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Oxidative stress induces PKR-dependent apoptosis via IFN-γ activation signaling in Jurkat T cells

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ABSTRACT

The dsRNA-dependent protein kinase, PKR, is a central component in antiviral defense. The biological importance of PKR is further remarked by its critical role in apoptosis induced by a variety of stresses. Here, we analyzed the implication of oxidative stress in the induction of PKR-dependent apoptosis in Jurkat cells. Our results revealed that reactive oxygen species (ROS) induced endogenous *pkr* gene expression at the transcriptional level by activating the interferon (IFN)-γ gene. However, IFN-γ siRNA expression abrogated the H_2O_2-mediated *pkr* induction. The radical scavenger *N*-acetyl-L-cysteine profoundly inhibited *pkr* induction via the reduction of IFN-γ expression. The treatment of cells with the specific JAK–STAT inhibitor, AG490, reduced the PKR expression, and suppressed PKR-dependent cell death. Finally, siRNA-mediated depletion of IFN-γ or *pkr* efficiently downregulated H_2O_2-mediated apoptotic cell death. These results indicated that oxidative stress induces PKR expression essentially via the IFN-γ activation signal, and causes apoptosis in Jurkat T cells.

The double-stranded RNA-activated protein kinase, PKR, is a member of the serine/threonine kinases [1]. In cellular antiviral responses, PKR is activated by binding to double-stranded viral RNA, and catalyzes eIF2α phosphorylation, thereby inhibiting protein synthesis. As the *pkr* gene is regulated under the TATA-less promoter, which harbors the IFN-stimulated response element (ISRE), kinase conserved sequence (KCS) and IFN-γ activated sequence elements (GAS), the IFN and Sp proteins are known to be critical regulators [2].

The Sp proteins (Sp1, 3) are expressed constitutively, and essential for basal PKR expression via interaction with the KCS element of 5′-flanking region of the *pkr* promoter [3,4]. However, the mechanism by which Sp proteins are activated in response to various stimuli has not yet to be clearly determined. On the other hand, IFNs bind with a specific receptor on the surface of plasma membrane. Each IFN receptor is composed of two subunits, IFNAR1 and 2, or IFNGR1 and 2, which are associated with Janus activated kinases (JAK1, 2) and tyrosine kinases 2 (TYK2) [4]. The activation of JAKs results in the tyrosine phosphorylation of the signal transducer and activator of transcription (STAT), and results in the formation of a protein complex among STATs and IFN regulatory factors (IRFs). The complexes are moved to the nucleus and bind to the responding element, ISRE or GAS, where they induce the *pkr* gene. It is generally believed that intracellular ROS generation involves increased electron leakage from the mitochondria or insufficient enzymatic antioxidants as the consequence of certain conditions, including viral infection, UV irradiation and stimulation by cytokines and growth factors. Among many viral proteins, HIV Tat has been known to generate oxidative stress and induce apoptosis in variety of cells [5,6]. However, the molecular mechanism by which Tat induces ROS generation remains unclear. Nevertheless, exposure to environmental ROS or induction of intracellular ROS may amplify a relevant signaling cascade, which is linked directly to the immunological functions of T cells, cell proliferation and death. Many reduction/oxidation (redox)-sensitive signaling proteins can be modified via direct oxidation or oxidative shifts in the thiol/disulfide status. Although the final destination, such as proliferation or apoptosis, remains subject to debate, the ROS-mediated modification of signaling molecules may cause the expression of a variety of genes in response to ROS in T cells.

The principal objective of the present study was to determine whether ROS could be an inducer of T_H cell-restricted cytokine, IFN-γ, and to further analyze the implication of IFN-γ induction in the PKR expression and apoptosis in response to ROS. We showed that oxidative stress induced an increase in IFN-γ levels, which in turn caused activation of a JAK2/STAT pathway, thereby finally inducing PKR expression and apoptosis. Because IFN-dependent PKR induction involves a variety of unique responses in T cells, an understanding of ROS-induced PKR-dependent apoptosis should provide new insights into ROS-mediated diseases and rationales for IFN therapeutic strategies.

