Mitochondrial signals initiate the activation of c-Jun N-terminal kinase (JNK) by hypoxia-reoxygenation

CHRISTOPHER J. DOUGHERTY,1 LORI A. KUBASIAK,1 DONNA P. FRAZIER,1 HUIFANG LI, WEN-CHENG. XIONG,† NANETTE H. BISHOPRIC, AND KEITH A. WEBSTER§,2

Department of Molecular and Cellular Pharmacology and §Vascular Biology Institute, University of Miami School of Medicine, Miami, Florida, USA; and 1Department of Pathology, University of Alabama, Birmingham, Alabama, USA

Abstract C-Jun N-terminal kinase (JNK) is part of the mitogen-activated protein kinase (MAPK) family of signaling pathways that are induced in response to extracellular stimuli. JNK is primarily a stress-response pathway and can be activated by proinflammatory cytokines and growth factors coupled to membrane receptors or through non-receptor pathways by stimuli such as heat shock, UV irradiation, protein synthesis inhibitors, and conditions that elevate the levels of reactive oxygen intermediates (ROI). The molecular initiators of MAPKs by non-receptor stimuli have not been described. Ischemia followed by reperfusion or hypoxia with reoxygenation represents a condition of high oxidative stress where JNK activation is associated with elevated ROI. We show here that the activation of JNK by this condition is initiated in the mitochondria and requires coupled electron transport, ROI generation, and calcium flux. These signals cause the selective, sequential activation of the calcium-dependent, proline-rich kinase Pyk2 and the small GTP binding factors Rac-1 and Cdc42. Interruption of these interactions with inactivated dominant negative mutant proteins, blocking calcium flux, or inhibiting electron transport through mitochondrial complexes II, III, or IV prevents JNK activation and results in a proapoptotic phenotype that is characteristic of JNK inhibition in this model of ischemia-reperfusion. The signaling pathway is unique for the reoxygenation stimulus and provides a framework for other non-receptor-mediated pathways of MAPK activation.—Dougherty, C. J., Kubasiak, L. A., Frazier, D. P., Li, H., Xiong, W.-C., Bishopric, N. H., Webster, K. A. Mitochondrial signals initiate the activation of c-Jun N-terminal kinase (JNK) by hypoxia-reoxygenation. *FASEB J.* 18, 1060–1070 (2004)

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The mitogen-activated protein kinase (MAPK) family comprises three signaling pathways each with distinct terminal kinases, including the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), and the p38 MAP kinase (for reviews, see refs 1–4). These pathways fulfill fundamental roles in cell growth, cell function, differentiation, and the response to stress by transmitting extracellular signals from the cell membrane to nuclear and cytoplasmic targets. Each MAPK pathway or module contains an evolutionarily conserved three-component cascade of dual specificity kinases that transmit an activation signal from its source through a phosphoryl relay chain to the final kinase. The component relay kinases are referred to as MAP kinase (MAPK), MAP kinase kinase (MKK), and MAP kinase kinase kinase (MKKK). Multiple individual kinases at the MKKK positions of each module confer differential affinity and selectivity for specific stimuli. Differential responses to any stimuli may be channeled by scaffold proteins that physically colocalize specific module components (5–7). Multiple MKKs in different modules may be activated by the same stimuli but by different degrees, so that the net outcome of any stimulation is a function of the relative activity of each MAPK (8, 9). The downstream targets include transcription factors, structural proteins, enzymes, other kinases, and multiple survival determination factors, each with a specific affinity for each terminal kinase. Activation of the ERK pathway typically confers growth and survival responses, while p38 and JNK may promote cell death so that the cellular fate in response to any stimulus is a function of the relative levels of activation of each MAPK (10, 11).

