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Pharmacokinetics of L-dopa

Special reference to food and aging

Abstract According to our data in rats, peripheral 3.4-dihydroxyphenylalanine (DOPA) kinetics are similar to striatal DOPA and dopamine kinetics. The measurement of plasma l-3.4-dihydroxyphenylalanine (L-dopa) concentration is thus useful to predict dopamine kinetics in the striatum and to treat the motor fluctuations of parkinsonian patients. In patients with Parkinson's disease (PD), long-term L-dopa therapy accelerated DOPA absorption and steepened features of L-dopa pharmacokinetics. In the senile-onset group, the pharmacokinetic pattern did not change even after long-term L-dopa therapy. The frequency of motor fluctuations is much lower in senile-onset patients with PD than in middle-onset patients. Differences in the pattern of L-dopa pharmacokinetics in the two groups may explain why the senile-onset group rarely develops 'wearing-off', even after long-term L-dopa therapy. L-dopa is transported by a saturable active transporter system, called the LNAA (large neutral amino acid) system, in the gut and blood brain barrier. L-dopa absorption is thus affected by food intake, especially a protein-rich diet. The slope of the time-concentration curve for L-dopa administered before a meal is steeper than if it is administered after a meal. Considering that pulsative stimulation of L-dopa may cause motor fluctuations, L-dopa should be given after meals whenever possible, even if it necessitates a higher L-dopa dose.

Key words Parkinson's disease · absorption · LNAA system · L-dopa · aging

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Introduction

L-dopa is the gold standard of antiparkinsonian pharmacotherapy; however, motor fluctuations, such as 'wearing-off', often develop during long-term L-dopa therapy. The definition of wearing-off is fluctuation of parkinsonian symptoms in line with L-dopa pharmacokinetics [1]. Therefore, the pharmacokinetics of L-dopa are very important in treating patients with Parkinson's disease (PD). The half-life ($T_{1/2}$) of L-dopa is short (1 h) and its absorption is greatly influenced by food intake and aging. Thus, these factors make PD therapy complicated.

Because L-dopa is used with a DOPA decarboxylase inhibitor (DCI), catechol-O- methyltransferase (COMT) is an important enzyme influencing peripheral L-dopa metabolism and L-dopa effects on parkinsonian symptoms. Therefore, nowadays, knowledge about L-dopa pharmacokinetics is increasingly important in treating PD.

In the present review, DOPA and dopamine kinetics in blood and brain, and food and aging effects on the pharmacokinetics of L-dopa are discussed. The correlation between DOPA and 3-O-methyl DOPA is also featured.

L-dopa and dopamine kinetics in peripheral blood and striatum

L-dopa pharmacokinetics are very important when treating PD, and the L-dopa concentration can be measured in blood. Dopamine kinetics in the brain are more

critical than peripheral DOPA kinetics; however, it is very difficult to measure dopamine in the brain of PD patients in vivo. Therefore, it is important to know how closely peripheral L-dopa kinetics reflect dopamine kinetics in the brain, especially in the striatum.

We measured DOPA and dopamine concentrations in the blood and striatum of normal rats after single and repeated L-dopa administration (Fig. 1) [2]. DOPA and dopamine kinetics in the striatum were well correlated with peripheral DOPA kinetics. We also showed that repeated L-dopa (L-dopa 50 mg/kg + benserazide 12.5 mg/kg) administration for 28 days increased the area under the concentration-time curve (AUC) and shortened $T_{1/2}$ and time to maximum plasma concentration (T_{max}) for both peripheral DOPA and central DOPA and dopamine in normal rats.

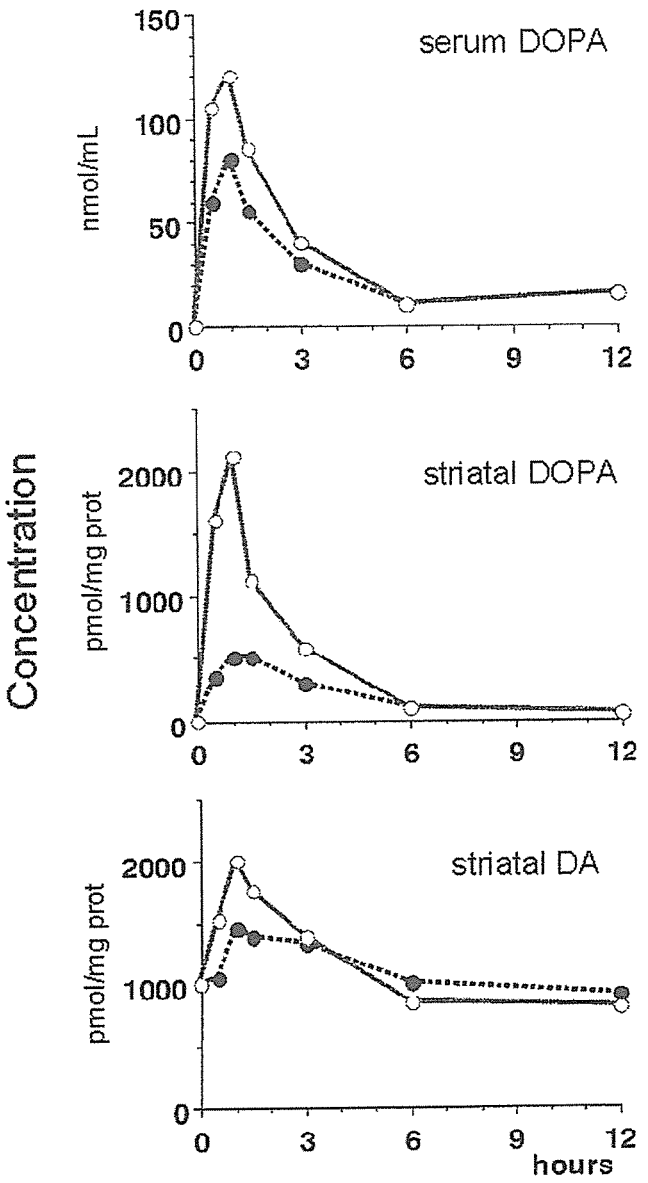

Fig. 1 Time course of serum L-dopa and striatal DOPA and dopamine (DA) after single and repeated administration of L-dopa in rats (-----●----- single administration, ——○—— repeated administration). Time course of serum L-dopa concentration is similar to that of striatal DOPA and dopamine. Repeated L-dopa administration increases C_{max} and AUC and shortens $T_{1/2}$

L-dopa pharmacokinetics in PD patients

PD patients were given L-dopa 100 mg plus benserazide 25 mg orally at 8:00 am after an overnight fast. Plasma DOPA concentrations were measured prior to treatment (baseline) and at 15 min, 30 min, 1 h, 2 h, 3 h and 4 h after medication using HPLC-ECD (L-DOPA test) [3]. In PD patients (onset age < 60 years old) who had received L-dopa therapy for longer than 5 years, the $T_{1/2}$ and T_{max} of L-dopa were much shorter than those measured in PD patients with a duration of L-dopa therapy of less than 5 years (Fig. 2a). In addition, the AUC was greater in the longer therapy duration group (Fig. 2a). These changes in the pharmacokinetics of L-dopa were significantly correlated with duration of L-dopa therapy and dose of L-dopa. A 4-year longitudinal study showed that four of five patients displayed an increased AUC and shortened $T_{1/2}$ and T_{max} at the second assessment [4]. Patients who demonstrated the wearing-off phenomenon had a significantly higher maximum plasma concentration (C_{max}) and greater AUC, and significantly shorter $T_{1/2}$ and T_{max} than those who did not display wearing-off. The pattern of L-dopa kinetics in those with wearing-off was obviously steeper than that of patients without wearing-off.

It is reasonable to suppose that these changes in pharmacokinetic features are due to changes in absorption or metabolism of L-dopa. Decreased metabolism of L-dopa can explain the increase in AUC and C_{max}, but cannot explain the shortening of T_{max} and $T_{1/2}$. Increased absorption, however, can explain the increase in AUC and C_{max} and the shortening of T_{max}. If the absorption system is saturable, increased absorption can also explain the shortening of $T_{1/2}$. L-dopa is transported by the saturable active transport system called the LNAA (large neutral amino acid) system in the gut and blood brain barrier (BBB) [5]. Furthermore, intravenous administration has demonstrated that the distribution and elimination of L-dopa was not changed after long-term L-dopa therapy [6]. Both monoamine oxidase (MAO) activity and COMT activity in the brain are unaffected by long-term L-dopa administration [2, 7]. Therefore, our results show that long-term L-dopa therapy alters its own kinetics by increasing the absorption of L-dopa. As early as 1971, Abrams et al. reported that long-term L-dopa therapy increases its own absorption [8]. At that time, L-dopa therapy involved L-dopa administered without a DCI, and liver DOPA decarboxylase (DDC) ac-

