ may act as an upstream signaling element in that response. To test the specificity of this response, lower doses of H$_2$O$_2$ were examined. Both 25- and 40-μM H$_2$O$_2$ treatments induced similar phosphorylation of p38 to that seen with 100 μM H$_2$O$_2$. This phosphorylation was not altered by myxothiazol and DIDS (Fig. 4B). These data...
indicate that myxothiazol and DIDS do not inhibit the ability of H2O2 to activate p38 MAPK phosphorylation, and they are consistent with the conclusion that ROS may act as the signal downstream from mitochondria. Cobalt chloride (100 μM) has been shown to induce ROS production via a nonmitochondrial mechanism in cells (6). Incubating cells with cobalt chloride also induced p38 phosphorylation, in accordance with this oxidant effect (Fig. 4A).

Effects of antioxidants on the phosphorylation of p38 MAPK during hypoxia. The data suggested that ROS released by the mitochondria during hypoxia or that exogenous H2O2 treatments during normoxia are sufficient to activate p38 phosphorylation. To test this further, the antioxidant NAC was used to attenuate intracellular levels of H2O2 during 2 h of hypoxia (1% O2). As shown in Fig. 5, 0.5 mM NAC abolished the hypoxia-induced p38 phosphorylation. However, this response was limited to this specific concentration of NAC.

Stress-induced pathway vs. mitogenic pathway. Several parallel signaling pathways lead to the activation of the MAPK. To confirm that the signaling pathway leading to the phosphorylation of p38 during hypoxia is a stress-induced pathway as opposed to a mitogenic pathway, cells were either made hypoxic (1% O2) for 2 h or exposed to H2O2 (100 mM) for 15 min in the presence or absence of PD-98059 (100 mM), an inhibitor of the mitogenic MEK/extracellular signal-regulated kinase signaling pathway (1). As shown in Fig. 6, pretreatment for 30 min with PD-98059 did not prevent the phosphorylation of p38 in response to either hypoxia or H2O2.

DISCUSSION

MAPK are key participants in signal transduction pathways activated by mitogenic stimuli, environmental stress, and inflammatory agents. The stress-activated protein kinase member p38 is activated by ROS generated in response to angiotensin II (33) and by exogenous H2O2 (8, 14, 27). Phosphorylation of p38 MAPK also occurs during hypoxia (9, 27, 32), although the mechanism underlying this response is not known. We previously reported that hypoxia elicits an increase in mitochondrial generation of ROS in cardiomyocytes (11) and in other cell types (6, 7). The present study therefore sought to determine whether hypoxia causes activation of p38 by stimulating the generation of ROS by mitochondria. We used phosphorylation of p38 MAPK as a surrogate marker of its activation.

Utilizing antibodies that can distinguish between the phosphorylated and nonphosphorylated forms of p38 MAPK, we detected phosphorylated p38 in cellular lysates within 1 h of exposure to hypoxia (P02 ~ 7 Torr, 1% O2); this signal persisted for up to 3 h of hypoxia. Phosphorylated p38 did not increase in cardiomyocytes kept normoxic (~21% O2), but those cells still contained the nonphosphorylated form of the protein. The phosphorylation of p38 during hypoxia was dependent on the level of O2, in that cells exposed to O2 levels between 8 and 21% did not exhibit significant phosphorylation, whereas cells exposed to O2 levels between 1 and 4% showed significant increases. These results support the conclusion that a p38 MAPK-dependent signaling pathway is activated in response to a physiological decrease in O2.

Fig. 5. Effects of the antioxidants N-acetyl-cysteine (NAC) on the phosphorylation of p38 MAPK during hypoxia in cardiomyocytes. Cardiomyocytes were exposed to 2 h of hypoxia (Hy, 1% O2) in the presence of NAC (0.5 mM). Lysates from each treatment group were probed with anti-phospho-specific p38 MAPK antibodies. Bands were compared with those lysates from normoxic and hypoxic cells not treated with antioxidants.

