

Link between Mitochondria and NADPH Oxidase 1 Isozyme for the Sustained Production of Reactive Oxygen Species and Cell Death*

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Although mitochondria and the Nox family of NADPH oxidase are major sources of reactive oxygen species (ROS) induced by external stimuli, there is limited information on their functional relationship. This study has shown that serum withdrawal promotes the production of ROS in human 293T cells by stimulating both the mitochondria and Nox1. An analysis of their relationship revealed that the mitochondria respond to serum withdrawal within a few minutes, and the ROS produced by the mitochondria trigger Nox1 action by stimulating phosphoinositide 3-kinase (PI3K) and Rac1. Activation of the PI3K/Rac1/Nox1 pathway was evident 4–8 h after but not earlier than serum withdrawal initiation, and this time lag was found to be required for an additional activator of the pathway, Lyn, to be expressed. Functional analysis suggested that, although the mitochondria contribute to the early (0–4 h) accumulation of ROS, the maintenance of the induced ROS levels to the later (4–8 h) phase required the action of the PI3K/Rac1/Nox1 pathway. Serum withdrawal-treated cells eventually lost their viability, which was reversed by blocking either the mitochondria-dependent induction of ROS using rotenone or KCN or the PI3K/Rac1/Nox1 pathway using the dominant negative mutants or small interfering RNAs. This suggests that mitochondrial ROS are essential but not enough to promote cell death, which requires the sustained accumulation of ROS by the subsequent action of Nox1. Overall, this study shows a signaling link between the mitochondria and Nox1, which is crucial for the sustained accumulation of ROS and cell death in serum withdrawal-induced signaling.

Reactive oxygen species (ROS)² such as H₂O₂ and O₂^{•−} act as key mediators of the cellular signaling induced by the ligation of

the cell surface receptors as well as by many classes of environmental agents (1–3). Cell stimulation by such agents has been shown to increase the cellular ROS levels, which regulate various cellular functions such as growth, differentiation, migration, and viability. Therefore, to better understand the regulatory mechanisms of diverse cell functions, it is essential that the cellular processes that lead to the induction of ROS be identified.

The mitochondria and the Nox family of NADPH oxidase have emerged as major sources of ROS induction (4, 5). The mitochondria generate ROS as byproducts of respiration, and inhibitors of the mitochondrial respiratory chain, such as rotenone and KCN, have been shown to attenuate the ability of hormones and cytokines to promote the production of ROS in various cell types (6, 7). Confocal microscopy has consistently shown increased mitochondrial ROS levels as a response to cell stimulation (7, 8). NADPH oxidase is a membrane enzyme that is responsible for the oxidative burst induced in activated phagocytes (9, 10). The proposed model suggests that phagocyte stimulation by fMLP results in the activation of phosphoinositide 3-kinase (PI3K), which then triggers the translocation of Rho GTPase Rac from the cytosol to the plasma membrane. The translocated Rac then interacts with and activates NADPH oxidase to generate ROS. Recent reports suggest that non-phagocytic cells can express isoforms of the catalytic subunit (Nox2) of NADPH oxidase, such as Nox1, -3, -4, and -5 (11–13). Although the physiological roles of these isozymes and the signaling events leading to their activation are largely unknown, Nox1 was shown to be involved in the production of ROS in the smooth muscle and epithelial cells induced by growth factors, such as angiotensin II and platelet-derived growth factor (14, 15). It was also reported that PI3K and Rac are involved in the platelet-derived growth factor-induced Nox1 stimulation in a mechanism similar to that proposed for phagocytic type Nox2 stimulation (15). However, Nox1 and Nox2 appear to use different Rac isoforms for their stimulation. Although the hematopoietic cell-specific Rac2 is believed to be the major isoform involved in Nox2 stimulation (10, 16–18), Nox1 stimulation was induced by an interaction with the ubiquitously expressed protein Rac1 (15, 19, 20). However, despite the proposed roles of the tested Nox family members and the mitochondria in the induction of ROS, there is little information available regarding the functional relationship between the two different types of ROS sources.

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² The abbreviations used are: ROS, reactive oxygen species; PI3K, phosphoinositide 3-kinase; PI3K-M, dominant negative mutant of PI3K; siRNA, small interfering RNA; LY, LY294002; DHR, dihydrorhodamine 123; CHX, cycloheximide; AD, actinomycin D; fMLP, formylmethionylleucylphenylalanine.

Serum withdrawal is a stimulus that can induce apoptosis by elevating the cellular ROS levels (21). Interestingly, it was proposed that serum withdrawal could induce the accumulation of ROS in a single cell type through two different mechanisms, PI3K-dependent and -independent pathways. This is based on the observation from human U937 monocytic cells, which showed that serum withdrawal begins to induce ROS prior to PI3K activation, and the induced ROS then activates PI3K. The activated PI3K was shown to promote the cellular process leading to the further accumulation of ROS (22). Therefore, the PI3K in this model does not simply mediate the stimulus-induced accumulation of ROS but amplifies and/or extends the ROS induction process. Given the unique features of serum withdrawal, further analysis of serum withdrawal-induced signaling was expected to provide new insights into how the accumulation of ROS is induced and regulated. Therefore, this study investigated the sources of serum withdrawal-induced ROS along with their functional relationship. To accomplish this, this study used human embryonic kidney 293T cells, which have a much higher efficiency of DNA transfection and RNA interference than U937 cells. The results show that both the mitochondria and Nox1 are involved in the serum withdrawal-induced accumulation of ROS in a single cell type. Importantly, it was found that they do not act independently but rather function in a cooperative manner to extend the production of ROS. The mechanism and functional significance of this phenomenon are discussed.

EXPERIMENTAL PROCEDURES

Antibodies

Antibodies raised against the p85 subunit of PI3K, Myc, and Lck were obtained from Upstate Biotechnology (Lake Placid, NY). Pharmingen/Transduction Laboratories (San Diego, CA) supplied the antibodies against Rac1, Lyn, and Hck. Anti-Ras and anti- α -tubulin were purchased from Calbiochem (La Jolla, CA). All of the other antibodies used in this study were supplied by Santa Cruz Biotechnology (Santa Cruz, CA).

