Signal transduction mechanisms activated during the early stages of necrotic cell death are poorly characterized. We have recently identified the Sterile 20 (Ste20)-like oxidant stress response kinase-1, SOK-1, which is a member of the Ste20 kinase family. We report that SOK-1 is markedly activated as early as 20 min after chemical anoxia induced by exposure of Madin-Darby canine kidney or LLC-PK1, renal tubular epithelial cells to 2-deoxy-glucose (2-DG) and any one of three inhibitors of the electron transport chain, cyanide (CN), rotenone, or antimycin A. Since oxidant stress activates SOK-1, we postulated that reactive oxygen species (ROS), which are produced by the electron transport chain during chemical anoxia, might be responsible for SOK-1 activation. The time course of CN2-DG-induced SOK-1 activation and of production of ROS, measured in cells loaded with dichlorodihydrofluorescein, were compatible with a role for ROS in SOK-1 activation. Furthermore, preincubation of LLC-PK1 cells with three unrelated scavengers of ROS, pyrrolidine dithiocarbamate, pyruvate, or nordihydroguaiaretic acid, reduced both cellular oxidant stress and activation of SOK-1 by CN2-DG. An increase in cytosolic free [Ca\(^{2+}\)] (Ca\(^{2+}\)) was necessary but not sufficient for CN2-DG-induced activation of SOK-1. Preincubation of cells with BAPTA-AM prevented activation of SOK-1. Incubation of cells with thapsigargin or the calcium ionophore, A23187, had no effect on SOK-1 activity, but preincubation of cells with either of these agents markedly enhanced CN2-DG-induced activation of SOK-1 (20-fold versus 7-fold). In summary, chemical anoxia activates SOK-1 via an oxidant stress-dependent mechanism that is both critically dependent upon and markedly amplified by an increase in Ca\(^{2+}\}). This requirement for dual inputs of oxidant stress and an increase in Ca\(^{2+}\}] may prevent inappropriate activation of the kinase by milder degrees of oxidant stress, which are insufficient to generate an increase in Ca\(^{2+}\}). The activation of SOK-1 may be one of the cell’s earliest responses to inducers of necrotic cell death.

1 The abbreviations used are: MAP, mitogen-activated protein; AM, acetoxymethyl ester; BAPTA, 1,2-bis-(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid/tetraacetoxymethyl ester; PDTC, pyrrolidine dithiocarbamate; MeSO, dimethyl sulfoxide; CN, cyanide; 2-DG, 2-deoxyglucose; DTT, dithiothreitol; SOK-1, Ste20-like oxidant stress response kinase-1; Ste20, Sterile 20; [Ca\(^{2+}\}], cytosolic free calcium concentration; ER, endoplasmic reticulum; SAPK, stress-activated protein kinases, or c-Jun N-terminal kinases, while not activated by periods of renal ischemia in vivo or chemical anoxia in vitro, are markedly activated after as little as 5 min of reperfusion of ischemic kidney or 5 min of re-exposure of anoxic cells to glucose. The SAPKs are the predominant c-Jun transactivation domain kinases and the predominant ATF-2 transactivation domain and DNA binding domain kinases in the post-ischemic kidney (7, 8).

Ischemia, caused by the interruption of blood flow to a tissue, is the cause of myocardial infarction and stroke, and is a major cause of acute renal failure. Ischemia is characterized by three major components: ATP depletion, acidosis, and metabolism accumulation (1). If blood flow is restored in whole or in part, a fourth component, production of reactive oxygen species, is introduced (2). In the kidney, the cells most adversely affected by ischemia are the tubular epithelial cells. Those cells that survive the ischemic insult may undergo dedifferentiation and, after a variable period of time, re-enter the cell cycle to replace cells which suffered necrotic or apoptotic cell death (1). The signal transduction pathways modulating these responses to ischemic injury are poorly understood.

