Obstructive Sleep Apnea Causes Systemic Inflammation and Metabolic Syndrome

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Apoptosis of Circulating Neutrophils and Alveolar Macrophages in COPD

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analysis[5] regarding the diagnostic accuracy of determining ADA vs interferon (IFN)-γ showed that IFN-γ is superior to ADA, although the difference is small. Therefore, establishing a diagnosis of tuberculosis pleuritis based only on pleural fluid ADA without pleural biopsy findings is still controversial.[6]

We do not intend to deny the usefulness of pleural fluid ADA for diagnosing tuberculosis pleuritis. Furthermore, we do not insist that pleural fluid INF-γ can replace pleural fluid ADA, or that pleural fluid INF-γ should be measured instead of measuring pleural fluid ADA for diagnosing tuberculous pleuritis. We would like to emphasize the usefulness of the measurement of pleural fluid INF-γ in addition to pleural fluid ADA, especially in low-incidence populations in developed countries, including Japan, because the measurement of IFN-γ is a relatively high-cost test, but has no associated complications.

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Apoptosis of Circulating Neutrophils and Alveolar Macrophages in COPD

To the Editor:

In a recent issue of *CHEST* (May 2004),[1] Noguera and coworkers reported that *in vitro* neutrophil apoptosis in patients with COPD occurred at a rate similar to that found in healthy individuals and smokers with normal lung function. Further, increased surface expression of Mac-1 (CD11b) and decreased surface expression of L-selectin (CD62L) were observed in the apoptotic neutrophils of patients with COPD.

It has been reported that neutrophil granulocytes show a reduced spontaneous apoptosis during acute exacerbations of COPD, but that increases progressively after treatment and clinical remission.[2] This raises the question of the importance of neutrophil apoptosis in the development and resolution of exacerbations of COPD. Thus, the current study may provide some scientific background to address the dynamics of apoptosis *in vivo* lung neutrophils. However, a number of questions remain to be solved.

First, apoptosis is induced by both oxidant production and the depletion of antioxidant in cells. Inversely, supplementation of antioxidant prevents apoptosis in lung-derived cells.[3] Thus, isolated circulating neutrophils from the blood stream that are not fully occupied with blood antioxidant are not a good candidate for the analysis of cell fate in the various inflammatory diseases.

Second, cigarette smoke (CS) contains approximately 4,000 various constituents, including numerous chemicals that result in the production of reactive oxygen species. CS causes apoptosis and necrosis in airway cells including alveolar macrophages (AMs).[4,5] CS-mediated depletion of lung glutathione is thought to lead to increased lipid peroxidation, neutrophil sequestration, and transcription of proinflammatory cytokine genes.[6]

We have reported that CS extract (CSE) induced apoptosis at lower concentrations (10 to 25%) and necrosis at higher concentrations (50 to 100%). We also examined the effects of glutathione S-transferase P1, one of the xenobiotic and antioxidant enzymes in the lung, against the cytotoxicity of CSE. Thus, the antioxidant status and antioxidant gene expression levels may have effects against CS in the airway cells.[7]

Third, although apoptosis is a universal process in the cells, the mechanism of apoptosis is not simple. In *in vitro* studies,[5] human AMs cultured with aqueous CSE undergo apoptosis. This apoptosis is associated with increased oxidative stress, Bax protein accumulation, mitochondrial dysfunction, and mitochondrial cytochrome c release, but is independent of p53, Fas, and caspase activation. These results may provide information to explain macrophage dysfunction and lung diseases in cigarette smokers.

Fourth, patients with bronchiectasis had a lower percentage of neutrophils that were neither apoptotic nor necrotic than in healthy control subjects.[8] The low levels of apoptosis observed in the patients may be associated with inhibition of apoptosis by inflammatory mediators such as interleukin (IL)-8 and tumor necrosis factor (TNF)-α.[9] High levels of TNF-α and IL-8 have consistently been found in the bronchial secretions of the patients. Because these cytokines have been known to be increased in patients with COPD, a similar mechanism may work in patients with COPD. Acute exposure to CS induces infiltration of neutrophils into the airways through nuclear factor-κB and IL-8 gene expression.[10]

Fifth, in COPD, plasma soluble Fas ligand (sFas) was within normal limits. Plasma soluble Fas/Apo-1 receptor (sFas) levels were similar among healthy control subjects, disease control subjects, and patients with mild-to-moderate COPD, but were significantly increased in severe COPD.[11] The increased plasma sFas is independent of hypoxemia, and increases in $PaCO_2$, TNF-α, IL-6, and inflammation may be associated with progression of COPD. Thus, the measurement of apoptosis of AMs and neutrophils in lungs in relation to the different pathologic stages of COPD may offer further information for the role of apoptosis of lung cells in the pathogenesis of COPD.

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Normal Polysomnography in Children and Adolescents

To the Editor:

Ng and colleagues[1] have raised several issues in their comments on our study.[2] Based on the study by Trang et al,[3] they questioned the validity and sensitivity of the thermistor to detect obstructive hypopneas. The use of a nasal cannula to monitor airflow and to detect apneas and hypopneas has become popular in recent years. This technique may be advantageous in many aspects; however, it has limitations. In their article, Trang and colleagues[3] showed that the time spent with an uninterpretable cannula signal was significantly longer than the time spent with an uninterpretable thermistor signal (mean uninterpretable time out of total sleep time for the thermistor, 0%, compared to 2 to 4% for the cannula). In addition, mouth breathing was a frequent cause for cannula signal unreliability. More studies are needed to compare the two techniques before the nasal cannula can become the "gold standard" and the only recommended method. The thermistor has been used in many published pediatric studies from the past few years.[4–7]

We think that the finding that only three subjects had a total of seven obstructive apneas (OAs) [one child had five of the seven OAs] precludes the possibility that the normal distribution of OAs over the 70 cases in the study is possible. Hence, calculating the SD for three cases would be meaningless.

The goal of the study was to establish normal values. Therefore, the study aimed to provide an upper limit value for OAs and obstructive hypopneas, such that all resulting values higher than that number would be considered abnormal. Because only 3 of 70 healthy subjects had a total of seven OAs, calculating the normal upper limit by dividing 7 by the total sleep time of all 70 cases combined will result with an OA index value that would define these three healthy children as abnormal. Using the method described in our study, we presented an upper limit value for the OA index that applies to any child who has OAs.

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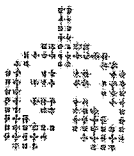

CORRESPONDENCE

Significance of chronic cough as a defence mechanism or a symptom in elderly patients with aspiration and aspiration pneumonia

To the Editors:

In a recent issue of the *European Respiratory Journal*, MORICE and the European Respiratory Society (ERS) Task Force committee members [1] comprehensively summarised the diagnosis and management of chronic cough in both adults and children. However, they have totally neglected the features, diagnosis and management of chronic cough in the elderly [1].

Although cough is one of the most common symptoms for which patients seek medical attention from primary care physicians and pulmonologists all over the world and is associated with deterioration in patients' quality of life [1], the concise and distinct guideline is not extensively introduced. Thus, the recent ERS Task Force report "The diagnosis and management of chronic cough" is very helpful and meaningful for chest physicians as well as primary care physicians. However, age-related changes in cough reflex and the protective roles of cough as the defence mechanism of aspiration in older patients are not argued in the report.

