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Figure legends

Figure 1 Cumulative event plots according to different dental status were estimated by the Kaplan-Meier method and compared using the log-rank test: survival curves for all causes of death by different levels of dental status. Group A indicates subjects with adequate dentition with natural teeth only or natural teeth and partial dentures. Group B indicates subjects with edentulous and denture wearers. Group C indicates subjects with inadequate dentition without dentures.

Table 1 Distribution of clinical characteristics and 2-year and 5-year mortality according to dental status

Variables	Total	Oral Status			*P*-value*
		Group A	Group B	Group C	
Number of patients	403	99	98	206	
Age, y	82.8±7.7	79.5±6.9**	84.3±6.8	83.7±8.0	< 0.001
Gender (male/female)	86/317	25/74	13/85	48/158	0.075
Dentition					
Natural teeth	5.1±7.4	12.7±8.9	0	3.9±5.3	< 0.001
Edentulousness, n/N (%)	192/403 (47.6 %)	0/99 (0 %)	98/98 (100 %)	112/206 (54.4%)	<0.001
Clinical basic conditions					
Cardiac disease, n/N (%)	68/403 (16.9%)	17/99 (17.2%)	18/98 (18.4%)	33/206 (16.0%)	0.874
Cerebrovascular disease, n/N (%	117/403 (29.0%)	21/99 (21.2%)	26/98 (26.5%)	70/206 (34.0%)	0.061
Diabetes Mellitus, n/N (%)	31/403 (7.7%)	11/99 (11.1%)	5/98 (5.1%)	15/206 (8.3%)	0.275
Arrythmia, n/N (%)	39/403 (9.7%)	8/99 (8.1%)	12/98 (12.2%)	19/206 (9.2%)	0.581
Medications, n/N (%)	166/403 (41.2%)	36/99 (36.4%)	42/98 (42.9%)	88/206 (42.7%)	0.533
Cognitive function					
MMSE (points)	10.6±9.5	15.4±9.4**	13.4±8.9**	7.6±8.3	< 0.001
Dementia, n/N (%)	346/403 (85.9 %)	72/99 (72.7%)	81/98 (82.7%)	193/206 (93.7%)	< 0.001
ADL					
Barthel Index (points)	15.8±7.1	19.2±6.3**	18.8±6.0**	12.8±6.7	< 0.001
Disability, n/N (%)	283/403 (70.2 %)	51/99 (51.5%)	57/98 (58.2%)	175/206 (85%)	< 0.001
Mortality					
2 years, n/N (%)	112/403 (27.8 %)	14/99 (14.1%)	21/98 (21.4%)	77/206 (37.4%)	< 0.001
5 years, n/N (%)	235/403 (58.3 %)	45/99 (45.5%)	54/98 (55.1%)	136/206 (66.0%)	0.002

Group A = adequate dentition with natural teeth only or natural teeth and partial dentures; Group B = edentulous and denture wearers;
Group C = inadequate dentition without dentures.
MMSE: Mini-Mental State Examination
Mean±SD for continuous variables and number (%) for categorical variables.
*One-way ANOVA for different dental status
**The post-hoc significant differences from corresponding values in Group C are indicated by $P<0.0001$

Table 2 Demographic variables among different age groups

Variables	65-74 year	75-84 year	85 ≤ year	*P*-value*
Number of patients	65	166	172	
Gender (male/female)	27/38	32/134	27/145	< 0.001
Dentition				
Natural teeth*, N	9.4±8.9**	5.6±7.4**	3.0±5.8	< 0.001
Edentulousness, n/N (%)	16/65 (24.6%)	67/166 (40.4%)	109/172 (63.4%)	< 0.001
Denture wearers in edentulous, n/N	11/16 (68.8 %)	42/67 (62.7 %)	45/109 (41.3 %)	0.008
Cognitive function				
MMSE (points)	13.7±9.5**	11.5±9.9**	8.7±8.7	< 0.001
Dementia, n/N (%)	51/65 (78.5%)	139/166 (83.7%)	156/172 (90.7%)	0.032
ADL				
Barthel Index (points)	16.7±6.8	16.5±6.9	14.9±7.4	0.059
Dependence, n/N (%)	44/65 (67.7%)	114/166 (68.7%)	125/172 (72.7%)	0.643

MMSE: Mini-Mental State Examination
Mean±SD for continuous variables and number (%) for categorical variables.
*One-way ANOVA for different dental status
**The post-hoc significant differences from corresponding values in Group C are indicated by $P<0.0001$

Table3 Unadjusted and adjusted 2-year and 5-year mortality ratio

		2-year mortality ratio				5-year mortality ratio			
		Unadjusted Hazard ratio (95% CI)	*P*-value	Adjusted Hazard ratio (95% CI)**	*P*-value	Unadjusted Hazard ratio (95% CI)	*P*-value	Adjusted Hazard ratio (95% CI)**	*P*-value
Oral status									
	A	1.0		1.0		1.0		1.0	
	B	1.55 (0.79-3.05)	0.202	1.31 (0.66-2.60)	0.47	1.35 (0.91-2.01)	0.134	1.13 (0.75-1.70)	0.554
	C	3.09 (1.75-5.46)	< 0.001	1.84 (1.01-3.36)	0.047	1.93 (1.38-2.71)	< 0.001	1.30 (0.90-1.88)	0.168
Age*									
	65-74	1.0		1.0		1.0		1.0	
	75-84	1.19 (0.60-2.35)	0.614	1.42 (0.71-2.84)	0.321	1.93 (1.17-3.17)	< 0.001	2.29 (1.38-3.81)	0.001
	85-	2.62 (1.39-4.96)	0.003	2.92 (1.51-5.66)	0	3.91 (2.41-6.34)	< 0.001	4.51 (2.74-7.44)	< 0.001
Gender	Male	1.0		1.0		1.0		1.0	
	Female	0.55 (0.37-0.81)	0.003	0.46 (0.30-0.70)	< 0.001	0.71 (0.53-0.96)	0.028	0.54 (0.39-0.73)	< 0.001
Cardiac disease	Absent	1.0		1.0		1.0		1.0	
	Present	0.96 (0.58-1.59)	0.87	1.10 (0.65-1.85)	0.72	0.94 (0.66-1.32)	0.705	1.01 (0.71-1.44)	0.96
Cerebrovascular diasease	Absent	1.0		1.0		1.0		1.0	
	Present	1.24 (0.84-1.84)	0.280	0.99 (0.66-1.48)	0.94	1.54 (1.18-2.02)	0.002	1.33 (1.00-1.77)	0.04
Diabetes mellites	Absent	1.0		1.0		1.0		1.0	
	Present	1.02 (0.52-2.02)	0.95	1.20 (0.60-2.42)	0.601	0.92 (0.56-1.51)	0.921	1.14 (0.69-1.89)	0.61
Cognitive function									
Dementia	Absent	1.0		1.0		1.0		1.0	
	Present	4.06 (1.66-9.95)	0.002	2.22 (0.87-5.65)	0.095	2.32 (1.46-3.67)	< 0.001	1.58 (0.97-2.58)	0.067
ADL									
Dependence	Absent	1.0		1.0		1.0		1.0	
	Present	3.32 (1.89-5.81)	< 0.001	2.22 (1.21-4.06)	0.010	1.89 (1.39-2,57)	< 0.001	1.44 (1.02-2.05)	0.040

