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Dehydroepiandrosterone increased oxidative stress in a human cell line during differentiation

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Abstract
Dehydroepiandrosterone (DHEA), a reversible inhibitor of glucose-6-phosphate dehydrogenase (G6PD), is increasingly taken as an antioxidative and anti-ageing supplement. This study investigated the effects of DHEA on the expression of G6PD and on the state of oxidative stress in a human promyelocytic leukaemia cell line, HL60, during the differentiation to neutrophil-like cell. This study differentiated HL60 with dimethyl sulfoxide (DMSO) in the presence (DMSO-HL60/DHEA) or absence (DMSO-HL60) of DHEA. During the differentiation, activity, mRNA and protein levels of G6PD were increased. DHEA increased these levels further. DHEA by itself suppressed the production of superoxide from DMSO-HL60 upon stimulation with phorbol myristate acetate (PMA). However, DMSO-HL60/DHEA stimulated with PMA in the absence of DHEA produced superoxide and 8-oxo-deoxyguanosine more than PMA-stimulated DMSO-HL60. After addition of H_2O_2, the ratio of reduced glutathione to oxidized glutathione was lower in DMSO-HL60/DHEA than in DMSO-HL60. These findings indicate that DHEA acts both as an antioxidant and as a pro-oxidant.

Keywords: *Dehydroepiandrosterone, Glucose-6-phosphate dehydrogenase, HL60, superoxide, GSH/GSSG ratio, 8-oxo-deoxyguanosine*

Abbreviations: *DHEA, dehydroepiandrosterone; G6PD, glucose-6-phosphate dehydrogenase; PPP, pentose phosphate pathway; R5P, ribose-5-phosphate; DMSO, dimethyl sulfoxide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species;* $O_2^{\bullet -}$*, superoxide; PMA, phorbol myristate acetate; 8-oxo-dG, 8-oxo-deoxyguanosine; GSH/GSSG, reduced glutathione/oxidized glutathione; SOD, superoxide dismutase; MPO, myeloperoxidase; D-PBS (−), Dulbecco's phosphate buffered saline (free of Ca and Mg ions); GPx, glutathione peroxidase; GR, glutathione reductase; dG, deoxyguanosine; FAS, fatty acid synthase; ACC1, acetyl-CoA carboxylase 1; HBSS-HEPES, Hanks' balanced salt solution containing 30 mM HEPES; X5P, xylulose-5-phosphate; ChREBP, carbohydrate-responsive element binding protein.*

Introduction

In humans, dehydroepiandrosterone (DHEA) is an abundantly produced adrenal steroid [1,2]. Higher plasma levels of DHEA-sulphate (DHEA-S), a major form of DHEA in human plasma, have been found in people who go on to live longer, thus DHEA has been associated with increased life expectancy [3]. While DHEA has become popular as an anti-ageing supple-

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ment, other reports have found no beneficial effects for healthy elderly people [4,5].

Biochemically, DHEA is a reversible inhibitor of mammalian glucose-6-phosphate dehydrogenase (G6PD), the first, and the rate-limiting, enzyme of pentose phosphate pathway (PPP), where it is a major source of ribose 5-phosphate (R5P) and the extra-mitochondrial reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) [6]. NADPH is a critical modulator of cellular redox state and serves as a reductant for several enzymes, such as: NADPH oxidase, a producer of reactive oxygen species (ROS) [7–10]; and glutathione reductase (GR), which reduces oxidized glutathione (GSSG) [11]. While ROS are necessary for inflammatory defense of the host against infectious agents [12], high levels of ROS are capable of damaging many critical cellular components, such as proteins, lipids and DNA, leading eventually to the kind of cellular injuries that are implicated in cardiovascular disease, diabetes, neurodegenerative disorders, cancer and various other age-related diseases [12–16]. DHEA is supposed to prevent age-related diseases by inhibiting G6PD and thus suppressing ROS formation [17]. Thus, DHEA is increasingly taken as an anti-oxidative and anti-ageing supplement. However, no reports had investigated extensively the effects of DHEA on the expression of either G6PD or those on the redox state at cellular level.

In this report we have investigated the effect of DHEA on the differentiating HL60, a human promyelocytic leukaemia cell line [18], to clarify the following points: (1) How does G6PD behave on its expression in the presence of its inhibitor, DHEA?; (2) Is the ability of ROS production changed by DHEA?; and (3) Does DHEA act as an anti-oxidant at cellular level? The reasons why we used HL60 are: (a) activity of G6PD in HL60 is reported to increase during the differentiation [19], (b) HL60 differentiates into neutrophil-like cell which produces large amounts of ROS [18,20] and (c) the status of oxidative stress is reliably evaluated in HL60 [20–23].

Materials and methods

Cell culture and treatments

HL60 was kindly supplied by the Japanese Cancer Research Resource Bank. It was grown in RPMI-1640 (SIGMA-Aldrich, St. Louis, MO) containing 10% heat-inactivated foetal calf serum (Life Technologies, Grand Island, NY). For the experiments, HL60 underwent differentiation for 1–7 days in a culture medium containing 1.3% dimethyl sulfoxide (DMSO; SIGMA-Aldrich) [20]. During the differentiation, cells cultured with or without 100 μM DHEA (Wako, Osaka, Japan) were defined as DMSO-HL60/DHEA or DMSO-HL60, respectively. In both groups, differentiation was initiated when the sample contained 0.3×10^6 cells/ml. Undifferentiated and differentiated cells were washed twice and resuspended in Hanks' balanced salt solution (Invitrogen, Carlsbad, CA) containing 30 mM HEPES, pH 7.4 (HBSS-HEPES) [20]. These washed samples were used for determination of superoxide ($O_2^{\bullet -}$) production, enzyme activities, 8-oxo-deoxyguanosine (8-oxo-dG) levels, reduced glutathione-to-oxidized glutathione (GSH/GSSG) ratio, amounts of NADPH and protein levels.