PALOMBINI *et al.* [2] reported that asthma, postnasal drip syndrome (PNDS) and gastro-oesophageal reflux disease (GERD), alone or in combination, were responsible for $\geqslant$90% of the causes of chronic cough. They proposed that asthma, PNDS and GERD should be called a pathological triad in chronic cough in adults [2]. However, in older patients, the causes of chronic cough may be more complicated. Age-related changes in cough reflex may affect the causes and therapeutic efficacy of chronic cough [3]. Furthermore, the protective role of cough as the defence mechanism of aspiration is very important for the pathogenesis of chronic cough in older patients. Owing to the increasing number of the elderly in the population, many pulmonologists and geriatricians recognised that silent aspiration might be very important for the pathogenesis of aspiration pneumonia and nosocomial pneumonia in older patients [4–7]. The prevalence of stroke, chronic obstructive pulmonary disease, sleep apnoea, gastro-oesophageal reflux, sedatives and/or hypnotics usage, post-gastrectomy and mechanical ventilation are increased in the aged population and these are believed to increase the risk of aspiration [4–7]. As pneumonia is in principle prevented by the defence mechanisms, such as upper airway reflexes, mucociliary clearance and phagocytosis by alveolar macrophages, age-dependent declines of upper airway reflexes may be one of the pathophysiological features of aspiration pneumonia in older subjects. Elderly persons appear to have slowed clearance of particles from the airway probably due to impaired mucociliary function that accompanies ageing. However, in our experience, cough reflexes rather than swallowing reflex or mucociliary clearance are of the utmost importance for preventing aspiration in elderly patients. In fact, a markedly decreased cough reflex was observed in elderly patients with aspiration pneumonia [3, 4]. Inversely, there is growing evidence that angiotensin-converting enzyme (ACE) inhibitors have beneficial effects on the prevention of pneumonia in elderly patients by improving both the impaired swallowing reflex and disturbed cough reflex [8, 9]. Although the elevated levels of bradykinin and substance P by ACE inhibitors are thought to be the source of the cough, bradykinin and substance P play a role in setting the threshold for the cough and swallowing reflex in humans, resulting in reduction of the occurrence of pneumonia in the elderly. The beneficial effects of ACE inhibitors for older subjects with the risk of aspiration pneumonia should be widely noted [8, 9].

In addition, we have recently demonstrated that recurrent silent aspiration causes diffuse aspiration bronchiolitis (DAB), which is characterised as a chronic inflammation of bronchioles accompanying a foreign body reaction [3, 10]. The patients with DAB mostly demonstrated signs of bronchorrhoea, cough, bronchospasm and dyspnoea in the case of food intake. The chronic cough in association with food intake is often misdiagnosed as bronchial asthma in the elderly. The chronic cough in DAB does not respond to β-adrenergic bronchodilators or to inhaled steroids. The swallowing rehabilitation and temporally *i.v.* alimentation are the most effective way to reduce the symptoms in DAB.

Although chronic cough is an untoward symptom in adults as well as elderly subjects, the protective roles of cough reflex on the development of aspiration pneumonia in older patients should be carefully considered by all physicians. Hopefully, the next European Respiratory Society Task Force report will include the clinical significance of chronic cough as a defence mechanism or a symptom in elderly patients with aspiration and aspiration pneumonia.

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From the author:

Teramoto and colleagues have completely misunderstood the purpose of the ERS Task Force on diagnosis and management of chronic cough [1]. The document deals with patients who have had a cough for >8 weeks. It is not about patients who can't cough. To suggest in their opening paragraph that we neglect cough in the elderly is simply disingenuous. We deliberately separated chronic cough in children from that in adults since the aetiology is different. However, in adults the causes and treatment of chronic cough are not age related and the elderly were frequent attendees in the 13 studies quoted in table 1 which presents the accumulated experience of specialist cough clinics [1].

Decreased cough and aspiration are important clinical problems but they were not the subject of our discussions. Clearly neurological illness [2, 3] and anatomical abnormality [4] can increase the likelihood of aspiration but this is neither age specific nor relevant to clinicians dealing with patients who present with isolated chronic cough.

Finally, an important function of documents such as the Task Force report is to provide a balanced overview of the literature. Teramoto and colleagues seem to have concentrated largely on their own work, which perhaps goes some way to explain the current debate.

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The hepatopulmonary syndrome: NO way out?

To the Editors:

The hepatopulmonary syndrome (HPS) is defined by the triad of chronic liver disease, abnormal pulmonary gas exchange (low arterial oxygen tension (Pa,O_2) and transfer factor of the lung for carbon monoxide), and intrapulmonary vascular dilatation [1]. The recent editorial on HPS [2] suggests that "hunting endogenous vasodilators that reduce pulmonary vascular tone logically became a sound strategy for those whose quest was to unravel the missing 'molecular' link between the diseased liver and the affected lung". But, is this strategy actually so logical? The key feature of the intrapulmonary vascular dilatation in HPS is the intrapulmonary shunt shown physiologically by a low Pa,O_2 after 100% oxygen breathing, and anatomically by the passage of radiolabelled albumin macroaggregates (20–60 μm in diameter), or echobubbles, through the pulmonary capillary bed [3]. The striking feature pathologically is gross dilatation of capillaries in the alveolar septum, diameters of 100 μm, as compared with the normal 7–15 μm being described [4]. Is it likely that endogenous vasodilators are responsible for "relaxing" alveolar capillaries to such an extent? Of course, endogenous vasodilators may play a part in "remodelling" these capillaries.

With regard to pulmonary gas exchange, two factors seem to operate in severe hepatopulmonary syndrome: 1) a

complaints were diagnosed with bronchitis. This resulted in a more frequent use of inhaled steroids and bronchodilators in Dutch children as compared with German children [2].

We cannot exclude the fact that a possible geographically heterogeneous worldwide *Chlamydia pneumoniae* pandemic could contribute to changes in asthma prevalences in different countries. However, it seems unlikely to us that this would be the sole explanation, as not all asthmatics (established or newly diagnosed) have *C. pneumoniae* present in bronchoalveolar lavage fluid. Moreover, the widespread use of (macrolide) antibiotics has not prevented a clear increase in asthma prevalence. On the contrary, it seems that a decrease in hospitalisation and mortality is strongly associated with an increase in the use of inhaled steroids [3], and there is no indication that this is associated with the use of antibiotics.

However, it is certainly worthwhile to pay attention to the so-called Chlamydia-asthma theory proposed by D.L. Hahn and to investigate the presence of *Chlamydia pneumoniae* or other infectious organisms in new asthma patients.

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Nasogastric tube feeding is a cause of aspiration pneumonia in ventilated patients

To the Editors:

In a recent issue of the *European Respiratory Journal*, KOSTADIMA *et al.* [1] reported that early gastrostomy is associated with a lower frequency of ventilator-associated pneumonia (VAP) compared with nasogastric tube (NGT) feeding in patients who are mechanically ventilated due to stroke or head injury. Since VAP is the most frequent and serious intensive care unit (ICU)-acquired infection among patients undergoing mechanical ventilation, and is associated with a 20–30% increase in the risk of death, the preventive strategy for VAP in mechanically ventilated patients is important to reduce the length of an ICU stay and overall mortality [2].

Although the classic theories, including the gastropulmonary hypothesis, are important to understand the mechanisms of VAP, the recent advancement of the pathophysiology of nosocomial pneumonia and aspiration pneumonia are not fully discussed in the paper by KOSTADIMA *et al.* [1].

There is growing evidence that oropharyngeal dysphagia plays a critical role in aspiration pneumonia and VAP in mechanically ventilated patients [3, 4]. Brain injury, severe stroke and unconsciousness, due to sedatives and hypnotics, disturb the swallowing reflex. This results in the development of aspiration pneumonia in humans and animals [5]. However, nosocomial pneumonia and aspiration pneumonia are prevented by the improvement of the swallowing reflex after administration of angiotensin-converting enzyme (ACE) inhibitors [6]. The elevated levels of bradykinin and substance P by ACE inhibitors play a role in setting the threshold for the cough and swallowing reflex in humans, resulting in the reduction of occurrence of pneumonia. Although KOSTADIMA *et al.* [1] speculated about the underlying mechanisms of risk of VAP in the patients with NGT feeding, they did not assess the swallowing reflex and cough reflex. We have developed a novel diagnostic test for the risk of aspiration pneumonia [7, 8]. The simple swallowing provocation test can be applied for all the ventilated patients as it is very easy and can be performed on bedridden patients without requiring their cooperation. The assessment of the swallowing reflex is the clue to the underlying mechanisms of VAP in critically ill patients. As it has been suggested that nosocomial maxillary sinusitis increases the occurrence of VAP, microaspiration of oropharyngeal materials, including maxillary sinus, is a significant cause of VAP [9].