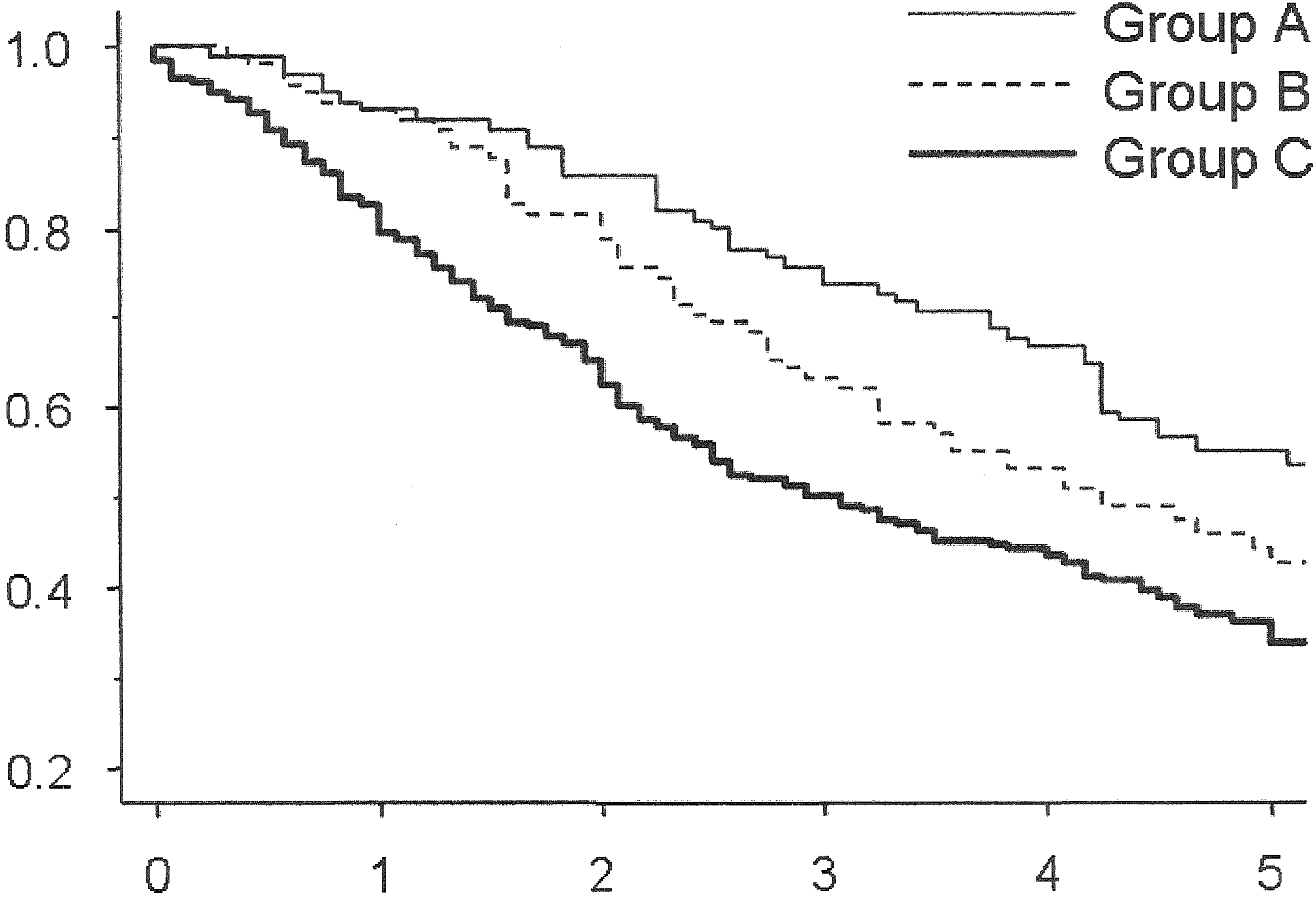
*Age adjusted odds ratios. Age was fitted 10 year age bands: 65-74; 75-84; 85 or more.

**Adjusted model includes the following variables: age; gender and severity of cognitive function and ADL.

Table 4 Underlying and immediate causes of death during 5-year follow-up

Causes of death	Group A (N=98) Number of deaths	Group A (N=98) Mortality rate (n/N, %)	Group B (N=99) Number of deaths	Group B (N=99) Mortality rate (n/N, %)	Group C (N=206) Number of deaths	Group C (N=206) Mortality rate (n/N, %)
All causes	**45**	**45.5**	**54**	**55.1**	**136**	**66.0**
Respiratory-tract infections	14	14.1	14	14.3	38	18.4
Senility without mention of psychosis	5	5.1	8	8.2	39	18.9
Ischemic heart disease	6	6.1	8	8.2	18	8.7
Cerebrovascular disease	8	8.1	3	3.1	10	4.9
Malignant neoplasms	2	2.0	4	4.1	4	1.9
Other infections*	0	0.0	4	4.1	3	1.5
Gastrointestinal bleeding	0	0.0	0	0.0	5	2.4
Cirrhosis of the liver	0	0.0	1	1.0	1	0.5
Renal failure	0	0.0	0	0.0	1	0.5
External and Unknown causes	10	10.1	12	12.2	17	8.3

*Other infections include septicemia (n=3) and infections of the kidney and urinary tract (n=4).


Group A
Group B
Group C
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5

M Asada, A Kikuchi

ELECTRONIC LETTER

Association of susceptibility to the development of pneumonia in the older Japanese population with haem oxygenase-1 gene promoter polymorphism

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Background: Oxidative stresses including cigarette smoking are implicated in the pathogenesis of cerebrovascular diseases, which are associated with pneumonia because of frequent aspiration. Haem oxygenase-1 (HO-1) acts in cytoprotection against oxidants, provides anti-inflammatory effects, and inhibits atherogenesis. A $(GT)_n$ dinucleotide repeat in the human *HO-1* promoter modulates *HO-1* gene expression and shows length polymorphism, which is grouped into three classes: class S (<27 repeats), class M (≥27, <33 repeats), and class L (≥33 repeats) alleles.

Objective: To investigate the correlation between the *HO-1* gene polymorphism and development of pneumonia in elderly Japanese.

Methods: The length of the $(GT)_n$ repeats was analysed in 200 elderly patients with pneumonia and 200 control subjects. The association of the *HO-1* gene polymorphism with risk of pneumonia was estimated by logistic regression.

Results: The proportion of allele frequencies in class L, and the proportion of genotypic frequencies in the L-allele carriers (L/L, L/M, and L/S), was significantly higher in patients with pneumonia than in controls (20% *v* 10% in class L, and 34% *v* 18% in L-allele carriers). After adjustment for potentially confounding factors, both cerebrovascular disorders and *HO-1* gene L-allele carriers were significant and independent risk factors for pneumonia. The adjusted odds ratio for L-allele carriers *v* non-L-allele carrier was 2.1 (95% confidence interval, 1.2 to 3.6).

Conclusions: The large size of a $(GT)_n$ repeat in the *HO-1* gene promoter may be associated with susceptibility to pneumonia in the older Japanese population.

Pneumonia is not only a common infection in older people, it is also the most common cause of death from nosocomial infection in the Japanese population.[1] Disorders of the central nervous system are more likely to develop in the elderly, and pneumonia has been estimated to occur in about one third of patients with stroke.[2] Cerebrovascular disease is associated with a high incidence of pneumonia owing to frequent aspiration.[3] As well as factors including diabetes mellitus, hyperlipidaemia, and hypertension, oxidative stresses such as cigarette smoking are also associated with the pathogenesis of cerebrovascular disease.[4] Genetic factors affecting antioxidants may be involved in the susceptibility to atherosclerosis of the cerebral arteries and the subsequent development of pneumonia in the elderly. Although the antioxidant enzymes inhibit the formation of atherosclerosis,[5] the roles of reduced expression of these enzymes on the development of pneumonia in elderly people are still uncertain.

Haem oxygenase (HO) oxidatively degrades haem to biliverdin, which is subsequently reduced to bilirubin, an efficient scavenger of reactive oxygen species (ROS), by biliverdin reductase.[6] HO-1, an inducible form of HO—and also a constitutive form of HO, including HO-2—provides cellular protection against haem mediated and non-haem-mediated oxidant injury.[6] HO-1 is thought to be an essential component in protection against various ROS.

A $(GT)_n$ repeat in the 5′ flanking region of the human *HO-1* gene is polymorphic,[7] and modulates human *HO-1* gene transcription by thermal stress[8] and hydrogen peroxide.[7] The size of the $(GT)_n$ repeat in the *HO-1* gene is associated with the antiapoptotic effects of HO-1 in lymphoblastoid cell lines.[9] We have shown that the size of the $(GT)_n$ repeat in the *HO-1* gene is associated with susceptibility to chronic pulmonary emphysema (CPE)[7] and lung adenocarcinoma,[10] and with longevity[11] in Japanese populations. This *HO-1* gene polymorphism is also associated with coronary artery disease, one of vascular diseases related to ROS.[12] However, the association between the size of the $(GT)_n$ repeat in the *HO-1* gene and the development of pneumonia in older populations is still uncertain.

In the present study, we screened allelic frequencies of the $(GT)_n$ repeats in the *HO-1* gene promoter in elderly people with and without pneumonia, and examined the association between the risk of senile pneumonia and length of the $(GT)_n$ repeats.