The ERK pathway is preferentially activated by mitogens and growth factors whereas the p38 and JNK pathways are more selective for environmental stresses, both physical and chemical, including inflammatory cytokines (1, 12). The upstream intermediates in the pathways for receptor-mediated MAPK activation have been more accessible to analysis than the non-receptor pathways; consequently these pathways are better described. Essentially complete pathways that couple growth factors and other receptor-mediated ligands to MAPK modules have been described (2, 3). In the classical scheme, ligand-activated transmembrane ty-
Rosine receptor kinases (e.g., EGF-R/PGF-R) recruit Src from the cell cytoplasm. Activated Src recruits GTP-Ras to the cell membrane, which in turn recruits and activates the serine/threonine kinase c-Raf or other MKKK. Activated c-Raf triggers the sequential phosphorylation of MEKS in the ERK module. A parallel pathway has been described for the activation of JNK by heterotrimeric G-protein-coupled receptors and angiotensin II (Ang II) (13–17).

Various environmental stresses can activate all three MAPK modules (2, 18). Oxidative stress is perhaps the most common environmental stress to be imposed on biological systems and may result from direct exposure to extracellular oxidizing agents such as hydrogen peroxide and menadione (12, 19, 20) or secondary to another primary stimulus such as UV irradiation or hyperglycemia (21, 22). Pathologically, the most common oxidative stress occurs when tissues are subject to ischemia and reperfusion associated with vascular disease and ischemia (23–26). ERK, JNK, and p38 MAPKS are all activated in cardiac myocytes or neuronal cells exposed to hydrogen peroxide treatment or after exposure to hypoxia and reoxygenation (24, 27–29). Because of the extreme clinical importance of these pathways, their regulation by oxidative stress has been under intense study for many years. As the cellular sensors of oxidative stress have not been identified, the intermediates that couple this oxidative stress to signal transduction cascades are also unknown. Indeed, schemes depicting signaling cascades often show extracellular oxidative stress connected directly to one of the MKKK intermediates (30). JNK is strongly induced by oxidative stress in cultured cardiac myocytes and in intact hearts (27, 31) and may be a critical determinant of ischemic injury in the myocardium as well as the brain (4, 32–34). Whereas it has been widely assumed that the signals that initiate JNK include ROI, neither the source of ROI nor the transduction intermediates have been identified.

Here we present evidence that electron transport-coupled calcium flux and ROI generated in the mitochondria provide the initiating stimuli for JNK activation in cardiac myocytes subjected to hypoxia-reoxygenation. These signals mediate phosphorylation and activation of the calcium-dependent, proline-rich protein kinase Pyk2 that constitutes the initiating kinase reaction of this cascade. Pyk2 becomes progressively tyrosine phosphorylated at different sites and recruits Rac-I and/or Cdc42. These intermediates are not used when anisomycin, antimycin A, or hydrogen peroxide activates JNK.

MATERIALS AND METHODS

Reagents

Antibodies to JNK, Pyk2, ASK-1, PAK-1, and HA were from Santa Cruz Biotech (Santa Cruz, CA, USA). Antibodies against ERK, phospho-ERK, phospho-JNK, p38, phospho-p38, phospho-Pyk2 were from New England Biolabs (Beverly, MA, USA). The Rac-1 activation kit and GST-MKK6 were from Upstate Biotech (Lake Placid, NY, USA). Hoechst 33342 and propidium iodide dyes were from CalBiochem (La Jolla, CA, USA). Anti-vertebrate sarcomeric myosin antibody (MF-20) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Polycationic liposomes (GeneFector™) were from VennNova, LLC (Pompano Beach, FL, USA) and LipoFectAMINE™ was from Life Technologies (Rockville, MD, USA). Recombinant adenoviral vectors including dnJNK, caMEKK-1, and dnPyk2 have been described (35–37). Plasmids encoding HA-JNK and dominant negative forms of Ras-I, Rac-I, Cdc42, and Rho A were gifts from Dr. S. Gutchkin (Oral and Pharyngeal Cancer Branch, NIDR, NIH, Bethesda, MD, USA). The lucigenin reagent (N-methyl-acridinium nitrate) was purchased from Molecular Probes (Eugene, OR, USA). Mitochondrial inhibitors and uncoupler carbonyl cyanide A trifluoromethoxyphenyldrazzone (FCCP) and antioxidant PBN were from Sigma (St. Louis, MO, USA). Nifedipine and (S)-BayK 8644 were also from Sigma.

