Hyperoxia activates the ATR-Chk1 pathway and phosphorylates p53 at multiple sites

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Das, Kumuda C., and Ravi Dashnamoorthy. Hyperoxia activates the ATR-Chk1 pathway and phosphorylates p53 at multiple sites. Am J Physiol Lung Cell Mol Physiol 286: L87–L97, 2004. First published September 5, 2003; 10.1152/ajplung.00203.2002.—Hyperoxia has been shown to cause DNA damage resulting in growth arrest of cells in p53-dependent, as well as p53-independent, pathways. Although H2O2 and other peroxides have been shown to induce ataxia telangiectasia-mutated (ATM)-dependent p53 phosphorylation in response to DNA damage, the signal transduction mechanisms in response to hyperoxia are currently unknown. Here we demonstrate that hyperoxia phosphorylates the Ser15 residue of p53 independently of ATM. Hyperoxia phosphorylated p53 (Ser15) in DNA-dependent protein kinase null (DNA-PK−/−) cells, indicating that it may not depend on DNA-PK for phosphorylation of p53 (Ser15). We show that Ser37 and Ser392 residues of p53 are also phosphorylated in an ATM-independent manner in hyperoxia. In contrast, H2O2 did not phosphorylate Ser37 in either ATM+/+ or ATM−/− cells. Furthermore, H2O2 failed to phosphorylate Ser15 in ATM−/− cells. Additionally, overexpression of kinase-inactive ATM-and-Rad3-related (ATR) in HEK293T cells diminished Ser15, Ser37, and Ser392 phosphorylation compared with vector-only transfected cells. In contrast, wild-type ATR overexpression did not diminish Ser15, Ser37, or Ser392 phosphorylation. We also show that checkpoint kinase 1 (Chk1) is phosphorylated on Ser345 in response to hyperoxia, which could be inhibited by caffeine or wortmannin, potent inhibitors of phosphoinositide 3-kinase-related kinases. Hyperoxia also phosphorylated Chk1 in ATM−/− as well as in ATM+/+ cells, demonstrating an ATM-independent mechanism in Chk1 phosphorylation. Together, our data suggest that hyperoxia activates the ATR-Chk1 pathway and phosphorylates p53 at multiple sites in an ATM-independent manner, which is different from other forms of oxidative stress such as H2O2 or UV light.

serine-15: ataxia telangiectasia mutated; ATM and Rad3 related; phosphoinositide 3-kinase-related kinases; DNA-dependent protein kinase; checkpoint kinase 1; hydrogen peroxide; ultraviolet; serine-392; serine-37

HYPEROXIA-MEDIATED GROWTH INHIBITION of lung cells is a hallmark of oxygen toxicity. Growth arrest in response to hyperoxia may be mediated by its inhibitory effect on cell cycle regulatory proteins and/or activation of cell cycle checkpoint proteins in response to DNA damage (23). DNA damage activates signaling pathways that can halt the cell cycle for subsequent repair or can induce cells to undergo programmed cell death. Therefore, understanding how cells respond to DNA damage is of paramount importance in understanding oxygen toxicity of lung cells. In addition to hyperoxia, the lung oxidant burden in various disease states also can induce a DNA damage response pathway resulting in the delay or inhibition of the repair process. Because hyperoxia produces reactive oxygen species (9, 39), many investigators use hydrogen peroxide (H2O2) as a surrogate for hyperoxia in their studies. Even though H2O2 oxidizes DNA, there is reason to believe that hyperoxia and H2O2 are not equivalent stimuli (23). For example, the genotoxic effect of H2O2 includes mutagenic single-strand breaks (10, 11), whereas hyperoxia induces chromosome aberration and sister chromatid exchange (13). Second, iron chelators such as desferrioxamine have no effect on hyperoxia-induced clonogenic survival, whereas they are protective to cells damaged by H2O2 (12). It also takes several days for hyperoxia to injure and kill the cell, whereas H2O2 takes immediate effect. Recent studies have demonstrated that H2O2 (37) or another peroxide (34) activates an ataxia telangiectasia-mutated (ATM)-dependent DNA damage signal transduction pathway. In this study, we investigated whether hyperoxia being an oxidant also uses ATM for transmission of DNA damage signal.

ATM and ATR (ATM-and-Rad3 related) are phosphoinositide 3-kinase-related kinases (PIKK) that play key roles in fundamental cellular processes, including proliferation and genomic surveillance (1). ATM is a 370-kDa protein kinase mutated in the human genetic disorder ataxia telangiectasia (17). Cell lines derived from patients lacking ATM are radiosensitive and exhibit defects in checkpoint responses to ionizing radiation (8), including p53-dependent G1 cell cycle arrest and p53-independent S and G2 cell cycle arrest (8). The kinase activity of ATM is induced in response to double-stranded DNA breaks (1), and it targets several effectors of checkpoint control, including Cds1 [also known as checkpoint kinase 2 (Chk2)], breast cancer 1 (BRCA1) (20), p53 (2), and Mdm2 (21).