METHODS

Clinical protocol and patient characteristics

We studied 200 elderly patients with pneumonia and 200 elderly control subjects without pneumonia, attending the departments of internal medicine in six hospitals in Miyagi prefecture. The hospitals were a university hospital, a Red Cross hospital, three public general hospitals, and a municipal hospital. All participants were Japanese and aged 65 and older. To evaluate whether *HO-1* genotypes are associated with the development of pneumonia in elderly Japanese people, we selected the subjects with a performance status of 2 or better[13] and in a stable state as potential participants, because those with too low a performance status ran a greater risk of infectious disease, which might mask the preventive effect of any genetic factors. Patients were given a score of 0 if they were fully active and asymptomatic, 1 if they were symptomatic but fully ambulatory, 2 if they were

Abbreviations: COPD, chronic obstructive pulmonary disease; CPE, chronic pulmonary emphysema; HO, haem oxygenase; HO-1, inducible haem oxygenase; ROS, reactive oxygen species; TNF, tumour necrosis factor

Table 1 Characteristics of the study subjects

Characteristics	Control subjects (n = 200)	Patients with pneumonia (n = 200)	p Value
Age (years)*	73.8 (0.7)	75.4 (1.0)	NS
Sex			
Male	99 (50%)	101 (50%)	NS
Female	101 (50%)	99 (50%)	
Performance status			
0–1	114 (57%)	108 (54%)	NS
2	86 (43%)	92 (46%)	
Smoking history (pack-year)*	18.2 (2.6)	19.3 (2.8)	NS
Cerebrovascular disease			
Yes	14 (7%)	101 (50%)	<0.0001
No	186 (93%)	99 (50%)	
COPD			
Yes	35 (18%)	38 (19%)	NS
No	165 (82%)	162 (81%)	
Congestive heart failure			
Yes	17 (9%)	28 (14%)	NS
No	183 (91%)	172 (86%)	
Hypertension			
Yes	43 (22%)	59 (30%)	NS
No	157 (78%)	141 (70%)	
Diabetes mellitus			
Yes	21 (10%)	34 (17%)	NS
No	179 (90%)	166 (83%)	
Hyperlipidaemia			
Yes	9 (5%)	10 (5%)	NS
No	191 (95%)	190 (95%)	

Values are n (%) or *mean (SD).
COPD, chronic obstructive pulmonary disease.

symptomatic and confined to bed or chair for less than 50% of their waking hour, 3 if they were symptomatic and confined to bed or chair for more than 50% of their waking hours, and 4 if they were completely bedridden. The study was approved by the Tohoku University ethics committee, and informed consent was obtained from each subject. This study was carried out between April 2002 and December 2004.

During the study period, 264 elderly patients with pneumonia were identified. Pneumonia was defined as pulmonary infiltrate on chest radiograph, cough, and a temperature higher than 38.0°C.[3] All patients with pneumonia had the features of pulmonary infiltrate on chest radiographs, cough, and a temperature above 38.0°C. The patients were enrolled consecutively. Among them, we selected for the case group those with a performance status of 2 or better and in a stable state. We excluded patients who were immunocompromised—for example, those with active malignant disease, on renal dialysis, receiving corticosteroid treatment, or with HIV-1 infection. Patients were also excluded if they had obvious swallowing dysfunction, chronic sepsis in pressure sores, venous ulcers, or an indwelling urinary catheter. After these selections and exclusions were applied, 200 elderly patients with pneumonia were enrolled in the case group.

Potential control subjects were 439 elderly patients who continued attending the departments of hospitals over the study period and who had never had pneumonia at any time in their life including the study period. Control subjects were excluded if their past history relating to pneumonia were unclear. After the same selection and exclusion criteria as in the case group were applied, 383 control subjects were available for frequency matching. To carry out a case–control study, we randomly selected 200 control subjects in a frequency matched manner from the control cohort. They were frequency matched on age (±5 years), sex, smoking history, and performance status with the patients with pneumonia. Physical characteristics, smoking history, and complications in patients with pneumonia and control subjects are shown in table 1.

Analysis of length variability of $(GT)_n$ repeats in HO-1 gene promoter

Genomic DNAs were extracted from leucocytes in peripheral venous blood by conventional procedures. The 5′-flanking region containing a poly $(GT)_n$ repeat of the *HO-1* gene was amplified by polymerase chain reaction (PCR)[7 11] with a

Table 2 Allele and genotypic frequencies of HO-1at polymorphic locus

	Control subjects (n = 200)	Patients with pneumonia (n = 200)	OR (95% CI) v all other classes or subjects	p Value
Allele class				
L	38 (10%)	79 (20%)	2.3 (1.5 to 3.5)	<0.0001
M	189 (47%)	159 (40%)	0.7 (0.5 to 0.9)	<0.05
S	173 (43%)	162 (40%)	0.9 (0.7 to 1.2)	NS
Genotype group				
L-allele carrier	36 (18%)	68 (34%)	2.3 (1.5 to 3.7)	<0.001
Non-L-allele carrier	164 (82%)	132 (66%)		

CI, confidence interval; OR, odds ratio.

Table 3 Multivariate analysis of risk factors related to pneumonia in older adults

Variable	OR (95% CI)	p Value
Haem oxygenase-1 genotype subgroup		
L-allele carriers v non-L-allele carriers	2.1 (1.2 to 3.6)*	<0.01
Cerebrovascular disease		
Yes v no	28.0 (13.3 to 58.6)†	<0.0001

*OR was calculated with the non-L-allele carriers as the reference group, and adjusted for age, gender, performance status, smoking history, and complications.
†OR was calculated with the patients without cerebrovascular disease as the reference group, and adjusted for age, gender, performance status, smoking history, *HO-1* genotype, and complications other than cerebrovascular disease.
CI, confidence interval; OR, odds ratio.

fluorescently labelled primer p1-s (5′-AGAGCCTGCAGCTTCTCAGA-3′) and an unlabeled antisense primer p1-as (5′-ACAAAGTCTGGCCATAGGAC-3′), which were designed according to the published sequence.[7 14] The PCR was carried out over 30 cycles of 20 seconds at 94°C, 10 seconds at 60°C, and 20 seconds at 72°C. The PCR products were analysed in a DNA sequencer (ALF express II DNA sequencer version 2.2, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Each size of $(GT)_n$ repeat in the PCR product was calculated with ALFwin fragment analysis version 1.03 (Amersham Pharmacia Biotech) using four cloned alleles as size markers, which were already sequenced with the ABI prism dye terminator sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, California, USA).[7] The repeat numbers of these size markers were 16, 23, 29, and 38, respectively. The investigators of genetic analysis were blinded with respect to the status of the subjects.

Carboxyhaemoglobin concentrations in patients with pneumonia

Blood samples were taken from the radial artery in patients with pneumonia on the first day of hospital admission. The patients for the carboxyhaemoglobin analysis were all non-smokers and consisted of five L-allele carriers and five non-L-allele carriers (L/L genotype and S/S genotype, respectively), who showed a similar C reactive protein concentration (15.0 to 20.0 mg/dl) and white blood cell (WBC) count (9500 to 12 500 cells/μl) at the time of analysis. The carboxyhaemoglobin concentrations were measured with a spectrophotometer (ASL System, Radiometer, Copenhagen, Denmark).[15]

Statistical analysis

In the analysis of *HO-1* gene polymorphism in this study, the patient and control groups were frequency matched by age, sex, performance status, and smoking history. For statistical analysis, age and smoking history (pack-year) between the two groups were compared using Student's *t* test, and sex, performance status, and the frequency of the complications between the two groups were compared using χ^2 tests (table 1), as described previously in coronary artery disease.[12] The proportion of allelic frequencies and genotypic frequencies between the two groups were also compared using the χ^2 test (table 2). Factors associated with the presence of senile pneumonia such as age, sex, performance status, smoking status, complications, and *HO-1* gene polymorphism (L-allele carrier) were examined with multivariate analysis by logistic regression analysis (table 3). Odds ratios (OR) and their 95% confidence intervals (CI) were calculated to assess the relative risk conferred by a particular genotype (L-allele carrier), and adjusted for age, sex, performance status, smoking history, and complications using logistic regression as described previously (table 3).[12] All the statistical analyses were undertaken using SYSTAT (version 10.2; SYSTAT Software, Richmond, California, USA). The values for age and smoking history (pack-year) are reported as means (SD). The *HO-1* genotype distributions were in Hardy–Weinberg equilibrium. Significance was accepted at $p<0.05$.

For statistical analysis in the study on the correlation between carboxyhaemoglobin level and *HO-1* genotype in the patients with pneumonia, the mean values for age (year), smoking history (pack-year), WBC number (cells/μl), C reactive protein (mg/dl), and carboxyhaemoglobin concentration (%) between the five L-allele carriers and the five non-L-allele carriers were compared using Student's *t* test and sex using the χ^2 test (table 4).

RESULTS

Allele frequencies of HO-1 gene in control and patients with pneumonia in older adults

There were between 16 and 39 $(GT)_n$ repeats in the human *HO-1* gene in the study subjects (fig 1). The distribution of the number of $(GT)_n$ repeats was trimodal, as previously reported, with two main peaks located at 23 and 30 GT repeats and another peak located at 33 GT repeats.[7 10 11] We therefore divided the alleles into three subclasses, as previously reported[7]: class S (<27 repeats), class M (≥27 and <33 repeats), and class L (≥33 repeats) alleles.

