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# Dehydroepiandrosterone augments sensitivity to γ-ray irradiation in human H4 neuroglioma cells through down-regulation of Akt signaling

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**Abstract**
Dehydroepiandrosterone (DHEA) modulates sensitivity to radiation-induced injury in human neuroglioma cells (H4) through effects on Akt signalling by glutathione (GSH)-dependent redox regulation. Previous treatment of H4 cells with DHEA for 18 h reduced the γ-ray-induced phosphorylation of Akt, activated $p21^{waf1}$ synthesis and up-regulated phosphorylation of Rb independent of p53. These reactions were followed by a decrease in cell number and an increase in apoptosis and $G_2$/M checkpoint arrest. The suppression of phosphorylation of Akt by DHEA was due to regulation of the dephosphorylation by protein phosphatase 2A (PP2A). DHEA up-regulated the expression of γ-glutamylcysteine synthetase, a rate-limiting enzyme of glutathione (GSH) synthesis, and the levels of GSH to maintain PP2A activity. The results suggested that DHEA increases the sensitivity of cells to γ-ray irradiation by inducing apoptosis and cell cycle arrest through GSH-dependent regulation of the reduced form of PP2A to down-regulate the Akt signalling pathway.

**Keywords:** *Dehydroepiandrosterone, radiation, Akt, protein phosphatase 2A, glutathione, γ-glutamylcysteine synthetase*

## Introduction

It is known that dehydroepiandrosterone (DHEA), a C-19 adrenal steroid, inhibits oxidative stress-induced cell damage. However, the mechanism behind the effect of DHEA on radiation-induced cell damage is not clear. DHEA and the sulphated prohormone of DHEA circulate at plasma concentrations higher than any other steroids and DHEA acts independent of estrogen receptors and androgen receptors. Animal experiments indicate that DHEA has a wide variety of beneficial biological and physiological effects on the prevention of ageing [1]. DHEA inhibits the progression phase of carcinogenesis by inducing cellular senescence [2]. However, the favourable effects of this hormone remain largely unclear. Radiation is a genotoxic agent. Radiation causes genotoxic damage in DNA, RNA, proteins and membrane lipids directly or by generating reactive oxygen species (ROS) [3]. The mechanisms of radiation-induced apoptosis have been studied extensively in terms of p53 status, the Bcl-2 gene family, the Fas-mediated pathway, the ceramide-mediated pathway, the caspase cascade and the ataxia-telangiectasia-mutated gene [4,5].

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Nevertheless, it remains unclear which macroscopic or molecular features determine the response of cells to irradiation.

Cellular responses to radiation vary broadly among cell types and are strongly affected by the spectrum of genes expressed, such as hormone receptors [6]. Among many factors related to radiosensitivity, Akt (Protein kinase B) is believed to play an important role in the regulation of cellular function against irradiation. The serine/threonine kinase Akt is a critical component of an intracellular signalling pathway that influences survival and apoptosis [7]. Inhibition of the Akt pathway together with $\gamma$-rays induces $G_2$/M cell cycle arrest [8]. The activity of Akt is regulated by redox [9]. The redox status of Cys297 and Cys311 of Akt is important for its activity. The phosphorylated Ser437 and Thr308 of Akt are dephosphorylated by protein phosphatase 2A (PP2A). The activity of PP2A is regulated by oxidative stress [10,11] and the expression of PP2A by $Ca^{2+}$ [12]. The glutathione (GSH)/glutathione disulphide (GSSG) equilibrium is the major redox buffer in cells. Apart from providing cells with a reducing environment ([GSH] >> [GSSG]) and maintaining proteins in a reduced state, the GSH redox couple dynamically regulates protein functions via a reversible disulphide bond formation [13]. The redox status of sulphydryl groups in proteins plays an important role in the regulation of cellular functions such as the synthesis and folding of proteins and regulation of the structure and activity of enzymes, receptors and transcription factors [14]. Furthermore, modifications of cysteine sulphydryls such as by sulphenic acids, S-nitrosylation and S-glutathionylation are known to be reversible and important to protect against irreversible oxidation [15].

In the present study, we show that DHEA increases the expression of $\gamma$-GCS. This increase in GSH-dependent redox potential stimulates PP2A to down-regulate the activity of Akt. In the present study, we investigated the role of DHEA in radiosensitivity and $\gamma$-ray-induced apoptosis, using human neuroglioma cells. We show here that DHEA modulates the radiosensitivity of H4 cells by suppressing Akt signalling for cell survival via alterations of PP2A activity by GSH-dependent redox state.