DNA-dependent protein kinase (DNA-PK) is another member of the PIKK family that has been shown to be activated in response to DNA double-stranded breaks (18). DNA-PK exhibits a strong preference for phosphorylating serine or threonine residues (19). DNA-PK phosphorylates p53 on Ser15 as well as on Ser37 residues. However, unlike ATM null cells, radiation-induced p53 protein induction and p53-dependent G1 checkpoint function appear normal in DNA-PK-deficient cells (19). Thus if DNA-PK plays any role in the regulation of p53 function in vivo, it seems that other kinases such as ATM or ATR fully compensate for this activity in cells that lack DNA-PK.

Unlike ATM, deletion of ATR in mice results in embryonic lethality, indicating that ATR is an essential gene (3). Cells lacking ATM are hypersensitive to ionizing radiation (IR), but not to ultraviolet (UV) radiation or hydroxyurea (HU) (7), whereas cells overexpressing a kinase-inactive form of ATR are sensitive to UV and HU (7), as well as to IR. This suggests that ATR plays a more prominent role than ATM during the cellular response to unreplicated DNA or to certain DNA-
damaging agents. ATM and ATR have overlapping substrate specificity. However, both prefer phosphorylating serine or threonine residues that are followed by glutamine (1).

The DNA damage transducer ATM kinase phosphorylates the amino terminus of p53 at Ser15 that blocks its association with Mdm2 (15, 21). Additionally, ATR phosphorylates Ser15, Ser37, and Ser392 in response to UV radiation (16, 36). Recent studies have reported that Chk1, which is activated by ATR in response to DNA damage, can phosphorylate p53 on Ser20 (38). Chk1 and Chk2 are serine-threonine kinases that are activated by phosphorylation on Ser345 or on Thr68 by ATR or ATM kinases, respectively (1). Activated Chk1 or Chk2 phosphorylates Ser216 on cdc25C, resulting in the inactivation of cdc25C phosphatase, which leads to the arrest of cells at the G2 transition (26).

Oxidative stress in the form of H2O2 or another peroxide has been demonstrated to activate the ATM-mediated DNA damage signaling cascade (34, 37). Additionally, ATM has been shown to be an oxidative stress sensor (30). Although hyperoxia increases p53 expression in vivo and in vitro (4, 23), little is known about how this occurs. Furthermore, hyperoxia-mediated DNA damage signal transducers have not yet been identified. In this report we demonstrate that hyperoxia activates the ATR-Chk1 pathway and phosphorylates multiple serine residues on p53 involving an ATM-independent mechanism. Additionally, hyperoxia-mediated Chk1 phosphorylation was found to be independent of ATM but was dependent on ATR.

**EXPERIMENTAL PROCEDURES**

**Cell culture, transfection, and exposure to hyperoxia.** A lung adenocarcinoma cell line derived from human alveolar type II cells (A549) was obtained from ATCC and grown in F-12K medium supplemented with 10% FBS and 100 units each of penicillin and streptomycin. GM03349 (ATM−/−) and GM02052 (ATM+/−) fibroblasts were obtained from the Corell Repository and were grown in minimal essential medium with 15% FBS and antibiotics. DNA-PK+/− (M059K) and DNA-PK−/− (M059J) cells were obtained from ATCC and propagated in F-12 and Ham’s media. Cells were exposed either to hyperoxia (95% O2 + 5% CO2) at a flow rate of 10 l/min for 10 min in humidified exposure chambers (Billups-Rothenburg) or to room air containing 5% CO2 (normoxia) in a CO2 incubator (Stericell, Forma Scientific) in 60- or 100-mm2 tissue culture dishes containing 5 or 10 ml of media for 24 h. At the end of the incubation, cells were processed either for total cell lysate preparation for Western blotting or for isolation of total RNA for microarray analysis. For UV exposure, dishes were placed in a UV stratalaration for 30 s. The supernatant was carefully aspirated, and the pellet containing the agarose beads was washed four times with the lysis buffer. Finally, the agarose beads were suspended in 40–50 μl of SDS sample buffer without reducing agents to prevent the interference of IgG heavy chain with p53 bands and boiled for 5 min (31). Aliquots of 5 or 10 μl were analyzed by Western blotting.

**Immunoprecipitations and Western blotting.** Total cellular lysate was prepared in lysis buffer [50 mM Tris-HCl at pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM PMSF, 30 μl/ml aprotinin (Sigma), and 1 mM Na3VO4]. One milligram of AT cell lysate, 800 μg of A549 cell lysate, or 1,500 μg of HEK293T cell lysate were immunoprecipitated with p53-agarose conjugate antibody (Santa Cruz Biotechnology). Briefly, cell lysates were incubated with 10 μl of anti-p53-agarose conjugate and incubated in the cold in a rotating mixer for 2 h. After incubation, lysates were centrifuged at 1,000 × g for 30 s. The supernatant was carefully aspirated, and the pellet containing the agarose beads was washed four times with the lysis buffer. Finally, the agarose beads were suspended in 40–50 μl of SDS sample buffer without reducing agents to prevent the interference of IgG heavy chain with p53 bands and boiled for 5 min (31). Aliquots of 5 or 10 μl were analyzed by Western blotting. For Chk1 or phospho-Chk1 Western blotting, 10 or 20 μg of protein were resolved in 10% SDS-PAGE and electroblotted onto nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (Bio-Rad). After washing, the membrane was incubated with primary antibody or phosphospecific antibodies (Cell Signaling Technologies) in TBS containing 0.1% Tween 20 and 5% BSA overnight with gentle shaking. For detection of Flag protein, anti-Flag polyclonal antibody was obtained from Sigma (St. Louis, MO). After incubation, the membrane was washed and incubated for 1 h with respective horseradish peroxidase-tagged secondary antibodies, and specific bands were detected by ECL (Amersham) LumiGlo reagent (Cell Signaling Technologies) or Super Signal West Femto reagent (Pierce Chemical).

