

# **Ionizing radiation-induced micronucleus formation is mediated by reactive oxygen species that are produced in a manner dependent on mitochondria, Nox1, and JNK**

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**Abstract.** Ionizing radiation (IR) is known to induce genotoxic damage to DNA, chromosomes, and the nucleus. However, the damage that IR causes to the nucleus has received much less attention. Given that reactive oxygen species (ROS) are involved in IR-induced DNA breaks and chromosomal aberrations, this study examined the role of ROS in IR-induced damage to the nucleus. Human Jurkat T cells were irradiated with  $\gamma$ -rays at a dose of 2.5 Gy, which resulted in a dramatic increase in both the cellular ROS levels and the number of micronuclei. This latter event was attenuated when the IR-induced ROS were eliminated through the exogenous application of an antioxidant enzyme catalase. The ability of IR to induce the accumulation of ROS and micronucleus formation was also reduced either when the cells were irradiated in the presence of rotenone, a mitochondrial respiratory chain inhibitor, or when the cellular Nox1 levels were reduced by RNA interference. These results suggest that IR stimulates both the mitochondria and Nox1 to produce ROS, and that these ROS are involved in the IR-induced formation of micronuclei. IR also activated c-Jun N-terminal kinase (JNK), which was reversed by catalase, rotenone, or Nox1 RNA interference. SP600125, a JNK-specific inhibitor, suppressed the IR-induced accumulation of ROS. This inhibitor consistently attenuated the IR-induced formation of micronuclei. Therefore, ROS and JNK appear to act in a positive mutual manner in IR-induced signaling processes. Overall, IR appears to induce the formation of micronuclei by inducing ROS through mitochondria, Nox1, and JNK.

## **Introduction**

Ionizing radiation (IR) is a genotoxic agent that is widely used in cancer treatment. IR-induced genotoxic damage has been observed in DNA, chromosomes, and nucleus. Examples of these include DNA breaks, chromosomal aberrations, and micronuclei formation (1-3). While the mechanisms underlying these effects are not completely understood, it is generally accepted that IR can induce DNA breakage either by directly ionizing DNA or by generating reactive oxygen species (ROS) (4,5). The role of ROS is supported by the observation that the exogenous application of antioxidants attenuated the ability of IR to induce DNA damage under a variety of experimental conditions (6,7). Since antioxidants have similar protective effects against IR-induced chromosomal aberrations (8), it appears that ROS are commonly involved in both the DNA and chromosomal damage induced by IR. However, it is unclear if ROS can also mediate the IR-induced nuclei damage.

The cellular ROS levels can be increased in response to many types of external stimuli, including growth factors, cytokines, and environmental stress (9-12). As the induced ROS can act as signaling messengers that regulate various cell functions such as growth, differentiation, and viability, many studies have focused on the cellular processes leading to the induction of ROS. Consequently, the mitochondria (13-15) and the Nox family of NADPH oxidase (16-18) have become major sources of ROS induced by external stimuli. The mitochondria generate ROS as byproducts of respiration, and rotenone, an inhibitor of the mitochondrial respiratory chain, has been shown to attenuate the ROS induction under diverse experimental conditions (13-15). NADPH oxidase is a membrane enzyme that is responsible for the oxidative burst induced in activated phagocytes (17,18). Recent studies have suggested that nonphagocytic cells can express the isoforms of the catalytic subunit (Nox2) of NADPH oxidase, such as Nox1, Nox3, Nox4, and Nox5 (19-21). Among these, Nox1 has been relatively well characterized, and shown to be involved in the growth factor-induced ROS production in smooth muscle and epithelial cells (22).

Given the role of the mitochondria and Nox isozymes in the induction of ROS, IR may induce the production of ROS

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by triggering their actions. It was indeed reported that rotenone could prevent the IR-induced accumulation of ROS (23), which suggests the involvement of the mitochondria in the generation of IR-induced ROS. Hence, it is possible that the mitochondria contribute to the genotoxic action of IR. It is also possible that Nox isozymes respond to IR to induce the generation of ROS with the resulting genotoxic effects. However, these possibilities have not been directly addressed, even though this information is essential for understanding the actions of IR and for the development of more efficient and safer strategies for the therapeutic use of IR. Therefore, this study investigated the possible role of ROS in IR-induced nuclei damage. The cellular components involved in the IR-induced generation of ROS were also determined, and tested for their roles in nuclei damage.

