Table 2 Profile before and after switch therapy in 32 patients who continued switch therapy for 4 months

Category and variables	At baseline	At end	*P*-value
Weight (kg)	60.5 ± 10.7	60.5 ± 11.2	0.94
A1C (%)	6.7 ± 1.3	5.9 ± 0.7	$P < 0.005$
Systolic blood pressure (mmHg)	122.3 ± 13.7	126.7 ± 15.8	0.16
Diastolic blood pressure (mmHg)	68.9 ± 1.0	69.3 ± 8.9	0.87
Triglyceride (mg/dL)	132.8 ± 6.9	132.5 ± 64.1	0.93
Creatinine (mg/dL)	1.0 ± 1.3	1.1 ± 0.3	0.08
Albumin (g/dL)	4.2 ± 0.4	4.3 ± 0.3	0.21
Blood urea nitrogen (mg/dL)	18.0 ± 6.6	19.0 ± 6.8	0.51
Total cholesterol (mg/dL)	180.8 ± 33.9	191.7 ± 34.4	0.19
HDL cholesterol (mg/dL)	45.1 ± 13.0	49.8 ± 17.1	0.06
LDL cholesterol (mg/dL)	107.1 ± 29.5	111.3 ± 25.9	0.53
Hematocrit	38.2 ± 5.2	36.9 ± 4.2	$P < 0.05$
AST (IU/L)	21.0 ± 5.7	22.0 ± 12.4	0.69
ALT (IU/L)	19.6 ± 11.6	18.4 ± 10.1	0.61

Data are mean ± standard deviation (SD). A1c, hemoglobin A1c; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 3 Baseline characteristics of subjects who succeeded and failed in the study

	Succeeded	Failed	*P*
n	30	6	NA
Male/female	14/16	3/3	NA
Age years	69.6 ± 11.4	58.7 ± 5.1	0.03*
BMI (kg/m^2)	24.1 ± 3.2	25.1 ± 6.2	0.53
Weight (kg)	60.1 ± 9.7	64.5 ± 14.4	0.37
Diabetes duration (years)	14.5 ± 11.9	22 ± 8.7	0.16
Insulin duration (years)	5.9 ± 8.2	7.8 ± 8.0	0.60
Insulin dosage (U/24 h)	25.7 ± 11.4	37.3 ± 9.8	0.03*
Insulin dosage (U/kg per 24 h)	0.42 ± 0.17	0.59 ± 0.10	0.048*
Fasting plasma glucose (mmol/L)	7.2 ± 2.0	6.5 ± 0.8	0.45
HbA1c (%)	6.7 ± 1.3	7.2 ± 1.0	0.43
Fasting IRI (μU/mL)	22.2 ± 21.3	29.7 ± 30.7	0.48
Fasting plasma C-peptide (ng/mL)	1.9 ± 0.8	2.1 ± 0.8	0.46
Urinary C-peptide excretion (μg/24 h)	49.5 ± 28.0	43.8 ± 19.9	0.65

Data are mean ± standard deviation (SD). BMI, body mass index; IRI, immunoreactive insulin; LDL, low-density lipoprotein; NA, not applicable.

The average post-prandial maximum blood glucose level was 12.2 ± 3.0 mmol/L on the day of the switch, ranging 5.7–19.0 mmol/L. No patient had a blood glucose level of more than 22.2 mmol/L on the switch day. No episode of severe hypoglycemia was encountered during the 4-month period after the switch.

In two cases, voglibose administration was suspended because of abdominal complications consisting of severe diarrhea and epigastric fullness. No patient suffered from either stroke or myocardial infarction during the 4 months after the switch.

To determine whether there was a difference in parameters between patients who succeeded and failed in the study, the data were assessed in those who achieved HbA1c of less than 7% (Table 3, "Succeeded") and those who did not (Table 3, "Failed"). Age was significantly younger in the failed subjects ($P < 0.05$). The insulin dose in both U/24 h and U/kg per 24 h, and the maximum blood glucose on the day of insulin switch were significantly higher in the failed patients ($P < 0.05$). No other parameters were statistically significantly different.

The data only including those who failed in the study excluding dropouts were also compared with those of patients who succeeded (data not shown). Although the age and insulin dose in U/kg per 24 h were not

statistically different, the insulin dosage in U/24 h and maximum blood glucose on the switch day were again statistically significantly higher ($P < 0.05$) in those who failed in the study.

Discussion

The present study showed that combination therapy comprising pioglitazone, glimepiride and voglibose efficiently and safely substituted insulin injection therapy among patients including a significant number ($n = 26$, aged >60 years) of elderly patients. Thirty out of 36 enrolled patients (83%) were successfully switched from insulin therapy to oral agent treatment. In 32 patients who continued the switch therapy for 4 months, blood sugar control was improved after the switch. The mean HbA1c value was significantly reduced from 6.7% to 5.9% after the switch in these 32 patients. The safety of this switch procedure was ensured by the fact that the maximum blood glucose level was less than 22.2 mmol/L on the day of insulin cessation and switch therapy initiation. No major side-effects were observed. Of the patients aged more than 60 years, 24 out of 26 patients (92%) were successfully switched from insulin therapy to oral agent treatment.

Because the proposed switch therapy was not a generally accepted approach and a significant number of elderly patients were included in the study, in order to ensure careful monitoring of blood glucose and to take immediate actions against severe hypoglycemia, all the enrolled patients were hospitalized. Fortunately, no patients showed severe hypoglycemia.

The UKPDS and Kumamoto studies showed that most patients on insulin therapy were treated at an insulin dose of 0.4–0.5 U/kg bodyweight per 24 h.[1,4] A recent survey conducted by the Japan Diabetes Clinical Data Management Study Group indicated that the average total daily dose of insulin for patients with type 2 diabetes was 26.3 U/24 h in Japan.[15] Because the average insulin dose was 0.43 U/kg bodyweight per 24 h and 25.7 U/24 h in the subjects who succeeded with switch therapy, the present approach may potentially cover the vast majority of patients under insulin injection therapy for possible oral agent switch in Japan and maybe also in other countries, if the inclusion criteria are met.

Recently, it has been reported that addition of pioglitazone to insulin therapy resulted in successful termination of insulin therapy in only a small proportion, 9%, of patients.[16] Concomitant use of pioglitazone with insulin injection as an add-on strategy may not efficiently reduce the insulin dosage. Rather, the abrupt cessation of insulin injection as shown in this study may bring about full activation of pioglitazone to improve insulin resistance, enabling the termination of insulin injection therapy.

The present study did not show any positive effect on the lipid profile or blood pressure control or any negative effect on bodyweight gain. As insulin has similar effects on lipid profile[17,18] and bodyweight control[19] compared to pioglitazone, the present observation does not contradict the reported effects of pioglitazone.[20] It is also possible that the observation period was too short to see any beneficial effects on lipid profile and blood pressure. Especially, the HDL cholesterol value might be significantly improved by switch therapy over a longer period of time, as its value showed a borderline ($P = 0.06$) increase after the switch (Table 2). Hematocrit was significantly reduced by 3.4% after the switch, consistent with the previous study.[20]

Assessment of data in the subjects who achieved and failed to achieve HbA1c of less than 7% suggested that the total insulin dose and maximum blood glucose on the switch day were significantly different, suggesting that these parameters determine the likelihood of success of switch therapy.

The high success rate of switching from insulin treatment to oral agent therapy is supposed to be mainly due to the pioglitazone- and possibly glimepiride-mediated improvement of insulin resistance. Even among those with the severely reduced amount of urine CPR of less than 20 μg/day (17 μg/day on average, $n = 6$), the success rate of switching was still high (83%, five patients out of six). It is reasonable to assume that patients treated with insulin show reduction of intrinsic insulin secretion and therefore the value of urine CPR under the condition of insulin treatment is smaller than that under a normal condition.

Pioglitazone may induce a beneficial effect on atherosclerosis[21,22] in contrast to insulin therapy, because treatment that improved insulin resistance reduced the recurrence of acute coronary syndrome more effectively than insulin upregulation therapy.[23] Hyperinsulinemia has been reported to be an independent risk factor for macrovascular disease.[24] Although intensive glucose-lowering therapy comprising insulin injection did not show any preventive effect on stroke,[1] pioglitazone significantly reduced the recurrence of stroke by 47%.[25] It is also possible that pioglitazone contributes to longevity by increasing the blood concentration of adiponectin.[26]

Several studies have identified hyperinsulinemia as a risk factor for accelerated cognitive decline and dementia.[27–29] Hyperinsulinemia may hinder the degradation of amyloid β peptide, whose accumulation in the brain is the main pathogenetic mechanism of Alzheimer's disease, by competition for the degradation enzyme common to insulin and amyloid β peptide.[30] It was reported that the risk of dementia is highest in people with diabetes treated with insulin.[31–33]

From the perspective of switching from insulin treatment to oral agent treatment especially in elderly

patients, it is prudent to carefully consider the suitability of the patient enrollment conditions described as inclusion criteria. Whether insulin dosage of 10 units or more/24 h, insulin injection duration of more than 3 months, and C-peptide in 24-h urine of more than 10 μg would be sufficient, or the state of blood sugar control including HbA1c of less than 10% should be additionally taken into account, is a matter of future study.

The Japan Diabetes Clinical Data Management Study Group reported that HbA1c value was higher in the insulin treatment group compared to that in the oral agent alone treatment group ($7.5 \pm 1.4\%$ vs $7.2 \pm 1.2\%$),[15] indicating that those who can be kept on oral agent therapy show better blood sugar control than those on insulin treatment. The proposed switch method may benefit a significant number of patients, especially elderly patients, because the results clearly showed a high success rate (92%) of switching from insulin therapy to oral agent therapy and the significant improvement of blood sugar control (reduction of HbA1c value from 6.5% to 5.8%, $P < 0.01$) 4 months after the initiation of the switch therapy in elderly patients. This approach may improve the blood sugar control of elderly patients with diabetes and contribute to a reduction of the overall cost of medical care.

Study limitation

The study design did not include a control arm. It is possible that enrollment into the study itself may have had a significant impact to improve lifestyle, leading to a positive effect on blood sugar control. A randomized controlled study is needed for accurate analysis of the efficacy of the present approach.

