evaluations as the narcolepsy group, including polysomnography and MSLT. Nevertheless, with any of the other neurologic disorders, a concomitant diagnosis of narcolepsy is relatively improbable based on their overall clinical presentations. Low CSF hypocretin levels were consistently found in all narcoleptic subjects. In addition, these levels were occasionally found prior to classic narcoleptic signs and symptoms. There may be a true, relatively independent diagnostic utility in measuring CSF hypocretin levels when narcolepsy is suspected in children.

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Aortic dissection presenting with transient global amnesia-like symptoms

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Diagnostic criteria of transient global amnesia (TGA) are witnessed attacks, clear-cut anterograde amnesia during the attack, absent clouding of consciousness and loss of personal identity, no accompanying focal neurologic symptoms or epileptic features, resolution of attacks within 24 hours, and no recent head injury or active epilepsy.[1] For the etiology of TGA, four main hypotheses have been considered: TIA, epilepsy, migraine, and transient venous ischemia.[1-3] None of these hypotheses fully explains the mechanism of this episodic disease, but the accepted neuroanatomic correlate of TGA is the mediobasal temporal lobe and hippocampus. We present two patients with aortic dissection who provide evidence for an ischemic pathogenesis in TGA.

Case reports. Patient 1. A 47-year-old man was found confused and disoriented. On examination at admission, he asked repetitive questions and was alert but completely disoriented to time and place and only partially oriented to person. The cranial nerve examination showed only a slight anisocoria. The pronator drift test revealed a discrete motor deficit of the left side, accompanied by a mildly increased reflex activity. The cranial CT was unremarkable. The EEG revealed no epileptic discharges. Some hours later, the patient was reoriented with an amnestic gap for the attack's duration, and the neurologic deficit had completely resolved. Because of persistent hypotension, a chest radiograph was taken, which revealed a widening of the mediastinum. A CT scan of the chest and abdomen revealed a dissecting aneurysm (Stanford type A) of the aortic arch starting at the aortic valve, involving both carotid arteries and the left subclavian artery, and continuing into both iliac arteries (figure).

Patient 2. A 61-year-old woman was taken to hospital because of acute chest pain. On admission, she had retrograde amnesia for the past few hours, anterograde amnesia with inability to learn new facts, and repetitious questioning. Neurologic examination revealed a mild right facial paresis. Cranial CT was unremarkable. Five hours after onset of symptoms, she was reoriented with an amnestic gap. Because of the initial thoracal pain and our knowledge of the first reported patient, an aortic dissection was considered. Chest radiograph was normal, but a CT scan of the chest and abdomen revealed a dissection of the aorta (Stanford type A) starting at the aortic valve, involving all supra-aortal branches, and ending above the left renal artery.

Discussion. Patients with TGA can be distinguished into three groups: "pure TGA" patients who fulfill all diagnostic criteria; patients with probable epileptic amnesia; and patients with probable transient ischemic amnesia. The third group includes patients with additional neurologic deficits during the attack as in our patients.[1] Although we found no proof of ischemic lesions in the cranial CT in our patients, the minor neurologic deficits suggested cerebral ischemia. MRI including diffusion-weighted MRI (DWI) would have been helpful to characterize the etiology, but both patients underwent immediate surgery without possibility for further diagnostics. We presume that an aortic dissection can cause a TGA subtype with ischemic etiology, which can be called transient ischemic amnesia. The underlying mechanism may be an embolic vascular occlusion in the posterior circulation, thus causing an embolic TIA with an unusual TGA-like TIA syndrome. The ischemic hypothesis in TGA was enforced by bitemporal hypoperfusion found in brain SPECT.[4] However, patients with TGA have fewer thromboembolic risk factors and smaller risk of cerebral infarction compared with those with TIA.[1] The DWI findings provide conflicting results concerning a possible ischemic mechanism.[5] In one study using DWI in 10 patients with TGA, 7 patients showed an elevated signal intensity in the left or in both temporomesial regions. This was interpreted as a hint of the possible etiologic role of spreading depression.[6] One-third of patients with TGA also have migraine. The low recurrence rate of ~8% in TGA and the different age distribution are arguments against migraine as a pathogenic mechanism.[1] The weakest evidence is

Figure. Multiplanar reconstructions from spiral CT: multiple dissection membranes within the aortic arch are demonstrated with involvement of the supra-aortic branches (arrows).

Enhanced Heat Loss and Age-Related Hypersensitivity to Diazepam

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Abstract: Whether elderly people suffer from age-related changes in pharmacokinetics and/or pharmacodynamics with administration of benzodiazepines is still a matter of controversy. We investigated the course of brain function and thermoregulation after oral administration of a standard benzodiazepine, diazepam (DZP), in 8 healthy young men (mean age, 19.8 years; range, 18 to 23 years) and 8 healthy middle-aged and older men (mean age, 60.9 years; range, 53 to 71 years). Placebo or DZP was administered in a single-blind crossover manner to the young men (placebo, 5-mg, 10-mg DZP) and to the older men (placebo, 5-mg DZP), and plasma DZP concentration, choice reaction time, proximal body temperature, and distal body temperature were monitored with high time resolution under a modified constant routine condition to exclude masking effects. Whereas there was no evidence of age-related alterations in pharmacokinetics between the 2 groups, the older subjects, in comparison to the young subjects, showed a more delayed choice reaction time in response to the same plasma DZP level, suggesting that hypersensitivity is related to increased age. DZP at 5 mg in the older subjects induced acute and transient hypothermia to the same degree as that induced by DZP at 10 mg in the young subjects. The distal-proximal body temperature gradient (difference between distal body temperature and proximal body temperature), an indicator of blood flow in distal skin regions, showed strong positive correlation with the delay in choice reaction time in both groups. These findings suggest that hypersensitivity to benzodiazepine in older persons may be due, at least in part, to age-related changes in thermoregulation, especially in the heat loss process.

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Benzodiazepine (BZP) is one of the most frequently prescribed drugs for elderly persons with anxiety or sleep disorders. Unfortunately, BZPs are often accompanied by undesirable side effects in the elderly, such as daytime sleepiness, cognitive decline, amnesia, rebound symptoms, ataxia, vertigo, and leading to falls and hip fracture.[1–5] An age-dependent increase in vulnerability of the elderly to these adverse effects has been discussed from the standpoint of pharmacokinetics (PK) and pharmacodynamics, that is, as the result of greater plasma concentrations of BZPs, increased sensitivity to BZPs, or a combination of the 2. Although numerous investigators have grappled with this issue, a definite conclusion has not been reached.[6–8]

Some studies showed age-related changes in the PK of BZPs such that elderly persons showed a higher plasma BZP concentration than that of young people.[9–13] Greenblatt et al[14] studied the PK and pharmacodynamics of orally administered triazolam in young and elderly subjects and concluded that the greater degree of sedation and impaired psychomotor performance observed in the elderly subjects was caused mainly by the reduced clearance and increased plasma concentration of triazolam rather than by increased intrinsic sensitivity to the drug. Another series of studies, however, showed that elderly persons might have greater sensitivity to BZP.[15–18] Platten et al[18] reported that midazolam induced more pronounced sedative effects in the brain, determined by choice reaction time (CRT) and visual analog scales, of elderly subjects compared with effects in young control subjects despite a lack of significant differences in PK parameters between the groups. This suggests altered pharmacodynamics, that is, increased sensitivity to BZPs with age. These conflicting study results may be due in part to differences in the drugs, to experimental protocols (including low time resolution), or to the limited number of available physiologic markers for exogenously administered BZP.

In the present study, we focused on the thermoregulatory response to the standard BZP, diazepam (DZP), as a reliable physiologic marker of sensitivity to BZP as well as a possible physiologic pathway by which brain function is impaired by DZP in the elderly. Many studies have consistently shown an intimate relation between thermoregulation and brain function, expressed in terms of alertness and psychomotor performance, not only under physiologic

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conditions[19–22] but also under various manipulations affecting sleep regulation: administration of melatonin, exposure to light, sleep deprivation, and passive body heating.[23–31] Recent studies have revealed that BZPs can also produce acute and transient changes in thermoregulation, that is, the heat loss process, in humans.[24,32–36] We aimed to investigate age-related changes in the sensitivity to DZP using thermoregulatory response as reliable physiologic marker. We performed CRT testing (responding to rare or frequent stimulation) with high time resolution and continuous monitoring of core body temperature (core BT) under a modified constant routine.[37] Under this routine, various masking effects on psychomotor performance and thermoregulation, produced by emotional stimulation, physical movement, posture, calorie intake, clothing, ambient temperature, and environmental light intensity are strictly controlled.

MATERIALS AND METHODS

Subjects

Healthy volunteers either younger than 25 years or between 50 and 75 years were each rigorously screened by 3 physicians via physical and psychologic evaluations. Volunteers were surveyed to exclude the following: irregularity in the sleep-wake pattern according to a self-registering 2-week sleep diary, history of physical disease that could affect sleep states, history of psychiatric disease identified by the Mini-International Neuropsychiatric Interview[38] or a structured diagnostic psychiatric interview for *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* disorders (including depression and anxiety disorders), use of medications (including BZP) that might modify sleep states during the previous 3 months, any airline travel during the previous 6 months that could cause jet lag, and any abnormal hematology or urinalysis findings. All older volunteers underwent Mini-Mental State Examinations (mean score, 29.3; range, 27 to 30) to rule out the presence of dementia. Finally, 8 young men (mean age, 19.75 years; range, 18 to 23 years) and 8 middle-aged and older men (mean age, 60.88 years; range, 53 to 71 years) gave written informed consent and participated in the study. All of the study subjects were paid volunteers. BMI in the young subjects was 20.8 ± 0.7 (SEM) (range, 17.6 to 23.5) and in the older subjects was 22.8 ± 1.0 (range, 17.3 to 26.8).

