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Increased Mitochondrial Mass in Cells with Functionally Compromised Mitochondria after Exposure to both Direct γ Radiation and Bystander Factors

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The bystander effect describes radiation-like damage in unirradiated cells either in the vicinity of irradiated cells or exposed to medium from irradiated cells. This study aimed to further characterize the poorly understood mitochondrial response to both direct irradiation and bystander factor(s) in human keratinocytes (HPV-G) and Chinese hamster ovarian cells (CHO-K1). Oxygen consumption rates were determined during periods of state 4, state 3 and uncoupled respiration. Mitochondrial mass was determined using MitoTracker FM. CHO-K1 cells showed significantly reduced oxygen consumption rates 4 h after exposure to 5 Gy direct radiation and irradiated cell conditioned medium (ICCM) and an apparent recovery 12–24 h later. The apparent recovery was likely due to the substantial increase in mitochondrial mass observed in these cells as soon as 4 h after exposure. HPV-G cells, on the other hand, showed a sustained increase in oxygen consumption rates after ICCM exposure and a transient increase 4 h after exposure to 5 Gy direct radiation. A significant increase in mitochondrial mass per HPV-G cell was observed after exposure to both direct radiation and ICCM. These findings are indicative of a stress response to mitochondrial dysfunction that increases the number of mitochondria per cell. © 2007 by Radiation Research Society

INTRODUCTION

Reports continue to accumulate that show that radiation-like damage occurs in cells that were never irradiated but were in the vicinity of irradiated cells or were exposed to medium from irradiated cells (1–4). These bystander effects are thought to be the result of a factor or number of factors

released from irradiated cells and sensed by “bystander” cells. These factors remain uncharacterized, though their effects are known to induce chromosome aberrations (5), micronucleus induction (6), changes in gene expression (7), sister chromatid exchanges (8), apoptosis (9), increases in reactive oxygen species (ROS) (10), and genomic instability (11). According to Mothersill and Seymour, (12) the bystander factor can pass through a 0.22- μ m filter, is present as early as 1 h after irradiation, and persists for several hours thereafter. Evidence suggests that at very low doses of radiation, any subsequent damage is induced predominantly as a result of the release of a bystander factor(s) and the direct effects of the radiation are negligible (13). There are currently three reported approaches to model bystander effects separately from the direct effect of radiation exposure *in vitro*. The first involves using low-fluence α particles, such that a small number of cells are traversed by a radiation track and the surrounding cells are thus considered as bystander cells (8). The second involves using a charged-particle microbeam to irradiate a specific cell or a specific region of a cell so that all neighboring cells are bystander cells (6, 14). The third involves the transfer of irradiated cell conditioned medium (ICCM) to previously unexposed, and thus bystander, cells (15).

Mitochondria are the only other location of genetic material outside the nucleus. They contain a circular double-stranded genome (16,569 bp) with no protective histone coat that is incredibly compact with some genes overlapping and only a small fraction of the genome is non-coding (16). There are approximately 2–10 copies of the mitochondrial genome per mitochondrion and tens to hundreds of mitochondria per cell, such that one cell may contain up to several thousand mitochondrial genomes (17). The human mitochondrial genome encodes 22 tRNAs, 2 rRNAs and 13 polypeptides that are all subunits of enzyme complexes of the oxidative phosphorylation (OXPHOS) pathway. This OXPHOS pathway consists of five enzyme complexes embedded in the inner mitochondrial membrane and thus is close to the mitochondrial genome in the matrix.

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Electrons are passed from complex to complex in the electron transport chain (ETC); like any biological system, this is never 100% efficient, and thus electrons are periodically lost into the matrix. The proximity of mitochondrial DNA (mtDNA) to this potential source of highly reactive species and its lack of any histone coat render it particularly susceptible to damage, even in "normal" conditions. Complexes I, III, IV and V of OXPHOS consist of both nuclear DNA (nDNA) and mtDNA encoded subunits, and therefore any phenotypic effect of mtDNA damage will be manifest in this pathway (only complex II is entirely encoded by nDNA).

Recent reports have shown an increase in ROS to be linked to an increase in mitochondrial mass (18–20) in what appears to be a cellular response to compensate for reduced mitochondrial function. Limoli *et al.* (18) reported that human-hamster hybrid unstable clones had a 15% increase in mitochondrial mass after exposure to ionizing radiation. ROS have also been shown to have a role in perpetuating bystander effects (21, 22). Lee *et al.* (19) showed an increase in mitochondrial mass in human osteosarcoma 143 B cells exposed to a high dose of hydrogen peroxide (H_2O_2). At these higher doses mtDNA content was reduced drastically, while lower doses of H_2O_2 resulted in a much higher mtDNA content. Other chemicals have been found to cause an increase in mitochondrial mass and are linked to mitochondrial damage. These include herbimycin A (23), genistein (24), taxol (25), aphidicolin (26), zidobudine (27) and mimosine and lovastatin (28). An increase in mitochondrial proliferation occurs frequently in aged individuals in whom defective respiratory chain activity is typical, resulting from mtDNA depletion and the progressive accumulation of mtDNA mutations and/or deletions (19). Murphy *et al.* (29) recently reported that mtDNA damage was induced by both direct γ radiation and bystander factors in HPV-G cells. mtDNA deletions are also associated with many human diseases such as chronic progressive external ophthalmoplegia (CPEO) and myoclonic epilepsy and ragged-red fiber (MERRF) (19). Rossignol *et al.* (30) showed that glucocorticoid-treated mice suffering from a mitochondrial myopathy had an increase in mitochondrial mass and suggested that this was a mechanism within the cell to compensate for an oxidative defect, facilitating maximum oxygen uptake and a higher yield of ATP in the cell.