Cell culture

All procedures involving animals were performed in accordance with institutional guidelines for the care and use of animals. Methods for primary culture of neonatal rat cardiac myocytes have been described (38, 39). In brief, enriched cultures of myocyte and nonmyocyte cells were obtained from 1- to 2-day-old neonatal rats by stepwise trypsin dissociation and plated at a density of 4 × 10^6/mmV. After 1-2 days, the cultures were rinsed three times in MEM and transferred to a defined serum-free DMEM or MEM supplemented with 5% fetal calf serum (MEM+5% FCS). After 3-5 days, cells were rinsed three times in MEM and transferred to a defined serum-free DMEM or MEM supplemented with transferrin, vitamin B12, and insulin. The final cultures contained >95% cardiac myocytes contracting at >200 beats per minute; 0.1 mM bromodeoxyuridine (BrdU) was included in the media for the first 3 days after plating to inhibit fibroblast growth.

Hypoxia and reoxygenation.

Details of our methods for exposing cells to hypoxia have been described previously (31, 40). Oxygen and pH were continuously monitored using appropriate electrodes and contractility was monitored by edge detection (38, 39). The medium oxygen concentration was maintained <10 mm Hg. For reoxygenation, plates were removed from the chamber and reoxygenated by replacing the medium with oxygenated medium or by gentle mixing without media change and incubating under 21% O2 (air/5% CO2).

Kinase assays

MAPK assays have been described in detail elsewhere (31, 40). Briefly, myocytes were lysed in 150 μL of ice-cold lysis buffer with protease inhibitors; equal amounts of preclarsed extracts were incubated on ice with 6 μL of antibody (anti-JNK1/JNK2, anti-ERK, anti-HA, anti-ASK-1), and protein A agarose beads. The beads were pelleted and washed twice with lysis buffer and resuspended in kinase buffer (25 mM HEPES pH 7.4, 25 mM MgCl2, 2 mM DTT, 0.1 mM sodium vanadate, 25 mM β-glycerophosphate) with 5 μg of purified c-Jun141 fusion protein, myelin basic protein (MBP), or MKK6 substrate, and ATP with 10 μM 32P-ATP. Samples were incubated at 30°C for 30 min and electrophoresed on 12% SDS polyacrylamide (31). Kinase activity was quantitated by...
analyzing densitometry of fragments on digitized images using NIH Image 1.60 with Adobe PhotoShop 4.0.

**Western blot and IP analyses**

Detailed procedures for Western blot analyses have been described (31, 40). Cultures were harvested in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 50 mM NaF) with freshly added 1 mM Na$_3$VO$_4$, 0.5 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin. Equal amounts of protein from cleared lysates were fractionated on 12 or 15% SDS-polyacrylamide gels and electroblotted to nitrocellulose (BioRad, Hercules, CA, USA). Blots were stained with Ponceau Red to monitor the transfer of proteins. Membranes were blocked for 1 h at room temperature with 5% non-fat milk in TBS (25 mM Tris, 137 mM NaCl, 2.7 mM KCl) containing 0.05% Tween-20 and incubated with specific antibodies for 2–4 h in the same buffer. After washing, the blots were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody and visualized using enhanced chemiluminescence (42). After subjection to exogenous ROI (superoxide) production was measured by lucinigen-ROS measurements

ROI (superoxide) production was measured by lucinigen-enhanced chemiluminescence (42). After subjection to experimental conditions, plates were treated with lucigenin at 200 μmol/L and the cells were dispersed directly into a luminometer tube. Chemiluminescence was recorded every minute for 20 min. For hypoxia-only conditions, luminometer tubes containing cells and lucigenin were sealed under hypoxia before reading. For reoxygenation ± mitochondrial inhibitors, the inhibitors were added to myocyte cultures under hypoxia 20 min before reoxygenation and lucigenin was added immediately before reoxygenation.

**Rac-1 activation assay**

Rac-1 activity was measured using a PAK-1 binding assay (43) supplied as a kit by Upstate Biotechnology. Cells were lysed in magnesium lysis buffer, mixed on ice with 10 μg PAK-1 agarose beads, and rocked gently at 4°C for 1 h. Beads were washed three times with magnesium lysis buffer, resuspended in 30 μL of 2X SDS buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, Tris-Cl pH 6.8), boiled, and fractionated on 15% SDS-polyacrylamide gels. Beads were incubated with GTPγS or GDP for positive and negative controls, respectively.

