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1 **Evaluation of mitochondrial redox status and energy metabolism of X-irradiated**
2 **HeLa cells by LC/UV, LC/MS/MS and ESR**

3

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20

21 **Running Head**

22 Mitochondrial redox status in X-irradiated tumor

23

24

25

1 **Abstract**

2 To evaluate the metabolic responses in tumor cells exposed to ionizing radiation, oxygen
3 consumption rate (OCR), cellular lipid peroxidation, cellular energy status (intracellular
4 nucleotide pool and ATP production), and mitochondrial reactive oxygen species (ROS),
5 semiquinone (SQ), and iron–sulfur (Fe–S) cluster levels were evaluated in human
6 cervical carcinoma HeLa cells at 12 and 24 h after X-irradiation. LC/MS/MS analysis
7 showed that levels of 8-iso PGF_{2α} and 5-iPF_{2α}-VI, lipid peroxidation products of
8 membrane arachidonic acids, were not altered significantly in X-irradiated cells, although
9 mitochondrial ROS levels and OCR significantly increased in the cells at 24 h after
10 irradiation. LC/UV analysis revealed that intracellular AMP, ADP, and ATP levels
11 increased significantly after X-irradiation, but adenylate energy charge (AEC = [ATP +
12 0.5 × ADP]/[ATP + ADP + AMP]) remained unchanged after X-irradiation. In low-
13 temperature electron spin resonance (ESR) spectra of HeLa cells, the presence of
14 mitochondrial SQ at g = 2.004 and Fe–S cluster at g = 1.941 was observed and X-
15 irradiation enhanced the signal intensity of SQ but not of the Fe–S cluster. Furthermore,
16 this radiation-induced increase in SQ signal intensity disappeared on treatment with
17 rotenone, which inhibits electron transfer from Fe–S cluster to SQ in complex I. From
18 these results, it was suggested that an increase in OCR and imbalance in SQ and Fe–S
19 cluster levels, which play a critical role in the mitochondrial electron transport chain
20 (ETC), occur after X-irradiation, resulting in an increase in ATP production and ROS
21 leakage from the activated mitochondrial ETC.

22

23 Keywords: electron spin resonance (ESR); tumor; mitochondrial electron transport chain
24 (ETC); ionizing radiation; semiquinone; Fe–S cluster

25

1 **Introduction**

2 It is well-known that cancer cells tend to convert glucose into lactate for energy
3 production rather than utilizing the mitochondrial electron transport chain (ETC), even
4 under oxygenated conditions (i.e., the Warburg effect) [1]. Recently, it has been reported
5 that most cancers still retain mitochondrial function [2] and inhibition of mitochondrial
6 ETC stimulates the apoptotic signaling pathways [3]. Lu *et al.* showed a rapid relocation
7 of the mammalian target of rapamycin (mTOR) to mitochondria and reprogramming of
8 biogenetics from glycolysis to mitochondrial oxidative phosphorylation due to the
9 mTOR-mediated inhibition of hexokinase II, a key enzyme in regulation of glycolysis, in
10 tumor cells after irradiation [4]. In our recent studies [5,6], inhibition of dynamin-related
11 protein 1 (Drp1), which controlled mitochondrial fission, reduced mitotic catastrophe in
12 mouse fibroblast NIH3T3 cells and mouse SV40-immortalized embryo fibroblasts
13 exposed to X-rays. It was also demonstrated that treatment with lipophilic
14 triphenylphosphonium cation (TPP⁺) derivatives, which inhibit mitochondrial ETC,
15 enhanced X-ray-induced cell death by increasing reactive oxygen species (ROS) release
16 from mitochondria and loss of intracellular ATP in human cervical carcinoma HeLa cells
17 [7]. Compared to the cytotoxicity induced by cisplatin alone, enhanced cytotoxicity was
18 observed when cisplatin was delivered to mitochondria of chemoresistant A2780/CP70
19 cells by nanoparticles (NP); the cisplatin-nanoparticle combination decreased mtDNA
20 levels and mitochondrial function [8]. In contrast, 3-methyl pyruvate, an activating agent
21 for mitochondrial ETC, enhances radiosensitivity by increasing mitochondria-derived
22 ROS levels in human lung carcinoma A549 cells and murine squamous carcinoma
23 SCCVII cells [9]. These reports strongly suggested mitochondria as novel targets for
24 radiation and chemotherapy in tumor tissue.

1 Several reports have shown that delayed production of ROS from mitochondria is
2 observed in human hepatocellular carcinoma HLE cells [10,11], HeLa cells [12,13],
3 human umbilical vein endothelial cells (HUVECs) [14], human leukemic cells K562 cells
4 [15,16], HL60 cells [16], normal human foreskin fibroblast BJ-hTERT cells [17], and
5 Chinese hamster ovary cells [18] after exposure to ionizing radiation. Moreover, it has
6 been reported that the antitumor genotoxic drugs cisplatin- [19] and doxorubicin-[20]
7 induced ROS release from mitochondria is linked to tumor apoptosis. These reports
8 indicated that delayed ROS release from mitochondria plays an important role in
9 cytotoxicity of tumor cells exposed to genotoxic stimuli. Regarding the mechanism
10 underlying DNA damage-induced increase in ROS release from the mitochondria,
11 simultaneous increases in the intracellular ROS levels and mitochondrial contents have
12 been closely linked in the cells of G2/M phase, which are arrested during the DNA
13 damage checkpoint [21]. Because intracellular mitochondrial content increases in the
14 order of G1, S, and G2/M phase [22] and the main source of intracellular ROS are
15 believed to be the complexes I and III of ETC [23,24], intracellular ROS level is
16 considered to be strongly dependent on mitochondrial content, which is regulated by the
17 cell cycle. In addition, Yoshida *et al.* showed that γ -ray irradiation induces mtDNA
18 damage and reduces NADH dehydrogenase activity, which is the most important enzyme
19 that regulates ROS release from mitochondrial ETC [25]. However, there is not enough
20 information concerning the redox status of mitochondrial ETC of tumor cells exposed to
21 genotoxic stimuli, although this is important to understand the mechanism of delayed
22 ROS release from the mitochondria.

23 Electron spin resonance (ESR) spectroscopy is widely utilized to evaluate
24 mitochondrial redox status such as semiquinone (SQ) and iron–sulfur (Fe–S) cluster in
25 various oxidative stress-related diseases, i.e., ischemia–reperfusion in cardiac muscles

1 [26], cardiomyopathy [27], sepsis [28], and tumor [29]. Ruuge *et al.* demonstrated that
2 ischemia–reperfusion induces an increase in ESR signal intensities of SQ ($g = 2.004$) and
3 Fe–S cluster of succinate dehydrogenase ($g = 2.02$) in isolated perfused hearts [26] and
4 that mitochondria isolated from ischemic–reperfused hearts exhibit significant
5 superoxide (O_2^-) capability than those from control hearts [30]. Burlaka *et al.* showed
6 that the intensity of the ESR signal for SQ increases significantly and is dependent on the
7 stage of gastric cancer, whereas that of Fe–S cluster ($g = 1.94$) in NADH dehydrogenase
8 decreases. Furthermore, spin trap experiments using 1-hydroxy-2,2,6,6-tetramethyl-4-
9 oxo-piperidine (TEMPONE-H) and Fe/DETC revealed that the production of O_2^- and
10 nitric oxide (NO) in tumor tissues increases with the stage of the disease [29]. From these
11 experiments, ESR spectroscopy for cells and tissues can be used as a powerful tool to
12 investigate the imbalance in mitochondrial redox status associated with energy
13 production and ROS leakage from mitochondria during oxidative stress.

14 In this study, to elucidate the role of mitochondrial function in tumor cells exposed
15 to X-rays, oxygen consumption ratio (OCR) and levels of mitochondrial ROS, F2-
16 isoprostane (as a marker of oxidative damage), and adenosine nucleotides (as an indicator
17 of intracellular energy status) were evaluated in X-irradiated HeLa cells. Furthermore, for
18 evaluation of the redox status, levels of mitochondrial SQ and Fe–S cluster were also
19 examined by low-temperature ESR measurements (103 K and 20 K).

20

21 **Materials and Methods**

22 ***Reagents***

23 ATP, ADP, and AMP were obtained from Sigma-Aldrich (St. Louis, MO, USA). NAD^+
24 and NADH were obtained from Nacalai Tesque (Kyoto, Japan). MitoSOX Red was
25 purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Tetra butyl ammonium

1 hydroxide was obtained from Wako Pure Chemical Co. (Osaka, Japan). 8-Iso PGF_{2α}, 8-
2 iso PGF_{2α}-d4, 5-iPF_{2α}-VI, and 5-iPF_{2α}-VI-d11 were obtained from Cayman Chemicals
3 (Ann Arbor, MI, USA).