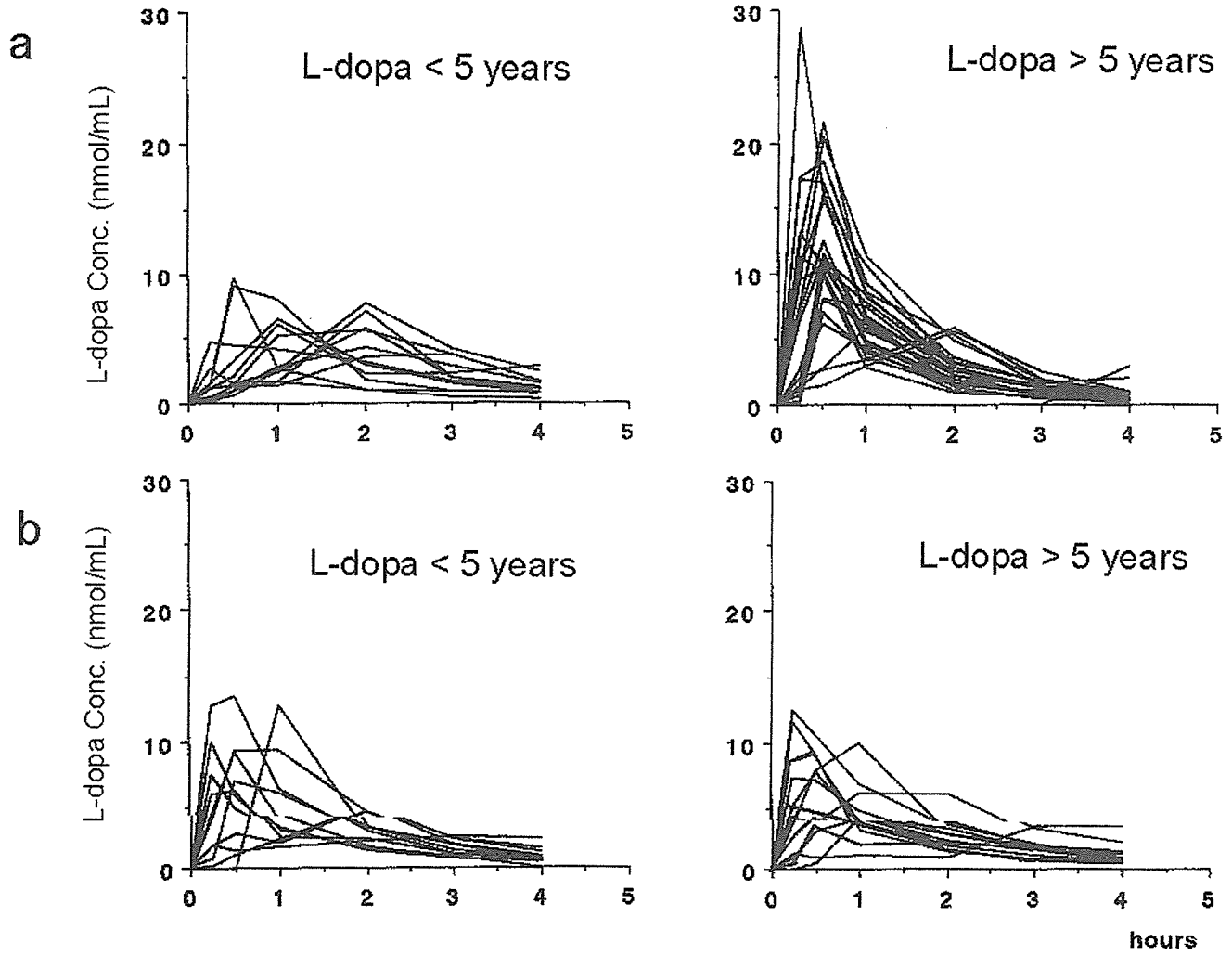

Fig. 2 L-dopa pharmacokinetics in parkinsonian patients with disease onset at < 60 years of age (**a**) and > 60 years of age (**b**) according to duration of L-dopa therapy. Long-term L-dopa therapy (> 5 years' duration) increases C_{max} and AUC and shortens $T^1/_2$. Pharmacokinetic changes after long-term L-dopa therapy are not seen in the senile-onset group

tivation was suggested as a cause of this phenomenon [9]. In fact, long-term L-dopa administration activates DDC in the liver but not in the brain, and no data has been published in the gut [10]. Our data was obtained using L-dopa with a DCI. It has been reported that plasma DDC is induced by administration of L-dopa with a DCI [11]. Therefore, the DDC activation theory cannot explain our results. We propose that long-term L-dopa therapy may induce the LNAA transporter system.

Aging and L-dopa pharmacokinetics

Although long-term L-dopa therapy steepened L-dopa kinetics, this change was less marked in senile-onset patients (onset age > 60 years old) than in younger onset patients (Fig. 2b). The frequency of wearing-off is much lower in senile-onset patients than in younger onset patients [12]. This suggests that changes in peripheral L-dopa pharmacokinetics after long-term therapy certainly contribute to the clinical expression of wearing-off.

Food and acidity effects on L-dopa absorption

L-dopa shares a saturable transporter system with other LNAA such as phenylalanine. Therefore, competitive inhibition of L-dopa absorption occurs with rising concentrations of neutral amino acids derived from food (Fig. 3). The L-dopa pharmacokinetic profile is steeper when intake occurs before a meal than after a meal. Considering that pulsative stimulation of L-dopa may cause motor fluctuations, L-dopa should be given after meals

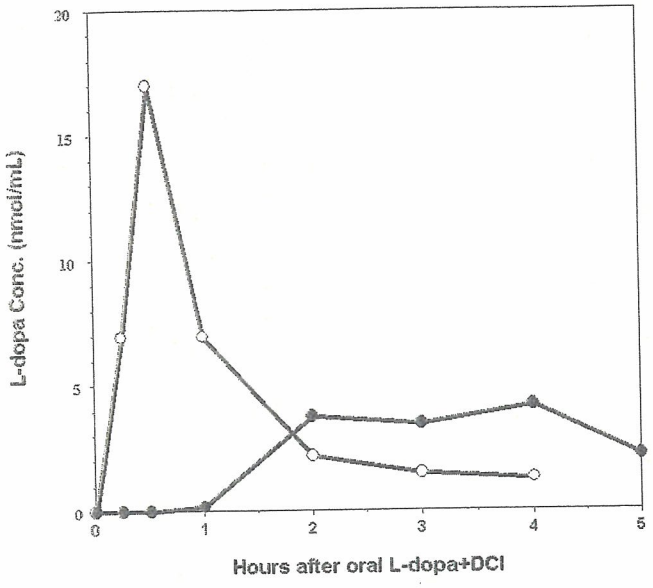

Fig. 3 Effects of a meal on L-dopa kinetics in a 55-year-old female patient with Parkinson's disease (○ L-dopa administration before meal, ● L-dopa administration after meal). C_{max} and AUC were markedly decreased and T_{max} was increased by L-dopa administration after a meal

whenever possible, even if it necessitates a higher L-dopa dose.

L-dopa is known to be easily soluble in acid environments and the pH of gastric juices affects the absorption of L-dopa. Fig. 4 shows the results of an L-DOPA test from a 65-year-old male patient with PD. The first test was performed using the ordinary method and, 1 year later, the second test was performed using duodenal infusion of L-dopa. Although the pH of duodenal juice is high, absorption was not impaired because L-dopa was administered dissolved in water. When it is dissolved in water, L-dopa is absorbed rapidly and adequately, even in alkaline duodenal juice.

L-dopa and 3-O-methyl DOPA

The main metabolite of DOPA is dopamine, formed by decarboxylation, and the COMT pathway is usually a rather minor pathway in the metabolism of DOPA. However, when L-dopa is used with a DCI, the COMT pathway is activated and a large amount of 3-O-methyl DOPA (3OMD) is synthesized. The $T_{1/2}$ of 3OMD (16 h) is much longer than that of DOPA; thus, the plasma concentration of 3OMD increases according to long-term L-dopa therapy (Fig. 5). Although the plasma concentration of 3OMD is usually closely correlated to daily L-dopa dose (Fig. 6), some patients show very low 3OMD concentrations relative to the L-dopa dose and plasma L-dopa concentration. These patients may obtain a good response with a COMT inhibitor. As COMT inhibitors will be approved for PD therapy in Japan this year, it will be important to assess this hypothesis soon.

3OMD also uses the LNAA transporter system in the gut and BBB. After protein-rich meals, competition between L-dopa, dietary LNAA and 3OMD for gut and BBB transport may further contribute to motor fluctuations.

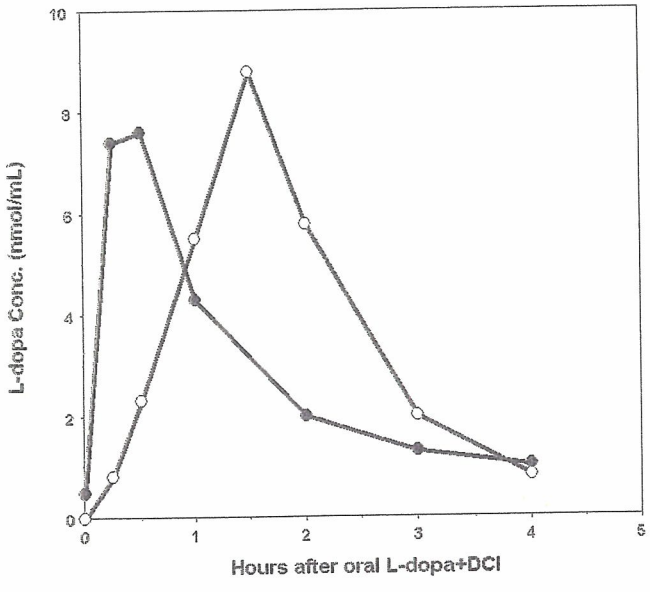

Fig. 4 Effects of duodenal infusion of L-dopa in a 65-year-old male patient with Parkinson's disease (○ L-dopa administration by tablet orally, ● L-dopa administration by duodenal infusion in water suspension). L-dopa concentration is rapidly and adequately increased by duodenal infusion

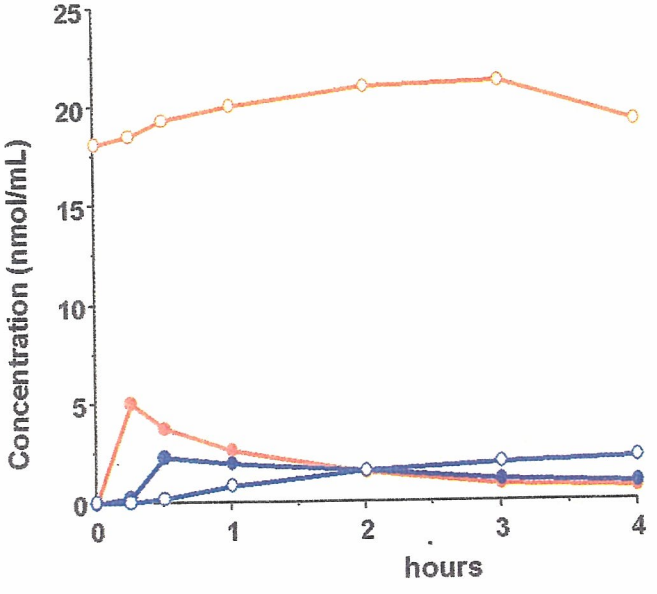

Fig. 5 Plasma concentration of dopa and 3OMD in PD patients (○ concentration of 3OMD, ● concentration of dopa, red line: long-term L-dopa therapy, blue line: L-dopa initial use)

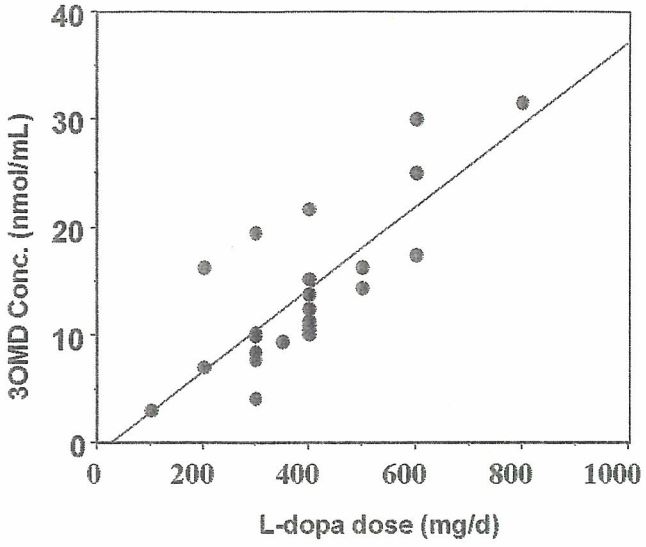

Fig. 6 Relationship between L-dopa daily dose and plasma concentration of 3OMD in PD patinets

Following L-dopa administration without a DCI, the plasma dopamine concentration is greatly increased and the 3OMD concentration is very low; however, L-dopa administration with a DCI results in synthesis of a large amount of 3OMD in plasma (Fig. 7) by activating the COMT pathway. If L-dopa is administered in combination with both a DCI and a COMT inhibitor, new metabolic pathways may be activated such as enhanced quinine formation [13].