**Analysis of apoptosis**

DNA fragmentation analyses (ladders) and quantitative apoptosis assays were implemented as described previously (31, 35, 40). Apoptotic cardiac myocytes were scored for morphologic evidence of apoptosis or necrosis after staining by TUNEL, Hoechst 33342, or propidium iodide) combined with immunostaining of sarcomeric myosin.

**Respiration measurements**

Oxygen consumption was measured using a Clark oxygen electrode as described previously (44). Cells (8×10$^5$ myocytes) were added to 500 μL of culture media in a micro water-jacketed cell with magnetic stirrer (Hansatech Instruments Limited, Norfolk, UK). Increasing concentrations of electron transport chain (ETC) inhibitors were added while recording oxygen tension to achieve complete inhibition of respiration or maximal uncoupling.

**Statistical analysis**

Results are expressed as mean ± se. Differences between means were evaluated by two-tailed Student t test. ANOVA was carried out using InStat 2.0 statistical software for Macintosh.

**RESULTS**

**JNK activation requires coupled electron transport and ROI**

We have shown that JNK activation by reoxygenation was blocked by antioxidant treatment before and during reoxygenation (27). These results implicate ROI as part of the initiating stimulus for JNK activation. To investigate the source of the activating ROI, we used selective inhibitors of the mitochondrial ETC, the oxidative phosphorylation uncoupler FCCP, and ATPase inhibitor oligomycin to inhibit mitochondrial functions before reoxygenation. The sites of ETC inhibition are shown in Fig. 1A; JNK activities in response to these treatments are shown in Fig. 1B. Rotenone, malonate, and azide inhibit at complexes I, II, and IV, respectively. The ubiquinone cycle at complex III (CIII) is detailed in Fig. 1A to show the sites of action of antimycin A (AA) and myxothiazol. Production of semiquinone, a highly reactive free radical, is enhanced by AA but blocked by myxothiazol. Therefore, inhibition of the ETC with AA but not myxothiazol (or other ETC inhibitors) stimulates superoxide production (45–47). Consistent with this, treatment of aerobic cultures with AA activated JNK whereas the other ETC inhibitors did not (Fig. 1B). Reoxygenation resulted in 9.50 ±0.50-fold induction of JNK compared with the aerobic control. Rotenone had no effect on JNK activation in air or reoxygenation (1.24±0.32-fold air; 8.25±0.84-fold reoxygenation). Malonate, myxothiazol, and azide all eliminated reoxygenation-induced JNK activation (respectively to 1.33±0.76-fold, 0.94±0.04-fold, 1.09±0.63-fold), indicating the importance of electron flow through complex II. Reoxygenation had no additional effect on JNK activation in the presence of AA (5.21±1.07-fold aerobic+AA, 4.80±1.15-fold reoxygenation+AA). Induction of JNK by AA treatment was both significantly higher than the aerobic control and significantly lower than the reoxygenation control (P<0.01 for both). These results suggest that electron transport, as well as ROI, is necessary for maximal stimulation of JNK by reoxygenation. The uncoupler FCCP blocked JNK activation by >90% (n=5, P<0.001) whereas the ATPase inhibitor oligomycin had no effect on basal aerobic levels of JNK (1.17±0.25-fold induction) or activation by reoxygenation.
JNK Activation by Oxidative Stress