General Setting

All subjects abstained from tobacco, alcohol, heavy exercise, and medications for at least 7 days before the beginning of the study. During this prestudy period, all subjects were asked to maintain their daily home routines but to keep light intensity under 10 lux during the time in bed. Sleep quality and regularity were assessed by means of an actigraph (AMI Inc., Ardsley, NY) fitted to each subject's nondominant wrist; the data were analyzed for computer-calculated sleep-wake determinations by using Cole et al's algorithm.[39] For each subject, sleep onset times during the prestudy period were averaged; average onset time was defined as 0000 hours. Young subjects participated in 3 experiments (2 drugs, 1 placebo), and older subjects participated in 2 experiments (1 drug, 1 placebo). Experiments were conducted at 2-week intervals.

On the day before each experiment, the subject entered the sleep laboratory 8 hours before 0000 hours (−0800 hours). Before −0600 hours, the subject donned a cotton gown provided by the laboratory to control for the influence of clothing on thermoregulation. At −0600 hours, a 750-kcal meal and as much water as desired were given to each subject. From the time after the meal until the end of the study at 1630 hours the next day, the subject rested in the supine position on a reclining seat during the awake period. Standing and walking were prohibited, and movement of the limbs was discouraged. An adjoining room was fitted with a portable toilet, movement to the toilet was assisted, and sitting was permitted while the toilet was being used. Subjects were allowed to recline and sleep on a bed in the laboratory only from 0000 to 0800 hours. During the sleep time, each subject underwent polysomnography and was confirmed to have no sleep disorder as defined by the International Classification of Sleep Disorders.[40] Sleeping was forbidden outside the sleep period. Participants were subjected to continuous electroencephalography monitoring, and at least 2 laboratory workers were always present to observe the subjects and provide assistance. Laboratory lighting was maintained at less than 10 lux during the sleep period and at 100 lux near the subject's eyes at other times to avoid the alerting and alleviating effects of bright light on sleepiness and psychologic performance.[41] Room temperature was maintained at 23°C ± 1°C throughout the study period.

Drug Administration

On the day of the experiment, each subject ingested a 100-kcal meal and 100 mL of orange juice at 0830 hours to ensure alimentary canal absorption of DZP and to suppress the influence of calorie intake on temperature and psychomotor performance. We tested responses to placebo (lactose), 5-mg DZP, and 10-mg DZP for young subjects and to placebo and 5-mg DZP for older subjects. All substances were packed in indistinguishable gelatin capsules and were administered with 100 mL water at 1200 hours. The study was conducted in a randomized, single-blind, crossover fashion.

Evaluation of Plasma Concentrations of DZP and Desmethyldiazepam

Blood samples were used to monitor plasma DZP and its active metabolite desmethyldiazepam (dmDZP). Samples were drawn every 20 minutes from 1200 to 1500 hours and

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again at 1600 hours via intravenous catheter placed in a forearm vein at 0900 hours. Blood samples were immediately centrifuged at 3000 rpm for 5 minutes, and the plasma was collected and frozen at −20°C for later assay. Plasma concentrations of DZP and dmDZP were determined by a high-performance liquid chromatography (HPLC) method previously described[42,43] but with some modifications. The apparatus used for HPLC was the EP-300 chromatography pump (EICOM, Kyoto, Japan) equipped with an EICOM NOD-10 NOx detector. The wavelength was set at 230 nm. Test samples were introduced with a 234 autoinjector (Gilson Inc., WI) at an effective volume of 75 μL. The HPLC column used was a Grand pack ODS-5 NK stationary phase (5 μm, Masis Inc., Owani, Japan). The column temperature was maintained at 25.0°C in an EICOM ATC-300 column oven. The mobile phase consisted of 0.5% KH_2PO_4 (pH 4.5)-acetonitrile (60:40, vol/vol), which was degassed in an EICOM DG-300 degasser before use. The flow rate was 1.0 mL/min. All solvents used were of HPLC grade (Wako Pure Chemical Industries, Osaka, Japan). After flunitrazepam (320 ng) in methanol (10 μL) was added to plasma samples (1 mL) as an internal standard, the plasma samples were diluted with 5 mL water, and the solution was mixed briefly. The mixture was applied to a Sep-Pak CN cartridge (Waters, Bedford, MA) that had been activated previously with 10 mL 100% acetonitrile and water. The cartridge was then washed with 10 mL water and 5 mL 20% acetonitrile in water. The desired fraction was eluted with 5 mL 70% acetonitrile in water. The eluate was evaporated in a vacuum at 40°C by a rotary evaporator (Tokyo Rikakikai, Tokyo, Japan). The residue was dissolved in 50 μL methanol, and 100 mL mobile phase was added. The samples were injected into the HPLC apparatus. The ratios of drug to internal standard were calculated from the recorded peaks. The results obtained from spiked plasma samples containing known amounts of drug were calculated on the basis of linear regression analysis.

Evaluation of Psychomotor Performance

Psychomotor performance was monitored every 20 minutes from 1000 to 1600 hours. During the first 5 minutes of each 20-minute epoch, CRT testing was applied as described previously.[44] Two different brief tones (2000 and 1000 Hz), each with a duration of 100 milliseconds, were generated by an automatic stimulator (Nihon Kohden SS-1449, Nihon Kohden, Tokyo, Japan) and presented at 60 dB (normal hearing level). The 1000-Hz tone was presented 80% of the time, and the target 2000-Hz tone was presented 20% of the time. Order of presentation was randomized, and length of the interstimulus interval was also randomized within a range of 1.0 to 3.0 seconds (mean, 2.0 seconds). Subjects were instructed to respond only to target stimuli (2000-Hz tone) by pressing with the thumb a button attached to the palm of the right hand ('oddball' paradigm). The button-press response was converted to an electrical signal recorded on a personal computer. Older subjects had been screened at time of recruitment for their ability to accomplish CRT testing to avoid as much as possible the influence of hearing disability due to aging; they responded to 165 2000-Hz tones and 660 1000-Hz tones through 5 test trials. CRT testing at 1200 hours was performed immediately before drug administration. Thirty-three reaction time latencies from target stimulus to button press were averaged as the CRT data at each point of measure. All other CRT data were expressed relative to the data obtained at 1200 hours. For further analysis, the change in CRT (ΔCRT) induced by DZP was defined as the difference in the corresponding values between the DZP and placebo experiments at each point of measure.

Evaluation of Heat Loss After DZP Administration

Between −0800 and −0600 hours, skin temperature thermistors were attached to both wrist areas and to the instep of each foot. A rectal thermistor (polyethylene-covered thermoprobe, accuracy within 0.01°C) was inserted 10 cm into the subject's rectum. The thermistors were connected to an ambulatory temperature monitoring system (Kohden Medical Inc., Tokyo, Japan), and sampling occurred at 1-minute intervals. All temperature recordings taken on the day of the experiment were later collapsed into 20-minute bins from 1100 to 1600 hours. The data at 1200 hours were averaged for 10 minutes just before drug administration to control for acute drug effects. After that, bin data obtained from each subject were expressed relative to the data at 1200 hours. Proximal body temperature (p-BT, rectal temperature) and distal body temperature (d-BT, average skin temperature from 4 sites) were used to calculate the distal-proximal body temperature gradient (DPG, difference between d-BT and p-BT) as an indicator of blood flow in distal skin regions.[26,45] For further analysis, the changes in p-BT, d-BT, and DPG (Δp-BT, Δd-BT, and ΔDPG, respectively) induced by DZP were defined as the difference in corresponding values between the DZP and placebo experiments at each point of measure.

Data Analysis

Results are shown as mean ± SEM. $P < 0.05$ was considered statistically significant. One-way analysis of variance or two-way analysis of variance (factors: time and dose) followed by Student t test was used to examine differences in BT and CRT values obtained for placebo and for each dose of DZP. Relations between psychomotor performance and plasma DZP concentrations or the DPG were determined by Pearson correlation coefficient. Analysis of covariance was used to examine the difference in the slopes of the simple linear regression lines between the young and older subjects.

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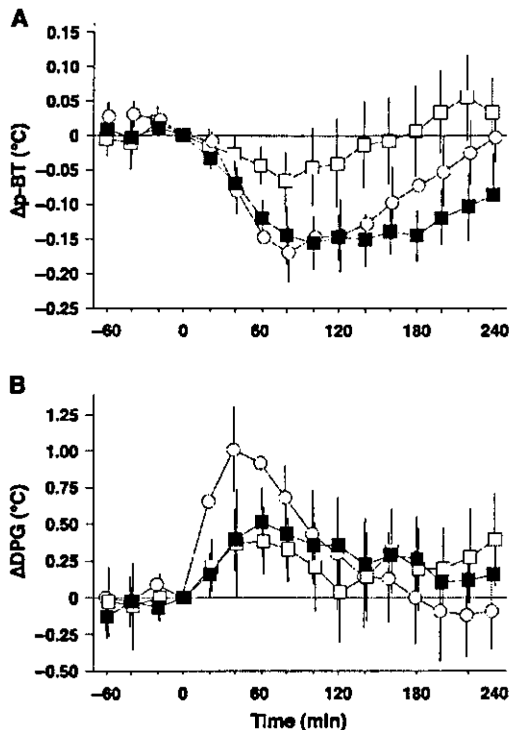

FIGURE 4. Changes in Δp-BT (A) and ΔDPG (B) in young and older subjects after DZP administration. White circles and white squares represent data for 10- and 5-mg DZP experiments in young subjects; black squares represent data for 5-mg DZP experiment in older subjects. Horizontal bars indicate time of drug administration relative to 1200 hours. Mean ± SEM values are shown. Δp-BT and ΔDPG were defined as difference in corresponding values between placebo and DZP experiments at each point of measure. Δp-BT and ΔDPG values are shown as data relative to value at 1200 hours.

the young ($r = 0.799$, $P < 0.0001$, Fig. 5A) and the older subjects ($r = 0.692$, $P < 0.01$, Fig. 5B).