Conclusion

Peripheral L-dopa kinetics closely reflects dopamine kinetics in the striatum so that measurements of L-dopa kinetics are useful for treating patients with PD. L-dopa is transported by a saturable active transporter system (LNAA system) in the gut and BBB. Onset age of PD, treatment duration, and food are greatly influence peripheral L-dopa kinetics. Food and onset age decrease C_{max} and prolong T_{max} and $T_{1/2}$. When L-dopa is administered with a DCI, the COMT pathway is activated and the COMT inhibitor becomes an important factor for peripheral L-dopa kinetics.

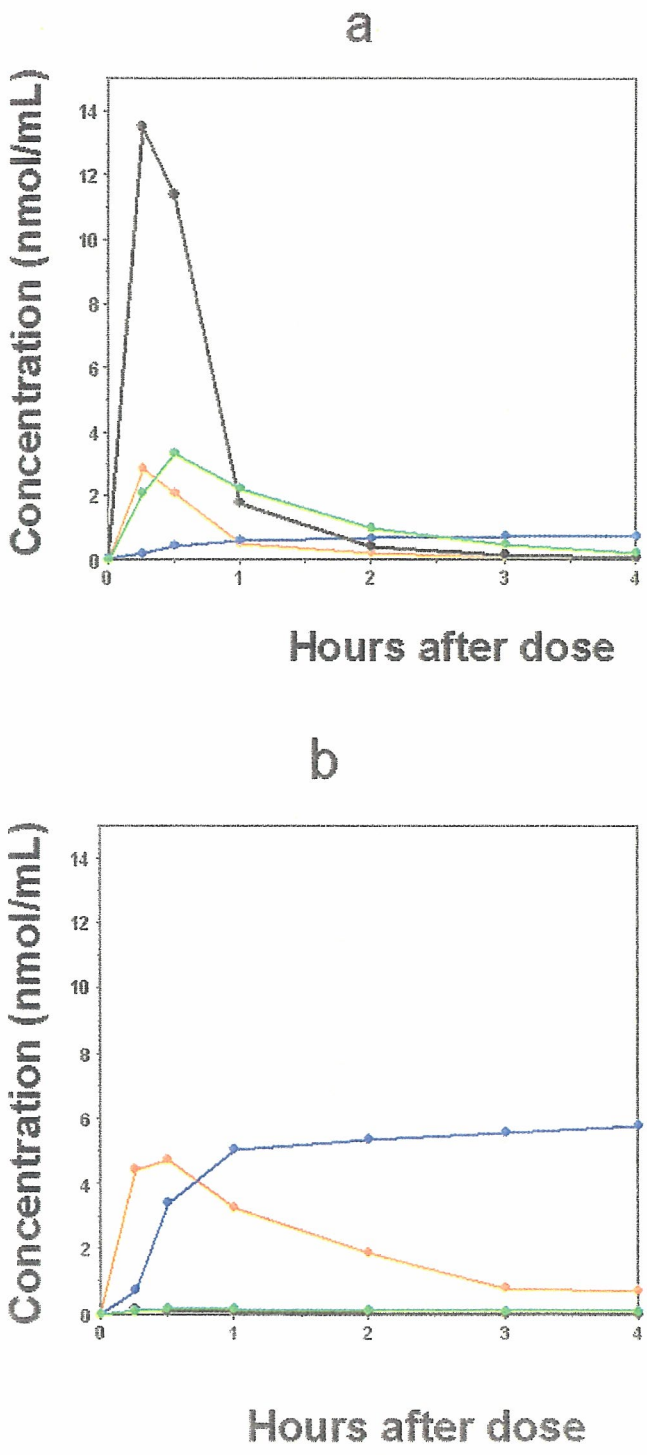

Fig. 7 Plasma concentration of DOPA and its metabolites after oral administration of L-dopa (100 mg) without DCI (**a**), and oral administration of L-dopa (100 mg) with DCI (benserazide 25 mg) (**b**) of the same PD patient (red: DOPA; black: dopamine; blue: 3OMD; green: homovanillic acid (HVA))

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CME

Zonisamide improves motor function in Parkinson disease

A randomized, double-blind study

Miho Murata, MD, PhD; Kazuko Hasegawa, MD, PhD; Ichiro Kanazawa, MD, PhD; and The Japan Zonisamide on PD Study Group*

Abstract—*Objective:* To evaluate the efficacy, safety and tolerability of daily doses of 25, 50, and 100 mg of zonisamide (ZNS) administered as adjunctive treatment in patients with Parkinson disease (PD). *Methods:* We conducted a multicenter, randomized, double-blind, parallel-treatment, placebo-controlled study in Japan. Patients with PD who showed insufficient response to levodopa treatment were given placebo for 2 weeks and then treated for 12 weeks with 25, 50, or 100 mg/day of ZNS or placebo, in addition to levodopa, followed by a 2-week dose-reduction period. The primary endpoint was change from baseline in the total score of the Unified Parkinson's Disease Rating Scale (UPDRS) Part III at the final assessment point. Secondary endpoints included changes from baseline in total daily "off" time; total scores of UPDRS Parts I, II, and IV; and Modified Hoehn and Yahr Scale score. Safety analysis was based on the incidence of adverse events. *Results:* There was significant improvement in the primary endpoint in the 25-mg and 50-mg groups vs placebo. The duration of "off" time was significantly reduced in the 50-mg and 100-mg groups vs placebo. Dyskinesia was not increased in ZNS groups. The incidence of adverse effects was similar between the 25-mg, 50-mg, and placebo groups but higher in the 100-mg group. *Conclusions:* Zonisamide is safe, effective and well tolerated at 25 to 100 mg/day as an adjunctive treatment in patients with Parkinson disease.

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Zonisamide (ZNS) (1,2-benzisoxazole-3-methanesulfonamide) is an antiepileptic drug with a long-half life ($T_{1/2}$ = 62 hours) that was originally synthesized in Japan.[1] ZNS has been used to treat epilepsy in Japan for more than 10 years; is currently approved for marketing in the United States, Europe, and Korea; and is generally well tolerated. We reported previously that ZNS has beneficial effects on PD in one patient with convulsion attacks.[2] Based on this finding, we subsequently performed an open trial in nine patients with PD and found that ZNS improved the main symptoms of PD, with particular benefits on motor fluctuation, known as "wearing-off."[2] Then, we conducted a small double-blind study that showed a daily dose of 50 to 100 mg of ZNS as an adjunct therapy significantly improved limb rigidity, tremor, and postural instability in patients with advanced PD and was well tolerated.[3]

In this study, we sought to confirm ZNS effectiveness as an adjunctive treatment for PD by evaluating the efficacy, safety, and tolerability of daily oral doses of 25, 50, and 100 mg of ZNS (once a day) in a large population of patients with PD who showed insufficient response to levodopa treatment.

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the January 2 issue to find the title link for this article.

Methods. This was a multicenter, randomized, double-blind, parallel-treatment, placebo-controlled study of ZNS as adjunctive treatment in patients with PD who showed insufficient response to levodopa (including dopa decarboxylase inhibitor: DCI combination drugs). Fifty-eight institutions throughout Japan participated in the study during the study period of January 15 to December 1, 2004.

Patients with PD of both sexes between ages 20 and 80 years were enrolled in the study. Patients who exhibited any problems based on levodopa therapy, such as wearing-off phenomena, "on"–"off" phenomena, and freezing phenomena, no-"on" and delayed-"on," or in whom the suboptimal dose of levodopa had been administered because of side effects or therapeutic strategy were not excluded from the study. Patients had received individual dosages of levodopa (plus a DCI) and were stable for at least 28 days before study initiation. Patients who fulfilled the above criteria were enrolled into the study by the investigators at each participating institution. Patients who met the above criteria and provided informed consent were randomized into the treatment groups of 25, 50, or 100 mg/day ZNS or placebo.

The study consisted of a 2-week run-in period of single-blind treatment with placebo, a 12-week double-blind treatment period, and a 2-week double-blind dose-reduction period (figure E-1 on the *Neurology* Web site at www.neurology.org), with the exception

*See the appendix for a full list of study participants.

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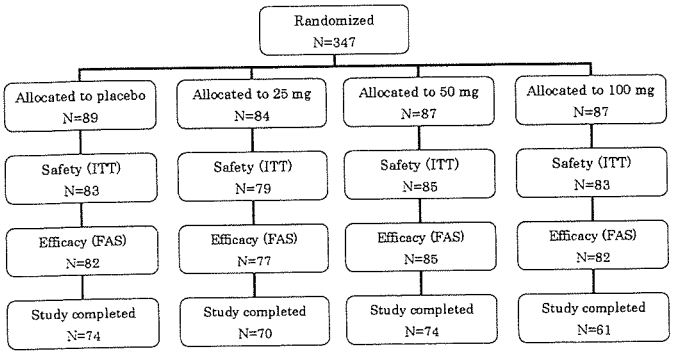

Figure 1. Patient disposition. ITT = intent-to-treat; FAS = full analysis set.

that the 25-mg group did not undergo dose reduction. Baseline assessment was conducted after a 2-week run-in period to reduce placebo effects. Clinical assessment including the Unified Parkinson's Disease Rating Scale (UPDRS) and Hoehn and Yahr staging was conducted at "on" state every 2 weeks.

The daily dosage was administered orally once a day in the morning as four tablets in the run-in and treatment periods and two tablets in the dose-reduction period. Study medication was dispensed as ZNS 25 mg tablets or matching placebo. Patients were randomized to one of the four treatment groups in blocks of size 8 (2 patients per group) during the run-in period using a randomization code generated by the study sponsor or designee. Study medication, indistinguishable by appearance, packaging, and labeling, was provided to each institution, and 1-week supplies were dispensed to patients according to the randomization code.