ROI production

To determine whether JNK activation correlated with ROI production, we measured ROI during hypoxia and reoxygenation in the presence and absence of mitochondrial inhibitors. Cultures of cardiac myocytes were treated with each of the mitochondrial-targeted drugs and assayed for superoxide anion ($O_2^-$) production under aerobic (controls), hypoxic, and reoxygenation conditions, as described in Materials and Methods. In these studies, whole cell oxygen consumption was undetectable at the inhibitor concentrations used (not shown). As shown in Fig. 2, none of the mitochondrial inhibitors except AA increased $O_2^-$ production of aerobic controls. AA treatment resulted in a 3.7 ± 0.8-fold increase in ROI. This result is consistent with the established property of AA to promote ROI formation and is quantitatively similar to measurements made with the $H_2O_2$ probe 2′,7′-dichlorofluorescin (45–48). ROI levels in hypoxic cells were <20% of the aerobic levels, and there were no detectable effects of any of the inhibitors under hypoxia (data not shown). Reoxygenation mediated a 5.08 ± 1.32-fold increase of $O_2^-$ compared with aerobic controls, which was decreased by all of the mitochondrial inhibitors except AA and oligomycin. Therefore, maximal activation of JNK correlates with coupled electron transport as well as ROI. ROI generation through ETC complex III may be necessary because myxothiazol eliminated JNK activation whereas AA did not. However, ROI production is not sufficient for maximal JNK activation because the...
combination of AA and reoxygenation, while generating the greatest ROI production, caused least JNK induction than reoxygenation alone.

**JNK activation is blocked by calcium chelators and calcium channel blockers**

All conditions that block electron transport or dissipate the mitochondrial membrane potential during reoxygenation inhibited JNK activation (Fig. 1). Membrane potential provides the driving force for calcium transport across the inner mitochondrial membrane (49, 50). Our group as well as others have reported enhanced intramyocyte calcium levels associated chronic hypoxia (51, 52) and a rapid increase of calcium uptake upon reoxygenation (53). To determine whether calcium has a role in this pathway, we treated cardiac myocytes with the calcium chelators EGTA and BAPTA and the L-type calcium channel blocker nifedipine. These results are shown in Fig. 3A–C. JNK activation was prevented by treatment with either calcium chelator and blocked in a dose-dependent manner by nifedipine. The lowest nifedipine concentrations that completely blocked JNK activation (200 nM) did not prevent contractility of cardiac myocytes as determined microscopically and by edge detection (data not shown). In further support of a calcium component in JNK activation, we found that treatment of cardiac myocytes with the L-type channel opener (S)-BayK 8644 added 10 min before reoxygenation increased JNK activation by 2.3-fold (compared with control reoxygenation) measured 1 h after reoxygenation (data not shown). Treatment of myocytes with the same nifedipine concentration that prevented JNK activation by reoxygenation did not block activation by AA (Fig. 3C; control: 5.86±0.11, +1 μM nifedipine: 5.21±1.07) and had no effect on the activation of ERK or p38 (latter data not shown). These studies demonstrate a unique requirement for both ROI and calcium flux for the activation of JNK by reoxygenation.

**JNK activation is channeled through Pyk2**

Pyk2 is a calcium-dependent kinase that is phosphorylated on tyrosine residues associated with the calcium binding domain and the catalytic domain. As shown in Fig. 4A, phospho-Pyk2 accumulated rapidly after reoxygenation peaking after 15 min and remaining elevated at 4 h. There was a slight lag in phosphorylation of the catalytic domain, but both sites were maximally phosphorylated within 30 min. Pyk2 phosphorylation was not activated in cultures pretreated with either 200 nM nifedipine or 0.5 mM EGTA (Fig. 4B). Pyk2 was activated by PMA but not by Ang II, anisomycin, or H2O2 at the concentrations used (Fig. 4C). To determine whether Pyk2 phosphorylation was a primary component of the reoxygenation pathway, cardiac myocytes were infected with adenovirus containing a kinase-dead, dominant negative Pyk2 cDNA, and JNK activity was measured after hypoxia-reoxygenation and aniso...
mycin treatments (Fig. 4D). Ad-dnPyk2 infection blocked the activation of JNK by reoxygenation but did not affect activation by anisomycin. The absence of any effect on the anisomycin pathway shows specificity for the reoxygenation pathway and indicates that the dominant negative does not indiscriminately sequester JNK pathway proteins. Taken together with the Pyk2 activity data, these results support a role for Pyk2 as the initiating kinase in the pathway of JNK activation by hypoxia-reoxygenation. In results not shown, dnPyk2 did not decrease the activity of ERK or p38 as measured by in vitro immunokinase assays with MBP substrate.