Acknowledgments

We are indebted to the 38 participants in this study whose cooperation made it possible. The authors thank Jean-Francois Jasmin PhD for assistance in the preparation of this manuscript. This work was supported by funding from Okhotsk-kai hospital.

References

1 UK Prospective Diabetes Study Group. Intensive blood-glucose control with sulphonylurea or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS33). *Lancet* 1998; **352**: 837–853.
2 Warren R, Frier B. Hypoglycaemia and cognitive function. *Diabetes Obes Metab* 2005; 7: 493–503.
3 Kanda Y, Yamada K, Harada Y *et al.* Clinical parameters for predicting possibility of insulin therapy withdrawal in patients with type 2 diabetes mellitus. *J Jpn Diabetes Soc* 2004; **47**: 271–275.
4 Shichiri M, Ohkubo Y, Kishikawa H *et al.* Long-term results of the Kumamoto study on optimal diabetes control in type 2 diabetic patients. *Diabetes Care* 2000; **23** (Suppl. 2): B21–B29.
5 Yki-Jarvinen H. Drug therapy: thiazolidinediones. *N Engl J Med* 2004; **35**: 1106–1118.
6 Kubota N, Terauchi Y, Kubota T *et al.* Pioglitazone ameliorates insulin resistance and diabetes by both adiponectin-dependent and -independent pathways. *J Biol Chem* 2006; **281**: 8748–8755.
7 DeFronzo R. Pharmacologic therapy for type 2 diabetes mellitus. *Ann Int Med* 1999; **131**: 281–303.
8 Dormandy J, Charbonnel B, Eckland D *et al.* Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial in macroVascular events): a randomized controlled trial. *Lancet* 2005; **366**: 1279–1289.
9 Koshiba K, Nomura M, Nakaya Y *et al.* Efficacy of glimepiride on insulin resistance, adipocytokines, and atherosclerosis. *J Med Invest* 2006; **53**: 87–94.
10 Marti S, Kolb H, Beuth J *et al.* Changes in patients' body weight after 12 months of treatment with glimepiride or glibenclamide in type 2 diabetes: a multicentre retrospective cohort study. *Diabetologia* 2003; **46**: 1611–1617.
11 Inoue K, Ikegami H, Fujisawa T *et al.* Less frequent body weight gain in elderly type 2 diabetic patients treated with glimepiride. *Geriatr Gerontol Int* 2003; **3**: 56–59.
12 Saito N, Sakai H, Suzuki S *et al.* Effect of an alpha-glucosidase inhibitor (voglibose), in combination with sulphonylureas, on glycaemic control in type 2 diabetes patients. *J Int Med Res* 1998; **26**: 219–232.
13 Ishida H, Kato S, Nishimura M *et al.* Beneficial effect of long-term combined treatment with voglibose and pioglitazone on pancreatic islet function of genetically diabetic GK rats. *Horm Metab Res* 1998; **30**: 673–678.
14 The Expert Committee on the Diagnosis and Classification of Diabetes Melittus. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 1997; **20**: 1183–1197.
15 Kobayashi M, Yamazaki K, Hirano K *et al.* The status of diabetes control and antidiabetic drug therapy in Japan-A cross-sectional survey of 17,000 patients with diabetes mellitus (JDDM1). *Diabetes Res Clin Pract* 2006; **73**: 198–204.
16 Schmitz O, Charbonnel B, Scheen A. Pioglitazone reduces insulin requirements and improves glycemic control in insulin-treated patients with type 2 diabetes: results from PROactive. Presented as a poster in the European Diabetes Association Conference in 2006.
17 Stern P, Haffner S. Dyslipidemia in type II diabetes. *Diabets Care* 1991; **14**: 1144–1159.
18 Agardh C, Nilsson-Ehle P, Schersten B. Improvement of the plasma lipoprotein pattern after institution of insulin treatment in diabetes mellitus. *Diabetes Care* 1982; **5**: 322–325.
19 Cheng A, Zinman B. Principles of insulin therapy. In: Kahn CR, Weir GC, King GL *et al.*, eds. *Joslin's Diabetes Mellitus*, 14th edn. Philadelphia, PA: Lippincott Williams & Wilkins, 2005; 659–669.
20 Waugh J, Keating G, Plosker G *et al.* Pioglitazone: a review of its use in type 2 diabetes mellitus. *Drugs* 2006; **66**: 85–109.
21 Langenfeld M, Forst T, Hohberg C *et al.* Pioglitazone decreases carotid intima-media thickness independently of glycemic control in patients with type 2 diabetes mellitus. *Circulation* 2005; **111**: 2525–2531.

22 Watanabe I, Tani S, Anazawa T *et al.* Pioglitazone on artheriosclerosis in comparison with that of glibencamide. *Diabetes Res Clin Pract* 2005; **68**: 104–110.
23 McGuire D, Newby L, Bhapkar M *et al.* Association of diabetes mellitus and glycemic control strategies with clinical outcomes after acute coronary syndromes. *Am Heart J* 2004; **147**: 246–252.
24 Despres J, Lamarche B, Mauriege P *et al.* Hyperinsulinemia as an independent risk factor for ischemic heart disease. *N Engl J Med* 1996; **334**: 952–957.
25 Wilcox R, Bousser M, Betteridge D *et al.* Effects of pioglitazone in patients with type 2 diabetes with or without previous stroke: results from PROactive (PROspective pioglitAzone Clinical Trial In macroVascular Events 04). *Stroke* 2007; **38**: 865–873.
26 Arai Y, Nakazawa S, Kojima T *et al.* High adiponectin concentration and its role for longevity in female centenarians. *Geriatr Gerontol Int* 2006; **6**: 32–39.
27 Kalmijn S, Feskens E, Launer L *et al.* Glucose intolerance, hyperinsulinaemia and cognitive function in a general population of elderly men. *Diabetologia* 1995; **38**: 1096–1102.
28 Luchsinger J, Tang M, Shea S *et al.* Hyperinsulinemia and risk of Alzheimer disease. *Neurology* 2004; **63**: 1187–1192.
29 Kuusisto J, Koivisto K, Mykkanen L *et al.* Association between features of the insulin resistance syndrome and Alzheimer's disease independently of apolipoprotein E4 phenotype: cross sectional population based study. *BMJ* 1997; **315**: 1045–1049.
30 Biessels G, Staekenborg S, Brunner E *et al.* Risk of dementia in diabetes mellitus: a systematic review. *Lancet Neurol* 2006; **5**: 64–74.
31 Ott A, Stolk R, van Harskamp F *et al.* Diabetes mellitus and the risk of dementia: the Rotterdam Study. *Neurology* 1999; **53**: 1937–1942.
32 Curb J, Rodriguez B, Abbott R *et al.* Longitudinal association of vascular and Alzheimer's dementias, diabetes, and glucose tolerance. *Neurology* 1999; **52**: 971–975.
33 Luchsinger J, Tang M, Stern Y *et al.* Diabetes mellitus and risk of Alzheimer's disease and dementia with stroke in a multiethnic cohort. *Am J Epidemiol* 2001; **154**: 635–641.

Free Radical Research, November–December 2008; 42(11–12): 957–965

informa
healthcare

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

TOMOHITO HIRAO[1], YOSHISHIGE URATA[1], KAN KAGEYAMA[1], MIDORI IKEZAKI[1], MIHO KAWAKATSU[1], MICHIKO MATSUSE[2], TAKAYUKI MATSUO[3], MASAHIRO AKISHITA[4], IZUMI NAGATA[3], & TAKAHITO KONDO[1]

[1]*Department of Biochemistry and Molecular Biology in Disease, and* [2]*Department of Molecular Medicine, Atomic Bomb Disease Institute,* [3]*Department of Neurosurgery, Nagasaki University Graduate School of Biomedical Sciences, 1-2-14 Sakamoto Nagasaki 852-8523 Japan, and* [4]*Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

Accepted by Professor N. Taniguchi

(Received 28 September 2008; revised 17 October 2008; accepted 17 October 2008)

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].

Correspondence: Takahito Kondo, Department of Biochemistry and Molecular Biology in Disease, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-2-14 Sakamoto Nagasaki 852-8523 Japan. Tel: +81-95-819-7097. Fax: +81-95-819-7100. Email: kondo@net.nagasaki-u.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760802566582

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 γ-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 γ-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 γ-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 γ-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 0.3×10^5 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 γ-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