## Materials and methods

### *Reagents*

Rabbit antibodies against retinoblastoma protein (Rb), phospho-p53(Ser15), phospho-Rb(Ser780), Akt and phospho-Akt(Ser473) antibodies were from Cell signaling technology (MA, USA). Anti-$p21^{waf1}$ was from Oncogene Research Products (Calbiochem, Germany). Anti-PP2A catalytic C subunit (PP2Ac) antibody was from BD Transduction Laboratories (CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from MBL (Nagoya, Japan). HRP-goat anti-mouse IgG was from Chemicon International (Temocula, CA). DHEA was from Wako Pure Chemicals (Osaka, Japan). GSH, GSSG, NADPH and N-acetylcysteine (NAC) were from Wako Pure Chemicals (Osaka, Japan).

### *Cell culture and treatments*

H4 (human, Caucasian, brain, nervous tissue glial tumour) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. Before reaching confluence, the cells were split, plated at low density in culture dishes containing DMEM with 10% FBS. The culture medium was replaced every 2 days. After attainment of confluence (70–80%), the cells were incubated in DMEM containing 0.5% foetal bovine serum (FBS) for 20–24 h. For experiments involving treatment with DHEA, a stock solution of DHEA (10 mM) was initially prepared in $Me_2SO$. This was diluted 50-fold with DMEM containing 0.5% FBS to obtain a working DHEA concentration of 200 nM.

### *Cell number and proliferation*

H4 cells were treated with 200 nM DHEA for 18 h and irradiated with 3-Gy of $\gamma$-rays. Subconfluent cultured cells were harvested by treating the cells with trypsin (0.05% trypsin and 0.5 mM EDTA, PBS). Cells were seeded in a series of 60-mm-diameter tissue culture dishes at $0.3\times10^5$ cells/dish in the medium with 0.2% FBS. The cells were cultured at 37°C in a humidified atmosphere composed of 95% air and 5% $CO_2$. Dishes were removed from the incubator at each of the indicated times (24–48 h); cells were detached after a brief exposure to 0.05% trypsin and suspended repeatedly to give a single-cell suspension. The number of cells was measured using a Nucleo Counter (M&S Techno Systems, Japan). The result at each time point shown in the growth curve represents the average for triplicate cultures.

### *Cell cycle analysis*

H4 cells were treated with 200 nM DHEA for 18 h and irradiated with 3-Gy of $\gamma$-rays. Cells were collected by trypsin 0, 3 and 6 h after being irradiated, washed in PBS and fixed in ice-cold 70% ethanol/PBS. The DNA was labelled with propidium iodide. Cells were sorted by flow cytometry and cell cycle profiles were determined using Cell Cycle software (Beckman Coulter, USA).

*Immunoblot analysis*

Cultured cells were harvested and lysed for 20 min at 4°C in lysis buffer B (20 mM Tris (pH 7.2), 150 mM NaCl and 1% Nonidet P-40, including protease inhibitors (200 μM phenylmethylsulphonyl fluoride, 50 μM pepstatin and 50 μM leupeptin). The protein concentration was determined using a BCA assay kit (Pierce, MA, USA). Protein samples were electrophoresed on SDS-polyacrylamide gels (7.5–15%) under reducing conditions. The proteins in the gels were transferred onto a nitrocellulose membrane. The membranes were blocked in Tris-buffered saline (TBS, 10 mM Tris-HCl (pH 7.5) and 150 mM NaCl) containing 0.1% (v/v) Tween 20 (TBST) and 5% (w/v) non-fat dry milk and then reacted with primary antibodies in TBST containing 5% (w/v) bovine serum albumin or 3% (w/v) non-fat dry milk overnight with constant agitation at 4°C. After several washes with TBST, the membranes were incubated with peroxidase-conjugated secondary antibodies. Proteins in the membranes were then visualized using the enhanced chemiluminescence (ECL) detection kit (GE healthcare Bioscience, Tokyo, Japan) according to the manufacturer's instructions.

*Protein phosphatase assay*

PP2A activity was assayed spectrophotometrically using the Ser/Thr phosphatase assay kit 1 (Upstate Biotechnology, IL, USA) according to the manufacturer's protocol. The phosphopeptide RKpTIRR (where pT is phosphothreonine) and p-nitrophenyl phosphate were used as phosphatase substrates.