In the control subjects, the distributions of the 400 alleles were 173 (43%) class S, 189 (45%) class M, and 38 (10%) class L (table 2); in the patients with pneumonia, the distributions were 162 (40%) class S, 159 (40%) class M, and

Table 4 Arterial blood carboxyhaemoglobin in patients with pneumonia

Patient	HO-1 genotype	Age (years)*	Sex†	Smoking history (pack-year)*	WBC (cells/μl)*	CRP (mg/dl)*	Arterial blood Hb-CO (%)‡
L-allele carrier 1	LL	71	M	0	12 300	18.3	0.57
L-allele carrier 2	LL	65	F	0	10 500	15.2	0.20
L-allele carrier 3	LL	79	F	0	9 700	15.7	0.80
L-allele carrier 4	LL	73	F	0	9 600	19.0	0.21
L-allele carrier 5	LL	76	F	0	10 020	19.4	1.20
Non-L-allele carrier 1	SS	65	M	0	12 400	18.5	1.50
Non-L-allele carrier 2	SS	77	M	0	11 000	16.3	1.20
Non-L-allele carrier 3	SS	79	F	0	10 500	19.2	1.02
Non-L-allele carrier 4	SS	65	F	0	9 900	15.6	1.10
Non-L-allele carrier 5	SS	75	F	0	9 600	19.5	0.90

*There was no significant difference in the mean value between L-allele carrier and non-L-allele carrier ($p>0.7$).
†There was no significant difference in the ratio between L-allele carrier and non-L-allele carrier ($p>0.5$).
‡There was a significant difference in the mean value between L-allele carrier and non-L-allele carrier ($p<0.04$).
CRP, C reactive protein; F, female; Hb-CO, carboxyhaemoglobin; M, male; WBC, white blood cell count.

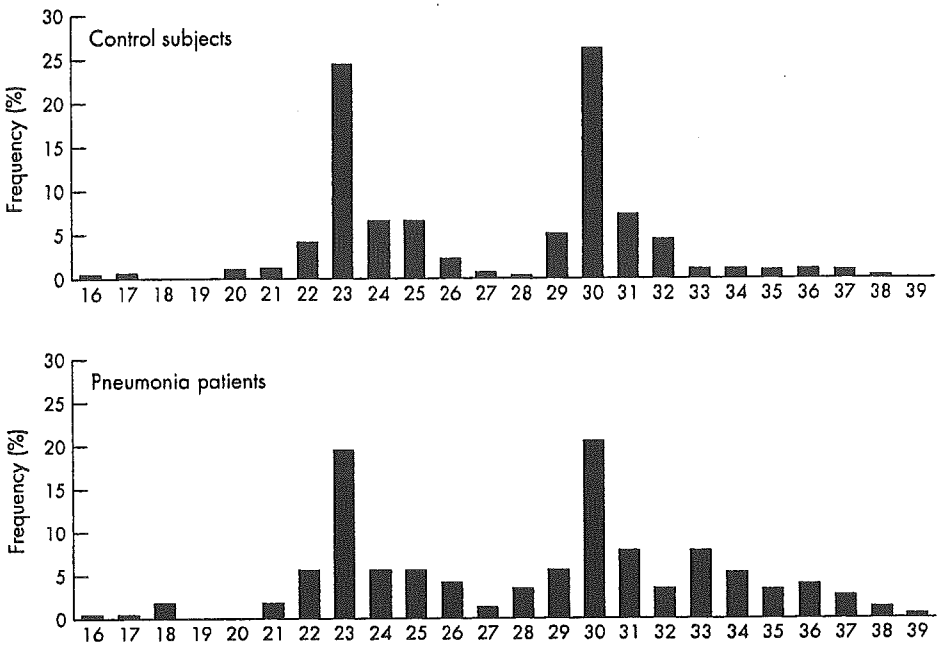

Figure 1 Frequency distribution of the number of $(GT)_n$ repeats in control subjects (n = 400 alleles) and patients with pneumonia (n = 400 alleles).

79 (20%) class L. The proportion of allelic frequencies in class L was significantly higher in all patients with pneumonia (n = 79, 20%) than that in all control subjects (n = 38, 10%) ($p<0.0001$). The odds ratio for pneumonia with L alleles *v* non-L alleles (class M allele + class S allele) was 2.3 (95% CI, 1.5 to 3.5) (table 2).

Genotypic frequencies of HO-1 gene in control and patients with pneumonia

Six genotypes (L/L, L/M, L/S, M/M, M/S, and S/S) of $(GT)_n$ repeats in the human *HO-1* gene promoter were divided into two subgroups according to allelic subclasses: L-allele carriers with a class L allele (L/L, L/M, L/S) and non-L-allele carriers without a class L allele (M/M, M/S and S/S).[7] The proportion of genotypic frequencies in L-allele carriers was significantly higher in all patients with pneumonia (n = 68, 34%) than that in all control subjects (n = 36, 18%) ($p<0.0001$). The odds ratio for patients with pneumonia with L-allele carriers *v* non-L-allele carriers was 2.3 (95% CI, 1.4 to 3.7) (table 2).

Risk factors for pneumonia

On multivariate analysis, cerebrovascular disease ($p<0.0001$) and *HO-1* genotype ($p<0.01$) were significantly and independently associated with the development of pneumonia (table 3), when the variables were adjusted by age, sex, performance status, smoking history, and complications including congestive heart failure, COPD, hypertension, diabetes mellitus, and hyperlipidaemia. The adjusted odds ratio (95% CI) was 2.1 (1.2 to 3.6) for *HO-1* genotype and 28.0 (18.3 to 58.6) for cerebrovascular disease (table 3).

Carboxyhaemoglobin concentrations in patients with pneumonia

To show the correlation between *HO-1* genotype and HO-1 activity caused by the inflammation of pneumonia, we examined the carboxyhaemoglobin concentration in several patients with pneumonia on their first day of hospital admission. The subjects for carboxyhaemoglobin analysis were five L-allele carriers and five non-L-allele carriers (L/L genotype and S/S genotype, respectively). There were no significant differences in age, sex, smoking history, WBC count, and C reactive protein concentration level between these two groups. However, the patients without the L-allele showed significantly higher carboxyhaemoglobin levels than those with the L-allele (1.14 (0.23)% *v* 0.5 (0.42)%, respectively; $p<0.04$) (table 4).

DISCUSSION

In this study we analysed *HO-1* gene polymorphism and showed that the proportion of allele frequencies in class L and the proportion of genotypic frequencies in the L-allele carriers (L/L, L/M, and L/S) were significantly higher in elderly people with pneumonia than in control subjects. The proportion of subjects with cerebrovascular disease in the pneumonia group was significantly higher than in the control group. With multivariate analysis, *HO-1* genotype and the presence of cerebrovascular disease were significant and independent risk factors for pneumonia. These findings suggest that the large size of a $(GT)_n$ repeat in the *HO-1* gene promoter may be associated with the development of pneumonia in older Japanese people with cerebral infarction.

Disorders of the central nervous system are more likely to develop in the elderly, and pneumonia has been estimated to occur in about one third of patients with stroke.[2] Basal ganglia infarction is associated with a high incidence of pneumonia owing to frequent aspiration[3] resulting from the reduction in the cough and swallowing reflexes.[16] In fact, in the present study, half these older patients with pneumonia also had cerebrovascular disease.

Oxidative stress such as cigarette smoking[4] is one of the important risk factors for cerebrovascular diseases, including basal ganglia infarction. Various ROS including superoxide and hydrogen peroxide induce lipid peroxide formation, which is a key process in atherosclerotic plaques in hypercholesterolaemia.[17] ROS are also involved in the brain tissue damage in stroke.[18] On the other hand, antioxidant systems such as glutathione, superoxide dismutase, and HO are suggested to protect the vascular disease caused by ROS.[19] The initial degradation of haem by microsomal HO involves the liberation of iron and CO and the formation of biliverdin, which is subsequently reduced to bilirubin by cytosolic biliverdin reductase.[6] Higher intracellular HO-1 activity may

increase the content of bilirubin, which is an efficient scavenger of ROS,[6] and a natural inhibitor of intimal hyperplasia after balloon injury.[20] In fact, Ishikawa *et al.* reported inhibitory effects of HO-1 on the atherogenesis in hyperlipidaemic rabbits.[21] Enhanced endothelial cell injury caused by oxidative stress was observed in a human case of *HO-1* deficiency.[22] Reduced expression of HO-1 might be partly associated with the development of stroke and subsequent pneumonia.