In tubular epithelial cells in culture, chemical anoxia, induced by inhibition of the electron transport chain combined with inhibition of glycolysis, recapitulates two key features of ischemia: ATP depletion and, if chemical anoxia occurs in a normoxic environment, oxidant stress due to enhanced mitochondrial generation of reactive oxygen species (3–5). Chemical anoxia, if prolonged, induces necrotic cell death, characterized by what appears to be intact chromatin, by mitochondrial swelling with loss of cristata structure, and loss of plasma membrane integrity (6). To identify signal transduction pathways that might modulate the cell’s response to impending necrotic cell death, we have examined activation of various MAP1 kinase cascades by renal ischemia and chemical anoxia. We have found that the stress-activated protein kinases (SAPKs, or c-Jun N-terminal kinases), while not activated by periods of renal ischemia in vivo or chemical anoxia in vitro, are markedly activated after as little as 5 min of reperfusion of ischemic kidney or 5 min of re-exposure of anoxic cells to glucose. The SAPKs are the predominant c-Jun transactivation domain kinases and the predominant ATF-2 transactivation domain and DNA binding domain kinases in the post-ischemic kidney (7, 8).

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Activation of the Ste20-like Oxidant Stress Response Kinase-1 during the Initial Stages of Chemical Anoxia-induced Necrotic Cell Death

REQUIREMENT FOR DUAL INPUTS OF OXIDANT STRESS AND INCREASED CYTOSOLIC [Ca\(^{2+}\)]

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SOK-1 (9), a human serine/threonine kinase from the germinal center kinase group of Ste20-like kinases which also includes hematopoietic progenitor kinase (10, 11). SOK-1 appears to be an environmental stress response kinase since it is not activated by ligand-receptor interactions but is activated by oxidant stress (9). Herein we report that chemical anoxia markedly activates SOK-1 during the early and potentially reversible stages of necrotic cell death, and describe the mechanisms responsible for activation.

EXPERIMENTAL PROCEDURES

Materials—The acetoxyethyl ester form of the intracellular Ca\(^{2+}\) chelator, 1,2-bis-(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid/ tetra(acetoxyethyl)ester (BAPTA-AM) was purchased from Calbiochem. 5-(and-6)-2',7'-Dichlorodihydrofluorescein diacetate was from Molecular Probes. A20187, thapsigargin, carbonyl cyanide p-trifluoro- methoxy)phenylhydrazide (FCCP), pyrroldine dithiocarbamate (PDTC), dimethyl sulfoxide (DMSO), deoxyxarnose, nordihydroguaiaretic acid (NDGA), EGTA, rotenone, antymycin A, sodium cyanide (CN), sodium pyruvate, 2-deoxyglucose (2-DG), menadione, and catalase were from Sigma. \(\gamma\)-\[^32\]P\]ATP (40,000 cpm/pmol) was from NEN Life Science Products. Luciferin and luciferase were from Boehringer Mannheim.

Induction of Chemical Anoxia—Madin-Darby canine kidney (MDCK) or LLC-PK\(_1\) renal tubular epithelial cells were grown to confluence in Eagle's minimum essential medium supplemented with glutamine (2 mM) and 10% fetal calf serum. Chemical anoxia was induced as described (7, 8, 12). Briefly, cell monolayers (in 10-cm dishes) were incubated in a Krebs-Henseleit buffer (115 mM NaCl, 3.6 mM KCl, 1.3 mM KH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), pH 7.4) for 30 min, followed by the addition of either 5 mM CN, 0.5 \(\mu\)g/ml rotenone, or 0.5 \(\mu\)g/ml antimycin A, and 5 mM 2-DG in the absence of dextrose. All incubations were performed at 37 °C in a 95% air, 5% CO\(_2\) incubator. With this protocol, during the CN/2-DG treatment, ATP content of cells was reduced to less than 5% of control within 10 min. Cells were released from chemical anoxia by washing once with phosphate-buffered saline, and then incubating them in Krebs-Henseleit buffer containing 5 mM glucose (7, 8).

