Loss of Gadd45a does not modify the pulmonary response to oxidative stress

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Loss of Gadd45a does not modify the pulmonary response to oxidative stress. Am J Physiol Lung Cell Mol Physiol 288: L663–L671, 2005. First published January 14, 2005; doi:10.1152/ajplung.00355.2004.—It is well established that exposure to high levels of oxygen (hyperoxia) injures and kills microvascular endothelial and alveolar type I epithelial cells. In contrast, significant death of airway and type II epithelial cells is not observed at mortality, suggesting that these cell types may express genes that protect against oxidative stress and damage. During a search for genes induced by hyperoxia, we previously reported that airway and alveolar type II epithelial cells uniquely express the growth arrest and DNA damage (Gadd45a) gene. Because Gadd45a has been implicated in protection against genotoxic stress, adult Gadd45a (+/+ ) and Gadd45a (-/- ) mice were exposed to hyperoxia to investigate whether it protected epithelial cells against oxidative stress. During hyperoxia, Gadd45a deficiency did not affect loss of airway epithelial expression of Clara cell secretory protein or type II epithelial cell expression of pro-surfactant protein C. Likewise, Gadd45a deficiency did not alter recruitment of inflammatory cells, edema, or overall mortality. Consistent with Gadd45a not affecting the oxidative stress response, p21Cip1/Waf1 and heme oxygenase-1 were comparably induced in Gadd45a (+/+ ) and Gadd45a (-/- ) mice. Additionally, Gadd45a deficiency did not affect oxidative DNA damage or apoptosis as assessed by oxidized guanine and terminal deoxyneucleotidyl transferase-mediated dUTP nick-end labeling staining. Overexpression of Gadd45a in human lung adenocarcinoma cells did not affect viability or survival during exposure, whereas it was protective against UV-radiation. We conclude that increased tolerance of airway and type II epithelial cells to hyperoxia is not attributed solely to expression of Gadd45a.

DNA damage; lung; mice; in situ nick-end labeling; 8-hydroxy-2’-deoxyguanosine

SUPPLEMENTAL OXYGEN THERAPY is often used to reduce hypoxemia in premature newborns, children, and adults suffering from respiratory distress (20). Unfortunately, exposure to elevated oxygen causes acute lung injury and under prolonged conditions can stimulate an inflammatory response that further injures and kills alveolar cells. Morphological studies in rodents and primates revealed hyperoxia injures and kills alveolar endothelial and type I epithelial cells (2, 25, 26). Because type I cells maintain the alveolar-capillary barrier, their loss is associated with fluid leak into the air space, respiratory failure, and mortality. Intriguingly, these studies also showed bronchiolar Clara and alveolar type II epithelial cells remain viable with intact cell membranes during exposures that kill microvascular endothelial and type I epithelial cells. Although cell death was not observed, Clara and type II cells clearly respond to hyperoxia as assessed by their respective decrease in mRNA and protein for Clara cell secretory protein (CCSP) and surfactant protein C (SP-C) (23, 39). Furthermore, alveolar type II cells exhibit mitochondrial swelling, increased vacuolization, and glycoprotein accumulation in lamellae, along with oxidized and fragmented DNA (39, 35). Mechanisms underlying selective cellular resistance and sensitivity during hyperoxia remain unknown.

Recent studies (8, 36, 47) have led to an appreciation that reactive oxygen species (ROS) produced by NADPH oxidase are involved in oxygen-induced cell death, whereas mitochondrial-derived forms appear less important. ROS produced during hyperoxia cause DNA strand breaks, sister chromatid exchanges, and other chromosomal aberrations (12, 13, 38). DNA strand breaks, as assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, and 8-hydroxy-2’-deoxyguanosine (8-oxoguanine) lesions have been observed in newborn and adult mice exposed to hyperoxia (4, 39). Although TUNEL staining is used to detect apoptotic DNA strand breaks, it has been detected in airway and type II epithelial cells (29, 31). Because DNA strand breaks are detected by Comet assay in viable type II cells isolated from hyperoxic mice, some TUNEL staining reflects oxidative damage (39). Consistent with oxidative damage to DNA, hyperoxia stimulates expression of the tumor suppressor p53 and its downstream target proteins p21Cip1/Waf1/Sdi1 and Bax. Although p53-deficient mice are not sensitive to hyperoxia (5), p21-deficient mice are highly sensitive and die within 72 h of exposure compared with 120 h for wild-type controls (34). In contrast to the protective effects of p21, hyperoxia kills cells through Bid-dependent activation of caspase 8 and mitochondrial translocation of Bax and Bak members of the Bcl-2 gene family (8, 43). Taken together, these observations suggest downstream targets of p53, such as p21 and Bax, modulate cell survival, and death during hyperoxia.