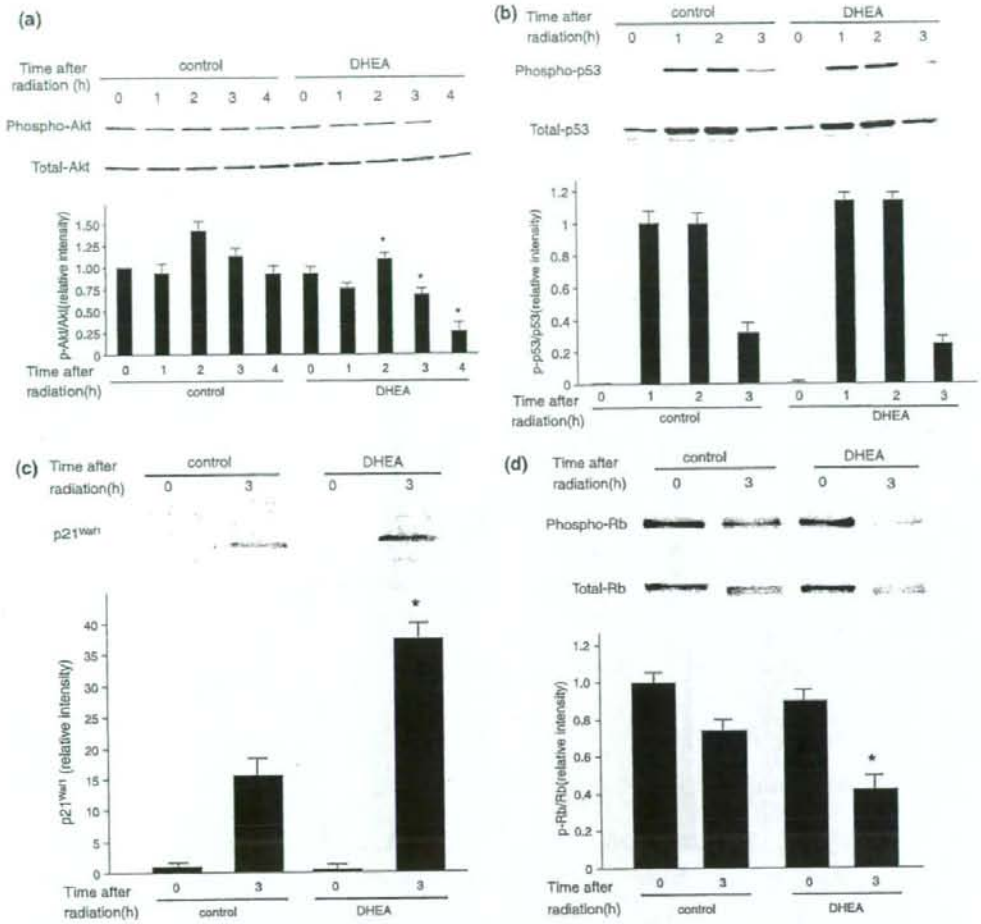

Figure 1. DHEA down-regulates the γ-ray-induced phosphorylation of Akt. H4 cells were serum-starved for 24 h. After prior treatment with 200 nM DHEA for 18 h, the cells were treated with 3-Gy of γ-rays. (A) Representative Western blot for the phosphorylation of Akt. The phosphorylation of Akt was estimated 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 γ-ray irradiation

H4 cells cultured with or without 200 nM DHEA for 18 h were irradiated with 3-Gy of γ-rays. The γ-ray-induced apoptosis was estimated by the TUNEL assay (Figure 2A). DHEA increased the γ-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 γ-ray-induced G_2/M checkpoint arrest by 1.3-fold compared to the control (right). The data indicate that DHEA promotes γ-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

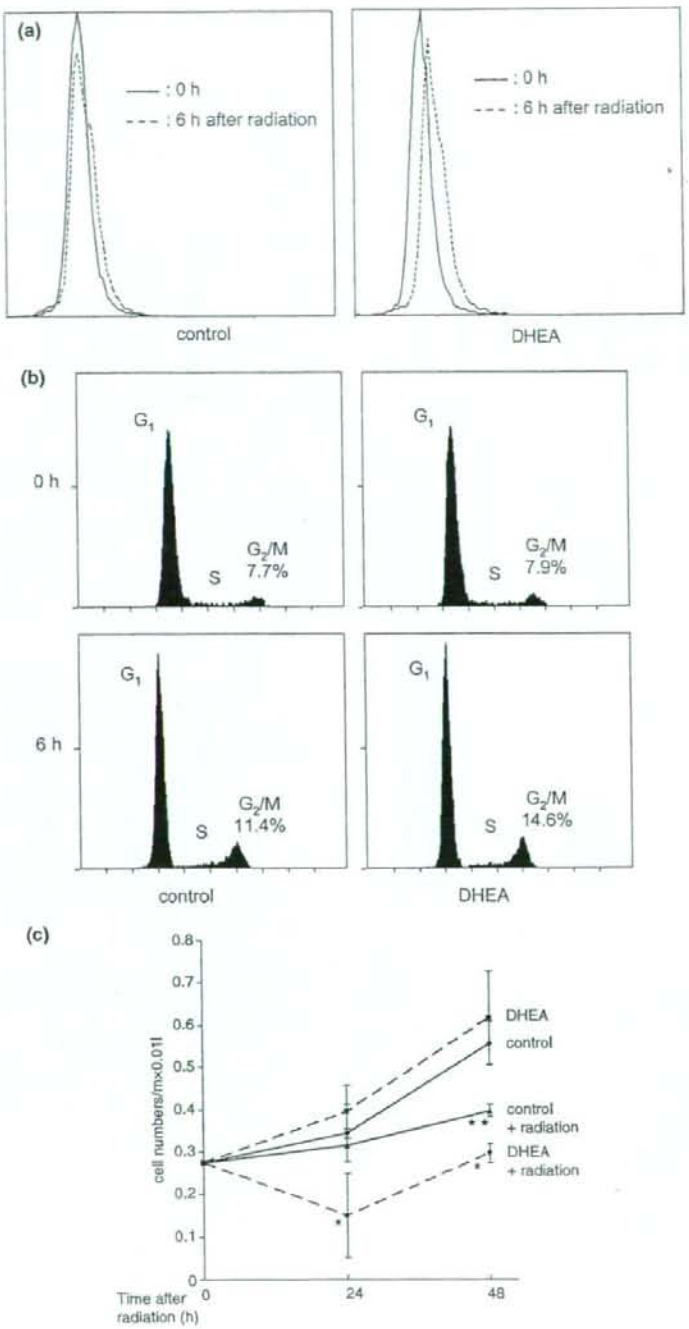

Figure 2 (*Continued*)

Figure 2. DHEA induces apoptosis and G_2/M arrest in response to γ-rays. The effect of DHEA on the γ-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 γ-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 γ-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.

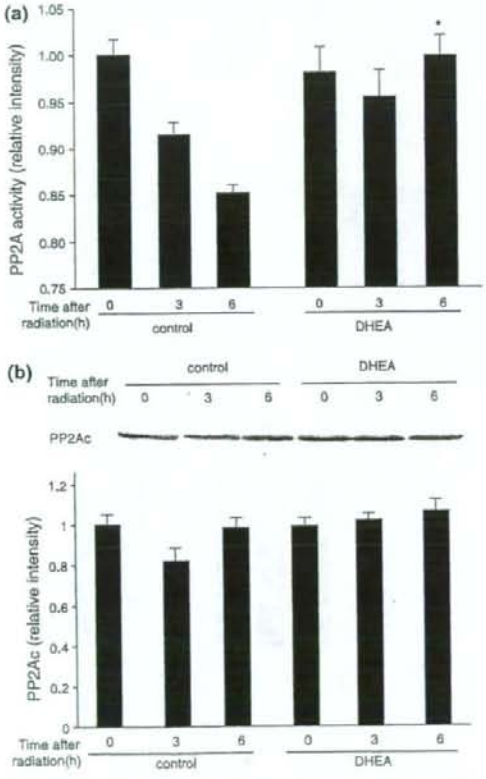

Figure 3. DHEA maintains the activity of PP2A. The effect of DHEA on the activity of PP2A was estimated as described in Materials and methods. (A) The effect of γ-rays on PP2A activity was estimated in cells previously treated with DHEA for 18 h. (B) Changes in the levels of PP2Ac protein were estimated 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 γ-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 γ-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 γ-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 γ-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 γ-ray-induced decrease of GSH and increase of GSSG in 6 h, to maintain the GSH/GSSG ratio. Then, the expression of γ-GCS was estimated by RT-PCR. DHEA increased expression of the γ-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 γ-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 γ-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.

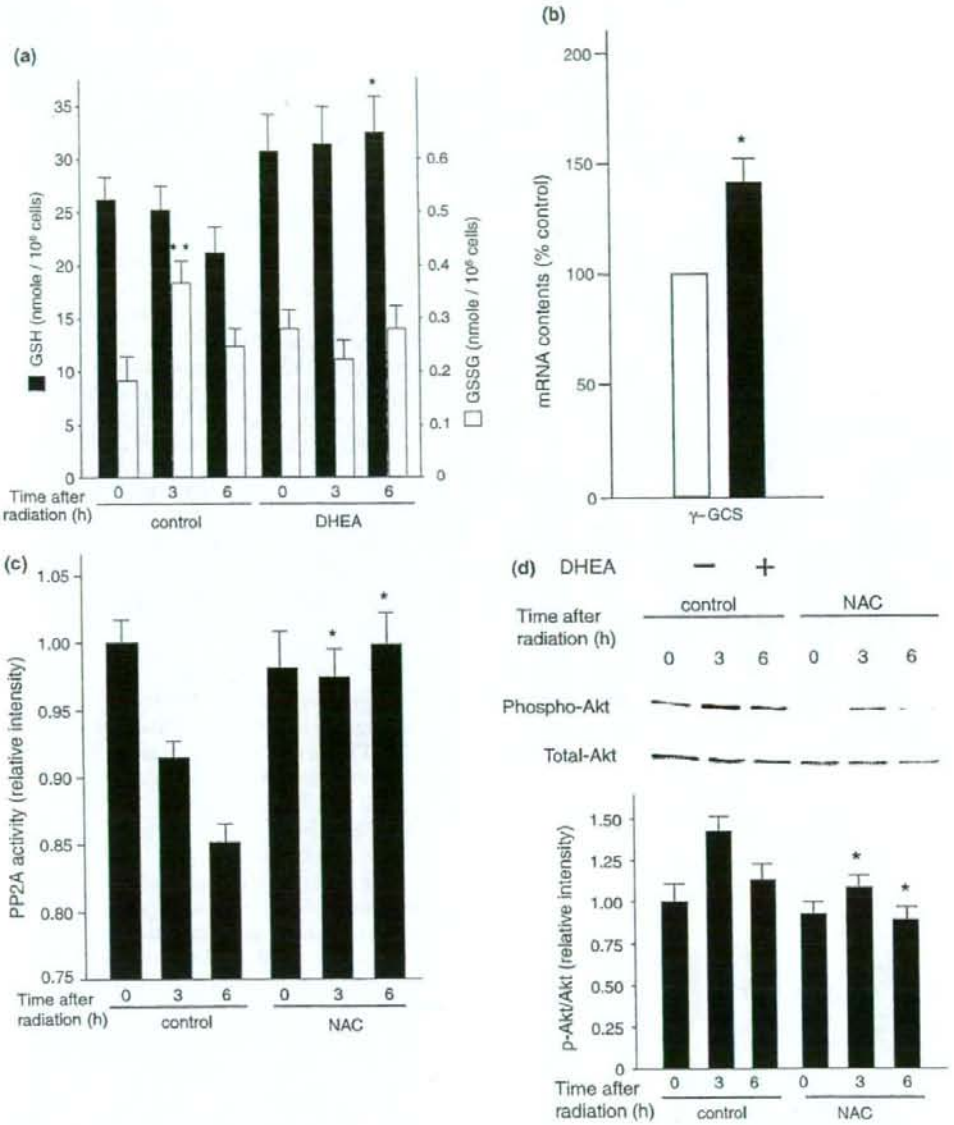

Figure 4. DHEA induces the expression of γ-GCS mRNA and increases the levels of GSH. The effect of DHEA on the expression of γ-GCS mRNA and the level of GSH was estimated. (A) H4 cells were treated with 200 nM DHEA for 18 h and the gene expression of the γ-GCS heavy sub-unit was analysed by quantitative 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 γ-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 γ-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 γ-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 γ-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 γ-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 γ-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 γ-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 γ-GCS by DHEA (Figure 4). However, the molecular mechanism by which the expression of γ-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.