Immunoprecipitation of SOK-1 and Immune Complex Kinase Assays—Cells were washed with cold Tris-buffered saline and then lysed in lysis buffer (20 mM HEPES, pH 7.4, 50 mM \(\beta\)-glycerophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 10 mM EGTA, 1 mM DTT) and then centrifuged (10,000 \(\times\) g, 10 min). Supernatants were matched for protein concentration (Bio-Rad) before immunoprecipitation with a polyclonal antiserum raised against amino acids 333–426 from the non-catalytic region of SOK-1 (9). After 2 h, the immune complexes were collected for 1 h with Protein G-Sepharose beads. Beads were washed three times in lysis buffer, twice in LiCl buffer (500 mM LiCl, 2 mM DTT, 100 mM Tris-HCl, pH 7.6), and three times in assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 1 mM DTT, 0.1% Triton X-100) (7). Kinase assays were started by the addition of myelin basic protein (250 \(\mu\)g/ml), \(\gamma\)-\[^32\]P\]ATP (100 \(\mu\)Ci, 3000–9000 cpm/pmol), and MgCl\(_2\) (10 mM). After 4 min at 30 °C, the kinase reactions were stopped with SDS sample buffer. After SDS-polyacrylamide gel electrophoresis and autoradiography, the bands corresponding to the substrate were cut out of the gel and radioactivity was determined by liquid scintillation counting. Data are presented as the mean ± S.E. for three separate experiments. All assays were performed in duplicate.

Determination of ATP Concentration—ATP concentration was determined using a luciferin-luciferase assay (12). Briefly, confluent LLC-PK\(_1\) cells on 10-cm plastic dishes were snap-frozen in a slurry of dry ice and ethanol, and ATP was extracted into 1 ml of 4% perchloric acid in 200 mM Tris. After 30 min on ice, the extract was titrated to pH 8.2 with 7 \(M\) KOH in 100 mM Tris, and then centrifuged (10,000 \(\times\) g, 10 min). Fifty microliters of supernatant were diluted 1:4 in luciferase buffer (50 mM Heps, 1 mM MgCl\(_2\), 1 mM EGTA, pH 8.2) containing 500 \(\mu\)g luciferin (final concentration), and then luciferase (5 \(\mu\)g) was added. Luminescence was measured in triplicate with a Luminos Expert, 505 luminoimeter, and ATP content was determined relative to a standard curve.

Determination of Cellular Oxidant Stress—Production of ROS was measured using the fluorescent probe 2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA). This probe is nonpolar, and is converted to the membrane-impermeant polar derivative, DCFH, by esterases when it is taken up by the cell. DCFH is minimally fluorescent, but is rapidly oxidized to the highly fluorescent 2',7'-dichlorodifu-
two other inhibitors that are chemically unrelated to CN. Both rotenone (an inhibitor of site I respiration) and antimycin A (an inhibitor of site III respiration) in combination with 2-DG activated SOK-1, albeit not as potently as CN/2-DG (Fig. 2).

ATP depletion is likely to be important for CN/2-DG-induced activation of SOK-1 since inclusion of 10 mM glucose in the media during the incubation of the cells with CN/2-DG completely abrogated SOK-1 activation (Table I). To determine whether SOK-1 activation was due to ATP depletion, we exposed LLC-PK<sub>1</sub> cells to either CN/2-DG or the uncoupling agent, FCCP, plus 2-DG, and determined SOK-1 kinase activity. Although FCCP/2-DG reduced cellular content of ATP to levels comparable to that induced by CN/2-DG (<5% of control), SOK-1 was not activated by FCCP/2-DG (1.0 ± 0.2-fold versus control) but was by CN/2-DG (5.6 ± 0.4-fold versus control). These data indicate that ATP depletion is not sufficient for activation of SOK-1 by chemical anoxia, and suggest that mechanisms in addition to ATP depletion were critical.

Our previous studies indicated that SOK-1 is activated by oxidant stress (9). Therefore, we explored whether ongoing production of reactive oxygen species by the electron transport chain during the period of chemical anoxia might be the mechanism by which SOK-1 was activated. We incubated LLC-PK<sub>1</sub> cells, which had been loaded with 7.5 μM DCFH-DA, with CN/2-DG and followed production of ROS for 45 min. In contrast to vehicle treatment (Fig. 3A), beginning at approximately 20 min after CN/2-DG, there was a discernible rise in DCF fluorescence compatible with production of ROS (Fig. 3B). Fluorescence continued to increase compared with control throughout the remainder of the experiment. Thus, CN/2-DG induced the production of ROS in LLC-PK<sub>1</sub> cells, and the time course of ROS production paralleled the time course of SOK-1 activation.