During a search for other p53-dependent genes whose expression changes during hyperoxia, we discovered hyperoxia stimulates expression of the growth arrest and DNA damage-inducible (Gadd45a) gene (33). Gadd45a was originally identified in a genetic screen for early response genes induced by ultraviolet radiation (11). It is a 21-kDa protein that shares homology to Gadd45b (Myd118) and Gadd45g (CR6/OIG37). Whereas all three family members are induced by genotoxic stress, Gadd45b and Gadd45g also participate in cellular dif-

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Gadd45a protects pulmonary epithelium from oxidative stress. H1299 cells overexpressing Gadd45a to investigate whether mice lacking Gadd45a and human lung adenocarcinoma compared with wild-type mice. These collective observations evidenced by increased expression of heme oxygenase-1 (HO-1) (29). Additionally, ability to be both upstream and downstream of MAPK signal transduction cascade. It can activate p38 and JNK MAPKs by binding and activating MEKK4/MTK1. Activated p38 and JNK in turn regulate p53, which stimulates Gadd45a expression (9, 16). Gadd45a also interacts with the DNA replication and repair proteins cdc2, p21, and PCNA, suggesting a potential role in cell cycle control and DNA repair (14, 44). Additionally, Gadd45a has been associated with core histones within damaged DNA (10). Consistent with a role in DNA repair, Gadd45a (−/−) mice display increased radiation and dimethylbenzanthracene-induced carcinogenesis (17, 18). Loss of Gadd45a in mouse embryo fibroblasts leads to genomic instability, single oncogene transformation, aberrant cell growth, and reduced DNA repair. These findings reveal Gadd45a protects cells from genotoxic stress.

As expected for a gene that protects against genotoxic stress, Gadd45a is selectively expressed by airway and alveolar type II epithelial cells during hyperoxia (33). Consistent with its ability to be both upstream and downstream of MAPK signaling, Gadd45a was highly induced in JNK1-deficient mice that exhibited increased sensitivity to hyperoxia (29). Additionally, JNK1 knockout mice have increased oxidative stress as evidenced by increased expression of heme oxygenase-1 (HO-1) compared with wild-type mice. These collective observations support the hypothesis that Gadd45a protects airway and type II epithelial cells during hyperoxia. In the present study, we use mice lacking Gadd45a and human lung adenocarcinoma H1299 cells overexpressing Gadd45a to investigate whether Gadd45a protects pulmonary epithelium from oxidative stress.

**MATERIALS AND METHODS**

**Exposure of mice to hyperoxia.** The generation and characterization of Gadd45a (−/−) mice have been described previously (18). In the present study, outbred Gadd45a (−/−) mice were backcrossed for 10 generations with inbred C57Bl/6J mice obtained from Jackson Laboratories. The wild-type and null alleles were identified by genomic PCR using specific primers (18). After mating to homozygosity, adult male and female C57B6 (8–12 wk) C57Bl/6J Gadd45a (+/+) and Gadd45a (−/−) mice were exposed to room air or 100% oxygen during 8 h (31). Animals were euthanized by intraperitoneal injection with 100 mg/kg pentobarbital sodium. The left lobe was inflation fixed through the trachea and sectioned for histology, whereas RNA or protein was obtained by homogenizing the right lobes in lysis buffer. Additional mice were lavaged 5 times with 1 ml saline and total protein was quantified using polyvinylpyrrolidone membrane. Membranes were blocked overnight at 4°C in 5% nonfat dry milk before incubating in anti-CCSP, anti-proSP-C (Chemicon International, Temecula, CA), anti-Gadd45a (Santa Cruz, Santa Cruz, CA), anti-HO-1 (Stressgen, Victoria, BC, Canada), or anti-β-actin (Sigma, St. Louis, MO) sera. Immune complexes were detected by chemiluminescence (Amersham, Arlington Heights, IL) and visualized using an Alpha Innotech Fluorchem 8900 gel documentation system (Alpha Innotech, San Leonardo, CA).