In the present study, we examined HPV-G and CHO-K1 cells, in which nonuniform bystander susceptibility has been observed previously (44→31), to determine whether direct irradiation and bystander factors compromised mitochondrial function, specifically OXPHOS, and whether any subsequent variation in mitochondrial mass could be observed.

MATERIALS AND METHODS

Cell Culture

Cells of two cell lines were used: HPV-G, a human keratinocyte cell line derived from human neonatal foreskin transfected with the HPV 16

virus (32), supplied as a kind gift by J. Di Paolo (NIH, Bethesda, MD), and CHO-K1, a spontaneously transformed Chinese hamster ovarian cell line (ECACC) (33). HPV-G cells were maintained in Dulbecco's modification of Eagle medium:F12 (1:1) (Sigma, Dorset, UK) supplemented with 10% fetal calf serum (Gibco, Irvine, UK), 1 U/ml penicillin/streptomycin (Gibco), 20 mM L-glutamine (Gibco), and 1 μ g/ml hydrocortisone (Sigma). CHO-K1 cells were maintained in Nutrient Mixture, F12 (Ham) (Sigma) containing 12% fetal calf serum (Gibco), 1 U/ml penicillin/streptomycin (Gibco), 20 mM L-glutamine (Gibco), and 25 mM Hepes buffer (Gibco).

Direct Irradiation

Cells were grown to 70–80% confluence in cell culture flasks (NUNC, Denmark). Cells were either directly irradiated or sham-irradiated at room temperature using a ^{60}Co teletherapy unit (St. Luke's Hospital, Rathgar, Dublin). The dose rate during the experiment was either 1.8 Gy/min at a source-to-flask distance of 80 cm (for 0.5 and 5 Gy) or 0.4 Gy/min at a source-to-flask distance of 170 cm (for 5 mGy). Flasks were exposed to either 0 Gy (sham), 5 mGy, 0.5 Gy or 5 Gy. The flasks were immediately returned to the incubator and were analyzed 4–96 h later.

Exposure to Irradiated Cell Conditioned Medium

Donor T-25 flasks (NUNC, Denmark) containing 5×10^5 cells in 5 ml of medium were either directly irradiated or sham-irradiated at room temperature using a ^{60}Co teletherapy unit (St. Luke's Hospital, Rathgar, Dublin). The dose rate during the experiment was either 1.8 Gy/min at a source-to-flask distance of 80 cm (for 0.5 and 5 Gy) or 0.4 Gy/min at a source-to-flask distance of 170 cm (for 5 mGy). Flasks were exposed to either 0 Gy (sham), 5 mGy, 0.5 Gy or 5 Gy. Medium was removed from each flask 1 h postirradiation, filtered through a 0.22- μ m sterile filter, and transferred to flasks of unirradiated cells at 70–80% confluence using T-25 flasks for mitochondrial mass analysis or T-75 flasks for polarographic analysis (T-75 recipient flasks received ICCM from $3 \times$ T-25 donor flasks). These ICCM recipient flasks were immediately returned to the incubator and were analyzed 4–96 h later.

Polarography

A Clarke-type oxygen electrode (Dual Digital, model 20) was used to measure oxygen consumption rates. The reaction chamber was maintained at 30°C throughout each experiment. A total of $3\text{--}5 \times 10^6$ cells were maintained in suspension in 400 μ l oxygen electrode buffer (OE buffer) containing 0.3 M sucrose, 5 mM $MgCl_2$, 10 mM KCl, and 10 mM KH_2PO_4 , pH 7.4. Succinate (20 mM) was added as an energy source. Digitonin was added at a concentration to selectively permeabilize cell membranes and not mitochondrial membranes. It was found that 7 or 5 μ g of digitonin was optimal to permeabilize 1×10^6 CHO-K1 cells or 1×10^6 HPV-G cells, respectively.

ADP (200 nM) was added to induce state 3 respiration (active respiration). Once all the ADP was exhausted, the respiration rate within the cells was considered state 4 respiration (inactive respiration). Then 50 μ M 2,4-dinitrophenol (DNP) was added to dissipate the mitochondrial membrane potential and induce uncoupled respiration (uncontrolled OXPHOS).

Cellular oxygen consumption rates were expressed as nanomoles of atomic oxygen per minute per 1×10^6 cells. OE buffer was estimated to contain 406 nM of oxygen/ml at 30°C (34). A total of 400 μ M KCN was added to the chamber to establish the percentage of cellular oxygen consumption resulting from OXPHOS.

