bodyweight.[3] As the average insulin dose used in general is approximately 0.4–0.5 U/kg bodyweight,[1,4] a more sophisticated method is needed to enable a successful switch from insulin injections to oral agent treatment.

Pioglitazone is a newly available agent that improves insulin resistance,[5,6] a core defect in type 2 diabetes.[7] Pioglitazone for the first time among hypoglycemic agents including insulin and sulphonylureas, significantly reduced the composite of mortality, stroke and myocardial infarction.[8]

Because pioglitazone has not been used as a major agent for switching, this study used this agent together with a sulphonylurea, glimepiride that improves insulin resistance[9] and has a less marked effect on bodyweight gain[10,11] and an α-glucosidase inhibitor, voglibose, that is effective for blood glucose control with a sulphonylurea[12] and pioglitazone[13] to develop a new approach for the substitution of insulin therapy.

Because insulin injection per se may exacerbate insulin resistance, we completely stopped insulin injections before the switch and then immediately began administration of oral agents in patients under long-term insulin injection in order to maximize the insulin-sensitizing capacity of pioglitazone.

Methods

A total of 38 type 2 diabetes patients on insulin injection therapy were recruited from our internal medicine division from May 2005 to December 2006. Type 2 diabetes was diagnosed according to World Health Organization criteria, by a 2-h post-load venous full blood glucose value of more than 10.0 mmol/L.[14]

This study protocol was approved by the local ethics committee, and all participants gave written informed consent before inclusion in the study.

Patient eligibility

Inclusion criteria were as follows: (i) aged 40–86 years; (ii) insulin dosage of more than 10 units/24 h; (iii) insulin injection duration of more than 3 months; (iii) C-peptide in 24-h urine of more than 10 μg; and (iv) fasting C-peptide immunoreactivity (CPR) of more than 0.5 ng/mL.

Exclusion criteria were as follows: (i) positive for glutamine acid decarboxylase antibody; (ii) alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) more than threefold the upper limit of normal; (iii) currently and/or previously suffering from heart failure; (iv) ejection fraction assessed by echocardiography of less than 40%; (v) malignancy on active therapeutic regimen or without complete remission or cure; (vi) concomitantly suffering from infection; (vii) planning to have surgery; (viii) more than 50% positivity for insulin antibody; (ix) diagnosis of type I diabetes; (x) pregnancy or breast feeding; (xi) under dialysis; (xii) concomitantly using pioglitazone; and (xiii) hemoglobin A1c (HbA1c) of 10% or more.

Two patients were excluded from the study, one because of heart failure and the other because of colon cancer. Thirty-six patients were enrolled in the study (Fig. 1).

Switch from insulin therapy to oral agents

On the day when insulin injection therapy was completely withdrawn together with concomitantly used oral hypoglycemic agents, combination therapy was initiated. Medication other than hypoglycemic agents was continued after the switch. Because the proposed switch therapy was not a generally accepted approach, in order to ensure careful monitoring of blood glucose and to take immediate actions against hypoglycemia, all the enrolled patients were hospitalized.

Pioglitazone was started at a dose of 15–30 mg, glimepiride at 1–3 mg and voglibose at 0.9 mg. The maximum dose of pioglitazone was 45 mg, and that of glimepiride was 4 mg. If fasting plasma glucose was less than 5.55 mmol/L and/or hypoglycemia developed, glimepiride was first reduced in dose and then pioglitazone.

In patients more than 65 years of age and in female cases, pioglitazone was initially used at 15 mg in order to avoid the development of heart failure.

When the post-prandial blood glucose level was less than 16.7 mmol/L, the patient was discharged and followed in the outpatient clinic on a monthly basis for 4 months.

Definition of success, failure and dropout in the study

Success was defined as HbA1c at 4 months after the switch of less than 7.0%. Failure was defined as: (i) on the switch day and the following days before discharge, blood glucose level reached more than 22.2 mmol/L at any point of the day; (ii) after discharge, the blood glucose level was more than 11.1 mmol/L in the fasting state or 16.6 mmol/L in the post-prandial state; (iii) heart failure developed; (iv) AST/ALT reached more than threefold the upper limit of normal. Countermeasures were taken to alleviate the conditions defined as failure. First, combined oral hypoglycemic agents were immediately stopped and the original therapy including insulin injection was reinitiated. Second, for the conditions (iii) and (iv), patients were referred to expert cardiovascular and hepatology clinicians.

