Protection of Cells in Physiological Oxygen Tensions against DNA Damage–Induced Apoptosis*

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Oxygen availability has important effects on cell physiology. Although hyperoxic and hypoxic stresses have been well characterized, little is known about cellular functions in the oxygen levels commonly found in vivo. Here, we show that p53-dependent apoptosis in response to different DNA-damaging agents was reduced when normal and cancer cells were cultured at physiological oxygen tensions instead of the usual atmospheric levels. Different from what has been described in hypoxia, this was neither determined by decreases in p53 induction or its transactivation activity, nor by differences in the intracellular accumulation of reactive oxygen species. At these physiological oxygen levels, we found a constitutive activation of the ERK1/2 MAPK in all the models studied. Inhibition of this signaling pathway reversed the protective effect in some but not all cell lines. We conclude that a stress-independent constitutive activation of prosurvival pathways, including but probably not limited to MAPK, can protect cells in physiological oxygen tensions against genotoxic stress. Our results underscore the need of studying cells in environments that are similar to those found in live tissues in the absence of stress or disease.

In that regard, the responses to DNA damage at physiological oxygen levels have been insufficiently characterized. The importance of oxygen availability in these processes is underscored by the fact that in hypoxic stress, tumor suppressor p53 is up-regulated (3, 8–10), but its transactivation capacity is inhibited (3, 11, 12). Also, hypoxic cells are known to be more resistant to damage because of a reduction in reactive oxygen species (ROS) generation (13, 14). However, it is not clear whether any of these effects can also be observed at nonhypoxic physiologically low oxygen tensions. To understand better the DNA damage responses of cells in vivo, we investigated the influence that microenvironmental levels of oxygen usually found in human tissues have on the induction of cell death in response to genotoxic stress.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (50 units/ml). IMR90 expressing a dominant negative p53 (IMR90 DNP53) were obtained using a R248W mutant of p53 subcloned with BamHI/AscI into a pBABE vector, as described (15). Briefly, amphotropic virus stocks were generated by transient

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cotransfection of 293T cells with 5 µg of the retroviral construct and 5 µg of pCL amphi packaging plasmid. Culture fluids were collected and filtered 48 h later, and stocks with titers of $>10^5$ selectable marker units were used for infections of IMR90 fibroblasts.

Doxorubicin (Sigma-Aldrich) and/or U0126 (Promega) were added to the medium and were not removed until analysis was performed or when medium was changed, as specified in the text. For UV treatments, medium was removed, and cells were kept under a UV lamp for a total of 50 mJ/cm². Incubations at 5% O₂ were performed in a Sanyo MCO-5 M cell incubator. Inhibition of ERK1/2 phosphorylation was achieved by transfecting On-Target plus SMARTpool small interfering RNA MAP2K1 and MAP2K2 (Thermo Scientific). Small interfering RNA against luciferase was used as control. Lipofectamine 2000 (Invitrogen) was used for transfection, following the manufacturer’s protocols.

FIGURE 1. Physiological oxygen tensions ameliorate responses to different DNA-damaging agents in HCT116. A, representative FACS analysis of PI-stained HCT116 cells exposed to 50 mJ/cm² UV radiation, 1 µg/ml doxorubicin, or 15 Gy of ionizing radiation and then incubated for 48 h in 20% O₂ or 5% O₂. Numbers indicate percentages of counts in sub-G₀ phase of the cell cycle (dead cells). B, representative FACS analysis of annexin/PI-stained HCT116 cells 48 h after being treated with 50 mJ/cm² UV radiation. Lower right quadrants (annexin-positive) represent early apoptotic, and upper right quadrants (annexin- and PI-positive) represent late apoptotic cells. Bottom left quadrant are annexin- and PI-negative cells (alive). Numbers indicate percentages in each quadrant. C, representative plates of a colony formation assay. 200 cells were seeded in each plate and, 24 h after passage, were treated with 1 µg/ml doxorubicin for 48 h before the medium was changed. Cells were allowed to grow for 14 days in 20% or 5% O₂ incubators before being stained with Giemsa. D, quantitation of the colony formation assays described in C. Results represent mean values of two duplicate experiments, and error bars show S.D.
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Fluorescence-activated Cell Sorter (FACS) Analysis—Fluorescent-stained cells were transferred to polystyrene tubes with cell strainer caps (Falcon) and subjected to FACS (BD Biosciences FACSCalibur or FACSCanto II) using Cell Quest 3.2 or FACSDiva 6.0 software (BD Biosciences) for acquisition and analysis.