4

5 ***Cell culture and treatment***

6 Human cervical carcinoma HeLa cells were maintained in DMEM medium (Thermo
7 Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum at
8 37°C in 5% CO₂. X-Irradiation was performed using an X-Rad iR-225 (Precision X-Ray,
9 North Branford, CT, USA). The dose rate was 1.37 Gy/min at 200 kVp, 15 mA, with a
10 1.0 mm aluminum filter. At indicated periods after 10 Gy of X-irradiation, cells were
11 trypsinized and collected for further analysis.

12

13 ***Measurement of OCR by ESR spectroscopy***

14 The peak-to-peak line width of the ESR spectrum of lithium 5,9,14,18,23,27,32,36-octa-
15 n-butoxy-2,3-naphthalocyanine (LiNc-BuO) shows a liner response to partial pressure of
16 oxygen (pO₂) [21]. LiNc-BuO was synthesized, according to the method described
17 previously [31,32]. At indicated periods after 10 Gy of X-irradiation, cells were collected
18 and washed three times with ice cold PBS. The cells were suspended in serum-free
19 medium containing LiNc-BuO and 2% dextran to avoid sedimentation of the cells and
20 LiNc-BuO. Thirty microliters of the cell suspension (1.25×10^7 cells/mL) was
21 immediately drawn into a glass capillary tube, which was then sealed at both ends. ESR
22 measurements were performed using a JEOL-RE X-band spectrometer (JEOL, Tokyo,
23 Japan) with a cylindrical TE011 mode cavity (JEOL). The cavity was maintained at 37°C
24 using a temperature controller (ES-DVT3; JEOL). The scanning parameters were as
25 follows: 1 mW incident microwave power, 100 kHz modulation frequency, 6.3 μT field

1 modulation amplitude, and 5 mT scan range. The spectral line width was analyzed using
2 a Win-Rad radical analyzer system (Radical Research, Tokyo, Japan). The ESR line
3 width versus pO₂ calibration curve was constructed from ESR measurements based on
4 LiNc-BuO equilibrated with oxygen/argon gas mixture.

5

6 ***Flow cytometric analysis of mitochondrial ROS levels in HeLa cells after X-irradiation***

7 The fluorescent probe MitoSOX Red was used for assessing mitochondria-derived O₂⁻.
8 At 12 h after 10 Gy of X-irradiation, cells were collected and washed three times with ice
9 cold PBS, then the cells were incubated in serum-free DMEM containing 2 μM MitoSOX
10 Red for 30 min at 37°C. Then, the cells were trypsinized and washed twice with PBS.
11 After re-suspending in PBS, the cells were analyzed using a BD FACSVerser flow
12 cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The mean MitoSOX Red
13 fluorescence intensity of each sample was normalized to that of a control sample to
14 calculate the relative MitoSOX Red intensity.

15

16 ***Measurement of cellular arachidonic acid oxidation products, F2-isoprostanes, by*** 17 ***LC/MS/MS***

18 At 12 or 24 h after 10 Gy of X-irradiation, cells were collected and washed three times
19 with ice cold PBS, and resuspended in 300 μL of PBS in a 1.5-mL eppendorf tube. After
20 2 ng of deuterated internal standards were added to cell suspension, the cells were
21 disrupted thrice by sonication (10 W; UR-20P, Tomy Seiko Co. Ltd, Tokyo, Japan) for 5
22 s. For measurement of F2-isoprostanes, the samples were subjected to solid phase
23 extraction (SPE; NH₂ Sep-Pak cartridges, Waters Corporation, Milford, MA, USA), as
24 described previously [33]. F2-isoprostanes in the aliquots were separated by Wakopak
25 Ultra C18-3 (φ2.0 × 100 mm, 3 μm, Wako Pure Chemical Co.) at 45°C. The mobile phase

1 comprised two eluents: 0.15% NH₄OH (eluent A) and 95% acetonitrile, 5% MeOH, and
2 0.0125% NH₄OH (eluent B), and the flow rate was 0.35 mL/min. F2-isoprostanes were
3 separated against a solvent gradient using 3% eluent B for 2 min followed by 30% of
4 elute B for 8 min; separation was further achieved with 95% elute B for 5 min, and the
5 solvent was maintained at 95% for 3 min. Column elute was directly coupled to a LCMS-
6 8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) fitted with an
7 electron spray ionization (ESI) operating in the negative ion mode. Multiple reaction
8 monitoring (MRM) was used to analyze the various isoprostanes. 8-Iso PGF_{2α} was
9 identified with a precursor-to-product ion transition m/z 353.4>193.1, 5-iPF_{2α}-VI with
10 transition m/z 353.4>115.1, 8-iso PGF_{2α}-d4 with transition m/z 357.4>313.2, and 5-iPF_{2α}-
11 VI-d11 with transition m/z 364.3>115.15 (Supplementary Figure 1). Isoprostane
12 concentrations were determined using their labeled internal standards. The extraction
13 rates of 8-iso PGF_{2α}, 5-iPF_{2α}-VI, 8-iso PGF_{2α}-d4, and 5-iPF_{2α}-VI-d11 by SPE extraction
14 procedures were approximately 77%, 77%, 77%, and 72%, respectively.

15

16 ***Measurement of cellular AMP, ADP, ATP, NAD⁺, and NADH levels by LC/UV***

17 Cellular AMP, ADP, ATP, NAD⁺, and NADH levels were measured following the
18 protocol published previously [34], with some modifications to the protocol. Briefly, at
19 12 or 24 h after 10 Gy of X-irradiation, the cells were collected and washed in manner
20 similar to that described for the aforementioned LC/MS/MS experiments, 300 μL of 0.5
21 M KOH was added. The cells were lysed by passing through a 23-gauge needle 10 times.
22 Cell lysate was neutralized by adding 120 μL of 10% phosphoric acid and centrifuged at
23 14,100 ×g for 30 min (4°C). The supernatant was separated by TSKgel ODS-80Ts (4.6 ×
24 150 mm, 5 μm, Tosoh, Tokyo, Japan). The HPLC system (Tosoh) consisted of an
25 autosampler (AS-8020), gradient pump (CCPM-II), and in-line degasser (SD-8022). The

1 mobile phase comprised two eluents: eluent A, 10 mM potassium phosphate (pH 5.0),
2 3% acetonitrile, and the ion pairing reagent tetra butyl ammonium hydroxide (TBAH; 2
3 mM); and eluent B, 10 mM potassium phosphate (pH 7.5) and 50% acetonitrile. The
4 nucleotides were separated using a gradient starting at 100% eluent A for 0.6 min, then
5 eluent B was increased to 25% for 1.5 min; eluent B was then further increased to 30%
6 for 11.4 min, 70% for 3 min, and finally to 95% for 1.5 min. The flow rate was 0.8 mL/min,
7 and detection was performed using a UV detector (UV-8020) at 260 nm. The
8 concentration of cellular AMP, ADP, ATP, NAD⁺, and NADH was calculated from the
9 calibration curve and expressed as amount per 1 × 10⁶ cells. Adenylate energy charge
10 (AEC) was calculated according to the following formula:

$$11 \quad \text{AEC} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \quad (1)$$

12

13 ***Measurement of the radicals derived from mitochondria by ESR***

14 Cells (3 × 10⁷) were trypsinized at 24 h after 10 Gy X-irradiation, collected by
15 centrifugation at 1,000 rpm for 5 min (4°C), and washed twice with PBS. Next, cells were
16 resuspended in 300 μL of PBS and transferred to natural quartz ESR tubes (ϕ5 mm × 250
17 mm, Tokyo Chemical Industry Co., Tokyo, Japan). For measurement at 103 K, ESR
18 spectra were recorded using a JEOL-RE X-band spectrometer (JEOL) with a nitrogen
19 temperature control system DVT-3 (JEOL). For measurement at 20 K, ESR spectra were
20 recorded using ELEXSYS E580 (Bruker GmbH, Mannheim, Germany) with a helium
21 temperature control system ER 4112HV (Bruker GmbH). The scanning parameters at 103
22 K and 20 K was as follows: 2 mW incident microwave power, 100 kHz modulation
23 frequency, 1.0 mT field modulation amplitude, 50 mT scan range or 0.63 mT field
24 modulation amplitude, and 5 mT scan range.

25

1 *Statistical analysis*

2 All results are expressed as mean \pm standard error (SE) of at least three independent
3 experiments. Statistical analyses were performed by Student's *t*-test. The minimum level
4 of significance was set at $P < 0.05$.