Acknowledgements

This work was supported in part by grants-in-aid for scientific research from the Ministry of Health, Labor, and Welfare of Japan (H17-Choju-017), and through the Groval Center of Excellence program from the Ministry of Education, Science, Sports, Culture, and Technology of Japan. We thank Hiromi Setoguchi for technical assistance.

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

References

[1] Williams JR. The effects of dehydroepiandrosterone on carcinogenesis, obesity, the immune system, and aging. Lipids 2000;35:325–331.

[2] Shikaitis A, Green A, Punj V, Steele V, Lubet R, Cristov K. Dehydroepiandrosterone inhibits the progression phase of mammary carcinogenesis by inducing cellular senescence via a p16-dependent but p53-independent mechanism. Breast Cancer Res 2005;7:R1132–R1140.

[3] Park E, Ahn GN, Lee NH, Kim JM, Yun JS, Hyun JW, Jeon YJ, Wie MB, Lee YJ, Park JW, Lee Y. Radioprotective properties of eckol against ionizing radiation in mice. FEBS Lett 2008;582:925–930.

[4] Zhivotovsky B, Joseph B, Orrenius S. Tumor radiosensitivity and apoptosis. Exp Cell Res 1999;248:10–17.

[5] Tribius S, Pidel A, Casper D. ATM protein expression correlates with radioresistance in primary glioblastoma cells in culture. Int J Radiat Oncol Biol Phys 2001;50:511–523.

[6] Matsuse M, Saenko V, Sedliarou I, Rogounovitch T, Nakazawa Y, Mitsutake N, Akulevich N, Namba H, Yamashita S. A novel role for thyroid hormone receptor beta in cellular radiosensitivity. J Radiat Res (Tokyo) 2008;49:17–27.

[7] Brazil DP, Hemmings BA. Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem Sci 2001;26:657–664.

[8] Park J, Jung H, Park S, Kang S, Yi M, Um H, Hong S. Combination of PTEN and γ-ionizing radiation enhances cell death and G_2/M arrest through regulation of AKT activity and $p21^{waf1}$ induction in non-small-cell lung cancer cells. Int J Radiat Oncol Biol Phys 2008;70:1552–1560.

[9] Muata H, Ihara Y, Nakmura H, Yodoi J, Sumikawa K, Kondo T. Glutaredoxin exerts an antiapoptotic effect by regulating the redox state of Akt. J Biol Chem 2003;278:50226–50233.
[10] Foley TD, Petro LA, Sterdny CM, Coppa TM. Oxidative inhibition of protein phosphatase 2A activity: role of catalytic subuinit disulfides. Neurochem Res 2007;32:1957–1964.
[11] Kim HS, Song MC, Kwak IH, Park TJ, Lim IK. Constitutive induction of p-Erk1/2 accompanied by reduced activities of protein phosphatases 1 and 2A and MKP3 due to reactive oxygen species during cellular senescence. J Biol Chem 2003;278:37497–37510.
[12] Okunaga T, Urata Y, Goto S, Matsuo T, Mizota S, Tsutsumi K, Nagata I, Kondo T, Ihara Y. Calreticulin, a molecular chaperone in the endoplasmic reticulum, modulates radiosensitivity of human glioblastoma U251MG cells. Cancer Res 2006;66:8662–8671.
[13] Quanungo S, Starke DW, Pai HV, Mieyal JJ, Nieminen A-L. Glutathione supplementation potentiates hypoxic apoptosis by S-glutathionylation of p65-NFκB. J Biol Chem 2006;282:18427–18436.
[14] Kanda M, Ihra Y, Murata H, Urata Y, Kono T, Yodoi J, Seto S, Yano K, Kondo T. Glutaredoxin modulates PDGF-dependent cell signaling by regulating the redox status of low molecular weight protein tyrosine phosphatase. J Biol Chem 2006;281:28518–28528.
[15] Shelton MD, Kern TS, Mieyal JJ. Glutaredoxin regulates nuclear factor kappa-B and intercellular adhesion molecule in Müller cells: model of diabetic retinopathy. J Biol Chem 2007;282:12467–12474.
[16] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.
[17] Urata Y, Ihara Y, Murata H, Goto S, Koji T, Yodoi J, Inoue S, Kondo T. 17β-Estradiol protects against oxidative stress-induced cell death through the glutathione/glutaredoxin-dependent redox regulation of Akt in myocardiac H9c2 cells. J Biol Chem 2006;281:13092–13102.
[18] Tan J, Hallahan DE. Growth factor-independent activation of protein kinase B contributes to the inherent resistance of vascular endothelium to radiation-induced apoptosis response. Cancer Res 2003;63:7663–7667.
[19] McKenna WG, Muschel RJ, Gupta AK, Hahn SM, Bernhard EJ. The Ras signal transduction pathway and its role in radiation sensitivity. Oncogene 2003;22:5866–5875.
[20] Kageyama K, Ihara Y, Goto S, Urata Y, Toda G, Yano K, Kondo T. Overexpression of calreticulin modulates protein kinase B/Akt signaling to promote apoptosis during cardiac differentiation of cardiomyoblast H9c2 cells. J Biol Chem 2002;277:19255–19264.
[21] Millward TA, Zolnierowicz S, Hemmings BA. Regulation of protein kinase cascades by protein phosphatase 2A. Trends Biochem Sci 1999;24:186–191.
[22] Junttila MR, Puustinen P, Niemelä M, Ahola R, Arnold H, Böttzauw T, Ala-aho R, Nielsen C, Ivaska J, Taya Y, Lu SL, Lin S, Chan EK, Wang XJ, Grènman R, Kast J, Kallunki T, Sears R. Kähäri VM, Westermarck J. CIP2A inhibits PP2A in human malignancies. Cell 2007;130:51–62.
[23] Rao RK, Clayton LW. Regulation of protein phosphatase 2A by hydrogen peroxide and glutathonylation. Biochem Biophys Res Commun 2002;293:610–616.
[24] Mallikarjuna G, Dhanalakshmi S, Singh RP, Agarwal C, Agarwal R. Silibinin protects against photocarcinogenesis via modulation of cell cycle regulators, mitogen-activatd protein kinases, and Akt signaling. Cancer Res 2004;64:6349–6356.
[25] Toillon RA, Magne N, Laïos I, Castadot P, Kinnaert E, Houtte V, Desmedt C, Leclercq G, Lacroix M. Estrogens decrease g-ray-induced senescence and maintain cell cycle progression in breast cancer cells independent of p53. Int J Radiat Oncol 2007;4:1187–1200.
[26] Lee SR, Yang KS, Kwon J, Lee C, Jeong W, Rhee SG. Reversible inactivation of the tumor suppressor PTEN by H_2O_2. J Biol Chem 2002;277:20336–20342.
[27] Huo YY, Li G, Duan RF, Gou Q, Fu CL, Hu YC, Song BQ, Yang ZH, Wu DC, Zhou PK. PTEN deletion leads to deregulation of antioxidants and increased oxidative damage in mouse embryonic fibroblasts. Free Radic Biol Med 2008;44:1578–1591.
[28] Eguchi T, Takaki T, Itadani H, Kotani H. RB silencing compromises the DNA damage-induced G_2/M checkpoint and causes deregulated expression of tyebECT2 oncogene. Oncogene 2007;26:509–520.
[29] Holmgren A. Thioredoxin and glutaredoxin systems. J Biol Chem 1989;264:13963–13966.
[30] Iwanaga M, Mori K, Iida T, Urata Y, Matsuo T, Yasunaga A, Shibata S, Kondo T. Nuclear factor kappa B dependent induction of gamma glutamylcysteine synthetase by ionizing radiation in T98G human glioblastoma cells. Free Radic Biol Med 1998;24:1256–1268.

This paper was first published online on iFirst on 28 November 2008.

European Journal of Pharmacology 589 (2008) 32–36

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Raloxifene analogue LY117018 suppresses oxidative stress-induced endothelial cell apoptosis through activation of ERK1/2 signaling pathway

Jing Yu [a,b], Masato Eto [a], Koichi Kozaki [a], Masahiro Akishita [a], Tetsuro Okabe [b], Yasuyoshi Ouchi [a,*]

[a] Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
[b] Department of Integrated Traditional Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

ARTICLE INFO

Article history:
Received 29 June 2007
Received in revised form 11 April 2008
Accepted 23 April 2008
Available online 5 May 2008

Keywords:
Endothelial cell
Apoptosis
Selective estrogen receptor modulator
Raloxifene
ERK1/2

ABSTRACT

A selective estrogen receptor modulator, raloxifene, has been shown to reduce cardiovascular events in relatively high-risk postmenopausal women with osteoporosis. However, the mechanisms by which raloxifene exerts a pharmacological effect on cardiovascular organs have not been fully elucidated. The present study was designed to examine whether the raloxifene analogue, 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(b) thien-3-yl-*p*-(2-(pyrrolidinyl)ethoxy phenyl ketone (LY117018), could inhibit apoptosis and to clarify the signaling pathway in vascular endothelial cells. LY117018 significantly inhibited hydrogen peroxide-induced apoptosis in bovine carotid artery endothelial cells. The anti-apoptotic effect of LY117018 was abolished by an estrogen receptor antagonist, 7α,7β-(9[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl) estra-1,3,5(10)-triene-3,17-diol (ICI 182,780). Mitogen-activated protein kinases (MAPK), including p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinase1/2 (ERK1/2), and Akt, have been shown to act as apoptotic or anti-apoptotic signals. Phosphorylation of p38, JNK, ERK1/2 and Akt was examined. LY117018 increased ERK1/2 phosphorylation but did not enhance the phosphorylation of p38, JNK, or Akt. The anti-apoptotic effect of LY117018 was prevented by treatment with 2-[2′-amino-3′-methoxyphenyl]-oxanaphthalen-4-one (PD98059), an upstream inhibitor of ERK1/2. LY117018 stimulated an increase in ERK1/2 phosphorylation, which was diminished by ICI 182,780. The activation of ERK/1/2 by LY117018 was not inhibited by the transcription inhibitor, actinomycin D. These results suggest that estrogen receptors and the ERK1/2 signaling pathway are involved in the anti-apoptotic action of LY117018 in vascular endothelial cells.