Patients were required to have concomitant administration with levodopa preparations including DCI combination drugs and were allowed to continue with other anti-Parkinson medications, such as dopamine receptor agonists (DAs), monoamine oxidase type B (MAO-B) inhibitors, anticholinergics, amantadine, or droxydopa, during the study. The dose regimens of these concomitant medications were to be maintained from 4 weeks before study initiation until the end of the dose-reduction period, except as required to alleviate dyskinesia or psychotic symptoms that were likely caused by dopaminergic-receptor hyperstimulation due to concomitant medication.

The primary endpoint was a change from baseline in the total score of UPDRS Part III (motor examination score) at the final assessment point. Secondary endpoints included a change from baseline in total daily "off" time as determined from patients' diaries, and changes from baseline in UPDRS Part I, II, and IV scores and Modified Hoehn and Yahr Scale score. Changes from baseline at the assessment point were analyzed by analysis of covariance using treatment group as a factor, and baseline value and treatment group scores were compared with placebo using the Dunnett test. A significance level of 0.05 (two-sided) was used for intergroup comparison, except for homogeneity assessment, when a significance level of 0.15 (two-sided) was used. The planned sample size of 80 patients per group (320 patients in total) was selected based on a requirement of 69 patients per group to achieve 80% power for comparison between placebo and each of the ZNS groups, assuming a between-group difference of 5.5 and an SD of 10.0 on the primary endpoint as seen in a preliminary study.[3] Multiplicity was taken into consideration in the primary analysis, but not in the secondary analysis or assessment of the dose–response relationship. Subgroup subset analysis was performed for the primary endpoint. Safety assessment was based on the incidence of adverse events including abnormalities of clinical/laboratory examinations and the incidence compared between the treatment groups by χ^2 test. Demographic and efficacy analyses were performed on the full analysis set (FAS), and safety assessments were performed on the intent-to-treat (ITT) population.

Results. Patient disposition is summarized in figure 1. Of the 347 screened and randomized patients, 279 patients (80.4%) completed the protocol as planned. There were no major differences between groups except that markedly fewer patients in the ZNS 100 mg group completed the study. The ITT population consisted of 330 patients (95.1%), with 83 patients in the placebo group, 79 in the 25-mg group, 85 in the 50-mg group, and 83 in the 100-mg group. A total of 6 patients in the placebo group, 5 in the 25-mg group, 2 in the 50-mg group, and 4 in the 100-mg group were not included in the ITT population because of withdrawal of consent or dosing regimen violation. The FAS consisted of the ITT minus 4 patients: 2 in the 25-mg group and 1 in each of the placebo and 100-mg groups because of no efficacy data during and after treatment period. Of the 326 patients (FAS), 279 patients completed the therapy period, and 47 patients discontinued therapy prematurely (8 patients in the placebo group, 7 in the 25-mg group, 11 in the 50-mg group, and 21 in the 100-mg group). The most common reason for discontinuation was adverse events (4 patients in the placebo group, 5 in the 25-mg group, 4 in the 50-mg group, and 9 in the 100-mg group). There were no Good Clinical Practice deviations in this study.

Table 1 shows the demographic background of patients in the placebo and ZNS treatment groups. There were no major differences between groups with respect to patients' background, including disease and treatment histories. The mean morbidity period was 8.6 years, and the mean modified Hoehn and Yahr Scale score ("on") was 2.5. The mean number of concomitant anti-Parkinson medicines was 3.2. The most common concomitant medications were DAs, which were used by 91.7% of the patients, and MAO-B inhibitors, which were used by 51.5% of the patients.

The changes (least-squares mean ± SE) in UPDRS Part III total score from baseline at final assessment were as follows: placebo group, −2.0 ± 0.8; 25-mg group, −6.3 ± 0.8; 50-mg group, −5.8 ± 0.8; and 100-mg group, −4.6 ± 0.8 (figure 2). All treatment groups showed decreases of UPDRS Part III total scores from baseline, but the improvement was significant for the 25-mg ($p = 0.001$, Dunnett test) and 50-mg ($p = 0.003$, Dunnett test) groups, vs the placebo group.

The proportions of responders, defined as patients with ≥30% reduction in UPDRS Part III total score from baseline at final assessment, were as follows: placebo group, 22.0% (18/82); 25-mg group, 35.1% (27/77, $p = 0.067$, χ^2 test vs placebo group); 50-mg group, 38.8% (33/85, $p = 0.018$, χ^2 test vs placebo group); and 100-mg group, 31.7% (26/82, $p = 0.158$, χ^2 test vs placebo group).

The degree of change for the primary endpoint were similar in the 25-mg and 50-mg groups, and these were greater than in the 100-mg group and significantly greater than in the placebo group. Subgroup analyses indicated no significant effects in subject baseline characteristics including with or without MAO-B inhibitor (table E-1) on the primary endpoint.

The mean decrease in total "off" time from baseline at final assessment is shown in figure 3. The mean changes in "off" time (hours) from baseline were as follows: placebo group, −0.20 (n = 61); 25-mg group, −0.22 (n = 58); 50-mg group, −1.30 (n = 68); and 100-mg group, −1.63 (n = 52). The duration of daily "off" time decreased for all treatment groups with improvement in the 50-mg ($p = 0.014$, Dunnett test) and 100-mg ($p = 0.013$, Dunnett test) groups compared with the placebo group.

Table 1 *Demographic and baseline characteristics of patients according to the dose of zonisamide*

		ZNS		
	Placebo	25 mg/day	50 mg/day	100 mg/day
n	82	77	85	82
Age, years	65.3 (7.5)	65.1 (8.5)	63.9 (9.4)	65.7 (8.6)
Older than 65 years	47 (57.3%)	42 (54.5%)	46 (54.1%)	53 (64.6%)
Men	41 (50.0%)	42 (54.5%)	51 (60.0%)	47 (57.3%)
Duration of PD, years	8.9 (5.8)	8.5 (4.6)	8.6 (6.0)	8.5 (5.6)
Dose of l-dopa, mg/day	351.2 (138.8)	355.5 (115.6)	363.9 (177.4)	327.7 (118.2)
Wearing-off	67 (81.7%)	64 (83.1%)	74 (87.1%)	62 (75.6%)
Dyskinesia	28 (34.1%)	18 (23.4%)	33 (38.8%)	22 (26.8%)
+ Dopamine agonist	80 (97.6%)	76 (98.7%)	85 (100.0%)	80 (97.6%)
+ MAO-B inhibitor	42 (51.2%)	38 (49.4%)	43 (50.6%)	45 (54.9%)
UPDRS Part III	22.9 (10.7)	26.5 (13.0)	22.5 (13.1)	22.7 (11.6)
H-Y ("on")	2.60 (0.72)	2.68 (0.76)	2.49 (0.80)	2.60 (0.77)
H-Y ("off")	3.52 (0.80)	3.64 (0.80)	3.49 (0.90)	3.40 (0.77)
"Off" time, hours	7.13 (3.45)	6.76 (3.13)	6.51 (2.30)	7.62 (3.03)

Data are mean (SD) or number (%).

ZNS = zonisamide; PD = Parkinson disease; H-Y = Modified Hoehn and Yahr Scale score.

There were no significant differences between the ZNS and placebo groups with respect to changes from baseline in UPDRS Parts I, II, and IV scores and in the Modified Hoehn and Yahr Scale score.

Some patients showed increased duration of dyskinesia with increase of "on" time; however, the frequency of dyskinesia was not increased in the entire ZNS group compared with the placebo group. Further analysis showed a decrease in disabling dyskinesia (UPDRS Part IV, No. 33) in the 50-mg group (table 2). In addition, the basal dose of levodopa did not correlate with worsening or improvement of dyskinesia.

There was no significant difference in incidence of adverse events between the 25-mg (a total of 164 adverse events reported by 70.9% [56/79] of the patients) and 50-mg (195 adverse events reported by 72.9% [62/85] of the patients) groups, compared with the placebo group (153 adverse events by 65.1% [54/83] of the patients). However, the incidence of adverse events was significantly higher in the 100-mg group (204 adverse events reported by 79.5% [66/83] of the patients) compared with the placebo group ($p = 0.037$, χ^2 test). Adverse events with an incidence of greater than 5% in the ZNS group are presented in table 3. Adverse events for which the incidence was greater in the total ZNS than in the placebo group were somnolence (10.9%), apathy (8.5%), decrease in body weight (6.9%), and constipation (6.5%). Adverse events for which the incidence in the total ZNS was less than that of the placebo

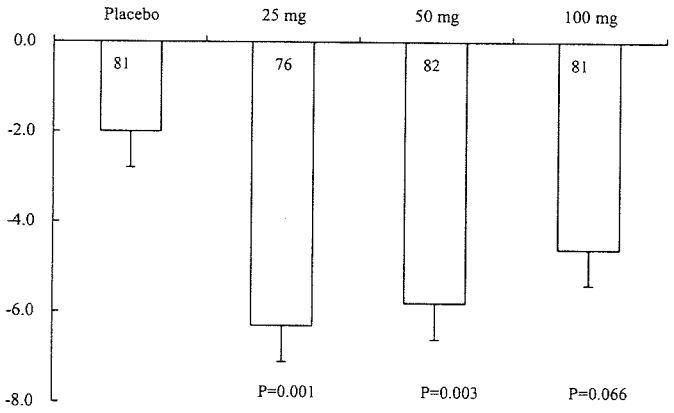

Figure 2. Changes in Unified Parkinson's Disease Rating Scale (UPDRS) Part III total score induced by zonisamide (ZNS) treatment from baseline to end of study (least-squares mean ± SE). Numbers indicate patient numbers. The total score of UPDRS Part III decreased after treatment in the 25-mg/day ($p = 0.001$) and 50-mg/day ($p = 0.003$) ZNS groups compared with the placebo group.

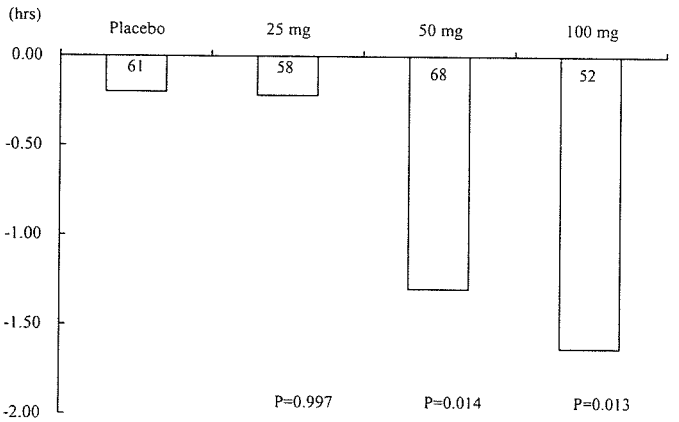

Figure 3. Changes from baseline in mean daily "off" time (hours) induced by treatment with zonisamide (ZNS). Numbers indicate patient numbers. "Off" time decreased after treatment with ZNS in the 50-mg/day (−1.3 hours, $p = 0.014$) and 100-mg/day (−1.63 hours, $p = 0.013$) groups compared with the placebo group.