Western blots were scanned, and image analysis was performed with NIH Image 1.61 software. Statistical analysis was performed by ANOVA and Tukey’s test for multiple comparison on the relative densities using In Stat statistical software (In Stat). Means with a significance level of <0.05 were considered significant.

**RESULTS**

**Microarray analysis of the cell cycle checkpoint gene expression in hyperoxia.** Although hyperoxia has been shown to cause DNA damage (5) and to induce p53 expression (4, 22),
the DNA damage response pathway is currently unknown. Therefore, to determine the status of the cell cycle checkpoint genes in response to hyperoxia, we examined 24 genes that are known to mediate cell cycle checkpoint control. We observed that p21, IκBα, and Gadd45 were strongly induced in hyperoxia (Fig. 1A). Although p53 was not transcriptionally induced in hyperoxia, p21 was strongly induced ($P < 0.001$), suggesting that posttranslational modification of p53 might cause increased p21 expression. In addition, there was significant ($P < 0.001$) induction of the IκBα (6) and Gadd45 genes (Fig. 1B). However, DNA damage transducers such as ATM, Chk1, or Chk2 were not transcriptionally upregulated in hyperoxia, suggesting that posttranslational modifications such as phosphorylation of these kinases may play a role in hyperoxia-mediated DNA damage signaling. Additionally, Rad family genes such as Rad9, Rad17, or Rad51 were not transcriptionally upregulated in hyperoxia. There was no significant decrease in any of the 24 cell cycle regulatory genes. These data demonstrate that the sensors of DNA damage, such as Rad family genes or the transducers of DNA damage, such as ATM, ATR, Chk1, or Chk2, are not induced or repressed in hyperoxia, whereas the effectors of DNA damage response such as Gadd45 or p21 are induced in hyperoxia.

Wortmannin, a PIKK inhibitor, decreases p53 Ser15, -20, -37, and -392 phosphorylation in hyperoxia in lung adenocarcinoma (A549) cells. Members of the PIKK such as ATM or ATR have been shown to be activated in response to DNA damage (1). To determine whether members of PIKKs are involved in hyperoxia-mediated DNA damage response, we used wortmannin, a potent inhibitor of PIKKs (27). We treated A549 cells with 15 μM wortmannin for 2 h followed by exposure of cells to hyperoxia containing wortmannin. ATM and ATR have been shown to phosphorylate Ser15 on p53 in response to DNA damage (1). Therefore, we measured the p53 Ser15 phosphorylation in response to hyperoxia in wortmannin-treated or wortmannin-untreated cells. As demonstrated in Fig. 2A, row B, wortmannin treatment decreased p53 Ser15 phosphorylation, demonstrating that phosphorylation occurs by a wortmannin-sensitive pathway. Additionally, the level of p53 protein was also increased in response to hyperoxia (Fig. 2A, row A), which is consistent with earlier studies in whole mouse lungs (24).

To evaluate whether other p53 residues are also phosphorylated in hyperoxia, we determined the phosphorylation of p53 on Ser392 and Ser37 residues in hyperoxia. Hyperoxia phosphorylated Ser392 (Fig. 2A, row E) and Ser37 (Fig. 2A, row D) on p53. Additionally, the levels of Ser37 and Ser392 phosphorylation in response to hyperoxia were decreased in response to wortmannin treatment, indicating a wortmannin-sensitive pathway.

ATM or ATR phosphorylates Ser20 on p53 in response to DNA damage (35). Additionally, recent studies have shown that Chk1 or Chk2 can also directly phosphorylate Ser20. Thus Ser20 phosphorylation is a target for multiple kinases (35). To

Fig. 1. A: microarray analysis of activation of cell cycle checkpoint genes in hyperoxia. A549 cells were exposed to normal air or 95% O₂ + 5% CO₂ for 24 h. After exposure, total RNA was prepared (Qiagen RNA isolation kit). Total RNA (10 μg) was reverse transcribed with GEAPRimer mix (Superarray kit). The hybridized membrane was washed and exposed to X-ray film (Kodak Biomax MS film) with an intensifying screen. The developed spots were identified with a grid card provided in the kit as described in EXPERIMENTAL PROCEDURES. B: densitometry was performed on 3 separate microarrays processed as described in A. Relative densities of genes were analyzed by ANOVA followed by multiple comparisons and Tukey’s test using In Stat statistical software. Chk, checkpoint kinase; ATM, ataxia telangiectasia mutated; Hus, Schizosaccharomyces pombe checkpoint homolog; MRE, meiotic recombination 11 homolog; RAD, S. pombe homolog; rpa, replication protein A3; BRCA1, breast cancer 1; MCM, minichromosome maintenance deficient (Saccharomyces cerevisiae); TIMP, tissue inhibitor of metalloproteinase; MAD, mitotic arrest deficient; NBS, Nijmegen breakage syndrome. **$P < 0.01$; ***$P < 0.001$. 