Mitochondrial Mass Analysis

Relative mitochondrial number per cell was measured using MitoTracker® Green FM (Molecular Probes, Leiden). MitoTracker is a fluorescent dye that localizes to the mitochondrial matrix regardless of the mitochondrial membrane potential and covalently binds to mitochondrial

proteins by reacting with free thiol groups of cysteine residues. Fluorescence was considered relative to mitochondrial number since analysis of a broad concentration range of MitoTracker confirmed that fluorescence intensity increases linearly in proportion with MitoTracker concentration over the concentration range used in this study (data not shown). Cells were exposed as described previously and incubated for 4–96 h after exposure. Growth medium was removed from cells, which were then rinsed with Mg^{2+}/Ca^{2+} buffer (1 mM $MgCl_2$ and 1 mM $CaCl_2$ in PBS). Then 150 nM MitoTracker (in Mg^{2+}/Ca^{2+} buffer) was added to each flask and incubated at 37°C for 20 min, after which cells were rinsed three times in Mg^{2+}/Ca^{2+} buffer. Cells were then harvested and counted using a Cell Counter (Coulter Z_2), and fluorescence intensity was measured in a fluorescent plate reader (TECAN GENios) using excitation and emission wavelengths of 485 nm and 535 nm, respectively. Fluorescence intensity measurements were normalized against cell numbers before being expressed as percentages of control values.

Statistics

Values are expressed as the means \pm SEM. Data are representative of three or more experiments. The multiple measures analysis of variance (ANOVA) was performed to determine significance, and values were considered significant if $P \leq 0.05$.

RESULTS

Polarography Analysis

OXPHOS was confirmed as the dominant contributory source of the cellular oxygen consumption rates we observed. State 4, state 3 and uncoupled respiration rates were measured to determine any differences in oxygen consumption rates induced in directly irradiated cells or ICCM-treated cells in any and/or all respiratory states. Polarography analysis from control cells showed the typical relative oxygen consumption rates during state 4, state 3 (active respiration) and uncoupled respiration, with state 3 respiration greater than state 4 and uncoupled respiration greater than state 3 (Figs. 1, 2). Control cell state 4, 3 and uncoupled respiration showed no significant variation of rates recorded 4, 12 and 24 h after sham exposures.

CHO-K1 cells exposed to 5 Gy γ radiation showed a significant loss of oxygen consumption during state 3 respiration only 4 h after exposure (Fig. 1A), an effect similarly observed in CHO-K1 cells 4 h after exposure to 5 Gy ICCM (Fig. 1B). Equally important was the response to ADP (lost) and DNP (retained) observed 4 h after direct irradiation and ICCM. State 3 respiration normally occurs in the presence of ADP, though it is reliant on both an intact proton gradient and a functional ATP synthase. DNP is an uncoupling agent, and if it is seen to increase oxygen consumption rate, it is evidence of an intact proton gradient across the inner mitochondrial membrane. At 12 h after direct 5 Gy irradiation and ICCM treatment (Fig. 1), it was observed that an apparent recovery in oxygen consumption rates occurred, that was sustained 24 h after exposure, at which time almost all respiratory states were significantly greater than control values in CHO-K1 cells.

HPV-G cells exposed to 5 Gy γ radiation showed a significant increase in oxygen consumption during state 4 res-

piration 4 h after treatment, with no change observed at state 3 or uncoupled respiration (Fig. 2A). At 12 and 24 h after direct irradiation, the oxygen consumption rates observed were not significantly different from control levels, although at 4, 12 and 24 h after direct irradiation, the ratio of the state 3 to state 4 oxygen consumption rate was reduced in these cells compared to that in control cells (Fig. 2A).

HPV-G cells exposed to ICCM showed a significant increase in uncoupled oxygen consumption rates 4 h after exposure that persisted 12 and 24 h later (Fig. 2B). State 4 and 3 respiration showed no significant change compared to control at any time, except that state 3 respiration was increased 24 h after ICCM exposure.

Mitochondrial Mass Analysis

There was a significant increase in mitochondrial mass in CHO-K1 cells 4 h after direct irradiation that reached a maximum at 24 h and was sustained at 96 h (Fig. 3A). Mitochondrial mass was not observed to increase significantly until 12 h after exposure to ICCM and continued to increase at 24 and 96 h, at which time the increase was comparable to that observed in directly irradiated CHO-K1 cells. At 96 h after direct exposures of 5 mGy to 5 Gy, CHO-K1 cells showed an increase in mitochondrial mass, with the greatest increase observed at the highest dose (Fig. 3B). At 96 h after exposure to ICCM, CHO-K1 cells showed similar significant increases in mitochondrial mass that were independent of dose (Fig. 3B).

A significant increase in mitochondrial mass in HPV-G cells was first observed 12 h after direct irradiation, reaching maximal levels 24–96 h later (Fig. 4A). A significant increase in mitochondrial mass in HPV-G cells was observed 4 h after exposure to ICCM, reaching a maximal level 12 to 24 h later that persisted at 96 h, although it was lower than the maximal level reached after direct irradiation (Fig. 4A).