We next determined whether several chemically unrelated agents that react with and thus inactivate ROS inhibited SOK-1 activation by chemical anoxia. We incubated cells with PDTC (100 μM) for 120 min before exposure to CN/2-DG. PDTC is a dithiocarbamate, which functions as an antioxidant by delivering thiol groups directly to the cell (17). PDTC abolished the CN-induced activation of SOK-1 and inhibited the CN/2-DG-induced activation by 70% (Fig. 4). An unrelated agent, sodium pyruvate, like other α-ketocids, reduces H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O while undergoing nonenzymatic decarboxylation at the 1-carbon position (18, 19). Pyruvate markedly inhibited SOK-1 activation by CN/2-DG (Fig. 4).

To confirm that PDTC and sodium pyruvate were functioning as expected to reduce cellular oxidant stress, we followed DCF fluorescence for 45 min after cells, previously treated with PDTC or pyruvate, were exposed to CN/2-DG. Both PDTC and pyruvate markedly reduced CN/2-DG-induced cellular oxidant stress as measured by DCF fluorescence (Fig. 3, C and D).

Three other redox-active agents, the antioxidant NDGA (20 μM) (20), the iron chelator deferoxamine (5 mM) (21, 22), which inhibits the production of hydroxyl radicals that derive from the Fenton/Haber-Weiss reactions, and MgSO<sub>4</sub> (50 mM), which avidly reacts with hydroxyl radicals (22), also inhibited activation of SOK-1 (60%, 55%, and 30% inhibition, respectively) (data not shown).

LLC-PK<sub>1</sub> cells were also incubated in media containing catalase (300 units/ml), or heat-inactivated catalase (55 °C for 5 min) for 3 h before CN/2-DG (13, 23). No cellular toxicity was observed at this concentration and duration of treatment (24). Catalase inhibited activation by 35%, but the heat-inactivated enzyme was ineffective (Table II). These data, taken together, strongly suggest that production of ROS is necessary for the activation of SOK-1 in response to chemical anoxia.

Oxidant stress and chemical anoxia produce an increase in cytosolic free [Ca<sup>2+</sup>] ([Ca<sup>2+</sup>]<sub>i</sub>) in part by releasing Ca<sup>2+</sup> from endoplasmic reticulum (ER) storage pools, by increasing Ca<sup>2+</sup>-influx, and by inhibiting cell membrane Ca<sup>2+</sup>-ATPase (24–28). The resulting increase in [Ca<sup>2+</sup>]<sub>i</sub> is believed to exacerbate cellular injury. Therefore, we examined the role of [Ca<sup>2+</sup>]<sub>i</sub> in the activation of SOK-1 by chemical anoxia. When [Ca<sup>2+</sup>]<sub>i</sub> was buffered by incubation of cells with BAPTA-AM (75 μM) for 60 min before the addition of CN/2-DG for 40 min, activation of SOK-1 was completely inhibited (Fig. 5A, top). Incubation of cells with BAPTA-AM alone had no effect on SOK-1 activity. Preincubation of cells with EGTA (2 mM) for 10 min also markedly inhibited activation of SOK-1 by CN/2-DG (Fig. 5A, bottom). This suggests extracellular Ca<sup>2+</sup> is important for SOK-1 activation. However, the prolonged (50 min) exposure to extracellular EGTA likely also resulted in some depletion of intracellular stores (29, 30). In addition, compromise of membrane integrity by chemical anoxia may have allowed extracellular EGTA to buffer [Ca<sup>2+</sup>]<sub>i</sub>, and thus to function similarly to BAPTA.

Although it is not clear whether intracellular stores, extracellular Ca<sup>2+</sup>, or both are necessary for the increase in [Ca<sup>2+</sup>]<sub>i</sub>, the data clearly suggested that the increase in [Ca<sup>2+</sup>]<sub>i</sub> was required for the activation of SOK-1 by chemical anoxia. Therefore, we determined whether the Ca<sup>2+</sup> ionophore, A23187 (2 μM), or thapsigargin (1 μM), which releases ER Ca<sup>2+</sup> stores by selectively inhibiting the ER Ca<sup>2+</sup>-ATPase, activated SOK-1. Although neither compound alone activated SOK-1, preincubation of cells with either agent markedly enhanced SOK-1 activation by chemical anoxia (Fig. 5B, top and bottom). If, however, EGTA (2 mM) was included in the preincubation with A23187, thus buffering any increase in [Ca<sup>2+</sup>]<sub>i</sub>, activation of
SOK-1 by CN/2-DG was completely prevented. These data suggest that an increase in [Ca^{2+}]_{i} markedly amplifies the activation of SOK-1 by chemical anoxia. The increase in [Ca^{2+}]_{i} is necessary but not sufficient for activation of SOK-1. Furthermore, it is the increase in [Ca^{2+}]_{i} and not depletion of intracellular Ca^{2+} pools that is the critical signal for SOK-1 activation, since A23187, which releases intracellular Ca^{2+} stores, was ineffective when [Ca^{2+}]_{i} was buffered.