Cell Cycle Analysis and Staining—Cells were stained with propidium iodide (PI), using the CycleTEST Plus DNA reagent kit (BD Biosciences), following instructions provided by manufacturer. Other cells were stained with the annexin V-Fluos staining kit (Roche Applied Science) following instructions provided by manufacturer.

Measurement of Intracellular ROS—Cells were incubated with 5 µg/ml of dichlorofluorescin diacetate (DCF) (Molecular Probes) for 30 min at 37 °C, then washed with phosphate-buffered saline, trypsinized, and collected in 1 ml of phosphate-buffered saline followed by FACS analysis. Values of mean fluorescence intensity were reported.

Measurement of Oxidative Damage to DNA—Cells were stained with an OxyDNA assay kit (Calbiochem) following the manufacturer’s instructions to detect the presence of 8-oxoguanine in DNA. Alternatively, relative levels of oxidative purine base damage were monitored using the human form-amidopyrimidine DNA glycosylase (fpg) comet assay as described previously (16), with the following modifications. After lysis, the slides were washed once with distilled water and immersed in three changes of enzyme digestion buffer (40 mM HEPES, 0.1 mM KCl, 0.5 mM EDTA, and 0.2 mg/ml bovine serum albumin (pH 8.0)), for 5 min each time, at room temperature. Fpg (Sigma-Aldrich) was added to the gel (50 µl/gel) at 1/500 or 1/1000 dilutions; gels were covered with a coverslip and incubated in a humidified chamber at 37 °C for 30 min. The coverslip was removed, and the slides were placed in a horizontal electrophoresis tank. DNA damage was expressed as the percentage of DNA in the comet tails.

p53 Lentiviral Transcriptional Enhanced Green Fluorescent Protein Reporter—The vector for lentiviral transcriptional enhanced green fluorescent protein reporter has been described previously (17). Briefly, three copies of p53-responsive element of mouse MDM2 gene were synthesized and ligated into a modified lentiviral vector using MluI and NheI. The sequence of p53-responsive element used is 5’-GGTCAAGTTCGGGACACGTCC-3’.

Immunoblot Analysis—Cells were washed twice with ice-cold phosphate-buffered saline and lysed in 10 mM Tris (pH 7.4), 30 mM Na2HPO4, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40, 0.5 mM Na3VO4, 10 mM β-glycerophosphate disodium salt hydrate, 0.1% SDS, and 1 µg/ml protease inhibitors mixture (Sigma). Lysates were cleared by centrifugation at 14,000 rpm for 20 min at 4 °C. Protein concentrations were then determined using the Bradford protein assay (Bio-Rad). 100 µg of total cell protein/sample was subjected to 12% SDS-PAGE and transferred to Protein nitrocellulose filter (Schleicher & Schuell). An ECL detection system (Amersham Biosciences) was used. p53 was detected with the 1801 antibody, p21 with the Ab-1 monoclonal antibody from Sigma) were used as loading factor. Other cells were stained with the annexin V-Fluos staining kit (Roche Applied Science) following instructions provided by manufacturer.