In HPV-G cells 96 h after direct exposure to 0.5 Gy and 5 Gy, mitochondrial mass was significantly greater than that of control cells. However, the most noticeable effect was observed after exposure to 5 mGy, when mitochondrial mass was approximately fourfold that of control cells (Fig. 4B). An increase in mitochondrial mass was also observed 96 h after exposure to ICCM-treated cells, although it was not as pronounced as in HPV-G cells 96 h after 5 mGy direct irradiation. Both exposure to various direct radiation doses and ICCM resulted in an increase in mitochondrial mass 96 h after exposure, with the greatest increase observed at the lowest dose (Fig. 4B).

DISCUSSION

A decrease in mitochondrial ATP production is often compensated for by increasing glycolysis, as is seen in most cancer cells, where OXPHOS is limited by hypoxic con-

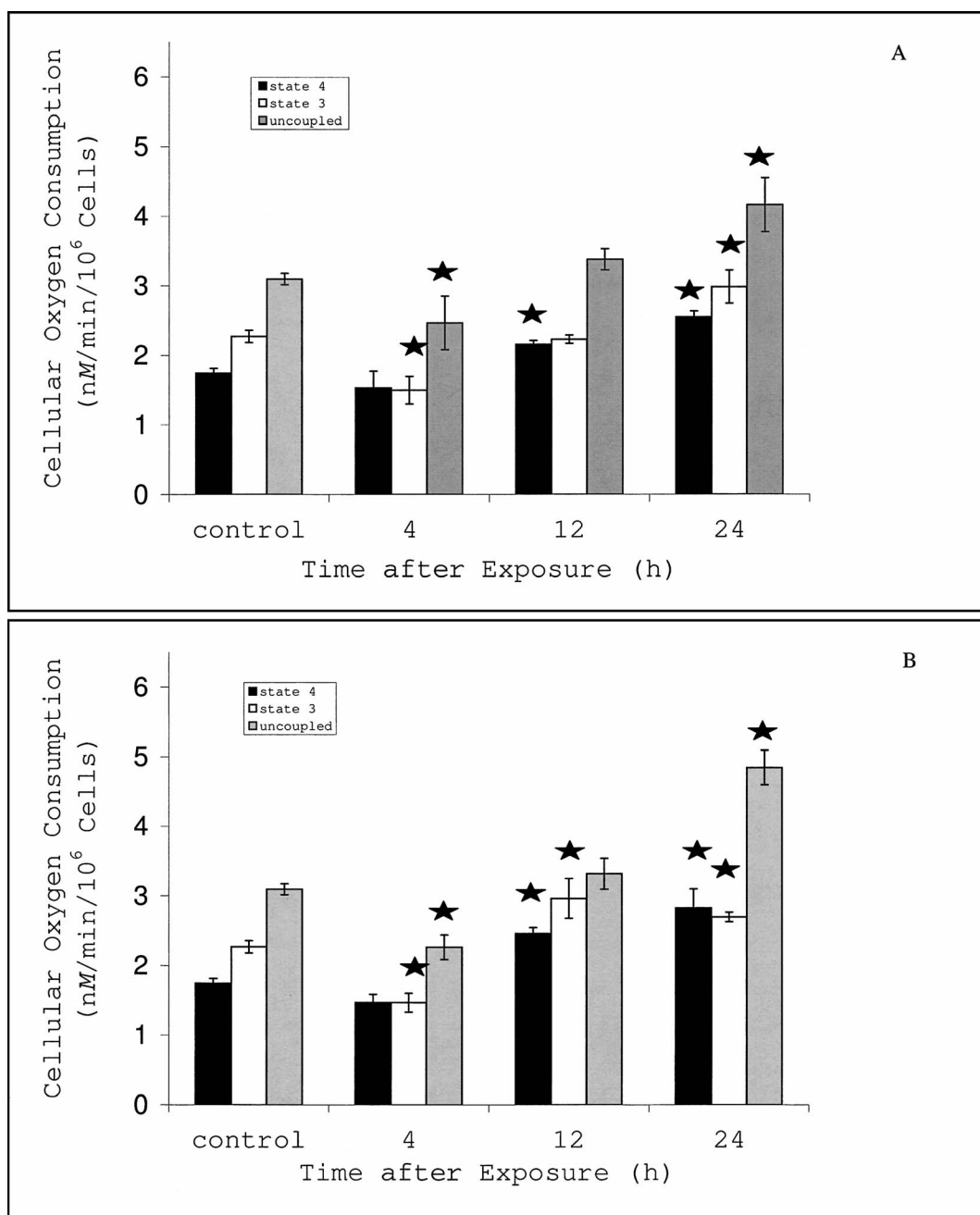


FIG. 1. CHO-K1 oxygen consumption rates in various respiratory states. Cells were treated with (panel A) 5 Gy direct irradiation or (panel B) irradiated cell conditioned medium (ICCM) and were assessed 4–24 h later in a Clarke-type oxygen electrode with additions of ADP and DNP inducing periods of state 3 and uncoupled respiration, respectively. State 4 respiration was considered to be that period of respiration after all ADP present was consumed. Control cell data recorded at 4, 12 and 24 h was pooled since no differences were found. Rates are expressed as nMO/min per 10⁶ cells. ★Change from control value statistically significant at $P \leq 0.05$.