*Quantitative RT-PCR*

Quantitative RT-PCR was performed using the One Step SYBR® RT-PCR kit (Perfect Real Time, TAKARA BIO. Inc. Japan) according to the manufacturer's directions. After the RT-PCR using Mx3000P (STRATAGENE, NY, USA), the products were analysed using SMxProTM Software version 3.00 (STRATAGENE). The isolation of cytoplasmic RNA was essentially performed as described by Sambrook et al. [16]. As material, 100 ng of total RNA extracted from the cells was used. The 546-bp oligonucleotides for the γ-GCS heavy sub-unit (human γ-GCS sequence, accession No. M90656) were obtained using as a forward primer, 5′-CCT TTG GAG ACC AGA GTA TGG GAG TTA C-3′, and as a reverse primer, 5′-CA GAT AGT AGC CAA CTG GTG ATC ATA AAG G-3′. The 404-bp oligonucleotides for β-actin (human sequence, accession No. HM-001101) were obtained using as a sense primer, 5′-GAG CTA GGA GCT GCC TGA CG-3′, and as an antisense primer, 5′-AGC ATT TGC GGT GGA CGA TG-3′.

*Determination of cellular glutathione levels*

Levels of GSH and GSSG were measured using a Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Inc, MD) according to the manufacturer's directions. Briefly, 5, 5′-dithiobis (2-nitrobenzonic acid) and GSH react to generate 2-nitro-5-thiobenzonic acid. The concentration of GSH in the sample solution was determined by measuring absorbance at 412 nm. For quantification of GSSG, cell lysates were treated with 2-vinylpyridine and triethanolamine to block the sulphydryl residue of GSH. GSSG in the sample solution was reduced to GSH using a reducing mixture containing GSSG reductase and NADPH as described [17] and the levels of GSSG were determined photometrically as for GSH.

*Statistical analysis*

Data were presented as means ± SD. Differences were examined by using ANOVA (StatView software). A value of $p < 0.05$ was considered significant.

## Results

*DHEA suppresses the activity of Akt in response to γ-ray irradiation*

The Akt cascade is known to mediate various functions, including the regulation of the cell survival and cell cycle in response to γ-ray irradiation [1]. Akt can phosphorylate Bad, caspase-9 and forkhead-related transcription factors, leading to an inhibition of apoptosis. We were interested in the possible role of DHEA in the regulation of sensitivity to γ-rays through Akt. As shown in Figure 1A, 3-Gy of γ-rays increased the phosphorylation of Akt (Ser473) with a peak at 2 h by 1.5-fold and returned to the control level in 4 h. Prior treatment with DHEA for 18 h resulted in a decrease in the γ-ray-induced phosphorylation of Akt. The phosphorylation showed a peak at 2 h after the irradiation being 80% and had declined to 0.25% of the levels of DHEA-untreated cells at 4 h. The levels of Akt protein did not change in the experiment. Figure 1B shows that the levels of p53 protein and the γ-ray-induced phosphorylation of p53 were not attenuated by DHEA. On the other hand, γ-ray-induced expression of $p21^{waf1}$, down-stream of the p53 signalling pathway, was enhanced by DHEA within 6 h after the irradiation (Figure 1C). It is known that the level of $p21^{waf1}$ is negatively regulated by Akt. The data suggest that DHEA stimulates the expression of $p21^{waf1}$ independent of p53. Similarly, phosphorylation of Rb, which is regulated by Akt, was down-regulated by DHEA in γ-ray-treated cells compared to the control (Figure 1D). In addition to down-regulation of Akt signals, DHEA decreased the γ-ray-induced phosphorylation of JNK, but did not affect that of ERK1/2 (data not shown). The effect of