RESULTS

Reduction of Apoptosis after DNA Damage in Physiological Oxygen Tensions—To study cellular responses to genotoxic stress in physiological oxygen conditions, we exposed wild type p53-containing HCT116 colon cancer cells to doxorubicin, UV or ionizing radiation, and immediately incubated them at 20% or 5% O2. 5% O2 was selected for being an oxygen tension commonly found in vivo that does not induce a hypoxic stress response (5). Indeed, untreated cells did not show any significant change in cell cycle profile or survival after 48 h at 5% O2 (Fig. 1A), indicating that this oxygen concentration did not impair basic cell physiology. Of note, we found significantly more dead cells in response to all damaging agents in cultures at 20% than in those at 5% O2 (Fig. 1A). This effect was reproducible with lower concentrations of doxorubicin (supplemental Fig. 1A). We further investigated this result using an annexin V/PI staining, which is specific for detecting apoptosis (18). As shown in Fig. 1B, there was a higher percentage of annexin-
positive cells at 20% O₂ after exposure to UV radiation, indicating that the cell death observed was due to apoptosis and confirming that it was much lower at physiological oxygen tensions. Similar results were obtained after treatment with different doxorubicin concentrations (supplemental Fig. 1B). We observed that this antiapoptotic effect was not dependent on preincubation of cells at 5% O₂ because a similar protection could be observed regardless of the amount of time that cells were equilibrated before treatment (supplemental Fig. 1, A and C).

Next, we extended our findings using other human cell lines. As shown in supplemental Fig. 2A, the reduction in cell death was also observed in IMR90 diploid fibroblasts, U2OS osteosarcoma cells, and MCF7 breast cancer cells, indicating that this phenomenon is likely common in both normal and cancer cells. Of note, all of these cells expressed wild type p53. Cells lacking a functional p53 were more resistant to DNA damage than their isogenic counterparts, as expected (supplemental Fig. 3). When death was eventually induced, no differences could be observed at 5% O₂, suggesting that p53-independent mechanisms of cell death were not affected by changes in oxygen levels.

Finally, we found that cells exposed to doxorubicin were able to form more colonies at 5% O₂ than 20% O₂ (Fig. 1, C and D), confirming the prosurvival effect of the lower oxygen tension. Of note, colonies at 5% O₂ were bigger in average, suggesting a growth advantage consistent with previous reports (6, 7). Taken together, these results show that physiological oxygen environments have a protective effect against p53-mediated apoptosis.

**FIGURE 3.** Reduction of p53-induced apoptosis at physiological oxygen tensions is not mediated by differences in intracellular ROS levels. A, ROS levels in HCT116 cultured at 20% or 5% O₂, as measured by DCF staining. Cells were treated with 15 Gy of ionizing radiation, and FACS analysis was performed 24 h later. B, ROS levels in HCT116 cells 24 h after treatment with 15 Gy of γ-radiation, as measured by FACS analysis of DCF-stained cells. Increases in mean fluorescence intensity in γ-radiation-treated cells compared with untreated cells are plotted. Results represent mean values of three different experiments, and error bars show S.D. p value between bars is 0.8218, calculated by Student’s t test. C, FACS analysis of HCT116 cells stained with the OxyDNA assay kit (Calbiochem) 2 days after being exposed to 50 mJ/cm² UV or 1 µg/ml doxorubicin (Dox). Cells were incubated at 20% (black bars) or 5% O₂ (gray bars) immediately after treatment. The percentage of increase in mean fluorescence intensity, compared with the basal fluorescence intensity of untreated cells (Control) cultured at 20% O₂, is plotted. Results represent mean values of three different experiments, and error bars show S.D. D, comet assay using HCT116 cells cultured at 20% or 5% O₂ for 24 h in the presence of 1 µg/ml doxorubicin. Experiments were performed in the absence (left) or presence (right) of fpg to determine specific damage due to oxidation (the difference in tail DNA percentage between the two conditions). Cells treated with 100 µM H₂O₂ for 10 min on ice right before analysis were used as positive controls of oxidative DNA damage. Results using fpg buffer (not shown) were comparable with samples without fpg. Mean percentage of tail DNA from three experiments is plotted. Error bars represent S.D.
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A

|          | HCT116 | IMR90 |
|----------|--------|-------|
| O₂ 20%  | +      | +     |
| 5%      | -      | +     |
| γR -     | +      | -     |

|          | P-MAPK | MAPK  |
|----------|--------|-------|

B

HCT116

20% O₂

|          | Control | +U0126 |
|----------|---------|--------|
| Control  | 5%      | 6%     |
| Doxorubicin | 46% | 63% |