Dropout was defined as: (i) insulin injection treatment was mandatory because of surgical operation, treatment of infectious disease or initiation of anticancer chemotherapy; (ii) the patient voluntarily returned

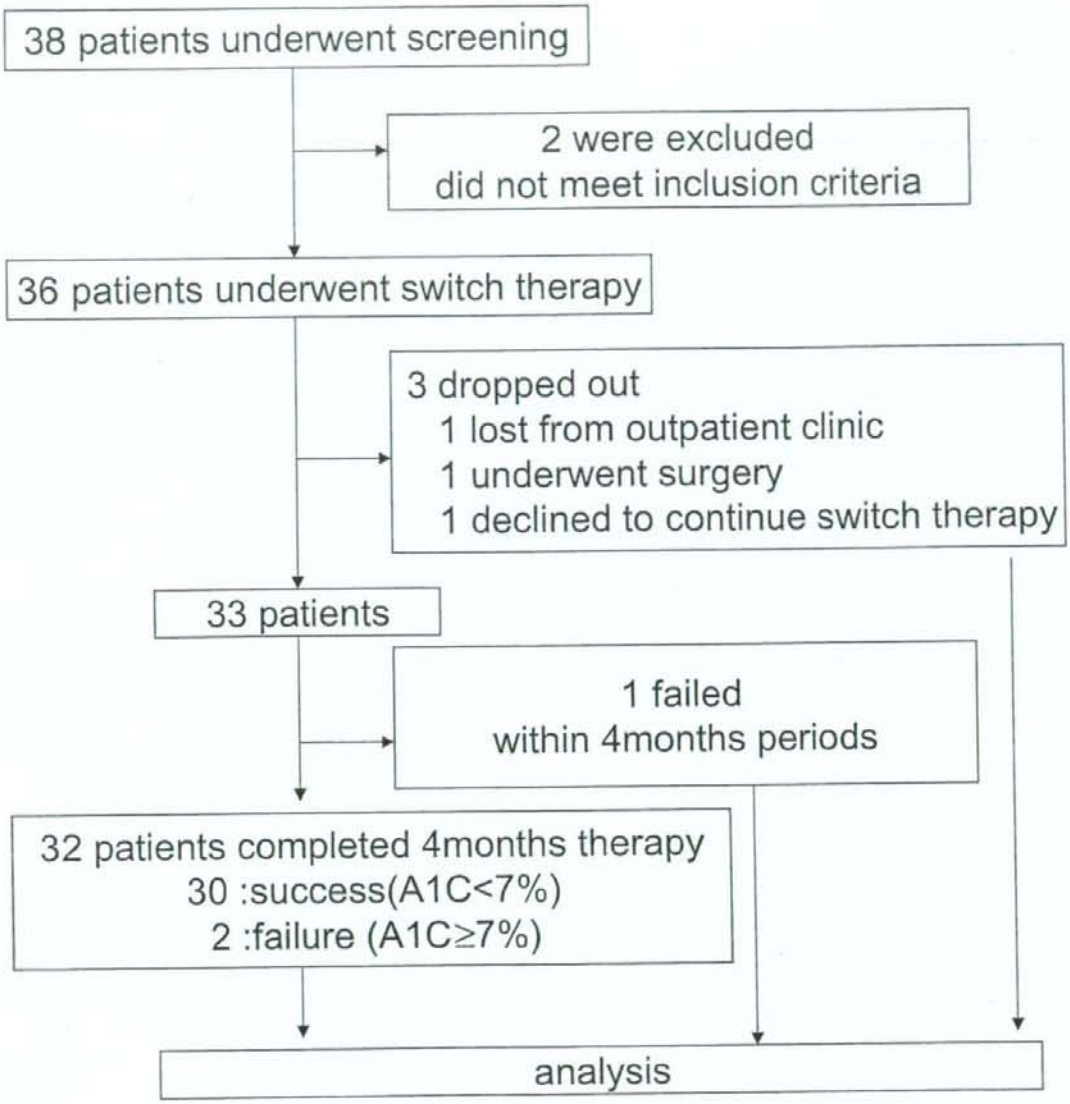

Figure 1 Enrollment and outcomes. A1c, hemoglobin A1c.

to insulin therapy; or (iii) the patient did not attend the outpatient clinic after the switch.