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also phosphorylated Ser15, -20, -37, or -392 in A549 cells similar to hyperoxia. However, the response of H2O2 was stronger with respect to Ser15, -37, and -20 compared with UV light. Although H2O2 has earlier been shown to induce Ser15 or Ser20 phosphorylation (37), the phosphorylation of Ser37 and Ser392 has not been previously reported. Because A549 cells have both ATM and ATR proteins, the response could be mediated by either ATM or ATR or both. Therefore, we used ATM+/− or ATM−/− fibroblasts (Corell Repository) to determine whether ATM mediates any or all of these p53 phosphorylations in hyperoxia.

Hyperoxia induces p53 Ser15, -20, -37, and -392 phosphorylation in ATM+/− and ATM−/− cells, whereas H2O2 does not phosphorylate Ser15, Ser20, or Ser37 phosphorylation in ATM−/− cells. Although hyperoxia increases p53 in vivo and in vitro, little is known about how this occurs. If hyperoxia phosphorylates p53 via an ATM-dependent pathway, we would expect inhibition of p53 phosphorylation in cells that lack a functional ATM or in cells where the ATM gene is functionally inactive due to mutation. Thus to determine the role of ATM in hyperoxia-mediated DNA damage response, we exposed ATM+/− or ATM−/− fibroblasts to 24 h in hyperoxia, followed by detection of p53 phospho-p53 Ser15, -20, -37, or -392 using anti-p53 or antiphosphospecific antibodies (Cell Signaling Technologies). As demonstrated in Fig. 3A, hyperoxia increased p53 protein levels in ATM+/− cells, as well as in ATM−/− cells (Fig. 3A, row A). In addition, hyperoxia induced Ser15 phosphorylation in ATM+/− cells, as well as in ATM−/− cells (Fig. 3A, row B). In fact, there was higher level of Ser15 phosphorylation in ATM−/− cells. To determine whether hyperoxia is similar to H2O2 or UV light in inducing p53 Ser15 phosphorylation, we treated ATM−/− or ATM+/− cells with H2O2 or UV light as described in experimental procedures and determined the p53 Ser15 phosphorylation. As demonstrated in Fig. 3B, row B, H2O2 did not induce Ser15 phosphorylation in ATM−/− cells. In contrast, UV light phosphorylated Ser15 in ATM−/− cells. These data indicate that hyperoxia-mediated Ser15 phosphorylation is independent of ATM, but different from that of H2O2, which depends on ATM for Ser15 phosphorylation.

To further determine the role of ATM in hyperoxia-mediated p53 phosphorylation, we analyzed Ser20, Ser37, and Ser392 phosphorylation in hyperoxia. As shown in Fig. 3A, rows C–E, hyperoxia caused Ser20, Ser37, and Ser392 phosphorylation in ATM+/− as well as in ATM−/− cells. However, the phosphorylation of Ser20 was significantly less in ATM−/− cells compared with ATM+/− cells. These results indicate that although hyperoxia-induced phosphorylation of Ser15, -37 and -392 is independent of ATM, phosphorylation of Ser20 in hyperoxia may depend on ATM. In contrast, H2O2 failed to induce Ser37 phosphorylation in either ATM−/− or ATM+/− cells, which suggests that Ser37 phosphorylation is not a target for H2O2 (Fig. 3B, row D). The effect of H2O2 on Ser37 phosphorylation has not been previously reported. Hyperoxia strongly phosphorylated Ser392 in ATM−/− cells compared with ATM+/− cells (Fig. 3A, row E), a response similar to UV (Fig. 3B, row E). However, H2O2 also phosphorylated Ser392 in ATM−/− or ATM+/− cells, which demonstrates that H2O2 can phosphorylate Ser392 in an ATM-independent manner.

Fig. 2. A: hyperoxia phosphorylates p53 Ser15, Ser20, Ser37, and Ser392, which is inhibited by wortmannin. A549 cells were exposed to hyperoxia or normoxia, and p53 was immunoprecipitated and subjected to Western blotting as described in experimental procedures. Lanes 1–3, cells exposed to normoxia; lanes 4–6, cells exposed to 95% O2 + 5% CO2; lanes 7–9, cells treated with 15 μM wortmannin and exposed to normoxia for 24 h; lanes 10–12, cells treated with 15 μM wortmannin and exposed hyperoxia. B: effect of UV light and H2O2 on p53 Ser15, Ser20, Ser37, and Ser392 phosphorylations in A549 cells. Confluent A549 cells were treated with 100 mJ/cm² (5 h postexposure) or to H2O2 (50 or 100 μM, 30 min), and p53 immunoprecipitates were subjected to Western blotting as described in experimental procedures. Lanes 1–3, UV exposure in triplicate; lanes 4 and 5, cells treated with 50 or 100 μM H2O2.
Hyperoxia induces p53 (Ser15) phosphorylation in DNA-PK<sup>+/+</sup> cells as well as in DNA-PK<sup>−/−</sup> cells. DNA-PK has been demonstrated to phosphorylate p53 (Ser15) (19). Therefore, to determine whether DNA-PK may mediate hyperoxia-induced Ser15 phosphorylation, we exposed DNA-PK<sup>+/+</sup> or DNA-PK<sup>−/−</sup> cells to hyperoxia and detected p53 or p53 (Ser15) by Western analysis. As demonstrated in Fig. 4A, hyperoxia phosphorylated p53 (Ser15) in DNA-PK<sup>+/+</sup> as well as in DNA-PK<sup>−/−</sup> cells, indicating that DNA-PK is not required for hyperoxia-mediated p53 phosphorylation.

