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Serum withdrawal kills U937 cells by inducing a positive mutual interaction between reactive oxygen species and phosphoinositide 3-kinase

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Abstract

Reactive oxygen species (ROS) can be generated following cell stimulation and function as intracellular signaling molecules. To determine signaling components involved in ROS induction, human U937 blood cells grown in 10% serum were exposed to serum-free media. It was previously reported that serum withdrawal (SW) killed cells by elevating cellular ROS levels. This study showed that SW activates phosphoinositide 3-kinase (PI3K). PI3K activation was evident after the ROS levels began increasing, and an antioxidant blockade of this increase resulted in PI3K activation suppression. Interestingly, the inhibition of PI3K activity/activation using either its specific inhibitor or dominant-negative mutant attenuated the subsequent additional increase in the ROS levels. These results suggest that SW-induced ROS activate PI3K, which in turn promotes the process leading to ROS accumulation. The present study also revealed that both ROS and PI3K support SW-induced cell death by activating stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). Overall, it appears that SW triggers a positive mutual interaction between ROS and PI3K, which amplifies signals required for the induction of an SAPK-dependent death pathway.

Keywords: Reactive oxygen species; Phosphoinositide 3-kinase; Serum withdrawal; Cell signaling; Cell death; SAPK/JNK

1. Introduction

Reactive oxygen species (ROS) such as H_2O_2 and $O_2^{\cdot -}$ have emerged as key mediators of intracellular signaling (for review, see Refs. [1–3]). Various types of extracellular stimuli, including growth factors, cytokines, and environmental stresses, were shown to elevate cellular ROS levels, and the prevention of this event using antioxidants resulted in a blockade of stimulus-dependent responses. The mechanisms underlying signaling actions of ROS have been well characterized. Consequently, it widely accepted that ROS can modulate cell functions by activating mitogen-activated protein kinases, phospholipase C, protein kinase C, and various other types of signaling components. However, in contrast to the downstream signaling events of ROS, much less attention has been paid to the upstream processes leading to the stimulus-dependent ROS accumulation. This is despite the fact that such information is essential for a better understanding of the ROS-dependent signaling pathway.

ROS induction is often accompanied by activation of phosphoinositide 3-kinase (PI3K), a lipid kinase that can support cell growth, migration, and survival [4–6]. This may

Abbreviations: ROS, reactive oxygen species; PI3K, phosphoinositide 3-kinase; LY, LY294002; SW, serum withdrawal; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; DPI, diphenyleneiodonium; PI3K-M, dominant-negative mutant of the p85 subunit of PI3K; DCFH-DA, 2′,7′ , dichlorofluorescein diacetate.

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reflect a signaling linkage between ROS and PI3K, which was indeed supported by recent reports. For example, LY294002 (LY), a specific inhibitor for PI3K, was shown to abolish chemokine-induced ROS generation in phagocytes [7]. This suggests that PI3K activity was required for ROS induction, which was further confirmed by studies using PI3K knockout mice [8]. It was similarly reported that the ROS accumulation induced by TNFα [9], PDGF [10], or VEGF [11] in various other cell types was suppressed when PI3K activity/activation was blocked by pharmacological or transfectional means. Therefore, PI3K appears to be commonly involved in the ROS accumulation induced by tested cytokines/growth factors. However, it is unclear whether or not PI3K can play a similar role in cell signaling induced by other stimulus types.

Despite the proposed role of PI3K in ROS induction, evidence that supports the opposite hierarchical relationship between ROS and PI3K exists. PI3K in various cell types was activated in response to the exogenous application of H_2O_2 [12–15]. Consistent with the ability of H_2O_2 to activate PI3K, the PI3K activation induced by UV irradiation [16] or Zn^{2+} treatment [17] was blocked by the addition of antioxidants. One simple explanation for these contradictory observations could be that the hierarchical order of ROS and PI3K varies depending on experimental settings. Alternatively, the ability of ROS and PI3K to enhance the level/activity of each component may reflect their positive mutual interactions, in a way that one of these components responds to an extracellular stimulus to enhance the level/activity of the other. This in turn elevates that of the initiator component. To prove this possibility, the ability of PI3K to elevate ROS levels and that of ROS to mediate the PI3K activation should be demonstrated in a single model system. Interestingly, the PI3K in this hypothetical model does not simply mediate the ability of an external stimulus to elevate the ROS levels, but amplifies and/or extends the process leading to ROS accumulation. Given that cell death is favored as either the intensity [18–20] or duration [21] of oxidative stress increases, such a stimulus, which kills cells via the ROS, appears to be a good model for investigating the possibility.

Serum withdrawal (SW) is one such stimulus that can kill cells by elevating cellular ROS levels [18,22]. Therefore, this study investigated the possible role of PI3K in cellular responses to SW. Human U937 monocytic cells were used as the model because these cells have been well characterized with respect to their responses to oxidative stress [18–20]. The data suggest that the SW-induced ROS activates PI3K, which in turn contributes to the subsequent further elevation in ROS levels. Consistent with the ability of PI3K to elevate ROS levels, PI3K was found to mediate cell death in this system. Moreover, we provide evidence showing that the lethal action of PI3K is mediated by the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). Importance of these findings is discussed.

2. Materials and Methods

2.1. Cell culture, DNA transfection, and treatments

U937 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and gentamicin (50 μg/ml). Cells were washed in phosphate-buffered saline and resuspended in serum-free media at a concentration of 3×10^5 cells/ml. Where specified, LY, catalase, and diphenyleneiodonium (DPI) were added at the indicated concentrations. For DNA transfection, the dominant-negative mutant of the p85 subunit of PI3K (PI3K-M) [23] was cloned into the pTRE vector, and was delivered into U937 cells by electroporation using the Tet-Off gene expression system (Clontech, Palo Alto, CA). The transfected cells were selected by using 1 mg/ml G418 sulfate, and were maintained in the presence of 2 μg/ml doxycycline to minimize the basal expression of cloned PI3K-M. The transfectants were washed, cultured in doxycycline-free media for 2 days to induce PI3K-M expression, and received the indicated treatments.

2.2. Analysis of cellular viability

The treated and untreated control cells were stained with propidium iodide (5 μg/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 [24]. All other populations were understood to be dead.

2.3. Analysis of cellular ROS levels

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

1. *DCF fluorescence assay.* Cells were exposed to 50 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) for 30 min in the presence or absence of the indicated inhibitors. The cells were washed and exposed to serum-free media. After the specified incubation times, the cell-associated DCF fluorescence levels were analyzed by flow cytometry [25].
2. *Chemiluminescence assay.* Cells were incubated in the presence or absence of the indicated inhibitors for 30 min. They were washed and resuspended in serum-free media containing 5 mM luminol (Sigma, St. Louis, MO). The chemiluminescence was continuously recorded on a luminometer (Berthold Technologies, Bad Wildbad, German) [26].

2.4. PI3K assay

Cells were lysed in Tris–HCl (20 mM, pH 8.0), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP-40, 10%