1. Introduction

The incidence of clinical coronary heart disease in premenopausal women is very low. However, following the menopause, atherogenic risk factors increase and the rate of clinical coronary events accelerates to the level observed in men (Kannel et al., 1976). This difference has been considered to be attributable to the protective effects of estrogen before the menopause (Clarkson, 2007). Recent randomized placebo-controlled trials of hormone replacement therapy, however, have not shown any benefit in either the secondary or the primary prevention of cardiovascular events (Hulley et al., 1998; Grady et al., 2002; Rossouw et al., 2002).

Much current interest is focused on the therapeutic potential of selective estrogen receptor modulators. Interestingly, drugs of this class show estrogen-antagonist effects in the mammary gland and uterus, while they have estrogen-agonist effects in bone and other tissues (Delmas et al., 1997; Grady et al., 2004; Johnell et al., 2004; Cox et al., 2004; Sporn et al., 2004). Thus, they are expected to overcome the adverse effects found with conventional hormone replacement therapy.

Recently, the MORE (Multiple Outcomes of Raloxifene Evaluation) study showed that a representative selective estrogen receptor modulator, raloxifene, significantly reduced cardiovascular events in relatively high-risk postmenopausal women with osteoporosis (Barrett-Connor et al., 2002). The death of endothelial and vascular smooth muscle cells is implicated in several pathological vascular conditions, such as atherosclerosis and aneurysm formation. Endothelial damage/dysfunction plays a central role in the clinical manifestation of coronary atherosclerosis (Ross, 1990; Ross, 1999). It has been reported that selective estrogen receptor modulators show a variety of direct actions on vascular cells via estrogen receptors (Simoncini et al., 1999; Simoncini et al., 2002). However, the effect of selective estrogen receptor modulators on endothelial apoptosis has not been clarified.

The aim of this study was to examine the effect of a raloxifene analogue, 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(b) thien-3-yl-*p*-(2-(pyrrolidinyl)ethoxy phenyl ketone (LY117018), on endothelial apoptosis and to clarify the mechanisms of action.

* Corresponding author. Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: +81 3 5800 8830; fax: +81 3 5800 6529.
E-mail address: youchi-tky@umin.ac.jp (Y. Ouchi).

0014-2999/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.ejphar.2008.04.052

2. Materials and methods

2.1. Chemicals and reagents

The raloxifene analogue LY117018 was provided by Eli-Lilly (Indianapolis, IN, USA). 1,3,5(10)-estariene-3,17β-diol (17β-estradiol), wortmannin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma (St. Louis, MO, USA). Phenol red-free Medium199 (M199) was from Gibco (NY, USA). 7α,7β-(9[(4,4,5,5,5-Pentafluoropentyl) sulfinyl]nonyl) estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) was purchased from AstraZeneca (Macclesfield, Cheshire, UK). Hydrogen peroxide (H_2O_2 30% solution) and actinomycin D were obtained from Wako (Osaka, Japan). The mitogen-activated protein/extracellular signal-regulated protein kinase (MEK)1 inhibitor, 2-[2′-amino-3′-methoxyphenyl]-oxanaphthalen-4-one (PD98059), and antibodies against Akt, phospho-Akt (Ser-473), c-Jun N-terminal kinase (JNK), phospho-JNK (Thr183/Tyr185), extracellular signal-regulated protein kinase1/2 (ERK1/2) and phospho-ERK1/2 (Thr202/Tyr204) were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against p38 (A-12) and phospho-p38 (D-8) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The JNK inhibitor anthrax [1, 9-cd] pyrazol-6(2H)-one (SP600125) and the p-38 inhibitor 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole (SB203580) were from Calbiochem (Darmstadt, Gemany). Fetal bovine serum (FBS) was from CCT (Sanko Junyaku Co., Ltd., Tokyo, Japan). Charcoal-stripped fetal bovine serum was from MultiSer (ThermoTrace Ltd., Melbourne, Australia). Nitrocellulose membranes were from Amersham (Buckinghamshire, UK). LumiGLO Reserve Chemiluminescent Substrate Kit was from KPL (Gaithersburg, MD, USA). Cell Death Detection ELISA ^{plus} was purchased from Roche (Mannheim, Germany).

2.2. Cell culture

Bovine carotid endothelial cells (BCEC) were provided by Dr. Sudoh and prepared as described previously (Sudoh et al., 2001; Akishita et al., 1998). Cells were cultured in a 37 °C humidified atmosphere of 95% air/5% CO_2 in DMEM containing 10% FBS and 100 units/ml penicillin/100 μg/ml streptomycin. For all experiments, BCEC were used at passages 5 to 7, and plated at a concentration of 10^4 cells/mL Raloxifene experiments were performed with phenol red-free M199. DMSO was used as a solvent for LY117018, 17β-estradiol, ICI 182,780 and PD98059. DMSO was present at equal concentrations (0.05%) in all groups, including the vehicle group.

2.3. Apoptosis induction

Apoptosis was induced by addition of hydrogen peroxide (H_2O_2). At 70–80% confluence, cells were washed with phosphate-buffered saline (PBS), and then replenished with phenol red-free M199 without serum, and proliferation was stopped. Cells were exposed to 100 μM H_2O_2 for 1 h after 6 h starvation, washed twice again with PBS (−), then replenished with phenol red-free M199 containing 5% DCC–FBS. In the same experiments, LY117018 or 17β-estradiol was added for 30 min before H_2O_2 stimulation in the apoptosis assay. In experiments on inhibitors, the inhibitors were added for 60 min before LY117018 addition. After 24 h of stimulation by H_2O_2, cell apoptosis was evaluated.

2.4. Assay of endothelial cell apoptosis (DNA fragmentation assay)

Cell apoptosis was quantified by means of DNA fragmentation, using a photometric enzyme-linked immunosorbent assay (Cell Death Detection ELISA ^{plus}) kit. Cells with each treatment were lysed in 300 μl lysis buffer, and a fraction of the supernatant was subjected to reaction for 2 h with the immunocomplex of anti-DNA conjugated with peroxidase, which binds to nucleosomal DNA, and antihistone–biotin, which interacts with streptavidin-coated wells in a microtiter plate. At the end of the incubation, substrate was added, and development was quantified at 405 nm wavelength.

2.5. Western blot analysis

After treatment with reagents, confluent monolayers of cells were washed two times in ice-cold phosphate-buffered saline and lysed with buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF. For Western blot analysis, total cell lysate was subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and proteins were transferred to a polyvinilidene difluoride (PVDF) membrane. The antibodies used in this study were anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-JNK (Thr183/Tyr185), anti-JNK, anti-phospho-p38, and anti-p38. Antibodies were detected by means of a horseradish peroxidase-linked secondary antibody. Immunoreactive bands were visualized using a LumiGLO Reserve Chemiluminescent Substrate Kit and quantified by densitometry in the linear range of NIH image 1.60.

2.6. Statistics

Values are expressed as means ± S.E.M. Statistical comparisons were performed by ANOVA followed by Fisher's protected least significance difference (PLSD) test. A probability value <0.05 was considered significant.

3. Results

3.1. Effect of LY117018 on endothelial cell apoptosis

On the basis of concentration- and time-response experiments (data not shown), H_2O_2 (100 μM) was added to BCEC for 1 h to induce apoptosis. BCEC apoptosis induced by H_2O_2 was significantly attenuated by treatment with LY117018 in a concentration-dependent manner (Fig. 1), while LY117018 per se did not show any effect on apoptosis (data not shown).

3.2. Involvement of MEK/ERK pathway in anti-apoptotic action of LY 110718

Phosphorylation levels of p38, JNK, ERK1/2, and Akt were examined because these kinases have been shown to regulate apoptosis (Xia et al.,

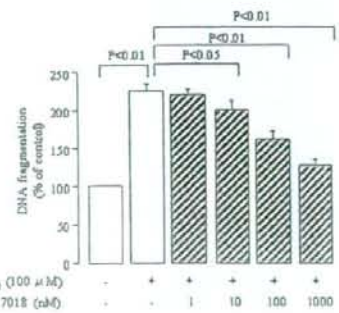

Fig. 1. Effect of LY117018 on H_2O_2-induced endothelial cells apoptosis. At 70–80% confluence, BCEC were starved and exposed to 100 μM H_2O_2 for 1 h as described in Materials and methods. Various concentrations of LY117018 (1 nM–1 μM) were added to the culture medium 30 min before H_2O_2 stimulation in the apoptosis assay. After a 24-h incubation, cell apoptosis was evaluated by means of DNA fragmentation (with a Cell Death Detection ELISA ^{plus} kit) as described in Materials and methods. Data are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$).

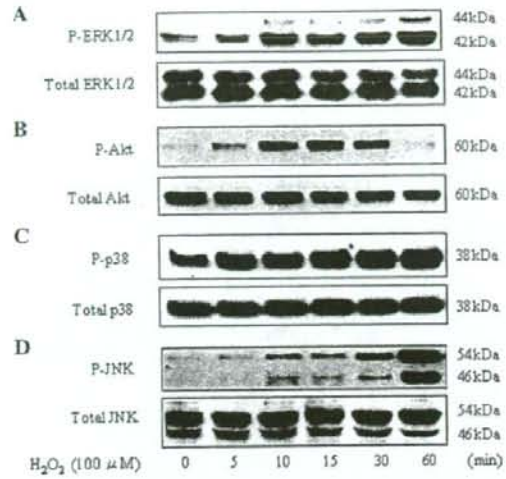

Fig. 2. Phosphorylation of p38, JNK, ERK1/2 and Akt induced by H_2O_2. Serum-starved cells were stimulated with H_2O_2 (100 μM) and harvested at the times indicated for Western blot analysis. Antibodies against (A) phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, (B) phospho-Akt (Ser473), Akt, (C) phopho-p38, p38, (D) phospho-JNK (Thr183/Tyr185) and JNK were used as described in Materials and methods.