5

6 **Results**

7 *X-Irradiation enhances OCR and ROS production by mitochondria*

8 Intracellular OCR is an important marker of mitochondrial energy metabolism because
9 ETC in the mitochondria requires oxygen to produce ATP. Cellular OCR in non-
10 irradiated and X-irradiated HeLa cells was measured by ESR oximetry using LiNc-BuO
11 as the oxygen-sensitive probe. When non-irradiated cells were mixed with LiNc-BuO
12 particles and ESR was performed at physiological temperature (37°C), the peak-to-peak
13 line width of ESR spectrum decreased gradually in a time-dependent manner (“Control”
14 in Figure 1A). Oxygen concentration calculated by the standard curve was plotted against
15 incubation time (closed circles in Figure 1B), and the obtained OCR from the slope of
16 this regression line was 6.0 ± 0.7 mmHg/ 1.25×10^5 cells. The presence of complex I
17 inhibitor, rotenone, abolished the time-dependent decrease in the peak-to-peak line width
18 of ESR spectrum in non-irradiated HeLa cells (“Rotenone” in Figure 1A) and reduced the
19 slope value to that of cell-free condition (open circles in Figure 1B), indicating that
20 oxygen consumption was primarily due to mitochondrial oxygen metabolism.
21 Furthermore, it was shown that the line width of the ESR spectrum (“X-irradiation” in
22 Figure 1A) obtained from HeLa cells at 24 h after irradiation rapidly decreased compared
23 to that (“Control” in Figure 1A) of the ESR spectrum of non-irradiated cells. In the OCR
24 data summarized in Figure 1C, OCR of X-irradiated cells (8.7 ± 0.5 mmHg/ 1.25×10^5
25 cells) was significantly higher, by approximately 1.5-fold, than that of non-irradiated cells

1 (6.0 ± 0.7 mmHg/1.25 × 10⁵ cells). In addition, rotenone inhibited the time-dependent
2 decrease of peak-to peak line width of ESR spectrum in both non-irradiated and irradiated
3 cells (“Rotenone” and “X-Irradiation + Rotenone” in Figure 1B) and there was no
4 significant difference in the OCR between non-irradiated and irradiated cells in the
5 presence of rotenone (Figure 1C). These observations indicated that X-irradiation induced
6 an increase in mitochondrial oxygen metabolism, including ETC activity.

7 Because it has been reported that the release of ROS from mitochondria is due to
8 O₂⁻ produced by the reaction of oxygen with the electrons leaked from complexes I and
9 III of ETC [23,24], there is a possibility that excess leakage of electrons from ETC,
10 mediated by X-irradiation, enhances intracellular ROS level including that of O₂⁻. To
11 examine this possibility, intracellular ROS levels of X-irradiated HeLa cells were
12 analyzed by flow cytometry with the O₂⁻-sensitive fluorescent probe, MitoSOX Red. As
13 shown in Figures 1D and E, cells collected 24 h after X-irradiation exhibited higher
14 MitoSOX Red fluorescence intensity than that exhibited by non-irradiated cells. In
15 addition, the treatment of rotenone increased MitoSOX Red fluorescence intensity
16 compared to that observed in the non-irradiated cells, and further enhanced the MitoSOX
17 Red fluorescence intensity of the irradiated cells. These results suggest that X-irradiation
18 induces mitochondrial ROS production concomitantly with the activation of
19 mitochondrial ETC.

20

21 ***Effects of X-irradiation on intracellular F2-isoprostane***

22 Next, to evaluate the effect of X-irradiation-induced ROS production on intracellular lipid
23 peroxidation levels, we analyzed F2-isoprostane levels, a lipid peroxidation product of
24 arachidonic acid, by LC/MS/MS. Arachidonic acid is the predominant polyunsaturated
25 fatty acid (PUFA) in mammalian cells and plays an important role in maintaining cell

1 membrane integrity. Isoprostanes are a series of prostaglandin-like compounds that are
2 formed non-enzymatically *in vivo* via the peroxidation of arachidonic acid by a free
3 radical-initiated mechanism [33]. We performed MRM to select the ion and have shown
4 the precursor and predominant fragment ions of standard materials in supplementary
5 Figures 1A (upper panel) and B (upper panel). Furthermore, the chromatograms of 8-iso
6 PGF_{2α} and 5-iPF_{2α}-VI are shown in supplementary Figures 1A (lower panel) and B (lower
7 panel), respectively. The retention time of 8-iso PGF_{2α} and 5-iPF_{2α}-VI was 7.8 min and
8 8.2 min, respectively, and the peaks of cell samples were determined from the retention
9 times of standard samples. Supplementary Figure 1C shows the chromatograms of
10 cellular 8-iso PGF_{2α} (upper panel) and deuterated internal standard 8-iso PGF_{2α}-d4 (lower
11 panel) from the non-irradiated cells, and supplementary Figure 1D shows the
12 chromatograms of cellular 5-iPF_{2α}-VI (upper panel) and deuterated internal standard 5-
13 iPF_{2α}-VI-d11 (lower panel) from the non-irradiated cells. Figure 2A shows the time
14 course of 8-iso PGF_{2α} contents after X-irradiation. 8-Iso PGF_{2α} contents remained
15 unaltered until 24 h after X-irradiation. Figure 2B shows the time course of 5-iPF_{2α}-VI
16 contents after X-irradiation. 5-iPF_{2α}-VI contents also remained unaltered until 24 h after
17 X-irradiation. Figure 2C shows the quantitative values of intracellular 8-iso PGF_{2α} from
18 area values. 8-iso PGF_{2α} level in the cells at 24 h after X-irradiation did not increase
19 significantly compared with that in non-irradiated cells. However, 8-Iso PGF_{2α} level
20 increased significantly in cells treated with rotenone compared with that in non-treated
21 cells. Similar tendencies were also observed for 5-iPF_{2α}-VI (Figure 2D). These results
22 indicate that X-irradiation-induced increase in intracellular ROS level was not enough to
23 significantly increase membrane oxidative damage.

24

25 ***Cellular homeostasis is maintained after X-irradiation***

1 Next, to evaluate whether X-irradiation-induced activation of mitochondrial ETC impacts
2 cellular energy metabolism, intracellular AMP, ADP, ATP, NAD⁺, and NADH levels
3 were measured by LC/UV. A typical chromatogram (control) obtained from HeLa cells
4 without X-irradiation is shown in the upper panel of Figure 3A. The retention times were
5 6.1 min, 11.4 min, 16.9 min, 21.0 min, and 23.3 min in the elution profile of NAD⁺, AMP,
6 NADH, ADP, and ATP, respectively, by comparison with retention times of control
7 substances. In HeLa cells at 24 h after X-irradiation, the elution profile (bottom panel of
8 Figure 3A) revealed that the peak height of AMP, ADP, and ATP apparently increased in
9 comparison with that of non-irradiated control. The time course of intracellular AMP,
10 ADP, ATP, NAD⁺, and NADH in HeLa cells after X-irradiation is denoted in Figures 3B
11 and C. Intracellular concentration of ATP started to increase at 12 h after irradiation and
12 that of ATP, ADP, and AMP significantly increased at 24 h after irradiation, although the
13 values of AEC in HeLa cells at 12 and 24 h after irradiation were maintained at levels of
14 the non-irradiated control. Next, to elucidate the involvement of mitochondrial F₀F₁-
15 ATPase/ATP synthase on radiation-induced increase in ATP levels, cells were incubated
16 with 2 ng/mL oligomycin for 12 h after irradiation. As shown in Figure 3C, treatment
17 with oligomycin did not influence the basal ATP level of non-irradiated cells; however,
18 this treatment completely abolished a portion of X-irradiation-induced increase in
19 intracellular ATP levels. Intracellular concentration of NAD⁺ significantly increased at
20 12 h after irradiation, and there were no significant changes in the intracellular
21 concentration of NADH. However, the NAD⁺-to-NADH ratio (NAD⁺/NADH) remained
22 unchanged until 24 h after X-irradiation, although an increasing tendency was observed
23 (Figure 3D). These data suggest that production of intracellular F₀F₁-ATPase/ATP
24 synthase-dependent ATP with increase in NAD⁺ levels is a response to irradiation.

25

1 ***X-Irradiation increases ESR signal intensity at $g = 2.004$ in HeLa cells***

2 Increase in rotenone-sensitive OCR and levels of oligomycin-sensitive ATP on X-
3 irradiation of HeLa cells (Figure 1A–C and Figure 3C) suggests that X-irradiation
4 activates mitochondrial functions. To analyze the mitochondrial ETC system after
5 irradiation, ESR was performed for whole cells. When the ESR spectrum of 3×10^7 HeLa
6 cells was recorded at 103 K, two distinct signals at $g = 1.941$ and $g = 2.004$ were primarily
7 observed (Figure 4A, upper panel). In previous ESR studies [35], it has been reported that
8 most normal tissues and their isolated cells yield similar ESR spectra, with prominent
9 peaks at $g = 2.004$ and $g = 1.94$. As shown in supplemental Figure 2, two peaks at $g =$
10 2.004 and $g = 1.941$ in heart tissue, liver tissue from mouse, and isolated mitochondria
11 from bovine heart were also observed in our experimental condition. Similar ESR signals
12 have been reported in many tumor cells, such as human cervical carcinoma HeLa cells
13 [36], lung adenocarcinoma A549 cells [37], and several gastric tumor cells (T2-4, N0-2,
14 M0-1, and G1-G4 cells) [29]. Emanuel reported that a narrow ESR signal at $g = 2.004$ is
15 due to radicals of SQ, which are primarily localized in the mitochondria, and the broader
16 signal at $g = 1.94$ originates from the non-heme iron of mitochondria containing sulfur
17 compounds in various types of cancer [38].