DISCUSSION

In this study, we aimed to clarify whether older persons possess hypersensitivity to BZPs. We used frequently recorded CRT and body temperature data as physiologic markers for psychomotor performance and thermoregulation under strict control of potential masking effects. Under the conditions of the present study, 4-hour plasma DZP concentration profiles were very similar between young and older subjects. Neither group showed significant differences in C_{max}, T_{max}, or area under curves for DZP and dmDZP after oral administration of DZP at 5 mg, suggesting that older subjects in the present study experienced no significant age-related changes in PK responses, at least after a single administration of the drug. Nevertheless, older subjects, in comparison to young subjects, showed significantly greater impairment of psychomotor performance after DZP administration. DZP at 5 mg in older subjects induced a significantly greater prolongation of CRT compared to that in the young subjects, resulting in decline in psychomotor performance similar to that induced by 10-mg DZP in the young subjects. Furthermore, analysis of covariance between plasma DZP concentration and increase in CRT (Fig. 2) revealed that the same degree of psychomotor performance impairment was induced by lower plasma DZP concentration in older subjects than in young subjects, at least within clinical dosage. Contrary to the present study, some previous studies have shown an age-related reduction in DZP clearance after single intravenous administration.[11,13] The lack of change in PK responses in our older subjects seemed to be caused partly by the smaller study group, shorter observation period (4 hours after administration), and lower mean age (60.9 years, with some subjects in middle age) compared to that of previous studies. Despite these limitations, our findings indicate increased sensitivity to DZP in older subjects and that the decline in psychomotor performance induced by DZP was due mainly to hypersensitivity to DZP.

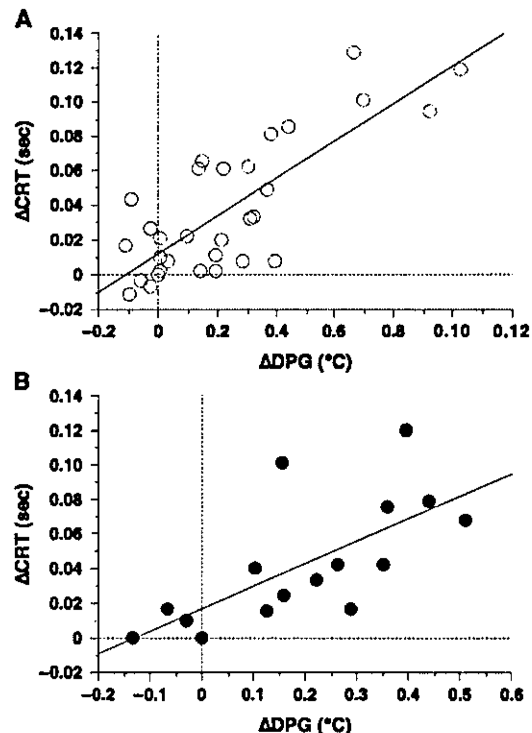

FIGURE 5. Relation between ΔDPG and ΔCRT in young (A) and older (B) subjects. Pearson correlation coefficient was determined for mean ΔDPG (x-axis) or ΔCRT (y-axis) at corresponding points of measure. Data in 5- and 10-mg experiments for young subjects were combined.

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We showed that heat loss might be a reliable physiologic marker of increased age-related sensitivity to BZPs and that it may to some extent explain the mechanism underlying increased impairment of psychomotor performance induced by DZP in elderly persons. Growing evidence suggests that thermoregulation, especially the process of heat loss, is a key generator of sedative effects in the brain, such as those producing sleepiness and impaired psychomotor performance, in humans. Recent studies have shown that the sleep-producing[21,24–26] and psychomotor performance-suppressing[31,36] mechanism in humans is preceded by increased heat loss. Various manipulations accelerating sleepiness or impaired psychomotor performance act, at least in part, by enhancing the heat loss mechanism.[23,24,26,29,31,36] Our study confirmed that a standard hypnotic drug, DZP, also induces an acute and transient decrease in p-BT (rectal temperature), as was previously reported in humans[24,32–35] and animals[46,47] for some other kinds of BZPs. The decrease in p-BT after administration of DZP at 10 mg was preceded by a prominent increase in distal skin temperature in the young subjects. Why the heat loss induced by 5-mg DZP in the young subjects did not last long and did not reach statistical significance remains unclear. As was discussed elsewhere,[36] it is possible that there is a threshold for the plasma concentration of DZP to cause an obvious heat loss reaction in humans. Additionally, some of the young subjects participated in the 5-mg DZP experiment only 2 weeks after participating in the 10-mg DZP experiment, and these subjects might therefore have developed a tolerance for DZP. By contrast, the older subjects exhibited significant decrease in p-BT, even with 5-mg DZP. This decrease was equal to that induced by 10-mg DZP, but not equal to that induced by 5-mg DZP in the young subjects; this supports the assumption that older subjects possessed thermoregulatory hypersensitivity to BZPs. Maximum suppression of mean p-BT (maximum difference between BT with placebo and BT with DZP) was 0.17°C ± 0.04°C for young subjects with 10-mg DZP and 0.14°C ± 0.04°C for older subjects with 5-mg DZP. Heat loss to this extent could significantly affect brain function because rapid decline in p-BT rather than the absolute value of the change in p-BT appears to be the critical factor in generating physiologic sleepiness in humans.[21] Our young and older subjects showed prominent daily p-BT rhythms with amplitudes of 0.77°C and 0.61°C in the placebo experiment (not shown in RESULTS), in which physiologic heat loss from peak (late afternoon) to nadir (early morning) took 12 hours 15 minutes and 11 hours 30 minutes, respectively. By contrast, the magnitudes of heat loss reactions with DZP, which were equivalent to approximately 22% of the amplitudes of the daily p-BT rhythms, occurred acutely within 60 to 100 minutes after DZP administration. It is interesting that our older subjects showed a lower magnitude of DPG after administration of 5-mg DZP despite their marked drop in p-BT. We assume that this was due to their diminished ability to produce heat. P-BT is determined by the balance between heat production and heat loss. Humans produce heat steadily by means of basal metabolism. When exposed to cold stress or heat loss stimulation, compensatory heat production is achieved mainly through shivering thermogenesis, in which the activity of skeletal muscles leads to involuntary contractions. Aging is associated with a decrease in the amount of skeletal muscle, diminishing both the capacity for shivering thermogenesis and basal metabolism.

The present study revealed that the heat loss induced by DZP might be intimately related to delayed CRT in humans. We found a strong positive relation between the magnitude of DPG, difference between distal and proximal BT, and impaired psychomotor performance induced by DZP in both young and older subjects. DPG has been shown to accurately reflect blood flow in distal skin regions regulated by anastomosis arteriovenosa.[45] This means that the greater the distal vasodilation after DZP administration, the greater the impairment of psychomotor performance. Krauchi et al[26] applied DPG as an indicator of distal heat loss and showed that it successfully predicts the reduction in sleep onset latency induced by various sleep-promoting modifications. Our findings show that the DPG induced by DZP might also be a potent physiologic marker to predict sedative effects on human brain function. We did not obtain enough data to determine to what extent the enhanced heat loss reaction observed in the older subjects contributed to their DZP-induced impaired psychomotor performance. We realize that our findings do not necessarily indicate a causative link between the heat loss process and impaired psychomotor performance. It is likely that at least some of the sedating effects of BZPs are due to their effect on several central activating systems that are likely not linked directly to thermoregulation. Additionally, whether the heat loss process could contribute to impairment of other cognitive functions including memory remains unresolved. To obtain more convincing evidence of an etiologic link, future study should focus on whether prevention of heat loss induced by BZPs results in attenuation of the sedative effect determined by various cognitive markers. Nevertheless, the present study provides a useful physiologic marker for predicting the magnitude of suppression of psychomotor performance with BZP in humans and important insight into the physiologic pathway of sedative/hypnotic effects induced by BZPs and the mechanisms underlying age-related hypersensitivity to BZPs.

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Similar profiles in human period1 gene expression in peripheral mononuclear and polymorphonuclear cells

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Abstract

Increasing amounts of data have indicated the physiological significance of circadian clock gene regulation in various peripheral cells. In the present study, we examined expression of the human homolog of period1 (*hPer1*) in peripheral mononuclear cells (MNCs) and polymorphonuclear neutrophils (PMNs) in seven healthy young male volunteers (mean age, 21.0 years; range, 19–24 years) under modified constant routine conditions. The expression of *hPer1* as determined by real-time PCR with gene-specific hybriprobes in MNCs and PMNs showed significant daily variations with similar acrophases and peak transcription in the subjective morning. The acrophases in *hPer1* expression rhythms in MNCs and PMNs were found to correlate positively with that of the serum melatonin secretion rhythms, which is a reliable phase marker of the suprachiasmatic nucleus (SCN), the circadian master clock. The present findings indicate that clock gene activity could be preserved across different peripheral blood cell types and support the assumption that peripheral clocks are entrained by the SCN.
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Keywords: Circadian rhythm; Suprachasmatic nucleus; Peripheral clock; Mononuclear cells; Polymorphonuclear neutrophils; Melatonin

The suprachiasmatic nucleus (SCN) is the site of the circadian pacemaker in mammals. Clock genes compose the translation–transcription feedback loop in the SCN and also in many peripheral tissues/cells, including liver, heart, kidney, and skin [1,12], suggesting that peripheral clocks are entrained by a "master" clock localized in the SCN. The circadian feedback loop consists of both positively regulating genes such as *Clock* or *Bmal1*, and negatively regulating genes such as *Period1-3*, *Cry1-2*, *Timeless*, and several other clock genes [6]. Recent findings strongly suggest that transcription of numerous genes related to metabolic and physiological processes are under the control of a circadian timing system [10,11]. A recent study revealed that clock genes directly regulate the cell cycle via modification of cell cycle-related gene expression in proliferating peripheral cells [9]. These findings support the assumption that clock genes contribute to the establishment of cell/organ-specific circadian rhythms in physiological processes. In other words, changes in transcription of clock genes could influence the circadian phase and/or amplitude of a physiological process at the single-cell level. The aim of the present study was to examine circadian phases in expression of the human homolog of period1 (*hPer1*; GenBank accession no. AB002107) in two representative fractions of peripheral blood cells, mononuclear cells (MNCs) and polymorphonuclear neutrophils (PMNs).