1995; Matsuzaki et al., 1999; Uchiyama et al., 2004). Phosphorylation levels of p38 (D-8), JNK (Thr183/Tyr185), ERK1/2 (Thr202/Tyr204), and Akt (Ser473) were elevated after exposure to H_2O_2, with no significant change in the total protein level (Fig. 2A, B, C, D). Maximal phosphorylation was observed at 15 min for Akt (Fig. 2B) and at 60 min for ERK, p38 and JNK (Fig. 2A, C and D).

We examined the effects of a p38 inhibitor, SB203580, and a JNK inhibitor, SP600125, on BCEC apoptosis. BCEC apoptosis was significantly decreased by the inhibitors of p38 and JNK (data not shown). We also examined the effects of a MEK1 (MEK is the immediate upstream regulator of ERK) inhibitor, PD98059, and a phosphatidylinositol-3 OH (PI3) kinase inhibitor, wortmannin, on BCEC apoptosis. PD98059 and wortmannin significantly enhanced H_2O_2-induced BCEC apoptosis (data not shown). These results suggest that p38 and JNK act as cell death signals, whereas ERK1/2 and PI3-kinase/Akt act as survival signals in the process of BCEC apoptosis. The induction of apoptosis by H_2O_2 may be regulated by the balance between death signaling and survival signaling.

Next, we examined the effects of LY117018 on the phosphorylation levels of p38, JNK, ERK1/2, and Akt. On the basis of time-response experiments (Fig. 2A, B, C, D), cells were stimulated with 100 μM H_2O_2 for 15 min for examination of Akt activity and for 60 min for examination of ERK1/2, p38 and JNK activity. Cells were pretreated with LY117018 for 30 min prior to exposure to H_2O_2. LY117018 significantly enhanced the phosphorylation level of ERK1/2 (Fig. 3A). However, no change in the phosphorylation of Akt (Fig. 3B), p38 (Fig. 3C), and JNK (Fig. 3D) was induced by LY117018.

We examined the effects of PD98059 on the anti-apoptotic activity of LY117018. The anti-apoptotic effect of LY117018 was prevented by PD98059 (Fig. 3E), while PD98059 alone did not induce BCEC apoptosis. These results suggest that the anti-apoptotic effect of LY117018 was not exerted by inhibition of cell death signals such as p38 or JNK, or by activation of a survival signal, PI3-kianse/Akt, but

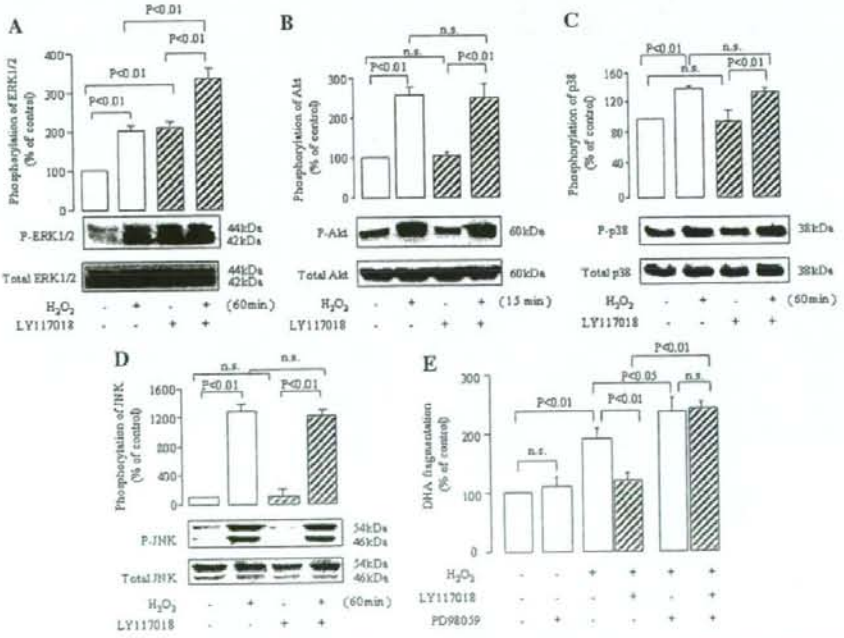

Fig. 3. Effect of LY117018 on H_2O_2-induced activation of p38, JNK, ERK1/2 and Akt. Serum-starved cells were pretreated with 1 μM LY117018 for 30 min. Then cells were stimulated with H_2O_2 (100 μM) for 15 min for determination of Akt activity (B) and for 60 min for determination of ERK1/2, p38 and JNK (A, C and D) activity. Cells were harvested, lysed and used for Western blot analysis. The activities of ERK1/2 (Thr202/Thr204), Akt (Ser473), p38 (D-8) and JNK (Thr183/Tyr185) were measured as described in Materials and methods. Representative blots and quantitative data evaluated by densitometry are shown ($n=3$). The data are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant. (E) In the PD98059 experiment, cells were pretreated with PD98059 (10 μM) for 1 h before addition of LY117018 (1 μM, 30 min), then stimulated with 100 μM H_2O_2 for 1 h. After a 24-h incubation, cell apoptosis was evaluated by means of DNA fragmentation as described in Materials and methods. Values are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$).

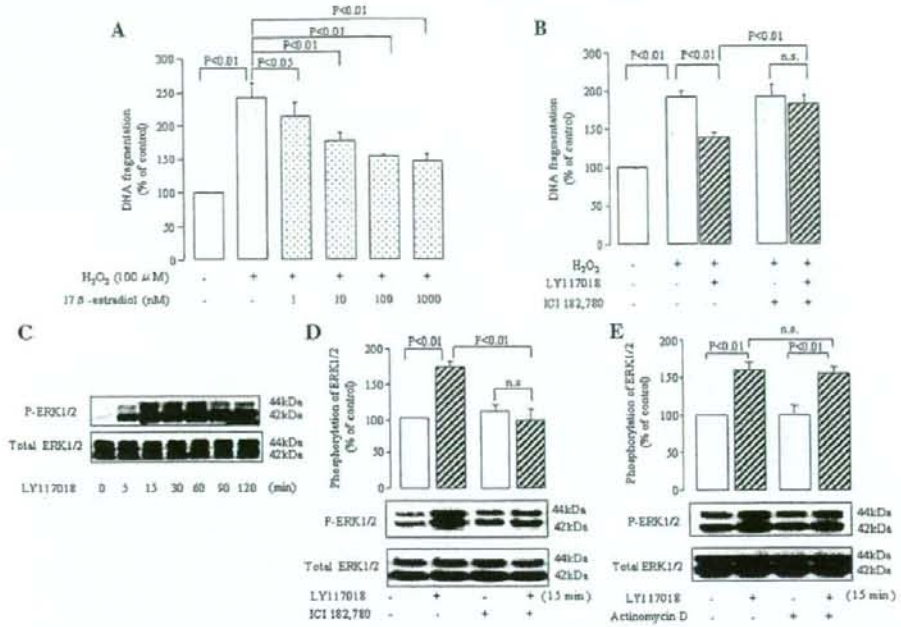

Fig. 4. Involvement of estrogen receptors in anti-apoptotic action of LY117018. At 70–80% confluence, BCEC were starved and exposed to H_2O_2 (100 μM) for 1 h as described in Materials and methods (A and B). (A) Various concentrations of 17β-estradiol (1 nM–1 μM) were added to the culture medium 30 min prior to exposure to H_2O_2 in the apoptosis assay. (B) In the estrogen receptor antagonist experiment, cells were pretreated with ICI 182,780 (10 μM) for 1 h before addition of 1 μM LY117018. Apoptosis was evaluated after 24 h of H_2O_2 treatment by means of DNA fragmentation (with a Cell Death Detection ELISA ^{plus} kit). Data are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant ($n=6$). (C, D and E) Serum-starved cells were stimulated with 1 μM LY117018 and harvested at the times indicated (C). In some groups, cells were pretreated with 10 μM ICI 182,780 (D) or 5 μg/ml actinomycin D (E) for 1 h before addition of LY117018 (1 μM, 30 min). Cell lysates were analyzed by Western blot as described in Materials and methods using a specific antibody against phospho-ERK1/2 (Thr202/Tyr204) or total ERK1/2. Representative blots and quantitative data evaluated by densitometry are shown ($n=3$). Values are expressed as means ± S.E.M. Differences with a value of $P<0.05$ were considered statistically significant.

was mediated through activation of another survival signal, the ERK1/2 pathway.

3.3. Involvement of estrogen receptors in anti-apoptotic action of LY117018

17β-Estradiol, an endogenous ligand for estrogen receptors, inhibited BCEC apoptosis in a concentration-dependent manner (Fig. 4A). 17β-Estradiol exerted an anti-apoptotic action at 1 nM, while LY117018 at 10 nM protected endothelial cells from apoptosis induced by H_2O_2 (Fig. 1). ICI 182,780, an estrogen receptor antagonist, significantly diminished the inhibitory effect of LY117018 on BCEC apoptosis (Fig. 4B). In addition, LY117018 per se rapidly increased the phosphorylation of ERK1/2 more than 5 min after its addition. Maximal phosphorylation was attained after 15 min of incubation (Fig. 4C). The LY117018-induced increase in ERK1/2 phosphorylation was significantly suppressed by ICI 182,780 (Fig. 4D). These results suggest that estrogen receptors are involved in the increased phosphorylation of ERK1/2 by LY117018.

To examine whether the LY117018-induced increase in ERK1/2 phosphorylation is due to a genomic or non-genomic action, the transcription inhibitor, actinomycin D, was added to BCEC prior to treatment with LY117018. The activation of ERK1/2 was not inhibited by actinomycin D (Fig. 4E). These results suggest that the anti-apoptotic activity of LY117018 is exerted through a non-genomic action.