Expression Constructs

The hemagglutinin-tagged dominant negative mutant of PI3K (PI3K-M) cloned in the pSR α 2 vector and the Myc-tagged dominant negative mutant of Rac1 (Rac1-M) in the pEXV vector (23) were generous gifts from Drs. L. C. Cantley (Harvard Medical School) and A. Hall (University College London), respectively. The following specific sequences of the 19 nucleotides were selected for the synthesis of small interfering RNAs (siRNAs): 5'-CCAGGATTGAAGTGGATGG-3' (residues 1130–1148 of the human Nox1 cDNA; 5'-GCCACCAATCTGAAGCTCA (residues 1291–1309 of the human Nox2), and 5'-GTCAACATCCAGCTGTACC-3' (residues, 1474–1492 of the human Nox4 cDNA) (24). As a negative control, the universal control sequence 5'-AGTTCAACGACCAGTAGTC-3', which has no significant homology to any known mammalian genome, was used (25). These oligonucleotides were cloned into the pSUPER vector (Oligoengine, Seattle, WA) according to the manufacturer's protocol.

Cell Culture, Transfection, and Treatments

The 293T cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and gentamicin (50 μ g/ml). The cells were transiently transfected using Lipofectamine 2000 (Invitrogen) (26) with the indicated expression construct or the chemically synthesized Lyn siRNA and its control RNA (Ambion, Austin, TX). To treat the 293T cells/transfectants, they were plated at 1×10^5 cells/ml, allowed to adhere overnight, washed, and then given serum-free medium. Where specified, LY294002 (LY) (Calbiochem), catalase, and inhibitors of the mitochondrial respiratory chain were added at the indicated concentrations.

Analysis of Cellular Viability

Treated and untreated control cells were stained with propidium iodide (5 μ g/ml) followed by flow cytometry analysis to monitor their staining intensity and size. The cells displaying both a normal size and a low permeability to propidium iodide were understood to be viable, as previously defined (27). All other populations were understood to be dead.

Analysis of Cellular ROS Levels

Cellular ROS levels were analyzed using the following two different methods.

Flow Cytometry—Treated and untreated cells were exposed to 50 μ M 2',7'-dichlorofluorescein diacetate (Molecular Probes, Eugene, OR) for 5 min, and cell-associated levels of 2',7'-dichlorofluorescein fluorescence were analyzed by flow cytometry (28).

Confocal Microscopy—Cells were treated with 10 nM Mito-tracker Red CMXRos (Molecular Probes) and incubated for 10 min at room temperature. Dihydrorhodamine 123 (DHR, 10 μ M) was then added, incubation was continued for 5 min, and cells were washed three times with phosphate-buffered saline. The confocal images were acquired and analyzed using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany).

PI3K Assay

Cells were lysed as described previously (22). Equal amounts of the lysate proteins (400 μ g) were immunoprecipitated with anti-p85. The immune complexes were washed and resolved in 20 mM HEPES, pH 7.4, 5 mM MnCl₂, 10 μ M ATP, 10 μ Ci [γ -³²P]ATP, and 2.5 mM EGTA. L- α -phosphatidylinositol (Sigma) (5 mg/ml) was used to initiate the kinase reactions. After 20 min of incubation, the reactions were quenched by adding 1 M HCl. The phospholipids were extracted using a 1:1 mixture of chloroform and methanol and separated by thin-layer chromatography.

Western Blot Analysis

The cells were lysed in a buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 20 mM β -glycerophosphate, 5 mM NaF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, and 100 μ M phenylmethylsulfonyl fluoride. Equal amounts of the proteins (50–100 μ g) were separated by SDS-PAGE and then electrotransferred to the Immobilon

membranes (Millipore, Bedford, MA), which were subsequently blotted using the indicated antibodies and visualized by chemiluminescence (ECL; Amersham Biosciences AB, Uppsala, Sweden).

Analysis of Rac1 Translocation and Its Binding to Nox1

The cells were resuspended in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml leupeptin and lysed by sonication. The lysates were then centrifuged at $100,000 \times g$ for 1 h at 4 °C. The pellet was resuspended in the above buffer, which also contained 0.1% Triton X-100, lysed by sonication, and centrifuged again at $100,000 \times g$ for 1 h at 4 °C to obtain the membrane fraction (supernatant). The Rac1 levels in these membrane fractions were analyzed by Western blotting to determine the extent of Rac1 translocation to the membrane. To determine the binding of the translocated Rac1 to Nox1, the membrane proteins were immunoprecipitated with anti-Nox1, and the levels of Rac1 in the precipitates were analyzed by Western blotting.

Statistics Analysis

Cellular viability and ROS levels were analyzed at least three times to obtain means \pm S.D. Results were analyzed for statistical significance using Student's *t* test. Differences were considered significant at *p* < 0.05.

RESULTS

Responses of 293T Cells to Serum Withdrawal—It was reported that serum withdrawal killed U937 cells by inducing the accumulation of ROS and PI3K activation (22). To confirm this in 293T cells, these cells were grown in 10% serum, washed, and exposed to serum-free medium. The treatment resulted in a dramatic increase in the cellular ROS levels, which was observed within 5 min of serum withdrawal and persisted for \sim 8 h without significant alterations (Fig. 1A). After this time, the ROS levels decreased gradually, reaching their original base at 16 h. Serum withdrawal did not re-induce the accumulation of ROS up to 72 h. The addition of catalase, an H_2O_2 -degrading enzyme, to serum-free medium greatly reduced the serum withdrawal-induced accumulation of ROS and cell death (data not shown), suggesting that ROS mediate serum withdrawal-induced 293T cell death. Serum withdrawal also induced the activation of PI3K in 293T cells, which in contrast to the induction of ROS, was evident at 4–8 h of serum withdrawal initiation, but not earlier (2.5 min–2 h) (Fig. 1B). Such a delay in PI3K activation was also reported when rat pheochromocytoma cells were exposed to hypoxic conditions (29). The serum withdrawal-induced activity of PI3K was abolished when PI3K-M was expressed in 293T cells (Fig. 1C, top). Although this treatment did not significantly influence the induction of ROS within 2 h of serum withdrawal, the later period (4–8 h) of ROS accumulation was effectively reduced by PI3K-M expression (Fig. 1A). Similar results were obtained using pharmacologic inhibitors for PI3K, such as LY (5–10 μ M) and wortmannin (0.25–1 μ M) (data not shown). Therefore, it appears that serum withdrawal increases the levels of ROS in 293T cells in both a PI3K-dependent and -independent manner, and that the latter