## Materials and methods

**Cell culture, transfection, and treatment.** Jurkat T cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and gentamicine (50  $\mu\text{g/ml}$ ). A specific sequence of 19 nucleotides (CCAGGATTGAAGTGGATGG, residues 1130 to 1148) of the human Nox1 cDNA was selected to synthesize small interfering RNAs (siRNAs) (24). The universal control sequence (AGTTCAACGACCAGTAGTC), which has no significant homology to any known mammalian genome, was used as a negative control (25). These oligonucleotides were cloned into the pSUPER vector (Oligoengine, Seattle, WA) according to the manufacturer's protocol. The vectors cloned were then introduced into the cells by electroporation. The transfected cells were selected using G418 sulfate (4 mg/ml). To treat the cells/transfectants, they were plated at a concentration of  $3 \times 10^5$  cells/ml, and then exposed to  $\gamma$ -rays from a  $^{137}\text{Cs}$   $\gamma$ -ray source (Atomic Energy of Canada, Mississauga, Canada) with a dose rate of 3.81 Gy/min. Where specified, catalase, LY294002, and SP600125 were added at the indicated concentrations.

**Analysis of cellular ROS levels.** The treated and untreated control cells were exposed to 50  $\mu\text{M}$  2',7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) for 5 min, and cell-associated levels of DCF fluorescence were analyzed by flow cytometry (26).

**Micronucleus assay.** After 24 h of irradiation, the cells received cytochalasin B (3  $\mu\text{g/ml}$ ) to inhibit cytokinesis, and were incubated for a further 29 h. The cells were harvested onto glass slides using a cytocentrifuge, air-dried, and fixed with methanol:glacial acetic acid (3:1 v/v). The slides were then stained with a Giemsa solution, and analyzed under a light microscope at a magnification of  $\times 1,000$ . The micronuclei were scored when their diameter was  $< 1/3$  of the diameter of the main nucleus. The number of micronuclei in 300 binucleated cells was determined.

**Reverse transcriptase-polymerase chain reaction (RT-PCR).** Total RNA was extracted using TRIzol (Sigma, St. Louis, MO). RT reaction was performed using total RNA as a template and a RT-for-PCR kit (Promega, Madison, WI). PCR amplification was carried out with the following primers:

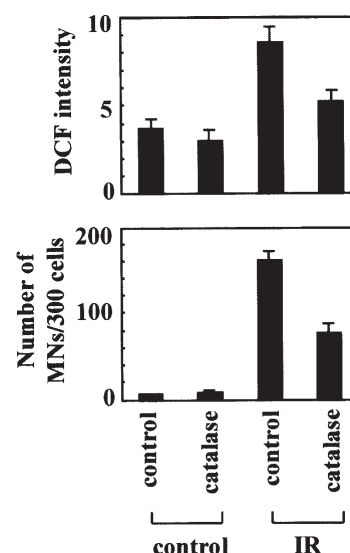


Figure 1. ROS mediate the IR-induced formation of the micronucleus. Jurkat cells were irradiated with  $\gamma$ -rays (2.5 Gy) in the presence or absence of catalase (1,000 U/ml). After 24 h of incubation, the cellular ROS levels were analyzed using DCF fluorescence (top panel). Alternatively, the number of micronuclei in the treated and untreated control cells was analyzed using a cytokinesis-block method (bottom panel). The values shown are the means of triplicate experiments. The error bars represent the standard deviations.

Nox1, 5'-ctgggtggttaaccactggtt-3' and 5'-accaatgccgtgaatccc taag-3';  $\beta$ -actin, 5'-gccagagcaagagagcat-3' and 5'-ggccatctc ttgctgaagt-3'.

**Western blot analysis.** The cell were lysed in Tris-HCl (40 mM, pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors (2  $\mu\text{g/ml}$  aprotinin, 2  $\mu\text{g/ml}$  leupeptin, and 100  $\mu\text{g/ml}$  PMSF). Equal amounts of the proteins were separated by SDS-PAGE, then electrotransferred to the Immobilon membranes (Millipore, Bedford, MA), which were subsequently blotted using the indicated antibodies and visualized by chemiluminescence (ECL; Amersham Pharmacia, Uppsala, Sweden).