4. Discussion

In the present study, we found that the raloxifene analogue, LY117018, inhibited BCEC apoptosis induced by H_2O_2. This inhibitory effect of LY117018 was concentration dependent. LY117018 at 10 nM protected endothelial cells from apoptosis by H_2O_2, while 17β-estradiol exerted an anti-apoptotic action at 1 nM. This may be explained by the difference in receptor ligand affinity between 17β-estradiol and LY117018. Indeed, the relative binding affinity of 17β-estradiol to estrogen receptor alpha is about 10 times higher than that of raloxifene in estrogen receptor-positive MCF-7 cells (Wijayaratne et al., 1999). The lower affinity of raloxifene for the estrogen receptor may be attributable to a structural difference. In addition, the concentrations of LY117018 used in our study might be relevant, because if we consider that the dose of raloxifene used in clinical settings is 120 mg/day, the serum concentration found in women treated with raloxifene is about 6 nM (Eli-Lilly, Indianapolis, IN, USA, unpublished data, 2003), which is close to the effective concentration of LY117018 in our experiments.

It has been reported that the inhibitory effect of raloxifene on bone absorption is mediated by direct binding with estrogen receptors. Endothelial cells express both estrogen receptor alpha (ER-alpha) and beta (ER-beta). In order to examine whether the anti-apoptotic effects of LY117018 are mediated by estrogen receptors, we examined the effects of a specific estrogen receptor antagonist, ICI 182,780. The anti-apoptotic effect of LY117018 was abolished by ICI 182,780. In addition, 17β-estradiol, an endogenous ligand for estrogen receptors, significantly inhibited apoptosis in BCEC. These observations suggest that LY117018 acts as an estrogen receptor agonist in endothelial cells, leading to endothelial cell survival. It has been reported that steroid hormones cause rapid responses, in minutes, through their membrane receptors. In recent years, several studies regarding the non-genomic actions of estradiol through estrogen receptors have been reported

(Razandi et al., 2003). In vascular cells, the roles of membrane estrogen receptors have been extensively investigated. Estrogen receptors mainly exist in the nucleus as ligand-dependent transcriptional factors, whereas a small amount of estrogen receptors in the cytoplasm do not enter the nucleus upon ligand stimulation and induce rapid signaling events (Pedram et al., 2002). LY117018 rapidly increased the phosphorylation of ERK1/2 after 5 min, and maximal phosphorylation was attained after 15 min of incubation. In addition, the increase in ERK1/2 phosphorylation was not inhibited by actinomycin D. These results suggest that the anti-apoptotic activity of LY117018 is exerted through a non-genomic action.

Recent studies support the idea that the induction of apoptosis by H_2O_2 is regulated by the balance between death signaling (p38, and JNK) and survival signaling (MEK/ERK1/2, and PI3-kinase/Akt) (Xia et al., 1995; Matsuzaki et al., 1999; Uchiyama et al., 2004). Indeed, in our study, H_2O_2 induced the phosphorylation of Akt, ERK1/2, JNK and p38. The p38 inhibitor, SB203580, and JNK inhibitor, SP600125, significantly decreased BCEC apoptosis induced by H_2O_2, whereas the PI3-kinase inhibitor, wortmannin, and MEK1 inhibitor, PD98059, significantly enhanced it. These results suggest that p38 and JNK act as cell death signals, whereas ERK1/2 and PI3-kinase/Akt act as survival signals in the process of BCEC apoptosis. Then we investigated the signaling pathways responsible for the anti-apoptotic effect of LY117018. Interestingly, LY117018 enhanced the phosphorylation level of ERK1/2 only, while it did not enhance the phosphorylation level of Akt or decrease that of p38 and JNK. In addition, PD98059 completely abolished the anti-apoptotic effect of LY117018, suggesting that the anti-apoptotic effect of LY117018 is mediated through enhancement of ERK1/2 signaling in vascular endothelial cells.

In conclusion, LY117018, an analogue of raloxifene, inhibits H_2O_2-induced endothelial apoptosis by activating ERK1/2, which is a non-genomic action via estrogen receptors. This study provides experimental evidence to support a novel therapeutic approach to pathological vascular conditions such as atherosclerosis.

Acknowledgments

This work was supported in part by a Grant-in-Aid (No. 15390-239) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology. We thank Eli-Lilly (Indianapolis, IN, USA) for providing LY117018.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2008.04.052.

References

Akishita, M., Kozaki, K., Eto, M., Yoshizumi, M., Ishikawa, M., Toba, K., Orimo, H., Ouchi, Y., 1998. Estrogen attenuates endothelin-1 production by bovine endothelial cells via estrogen receptor. Biochem. Biophys. Res. Commun. 251, 17–21.

Barrett-Connor, E., Grady, D., Sashegyi, A., Anderson, P.W., Cox, D.A., Hoszowski, K., Rautaharju, P., Harper, K.D., MORE Investigators, 2002. Raloxifene and cardiovascular events in osteoporotic postmenopausal women: four-year results from the MORE (Multiple Outcomes of Raloxifene Evaluation) randomized trial. JAMA 287, 847–857.

Clarkson, T.B., 2007. Estrogen effects on arteries vary with stage of reproductive life and extent of subclinical atherosclerosis progression. Menopause 14, 373–384.

Cox, D.A., Sarkar, S., Harper, K., Barrett-Connor, E., 2004. Effect of raloxifene on the incidence of elevated low density lipoprotein (LDL) and achievement of LDL target goals in postmenopausal women. Curr. Med. Res. Opin. 20, 1049–1055.

Delmas, P.D., Bjarnason, N.H., Mitlak, B.H., Ravoux, A.C., Shah, A.S., Huster, W.J., Draper, M., Christiansen, C., 1997. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N. Engl. J. Med. 337, 1641–1647.

Grady, D., Herrington, D., Bittner, V., Blumenthal, R., Davidson, M., Hlatky, M., Hsia, J., Hulley, S., Herd, A., Khan, S., Newby, L.K., Waters, D., Vittinghoff, E., Wenger, N., HERS Research Group, 2002. Cardiovascular disease outcomes during 6.8 years of hormone therapy: Heart and estrogen/progestin replacement study follow-up (HERS II). JAMA 288, 49–57.

Grady, D., Ettinger, B., Moscarelli, E., Plouffe Jr., L., Sarkar, S., Ciaccia, A., Cummings, S., 2004. Outcomes of raloxifene evaluation investigators. Safety and adverse effects associated with raloxifene: multiple outcomes of raloxifene evaluation. Obstet. Gynecol. 104, 837–844.

Hulley, S., Grady, D., Bush, T., Furberg, C., Herrington, D., Riggs, B., Vittinghoff, E., 1998. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. JAMA 280, 605–613.

Johnell, O., Cauley, J.A., Kulkarni, P.M., Wong, M., Stock, J.L., 2004. Raloxifene reduces risk of vertebral fractures and breast cancer in postmenopausal women regardless of prior hormone therapy. J. Fam. Pract. 53, 789–796.

Kannel, W.B., Hjortland, M.C., McNamara, P.M., Gordon, T., 1976. Menopause and risk of cardiovascular disease: the Framingham study. Ann. Intern. Med. 85, 447–452.

Matsuzaki, H., Tamatani, M., Mitsuda, N., Namikawa, K., Kiyama, H., Miyake, S., Tohyama, M., 1999. Activation of Akt kinase inhibits apoptosis and changes in Bcl-2 and Bax expression induced by nitric oxide in primary hippocampal neurons. J. Neurochem. 73, 2037–2046.

Pedram, A., Razandi, M., Aitkenhead, M., Hughes, C.C., Levin, E.R., 2002. Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. J. Biol. Chem. 277, 50768–50775.

Razandi, M., Pedram, A., Park, S.T., Levin, E.R., 2003. Proximal events in signaling by plasma membrane estrogen receptors. J. Biol. Chem. 278, 2701–2712.

Ross, R., 1990. Mechanisms of atherosclerosis: a review. Adv. Nephrol. Necker Hosp. 19, 79–86.

Ross, R., 1999. Atherosclerosis – an inflammatory disease. N. Engl. J. Med. 340, 115–126.

Rossouw, J.E., Anderson, G.L., Prentice, R.L., LaCroix, A.Z., Kooperberg, C., Stefanick, M.L., Jackson, R.D., Beresford, S.A., Howard, B.V., Johnson, K.C., Kotchen, J.M., Ockene, J., Writing Group for the Women's Health Initiative Investigators, 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 288, 321–333.

Simoncini, T. De., Caterina, R., Genazzani, A.R., 1999. Selective estrogen receptor modulators: different actions on vascular cell adhesion molecule-1 (VCAM-1) expression in human endothelial cells. J. Clin. Endocrinol. Metab. 84, 815–818.

Simoncini, T., Varone, G., Fornari, L., Mannella, P., Luisi, M., Labrie, F., Genazzani, A.R., 2002. Genomic and nongenomic mechanisms of nitric oxide synthesis induction in human endothelial cells by a fourth-generation selective estrogen receptor modulator. Endocrinology 143, 2052–2061.

Sporn, M.B., Dowsett, S.A., Mershon, J., Bryant, H.U., 2004. Role of raloxifene in breast cancer prevention in postmenopausal women: clinical evidence and potential mechanisms of action. Clin. Ther. 26, 830–840.

Sudoh, N., Toba, K., Akishita, M., Ako, J., Hashimoto, M., Iijima, K., Kim, S., Liang, Y.Q., Ohike, Y., Watanabe, T., Yamazaki, I., Yoshizumi, M., Eto, M., Ouchi, Y., 2001. Estrogen prevents oxidative stress-induced endothelial cell apoptosis in rats. Circulation 103, 724–729.

Uchiyama, T., Engelman, R.M., Maulik, N., Das, D.K., 2004. Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. Circulation 22, 3042–3049.

Wijayaratne, A.L., Nagel, S.C., Paige, L.A., Christensen, D.J., Norris, J.D., Fowlkes, D.M., McDonnell, D.P., 1999. Comparative analyses of mechanistic differences among antiestrogens. Endocrinology 140, 5828–5840.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., Greenberg, M.E., 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270, 1326–1331.

