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Original Contribution

p21^{Cip1} protects against oxidative stress by suppressing ER-dependent activation of mitochondrial death pathways

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ABSTRACT

Although it is well established that the cell cycle inhibitor p21 protects against genotoxic stress by preventing the replication of damaged DNA, recent studies have shown that the cytoplasmic form can also protect. It protects by delaying the loss of the antiapoptotic proteins Mcl-1 and Bcl-X_L; however, the mechanism of regulation is unknown. Utilizing hyperoxia as a model of chronic oxidative stress and DNA damage, p21 was detected in the nucleus and cytoplasm and cytoplasmic expression of p21 was sufficient for cytoprotection. p21 was enriched in a subcellular fraction containing mitochondria and endoplasmic reticulum (ER), suggesting that it may be coordinating ER and mitochondrial stress pathways. Consistent with this, p21 suppressed hyperoxic downregulation of BiP and subsequent activation of ER stress signaling, which affected Mcl-1, but not Bcl-X_L; though both inhibited hyperoxic cell death. Taken together, these data show that p21 integrates the DNA damage response with ER stress signaling, which then regulates mitochondrial death pathways during chronic genotoxic stress.

Oxygen homeostasis is vital for proper functioning, and fluctuations in oxygen metabolism can have detrimental consequences on biological organisms. An imbalance in oxygen levels causes the generation of reactive oxygen species (ROS), which promote oxidative stress by damaging macromolecules, contributing to the pathologies of Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, atherosclerosis, vascular disease, ischemia–reperfusion injury, congestive heart failure, stroke, and aging [1]. Organisms have evolved signaling pathways to deal with oxidative damage to macromolecules, particularly DNA. In response to genotoxic stress, cells initiate growth checkpoints to ensure the maintenance of genome integrity [2]. Damaged cells subsequently activate signaling pathways regulating DNA repair and/or cell death. A primary function of the tumor-suppressing protein p53 is the initiation of genotoxic pathways in response to oxidative stress by inducing gene targets, including p21$^{Cip1/Waf/Sdi1}$ (hereafter p21).

p21 is a member of the CIP/KIP family of cyclin-dependent kinase (cdk) inhibitors, along with p27 and p57. The CIP/KIP family has important functions in cell cycle regulation; however, p21 is also a downstream target of p53 and a major determinant of the cellular response to genotoxic stress. Growth arrest in G1 during oxidative stress is dependent on p21 inhibition of cyclin/cdk complexes and the DNA processivity factor, proliferation cell nuclear antigen (PCNA). p21 also has prosurvival functions in response to genotoxic stress by inhibiting the activation of caspase-3, caspase-9, stress-activated protein kinases (SAPK), and apoptosis signal-regulating kinase (ASK) 1, thus preventing cell death [3–6]. Recently, disruption of the p21 nuclear localization sequence (NLS) confirmed that its antiproliferative functions occur in the nucleus because cytoplasmic-targeted p21 had greatly reduced capacity to arrest the cell cycle [7]. Whereas the protective functions of p21 originate from cytoplasmic protein interactions, no studies have been performed to separate the growth inhibitory and survival roles of p21 by its localization.

Hyperoxia refers to a state of imbalance in which organisms are exposed to oxygen levels that are greater than atmospheric oxygen levels. Exposure to hyperoxia results in the persistent generation of ROS, causing DNA damage, p53 transactivation of p21, and increased mortality [8]. Mice and cells lacking *P21* had increased sensitivity to hyperoxic damage [9–11]. Expression of full-length p21 or the cdk- or PCNA-binding domains of p21 activated G1 growth arrest during hyperoxia; however, only full-length p21 had cytoprotective functions. In addition, expression of p21 at low doses was sufficient for growth inhibition and increased doses were required to inhibit cell death, thus uncoupling p21 growth arrest from survival [11]. Mitochondrial Bcl-2 proteins have been implicated in hyperoxic cell death pathways [12,13]. Hyperoxia downregulated the antiapoptotic Mcl-1 and Bcl-X_L and conditional expression of either Mcl-1 or Bcl-X_L prevented hyperoxic

Abbreviations: ASK, apoptosis signal-regulating kinase; cdk, cyclin-dependent kinase; ER, endoplasmic reticulum; NLS, nuclear localization sequence; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; UPR, unfolded protein response.

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death. Furthermore, expression of p21 delayed the loss of Mcl-1 and Bcl-X_L, thereby promoting survival [11,14]. Whereas it is clear that p21 can negatively inhibit activation of p53, possibly reducing apoptotic activation, p21 is able to promote survival in the absence of p53 [15]. This suggests that p21 may affect mitochondrial cell death through other signaling pathways, including MAPK/JNK, NF-κB, Fas, TRAIL, and the endoplasmic reticulum (ER) stress response [16–18].

This study makes use of *P53*-deficient H1299 cells that conditionally express p21 to investigate the dependence of p21 cytoprotection on its localization and to elucidate prosurvival p21 pathways utilizing hyperoxia as a model of chronic oxidative stress. Our studies demonstrate that hyperoxic activation of mitochondrial cell death is partly dependent on ER signaling and that cytoplasmic p21 exerts its cytoprotective functions by regulating these pathways independent of its growth inhibitory role.