NGT feeding is known to be a significant cause of aspiration pneumonia in stroke patients [10]. Since the NGT bypasses the small amount of gastric contents through to the oropharynx, the materials can be easily aspirated into lower airways in dysphagic patients with stroke. The mechanism is not related to the percutaneous endoscopic gastrostomy (PEG). This evidence supports the fact that NGT feeding, but not PEG, is a significant cause of VAP in critically ill patients. Although feeding *via* PEG is a very straightforward way to reduce aspiration and aspiration-associated pneumonia, the improvement of the swallowing reflex must be a fundamental approach to reduce VAP in patients. As the PEG procedure using gastroscopic fibre may also be a risk for aspiration in unconscious patients, the indication of early gastrostomy for

the patients should be very carefully assessed. The PEG feeding patients with dysphagia may be suffering from aspiration pneumonia [11].

Considered together, we believe that the prevention of aspiration by using oral care, angiotensin-converting enzyme inhibitors and swallowing rehabilitation may be an alternative approach in reducing the risk of ventilator-associated pneumonia in patients.

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From the authors:

We read with interest the letter from S. Teramoto and coworkers regarding the role of oropharyngeal dysphagia in the pathogenesis of ventilator-associated pneumonia (VAP). The presence of a nasogastric tube has been identified as an independent risk factor for VAP, mainly because of gastro-oesophageal reflux and aspiration [1, 2]. Aspiration is probably due to loss of anatomical integrity of the lower oesophageal sphincter, increased frequency of transient sphincter relaxation and oropharyngeal dysphagia *via* desensitisation of the pharyngoglottal adduction reflex [3, 4].

We speculate that the advantage of performing an early gastrostomy is the possibility of avoiding dysfunction of lower oesophageal sphincter due to the presence of a nasogastric tube [5]. JOHNSON *et al.* [6] have demonstrated an increase in lower oesophageal sphincter pressure following performance of percutaneous endoscopic gastrostomy and a decrease in gastro-oesophageal reflux score. Prevention of oropharyngeal dysphagia induced by the nasogastric tube may be another mechanism in reducing the risk of aspiration.

Of note, percutaneous endoscopic gastrostomy does not eliminate gastro-oesophageal reflux, mainly in patients with a pre-existing nasogastric tube [7]. For this reason, we selected the performance of early gastrostomy in our study. In a recent report, MCCLAVE *et al.* [8] found a decrease in the incidence of regurgitation in intensive care unit patients with early gastrostomy compared with those with a nasogastric tube.

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Lipoprotein Metabolism in Postmenopausal and Oophorectomized Women

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Objective: To investigate the mechanisms of accumulating cholesterol, and to analyze the metabolism of excess tissue cholesterol in women with low plasma levels of sex steroid hormones.

Methods: We measured plasma concentrations of cholesterol, triglyceride, apolipoproteins, sex steroid hormones, and lecithin cholesterol acyltransferase activity in 20 premenopausal, ten postmenopausal, and ten bilaterally oophorectomized women. Lipoprotein lipase and hepatic triglyceride lipase activities were measured in postheparin plasma. We compared the three groups and evaluated a correlation between lipid metabolism and sex steroid hormone concentrations.

Results: The mean plasma low-density lipoprotein (LDL) cholesterol level, lecithin cholesterol acyltransferase activity, and postheparin plasma lipoprotein lipase activity were higher in the postmenopausal and surgically menopausal groups. The mean plasma high-density lipoprotein (HDL) cholesterol concentration and postheparin plasma hepatic triglyceride lipase activity did not differ significantly among the three groups. The plasma LDL cholesterol level and postheparin plasma lipoprotein lipase activity showed a significantly negative correlation with plasma concentration of estrone (LDL: $r = 0.64$, $P < .001$; lipoprotein lipase: $r = 0.54$, $P < .005$) and estradiol (LDL: $r = 0.65$, $P < .001$; lipoprotein lipase: $r = 0.47$, $P < .01$), but not with that of testosterone. There was no significant relationship between postheparin plasma hepatic triglyceride lipase activity and plasma sex steroid hormones. Plasma lecithin cholesterol acyltransferase activity correlated significantly with plasma LDL cholesterol concentration, but not with levels of sex steroid hormones.

Conclusion: Because of low endogenous estrogens, enhanced postheparin plasma lipoprotein lipase activity may lead to an elevated plasma LDL cholesterol concentration in postmenopausal and bilaterally oophorectomized women. We demonstrated an accelerated cholesterol esterification in HDL cholesterol that may have been induced by LDL cholesterol accumulation, although the HDL cholesterol concentration remained unchanged. *(Obstet Gynecol 1995;85: 523–8)*

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Women become more susceptible to coronary heart disease with age. According to Castelli,[1] the incidence of this disease increases in women after 44 years of age at the same rate as in men. Premenopausal women who have had a bilateral oophorectomy also demonstrate a high incidence of the disease.[2,3] Two studies[4,5] have shown a high plasma level of low-density lipoprotein (LDL) cholesterol, a major risk factor for coronary heart disease, but no significant change in high-density lipoprotein (HDL) cholesterol, a protective factor against coronary heart disease in postmenopausal or bilaterally oophorectomized women. The higher incidence of coronary heart disease in women with advancing age has been attributed to a decrease in sex steroid hormones.[2,3]

Lipoprotein lipase, hepatic triglyceride lipase, and lecithin cholesterol acyltransferase are enzymes involved in lipoprotein metabolism. Lipoprotein lipase catalyzes the hydrolysis of very low-density lipoprotein (VLDL) to form intermediate density lipoprotein and HDL. Hepatic triglyceride lipase hydrolyzes the triglyceride in intermediate density lipoprotein to produce LDL, and converts HDL_2 to HDL_3. Lecithin cholesterol acyltransferase esterifies the free cholesterol in HDL that was transferred from the tissue. Many investigators[6–12] have demonstrated the therapeutic effects of the exogenous sex steroid hormones on the activity of these enzymes and on lipoproteins. However, only limited data are available on the effects of endogenous sex steroid hormones. According to Iverius and Brunzell,[13] the effects of endogenous sex steroid hormones on lipid metabolism differ from those such hormones given exogenously. Little is known about how and why women deficient in sex steroid hormones exhibit excessive amounts of plasma cholesterol.

For our study, we compared lipoprotein, sex steroid hormone concentrations, and enzymatic activities in premenopausal, postmenopausal, and surgically menopausal women, then evaluated the relationship between these characteristics to clarify the mechanisms of cho-

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lesterol accumulation and to analyze the metabolism of excess cholesterol in postmenopausal and surgically menopausal women.

Materials and Methods

We studied 40 healthy women: 20 premenopausal women 27–45 years old, with regular menstrual cycles; ten postmenopausal women 55–74 years old; and ten women with surgically induced menopause, 42–51 years old, who had bilateral oophorectomy before menopause. Each subject was selected randomly. Informed, written consent was obtained from each subject before entry to the study, and approval was granted by the Ethics Committee at Kochi Medical School. No subject smoked; used caffeine or alcohol; had a history of thyroid disease, liver disease, or diabetes mellitus; or was currently taking medication known to influence lipoprotein metabolism. Postmenopausal women had not had a menstrual period within the last year. Surgically menopausal subjects had bilateral oophorectomies at least 2 years before their participation in this study.