18 Previous reports have shown that saturation of the $g = 2.004$ signal in
19 mitochondrial SQ of *E. coli* [39] and bovine heart mitochondrial SQ at 77 K [40] occurred
20 at a very low microwave power level ($<10 \mu\text{W}$), with the power for half saturation ($P_{1/2}$)
21 being 10–100 μW . For the $g = 1.94$ signal of ferredoxin-type Fe–S cluster (2Fe–2S) at a
22 temperature of 12.5–20.7 K for various plant, bacterial, and adrenal mitochondria, the
23 values of $P_{1/2}$ were reported to be ranged from ≤ 0.1 mW to 0.4 mW [41]. These
24 observations indicate that the relaxation time of Fe–S clusters is relatively short compared
25 to that of SQ radicals. In fact, as shown in supplementary Figure 3, the value of $P_{1/2}$ of g

1 = 2.004 signal was 40 μ W at 103 K, and that of $P_{1/2}$ of $g = 1.941$ at 103 K and 20 K was
2 0.35 mW and 0.22 mW, respectively. These observations indicate that $g = 2.004$ and $g =$
3 1.941 signals observed in whole mammalian cells (Figure 4A, upper panel) originate from
4 mitochondrial SQ radical and mitochondrial ferredoxin-type Fe–S cluster, respectively.

5 Next, HeLa cells were irradiated with 10 Gy of X-rays, incubated for 24 h, and
6 the ESR spectrum of 3×10^7 whole cells were obtained at 103 K (Figure 4A, lower panel).
7 When the peak height of each ESR signal was measured, it was demonstrated that the
8 intensity of ESR signal at $g = 2.004$ was significantly enhanced as shown in Figure 4C.
9 Jong and Albracht [40] and Vinogradov *et al.* [42,43] demonstrated that activation of the
10 respiratory chain in bovine heart submitochondrial particles by NADH or succinate
11 enhances the intensity of ESR signal at $g = 2.004$ and that rotenone abolishes this response,
12 indicating that SQ radicals act as obligatory intermediates of ETC in the mitochondria.
13 To clarify the relationship between radiation-induced enhancement of ESR signal at $g =$
14 2.004 and mitochondrial functions, HeLa cells were incubated in the presence of a
15 complex I inhibitor, rotenone, in mitochondrial ETC systems after X-irradiation. X-
16 irradiation-induced increase in response of $g = 2.004$ signal was completely abolished by
17 incubation with rotenone (Figure 4B). In non-irradiated HeLa cells, quantitative analysis
18 revealed that the ESR signal intensity at $g = 2.004$ was attenuated to about half by
19 incubation with rotenone, indicating that the $g = 2.004$ signal was partly derived from
20 complex I. Furthermore, it was shown that the intensity of the $g = 2.004$ signal obtained
21 from X-irradiated HeLa cells with rotenone was quite similar to that of non-irradiated
22 HeLa cells with rotenone (Figure 4C). These data indicated that X-irradiation-enhanced
23 SQ was strongly associated with mitochondrial ETC systems.

24 In contrast, the intensity of ESR signals at $g = 1.941$ seemed to be not influenced
25 by X-irradiation as shown in Figure 4A. However, the quantitative measurement of the

1 ESR signal at $g = 1.941$ may be not accurate because the line width (7.5 mT) of this ESR
2 signal was too broad due to very short relaxation time at approximately 103 K. To obtain
3 more accurate data, the ESR spectra of HeLa cells without or with X-irradiation were
4 measured at 20 K. It was observed that the line width of ESR signals at $g = 1.941$ at 20 K
5 was 4.1 mT, and this ESR signal with high signal-to-noise ratio was suitable for
6 quantitative analysis (Figure 5A, upper panel). Moreover, it was clearly demonstrated
7 that the intensity of the ESR signal at $g = 1.941$ was not influenced by X-irradiation
8 (Figure 5B). These phenomena suggest that X-irradiation enhances SQ radicals but not
9 Fe-S cluster.

10

11 **Discussion**

12 Recent studies have demonstrated that exposure to radiation in human colorectal
13 carcinoma cell line HCT116, osteosarcoma cell line HPS11 [44], and human lung cell
14 carcinoma A549 [21] leads to the activation of mitochondrial energy metabolism and
15 mitochondrial ATP production. These reports suggested that the cellular switch
16 mechanism of energy metabolism in mitochondrial respiration provides additional
17 advantage for cell survival because several lipophilic triphenylphosphonium derivatives
18 enhance radiation-induced cell death via inhibition of mitochondrial energy metabolism
19 [7]. The present study also showed that radiation-induced increases in the rotenone-
20 sensitive OCR (Figures 1B and C) and oligomycin-sensitive ATP levels (Figure 3C) were
21 observed at 24 h after X-irradiation, whereas AEC values after X-irradiation were stable
22 (0.76–0.86). Extensive biochemical studies have shown that the narrow margin (between
23 0.7 and 0.95) of AEC values is preserved at physiological conditions in a wide variety of
24 eukaryotes and prokaryotes [45,46], and this value decreases during the pathological
25 conditions that lead to reduced energy levels, such as rotenone treatment [47], hypoxia

1 [48,49], and ischemic condition [50]. Moreover, after X-irradiation, NADH level was not
2 altered and NAD⁺ and ATP levels increased drastically, suggesting that there is ample
3 supply of NADH from the tricarboxylic acid (TCA) cycle or other routes after X-
4 irradiation. These results may indicate that radiation-induced increase in cellular NAD⁺
5 and ATP pool is operated under physiological homeostasis. Moreover, this radiation-
6 induced increase in cellular NAD⁺ and ATP pool (Figure 3) may act as an adaptive or
7 protective response against DNA damage after genotoxic stimuli, because decrease in
8 NAD⁺ and ATP levels triggered by poly(ADP-ribose) polymerase 1 (PARP1)-driven-
9 metabolic catastrophe has been reported to enhance radiation-induced programmed-
10 necrosis in human prostate NQO1 cancer positive cells (PC-3, DU145, and LNCaP)
11 exposed to β -lapachone [a substrate of NADH:quinone oxidoreductase 1 (NQO1)] [51].
12 This likely indicates that the mitochondrial ETC system related to radiation-induced
13 increase in NAD⁺ and ATP pool is an important target for radiosensitization in cancer
14 radiation therapy.

15 Recently, numerous DNA damaging agents, including X-irradiation and
16 anticancer drugs, have been reported to induce increase in mitochondrial ROS levels as a
17 late event in various cell lines [10-20], and this production of ROS is associated with
18 apoptosis [52] and senescence [53]. As shown in Figures 1D, 1E, and 2, the marginal
19 increase in radiation-induced response of mitochondrial ROS was confirmed in HeLa
20 cells, although lipid peroxides, such as 5-iPF_{2 α} -VI and 8-isoPGF_{2 α} were not influenced
21 until 24 h after X-irradiation. As shown in Figure 1E, inhibition of mitochondrial
22 respiratory chain complex I by rotenone elevated basal level of mitochondrial ROS
23 production and X-irradiation induced further increase of ROS production in the presence
24 of rotenone. This observation suggested that ROS was originated from Complex I, and
25 X-irradiation facilitated electron flow to complex I in ETC and the overflowed electron

1 reacted with molecular oxygen, resulting in X-irradiation-induced increase of ROS
2 production in Complex I. In Figures 2C and 2D, the production of 8-isoPGF_{2α} and 5-
3 iPF_{2α}-VI stayed similar level between untreated and X-irradiated cells, although there
4 was a significant difference in their production when the cells were treated with rotenone.
5 This observation may be explained by the existence of qualitative limitations of cellular
6 intrinsic antioxidants, i.e., vitamin E, ascorbate, GSH, SOD and catalase, against
7 oxidative stress. In other words, these phenomena suggested that cellular antioxidants
8 were enough existed to prevent cellular oxidative damages when the concentration of
9 cellular ROS is relatively lower level in the cells X-irradiated without rotenone. Whereas
10 cellular oxidative damages such as lipid peroxide might be significantly accumulated by
11 the reaction of biomolecules with ROS that could not be detoxicated by the intrinsic
12 antioxidants when the concentration of ROS is high level in the cells X-irradiated with
13 rotenone.