Additionally, UV light also induced p53 (Ser15) phosphorylation in DNA-PK<sup>+/+</sup> as well as DNA-PK<sup>−/−</sup> cells (Fig. 4B). In contrast, H<sub>2</sub>O<sub>2</sub> did not induce p53 (Ser15) phosphorylation in either DNA-PK<sup>+/+</sup> or DNA-PK<sup>−/−</sup> cells at a concentration of 50 μM (treated for 30 min, Fig. 4B). To determine whether DNA-PK cells are responsive to H<sub>2</sub>O<sub>2</sub> treatment, we treated DNA-PK<sup>+/+</sup> or DNA-PK<sup>−/−</sup> cells to 100, 200, or 300 μM H<sub>2</sub>O<sub>2</sub> for 1 h and assessed p53 (Ser15) phosphorylation by Western analysis. As demonstrated in Fig. 4C, DNA-PK<sup>+/+</sup>, as well as DNA-PK<sup>−/−</sup>, cells induced Ser15 phosphorylation in a dose-dependent manner.

Kinase-inactive ATR expression decreased Ser15, −20, −37, and −392 phosphorylation in response to hyperoxia. Studies to clarify the role of ATR have been hampered by the lack of a viable ATR-deficient animal or a specific chemical inhibitor. Therefore, kinase-inactive mutant ATR protein expression has been used as an effective tool to determine ATR-responsive signal transduction and has been utilized in several studies (7). By using a kinase-dead ATR (ATR<sub>kd</sub>) overexpression construct, one can produce the kinase-dead protein in excess of native wild-type protein in a dominant-negative manner. Because kinase-inactive protein would not phosphorylate downstream targets, we would expect to observe a decreased response in cells expressing kinase-inactive protein compared with wild-type protein-expressing cells, if the response is mediated by any of these proteins. Thus by overexpressing kinase-dead or wild-type proteins, we can determine whether ATR plays a role in hyperoxia-induced DNA damage signaling. Therefore, to delineate the role of ATR in DNA damage signaling in hyperoxia, we transiently transfected HEK293T cells with ATR<sub>kd</sub>, wild-type ATR (a generous gift of Dr. Karlene Cimprich, Stanford University), or with the vector.
alone construct (7). After 48 h of transfection, we exposed cells to hyperoxia (95% O₂ + 5% CO₂) for 24 h as described in EXPERIMENTAL PROCEDURES. Expression of ATRkd was confirmed by Western blotting of Flag protein using anti-Flag antibody (Fig. 4A, bottom). As demonstrated in Fig. 5A, row B, hyperoxia potently induced Ser15 phosphorylation in HEK293T cells transfected with vector alone. In contrast, cells transfected with a kinase-inactive ATR construct demonstrated significantly less (P < 0.05) Ser15 phosphorylation (Fig. 5A, row B, and 5B). However, HEK293T cells overexpressing wild-type ATR did not show diminished Ser15 phosphorylation, rather there was an increase in Ser15 phosphorylation in these cells.
(Fig. 4A, row B, and 4B). Overexpression of ATRkd also decreased the level of Ser37 and Ser392 (Fig. 5A, rows D and E; Fig. 5, C and D). In contrast, overexpression of wild-type ATR did not diminish the expression of these phosphorylations in hyperoxia. Additionally, the level of Ser20 phosphorylation was increased in vector-only transfected HEK293T cells exposed to hyperoxia. Cells treated with wild-type ATR showed significantly higher (P < 0.01) Ser20 phosphorylation compared with cells transfected with kinase-inactive ATR expression vector in hyperoxia. Additionally, although ATRkd-transfected cells demonstrated a lower level (25%) of Ser20 phosphorylation compared with vector-only transfected cells in hyperoxia, this difference was not statistically significant. Together, these data indicate that while Ser15, Ser37, or Ser392 are increased in response to hyperoxia in an ATM-dependent manner, Ser20 phosphorylation in hyperoxia may not fully depend on ATM activation.

As demonstrated in Fig. 5F, the expression of Flag protein was significantly decreased in hyperoxia. Thus the wild-type ATR expression was very high in 21% oxygen-exposed HEK293T cells, but there was a significant decrease in the expression of ATM wild type in 95% oxygen-exposed cells (P < 0.0001). Similarly, the expression of ATRkd was also decreased significantly (P < 0.0001) in 95% oxygen exposure compared with 21% oxygen exposure.