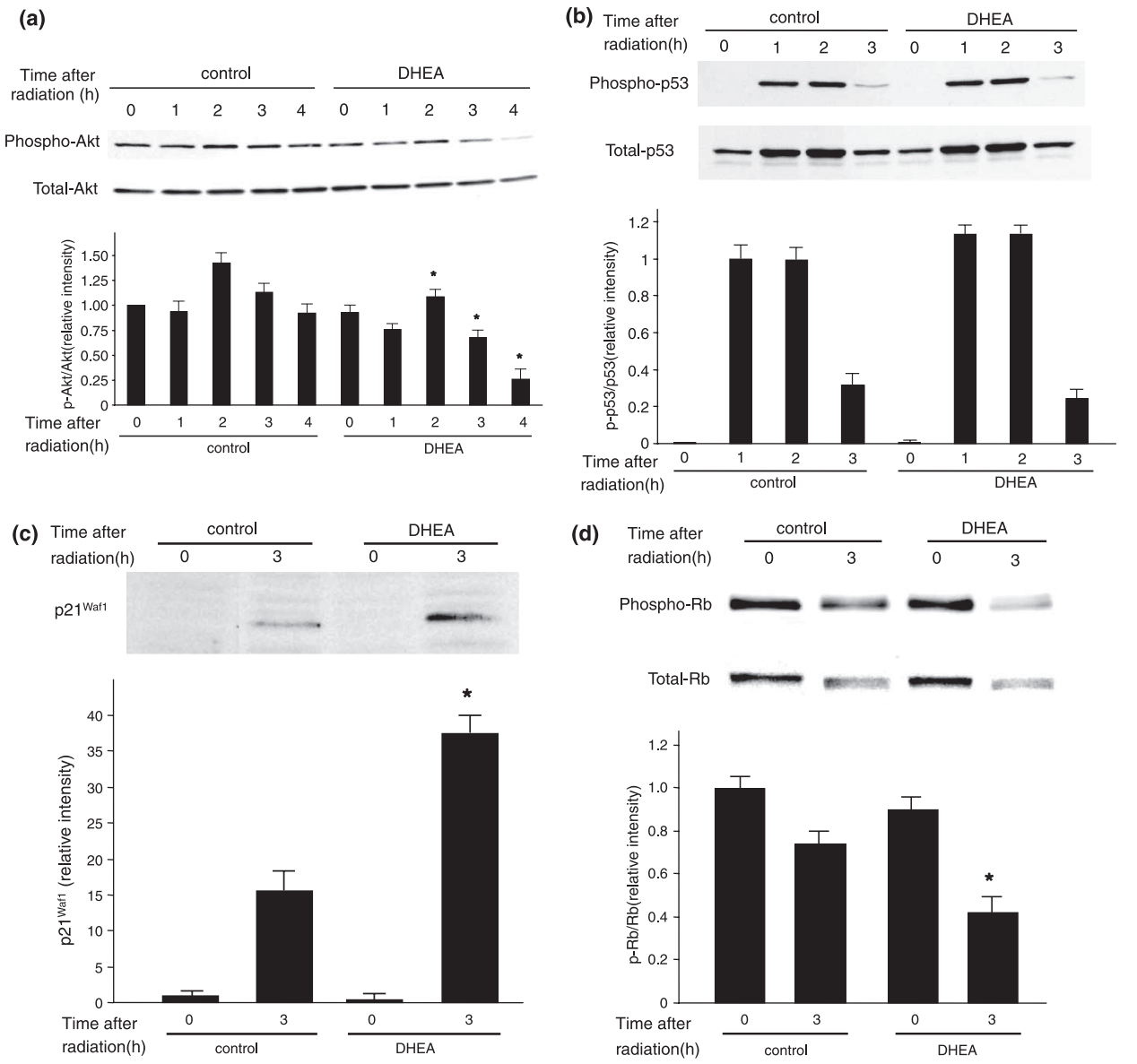


Figure 1. DHEA down-regulates the $\gamma$-ray-induced phosphorylation of Akt. H4 cells were serum-starved for 24 h. After prior treatment with 200 nM DHEA for 18 h, the cells were treated with 3-Gy of $\gamma$-rays. (A) Representative Western blot for the phosphorylation of Akt. The phosphorylation of Akt was estimated using rabbit antibodies against Akt and phospho (Ser473)-Akt and the band intensity was estimated densitometrically. The phosphorylation rate is expressed as the relative intensity of phosphorylated Akt to total Akt. (B) Representative Western blot for p53. The phosphorylation of p53 was analysed using rabbit antibodies against p53 and phospho-p53 and band intensity was estimated densitometrically. The phosphorylation rate is expressed as the relative intensity of phosphorylated p53 to total p53. (C) Representative Western blot for $p21^{Waf1}$. The level of $p21^{Waf1}$ was estimated using antibody against $p21^{Waf1}$ and the relative intensity is expressed compared to the control. (D) Representative Western blot for the phosphorylation of Rb. The phosphorylation of Rb was estimated using rabbit antibodies against Rb and phospho-Rb, and band intensity was estimated densitometrically. The phosphorylation rate is expressed as the relative intensity of phosphorylated Rb to total Rb. Each value represents the mean for three independent experiments. * $p < 0.05$ compared with DHEA-untreated cells.

DHEA on the activity of Akt was dependent on its concentration from 50–600 nM (data not sown).

*DHEA induces apoptosis in response to $\gamma$-ray irradiation*

H4 cells cultured with or without 200 nM DHEA for 18 h were irradiated with 3-Gy of $\gamma$-rays. The $\gamma$-ray-induced apoptosis was estimated by the TUNEL assay (Figure 2A). DHEA increased the $\gamma$-ray-induced apoptosis. Figure 2B shows results of a cell cycle analysis by flow cytometry. An apparent increase in $G_2$/M phase was observed 6 h after the radiation (left). DHEA enhanced the $\gamma$-ray-induced $G_2$/M checkpoint arrest by 1.3-fold compared to the control (right). The data indicate that DHEA promotes $\gamma$-ray-induced cell death and $G_2$/M arrest to enhance the radiosensitivity. The cells were further cultured for 24–48 h and cell numbers (%) were counted (Figure 2C). At 24 h after the radiation, the number had decreased by ~15% relative to the control. Prior treatment with DHEA