Laboratory studies

Concentrations of overnight fasting plasma glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured by enzyme methods, insulin, C-peptide and antibodies against glutamine acid decarboxylase and insulin by radioimmunoassay, glycosylated HbA1c by high-performance liquid chromatography, creatinine by the modified Jaffe method, blood urea nitrogen by urease indophenol method, albumin by the bacille Calmette–Guerin method, hematocrit by the erythrocyte pulse wave method, and alkaline phosphatase, AST and ALT by the JCCLS-SOP methods (Kishimoto Clinical Laboratory, Hokkaido, Japan).

Analysis of data

The primary outcome measure was the rate of success in the study. The secondary outcome measure was the difference in mean HbA1c from the baseline to the 4-month time point. Differences among values of serum lipid concentrations, blood pressure, bodyweight, hematocrit, albumin, blood urea nitrogen, creatinine, AST and ALT were assessed at months 0 and 4. Severe hypoglycemia was defined as an episode requiring intervention and either a blood glucose level of less than 36 mg/dL or the use of countermeasures. Differences among values of age, body mass index, weight, diabetes duration, insulin duration, insulin dosage, fasting blood glucose, initial HbA1c, fasting immunoreactive insulin, fasting plasma C-peptide, urinary C-peptide excretion, and maximum blood glucose on the switch day were assessed in subjects who achieved HbA1c levels of less than 7% and those who did not at the end of the study. Maximum blood glucose was defined as the highest postprandial 2-h blood glucose level on the day of the switch. First, dropouts were included in the analysis by adopting an intention-to-treat concept. Second, the datasets were compared between data including dropouts and data excluding dropouts.

Data are presented as mean ± standard deviation. Data were compared between two groups by the Student's *t*-test, and among more than two groups by one-way ANOVA followed by Bonferroni's test. $P < 0.05$ was considered significant.

Results

Of the 36 patients enrolled in the study, three patients dropped after the switch (Fig. 1). The first dropout case, aged 67 years, did not attend the outpatient clinic 2 months after the switch; the second case, aged 54 years, returned to insulin therapy in order to undergo thyroid resection surgery 2 months after the switch; and the third case, aged 55 years, voluntarily returned to insulin treatment 3 days after the switch.

Patient profile

The patient profiles of all 36 patients who underwent switch therapy are summarized in Table 1 (left column). The mean age was 67.8 years. The average diabetes duration was 15.7 years. The average insulin dose was 27.6 U/24 h (12–64 units) and 0.46 U/kg bodyweight per 24 h for an average duration of 6.1 years. HbA1c and fasting plasma glucose immediately before the switch were 6.8% and 7.1 mmol/L, respectively. The average C-peptide level was 1.97 ng/mL, with urinary C-peptide excretion per day of 48.4 μg.

The level of HbA1c within 2 months before the switch was 7.0 ± 1.2%, which was not statistically significantly different from that immediately before the switch ($P = 0.50$). These data indicate that the patients in the study maintained stable blood glucose control before the switch.

α-Glucosidase inhibitors and/or biguanides were used concomitantly with insulin before the switch in 18 and two patients, respectively.

Of patients aged more than 60 years, the mean age was 73.3 ± 7.5 years ($n = 26$, 11 men and 15 women). The average diabetes duration was 17.4 ± 12.6 years. The average insulin dosage was 24.9 ± 8.9 U/24 h and 0.42 ± 0.15 U/kg bodyweight per 24 h for an average duration of 6.1 ± 9.3 years. HbA1c and fasting plasma glucose immediately before the switch were 6.5 ± 0.9% and 6.9 ± 1.5 mmol/L, respectively. The average C-peptide level was 1.82 ± 0.59 ng/mL, with urinary C-peptide excretion/day of 45.7 ± 24.9 μg.

Outcome measures

As fasting plasma glucose reached more than 11.1 mmol/L 1 month after the switch in one male case aged 54 years with a daily insulin dose of 54 U, this case was determined to be a failure (Fig. 1).

The remaining 32 cases completed the planned 4-month study after the switch. Of these 32 cases, 30 achieved HbA1c of less than 7.0%. The primary outcome measure – the proportion of patients who achieved HbA1c of less than 7.0% at 4 months – was 83.3% (30/36). The average insulin dosage was 0.43 ± 0.17 U/kg bodyweight per 24 h and 25.7 ± 11.4 U/24 h for an average insulin therapy duration of 5.9 ± 8.2 years in these 30 patients. The results indicate a high rate of success in switching from insulin therapy to oral agents.