We collected blood samples between 8:00 and 10:00 AM after a 12-hour fast. In the premenopausal women, phases of the menstrual cycle were determined by basal body temperature. All women showed a biphasic pattern of basal body temperature. Blood samples were drawn at the mid-follicular phase (day 7–10) of the menstrual cycle. Blood was centrifuged immediately at 1500 × *g* for 20 minutes at 4C to separate the plasma for the assay of cholesterol, triglyceride, apolipoprotein, sex steroid hormone concentrations, and lecithin cholesterol acyltransferase activity. After the blood sampling, heparin (10 U/kg body weight) was administered intravenously, followed by 5 mL saline infusion to flush the line. Ten minutes after heparin administration, blood was collected and postheparin plasma was obtained by centrifugation at 1500 × *g* for 20 minutes at 4C for the assay of lipoprotein lipase and hepatic triglyceride lipase activity.

Plasma total cholesterol and triglyceride were measured enzymatically.[14] After precipitation of apolipoprotein B–containing lipoproteins with sodium phosphotungstate in the presence of magnesium chloride, HDL cholesterol was measured enzymatically.[14] Plasma LDL cholesterol was calculated using the Friedewald formula.[15] Plasma apolipoprotein A-I, A-II, and B concentrations were measured by turbidimetric immunoassay.[16] Plasma levels of estrone (E1), estradiol (E2), and testosterone were measured by radioimmunoassay.[17,18] Plasma lipid and lipoprotein assays were performed within 24 hours of storage at 4C. Sex steroid hormone and apolipoprotein assays were performed within 7 days of storage at −20C.

For the determination of lipoprotein lipase activity, 0.49 mL of assay mixture (50 mmol/L glyceryl trioleate, 15% gum arabic emulsion, 0.2 mol/L Tris-HCl at pH 8.2, 5% bovine serum albumin, 0.1% NaCl, 140 μL serum) was incubated for 80 minutes at 37C. Postheparin plasma and 100 mmol/L sodium lauryl sulfate mixture was incubated for 60 minutes at 26C. The reaction was started by the addition of 0.01 mL of the postheparin plasma–sodium lauryl sulfate mixture to the assay mixture; the result was then incubated for 60 minutes at 28C.[19] After incubation, 2.5 mL of an extraction mixture containing 40:10:1 of isopropyl alcohol, n-heptane and 1N H_2SO_4 was added to 0.5 mL of the incubation mixture and shaken vigorously. After standing for 10 minutes, the mixture was separated into two phases; each phase was then centrifuged. One milliliter of the upper phase was dried with nitrogen and eluted with 500 μL of 5% Triton X.[20] Fatty acid was measured in 500 μL of 5% Triton X by enzymatic determination.[21]

To determine hepatic triglyceride lipase activity, the reaction was started by adding 0.01 mL of a mixture of postheparin plasma and 0.2 mol/L Tris-HCl at pH 8.8 to 0.49 mL of the assay mixture (50 mmol/L glyceryl trioleate, 15% gum arabic emulsion, 0.2 mol/L Tris-HCl pH 8.8, 5% bovine serum albumin, 0.75 mol/L NaCl). After incubation for 60 minutes at 28C, fatty acid was measured as described in the lipoprotein lipase determination.

Plasma lecithin cholesterol acyltransferase activity was measured by enzymatic determination.[22] These enzyme assays were performed within 3 months of storage at −80C.

The within- and between-assay coefficients of variation were 0.9–4.0% for total cholesterol, 1.8–3.7% for triglyceride, 1.9–2.9% for HDL cholesterol, 1.4–1.8% for apolipoprotein A-I, 1.8–2.7% for apolipoprotein A-II, 1.3–1.6% for apolipoprotein B, 6.8–8.1% for E1, 2.5–3.0% for E2, 5.6–6.5% for testosterone, 1.0–1.7% for fatty acid, and 6.0–7.0% for lecithin cholesterol acyltransferase.

Data are reported as mean ± standard deviation (SD). Differences among the three groups were analyzed by one-way analysis of variance. Regression lines were determined by the least squares method. Statistical significance was accepted at $P < .05$.

Results

Analysis of variance showed significant differences in age, plasma total cholesterol, LDL cholesterol, and apolipoprotein B concentrations among the three groups. The mean age in the postmenopausal group

Table 1. Subject Characteristics, Plasma Lipids, Lipoprotein, and Apolipoprotein Concentrations

	Premenopausal (n = 20)	Postmenopausal (n = 10)	Surgically menopausal (n = 10)	P
Age (y)	42.75 ± 7.25	65.63 ± 5.58	43.67 ± 2.55	<.001
Body mass index (kg/cm^2)	21.09 ± 3.57	22.79 ± 5.39	21.88 ± 2.65	NS
Total cholesterol (mg/dL)	170.68 ± 30.37	220.38 ± 43.48	209.11 ± 37.82	<.005
Triglyceride (mg/dL)	96.45 ± 42.23	114.13 ± 39.42	100.33 ± 43.10	NS
HDL cholesterol (mg/dL)	62.70 ± 14.87	61.25 ± 12.74	65.00 ± 14.49	NS
LDL cholesterol (mg/dL)	89.85 ± 21.07	136.30 ± 39.00	120.46 ± 33.74	<.01
Apolipoprotein A-I (mg/dL)	139.64 ± 27.20	140.00 ± 31.16	144.65 ± 22.83	NS
Apolipoprotein A-II (mg/dL)	30.33 ± 6.84	34.65 ± 7.57	33.37 ± 2.93	NS
Apolipoprotein B (mg/dL)	65.60 ± 17.73	100.33 ± 29.57	97.69 ± 26.46	<.05

NS = not significant; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

was greater than that in the premenopausal and surgically menopausal groups. The mean plasma total cholesterol, LDL cholesterol and apolipoprotein B concentrations were greater in the postmenopausal and surgical menopausal groups. No significant differences were found in body mass index, plasma triglyceride, HDL cholesterol, and apolipoprotein A-I and A-II concentrations in the three groups (Table 1). Differences of plasma E1, E2, and testosterone levels among the three groups showed statistical significance. The mean of plasma E1, E2, and testosterone levels were lower in the postmenopausal and surgically menopausal groups (Table 2).

The plasma levels of E1 and E2 each showed a highly significant negative correlations with the plasma LDL cholesterol level (E1: $y = -0.63x + 118.81$, $r = 0.64$, $P < .001$; E2: $y = -0.74x + 119.85$, $r = 0.65$, $P < .001$) (Figure 1). However, no significant relationship was observed between the plasma level of testosterone and LDL cholesterol.

Differences of postheparin plasma lipoprotein lipase activity among the three groups approached significance. The mean of postheparin plasma lipoprotein lipase activity was increased in the postmenopausal and surgically menopausal groups (premenopausal group: 9.32 ± 1.40 μmol/mL per hour; postmenopausal: 11.03 ± 2.07 μmol/mL per hour; surgically menopausal: 12.61 ± 2.36 μmol/mL per hour) (Table 3). Significant negative correlations were determined between postheparin plasma lipoprotein lipase activity and either the plasma E1 or E2 level (E1: $y = -8.26x + 134.83$, $r = 0.54$, $P < .005$; E2: $y = -12.06x + 165.70$, $r = 0.47$, $P < .01$) (Figure 2). Plasma testosterone level was not significantly correlated with postheparin plasma lipoprotein lipase activity. No significant difference was found in postheparin plasma hepatic triglyceride lipase activity among the three groups (Table 3) or between postheparin plasma hepatic triglyceride lipase activity and plasma E1 and E2 levels.