**DISCUSSION**

*The Ste20 Family of Protein Kinases*

Epistasis analyses suggest that Ste20 is upstream of a MAP kinase in a cascade consisting of a MAP kinase kinase kinase (MAPKKK) (Ste11), a MAPKK (Ste7), and two MAP kinases (Kas1 and Fus3) in the pheromone response pathway of *Saccharomyces cerevisiae* (31–34). This pathway is activated by binding of mating factors to their cognate serpentine receptors, which are linked to heterotrimeric G proteins. Ste20 can also be activated by binding to the small G protein, Cdc42. The p21-activated kinases (PAKs) are human homologs of Ste20 and are activated by binding to GTP-loaded Rac and Cdc42Hs via a conserved sequence motif in the N-terminal regulatory domain of the kinases (35–37). Like Ste20, the PAKs are activated by ligands, including thrombin and the chemoattractant, f-Met-Leu-Phe, which bind to receptors linked to heterotrimeric G proteins (38). Downstream targets of the PAKs include the MAP kinases, p38, and the SAPKs (39–41).

A second group of Ste20-like kinases are not functional homologs of Ste20, have no Rac/Cdc42 binding domain, and, in contrast to Ste20 and the PAKs, have an N-terminal catalytic domain. They do, however, share a high degree of amino acid sequence identity with Ste20 and a Ste20-like kinase, Sps1 (42), indicating they too have been conserved throughout evolution. Three members of this group have been demonstrated to activate MAP kinase cascades, suggesting they are equivalent in this respect to the Ste20 proteins and PAKs. Specifically, germinal center kinase, hematopoietic progenitor kinase (HPK1), and Nck-interacting kinase activate the SAPKs via a Rac/Cdc42-independent mechanism (11, 43, 44). Physiologic activators of this family of Ste20-like kinases are not well

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**FIG. 3. Effect of CN/2-DG and antioxidant treatments on cellular oxidant stress.** Subconfluent LLC-PK_{1} cells on glass coverslips were loaded with DCFH-DA as described under “Experimental Procedures.” After a 2-min recording of base-line fluorescence intensity, cells were exposed to vehicle (A) or CN/2-DG (B). Intensity of DCF fluorescence (excitation 505 and 450 nm; emission > 515 nm) was determined over 45 min. The curve shown is the ratio of fluorescence intensity at excitation 505 nm over intensity at excitation 450 nm. Other LLC-PK_{1} cells were pretreated with PDTTC (100 μM, 120 min) (C) or sodium pyruvate (50 mM, 30 min) (D) before CN/2-DG. In C and D, the abrupt rise in the ratio at approximately 42 min is due to the addition of H_{2}O_{2} (3 mM). This was done to confirm that the cells could respond to oxidant stress with an increase in the 505/450 ratio.
Activation of SOK-1 by Chemical Anoxia

Mechanisms of Activation of SOK-1

Role of Oxidant Stress—Consistent with our prior studies, which suggested that oxidant stress was a relatively specific activator of SOK-1 (9), we found that chemical anoxia-induced activation of SOK-1 appeared to be dependent upon generation of reactive oxygen species. Two unrelated redox-active agents, the dithiocarbamate, PDTC (17), and sodium pyruvate, which scavenges $H_2O_2$ (19), markedly reduced CN/2-DG-induced cellular oxidant stress (as determined by DCF fluorescence) and inhibited activation of SOK-1. NDGA also markedly inhibited activation of SOK-1 by chemical anoxia. The iron chelator, deferoxamine, and Me$_2$SO, which readily scavenges hydroxyl radical, also inhibited CN/2-DG-induced activation of SOK-1, although the inhibition was less marked than with the other three agents. Exogenously added catalase, which is taken up by many types of cells in culture and can, therefore, scavenge cytosolic $H_2O_2$ (13, 23), also inhibited SOK-1 activation by chemical anoxia. Inhibition of SOK-1 activation by six chemically unrelated scavengers of ROS strongly suggests SOK-1 activation by chemical anoxia requires generation of ROS and the resultant cellular oxidant stress.