**Immunohistochemistry.** Lungs were inflated fixed through the trachea for 10 min with 10% neutral buffered formalin. Lobes were dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Paraffin sections (4 μm) for detection of 8-oxoguanine were stained as previously described (39). In situ DNA strand breaks were detected using a commercially available TUNEL assay kit (Serologicals, Norcross, GA). Sections were visualized with an E800 microscope (Nikon, Melville, NY) and images were captured with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

**Quantitative Immunohistochemistry.** Images of random, noncontiguous fields of total lung were captured as described in Immunohistochemistry. Five fields per lung were obtained from three separate animals for each genotype and each treatment. Quantification was performed with Metamorph software (Universal Imaging, Downingtown, PA). Metamorph was configured to measure total nuclei on the basis of the average area of a nucleus. TUNEL-positive cells were counted manually using Metamorph to mark the counted cells. For each animal, the counts from all fields were summed, and the ratio of TUNEL-positive cells/total cells was determined. Ratios for all animals at each time point were averaged as stated in RESULTS.

**Overexpression of Gadd45a in H1299 cells.** The Gadd45a open-reading-frame was amplified by RT-PCR of whole lung RNA isolated from wild-type mice exposed to 72 h of hyperoxia using forward 5′-GATCAAGCTTATAATGACTTTGGAGAATTG-3′ and reverse 5′-GATCCGAGCTCAGTCATTGGAGCATCC-3′ primers. A BglII restriction enzyme site and Kozak consensus sequence (5′-acc-3′) were added to the forward primer to enhance translation efficiency, and a SacI restriction site was added after the termination codon of the reverse primer. The amplified product was digested with BglII and SacI and ligated into the plasmid containing an internal ribosomal entry site (pRES)-enhanced green fluorescence protein (EGFP) vector (Clontech, Palo Alto, CA). Two fragments containing the Gadd45a-RES-EGFP sequence were obtained when the clone was digested with NheI and NotI. The fragments were sequentially ligated into the tetracycline-inducible pBri2i vector and oriented by PCR amplification. The plasmid was purified by Qiagen (Qiagen Sciences, Valencia, CA) preparation and transfected into H1299 cells using Genefect, a polycaitionic lipid transfection reagent (Molecula, Herndon, VA). Stable clones were selected using 200 μg/ml hygromycin (Invitrogen, Carlsbad, CA). Clones with inducible expression of EGFP were selected by treating cells with 2 μg/ml doxycycline (Sigma) and screening for green fluorescence. Fluorescent colonies were picked and propagated in the absence of doxycycline.
Cells were maintained at 37°C in 5% CO₂ in DMEM and F-12 medium with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin (GIBCO-BRL, Grand Island, NY). Experiments were initiated by plating cells in 100-mm dishes at a density of 5 × 10⁵ cells/ml overnight. Nonadherent cells were removed by washing and replenishing with fresh medium and cultured with or without doxycycline (2 μg/ml) for 24 h. Cells were then cultured in normoxia (room air with 5% CO₂) or hyperoxia (95% O₂-5% CO₂) in a Plexiglas box (Belco Glass, Vineland, NJ) (30) for the described times. The box was sealed and gas mixture delivered with a PROOX model 110 (Reming Instruments, Redfield, NY) at a flow rate of 5 l/min. For some experiments, additional cells were exposed to 30 J/m² UVC light using an UVC-515 Multilinker (Ultra-Lum, Carson, CA) and recovered for 12 h in room air. For viability determination, culture medium was removed from cells that were then trypsinized and pooled with the initial culture medium. Cells were pelleted, resuspended, and stained with trypan blue dye. Cells were counted with a hemacytometer, and viability was determined by dye exclusion. For clonogenic survival, cells were trypsinized, counted, and replated in fresh medium lacking doxycycline. After culturing cells for 14 days in room air, we stained plates with crystal violet, and colonies were scored.