14 In radiation response in glycolysis in tumor cells, Fujibayashi *et al.*, demonstrated
15 that the upregulation of glycolysis-associated gene products (glucose transporter protein
16 type 1 [SLC2A1] and hexokinase) and increase of uptake of [³H]-2-deoxy-D-glucose in
17 human colon adenocarcinoma LS180 cells occurred at 3 - 5 h after 30 Gy of X-irradiation
18 [54]. This radiation-induced increase of uptake of [³H]-2-deoxy-D-glucose was shown to
19 be completely diminished by the inhibitors of both mRNA (actinomycin D) and protein
20 synthesis (cycloheximide), indicating that the transiently elevated glucose metabolism
21 occurred via processes at the levels of gene expression. Recently, in human hepatoma
22 HepG2 cells and striated muscle HMCL-7304 cells, Wang *et al.*, have reported a
23 concomitant elevation of glucose 6-phosphate and the two pyruvate metabolites lactate
24 and alanine at 4 h after 2 Gy of γ -irradiation, suggesting induction of enhancement of
25 cytosolic aerobic glycolysis by X-irradiation [55]. In our preliminary experiment (data

1 not shown), we confirmed the increase of lactate concentration in the medium as a marker
2 of aerobic glycolysis at 24 h after exposure of 10 Gy X-rays to HeLa cells, suggesting
3 that X-irradiation also increase mitochondrial ETC but also aerobic glycolysis in our
4 present condition. However, as shown in Figure 3C, oligomycin completely abolished X-
5 irradiation-induced increase in ATP, meaning that X-irradiation-induced increase of ATP
6 was mainly derived from mitochondrial F_0F_1 -ATPase/ATP synthase in complex IV but
7 not aerobic glycolysis. From these data, it could be inferred that X-irradiation-induced
8 increase of aerobic glycolysis did not significantly contribute to total ATP production in
9 HeLa cells, because efficiency of ATP production (2 ATP per a glucose molecule) in
10 glycolysis was considerably smaller than that (36 ATP per a glucose molecule) in
11 oxidative phosphorylation.

12 Because the main source of mitochondrial ROS was believed to be leakage of
13 electrons from complexes I and III [23,24], effects of X-irradiation on SQ and Fe–S
14 cluster, which play a crucial role in these complexes of mitochondrial ETC, were
15 evaluated by ESR under low temperature. Based on the results of power saturation
16 experiments (Supplementary Figure 3) and rotenone treatment (Figure 4) in non-
17 irradiated HeLa cells, $g = 2.004$ and $g = 1.941$ signals were identified as SQ and
18 mitochondrial ferredoxin-type Fe–S clusters, respectively. Moreover, it was
19 demonstrated that X-irradiation enhanced the signal intensity of SQ but not Fe–S clusters.
20 Furthermore, it was clearly demonstrated that rotenone treatment reduced basal intensity
21 of SQ signals and abolished the increase in SQ signal induced by X-irradiation (Figure
22 4). De Jong and Albracht [40] and Burbaev *et al.* [42] reported that the short time reaction
23 (10 millisecond–15 s) of NADH with submitochondrial particles isolated from bovine
24 heart enhances the signal intensity of SQ at $g = 2.004$ but not Fe–S cluster at $g = 1.94$ and
25 this NADH-induced enhancement of SQ signal is abolished with rotenone treatment,

1 indicating that SQ form obligatory intermediates in the reaction of complex I with
2 ubiquinone. From these reports, our observation suggests the enhancement of electron
3 flow in rotenone-sensitive ETC in the mitochondria 24 h after X-irradiation. However,
4 the signal intensity of Fe–S cluster was not influenced by X-irradiation, although other
5 oxidative stress such as ischemia [56] and cardiomyopathy in mouse heart [27], alter the
6 signal intensity of Fe–S cluster by oxidation of the iron ion in Fe–S clusters. Burlaka *et*
7 *al.* showed that exposure to electromagnetic radiation of ultra-high frequency in rats
8 decreased the ESR intensity at the $g = 2.00$ and $g = 1.94$ signals in liver, cardiac, and aorta
9 tissues [57].

10 Our previous studies have demonstrated that mitochondria mass, mitochondrial
11 DNA (D-loop and cytochrome c oxidase subunit II [COXII] in A549 cells [21], and
12 NADH dehydrogenase subunit 6 [DN6] in NIH3T3 cells [6]), the expression of PPAR γ
13 coactivator-1 α (α mitochondrial biogenesis-related gene) in NIH3T3 cells were
14 enhanced by X-irradiation, although X-irradiation hardly influenced the expression of
15 two mitochondrial proteins, cytochrome c oxidase subunit IV and cytochrome c [6]. As
16 shown in Figure 4A and 5, the ESR data suggested the X-irradiation induced increase in
17 electron flow in complex I-related SQ but did not unchanged amount of Fe-S cluster.
18 Though the aerobic glycolysis (Warburg effect) seems to be enhanced by X-irradiation,
19 cellular NADH as an electron donor for the mitochondrial ETC was not altered until 24
20 h after X-irradiation as shown in Figure 3D. Taken together, X-irradiation induces
21 increase of mitochondrial mass and/or an imbalance of expression of some components
22 related with ETC, thereby increasing reaction of oxygen with the electrons leaked from
23 ETC to produce O_2^- . To clarify the precise mechanism for late production of ROS and
24 increase of ATP after exposure of X-rays to tumor cells, further experiments to examine

1 the radiation-induced response in energy metabolism of not only ETC but also glycolysis,
2 glutaminolysis and TCA cycle are necessary in the next step.

3 In summary, the present study clearly demonstrated that X-irradiation induced an
4 increase in OCR, ATP levels, and leakage of ROS at 24 h after X-irradiation, indicating
5 the activation of mitochondrial function. During this mitochondrial activation, the values
6 of AEC and NADH were maintained within the range of physiological condition.
7 However, X-irradiation induced an increase in SQ radical levels but not in Fe-S cluster
8 levels, suggesting redox imbalance in the mitochondria of X-irradiated cells. These
9 results suggested that the leakage of excess electrons triggered by this mitochondrial
10 redox imbalance reacted with molecular oxygen, leading to an increase in intracellular
11 O_2^- levels. In addition, the combined application of ESR oximetry and low-temperature
12 ESR spectroscopy to analyze whole cells showed that this combination is a powerful tool
13 for analyzing the mitochondrial redox status.

14

15

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18

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14
15

1 **Figure legends**

2

3 **Figure 1.** Oxygen consumption rate (OCR) and mitochondrial reactive oxygen
4 species (ROS) levels in HeLa cells after 10 Gy of X-irradiation without and with
5 10 μ M of rotenone. (A) Representative time-course of ESR spectra of the
6 extracellular oxygen probe LiNc-BuO in the presence of cells at 37°C without X-
7 irradiation and rotenone (Control), with X-irradiation (X-Irradiation), with rotenone
8 (Rotenone) and with X-irradiation and rotenone (X-Irradiation + Rotenone). After
9 irradiation, cells were incubated for 24 h, cells (1.25×10^7 cells/mL) were collected,
10 and re-suspended in ice-cold medium containing LiNc-BuO and 2% dextran in an
11 air-tight glass capillary tube. ESR spectra were measured from 1.5 to 31.5 min (at
12 intervals of 3 min) after heating up to 37°C. Two-faced arrow indicates line widths
13 of ESR signal at 1.5 min. (B) Time-dependent changes in pO_2 (mmHg/ 1.25×10^5
14 cells) calculated from ESR line width of LiNc-BuO in HeLa cells at 37°C without
15 (●, Control) and with 10 Gy of X-irradiation (■, X-Irradiation). Calibration curve
16 of line-width against pO_2 as described in our previous report [21] was used for
17 determining extracellular pO_2 . In case of rotenone treatments, rotenone (final
18 concentration 10 μ M) was added to the cell suspension just before ESR
19 measurement. ○, 10 μ M rotenone; □, combination of X-irradiation and rotenone.
20 (C) OCR (mmHg/min· 1.25×10^5 cells) of HeLa cells was calculated by regression
21 curves of (B). Data are expressed as means \pm SE of three experiments. * $P < 0.05$,
22 ** $P < 0.01$ (Student's *t*-test). (D) Mitochondrial ROS levels were measured by flow
23 cytometry using MitoSOX Red. Representative flow cytometric profiles were
24 obtained from the cells at 0 (control) and 24 h (X-rays) after X-irradiation. (E)
25 Summarized data of the relative mitochondrial ROS levels without and with 10 Gy

1 of X-irradiation. In case of rotenone treatments, rotenone (final concentration 1
2 μM) was added to the medium immediately after irradiation and cells were
3 incubated for 12 h. Data are expressed as means \pm SE of three experiments. $**P <$
4 0.01 (Student's t -test).