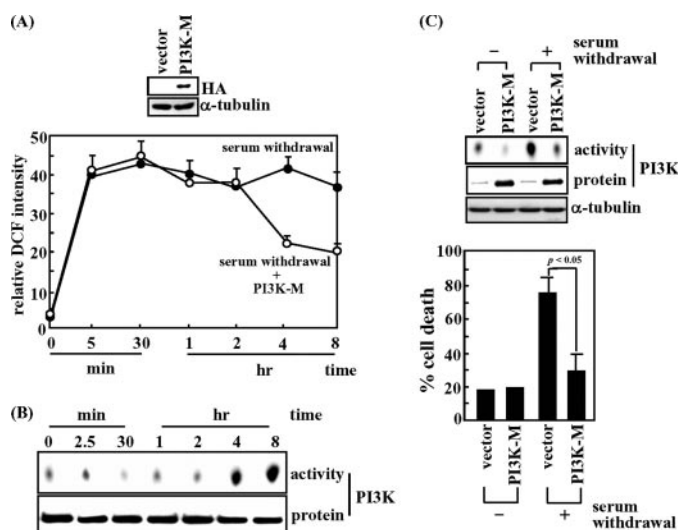


FIGURE 1. Serum withdrawal elevates the levels of ROS in 293T cells in two different ways. A, 293T cells were transfected with either the empty pSRA2 vector or the vector encoding hemagglutinin-tagged PI3K-M. The expression of the transfected gene was confirmed by Western blotting with anti-hemagglutinin, using α -tubulin as the loading control (top). Transfectants grown in 10% serum were washed and exposed to serum-free medium. At the end of the indicated times of incubation, the cellular ROS levels were compared by flow cytometry using 2',7'-dichlorofluorescein fluorescence. The values shown are the means of triplicate experiments; the error bars represent the S.D. (bottom). The untransfected 293T cells displayed a response similar to the control transfectants. B, the 293T cells were treated with serum-free medium for the indicated periods, lysed, and PI3K was immunoprecipitated with anti-p85. The PI3K activities in the precipitates were analyzed using L- α -phosphatidylinositol as a substrate (top). The levels of its p85 subunit in the samples were compared by Western blotting with anti-p85 (bottom). C, the control and PI3K-M-expressing transfectants were exposed to serum-free medium for 8 h. The PI3K activity and levels of its p85 subunit were analyzed (top). Alternatively, the cell viability was analyzed 72 h after initiating serum withdrawal (bottom).

mechanism precedes the former, as suggested for U937 cells (22). PI3K-M (Fig. 1C, bottom) and PI3K inhibitors also rescued the 293T cells from serum withdrawal, which suggests that the PI3K-dependent late phase of ROS accumulation is essential for the lethality of serum withdrawal.

Rac1 and Nox1 Are Involved in PI3K-dependent ROS Accumulation—Given the ability of PI3K to stimulate certain members of the Nox family (9, 15), this study focused on Nox isozymes to determine the sources of PI3K-induced ROS in this system. Western blotting showed that the 293T cells expressed Nox1, -2, and -4 (Fig. 2A). The expression of Nox1 and Nox4 in these cells has also been reported (15, 30). In contrast, real-time PCR experiments showed that there was little or no Nox3 and -5 expression in 293T cells (data not shown). To determine whether the Nox isozymes expressed were essential for serum withdrawal-induced ROS accumulation, the cellular levels of Nox1, -2, and -4 were specifically reduced by RNA interference (Fig. 2A). These cells and the cells that received the control RNAs were exposed to serum-free medium and incubated either for 30 min or 8 h to examine the early and late stages of ROS accumulation, respectively. Although both phases of ROS accumulation were not significantly influenced by reducing either the Nox2 or -4 levels, a decrease in the Nox1 levels resulted in a significant decrease in the induction of ROS 8 h (but not 30 min) after serum withdrawal (Fig. 2B). Serum withdrawal-induced 293T cell death was consistently attenuated

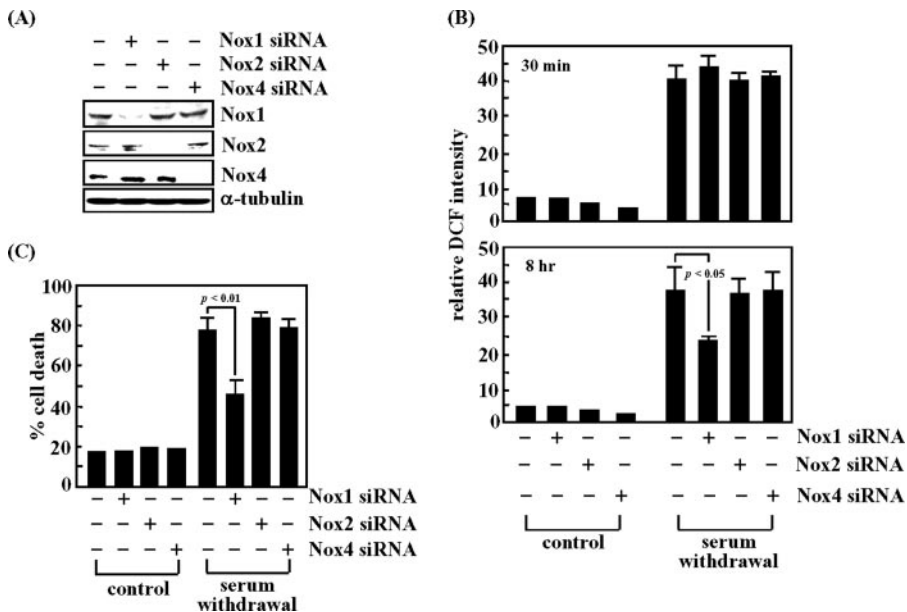


FIGURE 2. Nox1 is involved in the late stage of ROS accumulation. A, the 293T cells were transfected with the pSUPER vectors encoding either a nonspecific control RNA or the siRNA specific to Nox1, -2, or -4. The levels of Nox1, -2, and -4 in these transfectants were compared by Western blotting. B, the transfectants were exposed to serum-free medium, and the cellular ROS levels were analyzed at 30 min and 8 h after treatment. C, the treatment was extended to 72 h, and the cellular viability was determined.