Seven healthy young male volunteers (mean age, 21.0 years; range, 19–24 years) participated in this study. Subjects were surveyed for the following issues: no irregularities of sleep–wake patterns according to a self-registering sleep diary kept for 2 weeks, no history of psychiatric disease as assessed by Mini-International Neuropsychiatric Interview (MINI), no history of physical disease or use of medications during the past 6 months, no flights with jet lag during the past 6 months, and normal hematology and urinalysis data. Beginning 7 days before the start of the study, 8 h sleep beginning at the time of the volunteer's usual onset of sleep was enforced. During this pre-study period, all subjects were asked to keep an ordinary daily routine at home in which the light intensity during the time in bed was kept under 10 lux.

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Sleep quality and regularity were assessed with an actigraph (AMI Inc., Ardsley, NY) fitted to the non-dominant wrist of each subject. Data were analyzed for computer-calculated sleep–wake determinations by Cole's algorithm [5]. Night-time sleep parameters for each subject were defined as follows: sleep onset time (SOT), clock time of the onset of sleep; sleep efficacy (SE), the total time asleep as a percentage of the total time in bed; awake time (AT), the total number of waking episodes that continued for at least 10 min during the sleep period; and wake time after sleep onset (WASO), the accumulated time awake after the sleep onset. The average SOT during the pre-study period was defined as 0 h for each subject.

On the day before the experiment, subjects entered the sleep laboratory at 8 h before 0 h (−08:00 h). At −06:00 h, subjects were given a 750 kcal meal and as much water as desired. An intravenous catheter for blood sampling was placed in a forearm vein at −05:00 h. From −04:00 h until the end of the study at 10 h on the second experimental day (total, 39 h), subjects were under a modified constant routine condition in which the masking effects of physical movement, posture, calorie intake, clothing, ambient temperature, and environmental light intensity were controlled. Subjects rested in a supine position on a reclining seat during the waking time. Some physical movement, such as walking to the toilet in the next room and occasional stretching of the limbs, was allowed. Subjects slept on a bed in the same room from 00:00–08:00 h and 24–32 h. Sleep parameter values in the pre-study period, first and second experimental nights were as follows: SE (97.4 ± 0.9, 96.3 ± 1.3, 95.1 ± 1.3%); AT (0.29 ± 0.2, 0.60 ± 0.4, 0.33 ± 0.3); and WASO, (17.0 ± 5.5, 32.4 ± 15.4, 20.5 ± 6.1 min). No significant difference was observed in either SE, AT, or WASO among the three conditions. Subjects ingested a 200 kcal snack, free of caffeine or other stimulants, and 150 cm^3 of water every 2 h beginning at 08:00 h (except during the period of sleep from 24–30 h). Laboratory illumination was maintained at less than 10 lux during sleep and at 100 lux near the subjects' eyes at another times. The ambient temperature was maintained at 23 ± 1 °C throughout the study period.

Core body temperature was monitored at 1-min intervals throughout the study by an ambulatory temperature monitoring system (Kohden Medical Inc., Tokyo, Japan) with a polyethylene-covered thermistor (accuracy within 0.01 °C) inserted 10 cm into the subject's rectum. Blood collection for determination of serum melatonin levels, distribution of white blood cell (WBC) subsets, and *hPer1* mRNA expression was performed painlessly every 2 h from 09:00 h on the first experimental day until 33 h on the second experimental day (total 13 time points). Serum samples were collected and frozen at −80 °C for later radioimmunoassay (Buhlmann Lab AG, Basel, Switzerland). Distribution of WBC subsets was determined with ac diff (Beckman Coulter Inc., CA, USA), and MNCs and PMNs numbers are reported as the percentage of the total cell number for WBC. MNCs and PMNs were isolated from 5 mL of whole

Table 1
Primer and oligohybriprobe sets for *hPer*, *hBmal1a*, *β-actin*

hPer1	forward	5'-AgCAgCAgCCTCggTTTT-3'
hPer1	reverse	5'-CTCCTCCTCCATAgCCAAgT-3'
hPer1	Flu	5'-ATggCCTgTgTggACTgTggg-3'-Flu
hPer1	LC	5'-LC-gCAgCACCCAAgATCCTggTC-3'-p
hBmal1a	forward	5'-CCAggAggCAAgAAgATTT-3'
hBmal1a	reverse	5'-gATCCTTggTCgTTgTCA-3'
hBmal1a	Flu	5'-gCTCAggAgAACCCAggTTATCCATATT-3'-Flu
hBmal1a	LC	5'-LC-TgATAgTTCTTCTATTCTTggTgAgAACCCC-3'-p
ß-actin	forward	5'-CCAACCgCgAgAAgATgAC-3'
ß-actin	reverse	5'-ggAAggAAggCTggAAgAgT-3'
ß-actin	Flu	5'-CCTCCCCCATgCCATCCTgCgTC-3'-Flu
ß-actin	LC	5'-LC-ggACCTggCTggCCgggACCTgA-3'-p

Flu:Fluorescein, LC:LCRed640, p:phosphorylation.

blood by single-step separation over a Ficoll–Hypaque solution (Polymorphoprep, Axis-Shield PoC, Oslo, Norway). Each fraction was washed with phosphate-buffered saline and then promptly added to RNA/DNA stabilization reagent for blood/bone marrow (Roche Diagnostics Co. Ltd., Basel, Switzerland) and stored at −20 °C until later mRNA extraction. mRNA was extracted from each cell fraction with a magnetic particle separator method followed by conversion to first-strand cDNA with oligo-$(dT)_{15}$ primer and AMV reverse transcriptase. The primer and oligohybriprobe sets for *hPer1* and *ß-actin* (GenBank accession no. BC013380, internal standard housekeeping gene) for real-time PCR (LightCycler system, Roche Diagnistics KK, Tokyo) are listed in Table 1. Each cDNA-specific oligohybriprobe set was labelled with either donor fluorophore at the 3′-end (Fluorescein) or acceptor fluorophore (LC Red 640) at the 5′-end. Parameters for amplification of *hPer1* (*ß-actin*) were: 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s; 58 °C (60 °C) for 15 s; and 72 °C for 13 s (18 s). A series of 10-fold dilutions of plasmid DNA encoding each gene were prepared and included in each amplification reaction to generate a standard curve. All samples were tested in duplicate, and average of these duplicates was used for quantification. Relative expression of *hPer1* was defined as the ratio of *hPer1* mRNA expression to that of *ß-actin* for each blood sample. Gene expression data were then *z*-score normalized with respect to each subject's mean and standard deviation (S.D.) (*z*-score; mean = 0, S.D. = 1) to compare expression profiles between MNCs and PMNs.

Circadian phase and amplitude for each physiological rhythm for each subject were evaluated by cosinor curve-fitting analysis adapted to a 24 h-period with the method of least. One subject showed an irregular rhythm pattern in melatonin secretion and cosinor curve-fitting analysis could not be applied. The melatonin data for this subject was omitted from later analyses. Data in tables and figures are expressed as mean ± standard error of mean (S.E.M).

One-way ANOVA showed that the present subjects exhibited significant daily variations in serum melatonin levels

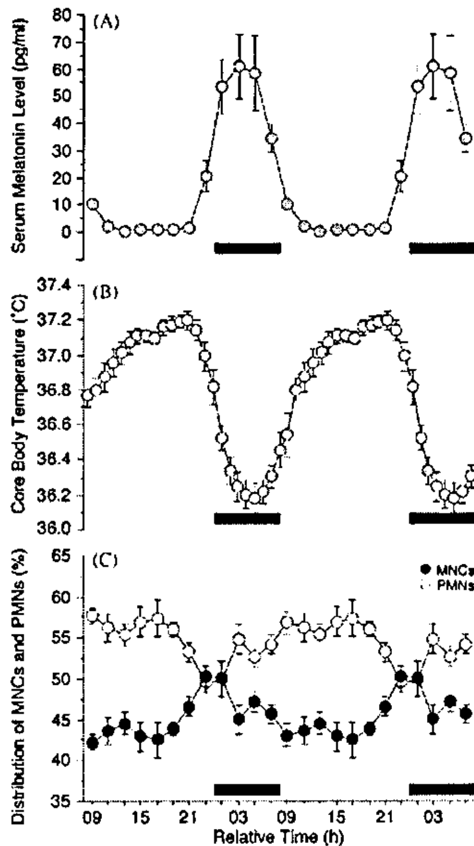

Fig. 1. Daily profiles of serum melatonin, core body temperature (A) distribution of MNCs and PMNs. (B) Horizontal axis indicates time relative to the average time of sleep onset (00:00 h) determined for each subject and (C) black bars indicate the sleep time. Data are double-plotted and shown as the mean ± S.E.M. values.