Differences of plasma lecithin cholesterol acyltransferase activity among the three groups showed significance. The mean of plasma lecithin cholesterol acyltransferase activity was increased in the postmenopausal and surgically menopausal groups (premenopausal group: 67.02 ± 17.28 nmol/mL per hour; postmenopausal: 78.20 ± 12.81 nmol/mL per hour; surgically menopausal: 90.59 ± 22.00 nmol/mL per hour) (Table 3). However, plasma

Table 2. Plasma Sex Steroid Hormone Concentrations

	Premenopausal (n = 20)	Postmenopausal (n = 10)	Surgically menopausal (n = 10)	P
Estrone (pg/mL)	68.60 ± 35.81	27.14 ± 11.94	34.00 ± 14.00	<.01
Estradiol (pg/mL)	56.45 ± 47.76	11.90 ± 2.19	16.48 ± 5.75	<.001
Testosterone (ng/dL)	73.35 ± 14.93	62.57 ± 17.58	57.17 ± 8.18	<.005

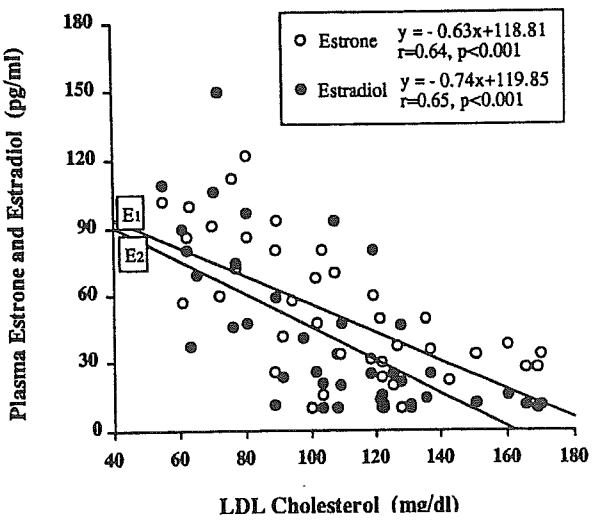

Figure 1. Relationship between low-density lipoprotein (LDL) cholesterol concentration and plasma concentrations of estradiol (E2) and estrone (E1) in premenopausal, postmenopausal, and surgically menopausal groups. Open and closed circles indicate plasma E1 and E2, respectively.

Table 3. Postheparin Plasma Lipoprotein Lipase, Postheparin Plasma Hepatic Triglyceride Lipase, and Plasma Lecithin Cholesterol Acyltransferase Activities

	Premenopausal (n = 17)	Postmenopausal (n = 10)	Surgically menopausal (n = 10)	P
Lipoprotein lipase (μmol/mL per hour)	9.32 ± 1.40	11.03 ± 2.07	12.61 ± 2.36	<.05
Hepatic triglyceride lipase (μmol/mL per hour)	9.23 ± 2.71	10.08 ± 3.03	9.51 ± 2.86	NS
Lecithin cholesterol acyltransferase (nmol/mL per hour)	67.02 ± 17.28	78.20 ± 12.81	90.59 ± 22.00	<.01

NS = not significant.

E1 and E2 levels did not show a significant correlation with plasma lecithin cholesterol acyltransferase activity. Plasma LDL cholesterol showed a strong positive correlation with plasma lecithin cholesterol acyltransferase activity ($y = 1.19x + 20.86$, $r = 0.66$, $P < .001$).

Discussion

Hypercholesterolemia is a leading risk factor for coronary heart disease. Some studies[4,5] have demonstrated elevated plasma total and LDL cholesterol levels in postmenopausal or oophorectomized women. In agreement with these studies, we found that plasma total cholesterol and LDL cholesterol levels were greater in postmenopausal and surgically menopausal groups. Apolipoprotein B concentration as a major component of the apolipoprotein in the LDL was also greater in those two groups. Our results indicate that the risk for coronary heart disease increases after natural or surgical menopause. The plasma LDL cholesterol concentration showed a significant negative correlation with plasma E1 and E2 levels, but was not correlated with testosterone concentration. This suggests that plasma LDL cholesterol concentration is linked to the plasma levels of estrogen, but not testosterone.

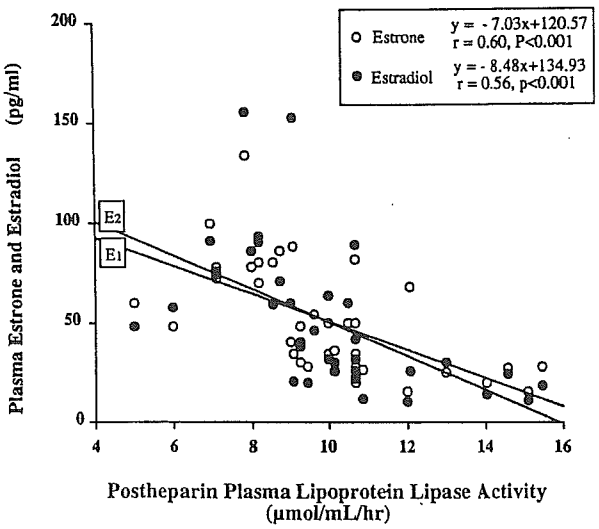

Figure 2. Relationship between postheparin plasma lipoprotein lipase activity and plasma estradiol (E2) and estrone (E1) concentrations in premenopausal, postmenopausal, and surgically menopausal groups. Open and closed circles indicate plasma E1 and E2, respectively.

Lipoprotein lipase and hepatic triglyceride lipase regulate the metabolism of the VLDL-LDL cascade. Analysis of the enzyme activities may help in understanding why the plasma LDL cholesterol level is elevated in postmenopausal and surgically menopausal women. According to Liu et al,[23] transgenic mice expressed human lipoprotein lipase, which resulted in expression of human lipoprotein lipase mass and an increase in postheparin plasma lipoprotein lipase activity, showed two to three times lower VLDL and 35% greater LDL concentrations in the plasma. Furthermore, Rutledge and Goldberg[24] created a vessel-perfused model and concluded that perfusion with lipoprotein lipase caused an increase in the rate of LDL accumulation and decreased the rate of efflux in perfused vessels. Clinical studies also demonstrated that there is a positive correlation between lipoprotein lipase activity and the obesity index[25,26] and that enhanced VLDL secretion leads to LDL accumulation in obese subjects.[27] These data indicate that lipoprotein lipase plays a principal role in the regulation of the VLDL-LDL cascade. We found that the activities of postheparin plasma lipoprotein lipase, a key enzyme in LDL production, was increased in postmenopausal and surgical menopausal women. Furthermore, we observed a negative correlation between postheparin plasma lipoprotein lipase activity and the levels of plasma E1 and E2. According to Iverius and Brunzell[13] circulating E2 is a regulator of lipoprotein lipase but not of hepatic triglyceride lipase. Sorva et al[28] demonstrated that endogenous estrogen is involved in regulating postheparin plasma lipoprotein lipase activity, and our data are in agreement. These results indicate that lowered plasma estrogen levels after menopause or postsurgical bilateral oophorectomy may lead to an increase in postheparin plasma lipoprotein lipase activity and that this increase may be one of the important factors for plasma LDL cholesterol accumulation.

Postheparin plasma hepatic triglyceride lipase activity did not differ significantly among the three groups. We observed no significant correlation between post-

heparin plasma hepatic triglyceride lipase activity and plasma estrogen concentrations. Based on these findings, we speculate that hepatic triglyceride lipase has a limited role in the hypercholesterolemia in postmenopausal and surgically menopausal women.