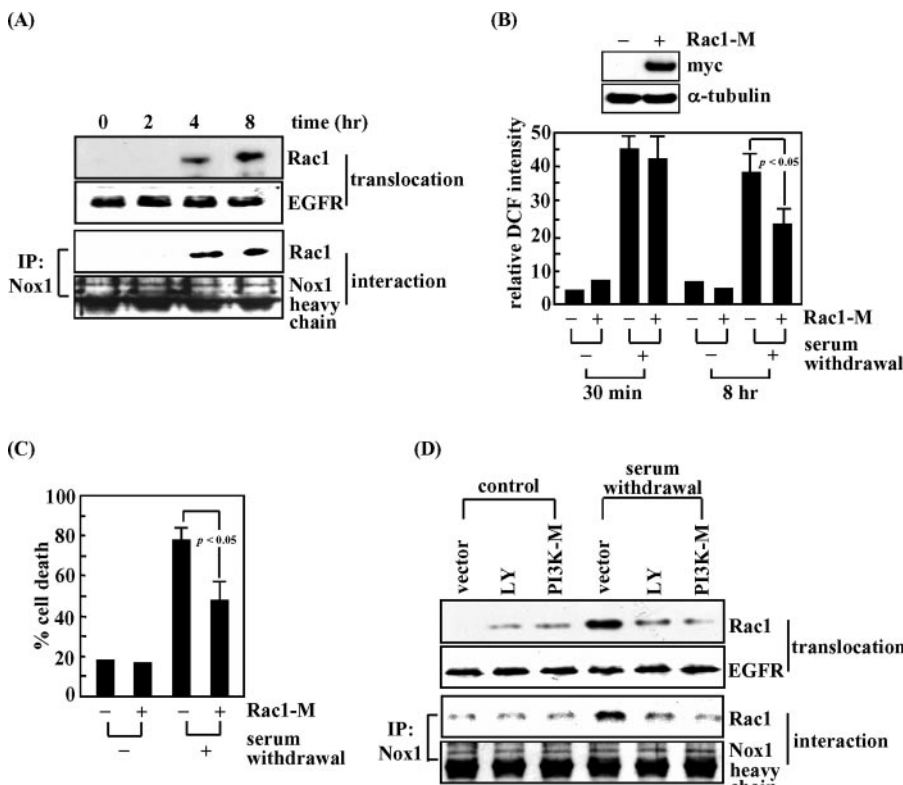


FIGURE 3. Role of Rac1 and Nox1 in the PI3K-dependent induction of ROS. A, the 293T cells were treated with serum withdrawal for the indicated periods. The cells were lysed, and the membrane fractions were prepared. The levels of Rac1 in the fractions were compared by Western blotting using the EGF receptor as a loading control (top two panels). Nox1 in the fractions was immunoprecipitated (IP), and levels of Rac1 in the precipitates were analyzed by Western blotting (bottom two panels). B, the 293T cells were transfected with the pEXV vector with (+) or without (–) the Myc-tagged Rac1-M. The expression of the cloned gene was confirmed by Western blotting with anti-Myc (top). The transfectants were serum-deprived for either 30 min or 8 h and analyzed for their ROS levels (bottom). C, the indicated transfectants were exposed to serum-free medium for 72 h and analyzed for their viability. D, the control and PI3K-M-expressing transfectants were treated with serum withdrawal for 8 h. LY was added where indicated. The membrane fractions were prepared, and the levels of Rac1 in the fractions and Rac1/Nox1 interaction were analyzed.

when the Nox1 levels (but not Nox2 and -4) were reduced (Fig. 2C). Therefore, Nox1 appears to be the major Nox isozyme involved in the serum withdrawal-induced accumulation of ROS. These results also suggest that Nox1 acts selectively in the late phase in a similar manner to PI3K.

In the case of growth factor-induced signaling, Rac1 was reported to translocate from the cytosol to the plasma membrane and interact with and stimulate Nox1 (15). To investigate the role of Rac1 in this system, membrane fractions were prepared from the cells treated with serum withdrawal for various periods. Western blot analysis of the samples showed that serum withdrawal induced the translocation of Rac1 to the membrane fractions (Fig. 3A, top two panels). Rac1 in these fractions was co-immunoprecipitated with Nox1, suggesting an interaction (Fig. 3A, bottom two panels). Interestingly, both Rac1 translocation and its interaction with Nox1 were observed at 4–8 h (but not earlier), which is similar to that observed with PI3K activation. The introduction of Rac1-M into the 293T cells consistently reduced the accumulation of ROS after 8 h of serum withdrawal (but not at 30 min) (Fig. 3B) and also attenuated serum withdrawal-induced cell death (Fig. 3C). Therefore, Rac1 appears to mediate the late (but not early) stage of ROS accumulation by interacting with Nox1.

Considering that the actions of Rac1 and Nox1 are kinetically associated with those of PI3K, it is likely that the PI3K in this system acts upstream of Rac1 and Nox1. Indeed, either LY or PI3K-M abolished the ability of serum withdrawal to induce the translocation of Rac1 and its interaction with Nox1 (Fig. 3D). Overall, serum withdrawal-activated PI3K appears to induce ROS by stimulating Rac1 and Nox1.

Mitochondria Are Involved in PI3K-independent Early ROS Induction—To determine the ROS source involved in the early phase,

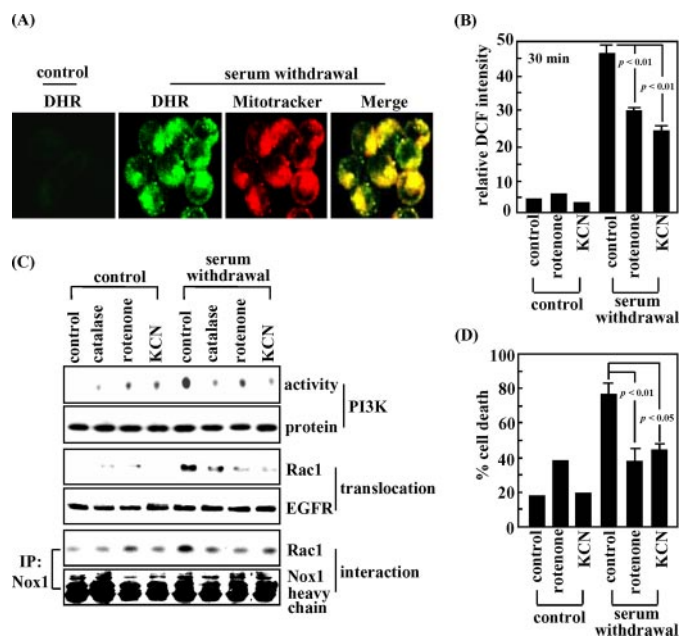


FIGURE 4. Mitochondria are involved in the early stage of ROS accumulation. A, the 293T cells were exposed to serum-free medium for 30 min. The treated and untreated control cells were stained with DHR and Mitotracker Red CMXRos and analyzed by confocal microscopy. B, the cells were treated with serum-free medium for 30 min with or without rotenone (0.1 μ M) or KCN (1 mM). The cellular ROS levels were compared by 2',7'-dichlorofluorescein fluorescence. C, the cells were treated with serum-free medium for 8 h with or without the indicated inhibitors/scavengers. The PI3K activity, levels of Rac1 in the membrane fractions, and the Rac1/Nox1 interaction were analyzed. D, the cells were serum-deprived for 72 h with or without the indicated inhibitors and analyzed for their viability. IP, immunoprecipitated.