**Phosphoinositide 3-kinase (PI3K) assay.** Cells were lysed as described previously (27). Equal amounts of the lysate proteins (400  $\mu\text{g}$ ) were immunoprecipitated with an antibody specific to the p85 subunit of PI3K (Upstate Biotechnology, Lake Placid, NY). The immune complexes were washed, and resolved in 20 mM HEPES (pH 7.4), 5 mM  $\text{MnCl}_2$ , 10 (M ATP, 10  $\mu\text{Ci}$   $\gamma$ -[ $^{32}\text{P}$ ]ATP, and 2.5 mM EGTA. L- $\alpha$ -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.

**c-Jun N-terminal kinase (JNK) assay.** Cells were lysed in a buffer defined previously (28). The lysate proteins (400  $\mu\text{g}$ ) were immunoprecipitated using anti-JNK (Santa Cruz Biotechnology, Santa Cruz, CA), and were resolved in 20  $\mu\text{l}$  of a kinase buffer containing 20 mM Hepes (pH 7.4), 10 mM  $\text{MgCl}_2$ , 20 mM  $\beta$ -glycerophosphate, 10 mM NaF, 1 mM DTT, 0.5 mM sodium orthovanadate, 50  $\mu\text{M}$  ATP, and 10  $\mu\text{Ci}$

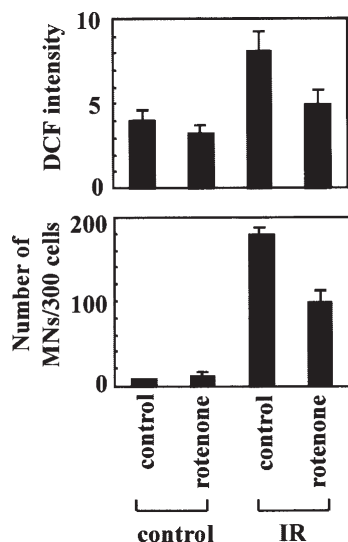


Figure 2. Role of the mitochondria in the IR-induced formation of micronuclei. Jurkat cells were irradiated with  $\gamma$ -rays (2.5 Gy) with or without rotenone (25 nM). The cellular ROS levels (top panel) and micronucleus formation (bottom panel) were analyzed 24 h after irradiation.

$\gamma$ -[ $^{32}$ P]ATP. The kinase reactions were initiated by adding 2  $\mu$ g of the recombinant c-Jun protein (New England Biolabs, Beverly, MA). After 30 min of incubation, the reactions were quenched by adding a boiled sample buffer, and the proteins were subsequently separated by 12% SDS-PAGE. A PhosphorImager using Tina 2.0 software was used to analyze the results.

## Results

**Role of ROS in IR-induced micronuclei formation.** To determine an optimal condition to analyze the role of ROS in IR-induced nuclei damage, Jurkat cells were irradiated with various doses of  $\gamma$ -rays. After 48 h of irradiation, the cellular viability was determined using a propidium iodide exclusion assay (29). The cells (>90%) were viable when they were irradiated with up to 2.5 Gy. However,  $\gamma$ -ray doses 5 Gy and higher induced cell death (data not shown). Therefore, the cells were irradiated with 2.5 Gy, incubated for 24 h, and their ROS levels were analyzed using DCF fluorescence. Irradiation with 2.5 Gy significantly increased the cellular ROS levels (Fig. 1, top panel). To determine if the treatment also induced nuclei damage, the irradiated cells were analyzed for the presence of micronuclei using a cytokinesis-block method. An analysis of 300 cells revealed <10 micronuclei in the case of the untreated control group. However, the number of micronuclei increased to 150-170 when the cells were irradiated with 2.5 Gy (Fig. 1, bottom panel). Given the co-induction of ROS and micronuclei, it appears likely that ROS mediated the IR-induced formation of micronuclei. To investigate this possibility, the cells were irradiated in the presence of catalase, a  $H_2O_2$ -degrading enzyme. Catalase almost completely abolished the IR-induced accumulation of ROS (Fig. 1, top panel), and effectively reduced the IR-induced formation of micronuclei (Fig. 1, bottom panel). Therefore, ROS appear to be involved in the IR-induced formation of micronuclei.