ditions (30), though cells in culture, even tumor cells, typically respire in an oxygen-rich environment. Polarographic analysis was used to measure total cellular oxygen consumption rates, which were confirmed to consist primarily of OXPHOS-related oxygen consumption. To have first isolated mitochondria from cells before polarographic analysis would have necessitated the use of over 10 times the amount of cultured cells used because mitochondrial iso-

lation from cells in culture is notoriously inefficient. Typical cellular oxygen consumption rates observed in this study are comparable to those reported previously at 30°C in a range of animal cell cultures (35) as well as HeLa cells (36) and C2C12 mouse myoblasts (37).

Previous studies have suggested that radiation has little effect on OXPHOS, and if a change was found it was due to a secondary factor such as ROS (38, 39). In the present

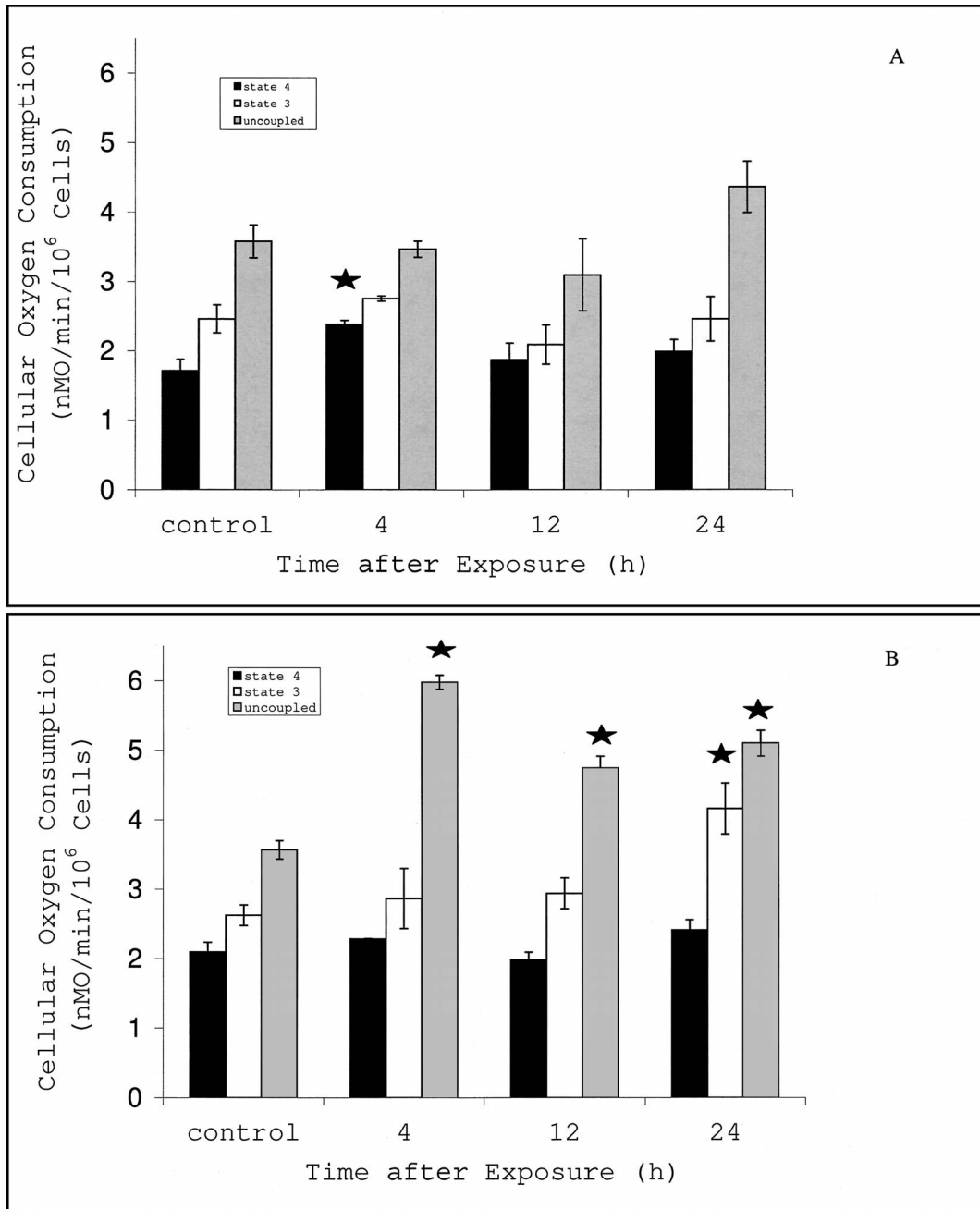


FIG. 2. HPV-G oxygen consumption rates in various respiratory states. Cells were treated with (panel A) 5 Gy direct irradiation or (panel B) irradiated cell conditioned medium (ICCM) and assessed 4–24 h later in a Clarke-type oxygen electrode with additions of ADP and DNP inducing periods of state 3 and uncoupled respiration, respectively. State 4 respiration was considered that period of respiration after all ADP present was consumed. Control cell data recorded at 4, 12 and 24 h were pooled since no differences were found. Rates were expressed as nMO/min per 10⁶ cells. ★Change from control value statistically significant at $P \leq 0.05$.