Table 2 *Changes in dyskinesia during 12-week treatment of zonisamide combined with anti-Parkinson disease drugs*

		ZNS		
	Placebo	25 mg/day	50 mg/day	100 mg/day
Dyskinesia				
Baseline	28	18	33	22
Final assessment	28	21	29	22
Post-ZNS improvement	4	4	8	0
Post-ZNS worsening	1	2	5	4
Appearance during ZNS	1	5	0	1
Disabling dyskinesia				
Baseline	19	7	17	11
Final assessment	17	9	12	11
Post-ZNS improvement	5	1	7	3
Post-ZNS worsening	3	0	0	0
Appearance during ZNS	1	2	0	3

Data are number of patients. Dyskinesia (UPDRS Part IV, No. 32), Disabling dyskinesia (UPDRS Part IV, No. 33).

ZNS = zonisamide.

group were dizziness (5.7%), decrease in appetite (10.1%), and increase in serum creatinine phosphokinase (7.3%).

Discussion. In this study, ZNS adjunctive therapy significantly improved PD symptoms vs placebo, as indicated by the significant improvement in the UDPRS Part III total score for the primary endpoint in the 25-mg and 50-mg groups and significant mean decrease in total "off" time in the 50-mg and 100-mg treatment groups. The improvement in "wearing-off" was similar to the effects seen with rasagiline and entacapone, although neither drug improved the UPDRS Part III total score.[4] Although the randomized patients of our study used many anti-Parkinson concomitant medicines, they did not meet the requirements of adequate treatment of PD because of the attenuation of the beneficial effects. ZNS treatment improved all main PD symptoms in these patients, including tremor, similarly to previous reports.[2,3,5]

Interestingly, administration of ZNS did not increase the frequency of dyskinesia, and the frequency of both dyskinesia and disabling dyskinesia improved in the 50-mg group. The reason for the improvement in parkinsonian symptoms and disabling dyskinesia is not known at present. ZNS is not a glutamate antagonist, but it reduces glutamate release[6] and increases neuronal transporter excitatory amino acid carrier 1.[7] These actions of ZNS on the glutamate system may mediate the improvement of dyskinesia seen in our patients.

In this study, the mean basal levodopa dose was approximately 350 mg/day, although it is lower than that used in Western countries. In Japan, many physicians are using lower doses from therapeutic strategy and patients' preference for not having troublesome side effects. Cultural difference between Japan and Western countries may also affect the maintenance dose. Furthermore, the effective dopa plasma level in Western PD patients is 2,000 to

Table 3 *Adverse effects associated with zonisamide treatment with an incidence of ≥5%*

		ZNS			
	Placebo	All patients	25 mg/day	50 mg/day	100 mg/day
Somnolence	4.8	10.9	1.3	15.3	15.7
Apathy	6.0	8.5	7.6	7.1	10.8
Dizziness	7.2	5.7	3.8	5.9	7.2
Reduced appetite	14.5	10.1	5.1	8.2	16.9
Weight loss	4.8	6.9	7.6	3.5	9.6
Constipation	3.6	6.5	6.3	8.2	4.8
Increased in serum CK	8.4	7.3	8.9	8.2	4.8

Data are presented as percentage of incidence.

ZNS = zonisamide; CK = creatinine phosphokinase.

4,000 ng/mL[8] but that of Japanese patients is around 500 (max 1,000) ng/mL (unpublished data; data from 200 Japanese PD patients). Race, amount of protein intake, and physique may explain the difference in the effective levodopa dose between Western countries and Japan. The above data indicate that our patients were not undertreated with anti-PD drugs. In fact, our patients, like other Japanese patients with PD, developed treatment-related adverse effects during maintenance therapy using levodopa with or without other drugs. Nevertheless, further studies are necessary to evaluate ZNS in patients with PD treated with anti-PD drugs at doses commonly used in Western countries.

We started the study with a run-in period of single-blind treatment with placebo to minimize placebo effects. To the best of our knowledge, this is the most rigorous study design used to date for the evaluation of anti-Parkinson effects. Our study design may explain the lower response rate in the ZNS groups (although still significantly higher than placebo in the 25-mg and 50-mg study groups) than that of previous reports for pramipexole.[9]

Although there was a higher incidence of adverse events in the 100-mg group than in the other treatment groups, the incidence of hallucination and dyskinesia, which are typically of concern with anti-Parkinson drugs, was the same across all treatment groups, indicating that a once-daily dose of 25 to 100 mg of ZNS is well tolerated.

Although the present study was only of 12 weeks' duration, our preliminary data showed that the benefits observed at 12 weeks were maintained for more than 1 year in all 17 patients in a study on the long-term effects of ZNS on PD. Another study that was designed to assess the long-term (up to 1 year) effects of ZNS on PD (n = 100) also showed that 12-week course of ZNS improved parkinsonian symptoms and that such effects were maintained for up to 1 year (manuscript in preparation).

It is notable that the typical dose of ZNS is 300 to 600 mg/day for epilepsy, but a significant improvement in motor symptoms was noted in our patients with PD with only 50 mg/day of ZNS. This suggests that the mechanism of action of ZNS in PD may be different from those in epilepsy. In this regard, ZNS has multiple mechanisms of action. The major effect of ZNS in epilepsy is modification of neuronal firing at high frequency through enhancement of sodium channel inactivation and reduction of T-type calcium current.[10-13] ZNS has no affinity to γ-aminobutyric acid (GABA) type A receptor or glutamate receptors[11] but is known to increase GABA[6] and glutamate[7] release. In the dopaminergic system, therapeutic doses of ZNS (20 and 50 mg/kg) increase intracellular and extracellular dopamine levels in the rat striatum.[14-16] Conversely, supratherapeutic doses of ZNS reduce intracellular dopamine. Thus, ZNS has biphasic effects on the dopamine system. We reported previously that at therapeutic levels, ZNS increased dopamine synthesis by increasing tyrosine hydroxylase (TH) activity and TH messenger RNA.[14] ZNS also affects MAO-B activity. The IC_{50} (50% inhibitory concentration) value of MAO-B in liver microsomal fraction was 600 μM, and that in striatal membrane fraction was 28 μM.[14,17] These data suggest ZNS inhibits striatal MAO-B activity but not peripheral MAO-B activity, and therefore ZNS may have little effect on peripheral MAO-B inhibition of functions such as blood pressure.

Zonisamide has no affinity to dopamine receptors (D1–D5) or dopamine transporter. ZNS also has no direct effects on glutamate receptors, adenosine receptors, or serotonin receptors, which have been suggested as possible sites of action for anti-PD drugs, other than the dopaminergic system.[14] We proposed previously that activation of dopamine synthesis and moderate inhibition of MAO-B are the main mechanisms that mediate the effects of ZNS in PD.[14] However, the present finding of lack of change in the efficacy of ZNS when coadministered with an MAO-B inhibitor suggests that MAO-B inhibition is not a main factor. We consider that the primary mechanism of action of ZNS in PD is to increase dopamine synthesis. Whether sodium channel inactivation or T-type calcium channel inhibition is involved in ZNS effects has not been elucidated yet. Further investigation is needed to clarify the mechanism of the beneficial actions of ZNS on PD.

Appendix

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Leucine-Rich Repeat kinase 2 G2385R variant is a risk factor for Parkinson disease in Asian population

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To assess the effect of genetic factors on sporadic Parkinson disease, we performed a case–control study of a variant (G2385R) in *Leucine-Rich Repeat kinase* 2 among the Japanese population. The G2385R (c.7153G>A) variant was reported as a risk factor for sporadic Parkinson disease in the Chinese population from Taiwan and Singapore. Genotyping was conducted in 448 Parkinson disease patients and 457 healthy controls. The frequency of A allele in Parkinson disease was significantly higher than in the control ($P=1.24\times10^{-4}$, odds ratio 2.63, 95% confidence interval 1.56–4.35). Our results suggest that the G2385R variant is a risk factor for sporadic Parkinson disease in the Asian population. *NeuroReport* 18:273–275 © 2007 Lippincott Williams & Wilkins.

Keywords: *Leucine-Rich Repeat kinase 2*, risk factor, single nucleotide polymorphisms

Introduction

Parkinson disease (PD) is one of the most frequent neurodegenerative diseases characterized by resting tremor, rigidity, bradykinesia, and postural instability. PD is thought to be a multifactorial disease caused by a combination of aging, environmental, and genetic factors. Although the majority of patients of PD are of sporadic type, some genes have been identified as a monogenic causative gene by molecular genetic studies for familial PD [1–6]. *Leucine-Rich Repeat kinase 2* (*LRRK2*) has been identified as a causative gene associated with autosomal dominant familial PD [7,8]. To date, many pathogenic substitutions in *LRRK2* have been identified in familial and sporadic PD [9]. The G2385R variant (c.7153G>A) in *LRRK2* was reported recently as a risk factor for sporadic PD in the Chinese population from Taiwan and Singapore [10,11]. This variant was identified originally as putative pathogenic mutation in a small Taiwanese PD family and was not found in Caucasians [12]. Thus, it is possible that the G2385R variant is a risk factor in Asian sporadic PD. To test this hypothesis, we conducted a case–control study to evaluate the association between the G2385R genotype and the risk for PD in the Japanese population.

Methods

Subjects and genomic DNA

Genomic DNA was isolated from 448 sporadic PD patients and 457 controls of the Japanese population by a standard protocol (Table 1). All PD patients had no family history of PD. PD patients with *parkin* or *PTEN-induced putative kinase 1 (PINK1)* mutation were not included in the study. Diagnosis of PD was adopted by the participating neurologists and was established on the basis of the United Kingdom Parkinson's Disease Society Brain Bank criteria [13]. This study was approved by the ethics committee of Juntendo University School of Medicine. All individuals gave an informed and signed consent form.

Genotyping

Exon 48 of *LRRK2* from each individual was amplified by polymerase chain reaction (PCR) using the primers and protocol described by Zimprich *et al.* [8]. The PCR products were sequenced directly using the BigDye Terminators v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). The reverse PCR primer was used as sequencing primer.