(F = 22.5, d.f. = 12, $P < 0.001$; Fig. 1A), core body temperature (F = 25.93, d.f. = 12, $P < 0.001$; Fig. 1B), and percentages of MNCs (F = 3.189, d.f. = 12, $P < 0.001$) and PMNs (F = 3.189, d.f. = 12, $P < 0.001$; Fig. 1C). The acrophase (peak time) and amplitude for each rhythm are shown in Table 2. The circadian phase and amplitude of melatonin or core body temperature rhythm in the present subjects were similar to those in young controls in many pre-

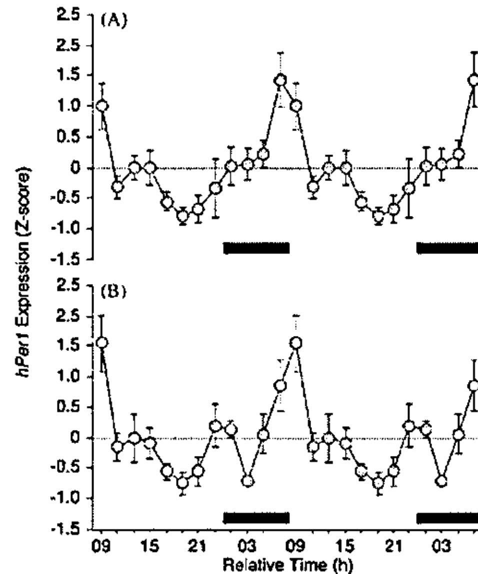

Fig. 2. Expression of *hPer1* in MNCs (A) and PMNs (B). Horizontal axis indicates time relative to the average time of sleep onset (00:00 h) determined for each subject. Black bars indicate the sleep time. Data for each subject was z-score normalized (mean = 0, S.D. = 1). Data are double-plotted and shown as the mean ± S.E.M. values.

vious studies (data not shown), suggesting that the circadian oscillations of the SCN and entrainment to the environmental light–dark cycle were stable in the present subjects.

hPer1 mRNA was detected in both peripheral MNCs and PMNs. Significant daily variations in *hPer1* expression were observed in MNCs (F = 4.451, d.f. = 12, $P < 0.001$; Fig. 2A) and PMNs (F = 5.336, d.f. = 12, $P < 0.001$; Fig. 2B) and peak transcription occurred between 07:00 and 09:00 h. The profile of *hPer1* expression in MNCs was consistent with that reported in the SCN and peripheral cells in rodents [6,13] and with that reported recently by Boivin et al. [3] in which the highest level of *hPer1* transcription in MNCs was observed in the subjective morning in humans. To our knowledge, the present study is the first showing that human peripheral PMNs and MNCs have the same acrophase of *hPer1* mRNA expression rhythm (Table 2). PMNs exhibited an additional smaller peak in *hPer1* expression in the

Table 2
Circadian properties in physiological markers

Variables	Acrophase h:m ± m	Amplitude	Analysis of variance		
			F-value	d.f.	P-value
hPer1 in MNCs	7:33 ± 50	–	4.451	12	<0.001
hPer1 in PMNs	7:43 ± 41	–	3.650	12	<0.001
Melatonin	3:33 ± 16	61.9 ± 11.7 pg/ml	22.50	12	<0.001
Core BT	16:53 ± 25	1.10 ± 0.08 °C	25.93	12	<0.001
MNCs	1:34 ± 37	–	3.189	12	<0.001
PMNs	13:33 ± 37	–	3.189	12	<0.001

BT, body temperature; MNCs, mononuclear cells; PMNs, polymorphonuclear cells. Data are shown as the mean ± S.E.M. values.

TABLE IV. *hClock* 3111T/C Genotype and Allele Frequencies in Japanese Subjects With Extreme Morning and Evening Preferences

	Genotypes			Allele frequency		3111T	
	3111T/T	3111T/C	3111C/C	3111T	3111C	Positive	Negative
Morning	24/30	6/30	0/30	0.894	0.106	30	0
Intermediate	25/30	5/30	0/30	0.913	0.087	30	0
Evening	19/30	7/30	4/30	0.796	0.204	26	4
P-value (χ^2)			0.055		0.001		0.015

sleep phases but not on ME preferences in humans. Physiological cross relationship between ME preferences, sleep phases and clock genotypes should be further assessed in future studies.

Iwase et al. [2002] reported that the 3111C allele frequency was lower in patients with DSPS than in controls. The finding that the 3111C allele was associated with evening preference in Caucasian and Japanese populations did not support the hypothesis that DSPS is simply an extreme form of evening preference. As was suggested in previous studies [Ozaki et al., 1996; Duffy et al., 1999; Uchiyama et al., 2000; Iwase et al., 2002], patients with DSPS could suffer from different pathological phase relationship between endogenous circadian phase and sleep timing from that for people with strong evening preference. Although the present study contained shift workers and the sample size for 3111C/C genotype was small (n = 12), our results suggest that a single nucleotide polymorphism may influence a human behavioral pattern and possibly lead to altered daytime brain performance.

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Resetting of peripheral circadian clock by prostaglandin E_2

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In mammals, the master circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN is thought to drive peripheral oscillators by controlling neuronal and humoral signals that can entrain the peripheral clocks. Here, we show that prostaglandin E_2 (PGE_2), a proinflammatory compound known to have diverse biological effects, is able to act as an *in vivo* clock-resetting agent. We find that in cultured NIH3T3 fibroblasts, PGE_2 is able to induce transient expression of Period 1 messenger RNA and the following circadian oscillation of clock gene expression. Furthermore, we demonstrate that intraperitoneal administration of PGE_2 results in the phase shift of circadian gene expression in mouse peripheral tissues in a time-dependent manner. This phase shift is also induced by the EP1/EP3 agonist sulprostone but not by the EP2 agonist butaprost. The PGE_2-induced phase shift is inhibited by the EP1 antagonist SC-51322. These results suggest that PGE_2 acts as an *in vivo* clock-resetting factor by means of the EP1 subtype of PGE receptors.
Keywords: circadian rhythm; clock; periphery; PGE_2; phase shift
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INTRODUCTION

In mammals, the master circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, but circadian oscillators also exist in most peripheral tissues (Reppert & Weaver, 2001). The SCN receives light information by means of the visual pathway from the retina to entrain the clock to the light–dark cycles of the environment, and it is thought to drive peripheral oscillators by controlling neuronal and humoral signals that can entrain the peripheral clocks (Yamazaki *et al*, 2000). Circadian gene expression can also be observed in cultured cell lines *in vitro* (Balsalobre *et al*, 1998; Akashi & Nishida, 2000). In these cell lines, various stimuli are known to induce the immediate expression of Period 1 (Per1) messenger RNA and the following circadian gene expression of clock and clock-controlled genes (Balsalobre *et al*, 2000a). However, *in vivo* clock-resetting factors that synchronize peripheral oscillators have not yet been fully clarified. It has been reported that glucocorticoid hormones, which are secreted in daily cycles, are able to reset the peripheral oscillators without affecting the SCN pacemaker (Balsalobre *et al*, 2000b). The glucocorticoid receptor, however, is dispensable for the entrainment of circadian gene expression in liver under steady-state conditions (Balsalobre *et al*, 2000b), indicating the existence of other intrinsic timing cues for the circadian oscillators *in vivo*. In the present study, we show that prostaglandin E_2 (PGE_2) is able to reset the rhythm-generating core feedback loops of circadian gene expression in murine peripheral tissues. PGE_2 is a proinflammatory compound known to have diverse biological effects, such as hyperthermia and waking effect (Narumiya *et al*, 1999; Yoshida *et al*, 2000; Bos *et al*, 2004), and PGE_2 secretion levels show diurnal variations in several tissues (Pandey *et al*, 1995; Yosipovitch *et al*, 1995). Our finding demonstrates that PGE_2 acts as an *in vivo* clock-resetting agent.

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RESULTS AND DISCUSSION

Circadian rhythms induced by PGE_2 in NIH3T3 cells

We searched for a new candidate for a clock-resetting agent and found that PGE_2 treatment of NIH3T3 cells is able to induce the acute and transient expression of mPer1 mRNA in a dose-dependent manner (Fig 1A). The immediate and acute induction of Per1 mRNA expression is strongly associated with the entrainment of circadian rhythms both *in vivo* and *in vitro* (Shigeyoshi *et al*, 1997; Balsalobre *et al*, 1998). To investigate the downstream signalling pathway participating in this transient expression of mPer1, we tested a wide range of inhibitors for their ability to prevent the PGE_2-induced mPer1 expression. As shown in Fig 1B, mPer1 expression was significantly inhibited by pretreatment of cells with the wide-spectrum kinase inhibitor staurosporine ($P<0.0001$ by Student's *t*-test), indicating that some kinase-dependent pathways contribute to the induction. Moreover, this mPer1 expression was strongly inhibited by the intracellular Ca^{2+} chelator BAPTA-AM ($P<0.001$ by Student's

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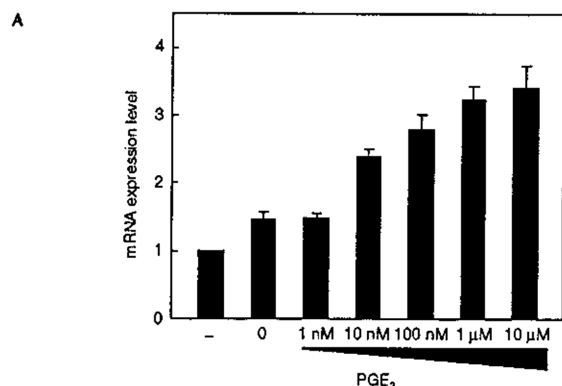

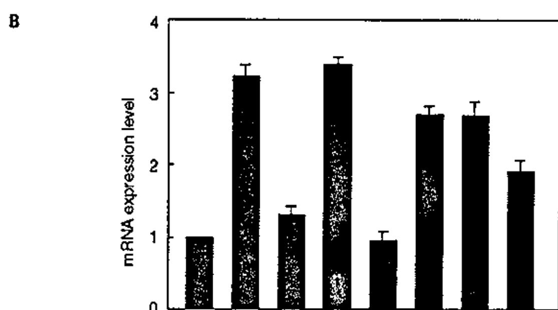

1 µM PGE2	–	+	+	+	+	+	+	+
1 µM staurosporine	–	–	+	–	–	–	–	–
1 mM EGTA	–	–	–	+	–	–	–	–
30 µM BAPTA-AM	–	–	–	–	+	–	–	–
20 µM KN-93	–	–	–	–	–	+	–	+
20 µM U0126	–	–	–	–	–	–	+	+

Fig 1 | Induction of mPer1 mRNA expression by prostaglandin E_2 (PGE_2) in NIH3T3 cells. (A) Dose-dependent induction of mPer1 mRNA by PGE_2 treatment of NIH3T3 cells. Relative levels of mRNA expression 1 h after the indicated treatment are evaluated by the real-time quantitative PCR method. Each value was normalized to mG3PDH. Values are mean ± s.e.m. from three experiments. (B) Effect of inhibitors on mPer1 induction by PGE_2 treatment. NIH3T3 cells are treated with the indicated agents 30 min before PGE_2 treatment. Values are mean ± s.e.m. from three experiments.