Statistical analysis. Values are typically expressed as means ± SD. Where appropriate, group means are expressed as means ± SE. Group means were compared by ANOVA using Fisher’s post hoc analysis, and P < 0.05 was considered significant.

RESULTS

Hyperoxia induces expression of Gadd45a. Gadd45a mRNA expression was investigated using a semiquantitative RT-PCR assay with β-actin used to verify equivalent amplification of the cDNA prepared by reverse transcription of RNA. Gadd45a mRNA was not readily detected in lungs exposed to room air (Fig. 1A). In contrast, Gadd45a mRNA was detected after 24 h of hyperoxia and continued to increase in abundance through 72 h of exposure. This assay confirmed Gadd45a (−/−) mice do not express Gadd45a (Fig. 1B).

Gadd45a deficiency does not affect the epithelial cell response to hyperoxia. Although airway and type II cells express Gadd45a and remain viable with intact cell membranes through 72 h of hyperoxia, they lose expression of CCSP and SP-C (23, 39). Because mice lacking these proteins exhibit increased sensitivity to hyperoxia (19, 22), it was of interest to use their expression as a marker of how Clara and type II cells respond to hyperoxia in the absence of Gadd45a. To determine whether increased tolerance of airway and type II cells was attributed to their ability to express Gadd45a, the expression of CCSP and SP-C was compared between Gadd45a (+/+) and Gadd45a (−/−) mice by RNase protection analysis (Fig. 2A). CCSP and SP-C mRNA significantly decreased after 72 h of hyperoxia but was not different between Gadd45a (+/+) and Gadd45a (−/−) mice. Whereas decreased CCSP and SP-C mRNA was detected in Gadd45a (−/−) mice exposed to hyperoxia for 48 h, it was not significantly different from Gadd45a (+/+) mice.

Western blot analysis was used to confirm that Gadd45a did not affect loss of CCSP and proSP-C during hyperoxia (Fig. 2B). CCSP was detected as a 10-kDa protein in Gadd45a
(+/+) and Gadd45a (−/−) mice exposed to room air. In both lines of mice, CCSP decreased ~50% after 48 h of hyperoxia and declined even further by 72 h. The full-length 21-kDa proSP-C precursor along with 18- and 16-kDa processing intermediates were detected with an antibody against the aminoterminus precursor region (6). In both Gadd45a (+/+) and Gadd45a (−/−) mice, hyperoxia markedly inhibited proSP-C expression by 48 h with minimal protein detected by 72 h. The loss of CCSP and proSP-C in both lines of mice was confirmed by immunohistochemistry (data not shown). Taken together, Gadd45a does not affect the loss of epithelial-specific lung genes during hyperoxia.

Gadd45a deficiency does not affect the overall pulmonary response to oxidative stress. Unlike Gadd45a that is induced in airway and alveolar type II epithelial cells, hyperoxia stimulates expression of the antioxidant enzyme HO-1 and the cell cycle inhibitor p21 throughout the parenchyma (32). Because these proteins are induced by and protect against hyperoxia, their expression was used to assess whether loss of Gadd45a affected the overall pulmonary response to oxidative stress. P21 mRNA was faintly detected by RNase protection analysis in Gadd45a (+/+) and Gadd45a (−/−) mice exposed to room air and increased markedly during hyperoxia (Fig. 3A). The induction of p21 by hyperoxia was temporally and qualitatively similar in Gadd45a (+/+) and Gadd45a (−/−) mice. HO-1 protein was faintly detected by Western blot analysis in lung tissue from Gadd45a (+/+) and Gadd45a (−/−) mice exposed to room air and was induced by hyperoxia (Fig. 3B). The induction of HO-1 by hyperoxia was comparable between Gadd45a (+/+) and Gadd45a (−/−) mice. Taken together, these findings suggest that Gadd45a does not affect the pulmonary response to oxidative stress through differential induction of the protective proteins p21 and HO-1.