Statistical analysis

Statistical analysis included the Hardy–Weinberg equilibrium test, χ^2 test, Fisher's exact test, odds ratio and its 95% confidence interval (95% CI), using SNPAlyze v5.1 software (Dynacom, Chiba, Japan). The *t*-test was performed using JMP 6.0 (SAS Institute Japan, Tokyo, Japan). In all statistical analyses, *P* values of 0.05 or less were considered statistically significant.

Results

We analyzed the frequency of the c.7153G>A (G2385R) substitution in 448 patients and 457 controls. Genotypes of the controls and patients were concordant with Hardy–Weinberg equilibrium. The frequency of A allele in the patients was significantly higher than in the controls ($P=1.24\times10^{-4}$, odds ratio 2.63, 95% CI 1.56–4.35, Table 2). We also detected homozygous substitution for the G2385R variant in two patients; however, we detected only the heterozygous substitution in the controls. Concerning the age at onset, the G2385R carriers were somewhat older than the noncarriers in total patients and in those <50 years of age. In contrast, the age at onset was not significantly different between carriers and noncarriers aged ≥50 years (Table 3). The disease duration was not significantly different between carriers and noncarriers (data not shown).

Discussion

In this study, we observed the *LRRK2* G2385R variant in 11.6% (52/448) of sporadic PD patients. So far, many putative pathogenic mutations have been reported including the G2385R. We detected G2385R in both patients and controls (22/457: 4.8%, Table 2); thus, this variant is not a pathogenic mutation, but a single nucleotide polymorphism. These results were similar to the allele frequencies in the Chinese [10,11]. It is estimated that mutations of *LRRK2* are the most frequent among the causative genes for autosomal dominant familial PD so far. Indeed, only one mutation (G2019S) accounted for ~6.6% of familial PD and ~1.6% of sporadic PD in Caucasians [14–16]. Interestingly, the frequency of the G2019S mutation is ~40% in the familial PD of North African Arabs [17] and ~30% in the familial PD of Ashkenazi Jews [18], whereas the G2019S mutation is a much less common mutation in Asians [19,20]. It is likely that some differences of genetic background exist among Caucasians, North African Arabs, Ashkenazi Jews, and Asians. Although G2385R has been detected only in Asian population, some risk variations in PD such as α-synuclein would be found in not only Asians but also all ethnic groups [21–24].

Among patients with age at onset <50 years, the G2385R carriers were somewhat older than noncarriers. This might indicate that G2385R has no influence on early-onset PD, and that PD of patients with early-onset might be influenced by other genetic and/or environmental factors. In addition, there were no differences in any clinical features including age at onset among carriers with homozygous or heterozygous G2385R substitution and noncarriers. Although the G2385R might increase the risk of development of PD, it does not seem to have a clear effect on modifying the symptoms or worsening the progression of the disease.

The amino-acid G2385 is located in the WD domain of LRRK2. This domain is known to bind various proteins [9]. The WD domain of LRRK2 appears to play an important role in neuronal cells. Indeed, oxidative-stress-induced cell death was more enhanced by the overexpression of G2385R variant than wild-type LRRK2 using culture cells [11]. More studies are needed to understand the functional significance of the substitution of glycine to arginine.

Conclusion

In this study, we identified that the G2385R variant in *LRRK2* is a risk for PD in Japanese population. To combine with the result of Chinese population [10,11], this variant increases the risk of PD in Asian population. So far, multiple genomic loci have been identified as susceptibility loci for PD [25], suggesting that many genes have a synergistic influence on the development of PD.

Table 1 Age characteristics of individuals

	Patients	Controls
Total sample, *n* (%)	448 (100)	457 (100)
Male, *n* (%)	217 (48.4)	240 (52.5)
Female, *n* (%)	231 (51.6)	217 (47.5)
Age at onset (years)[a]	50.7 ± 14.6 (5–89)	—
Male[a]	49.1 ± 14.8 (5–89)	—
Female[a]	52.2 ± 14.2 (7–82)	—
Age at sampling (years)[a]	59.4 ± 13.8 (15–93)	43.8 ± 16.0 (21–98)
Male[a]	57.8 ± 14.7 (15–93)	43.8 ± 14.5 (23–92)
Female[a]	60.9 ± 12.7 (22–88)	43.9 ± 17.5 (21–98)

[a]Data are mean ± SD (range).

Table 3 Comparison of age at onset of PD patients

Age at onset (years)	Carriers (*n*)	Noncarriers (*n*)	*P*-value
<50	42.5 ± 5.8 (17)	37.1 ± 9.4 (180)	0.003
≥50	59.9 ± 7.0 (33)	61.6 ± 7.8 (209)	0.24
Total	54.0 ± 10.6 (50)	50.3 ± 14.9 (389)	0.03

Data are mean ± SD.
Patients without information about age at onset (two of carriers and seven of noncarriers) were excluded from this analysis.
PD, Parkinson disease.

Table 2 Association analysis of *LRRK2* G2385R variant

	Genotype, *n* (%)			Allele, *n* (%)			
	G/G	G/A	A/A	G	A	χ^{2} [a]	*P*-value[a]
Patients (*n*=448)	396 (88.4)	50 (11.2)	2 (0.4)	842 (94.0)	54 (6.0)	14.74	1.24×10^{-4}
Controls (*n*=457)	435 (95.2)	22 (4.8)	0 (0)	892 (97.6)	22 (2.4)		

LRRK2, *Leucine-Rich Repeat kinase 2*.
[a]Compared with the control.

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〔特集：蛍光ディファレンスゲル二次元電気泳動〕

蛍光ディファレンスゲル二次元電気泳動による糖尿病病態解析

山下 亮・鏑木康志

SUMMARY

Two-dimensional differential in-gel electrophoresis technology (2D DIGE) technique is highly useful for differential analysis of protein spots in two-dimensional differential gels. Utilizing this technique, we attempted to search for diabetes-related drug targets and biomarkers. In human hepatoma cell line, HepG2, we analyzed secretome in the presence or absence of a Liver X receptor agonist, TO-901317, and identified one of the up-regulated proteins in response to LXR activation as apolipoprotein E. We also evaluated nuclear proteome of cultured cells overexpressing insulin receptor substrate proteins, in which insulin-stimulated cell cycle progression is differentially regulated, and the gel pattern indicated that insulin-induced phosphorylation of a nuclear protein may be impaired in cells overexpressing cell cycle-suppressive insulin receptor substrate-3. In addition, to search for urinary markers of diabetic nephropathy using 2D DIGE, we analyzed urine samples in which most abundant proteins were removed by immunoaffinity depletion. These findings indicate that the 2D DIGE-based approach is useful for the discovery of disease-specific drug targets and diagnostic biomarkers.

Key words: secreted protein, diabetes, insulin signal, subcellular proteome, urinary proteome.

糖尿病や脂質代謝異常をはじめとする生活習慣病の急激的な増加に伴い，これらの疾患に関する研究の重要性が高まっている．現在の研究の動向としては，ゲノム情報を基に作成した網羅的アプローチ，同定された遺伝子を改変したモデル動物による疾患関連遺伝子の役割の解析，マイクロアレイを用いた遺伝子発現解析といった遺伝子を中心としたアプローチが主流となっている．ところが代謝性疾患の発症には遺伝因子のみではなく環境因子の寄与が大であり，生体内で生命現象を担うタンパク質への環境因子の影響については，分子・細胞レベルのメカニズムはほとんど未解明である．

本稿では，本研究室で蛍光ディファレンスゲル二次元電気泳動（2D DIGE）を中心としたプロテオーム解析手法を用いて行っている糖尿病やインスリン抵抗性，脂質代謝異常を対象としたいくつかのプロジェクトを以下に紹介する．

細胞由来分泌タンパク質のプロテオーム解析

脂肪組織は主なホルモン標的組織であり糖尿病の基礎研究において重要な試料となる．これまでの脂肪組織の概念として，エネルギーの貯蔵が主な役割と考えられていたが，最近ではレプチンやアディポネクチン，レジスチンといったアディポサイトカインと称される脂肪細胞から分泌され，生理活性を持つタンパク質の存在が明らかとなり，脂肪組織が内分泌器官であるという新たな概念が定着してきている．またアディポサイトカインの分泌異常は糖尿病や動脈硬化などの病態に関与しているという報告が多くなされており，これらの液性因子の疾患における意義が盛んに研究されている[1,2]．また最近では脂肪細胞[3]のみならずマクロファージ[4]や血管平滑筋[5]などから分泌されるタンパク質の網羅的解析も行われており，様々な細胞間や臓器間での分泌タンパク質を介したシグナルの伝達が示唆されている．またこのような細胞から分泌されるタンパク質

Proteomic analysis to investigate the pathogenesis of diabetes using 2D DIGE.