The baseline profiles of these 32 patients who maintained the switch therapy for 4 months are shown in Table 1 (right column). No data were statistically significantly different between all the enrolled patients (left column in Table 1) and those who completed 4 months of switch therapy (right column in Table 1). These results indicate that these 32 patients had similar baseline characteristics to those of all the enrolled patients.

In these 32 patients, HbA1c gradually decreased every month after the switch (before switch, 6.7 ± 1.3%; after switch, 1 month, 6.4 ± 1.1%; 2 months, 6.4 ± 1.0%; 3 months, 6.2 ± 0.8%; 4 months, 5.9 ± 0.7%). The average HbA1c at 4 months after the switch was significantly lower than that before the switch ($P < 0.01$) and that 1 month after the switch ($P < 0.05$). HbA1c was also significantly lower at 3 months ($P < 0.05$) compared to the baseline value. The results showed that the switch therapy provided significantly better blood sugar control.

The average maintenance dose of pioglitazone was 26.4 ± 12.4 mg, that of glimepiride was 2.3 ± 1.2 mg, and that of voglibose was 0.84 ± 0.22 mg.

Table 1 Baseline characteristics of 36 patients who underwent switch therapy (left column) and 33 patients except for drop-outs (right column)

Age (years)		67.8 ± 11.3	68.6 ± 11.4
Male/female number		17/19	15/18
Weight (kg)		60.8 ± 10.7	60.9 ± 10.8
Body mass index (kg/m^2)		24.2 ± 3.8	24.6 ± 3.8
Diabetes duration (years)		15.7 ± 11.8	14.9 ± 11.7
Insulin treatment duration (years)		6.1 ± 8.2	6.4 ± 8.5
Insulin dosage (U/24 h)		27.6 ± 11.5	27.2 ± 12.4
Insulin dosage (U/kg per 24 h)		0.46 ± 0.17	0.45 ± 0.18
Fasting plasma glucose (mmol/L)		7.1 ± 1.9	7.0 ± 1.9
A1C (%)		6.8 ± 1.3	6.7 ± 1.3
Fasting IRI (μU/mL)		23.5 ± 23.4	21.9 ± 20.3
Fasting plasma C-peptide (ng/mL)		1.97 ± 0.76	1.95 ± 0.75
Urinary C-peptide excretion (μg/24 h)		48.4 ± 26.7	47.9 ± 27.2
Systolic blood pressure (mmHg)		121.8 ± 13.8	122.9 ± 13.8
Diastolic blood pressure (mmHg)		69.0 ± 10.6	69.3 ± 11.0
Triglyceride (mg/dL)		131.8 ± 56.1	132.2 ± 56.1
Creatinine (mg/dL)		1.1 ± 0.4	1.0 ± 0.3
Albumin (g/dL)		4.1 ± 0.4	4.1 ± 0.4
Blood urea nitrogen (mg/dL)		18.3 ± 6.7	18.1 ± 6.6
Total cholesterol (mg/dL)		185.1 ± 37.4	181.0 ± 33.3
LDL cholesterol (mg/dL)		110.5 ± 33.2	107.3 ± 29.1
HDL cholesterol (mg/dL)		46.6 ± 15.1	44.7 ± 13.0
Risk factor	Hypertension	24 (66.7)	22 (66.7)
	Hyperlipidemia	22 (61.1)	20 (60.6)
	Smoking	7 (19.4)	7 (21.2)
Complications	Retinopathy	20 (55.5)	19 (57.6)
	Nephropathy	22 (61.1)	19 (57.6)
	Neuropathy	22 (61.1)	20 (60.6)
	Cerebrovascular disease	5 (13.9)	5 (15.2)
	Ischemic heart disease	4 (11.1)	4 (12.1)
Treatments	Beta-blockers	2 (5.5)	2 (6.1)
	ACE inhibitors	7 (19.4)	7 (21.2)
	Angiotensin receptor blocker	11 (30.5)	11 (33.3)
	Statins	19 (52.7)	18 (54.5)
	α-Glycosidase	18 (50.0)	18 (54.5)
	Biganide	2 (5.6)	2 (6.1)

Data are mean ± standard deviation (SD) or *n* (%). A1c, hemoglobin A1c; ACE, angiotensin-converting enzyme; HDL, high-density lipoprotein; IRI, immunoreactive insulin; LDL, low-density lipoprotein.