Several studies[8–11] have reported that the oral administration of estrogen inhibits postheparin plasma lipoprotein lipase and hepatic triglyceride lipase activity in postmenopausal women. Although postheparin plasma hepatic triglyceride lipase activity is inhibited by exogenous estrogen,[8–11] its activity does not change with age, despite the decrease in endogenous estrogen levels. Because oral estrogen administration leads to a transient supraphysiologic concentrations of estrogen in the hepatic circulation, hepatic triglyceride lipase activity should be readily inhibited.

Very low-density lipoprotein, a substrate for LDL, is secreted by the liver. Triglyceride concentrations did not differ significantly among the three groups, indicating that there was no difference in plasma VLDL concentration. Therefore, an elevated plasma LDL cholesterol concentration may not be attributed to an enhanced VLDL secretion. However, we cannot exclude an increase in VLDL secretion because of an enhanced postheparin plasma lipoprotein lipase activity.

Exogenous estrogen stimulates the synthesis of LDL receptors and lowers the plasma level of LDL cholesterol in both animals[29,30] and humans.[31] A recent study[32] attributed hypercholesterolemia in postmenopausal women to a reduced activity of LDL receptors. Findings suggest that the lack of estrogen in postmenopausal and surgically menopausal women may enhance lipoprotein lipase activity and reduce LDL receptor activity, leading to the accumulation of plasma LDL cholesterol.

High-density lipoprotein readily removes excess tissue-free cholesterol via transfer from the cell membrane. Therefore, the plasma concentration of HDL cholesterol is related inversely to the risk of coronary heart disease. The HDL cholesterol level and apolipoprotein A-I and A-II concentrations as major apolipoproteins in HDL did not differ significantly between the three groups. An unchanged plasma HDL cholesterol level may also be one of the risk factors for coronary heart disease in women with a lack of sex steroid hormones because the plasma concentration of LDL cholesterol increases after natural and surgical menopause. Several routes lead to the production of HDL. It is secreted by the liver, intestine, and triglyceride-rich lipoproteins during the process of its degradation. In our study, no significant relationship was found between postheparin plasma lipoprotein lipase activity and plasma HDL cholesterol concentration. Because lipoprotein lipase facilitates the degradation of triglyceride-rich lipoproteins, this result indicates that the secretion of HDL is unlikely to occur via the degradation of triglyceride-rich lipoproteins. Hepatic triglyceride lipase helps to regulate the level of plasma HDL by delivering HDL from the circulation to the liver. In fact, the oral administration of estrogen inhibits postheparin plasma hepatic triglyceride lipase activity and elevates the plasma HDL concentration.[10,11] Our finding of no significant difference in hepatic triglyceride lipase activity between the three groups may have resulted in unchanged plasma concentration of HDL.

Plasma lecithin cholesterol acyltransferase activity was greater in the postmenopausal and surgically menopausal women, suggesting that cholesterol esterification is accelerated in plasma HDL. Plasma lecithin cholesterol acyltransferase activity is reportedly not influenced by exogenous estrogen,[33,34] but the relationship between its activity and the serum concentration of endogenous estrogen was not examined. In this study, we found a greater plasma lecithin cholesterol acyltransferase activity in the postmenopausal and surgically menopausal women. The activity positively correlated with the plasma concentration of LDL cholesterol but not with that of plasma estrogen. We propose that plasma lecithin cholesterol acyltransferase activity is enhanced by an increase in LDL cholesterol concentration but is unaffected by the endogenous estrogen concentration. We do not know why the plasma lecithin cholesterol acyltransferase activity is enhanced, whereas the plasma HDL cholesterol level remains unchanged. Further studies are required to clarify the metabolism of HDL in postmenopausal and bilaterally oophorectomized women.

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Subtle 17α-Hydroxylase/17,20-Lyase Deficiency with Homozygous Y201N Mutation in an Infertile Woman

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Steroid 17α-hydroxylase deficiency is characterized by failed sexual development and mineralocorticoid hypertension. Female patients usually exhibit primary amenorrhea. Some patients with partial deficiency are reported to have menses, yet they have hypertension and hypokalemia. We describe here a normotensive, infertile female patient with menses and minimal defects in secondary sex characteristics.

The patient experienced menarche at age 13, and her menstrual cycles were regular until age 18 and irregular thereafter. Pubic hair was present (Tanner stage 3), and breast maturation was within normal range (Tanner stage 5). The patient's resting blood pressure was normal, and hypokalemia was not observed despite high blood corticosterone levels and reduced plasma renin activity. Analysis of the CYP17 gene revealed that the patient was homozygous for the Y201N mutation. *In vitro* expression of the mutated Y201N enzyme revealed reduced activities of both 17α-hydroxylase and 17,20-lyase; however, these reductions were less than those of the F53/54DEL mutation, which also shows mild clinical deficiency of 17α-hydroxylase/17,20-lyase. Thus, the 17α-hydroxylase/17,20-lyase deficiency in the present case is very mild both clinically and enzymatically. This case raises the possibility that there are infertile, menstruating women with undiagnosed 17α-hydroxylase deficiency. (*J Clin Endocrinol Metab* 90: 2508–2511, 2005)

STEROID 17α-HYDROXYLASE DEFICIENCY (17OHD) is an autosomal recessive disorder characterized by failure of sexual development and mineralocorticoid hypertension (1–4). It is caused by a defect (5) in the steroidogenic enzyme cytochrome P450c17 gene (6), which has both 17α-hydroxylase and 17,20-lyase activities. Lack of sex steroids results in female external genitalia in genetically male individuals and the absence of sexual maturation, including primary amenorrhea, in genetically female individuals (7). Mineralocorticoid excess causes severe hypertension and hypokalemia. In a milder form of 17OHD, genetically male individuals exhibit ambiguous genitalia (8–10), and female patients sometimes menstruate (11, 12). Most patients with mild 17OHD have severe hypertension. When defects in the secondary sexual characteristics are minimal and severe hypertension is absent in female patients who menstruate, 17OHD may be overlooked.

We observed subtle 17OHD in a female patient with minimal defects in sexual maturation and detected a mutation in the CYP17 gene. This case suggests that there may be undiagnosed cases of mild 17OHD among normotensive menstruating women.

Patient and Methods

Clinical features

The patient was a 38-yr-old Japanese woman who was evaluated for low plasma renin activity. She was an offspring of a consanguineous marriage. She had menarche at age 13, and her menstrual cycles were regular until age 18 and irregular thereafter. After marriage, she was found to be infertile and was under the care of an obstetric clinic for several years. She visited Showa University Hospital at age 34 because of atypical genital bleeding. Her blood pressure at that time was 130/84. On gynecological examination, hypoplastic uterus was observed. Plasma LH and FSH levels were 17 and 22 mU/ml, respectively. Serum progesterone was 2.2 ng/ml, and serum estradiol was 107 pg/ml.

Four years later, when she visited the Division of Internal Medicine, her initial blood pressure was 156/80. It rose to 180/100 in the doctor's presence and then dropped to 150/90 after several deep breaths. Self-monitoring of blood pressure at home showed her regular blood pressure to be 120–130/70–80; however, it did increase in response to stress. Thereafter, her blood pressure at each visit to the outpatient clinic was almost normal. Although her initial hypertension was thought to be a form of white-coat hypertension, laboratory tests revealed that both plasma renin activity and aldosterone concentration were low, which prompted further endocrinological evaluations. She was 157 cm tall and weighed 45 kg. Pubic hair was present but relatively scarce (Tanner stage 3). Axillary hair had been present but was almost absent at the time of examination. Breast maturation was within normal limits (Tanner stage 5). Her karyotype was 46XX. Serum potassium concentrations were always within normal range. The patient's serum steroid hormone profile is shown in Table 1. A marked increase in corticosterone and a slight increase in deoxycorticosterone were observed. These steroid levels were markedly reduced with dexamethasone administration. Serum cortisol levels and plasma ACTH level were within normal limits, but the cortisol level did not respond well to ACTH load. Serum dehydroepiandrosterone sulfate was within the normal range, and serum androstenedione was low. Abdominal computed tomography revealed no adrenal tumor or hypertrophy of the adrenal glands.