Activation of Rac-1 and selective inhibition of JNK by dnRac-1

Pyk2 has been shown to interact with and activate Rac-1 and Cdc42 in some membrane receptor-mediated pathways (13–16). To determine whether Rac-1 lies downstream of Pyk2 in the reoxygenation–JNK pathway we measured the activity of Rac-1 using a PAK-1-linked binding assay as described in Materials and Methods. Rac-1 was activated within 5 min of reoxygenation; activity increased for 30 min (to 5.1±1.3-fold, n=3) (Fig. 5A) and remained above basal level for at least 2 h. The level of activation was at least as great as the positive controls (GTPγS). Rac-1 activation was blocked by 1 μM nifedipine (data not shown). To further investigate the role of Rac-1, cardiac myocytes were cotransfected with HA-JNK and a series of mutant signaling proteins with dominant negative functions (Fig. 5B). Transfected cultures were exposed to hypoxia-reoxygenation (left panel) or anisomycin (right panel) and the activity of HA-JNK was measured. JNK activation by reoxygenation was blocked by dnJNK, dnRac-1, and dnCdc42 but was minimally affected by dnRas or dnRho-1. JNK activation by anisomycin was not inhibited by dnRac-1, Cdc42, or dnRas. Quantita-

![Figure 4. Selective activation of Pyk-2 by hypoxia-reoxygenation. A) Cultures of cardiac myocytes were subjected to 24 h of hypoxia and harvested after hypoxia alone or after the indicated interval of reoxygenation. Samples containing equal amounts of protein were analyzed by Western blots probed with anti-phospho-Pyk-2 (ca and cd refer to the calcium binding and catalytic domain phosphorylation sites of Pyk2 respectively) or unphosphorylated Pyk2 antibodies; results are quantitated on the right (n=3). B) Cultures were treated with 200 μM nifedipine or 0.5 mM EGTA as indicated 30 min before reoxygenation as in Fig. 3, aerobic and hypoxic cultures were treated for 1 h before harvesting, positive control PMA samples were not treated. C) Samples treated with Ang-II (50 nmol/L), H2O2 (100 μmol/L), anisomycin (100 nmol/L), or PMA (100 μmol/L) were harvested after 30 min and analyzed as in 4A (n=3). D) Cardiac myocytes were infected with adenovirus expressing dnPyk2 or control (GFP) adenovirus 24 h before treatments as described in Materials and Methods. Cultures were subjected to hypoxia-reoxygenation or antimycin A treatments and harvested for JNK kinase assays as indicated. Data are representative of 3 separate experiments.](image-url)
tion of the effects of dominant negative mutants on HA-JNK activity is shown in Fig. 5C. These results support primary roles for Pyk2 and Rac-1 in the pathway of reoxygenation. The absence of any effect of dn-Rac-1 on anisomycin-induced JNK activation again indicates selectivity for the reoxygenation pathway rather than a general sequestration by the dn mutant of JNK pathway intermediates.

Inhibition of the JNK pathway with dn mutants increases apoptosis

In an earlier paper we demonstrated that JNK activation is protective in this model of hypoxia-reoxygenation and that blocking JNK activity with kinase-dead mutants increased cell death (35). Protection from apoptosis by JNK has also been reported in an in vivo model of pressure overload (34). To determine whether interference of JNK activation by other upstream dominant negative mutants affected the death pathway, we measured apoptosis in reoxygenated myocytes after transfection or infection with different blocking mutants. As shown in Fig. 6A, DNA fragmentation was visibly increased by infection with either Ad-dnJNK or Ad-dnPyk2 whereas infection with Ad-caMKK1 reduced fragmentation. Quantitative analyses of the effects of interfering vectors are shown in Fig. 6B. Significant increases in apoptotic death were produced by overexpression of dnPyk2, dnRac-1, or dnJNK-1. These results support roles for each of these intermediates in the pathway of JNK activation by reoxygenation as well as in the generation of a protective response to this type of oxidative stress.