Determination of $O_2^{\bullet -}$ production from HL60

$O_2^{\bullet -}$ production was measured as the reduction of cytochrome *c* at 37°C [20]. Briefly, undifferentiated and differentiated HL60 (2.5×10^6 cells/ml) were mixed with cytochrome *c* (Wako) and phorbol myristate acetate (PMA; Wako). Then absorbance changes at 550 nm were recorded for 10 min. The rate of $O_2^{\bullet -}$ production was calculated from the linear portion of the absorbance change. The absorbance changes at 550 nm after PMA stimulation were completely inhibited by superoxide dismutase (SOD) [20].

Measurement of G6PD, myeloperoxidase and anti-oxidative enzymes activities

To measure G6PD activity, cells were lysed with a lysis buffer (20 mM Tris-HCl, pH 7.5, containing 1% (v/v) Nonidet-P40, 10% (v/v) glycerol, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) [24]. Whole cell lysate (100 μg protein) was mixed with a buffer (50 mM Tris-HCl, pH 8.0, containing 10 mM $MgCl_2$,), 0.6 mM glucose-6-phophate (Oriental Yeast Co., Ltd, Osaka, Japan) and 0.2 mM of the oxidized form of nicotinamide adenine dinucleotide phosphate ($NADP^+$; Oriental Yeast Co., Ltd). The rate of increase in absorbance at 340 nm was then measured spectrophotometrically [25].

Myeloperoxidase (MPO) activity was measured using the guaiacol oxidation assay [26]. Cells were suspended in a buffer (25 mM HEPES and 5 mM NaH_2PO_4, pH 7.4, containing 10 mM glucose, 115 mM NaCl, 5 mM KCl and 1 mM $MgCl_2$). The cell suspension was frozen at −20°C and subsequently thawed. After the lysate was added to a reaction mixture containing 3.75 mM 3-amino-1,2,4-triazole, 0.02% (w/v) cetyltrimethylammonium bromide, 100 μM benzylsulphonyl fluoride, 0.1% (v/v) Triton X-100, 13 mM guaiacol and 670 μM H_2O_2, the rate of increase in absorbance at 470 nm was measured spectrophotometrically.

To measure anti-oxidative enzyme activities, the cells were suspended in ice-cold Dulbecco's phosphate buffered saline (free of Ca and Mg ions) [D-PBS (−)] and then homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10 000 *g* for 10 min at 4°C and the supernatants were used. SOD-like activity was measured using SOD Assay

Kit-WST (Dojindo, Kumamoto, Japan). Catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) activities were measured spectrophotometrically as described elsewhere [27–29].

Protein concentrations were measured using Bio-Rad protein assay kit with bovine serum albumin as a standard (Bio-Rad, Hercules, CA).

Western blot analysis of G6PD and p47^{phox}

Whole cell lysates, which were used for measurement of G6PD activity, were further treated with SDS lysis buffer [2% (w/v) SDS, 50% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 20 mM Tris, pH 6.8]. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 10% gel, transferred onto a PVDF membrane (Bio-Rad) and these samples were probed overnight at 4°C with one of the following antibodies: a rabbit anti-G6PD (1:2000; Bethyl Laboratories Inc., Montgomery, TX), a rabbit anti-p47^{phox} (1:2000; Cell Signaling Technology Inc., Beverly, MA) or a mouse anti-GAPDH (1:2000; Chemicon international Inc., Temecula, CA). Proteins were visualized with peroxidase-conjugated anti-rabbit (1:2000) or anti-mouse (1:10 000) IgG. All the first antibodies were diluted with an enhancer solution (Can Get Signal, Toyobo, Osaka, Japan). ECL kit (PIERCE, Rockford, IL) was used to detect the immunoreactive materials. Signals were analysed and quantified using Image-Pro Plus version 4.0 (Media Cybernetics, Silver Spring, MD).

Determination of 8-oxo-dG

After undergoing differentiation for 5 days, samples of DMSO-HL60 and DMSO-HL60/DHEA were treated with 0.1% (v/v) DMSO (unstimulated) or 1 μM PMA (stimulated) in 0.1% DMSO at 37°C for 60 min. The cells were then immediately chilled by ice-water bath, washed with ice-cold D-PBS (−) and then stored as cell pellets at −80°C until subsequent analysis. DNA was extracted from the cells and enzymatically digested to nucleosides under anaerobic condition [22]. The nucleosides were separated by an HPLC system, then 8-oxo-dG was detected by electrochemical detection and deoxyguanosine (dG) was detected by UV absorption as described before [23]. Oxidative DNA damage (8-oxo-dG levels) was expressed as the molar ratio of 8-oxo-dG per 10^5 dG [23].

Determination of GSH and GSSG

DMSO-HL60 and DMSO-HL60/DHEA after 5 days differentiation were treated with or without 50 μM H_2O_2 at 37°C for 10 min, The cells were then immediately chilled by an ice-water bath and washed with ice-cold D-PBS (−) containing 20 mM EDTA. The cell pellets were mixed with ice-cold 10% (v/v) trichloroacetic acid containing 5 mM EDTA. These samples were then centrifuged at 10 000 *g* for 10 min at 4°C. The supernatants were stored at −80°C until subsequent analysis. After neutralization with 0.5 M NaOH, treated with or without *N*-ethylmaleimide, the samples were reduced with dithiothreitol for oxidized or total glutathione measurement, respectively [30,31]. Then they were labelled with o-phthalaldehyde (OPA). The OPA-labelled GSH was measured quantitatively by HPLC as described [32].

NADPH measurement

DMSO-HL60 and DMSO-HL60/DHEA after 5 days differentiation cells were washed with ice-cold D-PBS (−) and then NADP+ and NADPH were extracted with NADP+/NADPH Quantification Kit (BioVision Research Products, CA). These samples were stored at −80°C until subsequent analysis. The amount of NADPH was measured using the kit according to the manufacturer's instructions.