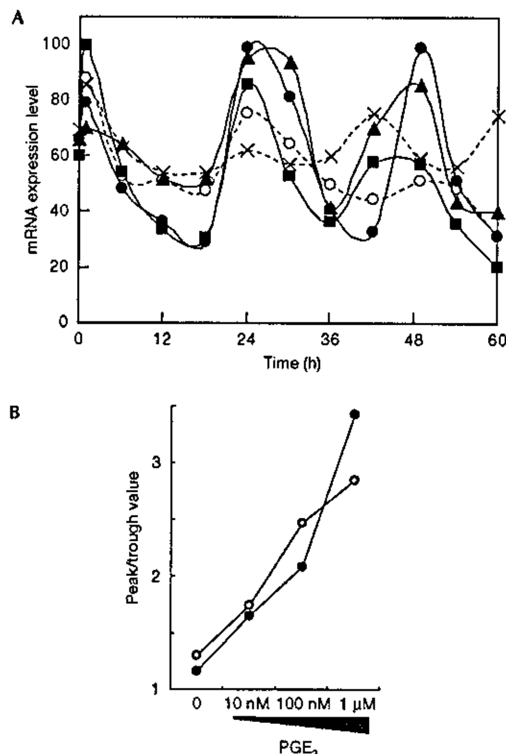

Fig 2 | Circadian gene expression induced by prostaglandin E_2 (PGE_2) in NIH3T3 cells. (A) Circadian oscillation of the expression level of mPer2 mRNA induced by varying concentrations of PGE_2. At time 0, NIH3T3 cells were treated with no stimulus (crosses), PBS (open circles) and 10 nM (squares), 100 nM (triangles) and 1 µM (filled circles) of PGE_2, and the mRNA expression levels were monitored by the real-time quantitative PCR method. Each value was normalized to mG3PDH. Data shown are representative of three independent experiments. (B) Robustness of circadian gene expression induced by PGE_2 in NIH3T3 cells. The peak and trough values of mRNA expression levels in the second cycle of oscillation were read from the graph and the extent of amplitude was plotted. Graphs shown are data for mPer2 (filled circles) and mDBP (open circles).

t-test), but not by the extracellular Ca^{2+} chelator EGTA, suggesting that intracellular Ca^{2+} store is responsible for the activation of mPer1 expression. We also found that both the Ca^{2+}/calmodulin-dependent kinase II (CaMK II) inhibitor KN-93 and the MEK inhibitor U0126 slightly but significantly inhibited the mPer1 induction ($P<0.05$ by Student's *t*-test), and cotreatment with KN-93 and U0126 resulted in more effective inhibition of this mPer1 induction by PGE_2 ($P<0.01$ by Student's *t*-test). In contrast, the PKA inhibitor H-89 or Rp-8-Br-cAMPS, the PKC inhibitor bisindolylmaleimide I, the PKG inhibitor KT5823, the epidermal growth factor (EGF) receptor kinase inhibitor AG1478, the casein kinase 1 inhibitor CKI-7, the PI3K inhibitor LY294002, the p38 inhibitor SB203580 and the JNK inhibitor SP600125 all failed to inhibit the induction of mPer1 expression by PGE_2 (data not shown). These results suggest that PGE_2 induces transient expression of mPer1 mRNA in NIH3T3 cells by increasing an intracellular Ca^{2+} level and activating relevant protein kinases including CaMK II and ERK MAP kinase. Both kinases are known to be activated downstream of Ca^{2+} signalling (Egea *et al*, 1999; Hudmon & Schulman, 2002). Among four PGE_2 receptor subtypes (EP1, EP2, EP3 and EP4), the EP1 and the EP3 subtypes are thought to be responsible for stimulation of intracellular Ca^{2+} mobilization (Negishi *et al*, 1995), although signalling pathways downstream of the EP receptors are fairly complicated and also cell type-specific (Bos *et al*, 2004). Thus, it is likely that PGE_2 induces

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the transient mPer1 expression mainly by means of the EP1 and/or the EP3 receptor.

To examine whether circadian oscillation of clock gene expression follows the acute and transient induction of mPer1 expression, we monitored the expression level of clock genes after PGE_2 treatment of NIH3T3 cells. As a result, circadian oscillation of mPer2 mRNA expression was clearly observed after PGE_2 treatment (Fig 2A). To verify the robustness of circadian oscillation of the mRNA expression level, the extent of amplitude (the difference between the second peak level in the graph and the second trough level) was evaluated. As shown in Fig 2B, the robustness of circadian expression of mPer2 is dose-dependently enhanced by PGE_2 treatment. Essentially similar results were obtained for mDBP, another clock-controlled transcription factor (data not shown; Fig 2B). Taken together, these results indicate that PGE_2 has the ability to induce robust circadian oscillation of clock gene expression in NIH3T3 fibroblasts.

PGE_2 induces the phase shift of peripheral clocks

We tested whether PGE_2 is able to reset the circadian clocks in peripheral tissues *in vivo*. Intraperitoneal administration of mice, which were maintained under 12:12 light:dark cycles, with PGE_2 at ZT21 (zeitgeber time; light-on at ZT0 and light-off at ZT12) resulted in marked phase shifts of mPer1 expression rhythms in liver, kidney and heart (Fig 3A). The marked immediate induction of mPer1 by PGE_2 was observed in kidney but not in liver or heart (Fig 3A). This may be due to the much higher expression level of the EP1 and the EP3 receptors in kidney than in liver or heart (Sugimoto *et al*, 1992; Watabe *et al*, 1993). We have also found that the phase shifts of the mPer1 expression rhythm induced by PGE_2 show significant variances that depend on the circadian time of the injection (Fig 3B). To enhance reliability, expression profiles of mDBP and mRev-erbα genes were also analysed (Fig 3B). These two genes are known to show robust and high-amplitude circadian oscillations in their mRNA expression levels (Yamaguchi *et al*, 2000; Preitner *et al*, 2002). All these results indicate that PGE_2 is able to shift the phase of the circadian rhythm in peripheral tissues in a time-dependent manner. The amplitudes of the phase advance or delay obtained from these data were plotted so as to clarify the dependency of phase shifts on the circadian time of PGE_2 injection (Fig 3C). The characteristics of PGE_2-induced phase shifts are similar among three types of tissue, that is, the phases of the rhythm advance in the early day and delay in the early night. The late day and the late night are thought to be transition phases of the shift direction. Again, the remarkable phase delays of mRev-erbα expression in kidney may reflect high expression levels of the EP1 and the EP3 receptors. The phase shifts throughout the 24-h day seem to be characteristic of peripheral oscillators, whereas the phase shifts of central clock by light are shown to be limited to the night time (Daan & Pittendrigh,

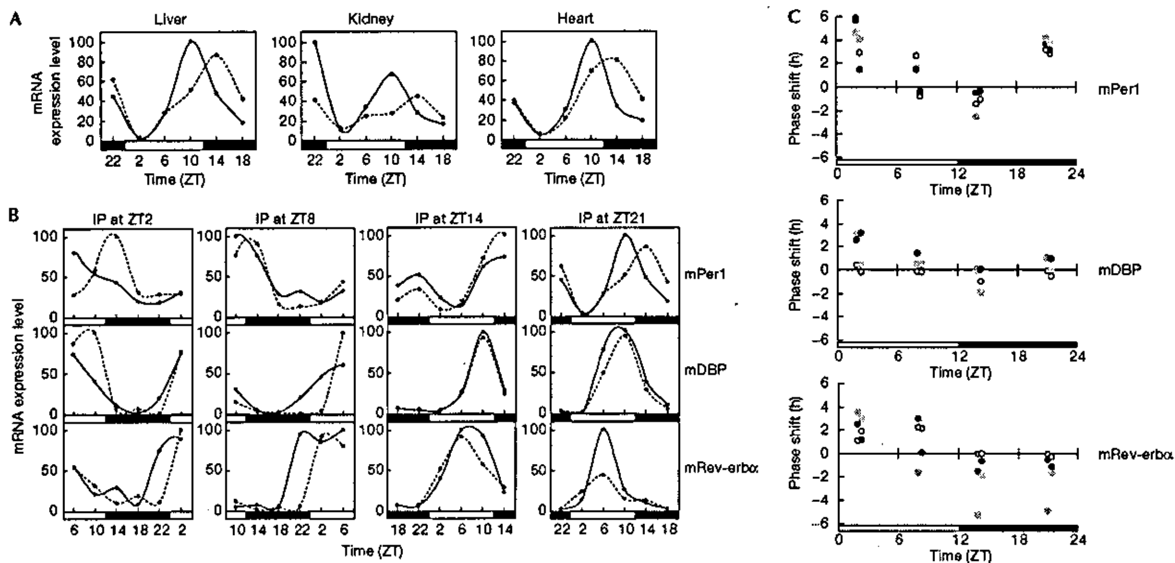

Fig 3 | Phase shifts induced by prostaglandin E_2 (PGE_2) in mouse peripheral tissues. (A) Phase shifts of circadian rhythm of mPer1 mRNA expression in peripheral tissues by PGE_2. Mice were entrained under a 12:12 light:dark cycle for 2 weeks and intraperitoneal administration of PGE_2 dissolved in PBS (solid line), or PBS alone (dotted line) was performed at ZT21. Two mice (one PGE_2 injected and the other PBS injected) were killed and mRNA was collected from tissues at each time point. The mRNA expression levels were evaluated by real-time quantitative PCR. Each value was normalized to mG3PDH. Data shown are representative of two independent experiments. (B) Variations of the phase shifts of circadian gene expression in liver induced by PGE_2 at different time points. Intraperitoneal (i.p.) administration of PGE_2 (solid line) and PBS (dotted line) was performed at different circadian time points. The mRNA expression levels of mPer1, mDBP and mRev-erbα were monitored. Data shown are representative of two independent experiments. (C) The amplitudes of phase shifts by PGE_2 at each circadian time point (ZT2, ZT8, ZT14 and ZT21). The amplitudes of phase shifts of mPer1, mDBP and mRev-erbα were evaluated in liver (black circles), kidney (grey circles) and heart (open circles). Data from two independent experiments were plotted.