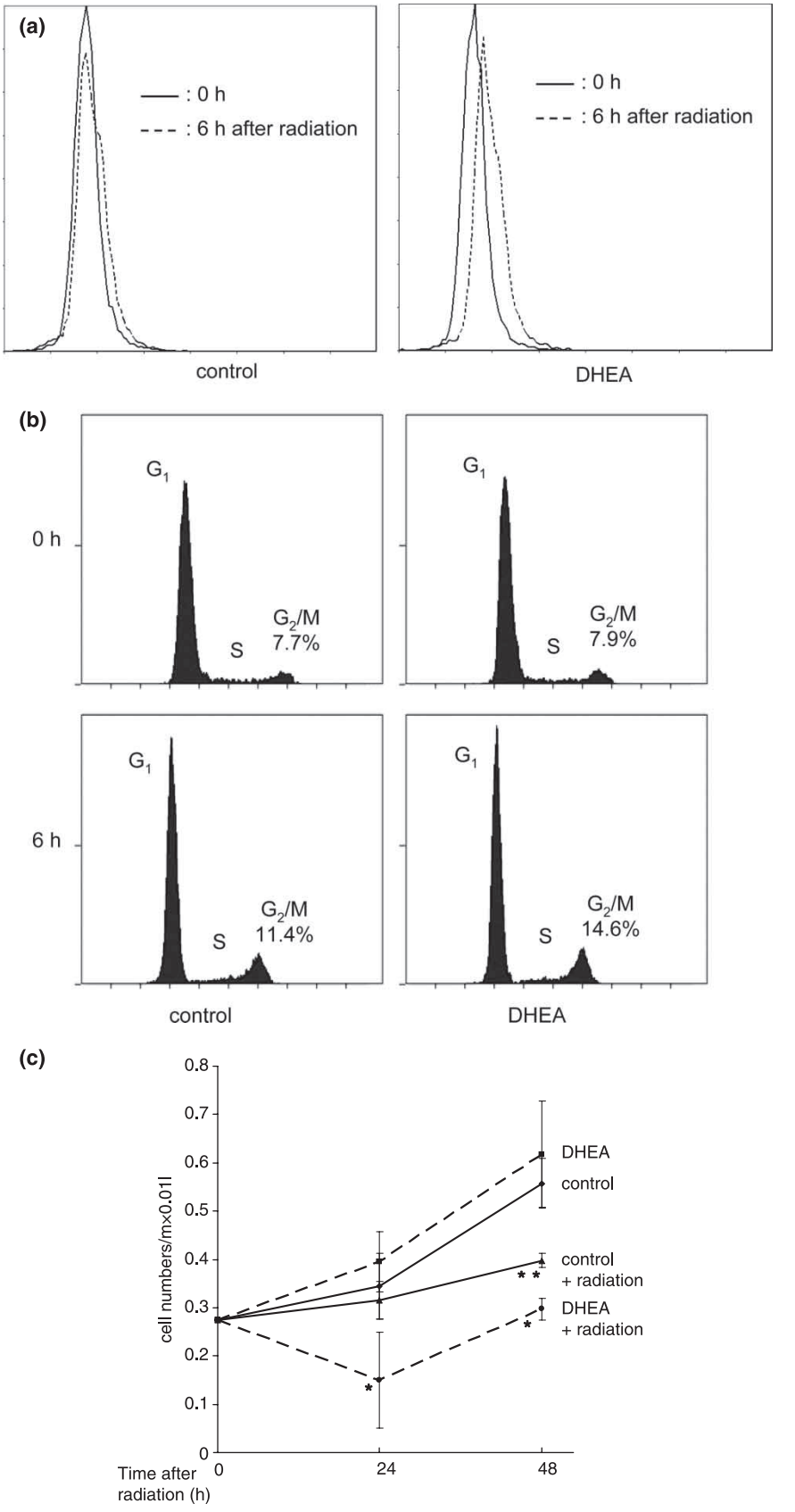


Figure 2 (*Continued*)

Figure 2. DHEA induces apoptosis and $G_2$/M arrest in response to $\gamma$-rays. The effect of DHEA on the $\gamma$-ray-induced cell damage was estimated. H4 cells previously serum-starved for 24 h, then cultured with or without 200 nM DHEA for 18 h, were irradiated with 3-Gy of $\gamma$-rays. (A) Apoptosis was evaluated by the TUNEL assay using a flow cytometer as described in Materials and methods. The increase in apoptosis was estimated 6 h after irradiation. (B) The cell cycle was analysed flow cytometrically using the PI staining of H4 cells at 0 h and 6 h after 3-Gy of $\gamma$-rays. Representative data are shown for the distribution of total cells in the M1–M4 gate in the flow cytommetric plot. (C) The number of cells was measured using a Nucleo Counter (M&S Techno Systems, Japan). The result at each time point in the growth curve represents averages from triplicate cultures. * $p < 0.05$ compared with DHEA-treated cells. ** $p < 0.05$ compared with control cells.

increased the cell number by 1.3-fold; however, it potentiated the radiation-induced decrease by ~ 38% compared to the non-irradiated cells with DHEA. The effect of DHEA on cell number among irradiated cells continued for 48 h.