293T cells were treated with serum withdrawal for 30 min. The treated and untreated control cells were stained with Mitotracker Red CMXRos and DHR, a ROS-sensitive probe that is localized in the mitochondria (31, 32). Confocal microscopy analysis of the stained cells revealed that serum withdrawal increased the staining intensity of DHR (Fig. 4A, green). The majority of DHR-positive cells became orange when the DHR images were merged with those of Mitotracker Red CMXRos. This shows the cell population with the induced ROS in mitochondria. Moreover, the inhibitors of the mitochondrial respiratory chain, rotenone and KCN, consistently suppressed the serum withdrawal-induced ROS accumulation after 30 min of serum withdrawal (Fig. 4B). This suggests that the ROS induced in the early PI3K-independent phase of serum withdrawal were generated, at least in part, by the mitochondria.

Mitochondrial ROS Are Required for PI3K-dependent ROS Induction—Having determined the actions of both the mitochondria and Nox1 in a single model system, the association between the two was next investigated. Given the ability of ROS to activate PI3K under various experimental conditions (22, 33, 34), it is likely that the ROS induced in the early phase stimulate the late PI3K-dependent events. Indeed, catalase suppressed the ability of serum withdrawal to induce PI3K activation, Rac1 translocation, and the Rac1/Nox1 interaction (Fig. 4C). Similar results were obtained using rotenone or KCN. These inhibitors consistently attenuated the accumulation of ROS not only after 30 min (Fig. 4B) but also at 8 h of serum withdrawal (data not shown) and suppressed serum withdrawal-induced cell death

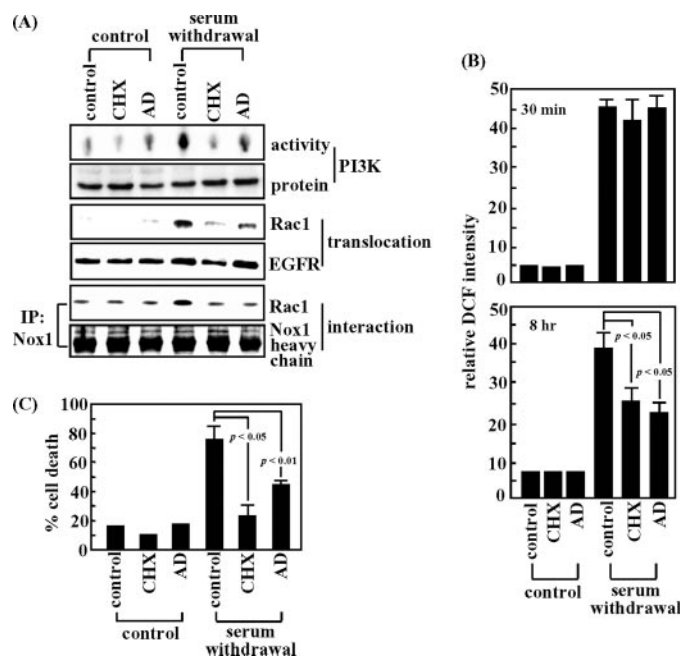


FIGURE 5. PI3K-dependent induction of ROS requires protein synthesis. A, the 293T cells were deprived of serum for 8 h in the presence or absence of CHX (1 μ g/ml) and AD (0.5 μ g/ml). The PI3K activity, levels of Rac1 in the membrane fractions, and Rac1/Nox1 interaction were analyzed. B, the cells were exposed to serum-free medium for either 30 min or 8 h in the presence of the indicated inhibitors and analyzed for their ROS levels. C, the treatments were extended for up to 72 h, and the cell viability was determined. IP, immunoprecipitated.

(Fig. 4D). This suggests that ROS, particularly those originating from the mitochondria, are essential for activating the PI3K/Rac1/Nox1 pathway as well as for accumulating ROS in the late stage and cell death.

PI3K-dependent ROS Induction Requires Protein Synthesis—Nevertheless, PI3K activation in 293T cells was observed \sim 4 h after the mitochondria began to generate ROS (Fig. 1). This time lag might reflect the need to accumulate additional factors for PI3K activation. Cycloheximide (CHX) and actinomycin D (AD), which inhibit protein synthesis and transcription, respectively, were used to examine this possibility. Either inhibitor significantly reduced the ability of serum withdrawal to induce PI3K activation, Rac1 translocation, and the Rac1/Nox1 interaction (Fig. 5A), suggesting that *de novo* protein synthesis is essential for the induction of these events. Both inhibitors consistently attenuated the accumulation of ROS at 8 h but had little effect at 30 min (Fig. 5B) and also suppressed serum withdrawal-induced cell death (Fig. 5C). Therefore, protein synthesis appears to be essential for the late (but not early) stage of ROS accumulation and thus for cell death. Overall, serum withdrawal appears to stimulate the synthesis of a protein crucial for activating the PI3K/Rac1/Nox1 pathway.