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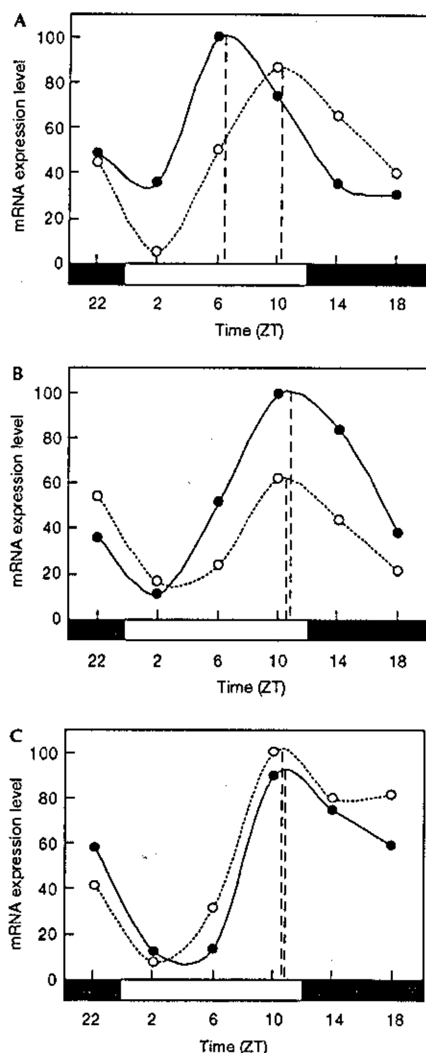

Fig 4 | The EP1 receptor is responsible for the phase shifts by prostaglandin E_2 (PGE_2). (A) Intraperitoneal administration of sulprostone (solid line) or PBS alone (dotted line) was performed at ZT21. mRNA was collected from liver at each time point and the expression levels of mPer1 mRNA were evaluated by real-time quantitative PCR. Each value of mRNA expression levels was normalized to mG3PDH. Data shown are representative of two independent experiments. (B) Intraperitoneal administration of butaprost (solid line) or PBS alone (dotted line) was performed at ZT21. The expression levels of mPer1 mRNA in liver were monitored as in (A). (C) Intraperitoneal administration of PGE_2 together with SC-51322 (solid line), or PBS alone (dotted line) was performed at ZT21. The expression levels of mPer1 mRNA in liver were monitored as in (A).

1976; Balsalobre *et al*, 2000b). Next, we investigated whether the PGE_2-induced phase shift is sustained over the second cycle after the injection at ZT21. As a result, the phase shift induced by intraperitoneal injection of PGE_2 was kept for only one cycle under light–dark cycles and disappeared in the second cycle (supplementary information online). This result indicates that the PGE_2-induced phase shift is an apparent phase shift as observed in the case of dexamethasone (Balsalobre *et al*, 2000b).

EP1 contribution to the phase shifts by PGE_2

We then examined which subtype of PGE_2 receptors is responsible for the phase shifts by PGE_2 *in vivo*. Intraperitoneal administration of an agonist of both the EP1 and the EP3 receptors, sulprostone, was able to induce the phase shift of the mPer1 expression rhythm in liver (Fig 4A). In contrast, an agonist of the EP2 receptors, butaprost, failed to shift the phase of the mPer1 expression rhythm (Fig 4B). Furthermore, an antagonist of the EP1 receptors, SC-51322, markedly inhibited the phase-shifting effect of PGE_2 in liver (Fig 4C). These results strongly suggest that the EP1 subtype of PGE_2 receptors is responsible for the entrainment of circadian clocks in peripheral tissues. Given the contribution of the EP1 receptors to Ca^{2+} signalling, the EP1 may have an important role in both transient upregulation of mPer1 mRNA expression and circadian phase shifts in peripheral tissues.

Locomotor activity rhythm is unaffected by PGE_2

To assess whether PGE_2 phase shifts the behavioural rhythm of mice, we monitored their locomotor activity before and after intraperitoneal injection of PGE_2. In this experiment, mice were maintained in constant darkness after the injection so as to eliminate the effect of light on their activity rhythm. As a result, no significant difference was observed in the activity onset time between PGE_2-injected and phosphate-buffered saline (PBS)-injected mice (Fig 5A), whereas a marked phase advance was observed in the mRNA expression rhythm of mPer1 in peripheral tissues (Fig 3A). To see detailed locomotor activity after PGE_2 injection, an averaged actogram for the 2 days following the injection was determined (Fig 5B). There was no significant effect of PGE_2 injection on the following activity onset time. This result suggests that PGE_2 does not affect the central circadian pacemaker that controls circadian behavioural rhythms. It is likely that PGE_2, similar to glucocorticoid hormones, functions as a transducer of the clock-resetting signal from the SCN to the peripheral clocks rather than as a direct resetting agent for the core pacemaker (Balsalobre *et al*, 2000b).

Several agents including glucocorticoid hormones are reported to be able to reset the internal circadian oscillators (Balsalobre *et al*, 2000b; McNamara *et al*, 2001). Our results suggest that PGE_2 also contributes to the synchronization of circadian oscillators *in vivo*. Detailed mechanisms showing how PGE_2 induces the phase shifts are yet to be shown. It is likely that PGE_2 acts directly on circadian oscillators via cell autonomous mechanisms, because PGE_2 is able to induce circadian gene expression in cultured NIH3T3 cells (Fig 2). It is also possible, however, that PGE_2 acts indirectly by inducing production of other factors. As PGE_2 is known to induce recruitment of a variety of cytokines (Hinson *et al*, 1996), these cytokines may participate in the resetting of the circadian rhythm *in vivo*. This study has shown a hitherto unidentified function of PGE_2, that is, resetting of the rhythm-generating core feedback loops of circadian gene expression in the periphery. The influence of PGE_2 on the master circadian pacemaker of the brain remains to be elucidated.

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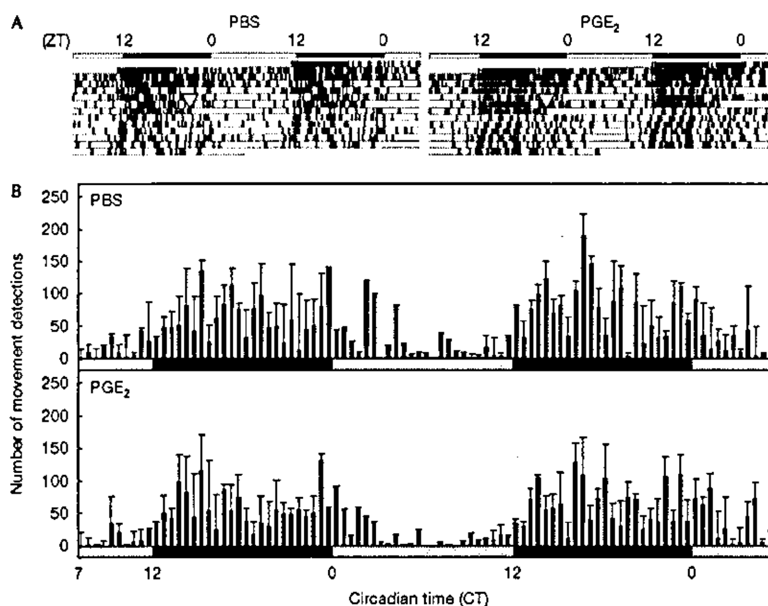

Fig S1 | **Prostaglandin E_2 (PGE_2) induces no significant alteration in circadian locomotor activity. (A) Representative double-plotted actograms show locomotor activity records of PBS alone (left) or PGE_2-injected (right) mouse at ZT21 (arrowheads). Mice were housed in a light–dark cycle for a week and injected with PBS or PGE_2 intraperitoneally at ZT21. Then, mice were maintained in constant darkness. (B) Activity profile over 2 days from 10 h after intraperitoneal injection of PBS or PGE_2 at ZT21. Bars represent mean ($\pm$s.e.m., $n = 4$) summed number of movement detections during 30 min of recording. Grey and black bars represent the subjective day and night, respectively. Shaded background also represents the subjective night. CT0 corresponds to ZT0 in constant dark conditions.**

Previous studies have suggested a role of PGE_2 in the regulation of body temperature and the sleep–wake cycle—two events that are closely related to an output pathway in circadian physiology and behaviour. Thus, PGE_2 may have a dual role in the circadian clock: a clock-resetting function in an input pathway and a circadian physiology-regulating function in an output pathway.

METHODS

Cell culture and time course experiment. NIH3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and antibiotics (100 U penicillin and 0.2 mg kanamycine/ml) at 37 °C under 5% CO_2. Cells were plated at a density of 2.5×10^5 cells per 35 mm dish. The cells reached confluence 2 days after the plating, and were kept for 2 days in medium containing 1% calf serum before the start of experiments. At time 0, PGE_2 (1 mM in PBS, Cayman Chemical, Ann Arbor, MI, USA) was added to the culture medium and then the cells were frozen in liquid nitrogen at indicated time points. Preparation of RNA samples and reverse transcriptase–PCR (RT–PCR) analysis were performed as described previously (Tsuchiya *et al*, 2003).