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の総体は“Secretome”と称され，プロテオーム解析やcomputational analysisによるシグナルペプチド予測などの解析から徐々に様々な細胞の分泌タンパク質が明らかになってきている．分泌タンパク質のプロテオーム解析には二次元電気泳動法や多次元クロマトグラフィー連結型タンデム質量分析法などが主に用いられている．先駆的に行われている脂肪細胞の分泌タンパク質のプロテオーム解析はいくつかのグループにより報告され，網羅的な探索と最近ではインスリンの刺激により変動する分泌タンパク質のLC-MS/MS法を用いたディファレンシャル解析も報告されている[6]．当研究室では脂肪組織と並んで主要なホルモン標的細胞である肝細胞から分泌されるタンパク質のプロテオーム解析を行っている．実験にはアルブミン合成能や糖新生などの肝特異的機能を持ち肝細胞のモデルとして汎用されているヒト肝癌由来HepG2細胞を用いた．HepG2細胞から分泌されるタンパク質のプロテオームマップはSWISS-2DPAGEで公開されているが約20種の比較的存在量が豊富なタンパク質だけで情報として乏しい[7]．われわれは，2D LC-MS/MS法によりHepG2細胞培養液中に含まれるタンパク質の網羅的な解析を行った．手法としては一般的なSecretome解析で行われるように，コンフルエント状態のHepG2細胞をphosphate-buffer saline（PBS）により数回の洗浄後に，fetal bovine serum（FBS）不含の培地にて24時間培養を行い，培地を回収した．さらに限外ろ過により濃縮・液置換した試料を解析に使用した．トリプシン消化したタンパク質試料は一次元目にイオン交換（SCX）クロマトグラフィー(Microtrap SCX, 1×8 mm 12 μm, Michrom BioResources)，二次元目に逆相（RP）クロマトグラフィー(MagicC18, 0.2×50 mm 5 μm, Michrom BioResources）を連結させた二次元HPLC（Magic 2002, Michrom BioResouces）で行った．イオン交換クロマトグラフィーはギ酸アンモニウムによる段階的な塩濃度勾配（0, 25, 50, 75, 100, 150, 250, 500 mM）による分離を行い，それぞれの溶出画分を0.1%ギ酸溶媒中に含むアセトニトリルを直線的な濃度勾配を用いた逆相クロマトグラフィーを行った．質量分析計にはエレクトロスプレーイオン源（AMR Inc.）を装備したLCQ-DECA XP Plus（Thermo Electron）を使用し，得られたMS/MSからBioworks™ソフトウェアにてタンパク質同定を行った．検索には，NCBIデータベースを用いた[8,9]．結果として89個のタンパク質が同定され，このうち分泌シグナルの有無をWebにて公開されているSignalIPにて検索を行ったところ，同定されたタンパク質の約半数はシグナルペプチドを持たないタンパク質であった．一般的なシグナルペプチドを持つ分泌タンパク質は小胞体で合成されゴルジ体に移行し細胞外に分泌されるという古典的な経路を介することが知られる．しかしながらN末端にシグナルペプチドをもたないで分泌されるタンパク質も多く存在することからその分泌過程は多岐にわたると考えられる．Volmerらも Secretome は古典的な経路と非古典的な経路などの様々な過程を介して培養液に分泌されるタンパク質を指すと，広義な定義をしている[10]．本研究からもHepG2細胞から既存の分泌タンパク質以外にも多くの存在が確認され，非古典的経路を介した分泌タンパク質の存在も示唆された．最近，核内受容体であるLXR（liver X receptor）の活性化によって肝細胞から分泌誘導され，生理活性を持つ分泌タンパク質の存在が報告されている[11]．LXRは脂肪酸中性脂肪代謝，コレステロール代謝を制御し，高脂血症や動脈硬化症などの疾患に深く関与すると考えられている．LXRの肝臓における脂質代謝の調節分子機構としては，主として肝臓において発現するLXRαアイソフォームが，RXR（retinoid X receptor）とヘテロダイマーを形成して，脂質代謝の調節関連遺伝子の転写調節を行うことが知られている．さらにLXRによって分泌誘導されるAngiopoietin like-3が血中トリグリセリドやコレステロールを調節することがわかり，肝由来の分泌タンパク質を介した脂質代謝調節機構も明らかになってきた[11]．われわれはLXRアゴニストをHepG2細胞の培養液に添加し，培養上清中のタンパク質についてディファレンシャル解析を行った（Fig. 1）．実験としてLXRアゴニスト（TO-901317）を最終濃度1 μMになるようにHepG2の培地に添加し，24時間後に回収した培養上清を限外ろ過・濃縮して試料とした．それぞれの試料を異なるCyDye（Cy3またはCy5）にてラベルし，すべての試料を混ぜたものをCy2でラベルし内部標準として，各培地間のタンパク質プロファイルの差異を2D DIGE法にて解析した．具体的には，固定化pH勾配ゲル（pH＝4–7, 24 cm）を使用した一次元目等電点電気泳動後に，SDS-PAGEによる二次元目展開を行い，Typoon9400にてゲルイメージを取得後にDeCyderソフトウェア（GE Healthcare）により内部標準サンプルを介したゲル間のスポットマッチング，ディファレンシャル解析を行った．有意に変化を認めたスポットについては，ゲルをSYPRO Rubyによる染色後に再マッチングを行い，タンパク質スポットを切り出した．In-Gel Digestion法による酵素消化の後に，得られたペプチドをLC-MS/MS法にて解析した．Fig. 2に二次元電気泳動像を示す．DeCyderによる統計解析の結果，TO-901317による刺激によって，矢印で示したスポットが有意に増加変動していた．データベース検索の結果，この全てのスポットがapolipoprotein E（apoE）として同定された．さらに抗apoE抗体を用いた2-D Western blottingを行った（Fig. 3）．刺激前後の上清タンパク質を二次元電気泳動後，PVDF膜に転写し，まず全タンパク質をCy5 Mono-reactive Dye（GE Healthcare）にて染色し，続いて抗体反応を行った．抗体の検出試薬にはECL Plus（GE Healthcare）を用いた．Typoon9400（GE Healthcare）にて，それぞれの蛍光波長に

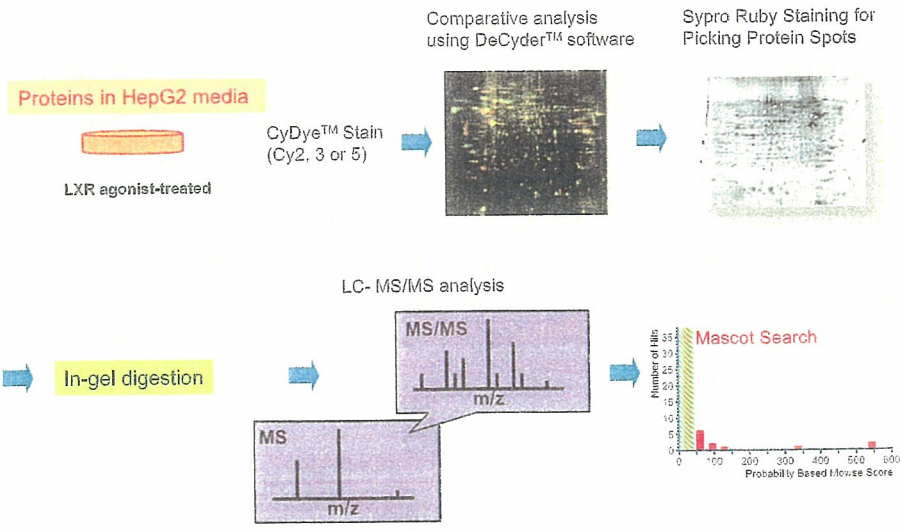

Fig. 1.　Flow chart of 2D DIGE-based proteomic analysis of secreted proteins in HepG2 cells.

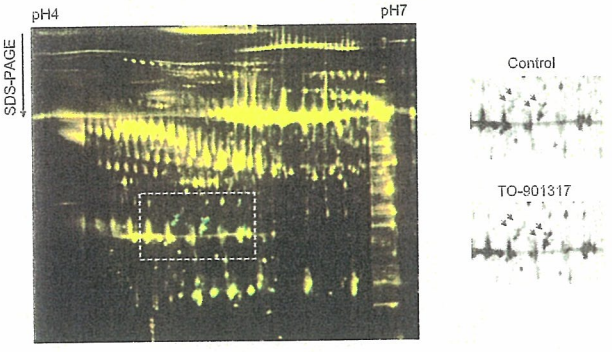

Fig. 2.　2D-DIGE analysis of secreted proteins in response to the LXR agonist T0901317 from HepG2 cells.

(A) Secreted proteins were labeled with Cy3 or Cy5, mixed, electrophoretically separated in the first dimension on pH 4–7 IPG strips and in the second dimension on a 10% polyacrylamide gel. Merge images of non-treated protein samples (red) and T0901317-treated protein samples (green). (B) Detailed 2-DE patterns of differentially expressed proteins in non-treated and T0901317-treated HepG2 cells.

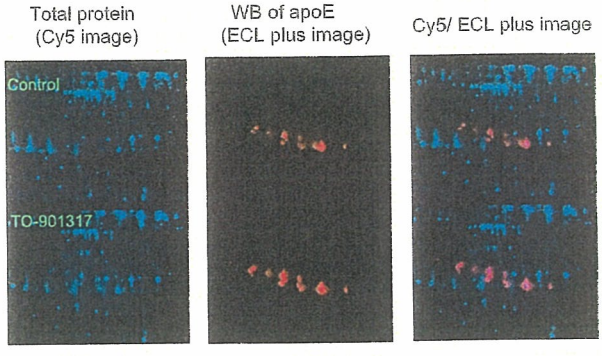

Fig. 3.　Detection of T0901317-induced increase in apoE secretion by 2-D Western blotting.

Secreted proteins in response to T0901317 treatment were separated by 2-DE, transferred to a membrane, and reacted with Cy5 mono-reactive dye (Cy5 image, blue) followed by probing with anti-apoE antibodies (ECL Plus image, red).

てスキャンし，ImageQuant ソフトウェア（GE Healthcare）にて画像解析した．Cy5 Mono-reactive Dye 染色と 2D DIGE 像とは，ほぼ同様の像が得られ，また同一メンブレン上で 2 波長にてスキャンすることで，全タンパク質と抗体反応と像の正確な重ねあわせが可能となる．Fig. 3 に示すように 2-D Western blotting の結果からも LXR リガンドによって apoE の分泌が増加していることが確認された（赤色：全タンパク質，青色：apoE）．さらに apoE は幅広い pI レンジと分子量にわたって複数のスポットとして点在していることも併せて確認され，このうちの一部は糖鎖等の修飾を受けていることが推定された．apoE は LDL レセプターのリガンドのひとつであり，脂質代謝に深く関与することが知られる．これより，LXR は肝臓からのいくつかの分泌タンパク質の誘導を促し，他の臓器においても遠隔的に脂質代謝の調節を行うことが予想された．

インスリンシグナルに関与するタンパク質のプロテオーム解析

インスリンシグナル伝達機構の障害はインスリン抵抗性として知られ，2 型糖尿病をはじめとした多くの生活習慣病の発症に関与する[12]．インスリン受容体チロシンキナーゼの主要な細胞内基質である IRS（insulin receptor substrate）ファミリーは肝臓，骨格筋，脂肪組織といった古典的標的細胞をはじめとした様々な細胞において発現しており，インスリン受容体との相互作用の結果，IRS の C 末端側にある複数のチロシン残基がリン酸化される．リン酸化されたチロシン残基の多くは，様々なアダプタータンパク質と結合し，それぞれに固有の伝達経路を形成する[13]．IRS は固有のシグナル経路を介して，様々な組織特異的な生理作用を持つことが知られる．例えば肝臓においては IRS-1 と IRS-

2が重要な役割を担っており，糖新生，グリコーゲンや脂質の合成などの肝に特徴的な機能を調節していることが知られている．さらにタンパク質合成や細胞増殖，アポトーシスなど組織に共通した生理作用を持つことも知られている．またIRSアイソフォームのIRS-1，IRS-2，IRS-3各々に固有の経路や作用の存在が示唆されている[13]．これまでわれわれはIRSの糖代謝，アポトーシス抑制，細胞増殖における役割を検討しさらに，IRSの各ドメインの詳細な機能解析を行ってきた[14～17]．フローサイトメトリーによる解析にて，IRS-3高発現CHO細胞はIRS-1高発現細胞に比べてインスリン刺激によるS期への誘導が抑制され（Fig. 4A），IRS-3に特異的な細胞周期に関する経路の存在が考えられることを報告している[16]．細胞周期に関わるインスリンシグナル下流の分子について解析を行ったところ，IRS-3は他のIRSと異なって，PKB，MAPKを含む主要な経路に影響を与えずに，cyclin D1，p21，c-Myc発現低下を介して細胞周期進行を抑制することを明らかにした（Fig. 4B）．しかしながら，IRS-3に特異的な経路を規定する直接的な分