Role of Lyn in Serum Withdrawal-induced Signaling—To identify this protein, we first examined PI3K. However, the levels of its p85 and p110 subunits were not increased for up to 8 h after serum withdrawal (Fig. 6A). Because PI3K can be activated in a small G protein (35, 36) or Src family protein kinase (37–39)-dependent manner, the potential roles of these proteins were next investigated. The small G proteins examined included Ras, Rac1, and Cdc42 (Fig. 6B), and the Src family

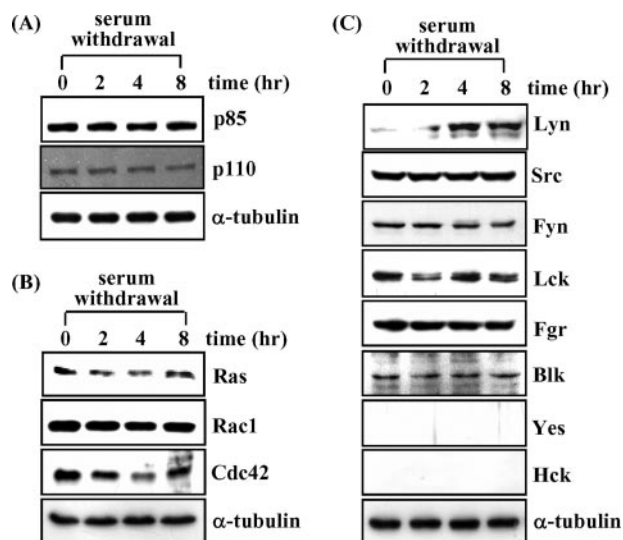


FIGURE 6. **Selective induction of Lyn by serum withdrawal.** The 293T cells were serum-deprived for the indicated periods. Western blot analysis was used to compare the levels of the PI3K subunits (A), small G proteins (B), and Src family protein kinases (C). In the case of Lyn, its two isoforms (p56 and p53) were detected.

protein kinases included Lyn, Src, Fyn, Lck, Fgr, Blk, Yes, and Hck (Fig. 6C). Of these, only the Lyn levels increased in response to serum withdrawal. Interestingly, this was observed only when there was significant PI3K activation (4–8 h), suggesting that Lyn is involved in the serum withdrawal-induced activation of PI3K. Therefore, Lyn siRNA was introduced into 293T cells to further examine this possibility. It was confirmed that the treatment reduced the serum withdrawal-induced Lyn expression level (Fig. 7A, top two panels). The treatment also attenuated the ability of serum withdrawal to activate PI3K (Fig. 7A, bottom two panels), to induce the accumulation of ROS at 8 h (but not at 30 min) (Fig. 7B), and also to kill the cells (Fig. 7C). This suggests that serum withdrawal-induced Lyn participates in the process leading to PI3K activation and thus to late ROS accumulation and cell death.

Lyn Is Induced in a ROS-independent Manner—Having determined that both mitochondrial ROS and Lyn are essential for serum withdrawal-induced PI3K activation, this study investigated whether or not ROS mediated the ability of serum withdrawal to induce Lyn. However, catalase, rotenone, or KCN had no significant effect on the serum withdrawal-induced Lyn expression, which was abolished by either CHX or AD (Fig. 7D). Therefore, serum withdrawal appears to increase the levels of Lyn in a ROS-independent manner.

DISCUSSION

To determine the cellular components involved in serum withdrawal-induced ROS accumulation, this study used 293T cells as a model. It was confirmed that, as in the case of U937 cells (22), serum withdrawal increases the levels of ROS in 293T cells in both a PI3K-dependent and -independent manner. This is based on the observations that, although ROS were induced immediately after serum withdrawal, the PI3K in 293T cells was activated 4–8 h later and that the inhibition of PI3K by PI3K-M or LY attenuated the induction of ROS at 4–8 h but not earlier. Therefore, the PI3K in this model

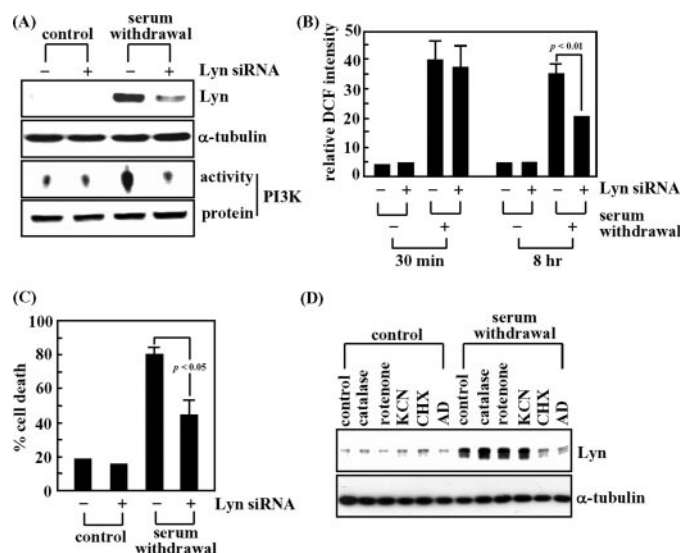


FIGURE 7. **Lyn is essential for PI3K-dependent ROS accumulation.** A, the 293T cells were transfected with a nonspecific control RNA or Lyn-specific siRNA. The cellular Lyn levels were compared by Western blotting using α -tubulin as the loading control (top two panels). The transfectants were treated with serum withdrawal for 8 h, and the PI3K activity and content were analyzed (bottom two panels). B, the transfectants were treated with serum withdrawal for either 30 min or 8 h. The cellular ROS levels were analyzed. C, this treatment was extended for up to 72 h, and the cell viability was analyzed. D, the 293T cells were exposed to serum-free medium in the presence or absence of catalase (1,000 units/ml), rotenone (0.1 μ M), KCN (1 mM), CHX (1 μ g/ml), and AD (0.5 μ g/ml). After incubation for 8 h, the cellular Lyn levels were analyzed by Western blotting.

appears to be selectively involved in the late (4–8 h) (but not early (0–4 h)) phase of ROS induction.

Our findings suggest that Rac1 and Nox1 are involved in the PI3K-induced accumulation of ROS in this system. First, it was shown that Rac1 responds to serum withdrawal by translocating to the membrane fractions and interacting with Nox1. These events were selectively induced during the late phase, and the late phase of ROS accumulation was attenuated by either Rac1-M or Nox1 siRNA. This suggests that Rac1 and Nox1 play a role in the late phase of ROS induction, as observed for PI3K. Moreover, the observation that PI3K-M or LY prevented serum withdrawal from inducing the translocation of Rac1 and the Rac1/Nox1 interaction, supports the view that PI3K acts upstream of Rac1 and Nox1 in this system. However, it should be noted that the PI3K/Rac1/Nox1 pathway might not be solely responsible for the late stage of ROS accumulation. This is based on the observation that a blockade of this pathway by targeting PI3K, Rac1, or Nox1 failed to completely stop the late stage of ROS accumulation. The alternative ROS sources do not appear to involve other Nox isozymes tested in this study, such as Nox2 and Nox4, because a reduction of their expression levels by RNA interference did not significantly influence the serum withdrawal-induced accumulation of ROS.