Hyperoxia causes phosphorylation of Chk1 (Ser345), which is sensitive to caffeine or wortmannin. ATR has been shown to phosphorylate its downstream target Chk1 on Ser345 in response to DNA damage (38). This phosphorylation activates Chk1, and the activated Chk1 phosphorylates cdc25C on Ser216 causing its sequestration in the cytoplasm and, hence, inhibition of cdc2 kinase activity (26). If hyperoxia activates the ATR signaling cascade then Chk1 is a likely target of ATR. Therefore, we determined the phosphorylation of Ser345 on Chk1 in hyperoxia. As demonstrated in Fig. 6A, hyperoxia phosphorylated Chk1 on Ser345. There was no increase in the Chk1 protein in hyperoxia, indicating that Chk1 phosphorylation is a specific response of hyperoxia (Fig. 6A, bottom). These results also agree with our microarray analysis that there was no increase in the Chk1 mRNA in response to hyperoxia (Fig. 1, A and B). To define the role of PIKKs in Chk1 phosphorylation, we treated cells with caffeine or wortmannin, inhibitors of PIKKs, followed by exposure of cells to hyperoxia. As demonstrated in Fig. 6A (lanes 7–9), caffeine abolished the phosphorylation of Chk1 in response to hyperoxia. Additionally, wortmannin also inhibited the phosphorylation of Chk1 in hyperoxia (Fig. 6B). These results indicate that hyperoxia activates Chk1 involving a wortmannin- or caffeine-sensitive pathway. Furthermore, these data also indicate that hyperoxia does not directly phosphorylate Chk1. In additional control experiments, we treated A549 cells with UV light or H2O2, as described in the legend of Fig. 5C, and determined Chk1 phosphorylation. As demonstrated in Fig. 6C, UV light or H2O2 phosphorylated Chk1 in a manner similar to hyperoxia in A549 cells. However, since A549 cells express ATM or ATR proteins, Chk1 phosphorylation in hyperoxia could be mediated by any or both of these signal transducers. Therefore, to determine the role of ATM in Chk1 phosphorylation in hyperoxia, we determined the effect of hyperoxia on Chk1 phosphorylation in ATM+/+ or ATM−/− cells.

Hyperoxia phosphorylates Chk1 (Ser345) in ATM+/+ as well as in ATM−/− cells. Next, we determined whether ATM plays a role in hyperoxia-mediated Chk1 phosphorylation. As demonstrated in Fig. 7A, hyperoxia activated Chk1 (Ser345) phosphorylation in ATM+/+ as well as in ATM−/− cells, indicating that Chk1 phosphorylation in hyperoxia is independent of ATM. Additionally, UV light or H2O2 also phosphorylated Chk1 in ATM−/− cells albeit to a lesser extent, indicating that ATM may be required for UV light- or H2O2-dependent Chk1 phosphorylation (Fig. 7B).

Kinase-inactive ATR expression decreases Chk1 phosphorylation in hyperoxia. To further determine whether hyperoxia-mediated Chk1 phosphorylation is dependent on ATR, we transfected HEK293T cells with wild-type or kinase-inactive ATR constructs as described in EXPERIMENTAL PROCEDURES, and expression of ATR was confirmed by Western blotting of Flag...
protein. We exposed these cells to normoxia or hyperoxia as described in experimental procedures. Hyperoxia significantly (P < 0.001) increased Chk1 phosphorylation compared with normoxia in vector-only transfected HEK293T cells (Fig. 8, A and B). Cells expressing kinase-inactive ATR demonstrated significantly less (P < 0.05) phospho-Chk1 compared with vector-only transfected cells. In addition, cells transfected with wild-type ATR expression plasmid demonstrated significantly (P < 0.001) higher level of Chk1 phosphorylation in normoxic cells compared with vector-only transfected normoxic cells (Fig. 8, A and B). Additionally, ATR wild-type transfected cells demonstrated a significantly higher (P < 0.01) level of Chk1 phosphorylation compared with ATRkd transfected cells in hyperoxia. Furthermore, there was no change in Chk1 protein expression in either hyperoxia or normoxia. These results demonstrate that hyperoxia-mediated Chk1 phosphorylation is dependent on ATR.