A $(GT)_n$ dinucleotide repeat in the 5′-flanking region of human *HO-1* gene shows length polymorphism.[7] We previously reported the influence of the number of the $(GT)_n$ repeats on the inducibility of the *HO-1* gene promoter under oxidative stimulus by transient transfection assay in human cell lines. The promoter activity of *HO-1* is modulated by the length variability of the $(GT)_n$ repeats, and large $(GT)_n$ repeats have a potent inhibitory activity on H_2O_2 induced gene expression of HO-1.[7] Furthermore, Epstein-Barr virus transformed lymphoblastoid cell lines were established from smokers with class L alleles (L/L) and with class S (S/S). When treated with H_2O_2, lymphoblastoid cells with the L/L genotype showed lower viability than those with the S/S genotype.[9] The GT dinucleotide repeat polymorphism has emerged as a potent genetic risk factor in various diseases, including vascular diseases such as coronary arteriosclerosis[12] and restenosis after balloon angioplasty.[23] These findings are consistent with the view that tissues of the non-L allele carrier could employ the antioxidant activity of HO-1 to a greater extent than that of the L-allele carrier when exposed to reactive oxygen species.[10] Large $(GT)_n$ repeats may affect the protective function against oxidant induced vascular endothelial injury and arteriosclerosis through the inhibition of HO-1 expression.

The results of our study suggest that the *HO-1* genotype is associated with susceptibility to pneumonia independently of cerebrovascular disease. Senile pneumonia is characterised by a high likelihood of aspiration pneumonia.[16] The severity of aspiration pneumonia is associated with the lung inflammation mediated by cytokines such as tumour necrosis factor α (TNFα).[24] On the other hand, it was reported that overexpression of the *HO-1* gene attenuated inflammation and decreased apoptosis of bronchial epithelial cells in a murine model of lung inflammation induced by *Pseudomonas aeruginosa*.[25] Furthermore, overexpression of the *HO-1* gene could reduce TNFα mediated apoptotic cell death in human endothelial cells.[26] These findings suggest that *HO-1* gene expression could be associated with the progress of aspiration pneumonia, and that reduced expression of the *HO-1* gene in elderly L-allele carriers might allow the development of pneumonia independently of cerebrovascular disease.

To examine the association between *HO-1* genotype and HO-1 activity in the pneumonia, we evaluated the carboxyhaemoglobin level in L-allele carriers and non-L-allele carriers with pneumonia. As a result, even after adjustment for the peripheral WBC count and C reactive protein level, patients without the L-allele showed higher carboxyhaemoglobin levels than those with the L-allele. Carbon monoxide (CO) is produced endogenously by HO and combines haemoglobin to form carboxyhaemoglobin complex. Therefore, the carboxyhaemoglobin concentration in the subject is a good marker of endogenous HO activity.[27] Furthermore, it has been reported that HO-1 is strongly induced in patients with bacterial infection.[28] We have already shown that arterial carboxyhaemoglobin increases at the onset of pneumonia in untreated patients returns to baseline on recovery after treatments.[15] We also showed that an increase in arterial carboxyhaemoglobin in pneumonia would be caused by carbon monoxide production in pulmonary inflammation, and that the arterial carboxyhaemoglobin is significantly correlated with disease severity in patients with bacterial pneumonia.[29] A study of lymphoblastoid cell lines by Hirai *et al* showed that mRNA level and activity of HO-1 were significantly higher in lymphoblastoid cells with the S/S genotype than in those with the L/L genotype after oxidant stimulation.[9] Therefore, analysis of the carboxyhaemoglobin level in pneumonia according to *HO-1* genotype would clarify the association between the *HO-1* genotype and HO-1 activity—that is, the HO-1 protein level, resulting from pneumonia. These findings suggest that HO-1 induction might be associated with the *HO-1* genotype (S>M>L).

In contrast to arterial blood carboxyhaemoglobin concentrations, we did not measure HO-1 activity in patients with pneumonia at the onset. However, we obtained new blood samples from eight people in the control group and seven in the pneumonia group after recovery from pneumonia, and analysed the serum HO-1 protein levels using enzyme linked immunosorbent assay methods as previously described.[30] There was no significant difference between these two groups when they were in good physical condition (2.6 (1.2) *v* 2.4 (1.0) ng/ml, $p>0.2$). These values were compatible with the results from a previous report.[30] Because the *HO-1* gene is inducible by inflammation or oxidative stress, the baseline expression of the this gene should be low regardless of the *HO-1* genotype, which was demonstrated in lymphoblastoid cell by Hirai *et al.*[9] Further studies are needed to clarify the relation between HO-1 activity and the *HO-1* genotype at the onset of pneumonia.

Conclusions

This is the first study to show that the 5′-flanking polymorphism in the *HO-1* gene is associated with the development of pneumonia in an older Japanese population with basal ganglia infarction. Increased susceptibility to developing pneumonia may be associated with sclerosis in the cerebral arteries.

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ACE inhibitors and protection against pneumonia in elderly patients with stroke

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Pneumonia is the most common cause of death from nosocomial infection in the elderly. The increased incidence of pneumonia and the high mortality are consequences of a number of age-related factors, including coexisting illnesses, therapeutic interventions, and the aging process itself.[1] Pneumonia has been estimated to occur in about one third of patients with stroke.[2] The most important factor contributing to the risk of pneumonia in patients with stroke is suggested to be dysphagia with aspiration.[1]

Angiotensin-converting enzyme (ACE) inhibitors have been shown to improve silent aspiration[3] and prevent pneumonia in elderly patients with stroke.[4] However, little is known about whether ACE inhibitors have a beneficial role in reducing the risk of pneumonia as compared to other classes of antihypertensive drugs in elderly patients with stroke. Thus, we investigated whether ACE inhibitors can reduce the risk of pneumonia as compared to other antihypertensive drugs.

Methods. We recruited patients with stroke who were followed up for more than 6 months after their ictus from eight outpatient clinics. We enrolled 1,190 patients in April 1999, and prospectively followed them for 35 months. The criteria for diagnosis of pneumonia and the patients' inclusion and exclusion criteria were described previously.[5] Eligible patients were those who received antihypertensive therapy, had a history of stroke, but were not bedridden.

We analyzed the incidence of pneumonia in three groups of hypertensive patients with stroke who were classified on the basis of treatment with antihypertensive drugs as follows: patients who received ACE inhibitors, calcium-channel blockers, and diuretics. Our hypertensive patients received only the same class of antihypertensive drugs. The control group consisted of non-hypertensive patients with stroke who did not receive any antihypertensive drugs. Follow-up data were available for all participants.

For the main analyses, we used the log-rank procedure and Cox's proportional hazards model to calculate the CI. Cumulative incidence curves were generated by the Kaplan-Meier method for endpoints in the ACE inhibitors, calcium-channel blockers, diuretics, and control groups. Significance was set at $p < 0.05$.

Results. There were no significant differences in age, sex, stroke severity as assessed by NIH Stroke Scale,[6] and poststroke duration among the four groups (table). During the follow-up, new pneumonia was diagnosed in 12 (2.8%) of the 430 patients in the ACE inhibitors group, 36 (8.8%) of the 409 patients in the calcium-channel blockers group, 29 (8.3%) of the 351 patients in the diuretics group, and 14 (8.8%) of the 160 patients in the control group. The patients in the ACE inhibitors group had a lower risk of pneumonia than those in the control group; the hazard ratio was 0.30 (95% CI 0.14 to 0.66, $p = 0.0013$). However, the risk in the calcium-channel blockers group (1.01, 95% CI 0.53 to 1.92, $p > 0.40$) or the diuretics group (0.94, 95% CI 0.48 to 1.83, $p > 0.30$) did not differ from that in the control group.

Discussion. We found a significantly reduced risk of pneumonia in patients receiving ACE inhibitors vs control patients. No such decreased risk was noted in users of calcium-channel blockers or diuretics. Silent aspiration reportedly disappears by treatment with ACE inhibitors in association with an increase in the serum substance P levels in hypertensive patients with stroke.[3] ACE inhibitors may increase the serum substance P levels, thereby reducing aspiration pneumonia in elderly patients with stroke.

A recent large-scale randomized trial has demonstrated that treatment with ACE inhibitors significantly reduced the risk of pneumonia among the participants of Asian ethnicity, although the protective effects of ACE inhibitors against pneumonia were not observed in the non-Asian participants.[7] However, this trial[7] included patients with a history of transient ischemic attacks and the mean Barthel index score of the patients was quite high. Since the incidence of pneumonia increased in association with a decrease in the Barthel index score,[5] the effects of ACE inhibitors against pneumonia might be underestimated by a population of patients with a high activity of daily life. Our present study only included patients with well-documented cerebral hemispheric strokes.

The present study supports the hypothesis that treatment with ACE inhibitors may be beneficial in reducing the risk of pneumonia in elderly patients with stroke.