Of the 26 patients aged more than 60 years, the remaining 25 subjects completed the planned 4-month study after the switch. Of these 25 cases, 24 achieved HbA1c of less than 7.0%. The primary outcome measure – the proportion of patients who achieved HbA1c of less than 7.0% at 4 months – was 92.3% (24/26). The results indicate a high rate of success in switching from insulin therapy to oral agents, even in elderly patients. In these 25 patients who completed the 4-month study, the average HbA1c at 4 months after the switch was 5.8 ± 0.8%. The value was significantly lower than that before the switch ($P < 0.01$). The results showed that the switch therapy provided significantly better blood sugar control also in elderly patients.

Safety measures

Bodyweight was not significantly different before and after the switch (Table 2). No cases were diagnosed as having heart failure or drug-induced liver dysfunction. All the other parameters were not statistically significantly different before and after the switch. Hematocrit significantly decreased after the switch.

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.[14] 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.

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

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

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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].

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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

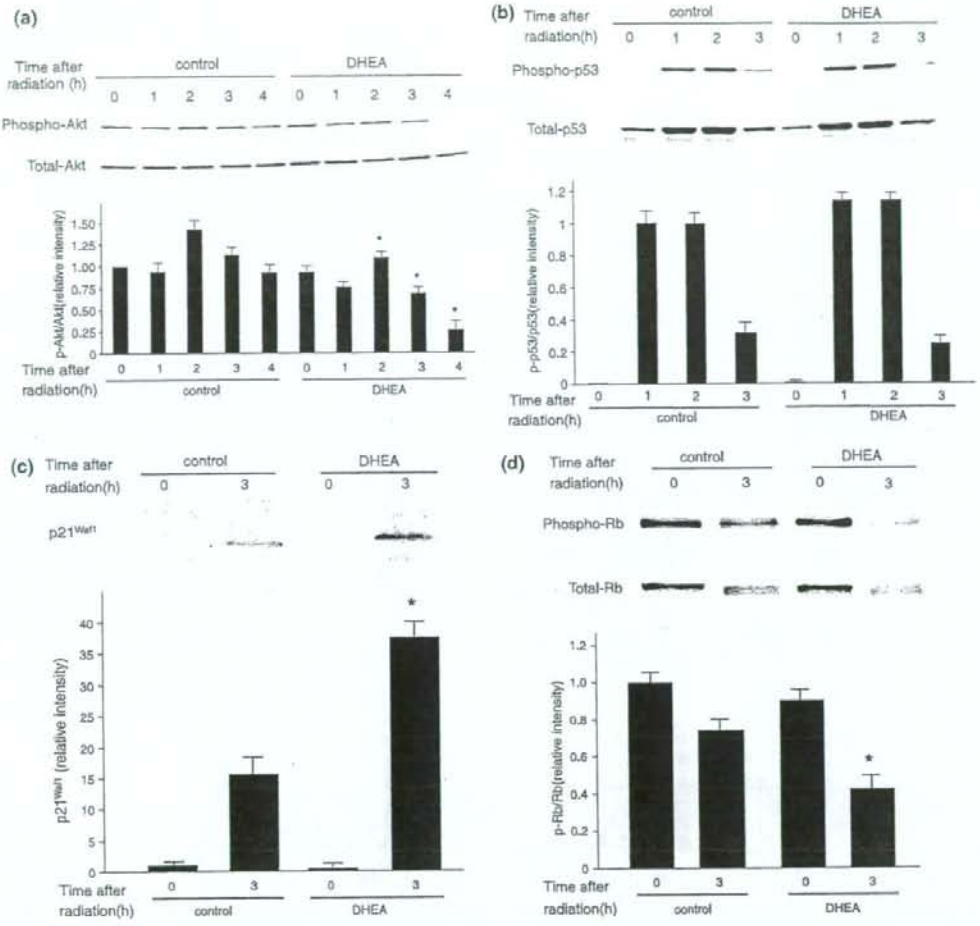

Figure 1. DHEA down-regulates the γ-ray-induced phosphorylation of Akt. H4 cells were serum-starved for 24 h. After prior treatment with 200 nM DHEA for 18 h, the cells were treated with 3-Gy of γ-rays. (A) Representative Western blot for the phosphorylation of Akt. The phosphorylation of Akt was estimated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