**DISCUSSION**

In the present study we have demonstrated that hyperoxia phosphorylates Ser15, Ser20, Ser37, and Ser392 residues of p53 in an ATM-independent manner. Hyperoxia phosphorylated p53 (Ser15) in DNA-PK−/− as well as DNA-PK−/− cells, indicating that DNA-PK is not required in hyperoxia-mediated p53 Ser15 phosphorylation. In addition, expression of kinase-inactive ATR decreased Ser15, Ser20, Ser37, or Ser392 phosphorylation in response to hyperoxia. Furthermore, Ser15, -20, -37, and -392 phosphorylation could be inhibited by wortmannin. We have also shown that H2O2 phosphorylates Ser15 and Ser20 in an ATM-independent manner but Ser392 in an ATM-dependent manner. Additionally, H2O2 did not phosphorylate Ser37 residue either in ATM+/+ or in ATM−/− cells. In contrast to H2O2, UV light phosphorylated Ser15, Ser37, and Ser392 in an ATM-independent manner but phosphorylated Ser20 in an ATM-dependent manner. We have also shown that Chk1 is phosphorylated in hyperoxia, and this phosphorylation is independent of ATM but dependent on ATR. In contrast, UV light or H2O2 phosphorylated Chk1 in an ATM-dependent manner (results summarized in Table 1). Our microarray analysis revealed that p53 mRNA does not increase in hyperoxia. A recent study has demonstrated that the mouse lung p53 mRNA level does not change in response to hyperoxia (24), which is in agreement with our data. Thus p53 is controlled in a posttranslational manner by phosphorylation in hyperoxia. This has several advantages for immediate “turning on” or “turning off” of p53-dependent genes. We have observed that p53 (Ser15) phosphorylation can occur as early as 4 h in response to hyperoxia (data not shown). However, the p53 level increases in hyperoxia with extensive phosphorylation on various serine residues.

Recent studies have demonstrated that H2O2 (37) or other peroxides (34) transduce DNA damage signaling in an ATM-dependent pathway. Additionally, ATM has been shown to be sensitive to oxidative stress (30). However, ATM and ATR have overlapping substrate specificity, but they are activated in response to distinct stimuli. Our present report demonstrates that hyperoxia transduces DNA damage response independently of ATM, which suggests that hyperoxia is a distinct form of oxidative stress, different from that of H2O2 or other peroxides.

The p53 tumor suppressor protein plays an important role in cellular responses to DNA damage and genomic surveillance
Table 1. Summary of phosphorylation of various p53 serine residues and Chk1 in A549, ATM, DNA-PK, and ATRwt or ATRkd transfected cells

<table>
<thead>
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<th>Hyperoxia</th>
<th>p53 (Ser15)</th>
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<th>p53 (Ser37)</th>
<th>p53 (Ser392)</th>
<th>pCHk1</th>
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<td>H2O2</td>
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<td>+</td>
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<td>ATRkd</td>
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Chk, checkpoint kinase; ATM, ataxia telangiectasia mutated; DNA-PK, DNA-dependent protein kinase; ATRwt, ATM and Rad3 related wild type; ATRkd, ATR kinase dead. NA, not applicable.

(2). Activation of p53 can lead either to cell cycle arrest and DNA repair or to apoptosis. In addition, p53 is phosphorylated at multiple sites by several different protein kinases (2). Phosphorylation of Ser15 on p53 impairs the ability of Mdm2 to bind p53, prompting both accumulation and functional activation of p53 in response to DNA damage (21). Recent studies have demonstrated that Ser20 on p53 is phosphorylated by Chk1 or Chk2, enhancing its tetramerization, stability, and activity (35) in response to DNA damage. Although hyperoxia has been shown to induce Ser15 phosphorylation on p53 (29), the signal transducer has not been identified previously. Our present study provided evidence that ATM may be a signal transducer in hyperoxia-mediated DNA damage signaling.

We have shown that ATM−/− cells can potently induce Ser15 phosphorylation in response to hyperoxia, suggesting that hyperoxia-mediated Ser15 phosphorylation is independent of ATM. Additionally, wortmannin inhibited Ser15 phosphorylation induced by hyperoxia, suggesting that PIKKs mediate this phosphorylation. Recent studies have demonstrated that while the IC50 of wortmannin for ATM is ~250 nM, for ATR it is ~1.8 μM (32, 33), suggesting that wortmannin inhibits ATR at a higher concentration. Sarkaria et al. (33) observed that ATR activity is not inhibited by exposure of cells to 100 μM wortmannin for 1 h in A549 cells. However, in our experiment, we pretreated A549 cells for 2 h followed by 24-h exposure to hyperoxia. Hence, inhibition of ATR by wortmannin may require a higher concentration as well as a longer time for interaction of the drug with ATR.

UV-mediated phosphorylation of Ser37 has been shown to be dependent on the activation of ATR (36). In contrast, ATM does not phosphorylate these residues in response to IR. Therefore, phosphorylation of Ser37 in hyperoxia suggests ATR-mediated DNA damage signaling. As demonstrated in Fig. 2A, we observed Ser37 and Ser392 phosphorylation in response to hyperoxia, which could be inhibited by wortmannin in a dose-dependent manner. These data strongly suggest that ATR, but not ATM, may be the DNA damage signal transducer in response to hyperoxia.