Determination of mRNA levels by real-time PCR

Real-time PCR was used to measure the mRNA levels of G6PD, fatty acid synthase (FAS), acetyl-CoA carboxylase 1 (ACC1), components of NADPH oxidase; p47^{phox}, p67^{phox}, p22^{phox}, gp91^{phox} and catalase. Briefly, total RNA was isolated from cells by Trizol reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA was synthesized by reverse transcription from total RNA using reverse transcriptase and oligo-dT_{20} (Toyobo). The resulting cDNA was amplified, using the FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH, Mannheim, Germany) under the following conditions: 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Real-time PCR was performed using a Thermal Cycler Dice® Real Time System (Takara Bio Inc., Otsu, Japan). The following primers were used: G6PD forward, 5′-CTG GAC CTG ACC TAC GGC A-3′ and reverse, 5′-CAG TGG GGT GAA AAT ACG C-3′ [33]; FAS forward, 5′-CTC TCC CAG GTA TGC GAC G-3′ and reverse, 5′-GTG ACC TCC GGT GGC AGG-3′; ACC1 forward, 5′-CAA ATC CGC AGC TTG GTC CA-3′ and reverse, 5′-GCC CTT TTC TCC AGA GAC AG-3′; p47^{phox} forward, 5′-AGC CCG CTC GAG GAG GAG-3′ and reverse, 5′-GCT AGC TGG GGA CTG CGC-3′; p67^{phox} forward, 5′-CCT GGT GTT ATC AAA GGT GAA T-3′ and reverse, 5′-CTT ACA AAC AAG TAA TAG GGC TT-3′; p22^{phox} forward, 5′-CTA CCT ACT GGC GGC TGT G-3′ and reverse, 5′-ACC GGG ATG GGG TTG ACC T-3′; gp91^{phox} forward, 5′-CAA GTC AAC ACC CTA ATA CCA G-3′ and reverse, 5′-CAT TTG GCA GCA CAA CCC AC-3′; catalase forward, 5′-GCG GTC AAG AAC TTC ACT GA-3′ and reverse, 5′-GCT AAG CTT CGC TGC ACA GGT-3′;

GAPDH forward, 5′-TGG ACC TGA CCT GCC GTC TA-3′ and reverse, 5′-CCC TGT TGC TGT AGC CAA ATT C-3′ [34]. The relative level of mRNA was calculated using cycle time (*Ct*) values, which were normalized against the value of GAPDH. Relative quantification (fold change) between different samples was then determined according to the $2^{-\Delta\Delta Ct}$ method [35].

Statistical analysis

Values are shown as means ± standard deviation (SD) or means + SD. Statistical analysis was performed using one-way ANOVA. Significant differences were determined using Dunnett's test for comparison with results from day 1 of each group (Figure 1) and Scheffé's *F*-test for multiple comparisons (Figures 1–8). Unpaired Student's *t*-test was also used. In Figure 3D, the Spearman correlation test was used. $p < 0.05$ was considered to be statistically significant.

Results

Cell growth and viability

In DMSO-HL60 cell number increased significantly after 2 days differentiation and the increase continued up to 5 days differentiation. Then the number tended to decrease. In DMSO-HL60/DHEA cell number increased after 5 days differentiation; however, it was less than that in DMSO-HL60 throughout 3–7 days (Figure 1A).

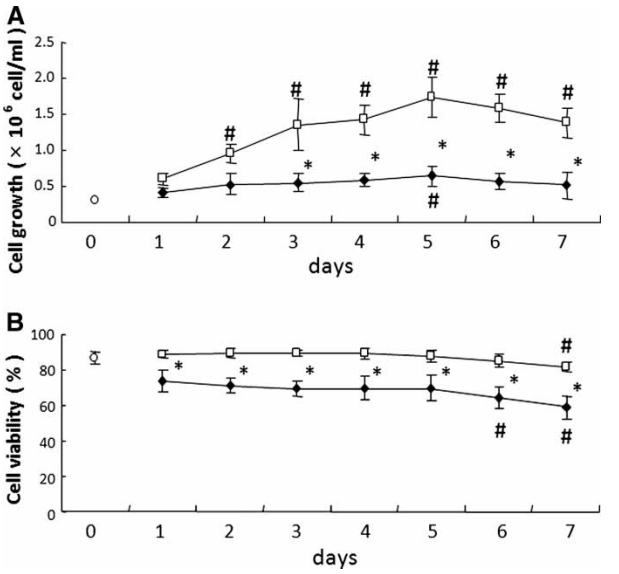

Figure 1. (A) Cell growth and (B) viability of HL60 during DMSO differentiation with and without DHEA. For up to 7 days, HL60 was differentiated with 1.3% DMSO in the presence (DMSO-HL60/DHEA, ◆) or absence (DMSO-HL60, □) of 100 μM DHEA. Open circle (○) indicates results for undifferentiated cells. The data are presented as means ± SD values from four-to-six independent experiments. *$p < 0.05$ compared with DMSO-HL60 on the corresponding day. #$p < 0.05$ compared with 1 day of each group.

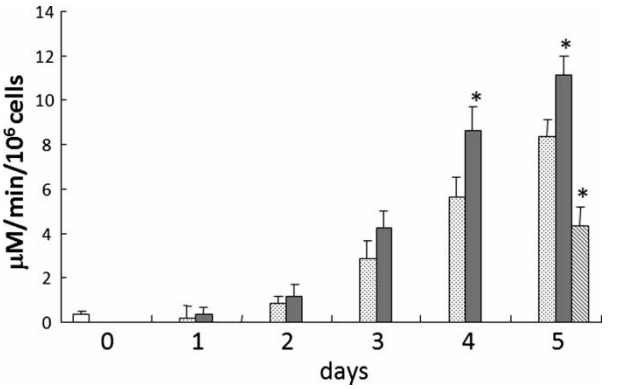

Figure 2. $O_2^{\bullet -}$ generation upon PMA stimulation from DMSO-HL60 (▨) and DMSO-HL60/DHEA (▬) between 1–5 days and undifferentiated cells (▭). DHEA (100 μM) was co-added with PMA to DMSO-HL60 at 5 days (▨). The data are presented as means + SD values from three-to-six independent experiments. *$p < 0.05$ compared with DMSO-HL60 on the corresponding days.