Most redox-active agents that limit cellular oxidant stress, including those used in this study, have additional effects on the cell which could account for the observed reduction in SOK-1 activation by CN/2-DG. This is the principal reason behind our choice of six unrelated agents. For example, while chelation of iron by deferoxamine will have effects on the cell beyond reduction in ROS production, the inhibition by the other agents of SOK-1 activation cannot be ascribed to iron chelation.

We believe the similar time courses of CN/2-DG-induced generation of ROS and activation of SOK-1, and the inhibition of SOK-1 activation (and reduction in the level of cellular oxidant stress) by unrelated ROS “scavengers,” especially when viewed in light of our previous work demonstrating direct activation of the kinase by $H_2O_2$ and by menadione, strongly suggests production of ROS by the electron transport chain is the primary mechanism of activation of SOK-1 by chemical anoxia. We did consider alternative mechanisms of activation of SOK-1, in addition to generation of ROS, which occur after chemical anoxia. ATP depletion per se is not likely to be the signal that activates SOK-1 since exposure of cells to FCCP plus 2-DG, which produced equivalent ATP depletion to that seen with CN/2-DG, did not activate SOK-1. Although not sufficient, ATP depletion may be necessary for CN/2-DG-induced activation of SOK-1 since activation was blocked if cells were incubated with CN/2-DG in the presence of glucose. These data suggest ATP depletion may be acting via secondary mechanisms to enhance SOK-1 activation by chemical anoxia, and the mechanism may, in part, be related to the inability of ER and cell membrane Ca$^{2+}$-ATPases to reduce [Ca$^{2+}$], (see below).

We also considered whether changes in intracellular pH could play a role in the chemical anoxia-induced activation of SOK-1 (45). To explore this possibility, LLC-PK$_1$ cells were incubated in media of pH 6.9, 7.4, or 7.9, and SOK-1 activity was assayed before and after CN/2-DG. Acidification or alkalization of media causes a corresponding change in intracellular pH (46). We found no effect of pH on either basal activity or activation of SOK-1 (data not shown).

Role of Increased [Ca$^{2+}$]i—A prominent early feature of chemical anoxia is a disruption of Ca$^{2+}$ homeostasis. [Ca$^{2+}$], begins to increase as early as 5–10 min after the onset of described. TNF$\alpha$ activates germinal center kinase (43), but no other physiologically relevant activators of this group are known and, thus, their role in the cell remains unclear. In yeast, most of the known MAP kinase cascades are not activated by ligand-receptor interactions, but by environmental stresses such as osmolar stress, heat, and nutritional starvation (31). We have recently reported the cloning and characterization of a Ste20-like kinase from the germinal center kinase group (9). This kinase, SOK-1, does not appear to be activated by ligand-receptor interactions, but is activated by oxidant stress and thus appears to be more similar in function to the SAPKs. Thus, activation of SOK-1 is not a consequence of impending cell death.

To better define the role that this kinase may play in the response to pathophysiologic processes, we examined whether it was activated by chemical anoxia. We found that SOK-1 was markedly stimulated by chemical anoxia. In distinct contrast to the SAPKs, we found that SOK-1 was activated during the period of chemical anoxia, but activity rapidly returned to control levels when cells were re-exposed to glucose. Thus, during chemical anoxia and recovery from it, SOK-1 appears to be “on” when the SAPKs are “off” and vice versa (7). These data are consistent with our observations that SOK-1 does not activate the SAPKs when plasmids encoding both kinases are transfected into COS7 cells (43).