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Table Characteristics and clinical features of the four groups

	ACE inhibitors	Calcium-channel blockers	Diuretics	Control
No.	430	409	351	160
Female/male	224/206	213/196	183/168	78/82
Mean age, y	75 (1)	75 (1)	75 (1)	76 (1)
Stroke severity	6 (1)	6 (1)	6 (1)	6 (2)
Poststroke duration, y	3.1 (1.1)	3.3 (0.9)	3.4 (1.1)	3.3 (1.2)

Values in parentheses are SD.

ACE = angiotensin-converting enzyme.

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Hypernatremia from a hunger strike as a cause of osmotic myelinolysis

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Too rapid correction of hyponatremia often causes osmotic myelinolysis. A rapid shift from normal to hypernatremia may also be dangerous. We report a hunger striker that developed an extreme serum sodium concentration followed by coma and radiologic abnormalities characteristic of osmotic myelinolysis.

Case report. A 19-year-old Algerian asylum seeker started a hunger strike after his residence permit had been rejected. His medical history was unremarkable. He used no medications, including lithium. One month before fasting, he was placed in isolation because of behavioral disturbances. At this time, the weather was hot, and the patient refused sufficient intake of water and food. One day after he stopped eating and drinking, he became confused; after another 5 days, he became somnolent. He was transferred to a penitentiary hospital. On admission, his serum sodium level was 187 mmol/L, creatinine 213 μmol/L, and glucose 6.8 mmol/L. Urine osmolality was not measured. A feeding tube rehydration regimen was started with 2 L/day of water. His sodium level was 172 mmol/L the next day. After 2 days, he became comatose and was referred to our intensive care unit. On admission, his blood pressure was 105/55 mm Hg, temperature was 38.5°C, Glascow Coma Scale score was 6 (E1M4V1), the pupillary light reflex was delayed, and the Achilles tendon reflexes were absent. Sodium level was 152 mmol/L, potassium 2.5 mmol/L, creatinine 91 μmol/L, urea 7.3 mmol/L, phosphate 0.41 mmol/L, magnesium 0.96 mmol/L, and albumin 26 g/L. CT of the brain and CSF analysis were normal. EEG showed diffuse slowing. A chest radiograph showed bilateral infiltrates. The patient was intubated and treated for aspiration pneumonia. Potassium and phosphate were replaced.

Because of the extreme hypernatremia, osmotic myelinolysis was considered. Brain MRI 5 days after admission was consistent with pontine and extrapontine myelinolysis (figure, A and B). MRI also revealed acute hydrocephalus (see the figure, B) and posterior fossa edema (see the figure, C). An external ventricular drain was inserted. The intracranial pressure proved normal. As the patient did not respond to 6 days of drainage, the drain was removed. Over the next days, the pupillary light reflex normalized and the patient regained consciousness. When asked, he was able to open, close, and move his eyes and slightly move his fingers. No other voluntary movements were possible. After 1 month, his neurologic condition gradually improved. After 4 months, he was able to speak and walk short distances. After 7 months, he was fully recovered but needed a cane while walking.

Discussion. We present a hunger striker that developed osmotic myelinolysis due to extreme hypernatremia from dehydration. The clinical presentation with confusion and coma several days after onset of the severe electrolyte disturbance followed by spontaneous recovery in the course of months is consistent with osmotic myelinolysis.[1]

Central pontine myelinolysis was first described in 1959, associated with alcoholism and malnutrition.[2] In 1976, it was first linked to hyponatremia.[3] In hypotonic hyponatremia, water initially enters brain cells, resulting in cerebral edema. The brain cells adapt by losing electrolytes and organic osmolytes, thus arresting a further influx of water. If chronic hyponatremia is cor-

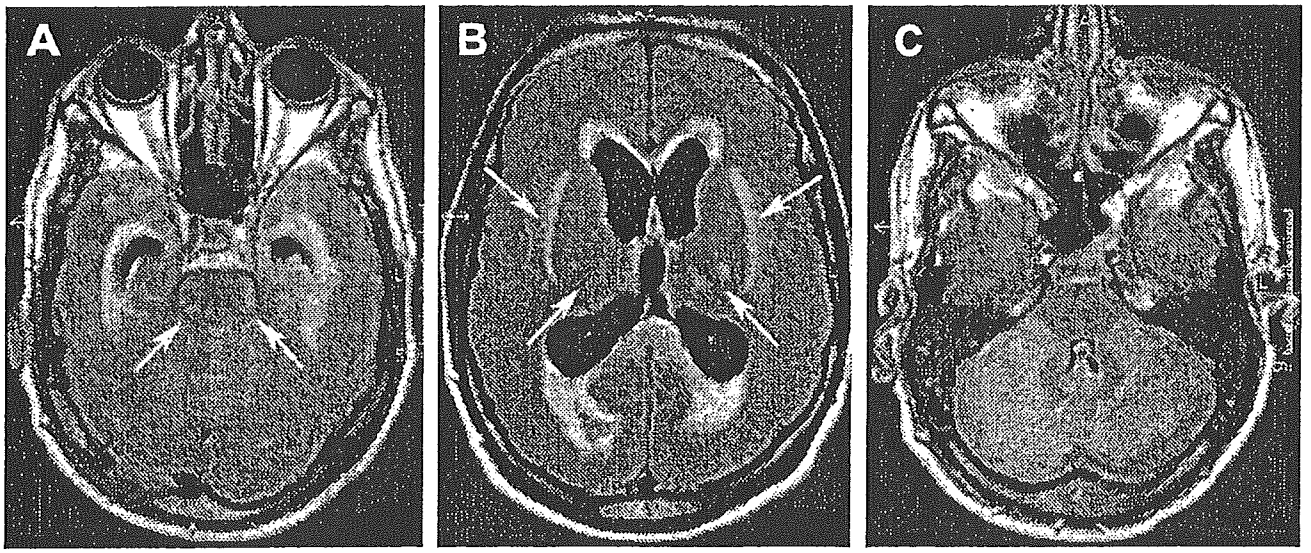

Figure. Axial fluid-attenuated inversion recovery MRI of the brain showing hyperintensities in the dorsolateral regions of the pons (A) and bilaterally in the thalamus, globus pallidus, and capsula extrema (B), consistent with osmotic myelinolysis. Also note the enlargement of the lateral and third ventricles with periventricular hyperintensities (B). This acute triventricular hydrocephalus was presumably caused by posterior fossa edema, yielding impaired CSF circulation (C).

In this case, the diagnosis of insulinoma was made in the examination of recurrent falls, and suspected because the falls always occurred after an overnight fast but in the absence of any other symptom. Also, the patient was alert and did not give the impression of frailty so often seen with recurrent falls. A careful history can identify unusual but treatable causes of recurrent falls.

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AN ADVERSE EVENT ASSOCIATED WITH HIP PROTECTORS

To the Editor: Hip protectors have been demonstrated to reduce fracture rates.[1] There are few descriptions of any adverse events associated with use of hip protectors. Of side effects described, most have been minor (e.g., not being comfortable (too tight/poor fit)).[2]

In January 2004, hip protectors were prescribed in a 74-year-old resident of an assisted living facility. One month later, she began to complain of right gluteal-area pain that radiated down her leg along the distribution of the sciatic nerve. The pain was worse after sitting for prolonged periods. Her history was noteworthy for mild Alzheimer's disease, treated depression, and hypothyroidism controlled with exogenous L-thyroxin. She got some relief of her pain from a Level 3 on the Functional Pain Scale to a Level 2 using acetaminophen and refecoxib.[3] Physical examination showed tenderness over the posterior superior iliac spine and an abnormal gait favoring her right leg. Tenderness was exacerbated in the seated position with external rotation of the leg and knee flexion. Within 6 weeks of discontinuing her hip protectors, the hip pain resolved completely.

Many clinicians have witnessed the delight of a patient who has had "wallet sciatica" resolve after simply discontinuing the practice of carrying a bulky billfold in the hip pocket. Other cases have been described of leg pain resolve after removal of piriformis muscle pressure.[4] This is believed to be the first case associating hip protectors with pyriformis syndrome or sciatica. Tight-fitting elastic or increased pressure from the protector pad over the pyriformis muscle adjacent to the sciatic nerve is likely to be the source of compression on the sciatic nerve resulting in sciatic neuropathy and should be considered when similar complaints arise in individuals who have been prescribed hip protectors.