5% O₂

|          | Control | +U0126 |
|----------|---------|--------|
| Control  | 8%      | 7%     |
| Doxorubicin | 26% | 60% |

C

HCT116

20% O₂

|          | Control | UV | U0126 | Dox | UV+U0126 | Dox+U0126 |
|----------|---------|----|-------|-----|----------|-----------|
| p53      |         |    |       |     |          |           |
| p21      |         |    |       |     |          |           |
| p21      |         |    |       |     |          |           |
| P-MAPK   |         |    |       |     |          |           |
| MAPK     |         |    |       |     |          |           |

5% O₂

|          | Control | UV | U0126 | Dox | UV+U0126 | Dox+U0126 |
|----------|---------|----|-------|-----|----------|-----------|
| p53      |         |    |       |     |          |           |
| p21      |         |    |       |     |          |           |
| p21      |         |    |       |     |          |           |
| P-MAPK   |         |    |       |     |          |           |
| MAPK     |         |    |       |     |          |           |

D

P-MAPK

Expression levels (OD/mm²)

- 20% O₂
- 5% O₂

Control | Dox | Dox + U0126

p53
in response to damage compared with atmospheric oxygen levels and that this occurs both in normal and cancer cells.

Reduction in p53-mediated Apoptosis Does Not Depend on Changes in p53 Induction or Activity—It has been reported that hypoxia can interfere with p53 transactivation properties (19). We reasoned that physiological oxygen tensions could have a similar effect, which could explain the decrease in apoptosis. However, we found that levels of induced p53 were not affected by oxygen tensions in HCT116 or the wild type p53 colon cancer RKO cell line (Fig. 2, A and B). Moreover, expression of different p53 target genes did not vary either (Fig. 2, A and B), indicating that p53 activity was not affected. This was further confirmed using a fluorescent reporter with a p53-responsive element, which showed comparable activation after damage at 20 and 5% O2 (Fig. 2C). Similar results were observed in normal fibroblasts (supplemental Fig. 4). We concluded that variations in the induction or the activity of p53 after DNA damage were not responsible for the reduction in apoptosis observed at 5% O2, which further stresses the differences between physiologically low oxygen tensions and hypoxia.

Physiological Oxygen Tensions Do Not Affect Intracellular ROS Levels or the Magnitude of Oxidative DNA Damage—UV, ionizing radiation, and doxorubicin induce ROS, which greatly contribute to their toxic effects (20–23). UV-treated fibroblasts show reduced levels of oxidation and apoptosis when cultured at 5% O2 (14), and squamous cell carcinomas have lower intracellular ROS levels in response to radiation (13). Also, murine fibroblasts maintained at 3% O2 have fewer oxidative lesions in their DNA than cells cultured at 20% (6). All of this suggests that the protection against apoptosis we observed could be dependent on a diminished induction of ROS after damage. To test this possibility, we stained HCT116 cells with DCF, a marker of accumulation of cellular oxidants (24). There were no statistically significant differences in ROS levels after ionizing radiation between cells at 20 and 5% O2 (Fig. 3, A and B). Basal ROS were also comparable (Fig. 3A). To confirm these results further, we used a fluorescent probe that binds to 8-oxoguanine in the DNA, a specific marker of oxidative DNA damage and thus an indirect measure of intracellular ROS accumulation. As shown in Fig. 3C, there were no significant differences in cells cultured at 20% or 5% O2, either before or after treatment with DNA-damaging agents. Finally, a fpg comet assay, which measures both strand breaks and oxidatively damaged purine base lesions in the DNA, also showed no differences before or after exposure to doxorubicin (Fig. 3D). These results together prove that physiological oxygen environments do not reduce basal or damage-induced intracellular ROS levels in HCT116 under the conditions explored and rule out a mechanism of cellular protection in these cells based on hampering ROS increases and the ensuing DNA damage that they cause.