It is important to note that at 20–40 min of chemical anoxia, when SOK-1 activity is greatest, damage to the cells is largely reversible and the vast majority of cells are viable (12). Thus,
chemical anoxia (47, 48). The production of reactive oxygen intermediates may cause a leak of Ca\(^{2+}\) from stores in the ER or other intracellular compartments, and an influx of extracellular Ca\(^{2+}\) (25–27, 49). ATP depletion can be expected to exacerbate any increase in \([\text{Ca}^{2+}]_{i}\) by preventing cell membrane and ER Ca\(^{2+}\) pumps from restoring \([\text{Ca}^{2+}]_{i}\) homeostasis. Our data suggest that the chemical anoxia-induced disruption in Ca\(^{2+}\) homeostasis is critical to the activation of SOK-1. Although an increase in \([\text{Ca}^{2+}]_{i}\) alone was not sufficient for activation of SOK-1, it was necessary for activation of SOK-1 by chemical anoxia and that activation was dramatically enhanced by increasing \([\text{Ca}^{2+}]_{i}\) with either A23187 or thapsigargin. These data suggest that dual inputs of generation of reactive oxygen species and an increase in \([\text{Ca}^{2+}]_{i}\) are required for activation of SOK-1 by chemical anoxia. With this model, oxidant stress alone, especially if it is mild, may not lead to activation of SOK-1 since it may not lead to an increase in \([\text{Ca}^{2+}]_{i}\). For example, although low concentrations of \(\text{H}_2\text{O}_2\) partially empty ER Ca\(^{2+}\) stores, they do not increase \([\text{Ca}^{2+}]_{i}\), presumably because the cell membrane Ca\(^{2+}\) pumps are able to restore homeostasis (25). The activation of SOK-1 by chemical anoxia may be so marked because in addition to oxidant stress, Ca\(^{2+}\) homeostasis is disrupted.

There are many possible mechanisms whereby increases in \([\text{Ca}^{2+}]_{i}\), may exert their effects. First, \([\text{Ca}^{2+}]_{i}\), could have a direct effect on the kinase. This is unlikely, however, since neither thapsigargin nor A23187 activated SOK-1 in the absence of CN/2-DG. In addition, performing SOK-1 kinase assays in standard assay buffer (very low \([\text{Ca}^{2+}]_{i}\)) or high \([\text{Ca}^{2+}]_{i}\) buffer (2 mM \(\text{CaCl}_2\)) had no effect on kinase activity (data not shown). Second, the increase in \([\text{Ca}^{2+}]_{i}\), caused by chemical anoxia likely leads to an increase in \([\text{Ca}^{2+}]_{i}\) in the mitochondria via uptake by the Ca\(^{2+}\) uniporter down an electrochemical gradient. An increase in mitochondrial \([\text{Ca}^{2+}]_{i}\) has been postulated to enhance generation of reactive oxygen species and formation of hydroperoxides (50). Thus, the increase in \([\text{Ca}^{2+}]_{i}\), could exert its effect on SOK-1 activation by enhancing CN2-DG-induced production of reactive oxygen species by the electron transport chain, thus worsening oxidant stress. It seems equally, if not more, likely that the increase in \([\text{Ca}^{2+}]_{i}\), synergizes with reactive oxygen species and ATP depletion to enhance activation of (or damage to) a putative sensor which effects the activation of SOK-1 (Fig. 6).
Ca\textsuperscript{2+} membrane integrity, allowing entry of signaling molecules, including cPLA\textsubscript{2}, might hasten, and the cell are minimized. Only recently has it become apparent death unless the inciting stimuli are removed or their effects on death has often been viewed as an inexorable progression to apoptotic death (51–55). In contrast, necrotic cell ways may be activated in the cell which modulate the participant in the progression to death. Furthermore, it has re-