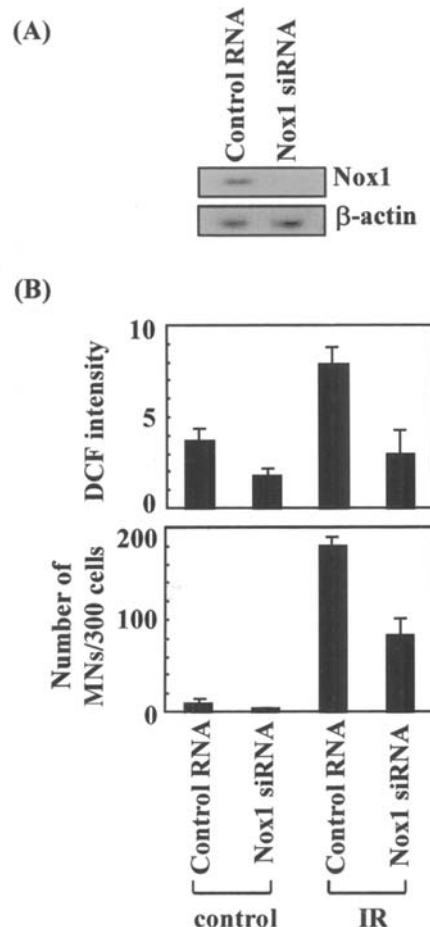


Figure 3. Role of Nox1 in the IR-induced formation of micronuclei. (A) Jurkat cells were transfected with the pSUPER vectors encoding either a nonspecific control RNA or siRNA specific for Nox1. The Nox1 levels in these transfectants were compared by RT-PCR using  $\beta$ -actin as the loading control. (B) The transfectants were irradiated with  $\gamma$ -rays (2.5 Gy), and the cellular ROS levels (top panel) and micronucleus formation (bottom panel) were analyzed 24 h after irradiation.

**Role of mitochondria and Nox1 in IR-induced micronuclei formation.** The mitochondria have been implicated in the IR-induced generation of ROS under other experimental conditions (23). In order to confirm this under the present experimental conditions, the cells were irradiated at 2.5 Gy in the presence of rotenone, an inhibitor of the mitochondrial respiratory chain. The addition of rotenone efficiently suppressed the ability of IR to induce both the accumulation of ROS (Fig. 2, top panel) and the formation of micronuclei (Fig. 2, bottom panel). This suggests that IR promotes the mitochondrial production of ROS, which then contributes to the formation of micronuclei.

Recent studies suggest that Nox1, a member of the Nox family of NADPH oxidase, is expressed in nonphagocytic cells, and responds to cell stimulation with growth factors by generating ROS (22). RT-PCR confirmed that Nox1 is expressed in Jurkat cells (Fig. 3A). A disruption of Nox1 expression by RNA interference attenuated the IR-induced accumulation of ROS and formation of micronuclei (Fig. 3B). Therefore, Nox1 appears to be an additional source of ROS that can damage the nuclei in this system.

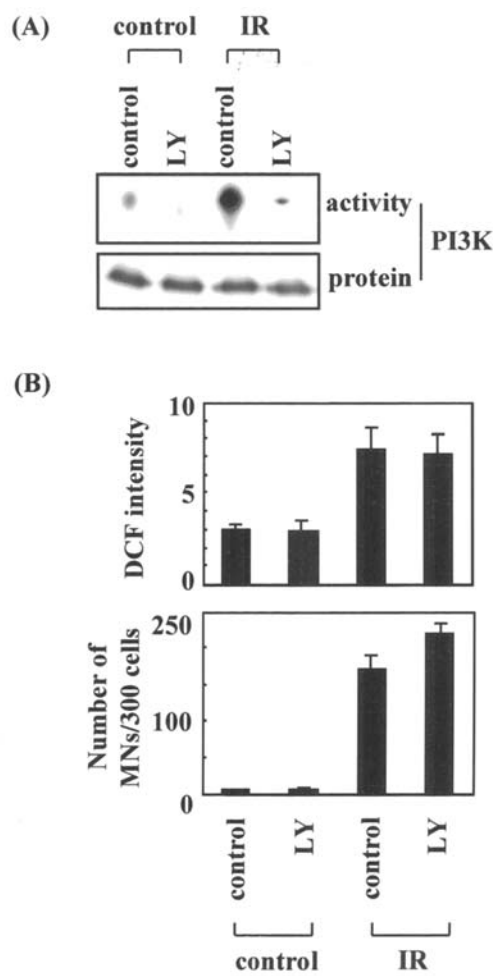


Figure 4. PI3K is not involved in the IR-induced formation of micronuclei. (A) Jurkat cells were irradiated with  $\gamma$ -rays (2.5 Gy) in the presence or absence of LY294002 (5  $\mu$ M). At the end of 15 min of incubation, the cells were lysed, and PI3K was immunoprecipitated with anti-p85. The PI3K activities in precipitates were analyzed using L- $\alpha$ -phosphatidylinositol as a substrate (top panel). The levels of its p85 subunit in samples were compared by Western blotting with anti-p85 (bottom panel). (B) The incubation was extended for up to 24 h, and the cellular ROS levels (top panel) and micronucleus formation (bottom panel) were analyzed.