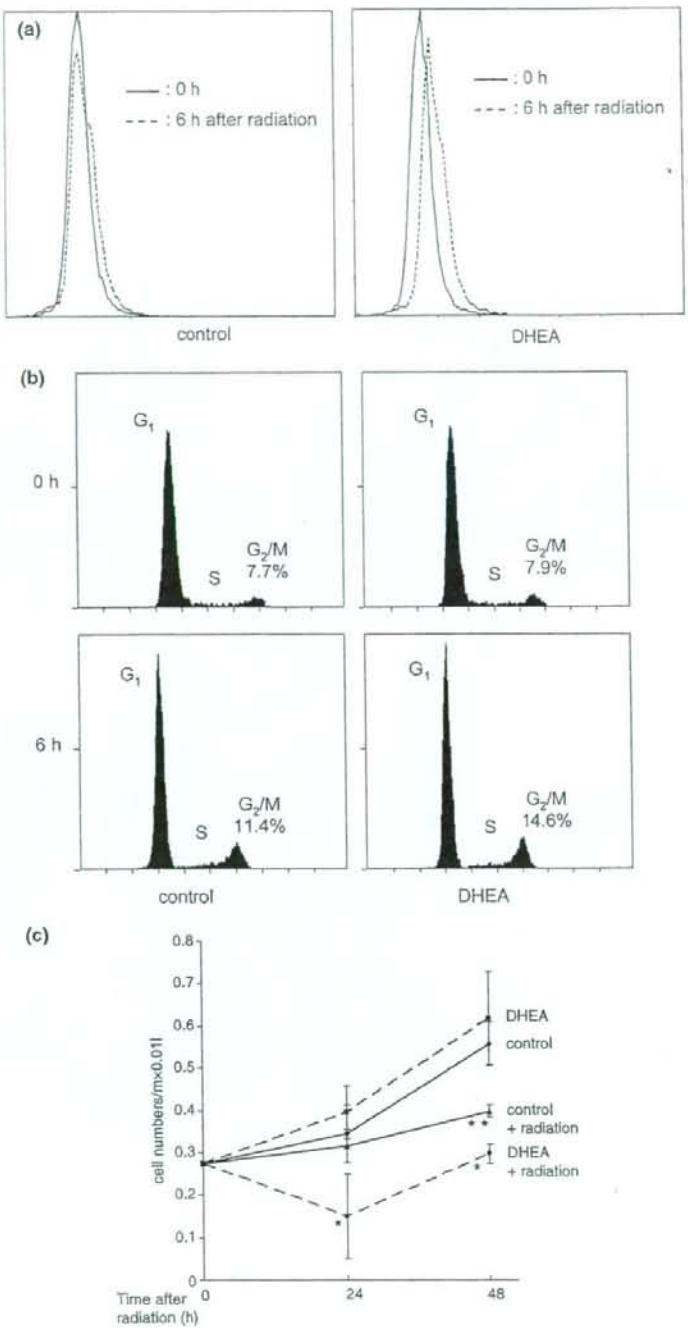

Figure 2 (*Continued*)

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.

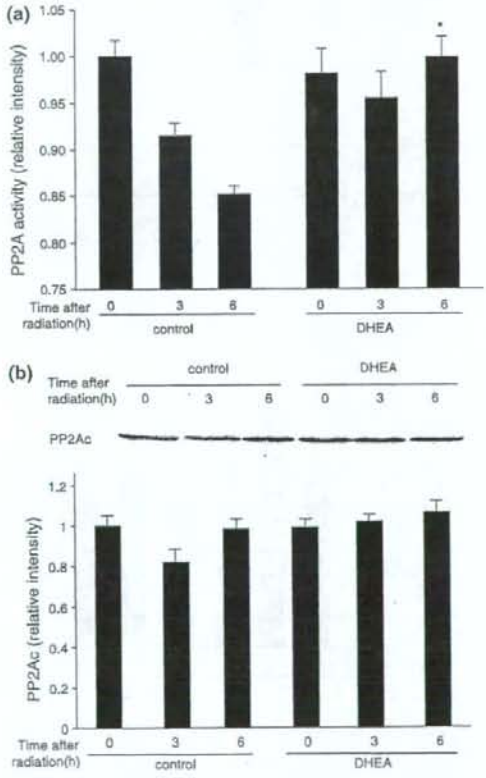

Figure 3. DHEA maintains the activity of PP2A. The effect of DHEA on the activity of PP2A was estimated as described in Materials and methods. (A) The effect of γ-rays on PP2A activity was estimated in cells previously treated with DHEA for 18 h. (B) Changes in the levels of PP2Ac protein were estimated 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.