8. Ivry RB, Spencer RM, Zelaznik HN et al. The cerebellum and event timing. Ann N Y Acad Sci 2002;978:302–317.
9. Rondi-Reig L, Burguiere E. Is the cerebellum ready for navigation? Prog Brain Res 2005;148:199–212.
10. Schmahmann JD, Sherman JC. The cerebellar cognitive affective syndrome. Brain 1998;121(Pt 4):561–579.

NOCTURIA IN ELDERLY PEOPLE WITH HYPERTENSION—NO INFLUENCE OF LOW-DOSE THIAZIDE ADDED TO LOSARTAN

To the Editor: Most guidelines recommend thiazide-type diuretics as the preferred initial drugs for the treatment of hypertension. Thiazides, used in lower doses in elderly people with hypertension, are useful especially when used with an angiotensin II receptor blocker (ARB), which can antagonize the potassium-excreting effect of thiazides. It is also reported that drug-related adverse reactions are lower when thiazide-type diuretics are added to ARB monotherapy than with other combinations.[1,2] However, in these studies, consideration of the influence of thiazides on the geriatric syndrome is lacking. There could be a particular concern with regard to nocturia, a component of the geriatric syndrome that influences general health and quality of life (QOL) in elderly patients.[3] Some epidemiological studies have reported that diuretics are one of the risk factors for urinary problems in elderly people.[4] With this in mind, the effect of low-dose thiazide added to losartan monotherapy on nocturia in elderly hypertensives was examined.

Fifteen elderly patients with hypertension (mean age ± standard deviation = 76 ± 8, female/male = 6/9) who had been receiving losartan monotherapy (50 or 100 mg/d) for more than 1 month and had systolic blood pressure (BP) of 140 mmHg or greater or diastolic BP of 90 mmHg or greater were enrolled. They did not have any history of heart failure or other cardiovascular disease. Hydrochlorothiazide (HCTZ) at 12.5 mg/d was added to losartan and maintained for 3 months. Other medication was unchanged during the study; four patients were taking a statin, one was taking a sulfonylurea, but no patient was taking medication for gout or an overactive bladder. BP was measured at home and in the doctor's office early in the morning. Laboratory tests were performed in the morning after an overnight fast, and questionnaires on nocturia and health-related QOL were given to all patients.

As shown in Table 1, addition of low-dose HCTZ significantly reduced office BP after 1 month. The BP-lowering effect was sustained at 3 months and confirmed by morning home BP. No patient experienced worsening of nocturia during the study. On average, the frequency of nocturia was less at 1 month. At 3 months, the frequency of nocturia was similar to that at baseline. The result was unchanged if the subjects without nocturia were excluded from the analysis (1.3 ± 0.6 episodes/night at baseline to 1.2 ± 0.6 episodes/night after 3 months; n = 11, $P = .17$). In the QOL questionnaire (visual analog scale graded 0–5), sufficiency of sleep (3.4 ± 1.5 to 3.3 ± 1.6) and satisfaction with health (3.1 ± 1.0 to 3.2 ± 0.9) did not change significantly during the study period. Although serum creatinine and uric acid were significantly higher after 3 months, the changes were slight, and no subject showed an abnormal rise beyond the normal range. Fasting plasma glucose, serum total cholesterol, triglycerides, urea nitrogen, and potassium did not change significantly (Table 1 and data not shown).

Table 1. Changes in Blood Pressure (BP), Serum Parameters, and Nocturia After Addition of 12.5 mg/Day of Hydrochlorothiazide to Losartan

Parameter	Baseline	After 1 Month	After 3 Months
	Mean ± Standard Deviation		
Office systolic BP, mmHg	153 ± 17	140 ± 18†	137 ± 18†
Office diastolic BP, mmHg	87 ± 9	81 ± 10*	75 ± 10†
Home systolic BP, mmHg	144 ± 11	—	132 ± 11†
Home diastolic BP, mmHg	81 ± 9	—	77 ± 8
Glucose, mg/dL	107 ± 14	—	107 ± 13
Creatinine, mg/dL	0.82 ± 0.13	—	0.88 ± 0.15*
Uric acid, mg/dL	5.3 ± 1.2	—	6.1 ± 1.8*
Potassium, mEq/L	4.3 ± 0.3	—	4.2 ± 0.2
Nocturia, times per night	0.97 ± 0.81	0.67 ± 0.82*	0.87 ± 0.74

P< †.01, *.05 vs baseline according to paired t-test.
— = not determined.

This study is preliminary in terms of its size and noncontrolled design, but the results suggest that addition of low-dose HCTZ to losartan monotherapy effectively inhibits BP without worsening of nocturia in elderly people with hypertension. Because an ARB/thiazide combination is one of the most frequently prescribed regimens, the findings of the present study may provide useful information on urinary problems in elderly people with hypertension.

Taro Kojima, MD
Masahiro Akishita, MD, PhD
Katsuya Iijima, MD, PhD
Masato Eto, MD, PhD
Yasuyoshi Ouchi, MD, PhD
Department of Geriatric Medicine
Graduate School of Medicine
University of Tokyo
Tokyo, Japan

ACKNOWLEDGMENTS

Conflict of Interest: The editor in chief has reviewed the conflict of interest checklist provided by the authors and has determined that the authors have no financial or any other kind of personal conflicts with this letter.

Author Contributions: T.K.: study design, analysis and interpretation of data, and preparation of manuscript. M.A.: study concept and design, and preparation of manuscript. M.E. and K.I.: acquisition of subjects. Y.O.: study concept and design.

Sponsor's Role: None.

REFERENCES

1. Oparil S, Barr E, Elkins M et al. Efficacy, tolerability, and effects on quality of life of losartan, alone or with hydrochlorothiazide, versus amlodipine, alone or with hydrochlorothiazide, in patients with essential hypertension. Clin Ther 1996;18:608–625.

2. Oparil S, Aurup P, Snavely D et al. Efficacy and safety of losartan/hydrochlorothiazide in patients with severe hypertension. Am J Cardiol 2001;87:721–726.
3. Asplund R. Nocturia in relation to sleep, health, and medical treatment in the elderly. BJU Int 2005;96(Suppl 1):15–21.
4. Johnson TM, Sattin RW, Parmelee P et al. Evaluating potentially modifiable risk factors for prevalent and incident nocturia in older adults. J Am Geriatr Soc 2005;53:1011–1016.

DISCLOSURE OF DEMENTIA DIAGNOSIS AND THE NEED FOR ADVANCE CARE PLANNING IN INDIVIDUALS WITH ALZHEIMER'S DISEASE

To the Editor: In a recent article in the *Journal of the American Geriatrics Society,* Carpenter and colleagues found that disclosure of a dementia diagnosis does not prompt a catastrophic emotional reaction in most people with dementia—not even in those who are cognitively only mildly impaired.[1] Companions of these patients also remained stable or even declined in their emotional stress immediately after the disclosure of dementia diagnosis. These findings suggest that physicians can provide a dementia diagnosis to a patient without fear of prompting strong emotional reactions.

In our experience, the disclosure of a diagnosis of dementia and adjustment to understanding all dimensions related to this information are a long-term process in which the patient's feelings develop and change over time. We examined the experiences of spousal caregivers of patients with Alzheimer's disease (AD) with many comorbidities and disabilities regarding the disclosure of dementia diagnosis and the subsequent need for advance care planning (ACP).

METHODS

The study included a survey of a random sample of 1,943 caregivers of persons with AD in Finland. All of the persons with AD had a confirmed diagnosis and received a compensation for AD medication from the state. Of the 1,434 respondents, 1,214 identified themselves as their spouse's caregiver. The mean age of the caregivers was 78.2, and that of the spouses with dementia was 80.5. Of the caregivers, 63% were female. The couples had long-lasting marriages (mean 52 years).[2] The mailed questionnaire included items on demographic characteristics and the physical and psychological symptoms and care needs of the persons with dementia. These questions and their validity have been described in detail elsewhere.[2]

The questions related to ACP had been used in previous interview studies in elderly people.[3,4] The ACP questions appear in Table 1.

RESULTS

Of the caregivers, 90% reported that dementia had been disclosed openly to their spouse; 97% also preferred that physicians openly inform the patients of the dementia diagnosis, although more than half of their spouses with AD had developed depressive symptoms after the disclosure. Of the caregivers, two-thirds felt that their awareness of their spouse's dementia caused them grief or symptoms of depression.[5]

After the disclosure of a diagnosis, a large proportion of caregivers felt a need for discussion about ACP with their physician. Of the caregivers, 59% expressed that they would like to discuss ACP with their spouse's physician, although only 6% reported that they had discussions related to ACP with a physician. Of the caregivers, fewer than one-third reported that they had discussed their spouse's medical care preferences with each other, and only 4% of the spouses with dementia had a written living will (LW). The couples in which the spouse with AD had a LW were generally better prepared for the loss of autonomy than the others.

DISCUSSION

This large epidemiological study of people with AD showed that physicians had openly disclosed their diagnosis to nearly all of the subjects, and depressive reactions over time were common in the patients with AD and their caregivers.

Table 1. Questions and Spousal Caregiver's Responses Relating to Advance Care Planning (ACP) of Home-Dwelling Persons with Alzheimer's Disease (AD) in Finland, 2005, According to Whether the Person with AD Had a Living Will (LW)

Question	Person with AD having a LW (n = 46)	Person with AD not having a LW (n = 1,127)	P-Value*
	Yes %		
Do you think that the follow-up care of your spouse's dementia is well arranged?	52.6	50.2	.77
Have you discussed your spouse's medical care preferences with each other?	63.2	26.1	<.001
Have you discussed these items with your spouse's physician?	31.0	5.1	<.001
Would you like to discuss ACP with your spouse's physician?	59.5	58.8	.93
Have you made a financial arrangement with your bank or some broader authorization to make your spouse's financial arrangements easier?	61.4	36.8	<.001
Have you requested for a legal guardian for your spouse to make financial or other arrangements easier?	13.6	3.8	<.001

*Differences in proportions between two groups were tested using the chi-square test.