In DMSO-HL60 cell viability decreased significantly after 7 days differentiation. In DMSO-HL60/DHEA cell viability decreased significantly after 6 days differentiation and it was lower than that in DMSO-HL60 throughout the differentiation (Figure 1B). Thus, we have investigated the effects of DHEA on the differentiation of HL60 up to 5 days.

$O_2^{\bullet -}$ production upon stimulation with PMA

After the culture medium was thoroughly washed with HBSS-HEPES, we measured $O_2^{\bullet -}$ production after PMA stimulation applied to undifferentiated cells, DMSO-HL60 and DMSO-HL60/DHEA. $O_2^{\bullet -}$ production was increased during differentiation in both DMSO-HL60 and DMSO-HL60/DHEA. However, DMSO-HL60/DHEA showed significantly higher rate of $O_2^{\bullet -}$ production than DMSO-HL60 after 4 days and 5 days differentiation. DMSO-HL60 after 5 days differentiation produced significantly less $O_2^{\bullet -}$, when DMSO-HL60 was stimulated in the presence of 100 μM DHEA (Figure 2).

G6PD activity and G6PD protein amounts

We measured G6PD activity of undifferentiated cells, DMSO-HL60 and DMSO-HL60/DHEA. G6PD activity was increased during differentiation in both DMSO-HL60 and DMSO-HL60/DHEA. However, DMSO-HL60/DHEA showed significantly higher G6PD activity than DMSO-HL60 after 4 days and 5 days differentiation. G6PD activity in DMSO-HL60 after 5 days differentiation significantly decreased, when DHEA (100 μM) was added in the measurement (Figure 3A).

We measured G6PD protein amounts in DMSO-HL60 and DMSO-HL60/DHEA. G6PD protein amounts were increased during differentiation in both DMSO-HL60 and DMSO-HL60/DHEA (Figure 3B).

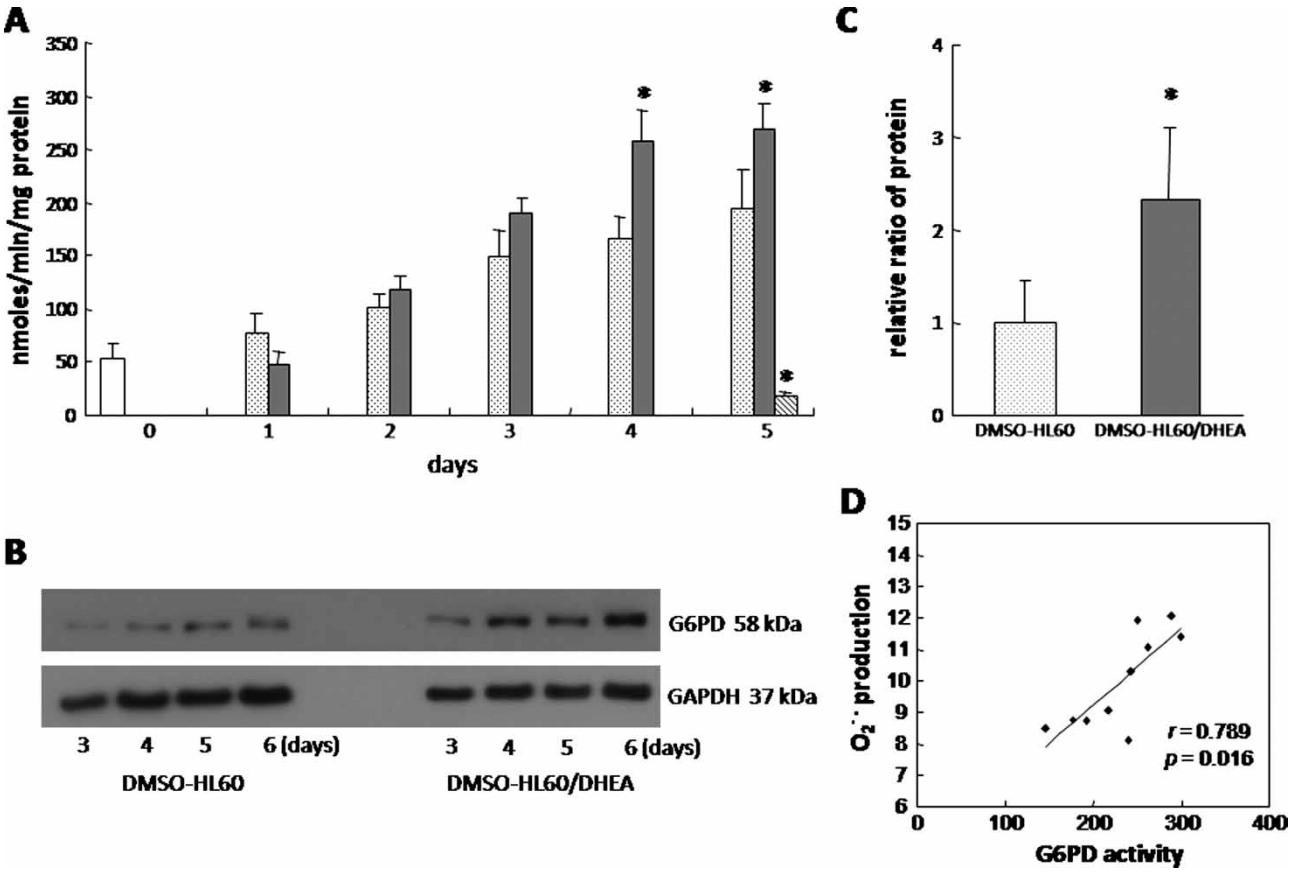

Figure 3. (A) Glucose-6-phosphate dehydrogenase (G6PD) activity in DMSO-HL60 (▨) and DMSO-HL60/DHEA (▬) between 1–5 days and undifferentiated cells (▭). DHEA (100 μM) was co-added to the lysate from DMSO-HL60 at 5 days (▨). The data are presented as means+SD values from four-to-five independent experiments. *$p<0.05$ compared with DMSO-HL60 on the corresponding days. (B) The representative data in protein levels of G6PD (upper part) and GAPDH (lower part) with Western blot analysis are shown. (C) The values of protein levels in DMSO-HL60 (▨) and DMSO-HL60/DHEA (▬) at 5 days are calculated by densitometric analysis. The value of DMSO-HL60 is set at 1.0. The data are presented as means+SD values from five independent experiments. *$p<0.05$ compared with DMSO-HL60. (D) Correlation plots of G6PD activity and $O_2^{\bullet-}$ production at 5 days are shown. Spearman correlation test was used. The analytical values as r (correlation coefficient) and p (significance) are presented.