In contrast to the late phase, ROS during the early stage appear to be generated, at least in part, in the mitochondria. Confocal microscopy revealed the co-localization of induced ROS and mitochondria during the early phase. It was also shown that inhibitors of the mitochondrial respiratory chain, such as rotenone and KCN, attenuate the early accumulation of ROS. Therefore, it appears that serum withdrawal increases the

cellular ROS levels initially through the mitochondria, which is followed by the action of Nox1. Although the ROS-inducing roles of the mitochondria (40, 41) and Nox isozymes (9, 15, 30) have been proposed for other stimuli, these findings suggest that they both can function in a single system. Importantly, the functions of the mitochondria and Nox1 appear to be linked. This is based on the observation that serum withdrawal failed to induce PI3K activation, Rac1 translocation, Rac1/Nox1 interaction, and the late stage of ROS accumulation when the ROS accumulation during the early stage was prevented by catalase, rotenone, or KCN. Therefore, ROS generated from the mitochondria during the early phase appear to trigger the late phase of ROS induction by stimulating the PI3K/Rac1/Nox1 pathway. To the best of our knowledge, this is the first report to show a direct signaling linkage between the mitochondria and Nox1 for ROS production.

The link appears to be influenced by an additional factor induced by serum withdrawal in 293T cells. This was suggested by the long interval between the mitochondrial production of ROS and the activation of the PI3K/Rac1/Nox1 pathway and was indicated more directly by the ability of CHX or AD to prevent the activation of the pathway and late accumulation of ROS. In contrast, the early induction of ROS was not significantly influenced by CHX or AD, suggesting that the induction of the late stage (but not the early stage) of ROS accumulation requires protein synthesis. Our data suggest that Lyn, an activator of PI3K (37, 42), is one such protein that is induced by serum withdrawal and participates in the process leading to PI3K-dependent ROS accumulation. First, serum withdrawal induced an increase in the cellular Lyn levels, which was observed only in the late phase. Moreover, the prevention of Lyn induction using its siRNA prevented PI3K activation and the late induction of ROS. This highlights the requirement of Lyn induction for PI3K-dependent ROS accumulation. Therefore, the time lag required to activate the PI3K-dependent pathway appears to reflect the need for Lyn accumulation. Given the finding that PI3K-dependent ROS accumulation requires both mitochondrial ROS and Lyn induction, this study examined the relationship between the two. However, the introduction of Lyn siRNA did not significantly influence early/mitochondria-dependent ROS induction. Moreover, neither catalase, rotenone nor KCN, blocked the serum withdrawal-induced accumulation of Lyn. This suggests that mitochondrial ROS and Lyn do not mutually influence their inductions. Therefore, it appears that serum withdrawal activates the PI3K/Rac1/Nox1 pathway by triggering at least two independent routes, one leading to the mitochondrial generation of ROS and the other to the induction of Lyn.

Although the mitochondria initiated the induction of ROS in the present system, the total levels of ROS induced were not further elevated by the subsequent additional action of Nox1 (Fig. 1A). Although the mechanism for this is unclear, it is possible that the ROS-inducing action of the mitochondria is slowed down as Nox1 begins to function. Regardless of the mechanism involved, Nox1 in this particular system appears to prolong the accumulation of ROS initiated by the mitochondria. In this regard, the mitochondria and Nox1 may be considered to be an initiator and a sustainer of ROS induction, respec-

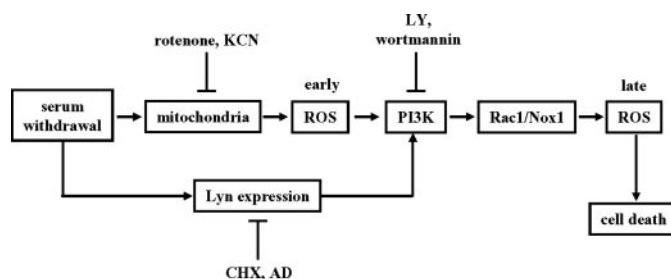


FIGURE 8. Schematic model outlining the serum withdrawal-induced signaling process leading to the sustained production of ROS and cell death. Serum withdrawal induces two pathways, one leading to the mitochondrial production of ROS and the other leading to Lyn expression. Although the former event occurs immediately after serum withdrawal, the latter is evident 4 h and later. PI3K is then activated in a mitochondrial ROS- and Lyn-dependent manner. The activated PI3K in turn stimulates Nox1 by inducing the translocation of Rac1 to membrane fractions and the Rac1/Nox1 interaction. Consequently, the accumulation of ROS is extended into the late phase (4–8 h), and cells lose their viability.

tively. Given that cell death is favored when an oxidative stress is protracted (43, 44), the sustained accumulation of ROS caused by the cooperative action of the mitochondria and Nox1 is expected to support cell death. Indeed, inhibitors of the mitochondrial respiratory chain, which abolished both the early and late stage of ROS accumulation, attenuated serum withdrawal-induced cell death. This suggests that both accumulation phases are essential for cell death. Interestingly, cell death was also suppressed when the late stage of ROS induction was selectively reduced by targeting Nox1, Rac1, PI3K, Lyn, or protein synthesis. Therefore, it appears that mitochondrial ROS are essential (but not sufficient) for cell death and that the accumulation of ROS must be extended by the subsequent action of Nox1 to induce cell death. Fig. 8 gives a schematic of this process.

In summary, this study has shown a link between the mitochondria and Nox1 for the production of ROS. The signaling molecules involved have also been determined. Finally, the functional significance of the link was verified by showing its key role in the sustained accumulation of ROS and cell death. These findings significantly advance our understanding of how ROS accumulation is induced and prolonged particularly in a cell death model.