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Materials and methods

Cell line and reagents. The Jurkat T human leukemia cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Jurkat cells were maintained in RPMI1640 medium under standard conditions. FBS, RPMI 1640, penicillin, glutamine and streptomycin were purchased from Sigma–Aldrich (St. Louis, MO). Hydrogen peroxide, *N*-acetyl-L-cysteine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) and human IFN-γ were purchased from Sigma–Aldrich (St. Louis, MO).

Immunoblotting, siRNA silencing and RT-PCR. Immunoblotting was conducted as described previously [7]. Anti-caspase 3, anti-PARP, and anti-cleaved PARP antibodies were purchased from Cell Signaling Technology (Denver, MA). Anti-PKR, anti-Sp1, anti-Sp3, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IFN-γ-specific dicer-substrate small interfering RNA (DsiRNA) and negative controls were purchased from Integrated DNA Technology (San Diego, CA). The first set of duplexes for IFN-γ siRNA was 5′-CAGGACAACCAUUACUGGGAUG CUCUU-3′ and the 5′-GAGCAUCCCAGUAAUGGUUGUCCTG-3′, and second set was 5′-AUCACAUAGCCUUGCCUAAUUAGUCAG-3′ and 5′-GACUAAUUAGGCAAGGCUAUGUGAT-3′. The first set of duplexes for PKR siRNA was 5′-GGAAAGACUUACGUUAUU ATT-3′ and the 5′-UAAAACGUAAGUCUUUCCTT-3′, and second set was 5′-GAUCU UAAGCCAAGUAAUATT-3′ and 5′-UAUUACUUGGCUUAAGAUCTT-3′. siRNA transfection was conducted with Lipofectamine 2000 (Invitrogen) or electroporation in accordance with the manufacturer's recommendations. RNA was subjected to semi-quantitative RT-PCR as described previously [8]. Total cellular RNA was extracted using Trizol (Invitrogen) in accordance with the manufacturer's recommendations.

Vector construction and luciferase assay. In order to construct the PKR-Luc, the 5′-flanking region of the *pkr* was isolated from Jurkat genomic DNA using the following primers: 5′-GATCCCCAGG CTTGTAAAGG-3′ and 5′-GAGACCCGCGGCTTCGGGA-3′. The amplified PCR products were digested with SacII, and blunted with Klenow fragments. The promoter fragment was inserted into the pGL3-Basic (Promega) reporter vector. Transient transfection was conducted via the DEAE-Dextran method [9]. Luciferase reporter assays were conducted in accordance with the manufacture's