DMSO-HL60/DHEA showed significantly higher G6PD amounts than DMSO-HL60 after 5 days differentiation (Figure 3C). G6PD activity was significantly correlated to $O_2^{\bullet-}$ production from the results of DMSO-HL60 and DMSO-HL60/DHEA after 5 days differentiation (Figure 3D).

mRNA levels of G6PD and various enzymes

We measured G6PD mRNA levels of undifferentiated cells, DMSO-HL60 and DMSO-HL60/DHEA. G6PD mRNA level was increased during differentiation in both DMSO-HL60 and DMSO-HL60/DHEA. However, DMSO-HL60/DHEA showed significantly higher G6PD mRNA level than DMSO-HL60 after 2–5 days differentiation (Figure 4A). The difference in the mRNA level was the largest after 3 days differentiation.

We also compared the mRNA levels of FAS and ACC1, between DMSO-HL60 and DMSO-HL60/DHEA after 3 days differentiation. DMSO-HL60/DHEA showed significantly lower FAS and ACC1 mRNA levels than DMSO-HL60 (Figure 4B). We also compared the mRNA levels of catalase between DMSO-HL60 and DMSO-HL60/DHEA. DMSO-HL60/DHEA showed significantly higher catalse mRNA levels than DMSO-HL60 (Figure 4C).

Oxidative DNA damage with or without PMA stimulation

We measured 8-oxo-dG levels of DMSO-HL60 and DMSO-HL60/DHEA after 5 days differentiation, with or without PMA stimulation. Without PMA stimulation, DMSO-HL60/DHEA showed similar 8-oxo-dG levels to DMSO-HL60. Both cells significantly increased 8-oxo-dG levels after stimulation with PMA. However, with PMA stimulation, DMSO-HL60/DHEA showed significantly higher 8-oxo-dG levels than DMSO-HL60 (Figure 5).

GSH/GSSG ratio with or without H_2O_2 addition

We measured GSH/GSSG ratio of DMSO-HL60 and DMSO-HL60/DHEA after 5 days differentiation with

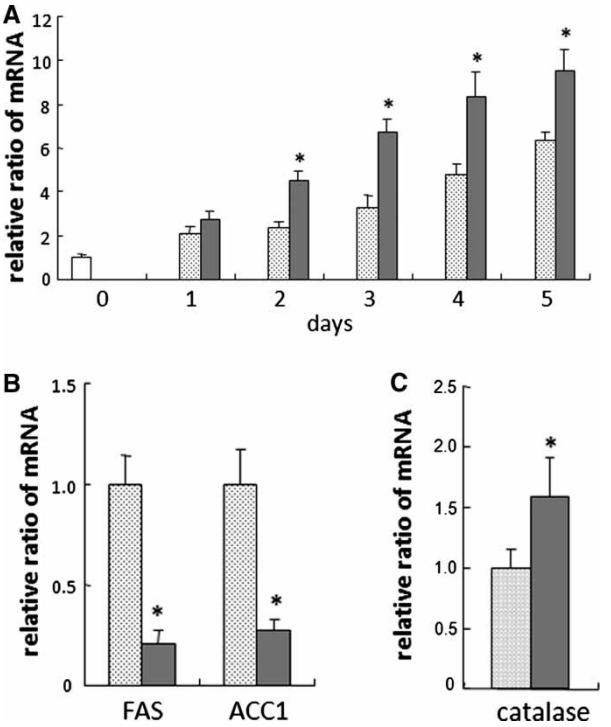

Figure 4. (A) G6PD mRNA levels in DMSO-HL60 (▒), DMSO-HL60/DHEA (▓) cells between 1–5 days and undifferentiated cells (□). The relative values of the undifferentiated cells are set at 1.0. The mRNA levels of (B) fatty acid synthase (FAS) and acetyl-CoA carboxylase 1 (ACC1) and (C) catalase in DMSO-HL60 (▒) and DMSO-HL60/DHEA (▓) cells at 3 days are shown. Corresponding values of DMSO-HL60 are set at 1.0. The data are presented as means+SD values from four-to-five independent experiments $*p<0.05$ compared with DMSO-HL60 on the corresponding days.

or without H_2O_2 addition. Without H_2O_2 addition, DMSO-HL60/DHEA showed a similar GSH/GSSG ratio to DMSO-HL60. Both cells significantly decreased GSH/GSSG ratio after the addition of H_2O_2. However, with H_2O_2 addition, DMSO-HL60/DHEA showed a significantly lower GSH/GSSG ratio than DMSO-HL60 (Figure 6).