Gadd45a deficiency does not affect oxidative DNA damage or DNA strand breaks during hyperoxia. Previous studies (4, 39) have shown that hyperoxia increases 8-oxoguanine and TUNEL staining in both airway epithelium and throughout the alveoli. Whereas increased 8-oxoguanine reflects oxidation to DNA, increased TUNEL staining has been suggested to reflect DNA damage caused by oxidation as well as during apoptotic cell death. To determine the effect of Gadd45a deficiency on DNA integrity, DNA strand breaks were assessed by TUNEL staining and quantified. A low level of TUNEL-positive cells were observed in lung tissue of Gadd45a (+/+) and Gadd45a (−/−) mice exposed to room air (Fig. 4, A and B). Quantification of the percentage of TUNEL-positive cells showed no difference in the baseline level of DNA strand breaks [9.6 ± 3.1% for Gadd45a (+/+), 11.0 ± 2.2% for Gadd45a (−/−), P = 0.59]. TUNEL-positive cells increased significantly in both strains of mice after 72 h of hyperoxia (P < 0.01) (Fig. 4, C and D), but quantification showed no significant difference [32.5 ± 3.5% for Gadd45a (+/+) , 34.7 ± 3.3% for Gadd45a (−/−), P = 0.41]. An additional marker of DNA damage, 8-oxoguanine lesion formation, was assessed in the lungs of Gadd45a (+/+) and Gadd45a (−/−) mice using a FITC-conjugated 8-oxoguanine binding protein. Low levels of 8-oxoguanine-positive cells were observed in both strains of mice in room air (data not shown). 8-oxoguanine DNA lesions increased after 72 h of hyperoxia (Fig. 4, E and F), although cell number, cell type, or staining intensity was not qualitatively different between Gadd45a (+/+) and Gadd45a (−/−) mice. These findings suggest that Gadd45a deficiency does not enhance DNA strand breaks or oxidative DNA lesion accumulation in response to hyperoxia.

Gadd45a deficiency does not affect inflammation and lung injury during hyperoxia. During exposure to hyperoxia there is a loss of air/blood barrier integrity as the microvascular endothelial and alveolar type I epithelial cells die. Cell death potentiates an inflammatory response characterized by an accumulation of polymorphonuclear leukocytes, monocytes, and macrophages. This inflammatory response, coupled with compromised air/blood barrier leads to an influx of serum proteins and fluid into the alveolar space, as well as interstitial edema in the perivascular and peribronchiolar areas of the lung parenchyma. To determine whether Gadd45a deficiency enhanced lung injury through an increased inflammatory response, total protein and inflammatory cell number were quantified in lavage fluid obtained from Gadd45a (+/+) and Gadd45a (−/−) mice exposed to room air or 72 h of hyperoxia (Fig. 5, A and B). Inflammatory cell number and protein in bronchoalveolar lavage fluid was low in mice exposed to room air and increased significantly during hyperoxia. However, there was no significant difference between Gadd45a (+/+) and Gadd45a (−/−) mice.
Lung tissue sections from Gadd45a (+/+) and Gadd45a (−/−) mice exposed to 72 h of hyperoxia were stained with hematoxylin and eosin, examined by a veterinary pathologist, and scored for toxicity using a previously described scale (3). Microscopic examination of tissue sections revealed an increase in perivascular and peribronchiolar edema in both Gadd45a (+/+) and Gadd45a (−/−) mice (Fig. 5, C and D). Additionally, there was an increase in necrosis of both the subterminal and terminal bronchioles in both strains of mice. There were no differences noted in the pathological markers of injury between Gadd45a (+/+) and Gadd45a (−/−) mice. Gadd45a deficiency does not effect overall survival during exposure to hyperoxia. In one survival study using 10 mice of each genotype, Gadd45a (+/+) mice died between 92 and 116 h of exposure with a mean survival of 112 ± 2.3 h. In contrast, Gadd45a (−/−) mice died between 116 and 138 h with a mean survival of 126.3 ± 2.3 h. This implies Gadd45a does not protect, but rather modestly reduces survival by ~12 h. Because this was difficult to confirm in subsequent survival experiments, we calculated the number of mice in all experiments that survived to 72 h. In contrast to the survival curve showing enhanced survival, 92% (35/38) of Gadd45a (−/−) mice survived to 72 h compared with 100% (39/39) of wild-type mice. This modest difference in survival at 72 h was not significant (P = 0.35). Based on the slight differences in survival coupled with the pathological indices of lung injury, we conclude Gadd45a does not play a major role in lung injury or mortality.