study, we identified both radiation- and bystander factor-induced loss of mitochondrial function by observing a reduction in oxygen consumption during state 3, and uncoupled respiration in CHO-K1 cells only. A notable observation was the loss of responsiveness to ADP but not DNP, which would be consistent with a loss of ATP synthase function and retention of the proton gradient across the inner mitochondrial membrane. The loss of mitochondrial

function in CHO-K1 cells 4 h after exposure to both ICCM and direct radiation appeared to be transient at first. If this observation is considered in the context of the mitochondrial mass results, then what is more likely is that cells 12 and 24 h after exposure contained increased amounts of functionally deficient mitochondria that were sufficient to more than counterbalance this damage.

HPV-G cells showed no apparent loss of mitochondrial

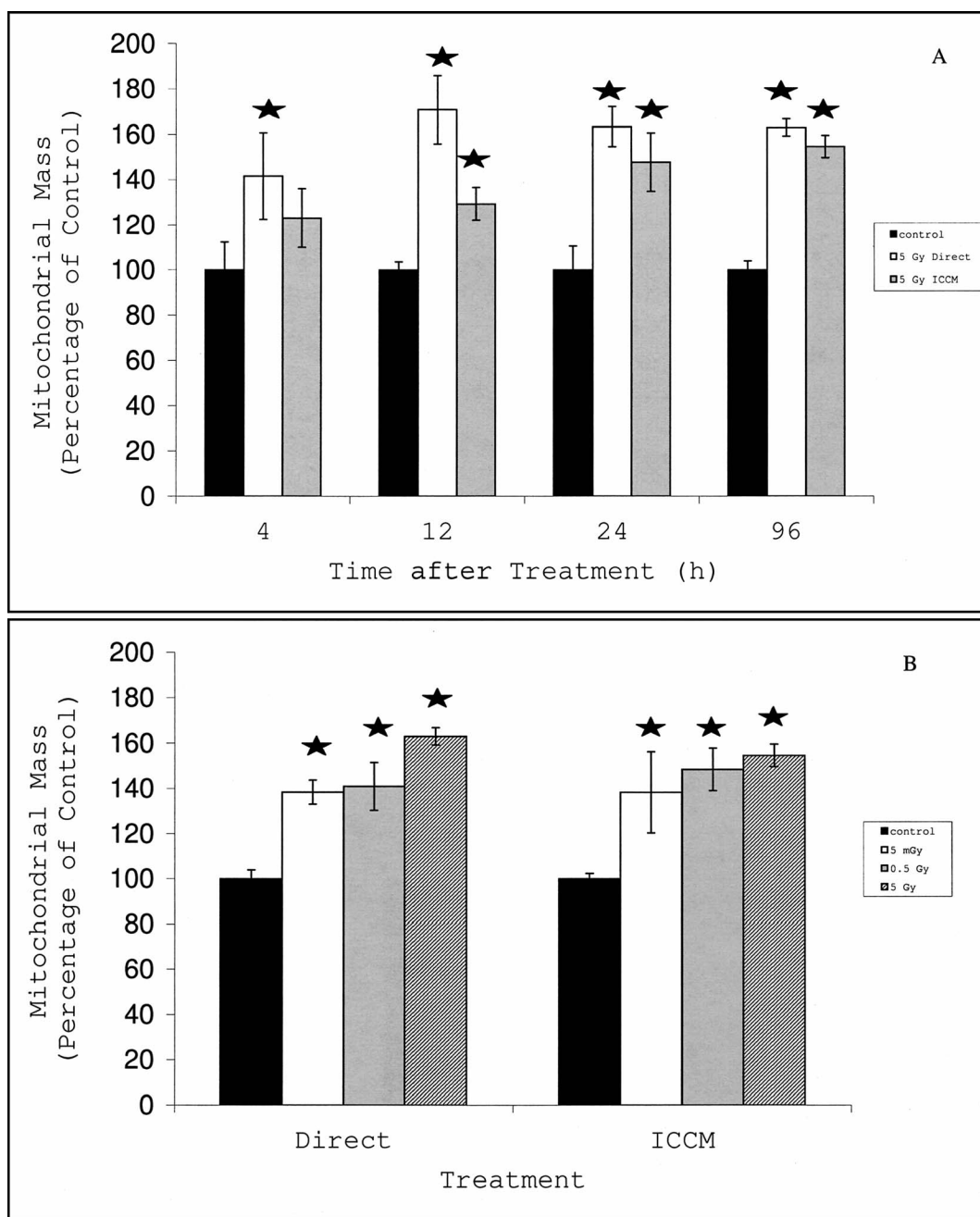


FIG. 3. Analysis of mitochondrial mass in CHO-K1 cells using MitoTracker Green. Cells were harvested (panel A) 4, 12, 4 and 96 h after treatment with 5 Gy and 5 Gy ICCM and (panel B) 96 h after treatment with 5 mGy, 0.5 Gy and 5 Gy. ★Change from control value statistically significant at $P \leq 0.05$.

function after exposure to either direct radiation or ICCM. Similarly, no pronounced loss of responsiveness to ADP was observed, unlike CHO-K1 cells. An increase in cellular oxygen consumption was observed in these cells during uncoupled respiration after ICCM exposure only; when considered in the context of the mitochondrial mass data, this would suggest that these cells did contain dysfunctional mitochondria, but the loss in oxygen consumption per mitochondrion was therefore more than compensated for at a cellular level.