Sequence analysis

Genomic DNA was extracted from peripheral blood leukocytes. The coding regions of exons 1–8 of the CYP17 gene, including exon-intron boundaries, were amplified from 1 μg genomic DNA by PCR with a set of gene-specific primers as described previously (13). Nucleotide sequences of purified PCR products were determined by direct sequencing

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Abbreviations: 17OHD, 17-Hydroxylase deficiency; RFLP, restriction fragment length polymorphism.

TABLE 1. Hormonal data

	Normal range	Basal	After dexa	ACTH load, 60 min
PRA (ng/ml·h)	0.20–3.90	<0.15–0.47		
Aldosterone (ng/dl)	3.6–24.0	2.3–6.7		
Progesterone (ng/ml)	<1.7	2.2–5.9	0.3	7.4
DOC (ng/ml)	0.11–0.40	0.40–0.59	0.04	1.0
Corticosterone (ng/ml)	0.21–8.48	23.8–132.0	3.0	312.0
18-OH DOC (ng/ml)	0.01–0.07	0.26		
17-OHP (ng/ml)	0.2–4.5	0.2	<0.1	1.0
Cortisol (μg/dl)	4.0–18.3	5.5–12.4		14.0
DHEA-S (ng/ml)	640–2030	769–1090		
Androstenedione (ng/ml)	0.6–2.2	0.2		
Estradiol (pg/ml)	10–366	90–333		
ACTH (pg/ml)	8.2–54.8	40		
LH (mIU/ml)	1.7–19.8	17–23		
FSH (mIU/ml)	1.1–13.6	22–33		
K (mEq/liter)	3.5–4.8	3.8–4.2		

Basal values with ranges are the results of multiple measurements. After dexa, overnight after 1 mg dexamethasone administration; PRA, plasma renin activity; DOC, deoxycorticosterone; 17-OHP, 17-hydroxyprogesterone; DHEA-S, dehydroepiandrosterone sulfate.

with an ABI Prism Dye Termination Cycle Sequencing Core Kit (Applied Biosystems, Foster City, CA).

PCR-restriction fragment length polymorphism (PCR-RFLP) analysis

The detected mutation eliminates a *Tsp*E1 restriction site. We performed PCR-RFLP analysis of genomic DNA from the patient and her parents. The mutated codon was amplified with primers 5′-GGCCAC-CCACAACGGACAGTC-3′ and 5′-GACTAGGTCCACCAGGCTGTC-3′. PCR consisted of 33 cycles of 94 C for 1 min, 58 C for 1 min, and 72 C for 1 min. PCR products were purified by ethanol precipitation, digested with *Tsp*E1 at 65 C for 4 h, and separated by agarose gel.

Site-directed mutagenesis, transfection of COS1 cells, and enzyme assays

A detected mutation was created in a human P450c17 cDNA in Bluescript (pBSH 17α-1), which was produced previously by Yanase *et al.* (14). Site-directed mutagenesis was performed with a BD Transformer Site-Directed Mutagenesis Kit (BD Biosciences Clontech, Tokyo, Japan) with a primer designed for the nucleotide change. The full-length mutant cDNA was sequenced to confirm the mutation. The *Bam*HI-*Hin*dIII fragment containing the full-length mutant cDNA was inserted into the pCMV expression vector at the *Bgl*II and *Hin*dIII sites.

Transfection of COS-1 cells and thin layer chromatography analysis of the catalytic properties of P450c17 were carried out as described previously (14). Enzyme activities were compared with those of wild-type enzyme, which was produced with pCMV17α-H, and another mutant with mild 17α-hydroxylase/17,20-lyase deficiency, F53/54DEL enzyme, which was produced with pCMVJG17α-H (14). Immunoblotting of cellular proteins with antibody raised against porcine testis P450c17 was carried out as previously described (14).

Results

Sequence of the CYP17 gene

A homozygous T→A transversion at nucleotide position 2472 in exon 3 of CYP17 (amino acid change, Tyr201 to Asn:Y201N) was detected in the proband (Fig. 1).

PCR-RFLP analysis

Because the T2472A mutation eliminates a *Tsp*E1 restriction site, we used PCR-RFLP analysis for genotyping (Fig. 2).

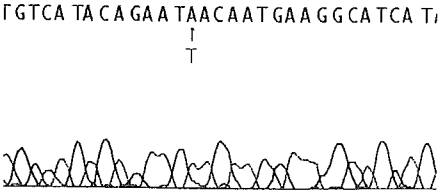

FIG. 1. Sequence analysis of the CYP17 gene revealed homozygous mutation of thymine to adenine at nucleotide position 2472 in exon 3, which causes amino acid substitution tyrosine 201 to asparagine (Y201N).

The expected PCR product was 184 bp. Digestion of the wild-type allele with *Tsp*E1 yielded 127-bp and 57-bp fragments. The patient carried only the mutant allele, whereas both her parents were heterozygous for the mutant allele.

In vitro expression study

To determine the functional significance of the Y201N mutation, the mutant cDNA was cloned into a eukaryotic expression vector, pCMV, and expressed in nonsteroidogenic COS-1 cells. Cells expressing the mutant cDNA(Y201N) produced the same amount of immunodetectable P450c17 protein as cells expressing wild-type P450c17 cDNA or mutant P450c17 cDNA (F53/54DEL) (data not shown). Although there was a similar amount of immunodetectable P450c17 protein in the various transfectants, the activities of P450c17 (Y201N), including 17α-hydroxylation and 17,20-lyase activity, were reduced. Comparison of the initial kinetics with progesterone and 17α-hydroxypregnenolone as substrates revealed that the 17α-hydroxylase and 17,20-lyase activities of P450c17 (Y201N) were less than 33% and less than 35%, respectively, those of wild-type P450c17. The activities of P450c17 (F53/54DEL) were less than 22 and 5.2%, respectively, those of wild-type (Table 2). Thus, P450c17 (Y201N) had greater 17α-hydroxylase and 17,20-lyase activities than did P450c17 (F53/54DEL), and both were reduced in parallel in P450c17 (Y201N), whereas there was a much greater reduction of 17,20-lyase activity relative to 17α-hydroxylase activity in P450c17 (F53/54DEL), as previously observed (14).

Discussion

The degree of deficiency in 17α-hydroxylase and 17,20-lyase activities in the present case was very mild. The manifestations possibly related to the enzyme defect were lack of

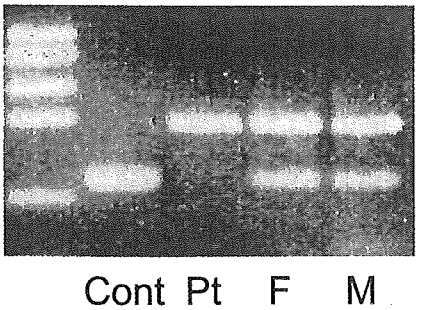

FIG. 2. PCR-RFLP analysis. Y201N mutation eliminates a *Tsp*E1 restriction site. Cont, Control; Pt, patient; F, father; M, mother. Patient had two mutant alleles, whereas each parent had one normal allele and one mutant allele.