Ectopic expression of Gadd45a in H1299 cells does not protect against hyperoxia. To test whether Gadd45a could protect cultured epithelial cells during hyperoxia, Gadd45a was overexpressed in human lung adenocarcinoma H1299 cells under tetracycline-dependent control. The Gadd45a open-reading-frame was expressed as a bicistronic mRNA upstream of the EGFP in the pBig2i bidirectional tet-on plasmid (Fig. 6A).

This vector contains the rtTA protein expressed under control of a minimal thymidine kinase promoter and the transgene of interest expressed in the opposite direction under control of a minimal cytomegalovirus promoter. The addition of doxycycline stimulates expression of both rtTA and the Gadd45a/EGFP bicistronic mRNA, whereas EGFP simplifies identification and purification of clones during the selection of stable colonies. In the absence of doxycycline, endogenous Gadd45a was weakly induced after 24 h of hyperoxia and remained detectable through 72 h (Fig. 6B). In the presence of doxycycline, Gadd45a was markedly induced and remained highly expressed through 72 h of hyperoxia. As confirmation of in vivo data showing that Gadd45a does not affect the p21
response to hyperoxia, lysates from induced and uninduced H1299 cells were immunoblotted with antibodies against p21. Overexpression of Gadd45a did not induce p21 in H1299 cells in the presence or absence of hyperoxia (data not shown). To determine the effects of overexpression of Gadd45a on cell viability during hyperoxia, inducible H1299 cells were cultured with and without doxycycline in room air or hyperoxia for 2, 4, or 6 days and viability was determined by trypan blue dye exclusion (Fig. 6C). Dye exclusion was used in place of the lactate dehydrogenase (LDH) assay because the LDH assay also measures mitochondrial output and membrane leakiness, two variables affected by exposure to hyperoxia. Viability was decreased in both uninduced and induced cells after 4 or 6 days of hyperoxia ($P < 0.01$); however, overexpression of Gadd45a was not protective. Overexpression of Gadd45a for 5 days also did not affect viability of cells exposed to room air.

Clonogenic survival was used as a more sensitive assessment of whether robust overexpression of Gadd45a could protect cells from hyperoxia. Clonogenic survival measures both replicative capacity as well as cell survival. In the absence of doxycycline, hyperoxia markedly reduced clonogenic survival (Fig. 6D). Surprisingly, survival of hypoxic cells was reduced even further when Gadd45a was overexpressed, although the trypan blue dye experiments ruled out toxicity from overexpression of Gadd45a. Gadd45a overexpression did, as expected, protect against UV radiation.

**DISCUSSION**

Of the nearly 40 different cell types that comprise the lung, microvascular endothelial and type I epithelial cells are selectively killed by prolonged exposure to a high amount of oxygen. Because most other cell types survive, they either are less damaged or express prosurvival molecules that stimulate DNA repair and block apoptosis. Consistent with the latter hypothesis, airway and alveolar type II epithelial cells exposed to hyperoxia selectively express Gadd45a, a protein that maintains genome stability after many types of genotoxic stress. If Gadd45a protected Clara and alveolar type II cells from oxidative damage, one would expect CCSP and proSP-C expression to decline faster in $Gadd45a^{-/-}$ mice. Because mice lacking CCSP or proSP-C are sensitive to hyperoxia, the rapid loss of these proteins in $Gadd45a^{-/-}$ mice should correlate with increased sensitivity. Contrary to our hypothesis, we provide evidence that loss of Gadd45a does not markedly affect the loss of these proteins or the pathological indices of oxygen toxicity. Like Gadd45a, loss of p53 does not sensitize mice to hyperoxia, whereas other p53-regulated targets such as p21 and Bax contribute to cell survival and death. Taken together, these findings indicate cellular protection against chronic oxidative stress involves only a subset of the p53-regulated genes.