Materials and methods

Cell culture

Human lung adenocarcinoma H1299 cells and A549 cells (ATCC) were cultured in 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (high glucose) with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin (Gibco), and 20 μg/ml gentamycin (Cellgro). Cells were maintained in tissue culture flasks and exposed to room air with 5% CO_2 or hyperoxia (95% O_2 with 5% CO_2) as previously described [10]. H1299 cells with conditional transgene expression were incubated with 2.0 μg/ml doxycycline (Sigma Chemical Co.) for 24 h in room air before exposure.

Cell lines

The EGFp21, EGFp27, and Bcl-X_L doxycycline-inducible constructs and stable H1299 cell lines have been previously described [11,19]. The EGFp21ΔNLS doxycycline-inducible construct was generated by PCR of the pEGFP-C1 plasmid containing the EGFp21ΔNLS sequence [7] using the forward 5′-ATGGTGAGCAAGGGCGA-3′ primer and the reverse 5′-TTAGGGCTTCCTCTTGGAG-3′ primer. A NheI site was added to the 5′ primer and a XhoI site was added to the 3′ primer. The NheI- and XhoI-digested fragments were ligated into the pBIG2i vector [20]. The human Mcl-1 open reading frame was amplified by RT-PCR using RNA obtained from H1299 cells with forward 5′-ATGTTTGGCCTCAAAAGAAA-3′ and reverse 5′-CTATCTTATTAGA-TATGCCAACCAGC-3′ primers. An EcoRI restriction site was added to the forward primer and a BamHI restriction site was added after the termination codon of the reverse primer. The amplified product was digested and ligated into the pBig2i N-terminal Met-FLAG containing the downstream IRES-EGFP sequence to produce Flag-tagged Mcl-1 IRES-EGFP. The plasmids were purified by Qiagen (Qiagen Sciences) preparation and transfected into H1299 cells using Genfect (Molecula). Cells were grown in 200 μg/ml hygromycin (Invitrogen, Carlsbad, CA, USA) and stable clones were initially selected based on EGFP fluorescence after treatment with 2 μg/ml doxycycline and visualization of green fluorescence with a Nikon TE2000-E inverted epifluorescence microscope.

Western blot analysis

Cells and whole lungs were lysed in 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 25 mM sodium fluoride, 25 mM sodium β-glycerophosphate, 0.1 mM sodium vanadate, 0.1 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 0.3% IGEPAL CA-630, 0.1 μg/ml pepstatin A, 1.9 μg/ml aprotinin, and 2 μg/ml leupeptin. Protein concentrations were determined by the BCA method (Pierce). Cell lysates were diluted in 3× Laemmli buffer and boiled for 5 min. Laemmli at 1× contains 50 mM Tris (pH 6.8), 1% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. The extracted protein was separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes (Pall Life Sciences). The membranes were then incubated with anti-EGFP (1:1000; Clontech), anti-p21 clone SX118

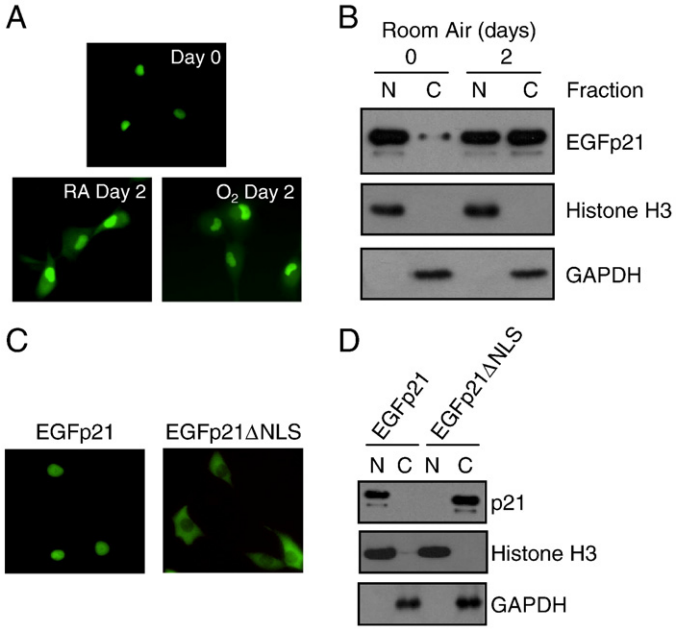

Fig. 1. p21 is detected in the nucleus and cytoplasm during persistent expression. H1299 cells with inducible expression of EGFp21 were cultured in the presence of doxycycline for 24 h (day 0) and then incubated in room air (RA) or hyperoxia (O_2) for 2 days. (A) Endogenous fluorescence of the EGFp21 transgene during culture in room air or hyperoxia. (B) Nuclear (N) and cytoplasmic (C) H1299 cell lysates after 0 and 2 days of culture after doxycycline treatment to induce EGFp21 expression immunoblotted for EGFp21, histone H3, and GAPDH. The NLS of EGFp21 ($RKR^{140–142}$) was mutated ($AAA^{140–142}$) to generate EGFp21ΔNLS, which was stably transfected into parental H1299 cells. H1299 cells with inducible expression of either EGFp21 or EGFp21ΔNLS were cultured in the presence of doxycycline for 24 h and (C) endogenous fluorescence was monitored and (D) protein was fractionated into nuclear or cytoplasmic fractions and immunoblotted for the p21 transgene with histone H3 and GAPDH used as fractionation controls. Immunoblots shown are representative of at least three separate experiments with similar results.