DISCUSSION

Results described here demonstrate that mitochondria generate the primary signals that initiate activation of the JNK pathway by hypoxia-reoxygenation. The pathway is distinct from other MAPK signaling pathways including hydrogen peroxide, antimycin A, and anisomycin in the source and nature of the initiating signals hypoxia-reoxygenation and harvested at the indicated time intervals for assays of Rac-1 as described in Materials and Methods. Control samples were incubated with the nonhydrolyzable GTP analog GTPγS or GDP. Equal amounts of protein were assayed in each case. B) Representative kinase assays from cardiac myocyte cultures cotransfected with HA-JNK and an empty pcDNA3 vector or with the different kinase mutants as indicated. Top panel: myocytes were cotransfected with HA-JNK (5 μg) and dominant negative mutants, and subjected to anisomycin, 10 μg/mL for 1 h. In each case HA-JNK activity was assayed after immunoprecipitation using anti-HA, and total HA-JNK was detected by Western blot. C) Quantitation of (HA) JNK activation; *P<0.05 compared with reoxygenation control (n=3).

Figure 5. Activation of Rac-1 by hypoxia-reoxygenation and effects of dn-Rac-1. A) Cardiac myocytes were subjected to
(ROI and calcium) and in the use of Pyk2 as the primary kinase. Pyk2 transmits the signal through Rac-1/Cdc42 to the JNK module.

Several lines of evidence support a dual component, ROI and calcium for maximal activation of JNK by hypoxia-reoxygenation. First, pretreatment with antioxidants or calcium blockers was equally effective in preventing JNK activation. Second, JNK activation did not correlate precisely with ROI production. Combined AA and reoxygenation generated the greatest ROI but did not maximally activate JNK. Activation of JNK by AA alone was not blocked by nifedipine (or dnPyk2), indicating distinctive pathways of JNK activation by AA and reoxygenation. Electron transport inhibitors malonate, myxothiazol, and azide or the uncoupler FCCP prevented JNK activation, indicating the requirement for coupled electron transfer through complexes II-IV. Nifedipine prevented JNK (and Pyk2) activation at concentrations that did not block myocyte contractions, suggesting that the pool of calcium that initiates the pathway is distinct from or in excess of that associated with the calcium transient.

Under the conditions of chronic hypoxia used, respiration and oxidative phosphorylation are inhibited but ATP levels are maintained by a compensatory increase in glycolysis (38, 39). Calcium accumulates slowly during hypoxia but rapidly during reoxygenation, when electron flow is resumed and mitochondrial membranes are repolarized (33–35). Intracellular calcium levels have been reported to increase >fivefold during reoxygenation with between two- and threefold increases of mitochondrial calcium (56). Our results suggest that this increase of calcium is a component of the signal that activates the JNK pathway. Inhibition of calcium entry during reoxygenation, either directly with nifedipine or indirectly by mitochondrial ETC inhibitors or an uncoupler, eliminated JNK activation. Pretreatment with the L-type calcium channel opener (S)-BayK 8644 augmented JNK activation. These results support a role for calcium in activating the JNK pathway. The absence of an effect of oligomycin indicates that production of ATP by oxidative phosphorylation is not required for JNK activation. It was reported recently that electron transport and ATP synthesis were both necessary for the induction of ERK by hydrogen peroxide (57). ERK activation involves sequential activation of protein kinase C, Src, Ras, and Raf (28, 29, 58). As discussed below, our results indicate that the pathway(s) activated by reoxygenation are distinct from those responding to extracellular H$_2$O$_2$.

Our results demonstrate that Pyk2 is essential for JNK activation by hypoxia-reoxygenation. Pyk2 phosphorylation increased >two- to fourfold within 15 min of reoxygenation and dnPyk2 eliminated JNK activation. The kinetics of Pyk2, Rac-1, and JNK activation are also consistent with a linear pathway. Pyk2 activity peaked after 10–15 min and remained activated for 2–4 h, Rac-1 activity increased over 30 min and remained above the basal aerobic level for at least 2 h. JNK activity peaks between 30 min and 1 h after reoxygenation and remains elevated for up to 4 h (27, 35). Consistent with this, Pyk2, Rac-1, and JNK activation by reoxygenation were each blocked by nifedipine or EGTA. In parallel experiments, Pyk2 was not significantly activated by anisomycin, Ang-II, or H$_2$O$_2$ but was activated by PMA. Dominant negative Pyk2 eliminated the activation of JNK by hypoxia-reoxygenation but had no effect on JNK activation by anisomycin or AA. This provides compelling evidence that inhibition by the dominant negative was selective for the hypoxia-reoxygenation stimulus and not a generalized sequestration of factors required for JNK activity. Similarly, Rac-1 was activated by reoxygenation downstream of Pyk2, and both dnRac-1 and dnCdc42 inhibited JNK activation. Transfection with dnRac-1 or dnCdc42 did not block JNK activation by anisomycin, again confirming the pathway-selective use of Rac-1/Cdc42 (59).