REFERENCES

1. Rhee, S. G. (1999) *Exp. Mol. Med.* **31**, 53–59
2. Kamata, H., and Hirata, H. (1999) *Cell. Signal.* **11**, 1–14
3. Martindale, J. L., and Holbrook, N. J. (2002) *J. Cell. Physiol.* **192**, 1–15
4. Fleury, C., Mignotte, B., and Vayssières, J. L. (2002) *Biochimie (Paris)* **84**, 131–141
5. Lambeth, J. D. (2004) *Nat. Rev. Immunol.* **4**, 181–189
6. Yoon, Y. S., Lee, J. H., Hwang, S. C., Choi, K. S., and Yoon, G. (2005) *Oncogene* **24**, 1895–1903
7. Felty, Q., Xiong, W. C., Sun, D., Sarkar, S., Singh, K. P., Parkash, J., and Roy, D. (2005) *Biochemistry* **44**, 6900–6909
8. Lam, M., Oleinick, N. L., and Nieminen, A. L. (2001) *J. Biol. Chem.* **276**, 47379–47386
9. Wymann, M. P., Sozzani, S., Altruda, F., Mantovani, A., and Hirsch, E. (2000) *Immunol. Today* **21**, 260–264
10. Brandes, R. P., and Kreuzer, J. (2005) *Cardiovasc. Res.* **65**, 16–27
11. Suh, Y. A., Arnold, R. S., Lassegue, B., Shi, J., Xu, X., Sorescu, D., Chung, A. B., Griendling, K. K., and Lambeth, J. D. (1999) *Nature* **401**, 79–82
12. Cheng, G., Cao, Z., Xu, X., van Meir, E. G., and Lambeth, J. D. (2001) *Gene* **269**, 131–140

13. Shiose, A., Kuroda, J., Tsuruya, K., Hirai, M., Hirakata, H., Naito, S., Hattori, M., Sakaki, Y., and Sumimoto, H. (2001) *J. Biol. Chem.* **276**, 1417–1423
14. Lassegue, B., Sorescu, D., Szocs, K., Yin, Q., Akers, M., Zhang, Y., Grant, S. L., Lambeth, J. D., and Griendling, K. K. (2001) *Circ. Res.* **88**, 888–894
15. Park, H. S., Lee, S. H., Park, D., Lee, J. S., Ryu, S. H., Lee, W. J., Rhee, S. G., and Bae, Y. S. (2004) *Mol. Cell. Biol.* **24**, 4384–4394
16. Heyworth, P. G., Bohl, B. P., Bokoch, G. M., and Curnutte, J. T. (1994) *J. Biol. Chem.* **269**, 30749–30752
17. Diebold, B. A., and Bokoch, G. M. (2001) *Nat. Immunol.* **2**, 194–196
18. Hordijk, P. L. (2006) *Circ. Res.* **98**, 453–462
19. Ueyama, T., Geiszt, M., and Leto, T. L. (2006) *Mol. Cell. Biol.* **26**, 2160–2174
20. Cheng, G., Diebold, B. A., Hughes, Y., and Lambeth, J. D. (2006) *J. Biol. Chem.* **281**, 17718–17726
21. Lee, S. B., Hong, S. H., Kim, H., and Um, H. D. (2005) *Life Sci.* **78**, 91–98
22. Lee, S. B., Cho, E. S., Yang, H. S., Kim, H., and Um, H. D. (2005) *Cell Signal.* **17**, 197–204
23. Lamarche, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1996) *Cell* **87**, 519–529
24. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) *Science* **296**, 550–553
25. Yu, L., Wu, G., Wang, L., Wang, H., and Zhang, G. (2006) *Tohoku J. Exp. Med.* **209**, 141–148
26. Kim, D. K., Cho, E. S., and Um, H. D. (2000) *Exp. Cell Res.* **257**, 82–88
27. Mangan, D. F., Welch, G. R., and Wahl, S. M. (1991) *J. Immunol.* **146**, 1541–1546
28. Bass, D. A., Parce, J. W., Dechatelet, L. R., Szejda, P., Seeds, M. C., and Thomas, M. (1983) *J. Immunol.* **130**, 1910–1917
29. Alvarez-Tejado, M., Naranjo-Suarez, S., Jimenez, C., Carrera, A. C., Landazuri, M. O., and del Peso, L. (2001) *J. Biol. Chem.* **276**, 22368–22374
30. Park, H. S., Jung, H. Y., Park, E. Y., Kim, J., Lee, W. J., and Bae, Y. S. (2004) *J. Immunol.* **173**, 3589–3593
31. Johnson, L. V., Walsh, M. L., and Chen, L. B. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 990–994
32. Emmendorffer, A., Hecht, M., Lohmann-Matthes, M. L., and Roesler, J. (1990) *J. Immunol. Methods* **131**, 269–275
33. Wang, X., McCullough, K. D., Franke, T. F., and Holbrook, N. J. (2000) *J. Biol. Chem.* **275**, 14624–14631
34. Eom, S. J., Kim, E. Y., Lee, J. E., Kang, H. J., Shim, J., Kim, S. U., Gwag, B. J., and Choi, E. J. (2001) *Mol. Pharmacol.* **59**, 981–986
35. Rodriguez-Vician, P., Warne, P. H., Vanhaesebroeck, B., Waterfield, M. D., and Downward, J. (1996) *EMBO J.* **15**, 2442–2451
36. Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997) *Nature* **390**, 632–636
37. Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994) *Science* **263**, 1606–1612
38. von Willebrand, M., Baier, G., Couture, C., Burn, P., and Mustelin, T. (1994) *Eur. J. Immunol.* **24**, 234–238
39. Kubota, Y., Tanaka, T., Kitanaka, A., Ohnishi, H., Okutani, Y., Waki, M., Ishida, T., and Kamano, H. (2001) *EMBO J.* **20**, 5666–5677
40. Vrablic, A. S., Albright, C. D., Craciunescu, C. N., Salganik, R. I., and Zeisel, S. H. (2001) *FASEB J.* **15**, 1739–1744
41. Aronis, A., Madar, Z., and Tirosh, O. (2005) *Free. Radic. Biol. Med.* **38**, 1221–1230
42. Ptasznik, A., Prossnitz, E. R., Yoshikawa, D., Smrcka, A., Traynor-Kaplan, A. E., and Bokoch, G. M. (1996) *J. Biol. Chem.* **271**, 25204–25207
43. Chiaramonte, R., Bartolini, E., Riso, P., Calzavara, E., Erba, D., Testolin, G., Comi, P., and Sherbet, G. V. (2001) *J. Cell. Biochem.* **82**, 437–444
44. Ventura, J. J., Cogswell, P., Flavell, R. A., Baldwin, A. S., Jr., and Davis, R. J. (2004) *Genes Dev.* **18**, 2905–2915

**Link between Mitochondria and NADPH Oxidase 1 Isozyme for the Sustained
Production of Reactive Oxygen Species and Cell Death**

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