*PI3K is not involved in IR-induced nucleus damage.* PI3K is a signaling enzyme that is involved in the generation of ROS induced by chemokines (30), growth factors (22), and serum withdrawal (27). The potential role of PI3K in IR-induced ROS accumulation was examined by analyzing the irradiated cells for their PI3K activity using an *in vitro* kinase assay. As shown in Fig. 4A, 2.5 Gy of IR induced the activation of PI3K. This event was abolished when cells were irradiated in the presence of LY294002, a PI3K inhibitor. However, LY294002 failed to significantly attenuate the IR-induced accumulation of ROS and the formation of micronuclei (Fig. 4B). Therefore, these events appear to occur independently of PI3K.

*Role of JNK in IR-induced ROS accumulation and micronucleus formation.* Given that JNK can mediate the production of ROS under certain experimental conditions (31,32), this study examined this role for JNK in the present system. An *in vitro* kinase assay revealed that IR activated JNK in Jurkat cells (Fig. 5A). This was prevented in the presence of

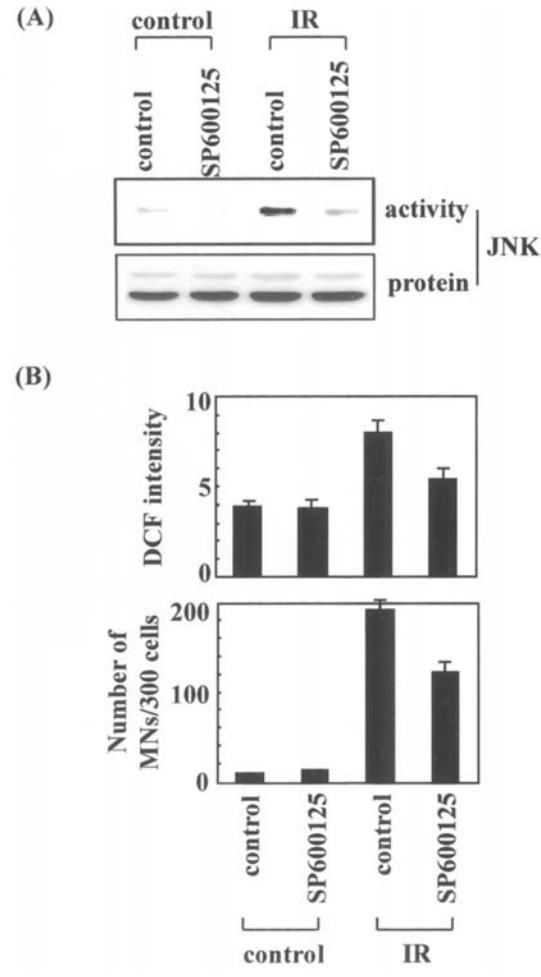


Figure 5. JNK mediates the IR-induced formation of micronuclei. (A) The Jurkat cells were irradiated with  $\gamma$ -rays (2.5 Gy) in the presence or absence of SP600125 (2.5  $\mu$ M). After 3 h of incubation, the JNK activity was analyzed by an *in vitro* kinase assay. (B) The incubation was extended for up to 24 h, and the cellular ROS levels (top panel) and micronucleus formation (bottom panel) were analyzed.

SP600125, an inhibitor of JNK, which resulted in a decrease in the IR-induced accumulation of ROS and the formation of micronucleus (Fig. 5B). Therefore, JNK appears to mediate the ability of IR to induce ROS and micronucleus formation.

The finding that JNK acts as an upstream inducer of ROS contrasts with a previous study showing that ROS mediate the activation of JNK under various experimental conditions (10,28). To confirm this latter event in the irradiated cells, the Jurkat cells were irradiated in the presence or absence of catalase and rotenone. The addition of either of these attenuated the IR-induced activation of JNK (Fig. 6). Similar results were obtained when the Nox1 levels were reduced by RNA interference. These observations suggest that the ROS induced by the mitochondria and Nox1 are essential for the IR-induced activation of JNK. Overall, it appears that ROS and JNK mutually interact to enhance the level/activity of the other in irradiated cells.