Although airway and alveolar type II cells remain viable during hyperoxia, they accumulate DNA strand breaks as measured by comet or TUNEL assay and 8-oxoguanine lesions (4, 39). Proliferation of airway epithelium is very low during recovery from hyperoxia, but these cells are not lost, indicating that they presumably repair DNA lesions. Like alveolar type II cells are progenitors for type I cells that die during exposure (1) they must also repair DNA that has been damaged by hyperoxia. The induction of Gadd45a specifically in these
cells made it an attractive molecule for promoting repair of oxidized cells. Indeed, studies with Gadd45a (−/−) mice and cell lines indicate Gadd45a plays a major role in nucleotide excision repair, the predominant pathway used to repair UV-induced DNA damage and other bulky adducts. The global genomic repair pathway of nucleotide excision repair is defective in Gadd45a (−/−) mouse embryonic fibroblasts, whereas transcription-coupled repair is not (28, 40). Through its ability to bind UV-induced DNA lesions and disrupt assembly of histones, Gadd45a may allow repair complexes to assemble at damaged sites (10). Its role in repair of oxidized lesions and DNA strand breaks, such as those caused by ionizing radiation, is less clear. Gadd45a (−/−) mice exhibit latent sensitivity to radiation significantly reduced clonogenic survival (*P < 0.05, n = 3).

Fig. 6. Overexpression of Gadd45a does not protect against hyperoxia. H1299 cells with tetracycline-inducible expression of Gadd45a were cultured in the absence (−) or presence (+) of doxycycline (DOX) for 1 day. Cells were then cultured in room air or hyperoxia for 48 h. A: schematic of the plasmid construct used to create stable expression of Gadd45a in H1299 cells. IRES, internal ribosomal entry site; EGFP, enhanced green fluorescent protein; TetO, tetracycline operon; CMV, cytomegalovirus; hph, hygromycin B phosphotransferase; TK, thymidine kinase; rTAN, reverse tetracycline transactivator. B: representative Western blot of Gadd45a and β-actin expression. C: trypan blue dye exclusion viability assay of cells exposed to room air for 1 or 5 days or hyperoxia for 2, 4, or 6 days (*P < 0.05). D: clonogenic survival of cells exposed to room air, 48 h of hyperoxia, or 30 J/cm² of UVC radiation. Whereas hyperoxia and UVR radiation significantly reduced clonogenic survival (*P < 0.01), overexpression of Gadd45a further reduced survival of cells in hyperoxia and enhanced survival of cells exposed to UV (†P < 0.05, n = 3).

In addition to its ability to affect DNA repair, Gadd45a stimulates JNK and p38 MAPK signaling. It was initially shown that Gadd45a binds and activates MTK1/MEKK4, a MAPK kinase kinase known to stimulate JNK and p38 (42). Activation of JNK and p38 by UV radiation is dependent on Gadd45a induction. Gadd45a also directly binds p38, and this association is necessary for p33-dependent suppression of H-ras transformation (9). Direct binding of Gadd45a with JNK or ERK MAPKs was not detected. Skin from Gadd45a (−/−) mice exposed to UV radiation exhibits prolonged JNK and p38 activity, failure to express p33 or execute cell cycle checkpoint control, and reduced apoptosis (16). Consistent with Gadd45a being both upstream and downstream of MAPK signaling, JNK1-deficient mice exposed to hyperoxia expressed fivefold higher levels of Gadd45a as measured by nylon array (29). Additionally, JNK1-deficient mice exhibited increased expression of the antioxidant enzyme HO-1. If Gadd45a deficiency affected JNK signaling, one would expect an increase in HO-1 expression due to increased oxidative stress. Our observations show that HO-1 levels increase in response to hyperoxia, but this increase was independent of Gadd45a status. With the exception of one report in human pulmonary artery endothelial cells (36), most studies find hyperoxia does not affect p38 MAPK signaling (for review see Ref. 27), and we were unable to detect phosphorylated p38 during hyperoxia either (data not shown). In contrast, the ERK1/2 pathway holds the most promise with studies showing it promotes death of cultured epithelial and macrophage cell lines during exposure and favors survival of type II cells isolated from rats recovering from hyperoxia (7, 37, 47). However, the role of ERK1/2 in cellular protection against hyperoxia remains unclear because it is not activated in human lung adenocarcinoma A549 cells...
As expected, hyperoxia increased phosphorylated ERK1/2 in mouse lung tissue, but there was no difference between Gadd45α (+/+ ) and Gadd45α (−/− ) mice (data not shown). Whereas hyperoxia can activate all three MAPK pathways, it appears to selectively activate different pathways that may be attributed to the type of cell and immortalization status. The activation of Gadd45α in airway and type II cells may help clarify which MAPK pathways become activated in these cell types.