Loss of function of components of the electron chain must reach a certain threshold level before the overall pathway is affected. Davey and Clarke (40) found that up to 72% of complex I activity could be lost before any effect is manifest in the overall flux through the pathway. Therefore, damage to one or more of the enzymes of OXPHOS is likely to have been substantial to induce the loss of oxygen consumption observed in this study. Rossignol *et al.* (30, 41) also found that threshold levels are tissue specific, with lower thresholds in tissues with higher energy de-

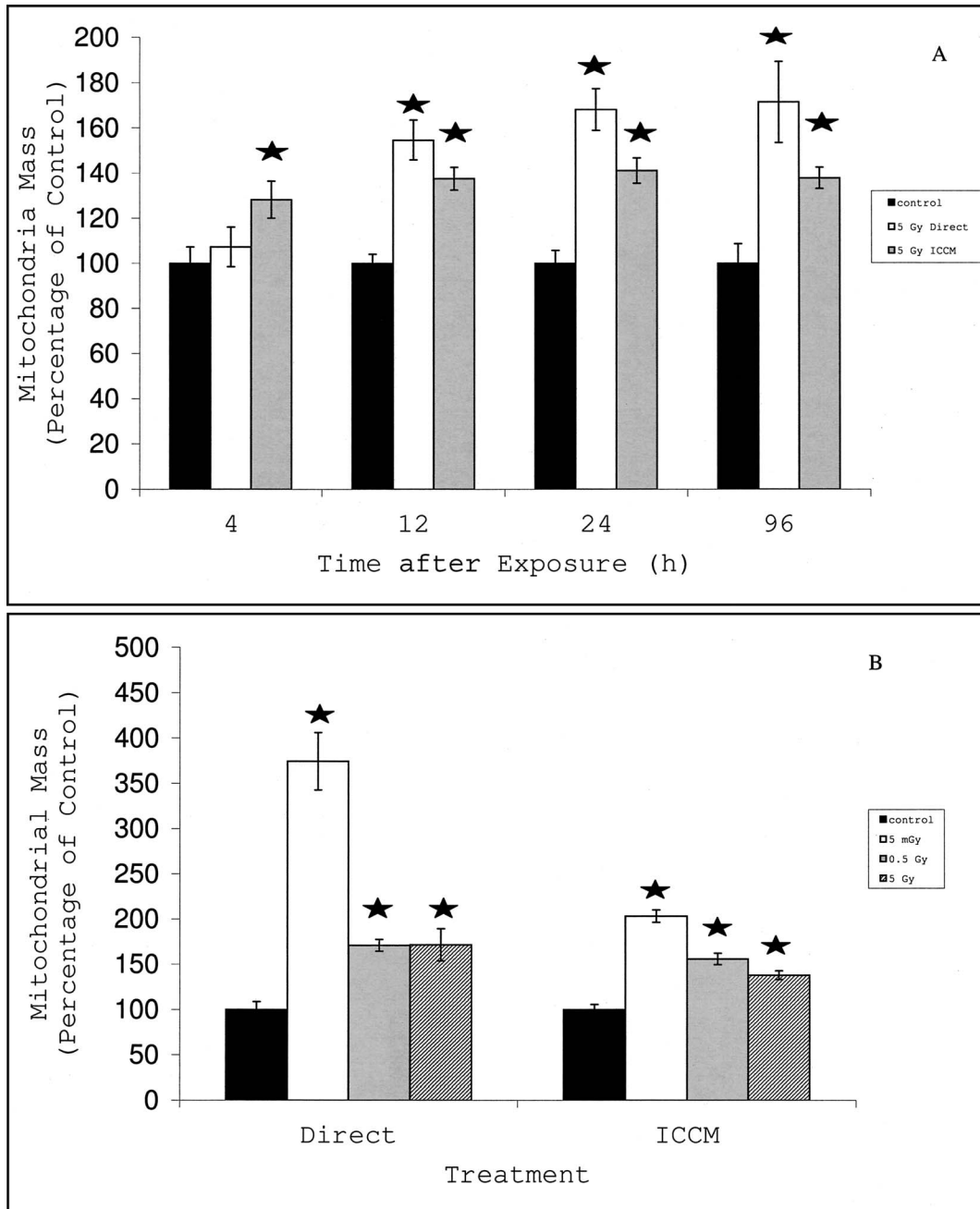


FIG. 4. Analysis of mitochondrial mass in HPV-G cells using MitoTracker Green. Cells were harvested (panel A) 4, 12, 4 and 96 h after treatment with 5 Gy and 5 Gy ICCM and (panel B) 96 h after treatment with 5 mGy, 0.5 Gy and 5 Gy. ★Change from control value statistically significant at $P \leq 0.05$.

mands; thus tissues such as muscle and nerve will typically have the lowest threshold levels.