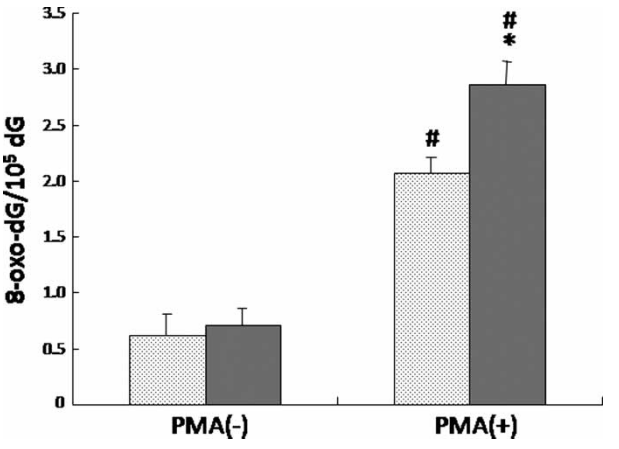

Figure 5. 8-oxo-deoxyguanosine (8-oxo-dG) levels in DMSO-HL60 (▒) and DMSO-HL60/DHEA (▓) at 5 days. 8-oxo-dG levels of DMSO-differentiated cells without [PMA (–)] or with PMA stimulation [PMA (+)] were measured. The data are presented as means+SD values from six independent experiments. $*p<0.05$ compared with DMSO-HL60. $\#p<0.05$ compared with PMA (–) condition of the corresponding cells.

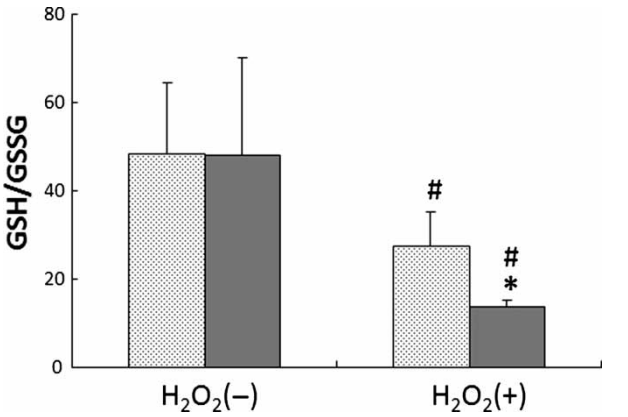

Figure 6. Reduced glutathione/oxidized glutathione (GSH/GSSG) ratio in DMSO-HL60 (▒) and DMSO-HL60/DHEA (▓) at 5 days. GSH/GSSG levels of the DMSO-differentiated cells under the conditions without [H_2O_2 (–)] or with 50 μM H_2O_2 addition [H_2O_2 (–)] were measured. The data are presented as means+SD values from six independent experiments. $*p<0.05$ compared with DMSO-HL60. $\#p<0.05$ compared with H_2O_2 (–) of the corresponding cells.

Activities of anti-oxidative enzymes and MPO

We measured activities of catalase, SOD-like, GPx and GR as anti-oxidative enzymes in undifferentiated cells, DMSO-HL60 and DMSO-HL60/DHEA after 5 days differentiation. DMSO-HL60/DHEA showed significantly higher catalase activity than DMSO-HL60. Both cells increased catalase activity after 5 days differentiation (Figure 7A). No significant differences were found in SOD-like activity or GPx activity among undifferentiated cells, DMSO-HL60 and DMSO-HL60/DHEA (Figure 7B and C). DMSO-HL60/DHEA showed significantly higher GR activity than DMSO-HL60. Both cells increased GR activity after 5 days differentiation (Figure 7D). DMSO-HL60 showed significantly lower MPO activity than undifferentiated cells. On the other hand, DMSO-HL60/DHEA showed similar MPO activity to undifferentiated cells. DMSO-HL60/DHEA showed significantly higher MPO activity than DMSO-HL60 (Figure 7E).

mRNA levels and protein amounts of NADPH oxidase components

We compared the mRNA levels of components of NADPH oxidase; $p47^{phox}$, $p67^{phox}$, $p22^{phox}$, and $gp91^{phox}$ among DMSO-HL60 and DMSO-HL60/DHEA after 3 days differentiation and undifferentiated cells. DMSO-HL60/DHEA showed significantly higher $p47^{phox}$, $p67^{phox}$ and $gp91^{phox}$ mRNA levels than DMSO-HL60. Both cells significantly increased $p47^{phox}$, $p67^{phox}$ and $gp91^{phox}$ mRNA levels compared to undifferentiated cells.