Besides its cell type restricted expression, Gadd45α induction by hyperoxia does not require p53 (33). In contrast, Gadd45α induction after IR is strictly dependent on a functional p53 binding site within the third intron (45). Because most of the damage caused by IR requires an oxidized state, it has been suggested IR and hyperoxia induce the same type of DNA lesions. However, HeLa and Chinese hamster ovary cells that have been made tolerant to hyperoxia by incremental increases in oxygen tension remain sensitive to IR, whereas radio-resistant cells remain sensitive to hyperoxia (21). The observation that cross-tolerance does not occur with these different types of oxidizing agents is consistent with IR stimulating p53-dependent and hyperoxia stimulating p53-independent expression of Gadd45α. Interestingly, p53 contributes without being essential for expression of Gadd45α after UV, medium starvation, or exposure to chemical alkylating agents (46). Because UVB radiation cross-links DNA and stimulates cellular oxidation, it is intriguing to consider whether hyperoxia induces similar types of DNA lesions as those seen with UVB.

Whereas Gadd45α may not play an essential or observable role in hyperoxic lung injury, overexpression in H1299 cells potentiates hyperoxia-induced cell death while protecting against UV radiation. H1299 cells are deficient in p53 and therefore fail to express p21 or arrest in G1 and they slowly accumulate in S phase when exposed to hyperoxia (15). Because Gadd45α modifies DNA accessibility when UV damages chromatin, it may enhance oxidative damage to DNA through its ability to interact with damaged histones (10). In contrast, the mitotic index of the adult lung is <1%, which may explain why phenotypic differences were not readily detected in Gadd45α (−/− ) mice. One might predict Gadd45α to play a more prominent role in newborn mice exposed to hyperoxia because the mitotic index of the postnatal lung is much higher. Surprisingly, hyperoxia does not induce Gadd45α in newborn mice (M. A. O’Reilly, unpublished observations). A better understanding of the age-restricted expression of Gadd45α could provide insight into why newborns are significantly more tolerant to hyperoxia than adults.

In summary, the studies presented here demonstrate that loss of Gadd45α does not significantly affect the pulmonary response to prolonged oxidative stress. This conclusion is obviously limited by our inability to detect differences in the parameters studied. The possibility remains that Gadd45α plays a critical role in cellular events that were not investigated and that do not result in detectable pathology. Alternatively, the role Gadd45α plays in maintaining genome stability is so critical that alternative pathways exist to ensure that its loss is not detrimental. Clearly the p53-independent induction of p21 is one example of such a process. Furthermore, two other isoforms, Gadd45b and Gadd45g, have overlapping yet distinct activities from Gadd45α and may act to ensure Gadd45α functions are met in its absence. Future studies using gene array tools should permit the identification and classification of gene networks that selectively regulate individual cell survival and death during hyperoxia.

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REFERENCES

1. Adamson IY and Bowden DH. The type 2 cell as progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen. Lab Invest 30: 35–42. 1974.


GADD45A DURING HYPEROXIA

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