5

6 **Figure 2.** LC/MS/MS measurements of cellular F2-isoprostane in HeLa cells after
7 X-irradiation. Time-dependent changes in the amount of 8-iso $\text{PGF}_{2\alpha}$ (A) and 5-
8 $\text{iPF}_{2\alpha}\text{-VI}$ (B) in HeLa cells after irradiation were evaluated by LC/MS/MS with ESI.
9 Effect of rotenone on cellular 8-iso $\text{PGF}_{2\alpha}$ (C) and 5- $\text{iPF}_{2\alpha}\text{-VI}$ levels (D) in non-
10 irradiated and irradiated HeLa cells at 24 h was evaluated by LC/MS/MS analysis.
11 Treatments with rotenone are similar to those described in Figure 1E. Data are
12 expressed as means \pm SE of three experiments. $*P < 0.05$, $**P < 0.01$ (Student's t -
13 test).

14

15 **Figure 3.** Cellular AMP, ADP, ATP, NAD^+ , and NADH levels in HeLa cells after
16 X-irradiation measured by reverse-phase HPLC equipped with a UV detector
17 (LC/UV). (A) LC/UV chromatograms obtained from non-irradiated cells (upper
18 panel) or cells at 24 h after X-irradiation (lower panel). After X-irradiation at 10
19 Gy, cells were incubated for 12 or 24 h and collected. Intracellular AMP, ADP,
20 ATP, NAD^+ and NADH levels were measured by HPLC. Representative
21 chromatograms of three experiments are shown. (B) Time course of the intracellular
22 AMP (\blacktriangle), ADP (\blacksquare), and ATP (\blacklozenge) levels as well as adenylate energy charge (AEC;
23 \bullet). AMP, ADP, and ATP levels were calculated from the calibration curve created
24 by the measurements of standards. AEC was calculated by the following equation:
25 $\text{AEC} = ([\text{ATP}] + 0.5[\text{ADP}])/([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$. (C) Effect of oligomycin

1 on cellular ATP production induced by X-irradiation. Oligomycin (2 ng/mL) was
2 applied to cells for 12 h immediately after X-irradiation. (D) Time course of
3 intracellular NAD⁺ (△) and NADH (◇). NAD⁺ and NADH levels were estimated
4 from the calibration curve created by the measurements of standards. Data are
5 expressed as means ± SE of three experiments. **P* < 0.05, ***P* < 0.01 (Student's *t*-
6 test).

7

8 **Figure 4.** Mitochondrial electron transport chain-related substance levels in HeLa
9 cells after X-irradiation measured by ESR. (A) Typical ESR spectra at 103 K
10 obtained from HeLa cells without (upper panel, control) and with 10 Gy of X-
11 irradiation (lower panel, X-rays). After X-irradiation at 10 Gy, cells were incubated
12 for 24 h, ESR spectra were then recorded. (B) Effect of rotenone on ESR signal
13 intensity at *g* = 2.004 (SQ) in HeLa cells. Typical ESR spectra at 103 K were
14 obtained from rotenone-treated cells without (upper panel, control) and with 10 Gy
15 of X-irradiation (lower panel, X-rays), respectively. For rotenone treatment,
16 rotenone (final concentration 1 μM) was added to the medium immediately after
17 irradiation and cells were incubated for 24 h. The ESR scanning parameters were
18 as follows: 2 mW incident microwave power, 9.05 GHz modulation frequency, 1.0
19 mT field modulation amplitude, and 50 mT scan range. (C) Relative ESR signal
20 intensity of *g* = 2.004 obtained in HeLa cells after X-irradiation from three
21 independent experiments are summarized. Data are expressed as means ± SE of
22 three experiments. **P* < 0.05 (Student's *t*-test).

23

24 **Figure 5.** Measurement of mitochondrial electron transport chain-related
25 substances at 20 K. (A) Typical low temperature ESR spectra at 20 K obtained from

1 HeLa cells without (upper panel, control) and with 10 Gy of X-irradiation (lower
2 panel, X-rays). (B) Relative ESR signal intensity at $g = 1.941$ obtained from three
3 independent experiments at 20 K are summarized. Data are expressed as means \pm
4 SE for three experiments. N.S., not significant (Student's t -test).

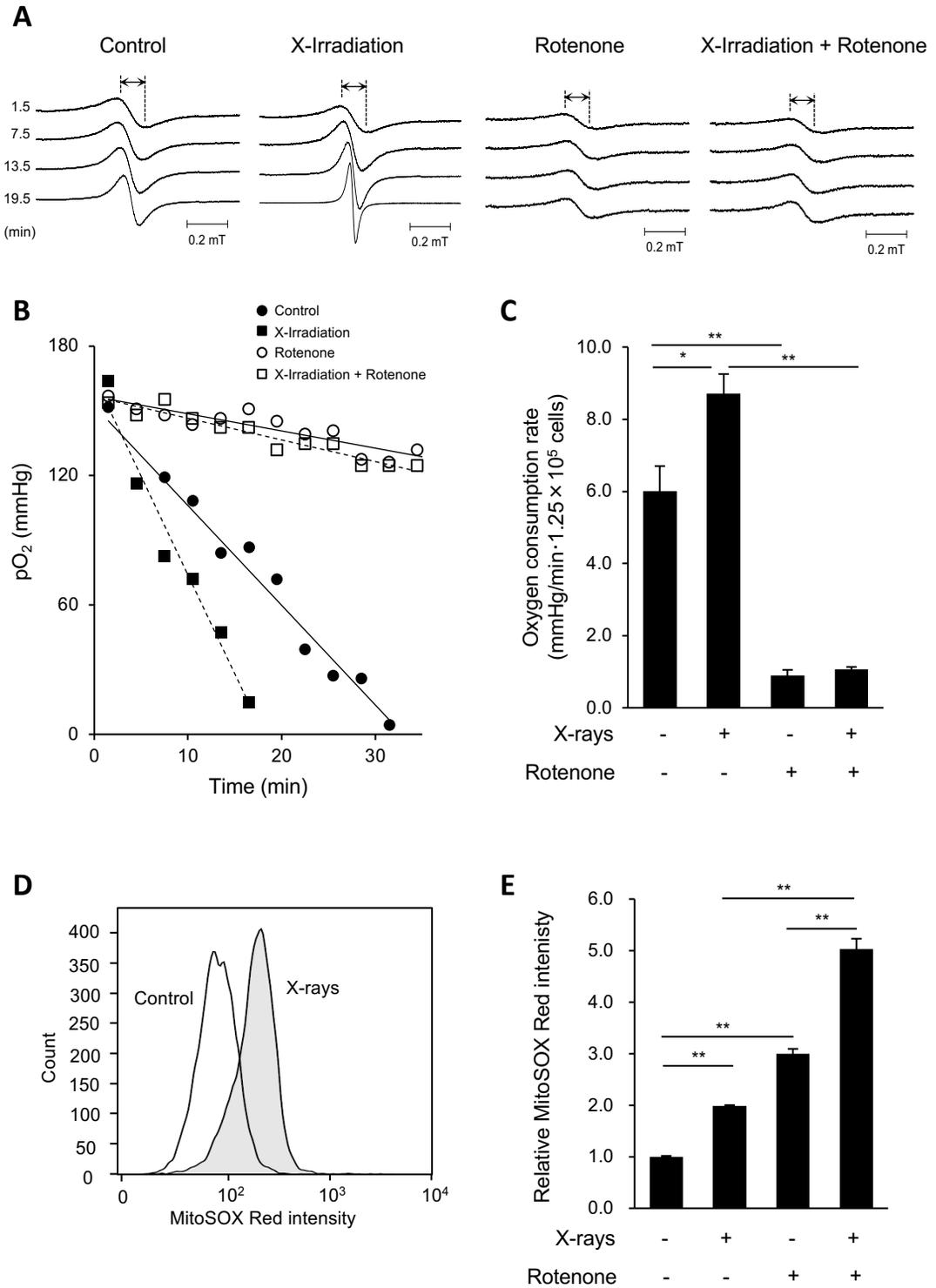


Fig. 1. Yamamoto et al.