In this study, an increase in mitochondrial mass in CHO-K1 cells with time after both direct irradiation and ICCM exposure was observed, with levels peaking 24–96 h later at comparable levels in both directly irradiated and ICCM-treated CHO-K1 cells. It must be noted that mitochondrial mass measurements, as determined in this study, are dependent on the absence of both pronounced irregular cell size formation and pronounced variation in thiol residue

availability. Mitochondrial mass was also observed to increase in a dose-dependent manner after direct irradiation although it was independent of ICCM dose. The trends in mitochondrial mass increase observed in HPV-G cells were not similar to those seen in CHO-K1 cells. Mitochondrial mass peaked at 12–24 h after exposure, with increases after ICCM exposure being significantly less than after direct irradiation. Indeed, mitochondrial mass was observed to increase in an inverse dose-dependent manner, with the remarkable observation of a fourfold increase in mitochon-

drial mass after 5 mGy direct irradiation, an increase three-fold higher than that observed in cells after either 0.5 Gy or 5 Gy. This compares favorably with Murphy *et al.* (29), who reported that HPV-G cell mtDNA was most sensitive to the lower direct γ -radiation dose when examined 96 h after exposure to either 5 mGy, 0.5 Gy or 5 Gy for the induction of the mtDNA⁴⁸⁸¹ deletion. Maguire *et al.* (42) observed a similar increase in HPV-G cell mitochondrial mass after exposure to ICCM that was dependent on dose and suggested that the expression of BCL2 was linked to a loss of mitochondrial proliferation. There appears to be a threshold of insult to the mitochondria above and below which not only the amplitude of response but also the nature of the response is different.

This increase in the rate of mitochondrial proliferation is a probably a cellular response mechanism to counteract the loss of mitochondrial function and recover ATP synthesis capacity. However, this is undoubtedly a short-term gain, because in the long term, more mitochondria not only are a strain on the cell because of the resources needed to sustain this accelerated proliferation but also are likely to provide for a substantial increase in free radicals originating from these misfiring mitochondria. A self-perpetuating cycle may result because an increase in ROS is a reported stimulus of mitochondrial proliferation (19, 22, 25). CHO-K1 cells appear more radiosensitive than HPV-G cells in the context of mitochondrial damage, but the amplitude of the HPV-G cell response is greater than that seen in CHO-K1 cells in the context of the cellular response of increasing mitochondrial mass. It has also been reported previously that the bystander effect is more pronounced in irradiated HPV-G cells than in irradiated CHO-K1 cells (43), an observation that would concur with the finding in this study that the maximal CHO-K1 mitochondrial mass after exposure to 5 Gy ICCM was comparable to that after 5 Gy direct irradiation, whereas the maximal HPV-G mitochondrial mass after 5 Gy ICCM was lower than after 5 Gy direct irradiation. The nonuniform sensitivity of the mitochondrial populations of the two cell types to ICCM must also be considered in the context of the previous finding that the energetic status of mitochondria, namely their capacity for ATP synthesis, has been observed to be a potentially critical factor in the bystander effect (31). With this in mind, it is notable that irradiated HPV-G cells showed increased oxygen consumption when ADP was added, whereas irradiated CHO-K1 cells showed no increase when ADP was added. This would indicate a loss of ATP synthase activity (and not a complete loss of membrane potential since oxygen consumption increased in the presence of DNP, which would dissipate any mitochondrial membrane potential). It is therefore likely that this retention of ATP synthase activity in HPV-G cells and not CHO-K1 cells is a contributory factor in HPV-G cells demonstrating a more pronounced bystander effect than CHO-K1 cells.

When the polarographic data in cells exposed to direct radiation are considered in the context of previous clono-

genic analysis of these cell types exposed to 5 Gy direct γ radiation, where cell death was equal in both HPV-G and CHO-K1 cells (43), no direct correlations may be drawn, suggesting that the radiosensitivity of cells may not necessarily be indicative of the radiosensitivity of the mitochondria contained therein.

In conclusion, the results in this study highlight a non-uniform sensitivity to direct radiation and ICCM damage in the mitochondrial populations of the two cell types examined. In general, the effects of direct radiation and ICCM on mitochondria were relatively comparable. A striking result was the immediacy with which mitochondrial dysfunction occurred (as soon as 4 h after exposure), and also the observation that no dose that we examined was too low to induce an increase in mitochondrial mass in either cell type. This increase in mitochondrial replication rate may simply serve to accelerate the increase in the frequency of deficient mitochondria in the overall mitochondrial population. This has grave implications for the distal progeny of cells that survive irradiation, because the mitochondrial genome has the ability to carry significant heterogeneity without loss of function, although a threshold will ultimately be reached, beyond which the fate of these cells is uncertain. Further study of the distal progeny is therefore warranted in future studies.

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