The reoxygenation stimulus differs from that mediated by H$_2$O$_2$ or AA in the selection of Pyk2. These stimuli all involve ROI but the reoxygenation stimulus...
Figure 7. Contrasting pathways of JNK activation by reoxygenation and H2O2. Steps in the activation of JNK by treatment of cells with H2O2 include stimulation of the TNFα receptor (TNFαR) and TRAF2 at the plasma membrane followed by sequential phosphoryl transfers between PKC, Src, Gab1, Ras, ASK-1, MKK 6, and JNK ((29, 61, 62). The Ras binding proteins Raf-1, Rac-1, and Cdc42 may also be involved. The reoxygenation signal is initiated in the mitochondria and requires coupled electron transport stimulated by the re-entry of oxygen. Combined calcium and ROI activate Pyk2 stimulating recruitment of Rac1 and/or Cdc42. AA stimulates ROI production and activates JNK but not Pyk2. Nif = nifedipine; dn = dominant negative.

requires both ROI and calcium. It is possible that ROI generation and calcium flux during reoxygenation are coupled. JNK activation by AA and H2O2 are not calcium dependent. Other studies have shown that H2O2 stimulates the transmembrane tumor necrosis factor-α receptor (TNFαR) and the signal is channeled through Src, Gab1, and ASK-1 (60–62). In results not shown here, we confirmed that ASK-1 was also activated by treatment of cardiac myocytes with 200 μM H2O2 for 30 min but was not activated by reoxygenation. The activation of distinct signaling pathways by reoxygenation and H2O2 treatment may be a consequence of the source of the signal as well as the nature of the ROI. H2O2 treatment delivers oxidative stress to the receptor side of the plasma membrane whereas reoxygenation delivers a stress that is initiated inside the cell by the mitochondria and involves superoxide (see Fig. 7). We have reported that neutral sphingomyelinase is activated rapidly after reoxygenation in this model and that increased ceramide levels correlated with JNK activation (63). Because Pyk2 is activated by protein kinase C, it is possible that ceramide-activated PKC contributes to hypoxia–reoxygenation activation of Pyk2. We are currently testing this possibility.

The relative degree of activation of the different MAPKs may determine cell fate under stress (10). ERK activation is generally associated with survival signals whereas JNK and p38 are often, but not always, involved in pro-death pathways (4, 34, 64). Oxidative stress is generated internally or externally by a diverse set of conditions, including UV irradiation, hyperglycemia, H2O2, hypoxia, reperfusion, and exposure to xenobiotics (4, 21, 25, 65). These stresses activate the MAPK modules differentially in a stimulus and cell-specific manner. The level of activation of each pathway is determined primarily by selection of the initial kinases and G-proteins are in turn determined by the species, magnitude, and cellular location of reactive oxygen. Our results suggest that reoxygenation is primarily a calcium-initiated stimulus generated by the resumption of coupled mitochondrial electron transport after hypoxia. JNK activation is a key feature of reperfusion in multiple tissues, including heart, brain, liver, and kidney, and the activation has been assigned positive and negative survival roles (34, 35, 66). The present study confirms our previous report that JNK activation can be protective and that blocking JNK activity at multiple points of the pathway interferes with JNK activation and promotes apoptotic cell death (35).

Preliminary reports of this work have been published in abstract form (C. J. Dougherty et al., Activation of c-Jun N-terminal kinase through the Rac1/cdc42-TAK-1 pathway promotes survival of reoxygenated cardiac myocytes. Circulation 102, 2000; L. A. Kubasiak et al., Roles of mitochondrial complexes II and III and the permeability transition pore, respectively in hypoxia-reoxygenation and hypoxia-acidosis of cardiac myocytes. Circulation 102, 2000).

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