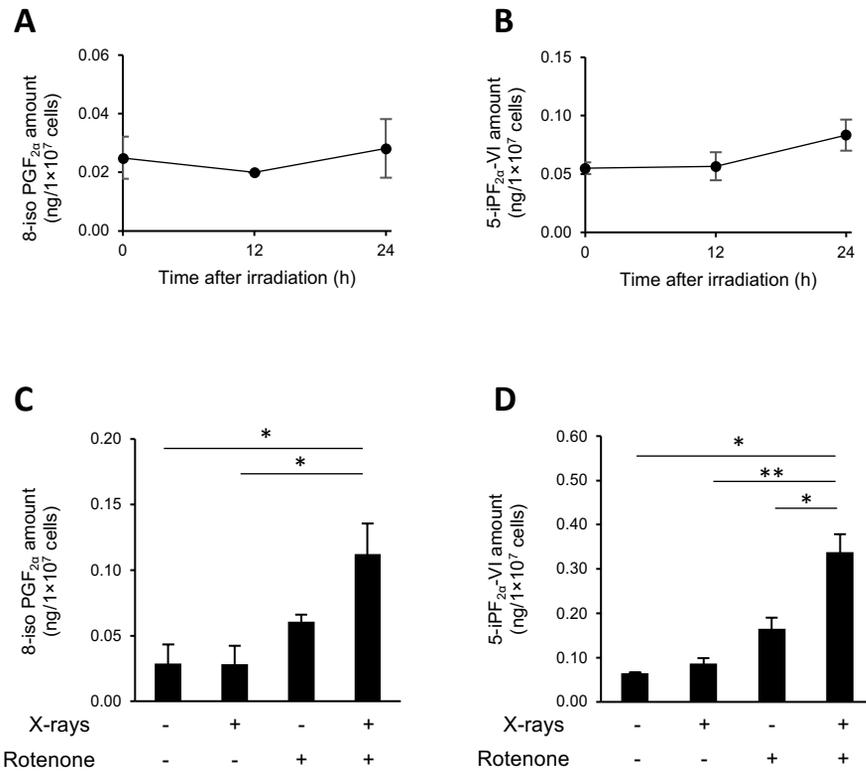


Fig. 2. Yamamoto et al.

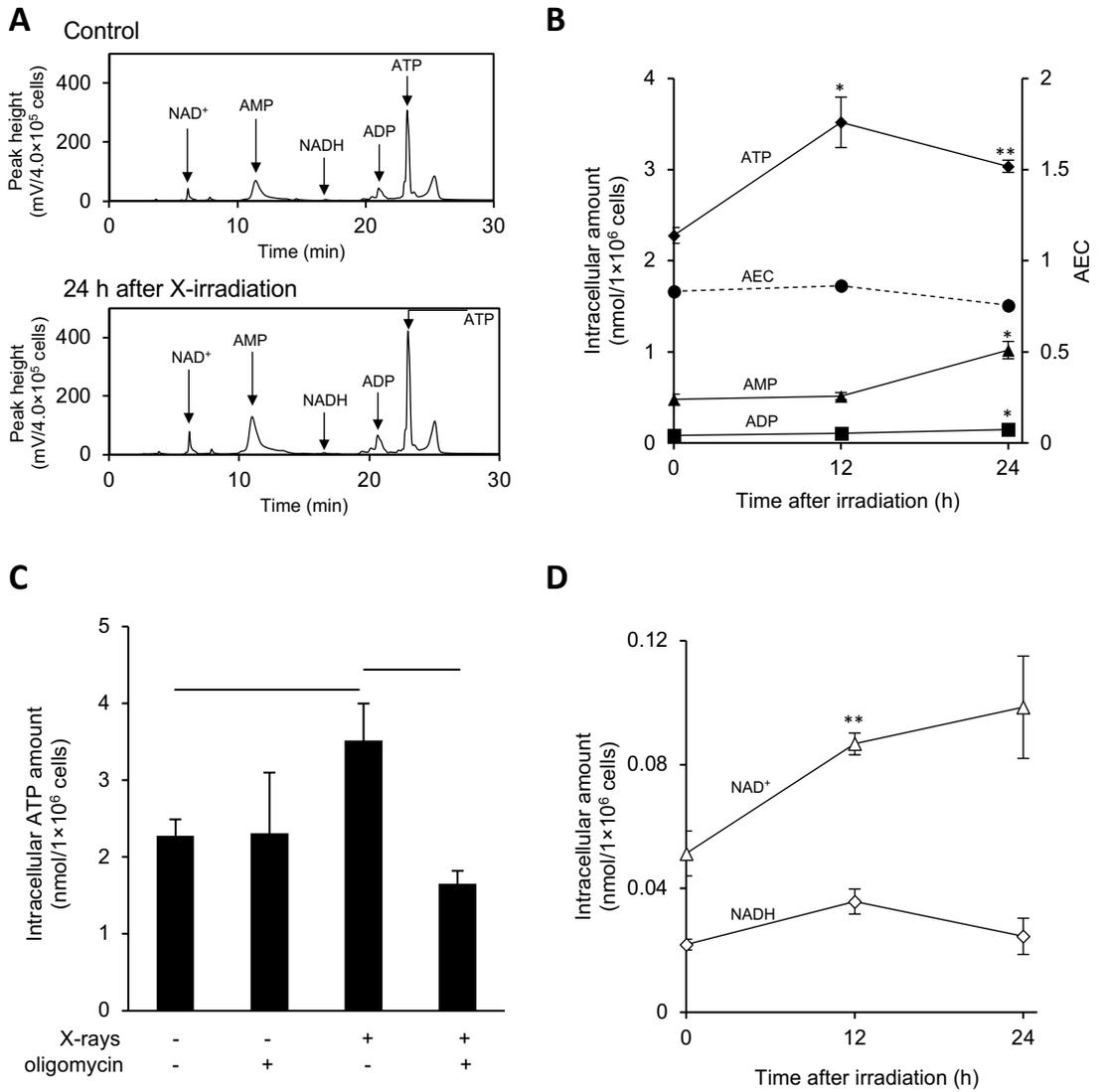


Fig. 3. Yamamoto et al.

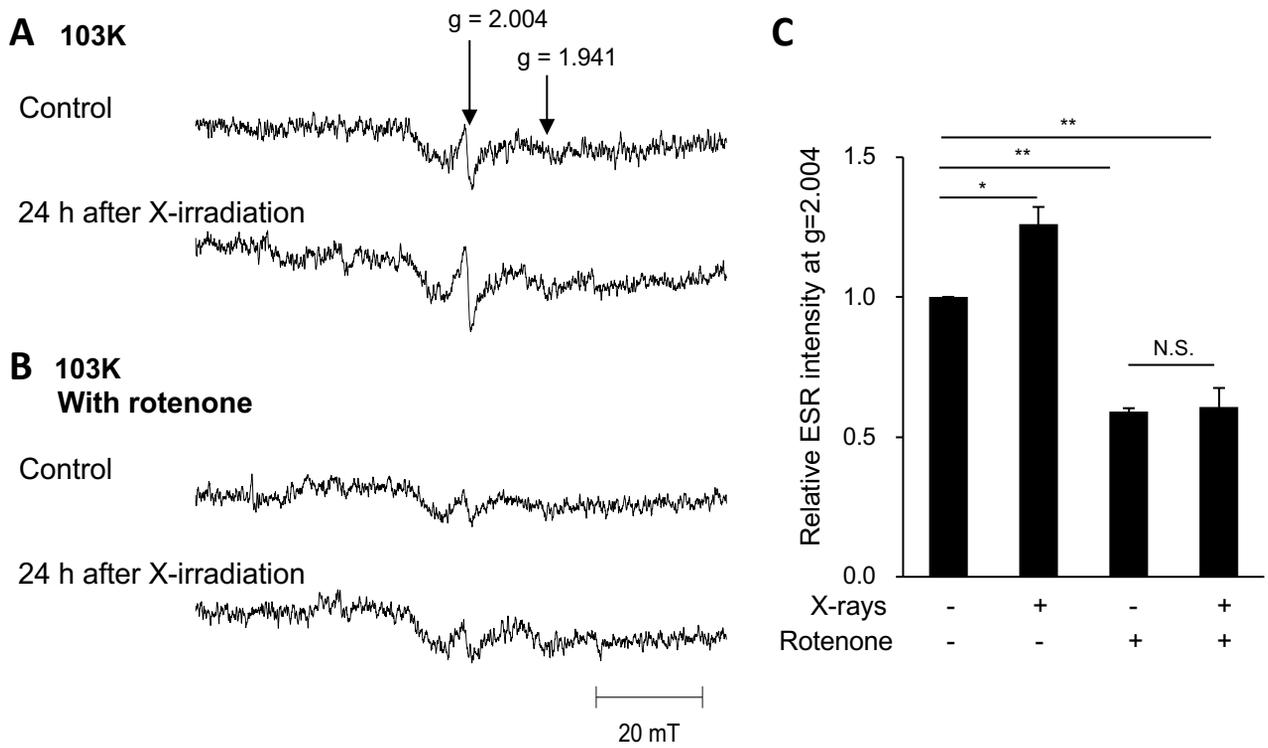


Fig. 4. Yamamoto et al.