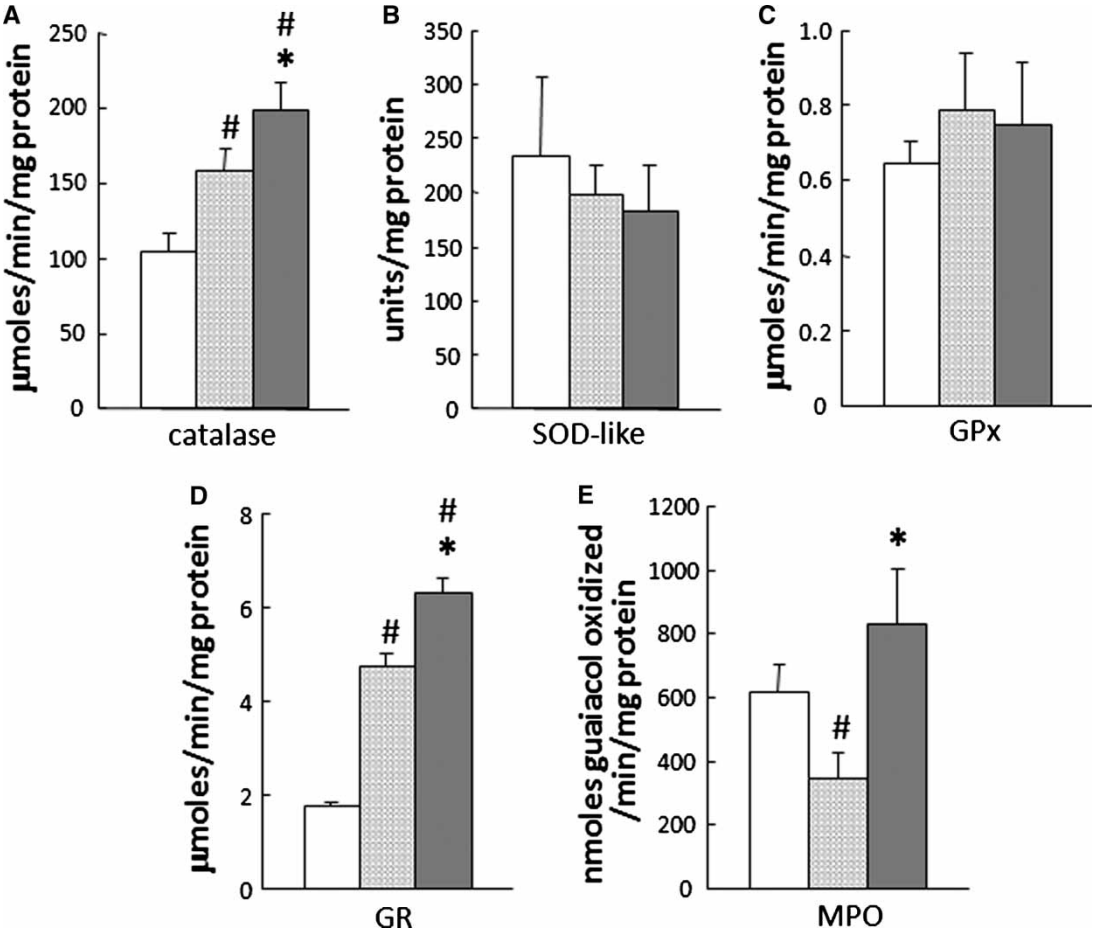

Figure 7. Activities of anti-oxidative enzymes and myeloperoxidase (MPO) in DMSO-HL60 (▨) and DMSO-HL60/DHEA (▬) at 5 days and undifferentiated cells (▭). Results for (A) Catalase, (B) SOD-like, (C) GPx, (D) GR and (E) MPO activity are shown. The data are presented as means+SD values from four-to-six independent experiments. *$p < 0.05$ compared with DMSO-HL60. #$p < 0.05$ compared with undifferentiated cells.

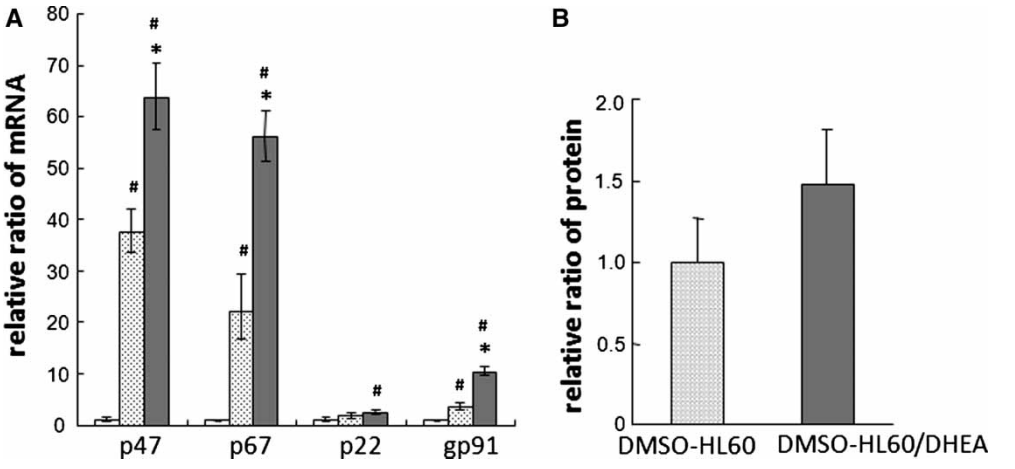

Figure 8. (A) The mRNA levels of components of NADPH oxidase; $p47^{phox}$, $p67^{phox}$, $p22^{phox}$ and $gp91^{phox}$ in DMSO-HL60 (▨) and DMSO-HL60/DHEA (▬) cells at 3 days and undifferentiated cells (▭). The relative values for the respective mRNAs of undifferentiated cells are set at 1.0. The data are presented as means+SD values from four-to-five independent experiments *$p < 0.05$ compared with DMSO-HL60. #$p < 0.05$ compared with undifferentiated cells. (B) The values of $p47^{phox}$ protein levels in DMSO-HL60 (▨) and DMSO-HL60/DHEA (▬) at 5 days are calculated by densitometric analysis. The value of DMSO-HL60 is set at 1.0. The data are presented as means+SD values from four independent experiments.

DMSO-HL60/DHEA showed similar $p22^{phox}$ mRNA levels to DMSO-HL60. However, DMSO-HL60/DHEA showed significantly higher $p22^{phox}$ mRNA levels than undifferentiated cells (Figure 8A). We measured $p47^{phox}$ protein amounts in DMSO-HL60 and DMSO-HL60/DHEA after 5 days differentiation. DMSO-HL60/DHEA showed higher $p47^{phox}$ amounts than DMSO-HL60, but the difference was not statistically significant ($p = 0.074$) (Figure 8B).

Discussion

DHEA has come to be widely taken as an anti-ageing supplement [4,17]. Biochemically, DHEA is a reversible inhibitor of G6PD; however, its effects on the expression of G6PD have not been investigated thoroughly. DHEA is reported to decrease NADPH through G6PD inhibition [6] and NADPH regulates cellular redox state [7–11]. Thus, the effect of DHEA on redox state should be comprehensively studied, however, it has not been studied at cellular level either. In the present study, because G6PD activity [19] and $O_2^{\bullet -}$ production [20] are increased in HL60 during differentiation, we sought to reveal the effect of DHEA on the behaviour of G6PD as well as on the redox state of HL60 during differentiation.