Role of MAPK in Cell Viability at Physiological Oxygen Tensions—p53-dependent apoptosis is ameliorated by prosurviving signals such as MAPK (25), which can be induced in low oxygen conditions (26). We reasoned that activation of the MAPK pathway could be contributing to a reduction in cell death responses at 5% O2. We explored this hypothesis by measuring phosphorylation of MAPK (ERK1/2) in cells treated with DNA-damaging agents. Consistent with previous reports (14, 25), MAPK phosphorylation increased due to DNA damage (Fig. 4A). However, we could also observe an increase in MAPK phosphorylation in the absence of any stress in both normal and cancer cells cultured at 5% O2 (Fig. 4A; see also supplemental Fig. 5D). The levels of activated MAPK after treatment were also proportionally higher in 5% than in 20% O2. These data show a constitutive activation of MAPK in cells cultured at physiological oxygen tensions and suggest that this prosurvival pathway could interfere with the induction of apoptosis.

To test this possibility, we blocked MAPK activation in HCT116 with the specific chemical inhibitor U0126 (27). We found that this abrogated the protective effects of 5% O2 after treatment with doxorubicin (Fig. 4B). As reported previously (25), MAPK inhibition also increased p53-induced apoptosis in cells cultured at atmospheric oxygen tensions. Of note, although MAPK phosphorylation was completely blocked, expression of p53 and p21 was not affected (Fig. 4, C and D). Because U0126 could, in principle, induce nonspecific inhibitions of other kinase pathways, we also suppressed MAPK phosphorylation using small interfering RNA against the upstream kinase, MAPK/ERK kinase (MEK). This similarly reversed the antia apoptotic effect of 5% O2 in HCT116 (supplemental Fig. 5, A and B). However, repression of MAPK phosphorylation was not sufficient to restore cell death in U2OS cells cultured at 5% O2 (supplemental Fig. 5, C and D). Of note, chemical inhibition of MAPK phosphorylation in these cells was not as complete as that observed in HCT116. These results together show that the resistance to DNA damage in HCT116 when cultured at physiological oxygen tensions depends on MAPK activation. This is likely to be determined by cell context because other cell lines did not respond similarly.

Finally, to assess the importance of MAPK in the increased cell growth observed at 5% O2 (6, 7) (see Fig. 1, C and D) we performed a colony formation assay in the presence of the MAPK inhibitor. As shown in supplemental Fig. 6, the increase in colony size observed at 5% O2 was suppressed in the absence of MAPK activity. Of note, the inhibitor equally diminished the number of colonies that could be formed at 20 and 5% O2. These results indicate that the growth advantage of cells cultured at physiologic oxygen tensions in HCT116 is also dependent on MAPK signaling.

FIGURE 4. Role of MAPK in the prosurvival effects of physiological oxygen environments. A, left, immunoblot analysis of HCT116 cells 48 h after being treated with 15 Gy of γ-radiation. Cells were transferred to 20% or 5% O2 incubators immediately after treatment. A, right, immunoblot analysis of IMR90 treated with 50 μl/cm2 UV or 1 μg/ml doxorubicin and incubated at 20% or 5% O2. Lysates were obtained 24 h after treatment. B, representative FACS analysis of annexin V-stained HCT116 treated with 1 μg/ml doxorubicin (Dox) in the presence or absence of 5 μM MAPK inhibitor (U0126). Cells were incubated for 48 h in 20% or 5% O2 immediately after chemicals were added to the medium. Percentages indicate number of annexin V-positive cells (apoptotic). C, immunoblot analysis of HCT116 cells treated with 50 μl/cm2 UV or 1 μg/ml doxorubicin in the presence or absence of 5 μM MAPK inhibitor (U0126) and incubated at 20% or 5% O2 for 24 h immediately after chemicals were added to the medium. Lysates were obtained 24 h after treatment. D, quantitation by densitometry of the protein levels of P-MAPK and p53 of cells in C.
**DISCUSSION**

Human tissues are normally exposed to a range of low oxygen concentrations (5, 28), most of them insufficient to elicit a cytotoxic stress. These conditions are often overlooked because cell culture studies are generally performed at atmospheric oxygen tensions. Thus, although hypoxic stress has been thoroughly studied, little is known about cell physiology in the most common in vivo oxygen tensions. We analyzed the influence of oxygen in the response of normal and cancer cells to different DNA-damaging agents. Our results underscore the impact that normal microenvironmental oxygen levels have on cellular functions and stress the importance of taking them into account when studying genotoxic stresses.