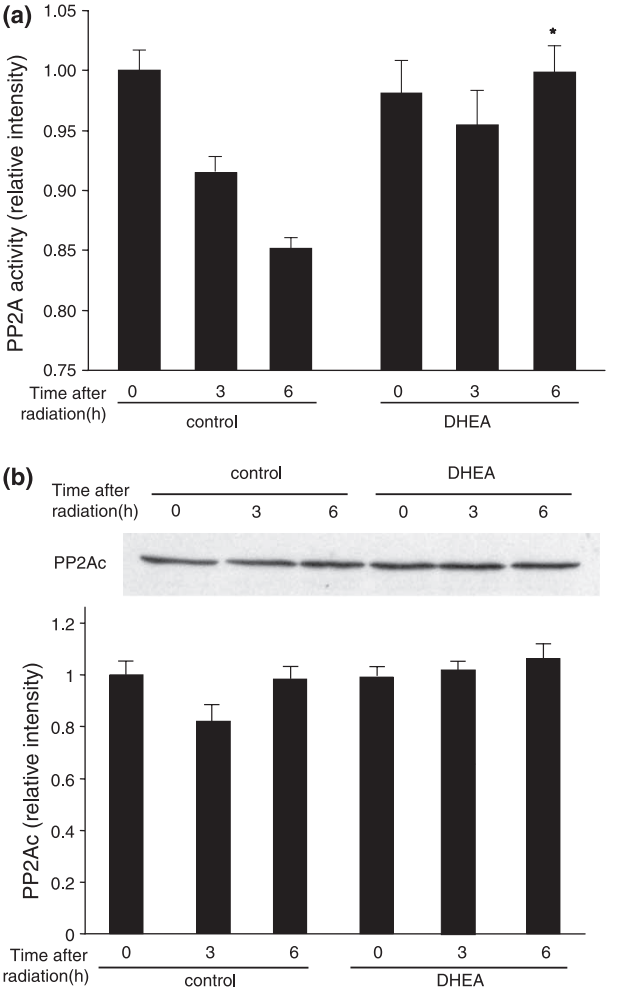


Figure 3. DHEA maintains the activity of PP2A. The effect of DHEA on the activity of PP2A was estimated as described in Materials and methods. (A) The effect of $\gamma$-rays on PP2A activity was estimated in cells previously treated with DHEA for 18 h. (B) Changes in the levels of PP2Ac protein were estimated immunologically in the cells as for (A). Proteins were separated by 12.5% SDS-PAGE and blotted to nitrocellulose membranes. Proteins in the membranes were visualized by immunoblotting. Each value represents the mean for three independent experiments. * $p < 0.05$ compared with DHEA-untreated cells. ** $p < 0.05$ compared with control cells.

*Activity of PP2A is up-regulated by DHEA*

To know the mechanism by which DHEA suppressed the Akt activity in response to $\gamma$-rays, we examined the involvement of PP2A in the regulation of Akt activity. The unphosphorylated form of Akt is virtually inactive and dephosphorylation of Akt is regulated by PP2A. Figure 3A shows the effect of DHEA on the activity of PP2A of 3 and 6 h after 3-Gy of radiation. The radiation decreased the activity of PP2A. However, H4 cells previously treated with DHEA retained the activity for 6 h. The levels of PP2Ac did not change with or without $\gamma$-ray irradiation and DHEA (Figure 3B). The data suggest that the maintenance of PP2A activity by DHEA plays a role in the suppression of Akt activity.

*DHEA induces $\gamma$-GCS*

It has been reported that the activity of PP2A is regulated by the redox status of the catalytic subunit of PP2A (PP2Ac) [10]. Figure 4A shows that $\gamma$-rays gradually decreased the level of GSH to 80% of the control in 6 h and the level of GSSG increased by ~ 2.5-fold. These changes by irradiation led to a decrease in the GSH/GSSG ratio. On the other hand, DHEA protected the $\gamma$-ray-induced decrease of GSH and increase of GSSG in 6 h, to maintain the GSH/GSSG ratio. Then, the expression of $\gamma$-GCS was estimated by RT-PCR. DHEA increased expression of the $\gamma$-GCS heavy sub-unit (catalytic sub-unit) by 1.5-fold (Figure 4B). The results suggest that DHEA increases the level of GSH through up-regulation of the GSH synthesis and maintains the GSH/GSSG ratio in response to $\gamma$-ray irradiation.