TABLE 2. Enzymatic activities of mutant enzymes

	17α-hydroxylase (%)	17,20-lyase (%)
Wild type	180.5 (100)	73.3 (100)
Y201N	56.5 (31.3)	24.8 (33.7)
F53/54DEL	37.8 (20.9)	3.8 (5.2)

All values are the mean of two independent measurements. Units for all measurements are picomoles per dish.

axillary hair, irregular menstruation, hypoplastic uterus, infertility, and occasional hypertension. However, biochemical analyses suggested that the patient had 17OHD. Plasma renin activity and aldosterone level were low, whereas plasma corticosterone was high and suppressed by dexamethasone. We were not sufficiently confident to diagnose 17OHD at that time because hypokalemia was absent and plasma cortisol and corticotropin levels were within normal limits. Because consanguinity supported recessive inheritance, we sequenced the CYP17 gene and found that the proband was homozygous for a novel missense mutation. Each parent carried one mutant allele. *In vitro* expression analysis revealed that the Y201N mutation partially reduces the activities of both 17α-hydroxylase and 17,20-lyase. The 17α-hydroxylase activity and 17,20-lyase activity of mutant P450c17 were less than 33% and less than 35%, respectively, those of wild-type P450c17. This finding is consistent with the patient's clinical 17α-hydroxylase/17,20-lyase activities. It appears that a 17α-hydroxylase activity that is 30% of that of normal may be sufficient to maintain a normal blood pressure. The patient's 17,20-lyase activity (30% of normal) was higher than a previously reported patient with the F53/54DEL mutation, whose 17,20-lyase activity was 5% of that of normal and with regular menstruation (14). Therefore, this mutant may allow for regular menstruation. Although Tyr201 is located in the F helix that forms the F-G loop, the structural and functional significance of this region of the molecule remains unclear (15).

The clinical manifestations of 17α-hydroxylase/17,20-lyase deficiency reflect the activities of the mutated enzymes. Mutations that result in complete loss of enzymatic activities yield the phenotypes associated with complete deficiency (16). Partial deficiency is related to mutations that only partially affect activities (10, 17). Isolated 17,20-lyase deficiency, in which only phenotypes related to sex hormone deficiency are present and those of mineralocorticoid excess are absent, is caused by specific mutations such as R347H and R358Q (18). These mutations cause only 17,20-lyase defects, and 17α-hydroxylase activity is essentially unaffected (19).

There are generally three opportunities for diagnosis of 17OHD. Ambiguous genitalia at birth give the opportunity to check for genetically male infants with partial deficiency. Genetically male individuals with severe deficiency and genetically female individuals are both phenotypically female, and sex hormone deficiencies are typically recognized at puberty. Primary amenorrhea and lack of female secondary sex characteristics, including breast development and pubic hair, may require evaluation. In other cases, the disease is detected during the course of evaluation of severe hypertension. Genetic females with 17OHD who menstruate are typically identified in this manner (11, 12). To date, approximately 10 patients with 17OHD and menstruation have been reported including three patients with homozygous F53/54DEL mutation (Refs. 11, 12, 21, 22, and references in Ref. 2). Although these patients menstruated, only three had pubic hair and mature breast development. Moreover, with the exception of the patient described herein, all had severe hypertension and hypokalemia. Cases of 17OHD without hypertension (Ref. 23 and references in Ref. 2) or with mild hypertension (7) have been reported; however, the patients had primary amenorrhea. Thus, our case appears to be very rare. However, mild cases may be overlooked because the patients are not evaluated for mineralocorticoid excess or sex hormone deficiencies. Such infertile patients with mild 17OHD have been reported (24). Some specific mutations are reported to be widespread in the particular geographic area (25). It is conceivable that undiagnosed patients with subtle 17OHD with mild deficiency of enzyme activities are present.

It was reported that administration of testosterone as an aromatizable substrate to a patient with 17OHD resulted in follicular maturation and ovulation (26). Thus, identification of 17OHD is essential not only for diagnosis of the cause of infertility but also for developing possible strategies to treat this type of infertility. It seems valuable to screen for 17OHD by plasma renin activity or by corticosterone or progesterone level (27) in infertile women with only minimal defects on sexual maturation.

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Impaired Nuclear Translocation, Nuclear Matrix Targeting, and Intranuclear Mobility of Mutant Androgen Receptors Carrying Amino Acid Substitutions in the Deoxyribonucleic Acid-Binding Domain Derived from Androgen Insensitivity Syndrome Patients

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Context: Recent imaging studies revealed that androgen receptor (AR) is ligand-dependently translocated from the cytoplasm into the nucleus and forms intranuclear fine foci. In this study, we examined whether intracellular dynamics of mutant ARs detected in two androgen insensitivity syndrome (AIS) patients was impaired.

Objective: ARs with mutations in the DNA-binding domain were functionally characterized and compared with the wild-type AR.

Patients: In a complete AIS patient (subject 1), cysteine residue 579 in the first zinc finger motif of AR was substituted for phenylalanine (AR-C579F). Another mutation (AR-F582Y) was found in a partial AIS patient (subject 2).

Results: AR-F582Y retained less than 10% of the transactivation activity of the wild-type AR, whereas no ligand-dependent transactivation was detected for AR-C579F. Image analyses of the receptors fused to green fluorescent protein showed that the wild-type AR was ligand-dependently translocated into the nucleus in which it formed fine subnuclear foci. Surprisingly, after the addition of dihydrotestosterone, the two mutant ARs initially formed large cytoplasmic dots, many of which were found to be close to mitochondria by electron microscopy. Subsequently, a part of the ligand-bound mutant ARs gradually entered the nucleus to form a smaller number of larger dots, compared with the wild-type AR. Fluorescence recovery after photobleaching analysis revealed that the intranuclear mobility of the mutant ARs decreased, compared with that of the wild-type AR.

Conclusions: These results suggest that the abnormal translocation, localization, and mobility of the mutant ARs may be the cause of AIS in these subjects. (***J Clin Endocrinol Metab* 90: 6162–6169, 2005**)

ANDROGENS PLAY AN essential role in the expression of the male phenotype. The actions of androgens are mainly mediated by the androgen receptor (AR). The AR belongs to the nuclear receptor superfamily, a large group of transcription factors whose members share basic structural and functional homology (1, 2). The N-terminal domain of the AR contains the major transactivation function region, AF-1, which acts in a ligand-independent fashion. The centrally located DNA-binding domain (DBD) is highly conserved among steroid hormone receptors and consists of two zinc finger clusters. The first zinc finger motif is involved in direct DNA-binding and contains the P-box for specific recognition of the androgen-responsive elements of target genes (3). The C-terminal ligand-binding domain (LBD) contains transactivation function domain 2 and functionally interacts with intermediary factors and nuclear cofactors. In the absence of an agonist, the LBD is believed to prevent the transactivation function of the N-terminal domain through an intramolecular interaction (4).

Unliganded ARs are located in the cytoplasm, in which they are sequestered with heat shock proteins. After ligand binding, a conformational change of the receptor protein results in unmasking of both the dimerization motif and the nuclear localization signal that allows translocation into the nucleus (1). Upon nuclear entry, the ligand-receptor complexes appear to move into subnuclear compartments, which are common congregation sites for steroid hormone receptors and other associated factors, such as nuclear receptor coactivators, that are required for transcriptional activation of the target genes. Complete subnuclear foci formation seems to be essential for steroid hormone receptor-mediated transactivation (4–6).

Because the human AR gene is located on the X chromosome at Xq11–12 (1, 4, 7), just a single allele mutation in the AR gene causes dysfunction of the receptor in 46, XY individuals, resulting in androgen insensitivity syndrome (AIS) (3, 8–10). Despite a high or normal level of serum testoster-

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Abbreviations: AIS, Androgen insensitivity syndrome; AR, androgen receptor; CAIS, complete androgen insensitivity syndrome; 3D, three-dimensional; DBD, DNA-binding domain; DHT, dihydrotestosterone; FBS, fetal bovine serum; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; GR, glucocorticoid receptor; LBD, ligand-binding domain; PAIS, partial androgen insensitivity syndrome; $t_{1/2}$, half-recovery time; YFP, yellow fluorescent protein.

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