Fig. 6. Stress-induced pathway vs. mitogenic pathway. Lysates from cardiomyocytes exposed to 2 h of hypoxia (1% O2) or 15 min of H2O2 (100 μM) with or without 30-min pretreatment with PD-98059 (50 μM) were probed with anti-phospho-specific p38 MAPK antibodies.
Our previous studies demonstrate that hypoxia activates ROS generation in cardiomyocytes and that these oxidant signals originate from the mitochondrial electron transport chain (11). To determine whether mitochondrial ROS are required for p38 MAPK activation during hypoxia, inhibitors were used to attenuate electron flux in different regions of the electron transport chain. Myxothiazol, rotenone, and TTFA abolished the response to hypoxia, presumably by attenuating the generation of superoxide. These compounds inhibit the formation of ubiquinone at complex III, which appears to be the primary site of superoxide generation (31). By contrast, antimycin A augments ROS generation at complex III by prolonging the lifetime of ubiquinone (29), and it failed to block the response to hypoxia. Interestingly, myxothiazol, rotenone/TTFA, and antimycin A all inhibit mitochondrial ATP production because they block electron transport, yet only myxothiazol and rotenone/TTFA abolished the response to hypoxia. This indicates that mitochondrial ATP is not required for the phosphorylation of p38 MAPK and that electron supply in the proximal but not the distal region of the electron transport chain is responsible for the increase in ROS generation during hypoxia.

The compound DIDS is an inhibitor of anion channels in the mitochondrial inner membrane, through which superoxide anions presumably must pass to reach the cytosol (3). DIDS abolished the response to hypoxia, which suggests that superoxide transit through anion channels may be required for the phosphorylation of p38 during hypoxia. Collectively, these results indicate that mitochondrial ROS participate in the signaling pathway leading to p38 phosphorylation during hypoxia in cardiomyocytes.

If mitochondria activate a signal transduction system during hypoxia by generating superoxide in response to a change in redox of the electron transport proteins, then other inhibitors that mimic the redox changes of hypoxia should mimic the effects of hypoxia. Azide, a noncompetitive inhibitor of cytochrome oxidase, was therefore used to examine the role of mitochondrial ROS in the p38 MAPK phosphorylation pathway. At concentrations of 1–2 mM, azide induced a modest increase in the phosphorylation state of p38 compared with normoxic cells. The intensity of these bands was not as great as that of cardiomyocytes exposed to H2O2. However, the oxidant signal generated by these concentrations of azide was previously found to be less than those generated during hypoxia (11).

Hence, the moderate degree of p38 phosphorylation could be explained by the smaller ROS signal in response to azide. These results demonstrate that modulation of mitochondrial redox can mimic the effect of hypoxia on the phosphorylation state of p38 MAPK, strengthening the link between mitochondrial ROS generation and the modification of this protein.

Superoxide anion is primarily degraded by conversion to H2O2 by superoxide dismutase, and low concentrations of exogenous H2O2 induced p38 activation during normoxia. It is therefore conceivable that ROS produced in mitochondria during hypoxia are released to the cytosol and subsequently converted to H2O2, which then acts as the downstream signal leading to p38 phosphorylation. In the cytosol, H2O2 is degraded primarily by the glutathione peroxidase system. To determine whether H2O2 is required for the phosphorylation of p38, the nonspecific ROS scavenger NAC was used to accelerate its clearance. NAC abolished the p38 phosphorylation during hypoxia, albeit at a single concentration. This suggests that H2O2 is sufficient to induce the phosphorylation of p38 MAPK.

To determine whether p38 phosphorylation during hypoxia reflects activation of a stress-induced or a mitogenic pathway, the compound PD-98059 was used to block phosphorylation of the traditional MAPK (p42/p44) by MEK (1). If p38 MAPK activation in hypoxia requires MEK, then the inhibitor should have abolished p38 phosphorylation in response to hypoxia or to H2O2. However, PD-98049 failed to do so, suggesting that p38 phosphorylation during hypoxia is part of a separate signaling pathway. These results are consistent with the idea that p38 MAPK is activated in response to cellular stress, rather than the mitogenic pathway.

Activation of p38 MAPK has been implicated in the early phase of cardiac preconditioning (35), but considerable controversy exists regarding whether preconditioning truly leads to activation of p38 MAPK and whether this activation is required for the subsequent protection (22). Our previous studies revealed that mitochondrial oxidant signals are generated during hypoxic preconditioning of cardiomyocytes and that these ROS contribute to the triggering of ischemic preconditioning (34). However, it is not clear whether the ROS signal generated during the brief period of hypoxic preconditioning is sufficient to trigger p38 MAPK activation, and a full understanding of the significance of p38 MAPK in preconditioning requires further study.

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