To further confirm the effect of thiols on the activity of PP2A, H4 cells were pre-treated with 5 mM NAC for 18 h. Figure 4C shows the effect of NAC on the activity of PP2A. NAC maintained the activity of PP2A similar to the effect by DHEA. Concomitantly, the $\gamma$-ray-induced activation of Akt-phosphorylation was down-regulated in the cells pre-treated with NAC (Figure 4D). The results suggest that PP2A activity is regulated by GSH-dependent redox status. They also strongly suggest that the phosphorylation of Akt is regulated by PP2A and that DHAE induces GSH synthesis to maintain the redox state of PP2Ac following dephosphorylation of Akt.

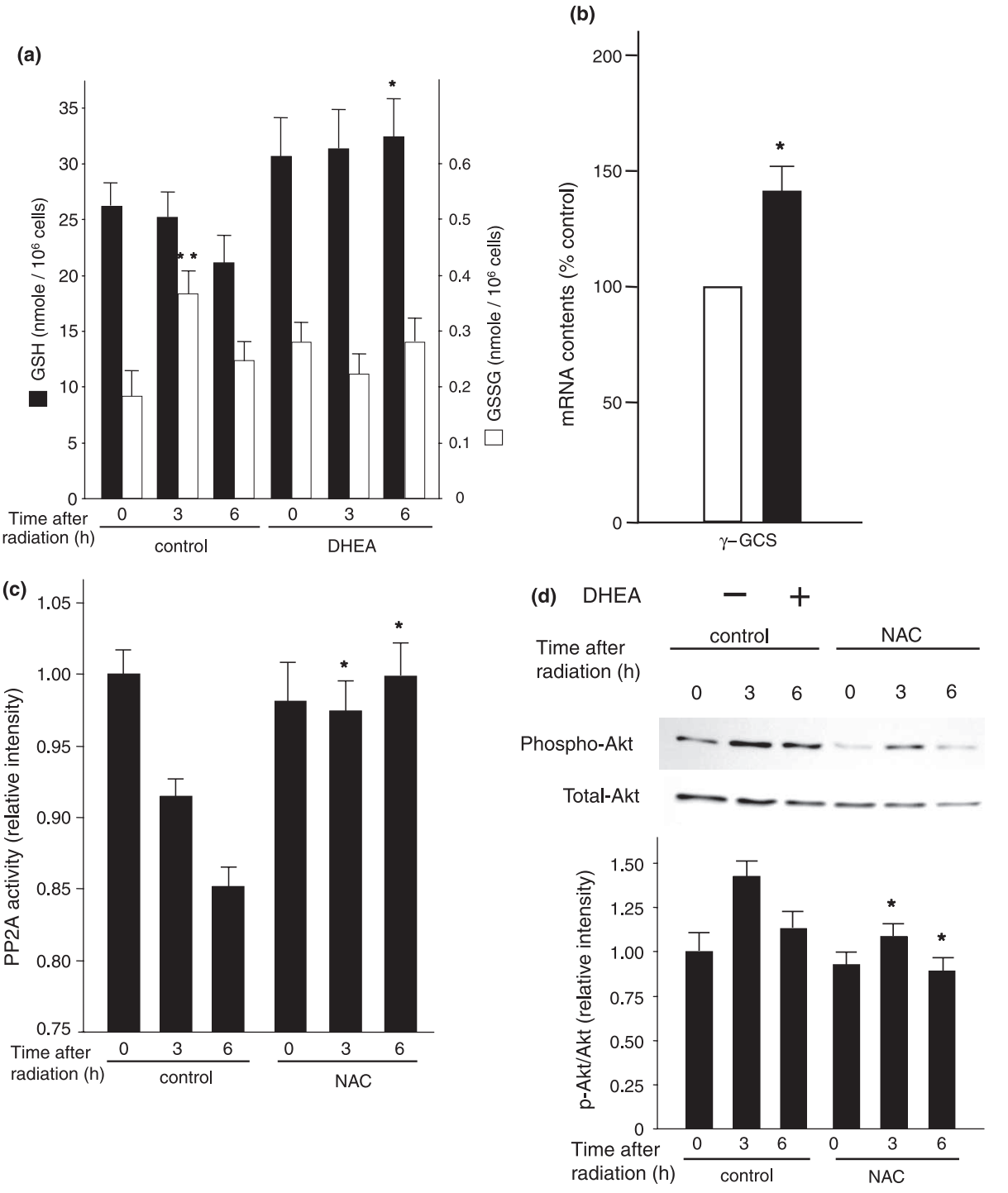


Figure 4. DHEA induces the expression of $\gamma$-GCS mRNA and increases the levels of GSH. The effect of DHEA on the expression of $\gamma$-GCS mRNA and the level of GSH was estimated. (A) H4 cells were treated with 200 nM DHEA for 18 h and the gene expression of the $\gamma$-GCS heavy sub-unit was analysed by quantitative RT-PCR. The expression was expressed as relative intensity compared to the control. (B) Concentrations of GSH were estimated using a Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Inc, MD) according to the manufacturer's directions and were expressed as relative intensity compared to the control. (C) Effect of NAC on the PP2A activity was estimated. Cells previously treated with 5 mM NAC for 18 h were irradiated by 3-Gy of $\gamma$-rays. (D) Change in the phosphorylation of Akt was estimated in cells in the same condition as (C). Each value represents the mean of three independent experiments. * $p < 0.05$ compared with DHEA-untreated cells.

## Discussion

The Akt signalling pathway is an important cell survival and anti-apoptotic signal in $\gamma$-ray-induced apoptosis [18,19]. In this study, we found that the pathway was significantly suppressed in the DHEA-treated cells after the radiation. Moreover, we found that the activity of PP2A was maintained in DHEA-treated cells compared with control cells. PP2A is

known to modulate the activities of several kinases and is responsible for the dephosphorylation and inactivation of Akt [20,21]. Therefore, these results suggest that Akt signalling was suppressed by the up-regulation of PP2A activity in the cells irradiated with $\gamma$-rays. PP2A is a widely conserved protein serine/threonine phosphatase that function s as a trimeric protein complex consisting of PP2Ac, a scafford subunit (PP2Aa), and an alternative regulatory B subunit [22]. The expression and activity of PP2A are regulated by many factors such as $Ca^{2+}$, oxidative stress and glutathionylation [23]. Reduction of the activity of PP2A by ROS plays a role in the progression of cellular senescence [10,11]. These reports suggest that the activity of PP2A is regulated by a GSH-dependent redox system and plays a role in the regulation of $\gamma$-ray-induced cell cycle arrest and apoptosis.