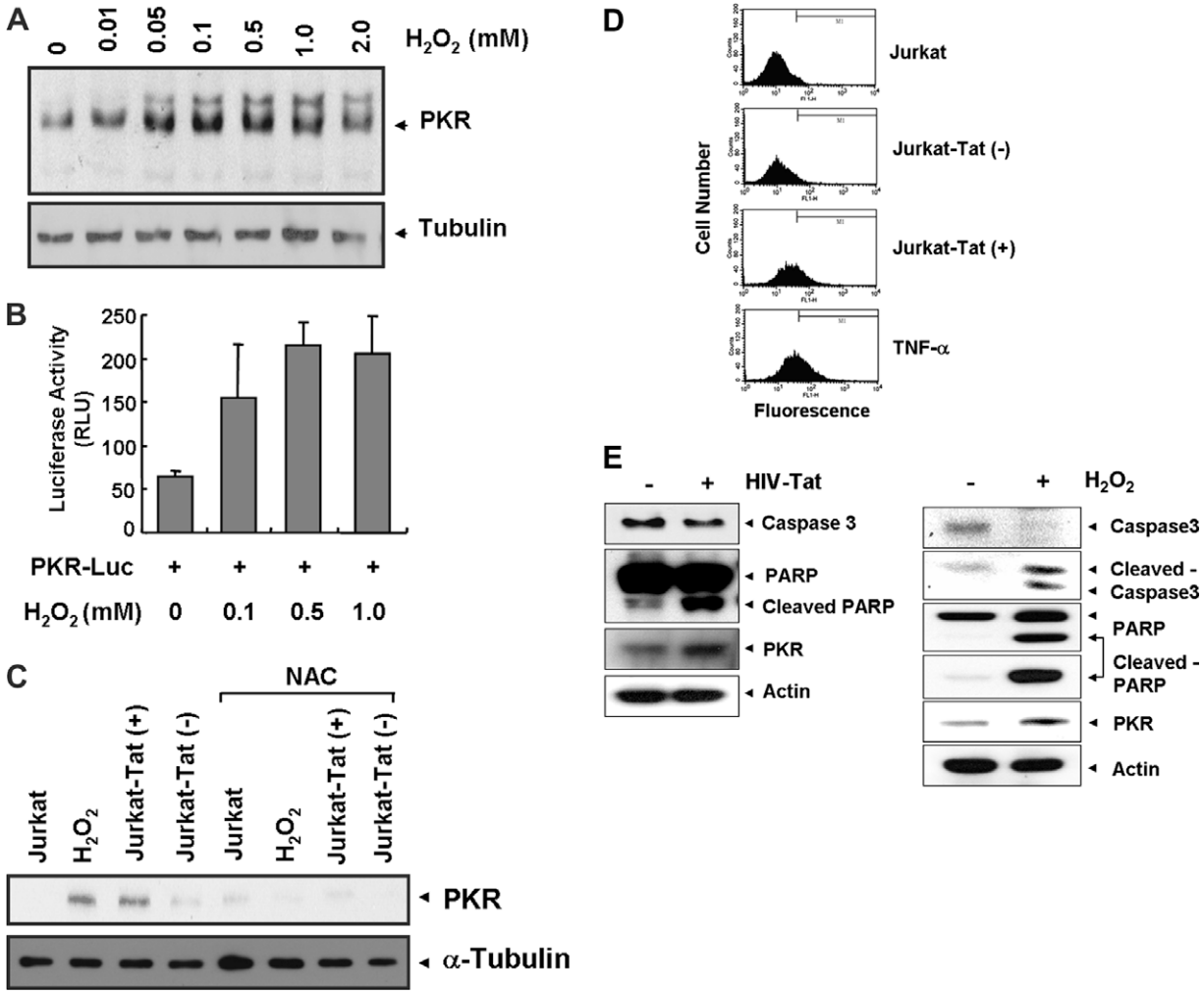

Fig. 1. H_2O_2 induces endogenous PKR expression and *pkr* promoter activation. (A) Jurkat cells were treated with H_2O_2 (0.5 mM) for 4 h. After harvest, total lysates (15 μg) were analyzed for PKR expression level via immunoblotting. (B) Plasmid PKR-Luc was transfected into the cells. After 18 h, the cells were treated with H_2O_2 (0.5 mM) for 4 h, and the luciferase activity was assessed. Each bar represents the mean ± SE of triplicate samples from three different experiments. (C) Jurkat cells were infected with the Tat-expressing virus (+) or empty virus (−) as described in Materials and methods. After infection, the cells were preincubated with or without NAC (20 mM) for 6 h, followed by 4 h of incubation with H_2O_2 (0.5 mM). PKR expression levels were analyzed via immunoblotting analysis. (D) The ROS levels of indicated cells were determined by FACS. The cells were stained with DCFH-DA (10 μM), and run on flow cytometry. As a positive control, the cells were treated for 18 h with 100 ng/ml of TNF-α. (E) Jurkat cells infected with the Tat-expressing virus (+) or empty virus (−) (left panel) and Jurkat cells incubated with 0.5 mM of H_2O_2 (right panel) were subjected to immunoblotting analysis with the indicated antibodies. The β-actin and α-tubulin levels were detected for normalization.