We have observed that Ser20 phosphorylation in response to hyperoxia could be inhibited by wortmannin. Ser20 has been shown to be phosphorylated by ATM, ATR, Chk1, or Chk2 (1, 17, 35). The inhibition of Ser20 phosphorylation by wortmannin suggests that PIKKs are involved in Ser20 phosphorylation in hyperoxia. However, phosphorylation of Ser20 was significantly lower in ATM−/− cells in hyperoxia compared with ATM+/+ cells, which suggests that ATM may be involved in Ser20 phosphorylation in hyperoxia. Additionally, Ser20 phosphorylation in hyperoxia was increased in ATR wild-type transfected cells, but decreased in ATRkd transfected cells. This decrease was not statistically significant. Together, these studies suggest that hyperoxia-mediated phosphorylation of Ser20 may involve both ATM and ATR, with the predominant role played by ATM. A recent study has demonstrated the polo-like kinase-3 (Plk3) phosphorylates the Ser20 residue of p53 in response to H2O2 treatment (37). In addition, this phosphorylation was caffeine sensitive and ATM dependent (37). The study also demonstrated that UV light, H2O2, or adriamycin can increase the activity of Plk3 (37). We observed that ATM−/− cells failed to phosphorylate Ser20 in response to UV light or H2O2, suggesting that these stimuli depend on ATM for Ser20 phosphorylation (Fig. 3B, row C). Additionally, these studies also demonstrate that Plk3 may phosphorylate Ser20 in response to UV or H2O2 in an ATM-dependent manner. However, our data indicate that ATR may, in part, phosphorylate Ser20. Interestingly, H2O2 did not phosphorylate Ser37 either in ATM+/+ or in ATM−/− cells, which is consistent with IR, but in contrast with the UV response. In addition, Ser392 phosphorylation by H2O2 was independent of ATM, which has not been previously reported (Fig. 3B, row E).

Chk1 and Chk2 are serine threonine kinases that have been shown to act downstream of ATM or ATR as signal transducers in DNA damage signaling. Chk1 has been shown to be phosphorylated by ATR (38), whereas ATM phosphorylates Chk2 (1). Therefore, activation of either Chk1 or Chk2 in hyperoxia would shed light on the activation of its upstream signaling molecule. To evaluate this possibility, we determined...
Chk1 phosphorylation in hyperoxia. As demonstrated in Fig. 6A, hyperoxia caused Chk1 phosphorylation, and this phosphorylation was inhibited by caffeine (Fig. 6A) or wortmannin (Fig. 6B). Caffeine has been shown to be a potent inhibitor of ATR (14, 32, 33). Therefore, our data demonstrate that Chk1 phosphorylation in hyperoxia is dependent on a caffeine-sensitive pathway. Chk1 phosphorylation in hyperoxia was independent of ATM as demonstrated in Fig. 6A. Additionally, overexpression of kinase-inactive ATR expression decreased Chk1 phosphorylation in hyperoxia. Together, our data demonstrate that hyperoxia induces ATR-Chk1 signaling, which is different from that of H2O2 or UV light. Interestingly, we have observed that UV light-induced Chk1 phosphorylation does not occur in ATM−/− cells, whereas ATM+/+ cells phosphorylate Chk1. UV light has been shown to phosphorylate Chk1 in an ATR-dependent manner (38). Therefore, our observation that UV light failed to induce Chk1 phosphorylation in ATM−/− cells is rather surprising.

It is important to mention here that our experiments demonstrating the effect of ATRkd expression on p53 phosphorylation in HEK293T cells in hyperoxia may underestimate the level of inhibition. It appears that although the expression of wild-type ATR is very strong in HEK293T cells exposed to 21% oxygen, as demonstrated by immunoblotting of Flag, the expression of wild-type ATR significantly (P < 0.001) decreased in 95% oxygen (Fig. 6A, bottom). In addition, expression of ATRkd was also significantly (P < 0.001) less in 95% oxygen-exposed cells compared with 21% oxygen-exposed ATRkd transfected cells (Fig. 5F). Therefore, the inhibition of p53 phosphorylation in the presence of less expression of ATRkd in 95% oxygen may actually underestimate the level of inhibition. Similarly, less expression of wild-type ATR in hyperoxia may also underestimate the effect of overexpression of wild-type ATR on p53 phosphorylations. We do not know the reason for decreased expression of ectopically expressed ATR in hyperoxia. We also do not know whether endogenous ATR expression is decreased in hyperoxia. However, experiments are under way to determine the effect of hyperoxia on ATR expression.

Although hyperoxia has been shown to cause growth arrest of a variety of cells (22, 25), the mechanism of growth arrest has not been elucidated. Additionally, although hyperoxia is known to cause DNA damage (4), the signaling pathway has not been delineated. Our present study demonstrates that hyperoxia can activate a p53-dependent and/or p53-independent pathway in response to DNA damage. A p53-dependent DNA damage response may be transmitted through a p21- or Gadd45-mediated pathway, resulting in G1/S phase arrest of cells. In contrast, a p53-independent pathway may involve ATR-Chk1 activation, resulting in the inactivation of matura tion-promoting factor and arrest of cells at the G2/M boundary. Recent studies have shown that hyperoxia causes growth arrest of A549 cells at the G2/M phase boundary (20), supporting our present finding that Chk1 activation may mediate G2 arrest of cells in hyperoxia. In addition, a recent study has demonstrated that DNA strand breaks that occur in exposure of cells to hyperoxia may be due to a replication blockade (28). Because ATR is induced in response to stalled replication, our present study suggests that hyperoxia may activate ATR-mediated signaling in response to a replication block. Additionally, our study demonstrates that hyperoxia is a unique stress that is different from H2O2 or other peroxides in its response to DNA damage.

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