As shown in Figure 2, co-addition of DHEA suppressed $O_2^{\bullet -}$ production in DMSO-HL60 after PMA stimulation. Unexpectedly, our study also showed that DMSO-differentiated HL60 in the presence of DHEA (DMSO-HL60/DHEA) stimulated with PMA in the absence of DHEA produced a significantly higher amount of $O_2^{\bullet -}$ than DMSO-HL60 (Figure 2). To elucidate the underlying mechanism, we examined the effects of DHEA on the expression of G6PD. During differentiation, the mRNA level and activity of G6PD in HL60 increased and the increases corresponded well with the increased production of $O_2^{\bullet -}$ (Figures 2 and 3A and 4A). DHEA further increased G6PD mRNA, protein and activity, indicating that DHEA promoted the expression of G6PD, which gave rise to the increased production of $O_2^{\bullet -}$. To our knowledge, this is the first report to describe the effect of DHEA on G6PD expression. DHEA increased the expression of G6PD, although the exact mechanism is not known. DHEA increased expression of all components of NADPH oxidase except $p22^{phox}$. $p47^{phox}$ is the most important component of NADPH oxidase for $O_2^{\bullet -}$ production [36]. DHEA significantly increased $p47^{phox}$ mRNA level. $p47^{phox}$ protein level of DMSO-HL60/DHEA was higher, but not significantly, than DMSO-HL60. Between DMSO-HL60 and DMSO-HL60/DHEA, the increased rates of mRNA and protein of $p47^{phox}$ were relatively smaller compared with G6PD (Figure 3C, 4A, 8A and B). We also determined the amounts of NADPH and the ratio of NADP/NADPH, however, we found no difference between DMSO-HL60/DHEA and DMSO-HL60 (data not shown). Besides results from DMSO-HL60 and DMSO-HL60/DHEA at 5 days differentiation showed that G6PD activity significantly correlated to $O_2^{\bullet -}$ production (Figure 3D). Therefore, we concluded that increased $O_2^{\bullet -}$ production is mostly due to increased G6PD expression.

We then examined whether DHEA modulates the state of oxidative stress in HL60 during differentiation by comparing DMSO-HL60/DHEA and DMSO-HL60 for DNA damage, assessed by measuring 8-oxo-dG levels, which are typically associated oxidative DNA damage [20–23,37–39]. Without PMA stimulation, we found no difference in 8-oxo-dG levels between the two sites of cells. With PMA-stimulation, however, DMSO-HL60/DHEA had higher 8-oxo-dG levels than DMSO-HL60 (Figure 5). We also investigated whether DHEA increased sensitivity to ROS by decreasing the activity of anti-oxidative enzymes. We found that DHEA increased activity of catalase, an enzyme decomposing H_2O_2 to H_2O, but that DHEA had no effects on SOD-like nor GPx activities (Figure 7A, B and C). In DMSO-HL60 8-oxo-dG production has been reported to be closely correlated to $O_2^{\bullet -}$ production [20,22]. These results suggest that, rather than being due to alteration of anti-oxidative enzymes activities, increased oxidative DNA damage results from increased $O_2^{\bullet -}$ production mostly via increased G6PD expression. The increased catalase activity coincided with the increased expression of catalase (Figure 4C). However, further study is required to clarify why only catalase activity was increased among anti-oxidative enzymes.

NADPH is required both for the production of $O_2^{\bullet -}$ and the reduction of GSSG. So next we investigated whether DHEA modulates GSH/GSSG, an indicator of cellular redox state [11,40]. No differences in GSH/GSSG were found when plain DMSO-HL60/DHEA and DMSO-HL60 were used. After addition of H_2O_2, however GSH/GSSG was significantly lower in DMSO-HL60/DHEA than in DMSO-HL60 (Figure 6). Evaluating GR activity, which reduces GSSG to GSH [11], we were surprised to find higher GR activity in DMSO-HL60/DHEA than in DMSO-HL60 (Figure 7D).

Hoping to account for this interesting finding, we then evaluated the activity of MPO, which converts H_2O_2 to HClO, and consequently decreases GSH/GSSG [41,42]. During differentiation, the activity of MPO has been reported to decrease in both HL60 cells and in neutrophil of human peripheral blood [43,44]. Our DMSO-HL60 samples showed significantly lower MPO activity than undifferentiated cells. Meanwhile, DMSO-HL60/DHEA showed no decreases in MPO activity and significantly higher MPO activity than DMSO-HL60 (Figure 7E).

Increased MPO activity may cause the decreased GSH/GSSG in DMSO-HL60/DHEA after H_2O_2 addition. This finding, together with the increased production of $O_2^{\bullet -}$ and 8-oxo-dG, indicates that DHEA plays a role as a pro-oxidant rather than an antioxidant.

To investigate DHEA effects on HL60, we used 100 μM concentration, 7–9-times greater than the DHEA-S concentrations found in healthy young people [1,2]. Even so, people taking DHEA have presented plasma DHEA-S levels of 65–80 μM [45,46]. Thus, the concentration used in this experiment could occur *in vivo* in humans. While oxidative stress may be suppressed while DHEA is taken, after cessation of DHEA, oxidative stress may increase.

In vivo, DHEA is known to affect mRNA levels in genes related to fatty acid and cholesterol synthesis [47,48]. We found that DHEA decreased the expression of FAS and ACC1 (Figure 4B), which are related to fatty acid synthesis [47]; because cells require more fatty acids to proliferate, the reduction may be related to DHEA suppression of cell proliferation (Figure 1) [49,50]. Inhibition of G6PD by DHEA is reported to decrease R5P [51]. R5P is converted to xylulose-5-phosphate (X5P) and X5P can modulate carbohydrate-responsive element binding protein (ChREBP), a transcriptional factor involved in FAS and ACC1 expression [52,53]. Thus, the reduction of FAS and ACC1 expression may be due to the probable decrease in X5P. DHEA more than simply inhibits G6PD it also affects expression of several genes through its capability of altering transcriptional factors, such as ChPEBP regulated by X5P.

In conclusion, by itself, DHEA inhibits G6PD and suppressed $O_2^{\bullet -}$ production. Thus, DHEA is widely advocated as a supplement to reduce oxidative stress. However, DHEA increased the expression of G6PD as well as components of NADPH oxidase and made cells acquire a higher ability to produce ROS. Furthermore, after removing DHEA and the assaults of ROS, cells that had been differentiated in the presence of DHEA showed higher oxidative damage than those differentiated in its absence. To determine how DHEA should be used as a supplement, more comprehensive studies on the effects, mechanism and safety of DHEA are required.

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