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HOMICIDES OF DISABLED OLDER PERSONS BY THEIR CAREGIVERS IN JAPAN

To the Editor: Following the lead of the Netherlands and Germany, in April 2000 Japan launched a long-term care (LTC) insurance system nationwide in a courageous attempt to comprehensively solve the problems of caring for frail older people.[1,2] This new system was unprecedented in making the government rather than the family responsible for the care of the disabled elderly. Specifically, the goals of the LTC insurance system are to allocate limited resources to impaired elderly in a way that adequately reflects need, support home care, and reduce caregiver burden.[3,4] Under this care, the level of services provided are keyed to the degree of a recipient's impairment. To assess the effectiveness of the new insurance system, we investigated the prevalence of caregivers murdering frail older recipients because of exhaustion from the care burden before and after the inception of the insurance system in Japan. We employed the key words of "kaigo" (care) and "satsujin" (homicide) and collected more than 600 articles associated with caregiver murders between January 1997 and December 2003 using a computerized surveillance system (http://www.asahi.com/) provided by a major Japanese newspaper, the *Asahi Shinbun*. Thereafter, we carefully analyzed the eligibility of the articles to this study one by one and summed up the yearly cases accordingly. Eligible recipients were disabled persons who were aged 40 and older living with their caregivers in their own home and were killed by their caregivers because of exhaustion associated with the care burden (confirmed by police records). Cases were excluded if caregivers were drug abusers or had psychological disorders such as depression and schizophrenia.

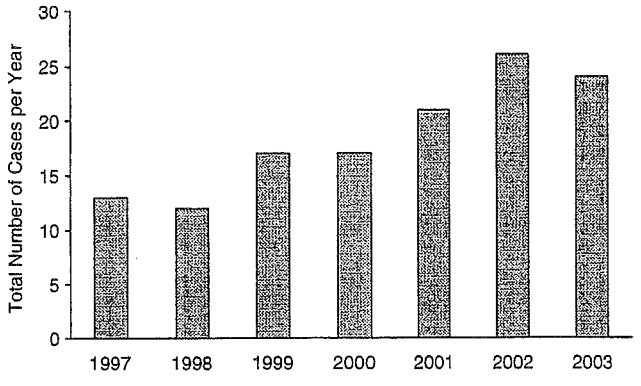

Figure 1. Annual prevalence of caregiver murders.

Finally, we found 130 cases eligible for this study. The mean age ± standard deviation of the caregivers was 66.3 ± 13.4 and that of the recipients was 72.5 ± 11.9. The most frequent cases were murder between sons or daughters and their parents (50%), followed by husbands and wives (47%) and others (3%). As shown in Figure 1, the annual prevalence of caregiver murder seems to be increasing even though the new LTC insurance was implemented in 2000. The recipients' principal physical conditions were dementia (57%), bedridden condition due to stroke with or without dementia (40%), and others (3%). The murders were by strangulation (68%), stabbing (13%), striking (9%), and other (10%). We could find no significant difference in any parameters described above before and after the new insurance system was begun.

We demonstrated that the current prevalence of murder by exhausted caregivers to the frail recipients was increasing rather decreasing after the inception of the new LTC insurance system. Now, 4 years after its inception, it seems necessary to ask whether the program has developed a fair and appropriate way of allocating limited resources to people with different diagnosis. Services are allocated based on the Government-Certified Disability Index.[1,3] Recent reports describe that the needs of demented elderly are often underestimated because of a lack of field-proven items to accurately assess their cognition and behavioral problems under the current insurance system.[3,5] This might be one explanation of the unexpected result of the present study, because most of the killed frail elderly suffered from dementia. The induction of Japan's new LTC system was quite significant in making clear the plight of the aged and their caregivers, but the system should be improved to lighten the caregiver's burden, especially in caring for demented people who require a great amount of assistance from family caregivers to live in the community.

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TEACHING THE OLDER ADULT

To the Editor: Older adults learn in the same way as do all other adults.[1] They must be motivated and be in an environment that is conducive to learning (i.e., it must be quiet, well lit, at a comfortable temperature, and have a relaxed atmosphere).[2] The best motivator for adults is when they have an immediate need or requirement for the information. This is further enhanced when their personal goals and objectives are clarified and addressed before the information is presented. The older adult can benefit from certain adjustments to the teaching methodologies that address the various sensory impairments and cognitive changes common to this age group. (It is important to remember that not all older adults suffer from these.)

Visual deficits can be accommodated for by providing larger typeface (12 point or larger) and using a nonserif font with high contrast between type color and background. Auditory deficits are overcome by speaking distinctly and slowly, in a slightly louder voice, and at a deeper pitch than usual, while looking directly toward the person. The use of multiple-sensory input (i.e., written and auditory (also tactile and olfactory, if appropriate)) is helpful. Also, avoiding the necessity of writing or using small dials or buttons to access the information and eliminating background "noise" (auditory and visual) enhances accessibility of the information.[3]

Building upon previously acquired knowledge can accommodate cognitive changes, as can providing new information in shorter, simpler segments (no more than 3–5 points in one session). Use the same terms to refer to the same points each time, allowing the person to problem solve to come to conclusions on his/her own. Focus on problem solving and limit information to that necessary to make learning and retention easier. Frequent repetition of previously learned information (concepts) through different sensory channels and reducing the necessity of abstract thinking are also useful techniques to employ. Allow more time for older adults to absorb new information and permit them to set their own pace. Encouraging active participation and providing ways to reinforce memory can also improve learning and retention (e.g., posted lists/steps).[3]

Most older adults still prefer to learn in familiar ways: one-to-one oral instruction, written material, and classroom instruction—if it is relaxed and if they are treated with respect and dignity. However, the fastest-growing group of people learning to use the Internet is those aged 55 and older. This is likely to become a common method of learning for many older adults in the near future—within the next 10 years. It appeals especially because of its availability for those who may not be as mobile as they had been and because of its instant availability to fit into the increasingly busy lifestyle

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ORIGINAL ARTICLE

The entorhinal cortex regulates blood glucose level in response to microinjection of neostigmine into the hippocampus

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Abstract

OBJECTIVE: Microinjectin of neostigmine, an inhibitor of acetylcholine esterase, into the rat hippocampus elicited stress-like responses reflected by the release of adrenocorticotropic hormone (ACTH) and blood glucose elevations. The entorhinal cortex is regarded as an interface between the hippocampus and neocortex. The current study was designed to examine the role of the entorhinal cortex in regulation of blood glucose elevation induced by hippocampal neostigmine injection.

MARERIAL AND METHODS: We produced the entorhinal cortex lesions in 9 week-old male Wistar rats by the bilateral injections of the cell-selective neurotoxin, ibotenic acid (15 μg / μl). Two weeks after the injections, neostigmine methylsulfate (sigma, $5x10^{-8}$ mol) was microinjected into the rat hippocampus in a volume of 1 μl for 1 min using a CMA/100 microinjection pump.Plasma ACTH levels were measured by radioimmunoassay. Plasma glucose concentrations were determined by the immobilized enzyme membrane/H_2O_2 method with a compact glucose analyzer Antsense II (Bayer Medical Co.Ltd,Tokyo, Japan).

RESULTS: Compared with sham-operated control rats, the entorhinal lesions produced by ibotenic acid significantly attenuated the elevations of blood glucose evoked by the microinjection of neostigmine into the hippocampus. However, no significant difference of plasma ACTH in response to the injection was observed between the entorhinal-lesioned rats and controls.

CONCLUSION: The results of the present study indicate that the entorhinal cortex plays a role in the central nervous systems regulation of blood glucose and may be involved in a stress response presumably via an alternative pathway.

Abbreviations:

ACTH	adrenocorticotropic hormone
EC	entorhinal cortex
CNS	central nervous systems
CRH	corticotropin releasing hormon
PVN	paraventricular nucleus
HPA	hypothalamic-pituitary-adrenal
BNST	bed nucleus of the stria terminalis