Cell cycle inhibition and the induction of apoptosis are common mechanisms proposed for prevention of radiation-induced carcinogenesis or tumour cell progression. Inhibition of Akt signalling causes protection of cells against photocarcinogenesis via modulation of the cell cycle [24]. On the other hand, estradiol down-regulates $p21^{waf1}$ synthesis and dephosphorylates Rb to decrease $\gamma$-ray-induced cell cycle arrest independent of p53 [25]. PTEN is a member of the protein tyrosine phosphatase family and reverses the action of phospoinositide 3-kinase [26] and its depletion prevents the tumour suppression through activation of the PI3K/Akt pathway [27].

$p21^{waf1}$ is the most important protein involved in cell-cycle arrest at both $G_1$ and $G_2$/M check point. The synthesis of $p21^{waf1}$ is regulated by Akt and negatively regulated by p53. In the present study, the synthesis of $p21^{waf1}$ was suppressed by DHEA. Phosphorylation of Rb has been shown to play a key role in cell cycle progression the $G_1$ to S phase; furthermore, a recent study indicated that Rb also regulates the $G_2$/M check point [28]. Down-regulation by DHEA of the x-ray-induced phosphorylation of Rb is consistent with other findings regarding the role of DHEA in radiation-induced cell damage.

To maintain the cellular thiol-disulphide redox status under reducing conditions, cells possess the thioredoxin/thioredoxin reductase system and the GSH/glutaredoxin system [29]. These or other systems are thought to be involved in a variety of cellular events such as signal transduction, stress response and metabolic regulation by regulating the redox status of various cellular proteins including Akt [9,16]. Previously, we reported that the radiation up-regulates the expression of $\gamma$-GCS [30]. In response to oxidative stress, the GSH/GSSG ratio is regulated by GSH synthesis, transport outside the cells or catalytic activity of GSH reductase and GSSG peroxidase. In the present study, the GSH/GSSG ratio decreased by the radiation (Figure 4). On the other hand, treatment with DHEA protected $\gamma$-ray-induced-decrease of the GSH/GSSG ratio. One of the mechanisms was thought to be due to up-regulation of the expression of the $\gamma$-GCS by DHEA (Figure 4). However, the molecular mechanism by which the expression of $\gamma$-GCS genes is regulated by DHEA is not clear. Also, involvement of other redox regulating proteins such as glutaredoxin and thioredoxin has not been clarified. Further study therefore is needed. In summary, DHEA regulates radiosensitivity to induce cell cycle arrest and apoptosis in tumour cells through the GSH-dependent down-regulation of Akt signalling.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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