Mice. C57BL/6CrSlc wild-type mice were maintained under a 12:12 light:dark cycle for 2 weeks before the start of experiments. Male mice aged 11–13 weeks were used. Animal care was in accordance with institutional guidelines. Intraperitoneal injection of 3 μg/g body weight of PGE_2, sulprostone (Cayman), butaprost (Cayman) or SC-51322 (Biomol International LP, Plymouth meeting, PA, USA), all dissolved in PBS or ethanol, was performed under dim red light (during the night) or under ambient light (during the day). At indicated time points, mice were killed by cervical dislocation and tissues were excised and immediately frozen in liquid nitrogen and stored at −80 °C. Frozen tissues were homogenized by using QIAshredder (Qiagen, Japan) and then total RNA was extracted by using the RNeasy kit (QIAGEN). RT–PCR analysis was performed as described previously (Tsuchiya *et al*, 2003). Mouse locomotor activity was measured by using an infrared sensor. The number of movement detections was counted and summed over the subsequent 3 min by the counting processor.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS
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ORIGINAL ARTICLE

Acceptance and short-term tolerance of nasal continuous positive airway pressure therapy in elderly patients with obstructive sleep apnea

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Abstract

Short-term compliance of nasal continuous airway pressure (nCPAP) therapy in elderly patients (65 years or older; *n* = 115) with moderate to severe obstructive sleep apnea was investigated. When nCPAP therapy was offered to elderly patients with an apnea–hypopnea index greater than 20/h, the acceptance rate of nCPAP therapy was 70%. Of those, the short-term tolerance rate of nCPAP therapy (usage over 3 months after initial trial) was 83%. The patients who refused or abandoned nCPAP therapy were less severe (in terms of the apnea–hypopnea index), less sleepy and there were a lower number with hypertension. Considering the short-term compliance of nCPAP therapy reported for middle-aged patients in the literature, it is found that acceptance and short-term tolerance of nCPAP therapy in elderly patients is high, especially in patients with hypertension and excessive daytime sleepiness.

Key words: acceptance, continuous positive airway pressure, elderly, sleep apnea, tolerance.

INTRODUCTION

It is reported that 30% of patients with severe obstructive sleep apnea (OSA) refuse nasal continuous positive airway pressure (nCPAP) therapy[1] and 20–30% of patients abandon this therapy after an initial trial, which is usually just after the titration night or during the first few months of home therapy.[2,3] Major reasons for the refusal or discontinuation of nCPAP therapy are lack of perceived benefits and obvious drawbacks of the nCPAP system, such as discomfort, claustrophobia and noise of the system.[4] Recently, it has been elucidated that the population of elderly patients with sleep apnea is very large.[5] However, the number and the characteristics of the elderly patients who refuse or abandon nCPAP therapy have not been reported. In the present study, acceptance and short-term tolerance of nCPAP therapy in elderly OSA patients (defined as 65 years and older) were retrospectively investigated.

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METHODS

Of the 175 consecutive subjects over 65 years of age who were referred to our sleep center because of suspected OSA between March 1999 and July 2001, 115 (93 male and 22 female) who exhibited an apnea–hypopnea index

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Table 1 Acceptors and refusers of nasal continuous airway pressure therapy in elderly obstructive sleep apnea patients

	Acceptors ($n=80$)	Refusers ($n=35$)	P
Age (years)	69.9±3.9	69.9±3.3	NS
Body mass index (kg/m^2)	25.2±4.0	24.6±4.1	NS
Apnea–hypopnea index (/h)	49.0±23.9	37.1±20.5	<0.05
Lowest SpO_2 (%)	74.1±12.9	78.4±9.9	NS
Epworth Sleepiness Scale	8.9±5.3 ($n=62$)	6.1±3.9 ($n=25$)	<0.05
Hypertension (%)	33.8%	8.6%	<0.05
Female (%)	21.4%	22.9%	NS

Data are shown as mean ± SD. NS, not significant.

(AHI) greater than 20 events per hour were included in the present study. The subjects were asked about their history of hypertension and medication used. Those subjects who had a history of hypertension, those who were receiving any antihypertension medication, and those whose blood pressure was more than 140/90 mmHg were considered to have hypertension.

All subjects underwent a polysomonography or cardiorespiratory sleep study to determine AHI, with manual scoring by our experienced sleep technicians. Apnea was defined as a cessation of airflow for >10 s and hypopnea was defined as a reduction of >50% in chest movement for >10 s with over 3% desaturation. After this diagnostic study, patients with an AHI greater than 20/h were informed of the nature and the consequences of OSA. And, nCPAP therapy was offered as the most efficient mode of treatment for their disease. If the patients agreed to try nCPAP therapy, treatment was initiated with an additional two nights for patients to become familiar with nCPAP and another night for polysomonography with manual titration. Patients who accepted a nCPAP trial and continued therapy for at least 1 week were considered as acceptors. Those patients who abandoned nCPAP therapy within 3 months after the trial were considered intolerant of this therapy, and they were compared with the patients who had continued this therapy by the end of December 2002.

The two-tailed unpaired Student's t-test was used for comparison of unpaired samples with normally distributed data (age, body mass index). The Mann-Whitney U-test was used for comparing unpaired samples without the assumption of normally distributed data (apnea–hypopnea index, Epworth sleepiness scale, lowest oxygen saturation). Table analysis with a Pearson chi-squared test was used for proportional comparison. Two-tailed P values <0.05 were considered to be significant.

Table 2 Acceptance rate of nasal continuous airway pressure therapy in elderly obstructive sleep apnea patients (OSA)

	n	Acceptance rate (%)
Severity of OSA		
AHI ≥ 30	80	78.8
20 ≤ AHI < 30	35	48.6
Daytime sleepiness		
ESS ≥ 11	24	91.7
ESS < 11	63	61.9
Hypertension		
+	30	90.0
–	85	62.4

AHI, apnea–hypopnea index; ESS, Epworth Sleepiness Scale.

RESULTS

Out of 115 consecutive elderly patients with an AHI greater than 20/h, 84 agreed to have a trial on nCPAP (73%). Four patients stopped therapy within 1 week after the trial, resulting in a 70% acceptance rate for elderly patients with an AHI greater than 20/h receiving nCPAP therapy.

Table 1 shows the characteristics of the patients who accepted ($n=80$) and refused ($n=35$) nCPAP therapy. Acceptors and refusers did not differ in age, body mass index, or sex distribution. However, AHI, Epworth sleepiness scale (ESS) and prevalence of hypertension were significantly higher in acceptors, although ESS was obtained from 87 of 115 subjects. More than 90% of patients accepted nCPAP therapy when they had excessive daytime sleepiness or hypertension (Table 2).

Because 64% of the patients who abandoned nCPAP therapy within the first 12 months gave up the use within 3 months, those patients were considered to be intolerant of this therapy (intolerant group). The proportion of patients who were intolerant of nCPAP

Table 3 Elderly obstructive sleep apnea patients tolerant and intolerant of nasal continuous airway pressure (nCPAP) therapy

	Tolerant group (n = 40)	Intolerant group (n = 14)	P
Age (years)	69.8 ± 3.7	70.6 + ± 3.9	NS
Body mass index (kg/m^2)	25.2 ± 4.3	24.1 ± 5.6	NS
Apnea–hypopnea index (/h)	47.7 ± 20.2	41.0 ± 27.9	NS
Lowest SpO_2 (%)	73.3 ± 15.4	79.3 ± 9.5	NS
Epworth Sleepiness Scale	9.2 ± 5.8 (n = 34)	6.5 ± 4.7 (n = 11)	NS
CPAP pressure (cmH_2O)	8.3 ± 2.3	6.3 ± 1.3	<0.05
Hypertension (%)	37.5%	14.3%	NS
Female (%)	22.5%	42.9%	NS

Data are shown as mean ± SD. NS, not significant.

therapy was 17% (n = 14) of all the patients who tried nCPAP therapy. In the end, 37% of patients (n = 31) eventually abandoned nCPAP therapy. Excluding the patients who transferred to other hospitals (n = 13), 48% of the patients (n = 40) continued nCPAP therapy for 21.4 ± 9.4 months by December 2002 (mean ± SD, tolerant group). Table 3 shows the comparison of the tolerant group with the intolerant group. Although there were no significant differences between the two groups, except for nCPAP pressure, patients from the intolerant group tended to be less severe (in terms of the apnea–hypopnea index), less sleepy and there were a lower number with hypertension.

DISCUSSION

In the present study, it was shown that 70% of the patients accepted nCPAP therapy when offering this therapy to elderly patients with AHI greater than 20/h and 17% of those patients abandoned nCPAP therapy within 3 months after initial trial of the therapy. The characteristics of the patients who refused or abandoned nCPAP therapy were less severe and less sleepy, and there were a lower number with hypertension.

The acceptance rate of nCPAP therapy in elderly patients was fair, compared with the acceptance rate of 50 and 70% reported by Rauscher *et al.* when offering nCPAP therapy to the patients with an AHI of 15 or greater and 30 or greater, respectively, in adult OSA patients (30–69 years old, n = 94).[1] They also reported that the acceptance of nCPAP therapy is strongly related to the patient's subjective excessive daytime sleepiness.[1] In the present study, the acceptance rate of nCPAP therapy was considerably high in the patients with hypertension as well as in those with daytime sleepiness. Because hypertension is considered to be one of the deteriorating factors of the prognosis of OSA,[6] well-educated patients might have accepted this therapy. In fact, the acceptance of nCPAP therapy is considered to depend on the education of the patients about the need for treatment and the consequence of the illness.[7]

The proportion of patients in the elderly population who were intolerant of nCPAP therapy after an initial nCPAP trial is similar to that reported in literature on middle-aged OSA patients.[2,3] Although there has been a controversy on the relationship between age and tolerance of nCPAP therapy,[8,9] elderly patients were found able to tolerate short-term periods of nCPAP therapy. Those patients who were intolerant of nCPAP therapy had similar characteristics to those who refused the therapy. Because the reason why they gave up nCPAP use is mostly due to mask discomfort including pressure, it is essential to increase acceptance and tolerance by considering the following: careful mask fitting, the use of humidification if mucosal dryness or a mouth leak is prominent, pretreatment of nasal congestion, and slow upward titration of pressure,[7] especially in elderly patients.

In conclusion, acceptance and short-term tolerance of nCPAP therapy in elderly patients with OSA are considerably high, especially in patients with hypertension and excessive daytime sleepiness.

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