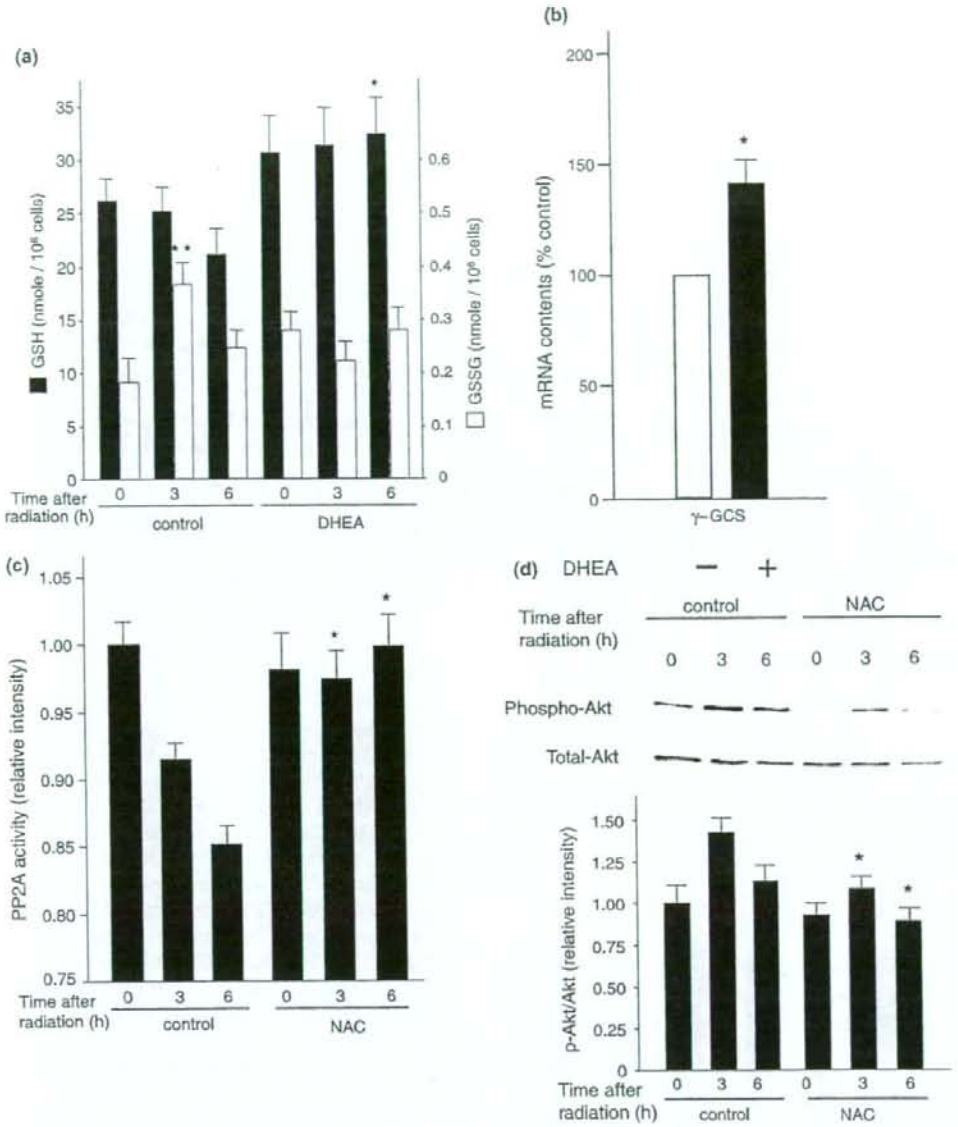

Figure 4. DHEA induces the expression of γ-GCS mRNA and increases the levels of GSH. The effect of DHEA on the expression of γ-GCS mRNA and the level of GSH was estimated. (A) H4 cells were treated with 200 nM DHEA for 18 h and the gene expression of the γ-GCS heavy sub-unit was analysed by quantitative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

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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

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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 (*M*ultiple *O*utcomes of *R*aloxifene *E*valuation) 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.