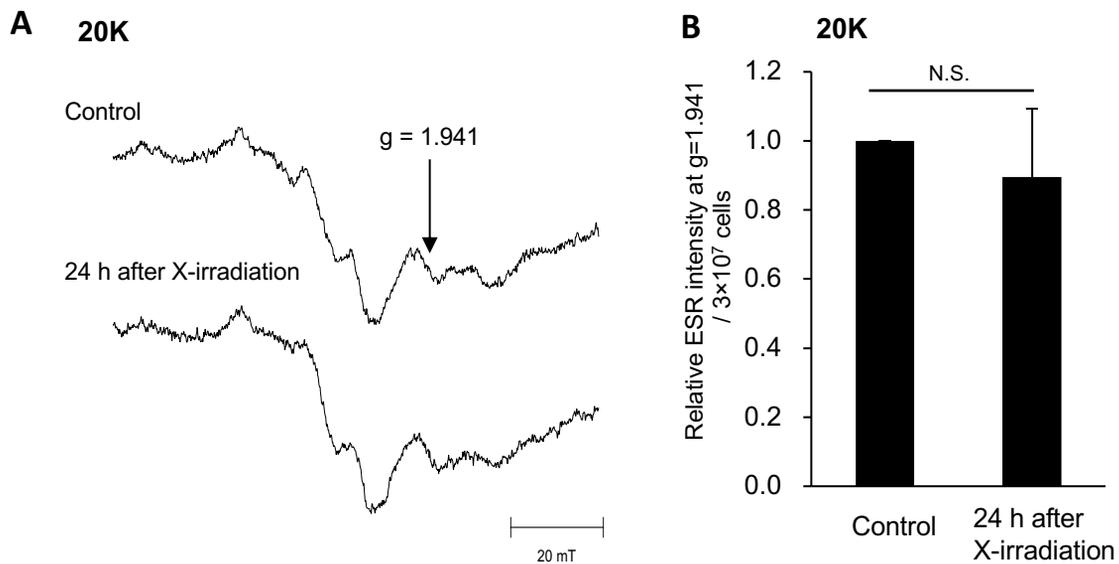
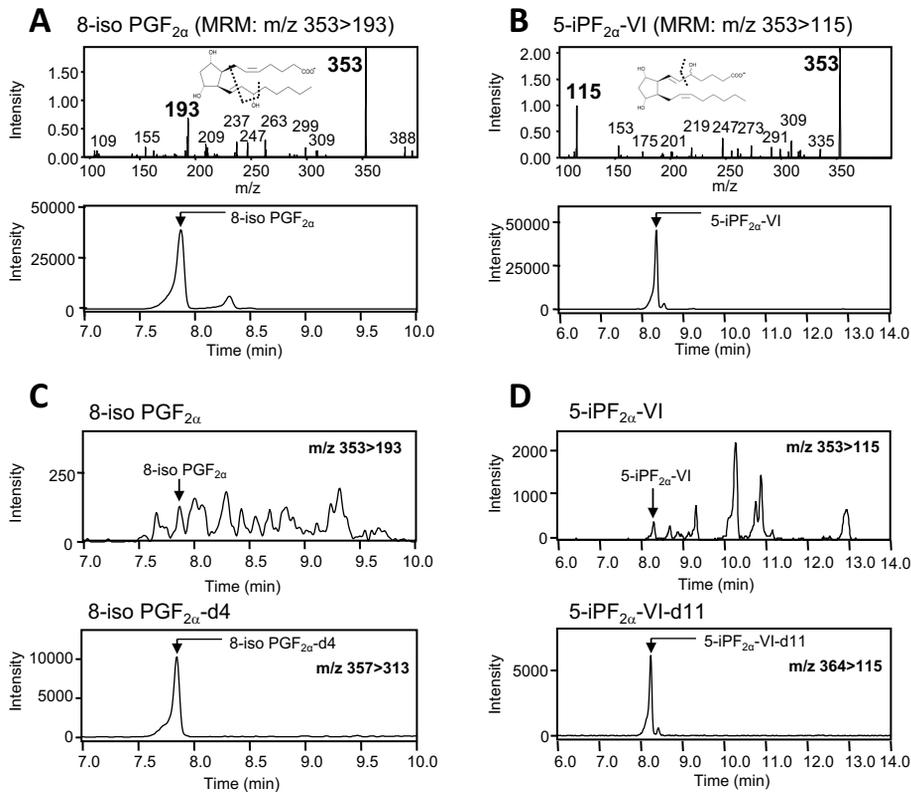
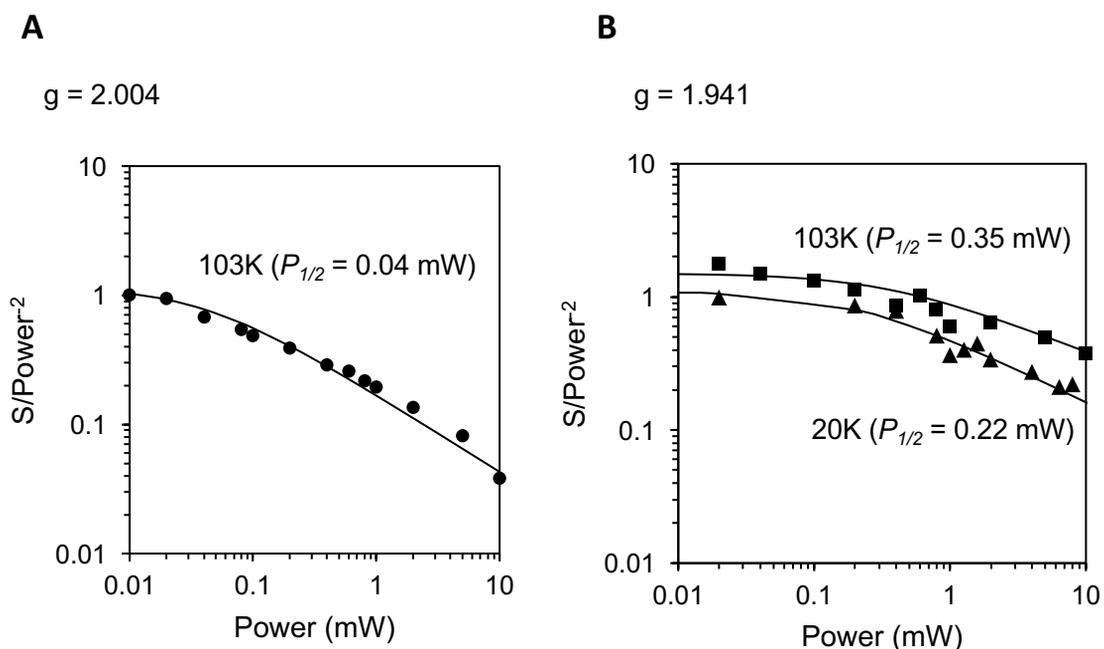


Fig. 5. Yamamoto et al.

Supplementary Materials



Supplemental Figure 1. LC/MS/MS measurement of cellular F2-isoprostane level in HeLa cells after X-irradiation. (A) Product ion scan (upper panel) and multiple reaction monitoring (MRM) chromatogram (lower panel) of 8-iso PGF_{2α} standard sample (*m/z* 193). (B) Product ion scan (upper panel) and MRM chromatogram (lower panel) of 5-iPF_{2α}-VI standard sample (*m/z* 115). (C) LC/MS/MS chromatograms of cellular 8-iso PGF_{2α} (upper panel) or deuterated internal standard (lower panel); 8-iso PGF_{2α}-d4 obtained from non-irradiated cells. (D) LC/MS/MS chromatograms of cellular 5-iPF_{2α}-VI (upper panel) or deuterated internal standard (lower panel); 5-iPF_{2α}-VI-d11 obtained from non-irradiated cells.



Supplemental Figure 2. Microwave power saturation curves of the ESR signals of HeLa cells at $g=2.006$ (A) and $g=1.941$ (B) in HeLa cells. The ESR spectra were measured at various microwave power (0.01~10 mW). Log (Signal amplitude/Power^{0.5}) was plotted against log (Power). Padmakumar and Bamerjee [J. Biol. Chem., 270:9295-9300, 1995] have given an equation that the ESR signal amplitude (S) is related to the microwave power (P) by $\log S/(P)^{0.5} = \log A/(1 + P/P_{1/2})^{0.5b}$, where $P_{1/2}$ and A refer to the power for half saturation and a scaling factor, respectively. b refers to the inhomogeneity parameter, which can vary from 1.0 for inhomogeneous broadening to 2.0 for homogeneous broadening. The value of $P_{1/2}$ was estimated by fitting the experimental saturation data (EPR signal amplitude as a function of incident microwave power) to this equation. Data fitting by a least squares method was performed by a personal computer with Microsoft Excel.