Discussion

The present study showed that ROS act as mediators of IR-induced micronucleus formation. This was initially suggested



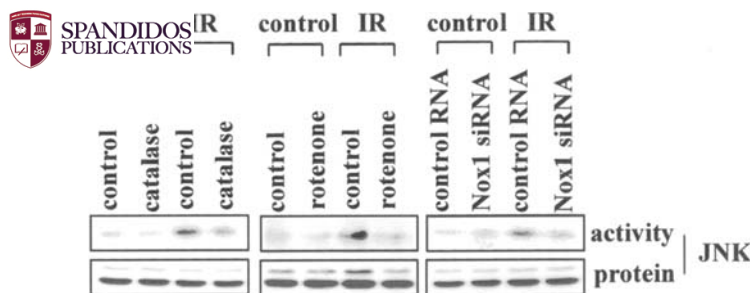


Figure 6. ROS mediate IR-induced JNK activation. Untransfected Jurkat cells or indicated transfectants were irradiated with  $\gamma$ -rays (2.5 Gy). Where indicated, catalase (1,000 U/ml) and rotenone (25 nM) were added. The JNK activity was analyzed by an *in vitro* kinase assay 3 h after irradiation.

by the observation that the IR-induced accumulation of ROS is accompanied by the formation of micronuclei, and was more directly supported by the finding that the elimination of the IR-induced ROS using catalase reduced the formation of micronuclei. Although the role of ROS in IR-induced genotoxic damage has been proposed at the levels of DNA and chromosomes (6,7), this is the first study to show that ROS are also involved in the gross nuclei damage.

Despite the well-recognized ability of IR to induce the generation of ROS (6,8,23), the cellular processes leading to this event are not completely understood. Under the present experimental conditions, the IR-induced accumulation of ROS was attenuated either when rotenone was added to the culture or when the cellular Nox1 levels were reduced by RNA interference. This suggests that IR can stimulate both the mitochondria and Nox1 to produce ROS in a single cell type. Neither rotenone nor Nox1 siRNA reduced the IR-induced formation of micronucleus, suggesting that the ROS involved in this process are generated, at least in part, by the mitochondria and Nox1. While IR has long been considered to possess high energy that can attack the cellular components directly, these results suggest that IR can destroy the nuclei through biological processes.

An additional finding of this study is that cellular signaling molecules can also participate in the process leading to the IR-induced damage to the nucleus. Given that JNK (31,32) and PI3K (22,27,30) can mediate the induction of ROS under other experimental conditions, this study investigated their roles in this system and found that JNK is involved in the IR-induced generation of ROS and micronucleus formation. IR activated JNK in Jurkat cells, and the prevention of this event using its specific inhibitor SP600125 attenuated the ability of IR to induce the accumulation of ROS and formation of micronuclei. These effects were not observed using LY294002, a PI3K inhibitor, although IR induced the activation of PI3K in Jurkat cells. Therefore, PI3K does not appear to be involved in the accumulation of ROS induced under these experimental conditions. It is unclear how JNK induces the generation of ROS in this system. The IR-activated JNK might stimulate the mitochondria, Nox1, or an alternative unidentified source of ROS. We are currently attempting to establish biochemical markers for the mitochondria and Nox1 stimulation to directly assess this possibility.

These results suggest that IR activates JNK in a ROS-dependent manner. This is consistent with the ability of ROS to induce the activation of JNK under various experimental settings (10,28), and is supported by the observation that catalase blocks the IR-induced activation of JNK in this system. This effect was also observed using rotenone or Nox1 siRNA. Therefore, the ROS generated by both the mitochondria and Nox1 appear to be involved in the IR-induced activation of JNK. The finding that JNK and ROS enhance the level/activity of each component in a single model, suggests their positive mutual interaction in a way that either ROS or JNK responds to IR to enhance the level/activity of the other. This in turn elevates that of the initiator component. In this regard, JNK might not simply mediate the ability of IR to induce ROS but amplify the ROS-inducing process.

In summary, this study showed that ROS contribute to the IR-induced formation of micronuclei. The cellular components involved in this process were also determined. These findings significantly advance our understandings of how IR induces genetic instability.

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