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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.,

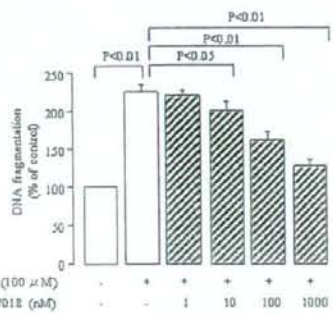

Fig. 1. Effect of LY117018 on H_2O_2-induced endothelial cells apoptosis. At 70–80% confluence, BCEC were starved and exposed to 100 μM H_2O_2 for 1 h as described in Materials and methods. Various concentrations of LY117018 (1 nM–1 μM) were added to the culture medium 30 min before H_2O_2 stimulation 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 ($n=6$).

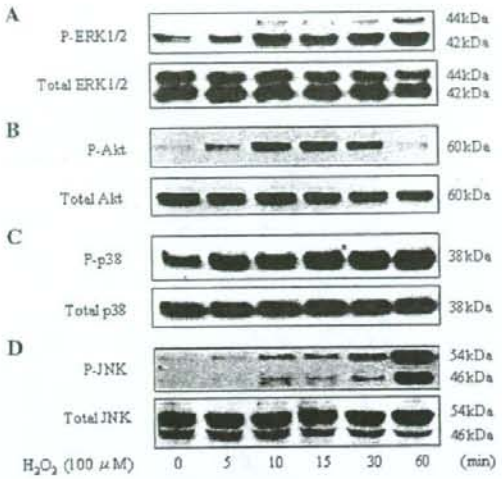

Fig. 2. Phosphorylation of p38, JNK, ERK1/2 and Akt induced by H_2O_2. Serum-starved cells were stimulated with H_2O_2 (100 μM) and harvested at the times indicated for Western blot analysis. Antibodies against (A) phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, (B) phospho-Akt (Ser473), Akt, (C) phopho-p38,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

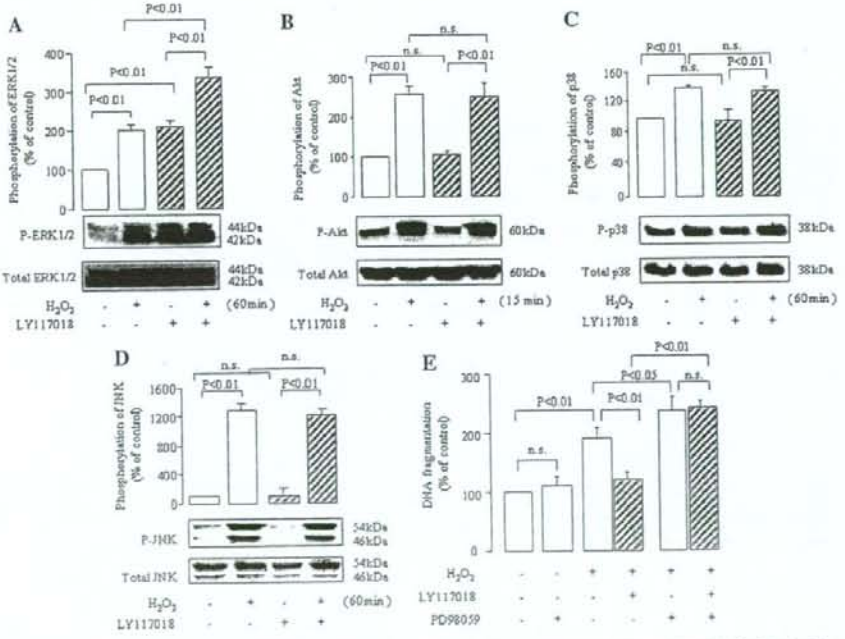

Fig. 3. Effect of LY117018 on H_2O_2-induced activation of p38, JNK, ERK1/2 and Akt. Serum-starved cells were pretreated with 1 μM LY117018 for 30 min. Then cells were stimulated with H_2O_2 (100 μM) for 15 min for determination of Akt activity (B) and for 60 min for determination of ERK1/2, p38 and 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 ($n=6$).