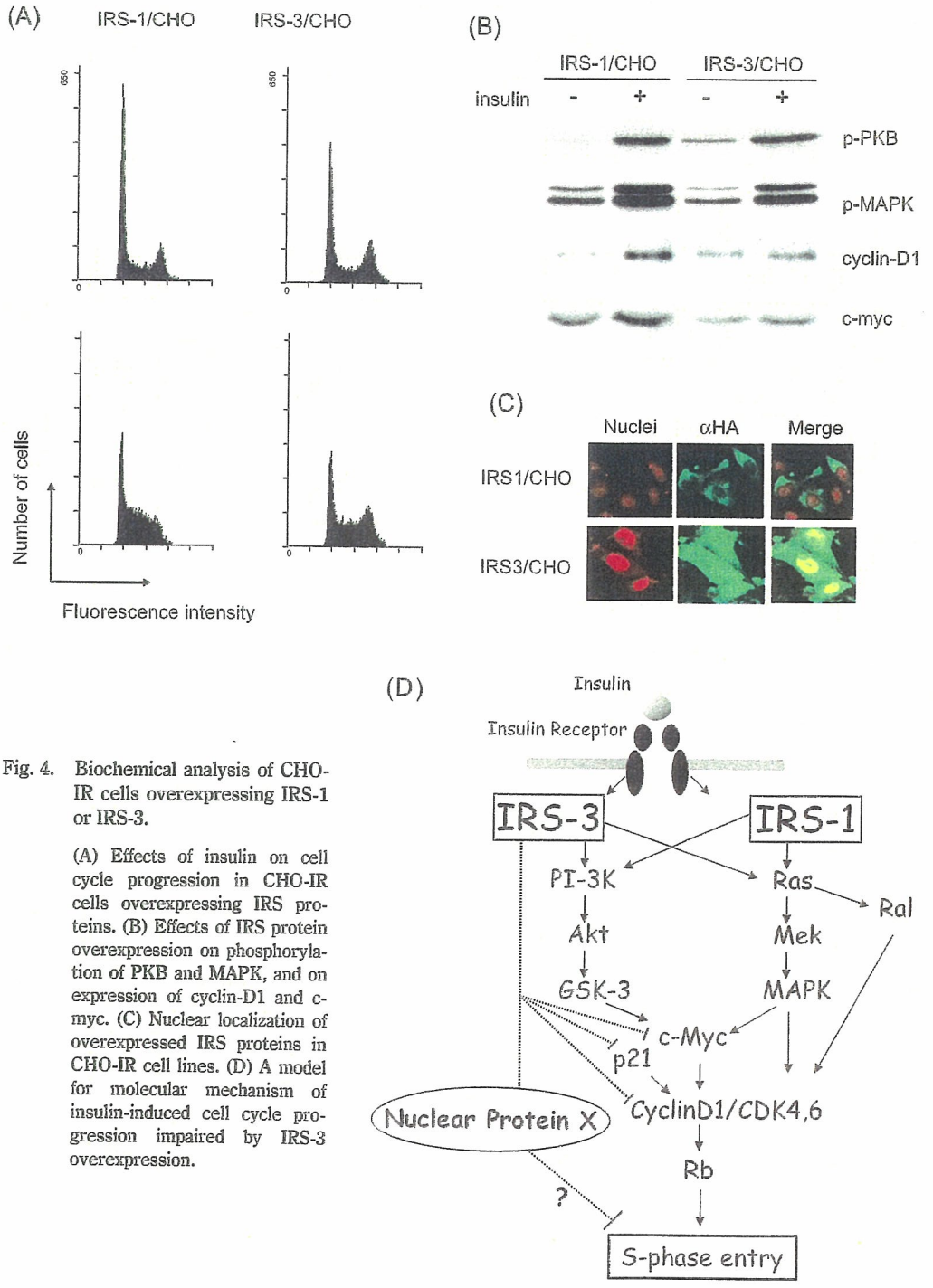

Fig. 4. Biochemical analysis of CHO-IR cells overexpressing IRS-1 or IRS-3.

(A) Effects of insulin on cell cycle progression in CHO-IR cells overexpressing IRS proteins. (B) Effects of IRS protein overexpression on phosphorylation of PKB and MAPK, and on expression of cyclin-D1 and c-myc. (C) Nuclear localization of overexpressed IRS proteins in CHO-IR cell lines. (D) A model for molecular mechanism of insulin-induced cell cycle progression impaired by IRS-3 overexpression.

子の存在が推定されるがこれまでのところわかっていない．さらにわれわれは免疫染色（Fig. 4C）にてIRS-3が核へ選択的に局在することを明らかにし，核内におけるタンパク質との相互作用などによってIRS-3特異的なシグナル経路が形成される可能性を示唆してきた（Fig. 4D）．このような標的タンパク質を検索するために，インスリンにて刺激をしたIRS-3高発現細胞のプロテオーム解析を行った．しかしながら全タンパク質を試料とした2D DIGE解析を行ったところ，IRS-3の作用に関係あるような有用な情報を得ることができなかった．最近，細胞内小器官を分画・精製しプロテオーム解析を行う，いわゆるフォーカスドプロテオミクスの手法が，より微量なタンパク質や器官に特異的なタンパク質の検出を目的として多用されてきている．また，前述したようにIRS-3は核においての機能が推測されることから，IRS-3高発現細胞の核抽出液にて2D DIGEを行うことにした．解析結果より，IRS-3高発現細胞に特異的な変動を認めるスポットが数多く見られ，その中からFig. 5に示したスポットに着目した．インスリン刺激に応じてスポットAとBが有意に変動していたが，この現象はIRS-1高発現細胞においてのみ観察され，IRS-3高発現細胞では変動がなかった．さらに3D画像からわかるように，スポットBからA，つまり酸性側にシフトする傾向があることから，このタンパク質がリン酸化されていることが推定された．LC-MS/MSによるこのスポットのタンパク質同定から，核に局在をもつタンパク質が同定された．これまでのところこのタンパク質がインスリンを含む外的な刺激でリン酸化される報告はなく，IRS-3に固有な細胞周期進行を阻害する経路に関与する可能性のあるタンパク質とみてさらなる解析を行っている．

尿タンパク質のプロテオーム解析

近年，糖尿病の増加に伴って，糖尿病性腎症に罹患する患者数が増加しており，同時に糖尿病性腎症を基礎疾患として持つ慢性透析患者の割合も多くなっている．現在，糖尿病患者における腎機能障害の最初の臨床兆候は微量アルブミン尿にて診断される．さらに糖尿病性腎症に特異性の高いⅣ型コラーゲンなども尿中マーカーとして用いられているが，従来のマーカーによる診断時点で腎症がすでに進行している可能性もあることから，新規の糖尿病性腎症のマーカーになりうるタンパク質やペプチドの発見が期待されている[18]．また微量アルブミン尿はさらに脳梗塞や心血管系疾患の予測因子としても知られており，他の尿中のタンパク質も疾患マーカーとして潜在的な可能性を持つと考えられる[19,20]．尿タンパク質のプロテオーム解析は二次元電気泳動法やLC-MS/MSを用いた解析により多数のグループが網羅的な同定結果を報告している．Pieperらは正常人の男女から回収した尿から分子量によりタンパク質を分画の後，二次元電気泳動を行い，MALDI-TOFとLC-MS/MSにて150種類のタンパク質を同定している[21]．またOhらも二次元電気泳動による尿プロテオームマップを作成し，113種類のタンパク質を同定している[22]．Tantipaiboonwongらは肺がんの患者の尿のプロテオーム解析を行っているが，この中で尿中のタンパク質の前処理法を検討している[23]．アセトンやTCAなどの有機溶媒を用いたタンパク質沈殿法に比して，限外ろ過膜を用いてタンパク質を濃縮し，二次元電気泳動を行うことでより多くのタンパク質スポットを検出している．最近では，Sharmaらが糖尿病性腎症の患者の尿タンパク質の2D DIGE解析を行い，alpha-1 antitrypsinが患者群において有意に尿中で高値を示すことを見出している[24]．この中で著者らは尿中のアルブミンやIgGがゲルのストリーキングを引き起こすことを問題点として提起している．

われわれの研究室では腎症をきたしていない糖尿病患者の尿のプロテオーム解析により，糖尿病性腎症への進行の臨床指標となりうる新規の早期診断マーカーや心血管系疾患などの予測マーカーの探索を目標としてプロテオーム解析を行っている．最初に尿タンパク質の2D DIGE解析応用への条件の最適化を検討した．タンパク尿をきたさない人の尿中のタンパク質は微量であり，一般的な二次元電気泳動法による解析では大量の尿が必要となる．2D DIGE法を用いる利点として，CyDyeによるタンパク質のラベルには数十μgのタンパク質があれば可能であり，少量の尿の回収で解析が可能になる．一方で，アルブミンなどの存在量の豊富なタンパク質があると多くのCyDyeがこのタンパク質のリジン残基にとられてしまうことが推定され，正確な分析を行うためにも豊富なタンパク質の除去が必要になると考えられる．このような問題を解決するため尿中の回収した尿を限界ろ過にて濃縮を行い，Albumin and IgG Removal Kit（GE Healthcare）またはMultiple Affinity Removal Spin（MARS）Cartridge（Agilent）にて高含有量タンパク質の除去を試みた．試料を再濃縮・バッファー置換を行った後に

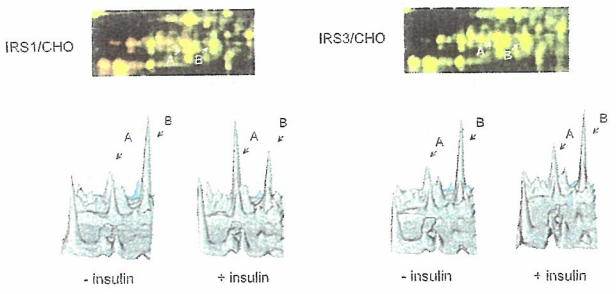

Fig. 5.　2D DIGE analysis of CHO-IR cells overexpressing IRS-1 or IRS-3 treated with insulin.

Merge images of non-treated cells (green) and insulin-treated cells (red). The 3-D views corresponding to spot A and B on 2-DE images.