We compared the toxic effects of a chemotherapeutic compound and radiation on cells cultured at atmospheric (20%) and physiological (5%) oxygen tensions. We uncovered substantially different responses. Normal and cancer cells were more resistant to p53-induced apoptosis when cultured at physiological oxygen tensions, and this was independent of changes in protein levels or transcriptional activity of p53. Our data are consistent with previous reports showing that 5% O₂ can delay DNA fragmentation in calcium-mediated apoptosis (29). It is, however, the opposite of what has been described in hypoxia, in which p53 expression is induced while simultaneously its transactivation functions are suppressed (11, 12). Importantly, these results show that a reduction of oxygen up to 5% does not trigger the stress pathways activated in hypoxia in the models tested, inducing a previously uncharacterized prosurvival response instead.

It has been proposed that cells at physiological oxygen tensions have a lower level of oxidative damage after exposure to toxic agents (6, 13, 14). In contrast, we did not observe any change in basal or induced intracellular ROS levels or the subsequent oxidative damage to DNA in the models studied. Our data are consistent with the fact that very drastic reductions in oxygen availability (<0.22%) are required to interfere biochemically with the generation of oxygen radicals in response to damaging agents such as ionizing radiation (5). This suggests that although microenvironmental oxygen reductions may hinder ROS generation in some tissues, they do not play a role in others. Our results indicate that these tissues are nevertheless protected from genotoxic stress.

The reasons for this protection still need to be fully elucidated. We observed that culturing cells at 5% O₂ induced ERK1/2 MAPK phosphorylation in normal and cancer cells, even in the absence of any damage. The prosurvival and antiapoptotic effects of MAPK are well known. For instance, we have shown that p53 itself can activate MAPK and that this compensates the induction of apoptosis (25). Inhibition of MAPK phosphorylation suppressed the prosurvival effects of 5% O₂ in HCT116, confirming that the MAPK pathway participates in blocking apoptosis in these cells. This effect was not observed in other cell lines tested. This could be in part explained by the difficulty of inhibiting MAPK phosphorylation in some cells. However, it is likely that cell-specific factors determine the involvement of the 5% O₂-dependent MAPK activation in protecting against apoptosis. For instance, HCT116 has a ras mutation in codon 13 (30) that could determine their dependence on MAPK signaling. Other prosurvivals factors that may be constitutively activated at physiological oxygen tensions need to be investigated to understand what factors may contribute to the effect in other cell lines. Moreover, the mechanisms by which physiological oxygen tensions trigger MAPK signaling and maybe other prosurvival pathways are not yet clear. Hypoxia results in MAPK activation in certain models (31–33), suggesting that in some tissues MAPK could be closely involved in prosurvival signaling when oxygen is reduced. This opens new avenues of research that fall beyond the scope of this study and will be explored in the future.

5% is a concentration of oxygen frequently found in vivo (12, 34, 35). In other studies it has been considered to induce a hypoxic stress (13, 14). We showed that it is insufficiently low to induce damage in the models we tested. Moreover, it increased cell survival and growth. It is possible that these oxygen conditions, although not toxic, may be slightly below the metabolic needs, prompting the constitutive induction of prosurvival mechanisms that would help the cells adapt to the environment. Thus, cells in physiologically low oxygen tensions would have a survival advantage when exposed to damage compared with those in environments richer in oxygen. On the other hand, they could also be more sensitive to transformation because tumor suppressor mechanisms are not as efficient at removing damaged cells.

The present study provides novel insights on how cells from different tissues react to chemicals and radiation and what defines their specific sensitivity to toxic agents. Our data are especially relevant in colon cancers with Ras activation, such as those represented by HCT116. This could be important to explain the increased resistance of some cells to certain drugs or their susceptibility to oncogenic processes, thus opening the possibility of designing improved therapies to cancer and other diseases.

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