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27. McCoy, C. E., Selvaggio, A. M., Alexander, E. A., and Schwartz, J. H. (1988) *J. Clin. Invest.* 82, 1326–1332
28. Josephson, R. A., Silverman, H. S., Lakatta, E. G., Stern, M. D., and Zweier, J. L. (1991) *J. Biol. Chem.* 266, 2354–2361
29. Force, T., Hyman, G., Hajjar, R., Sellmayer, A., and Bonventre, J. V. (1991) *J. Biol. Chem.* 266, 4295–4302
30. Force, T., Bonventre, J. V., Flannery, M. R., Gorn, A. H., Yamin, M., and Goldring, S. (1992) *Am. J. Physiol.* 262, F1110–F1115
31. Herskowitz, I. (1995) *Cell* 80, 187–197
32. Elion, E. A. (1995) *Trends Biochem. Sci.* 5, 322–327
33. Levin, D., and Errede, B. (1995) *Cell Biol.* 7, 197–202
34. Leberer, E., Dignard, D., Harcus, D., Thomas, D. Y., and Whiteway, M. (1993) *EMBO J.* 11, 4815–4824
35. Manser, E., Leung, T., Salihuddin, H., Zhao, Z., and Lim, L. (1994) *Nature* 367, 40–46
36. Burbelo, P. D., Drechsel, D., and Hall, A. (1995) *J. Biol. Chem.* 270, 29071–29074
37. Martin, G. A., Bollag, G., McCormick, F., and Abo, A. (1995) *EMBO J.* 14, 1970–1978
38. Kraus, U., Morris, S., Dong, H. J., Chernoff, J., and Bokoch, G. M. (1995) *Science* 269, 221–223
39. Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J., and Bokoch, G. M. (1995) *J. Biol. Chem.* 270, 23934–23936
40. Pulverino, A., Frost, J., Yang, P., Hutchinson, M., Neiman, A. M., Cobb, M. H., and Marcus, S. (1995) *J. Biol. Chem.* 270, 26067–26070
41. Kyriakis, J. M., and Avruch, J. (1996) *J. Biol. Chem.* 271, 24313–24316
42. Friessen, H., Lanza, R., Doyle, S., and Segall, J. (1994) *Genes Dev.* 8, 2162–2175
43. Pombo, C., Kehrli, J., Sanchez, I., Katz, P., Avruch, J., Zon, L., Woodgett, J., Force, T., and Kyriakis, J. M. (1995) *Nature* 377, 755–758
44. Su, Y. C., Han, J., Xu, S., Cobb, M., and Skolnick, E. Y. (1997) *EMBO J.* 16, 1279–1290
45. Fish, E. M., and Molitoris, B. A. (1994) *Am. J. Physiol.* 267, F566–F572
46. Bonventre, J. V., and Cheung, J. Y. (1985) *Am. J. Physiol.* 249, C149–C159
47. Carroll, J. M., Toral-Barza, L., and Gibson, G. (1992) *J. Neurosci.* 59, 1836–1843
48. Dubinsky, J. M., and Rothman, S. M. (1991) *J. Neurosci.* 11, 2545–2551
49. Lam, M., Dubyk, G., Chen, L., Nusin, G., Miesfeld, R. L., and Distelhorst, C. W. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 6569–6573
50. van de Water, B., Zoeteweij, J. P., de Bont, H. J. G. M., Mulder, G. J., and Nagelkerke, J. F. (1994) *J. Biol. Chem.* 269, 14546–14552
51. Xia, Z., Dickens, M., Rainaurod, J., Davis, R. J., and Greenberg, M. E. (1995) *Science* 270, 1332–1331
52. Verheij, M., Bose, R., Hua Lin, X., Yao, B., Jarvis, W. D., Grant, S., Bjerre, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Fuku, Z., and Kolesnik, R. N. (1996) *Nature* 380, 75–79
53. Johnson, N. L., Gardner, A. M., Diener, K. M., Lange-Carter, C. A., Galey, J., Jarpe, M. B., Minden, A., Kerin, M., Zon, L. I., and Johnson, G. L. (1996) *J. Biol. Chem.* 271, 3229–3237
54. Cleveland, J. L., and Ihle, J. N. (1995) *Cell* 81, 479–482
55. Shimon, S., Eguchi, Y., Kosaka, H., Kaniike, W., Matsuda, H., and Tsujimoto, Y. (1995) *Nature* 374, 811–813
56. Jacobson, M. D., and Raff, M. C. (1995) *Nature* 374, 814–816
57. Hockenbery, D. M., Oltval, Z. N., Yin, X. M., Milliman, C. L., and Korsmeyer, S. J. (1993) *Cell* 75, 241–251
58. Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T., and Bredesen, D. E. (1993) *Science* 262, 1274–1277
59. Baffy, G., Miyashita, T., Williamsen, J. R., and Reed, J. C. (1993) *J. Biol. Chem.* 268, 6511–6519
60. Muscat, R. J., Bernard, E. J., Garza, L., McKenna, W. G., and Koch, C. J. (1995) *Cancer Res.* 55, 995–998