Introduction

Stress is common to all living creatures regardless of differences in its quality or intensity. The imposition or perception of environmental or physical change, negative or positive, elicits a spectrum of physiologic changes that can be construed as adaptive to the organism. Prominent among these is the release of glucocorticoids by the adrenal glands, which serves to alert the organism to environmental or physiologic changes and to preserve homeostasis. Levine and Ursin [10] provided a definition of stress that consists of three elements: stimulus input, central processing system, and response output; with biological and psychological processes viewed as integral parts of the general homeostatic principle. The brain perceives inputs of various stressors and responds via the nervous, endocrine and immune systems, which are called stress responses [17]. In this sense, the brain plays a role in governing the stress responses. Elevations of corticotropin-releasing factor, ACTH and glucocorticoids are the main features of reactions to diverse and acute stressful stimuli [2, 24]. During stress, neurons of the hypothalamic paraventricular nucleus (PVN) release corticotropin-releasing hormone into the pituitary portal circulation, and ACTH secreted from the anterior pituitary gland in response to corticotropin-releasing hormone, stimulates the secretion of glucocorticoids from the adrenal gland. This constitutes the hypothalamic-pituitary-adrenal (HPA) axis, which is the major regulator of neuroendocrine stress responses [1,5,7,11,13,15]. Involvement of the limbic system in neuro-endocrine responses to some stressors has been documented. A wealth of evidence suggests that the hippocampal cholinergic system is involved in some stress responses [9,12,21]. In particular, the cholinergic system in the hippocampus plays a role in regulating the peripheral metabolism of glucose and catecholamines [7,21]. Under stress, the release of acetylcholine in the hippocampus increases, which coincides with the elevation of plasma glucose and catecholamines [19]. In our previous experiments, we observed that the administration of neostigmine, an acetylcholine esterase inhibitor, into the hippocampus elevates the levels of blood glucose and ACTH. Thus, we concluded that the microinjection of neostigmine into the hippocampus is a potential experimental model for acute stress responses [7,8]. The entorhinal cortex is a gateway to the hippocampus. Many sensory inputs and other information reach the hippocampus via the entorhinal cortex. It receives inputs from the neocortex, including the temporal and frontal lobes, amygdala and olfactory bulbs [3]. Information enters the hippocampal formation via the entorhinal cortex and exits via the fornix. Also, the entorhinal cortex is the primary supplier of converging neocortical sensory input to the ipsilateral dentate gyrus of the hippocampal formation [20]. We previously reported on the involvement of the entorhinal cortex in the stress response to immobilization [22]. Lesions in this area produced by ibotenic acid attenuate ACTH elevation during immobilization stress but not during insulin-induced hypoglycemia. The aim of this study was to investigate the role of the entorhinal cortex in stress responses. We produced bilateral entorhinal lesions using ibotenic acid in rats, and observed the peripheral responses of stress markers induced by microinjections of neostigmne into the hippocampus.

Material and Methods

Subjects: We used 9 week-old male Wistar rats (200–300 g) for the experiment. The animals were individually housed under standard laboratory conditions in temperature-controlled rooms (25 °C), and were maintained under a 12 h light/dark cycle (light on at 06.00) with food pellets and water available *ad libitum*. The rats were cared for in accordance with the ethical guidelines approved by the Animal care and Use Committee of Nagoya University.

Experimental protocol: Rats were randomly assigned to one of two major groups: unlesioned or lesioned.

The rats in each group were then divided into two subgroups: Group 1: unlesioned neostigmine-injected, Group 2: unlesioned saline-injected, Group 3: lesioned neostigmine-injected, and Group 4: sham-operated rats, neostigmine-injected.

Surgery: The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and mounted in a stereotaxic frame (Narishige Scientific Instruments Laboratory, Tokyo, Japan). For insertion of the stainless steel needle, the skull was exposed and a burr hole was drilled overlying the injection coordinates. Ibotenic acid was injected through a stainless steel needle (outside tip diameter of 28 μm), which was connected to a 1.0 μl syringe via a 30 cm tube filled with the injection solution. Coordinates for the entorhinal cortex were calculated relative to Bregma with the incisor bar set at −3.30 mm. The coordinates used were anterior-posterior −6.04 mm, medial-lateral ±6.50 mm and dorsal-ventral 7.00 mm from the skull surface in accordance with the atlas of Paxinos and Watson [14]. Entorhinal cortex lesions were produced by pressure-injection of 0.1 μl of ibotenic acid (15 μg / μl in 0.9% NaCl, Sigma Chemical Co., St Louis, MO, USA) bilaterally over 5 min. The tip was allowed to remain in the brain for 5 min after injection to minimize dorsal diffusion of the drug along the needle tract. Sham-operated rats were treated in an identical manner to the ibotenic acid-lesioned rats but were injected with the same volume of saline without ibotenic acid.

A recovery period of 7 days was given to the above operated rats (Group 3, 4), otherwise all rats were anesthetized one week before the experiment to stereotaxically implant a guide cannula (Bas, Tokyo, Japan) into the left dorsal hippocampus at the following coordinates: anterior-posterior -2.0 mm, medial-lateral 1.5 mm, dorsal-ventral 3.5 mm in accordance with the Paxinos and Watson atlas [14] one week before the experiments.

The day before the experiments, the rats were anesthetized with diethyl ether (Kanto Chemical Co. Inc, Tokyo, Japan), and a catheter was inserted into the jugular vein for repeated blood sampling. A 2 cm longitudinal incision was made in the neck directly over the trachea. The underlying muscles were separated using blunt dissection and the right jugular vein was catheterized with Silastic tubing (Shiniest Polymer, Nagoya, Japan) filled with heparinized saline. The catheter was threaded through the vein over a distance of 2.5 cm, which allowed the tip of the cannula to rest in or near the atrium. The free end of the catheter was plugged with a knot and the catheter exteriorized and secured at the back of the neck with a special cap.The rats were kept in individual cages with free access to water and food.

Procedures: Two weeks after developing entorhinal cortex lesions, saline containing neostigmine methylsulfate (sigma, $5x10^{-8}$ mol) was microinjected in a volume of 1µl for 1 min using a CMA /100 microinjection pump (BSA, Tokyo, Japan) through the guide cannula into the left dorsal hippocampus of free moving rats. To determine the plasma concentration of ACTH and glucose, blood was intermittently sampled (0.8 ml), starting at time 0, just before injection, and at 10, 30, 60 and 120 min after. To minimize the effect of volume loss, an equal volume of heparinized saline was retuned to the general circulation at each sampling. The blood samples were kept on ice, centrifuged, and the plasma was removed and stored at -20 °C in 400 µl aliquots for subsequent determination of ACTH by radioimmunoassay [16]. Plasma glucose concentrations were determined by the immobilized enzyme membraine/H_2O_2 method with a compact glucose Antsense II analyzer (Bayer Medical Co. Ltd, Tokyo, Japan) (21). All experiments were completed between 10.00 h and 13.00 h to minimize variability resulting from circadian rhythm. Two hours after neostigmine injection, the rats were deeply anesthetized with a lethal dose of sodium pentobarbital and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). They were postfixed over night and cryoprotected in phosphate-buffered saline containing 30% sucrose for 2 days.

Histological verification: For the verification of lesions and the effect of the vehicle, the postfixed brains were frozen with powdered dry ice and serial sections 20 µm in thickness were processed from the region of the entorhinal cortex and mounted on glass slides. Selected regions were stained with Cresyl Violet to assess the extent of the lesions in the entorhinal cortex.

Data and statistical analysis: All sections were assessed by means of microscopic examination using an Olympus BX50 microscope (Tokyo, Japan). Photographs were made of representative lesions and vehicle injection sites in the entorhinal cortex. Blood glucose concentrations were expressed as means ±S.E.M, and differences between the four experimental groups were assessed using repeated measures of one factor ANOVA. Plasma ACTH levels were measured by radioimmunoassay [16], and plasma glucose concentrations were determined by the immobilized enzyme membrane/H2O2 method with a compact glucose Antsense II analyzer (Bayer Medical Co. Ltd, Tokyo, Japan) [18].

Results

Figure 1 shows Nissle staining of representative sections including the entorhinal cortex. The significant loss of neurons accompanied by extensive glial proliferation was observed in the entorhinal cortex sections of animals that received ibotenic acid injections (Group 3) (Fig. 1D,E). Animals that received injections of vehicle in the entorhinal cortex did not show any histological signs of neuronal damage (Fig. 1B,C). Figure 2 shows the ACTH and blood glucose concentrations for the lesoined and unlesioned rats following microinjectin of neostigmine into the hippocampus. Figure 2A shows the blood glucose concentration after microinjection of neostigmine into the hippocampus. For Group 1, the plasma concentration of glucose increased after 10 min and reached a peak after 60 min. The saline injected group (Group 2) showed no effect. For the lesioned groups, blood glucose levels for Group 3 were significantly lower than those of Group 4 (Fig. 2A). ANOVA showed that there was a statistically significant difference among the groups ($p<0.0001$), and Scheffe's post-hoc analysis indicated that ibotenic acid lesions significantly attenuated blood glucose release evoked by the microinjection of neostigmine into the hippocampus (Fig. 2A). No significant difference was observed in the plasma ACTH concentration between Group 1, 3 and 4 after the microinjection of neostigmine into the hippocampus (Fig. 2B).

Discussion

In the current study, we have discovered that the entorhinal cortex is involved in the regulation of stress-like responses induced by hippocampal neostigmine injection. The lesions in this area significantly attenuated the blood glucose elevation but did not affect ACTH secretion. No significant difference of weight was observed before and after lesion.

During stress, an adaptive or compensatory response by the organism is activated to sustain homeostasis. Stress induces adaptation through the produc-